PLANT SCIENCE'S CONTRIBUTION TO FIGHTING VIRAL PANDEMICS: COVID-19 AS A CASE STUDY

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PLANT SCIENCE'S CONTRIBUTION TO FIGHTING VIRAL PANDEMICS: COVID-19 AS A CASE STUDY

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EDITORIAL
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Editorial: Plant Science's Contribution to Fighting Viral Pandemics: COVID-19 as a Case Study

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Editorial on the Research Topic

Plant Science's Contribution to Fighting Viral Pandemics: COVID-19 as a Case Study

The use of plants to treat human diseases can be traced back to 6,000 years ago. The enormous biochemical arsenal of plants known as secondary (or specialized) metabolome has enabled the production of medicinal compounds to cure and/or ameliorate the symptoms of many human and animal diseases that are still prevalent today.

The enormous progress of plant biotechnology in the last 30 years has further boosted the potential of plants to help us cope with diseases, thanks to the advancement of molecular pharming, i.e., the use of plants to produce, by transient or stable transformation, biopharmaceuticals.

Given the current pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the international scientific community has gathered together in an urgent search for solutions to the current coronavirus disease-2019 (COVID-19) outbreak. To date, the plant-derived compounds chloroquine phosphate and artemisinin, which were previously used against malaria, have been repurposed for the treatment of COVID-19. Medicago is developing a candidate vaccine in plant factories, based on Virus-Like Particles (VLPs) of SARS-CoV-2, that is currently in phase III of its clinical trial, and promises to be one of the first subunit vaccines to enter the market. Furthermore, ongoing research supports the use of plants as bio-factories for the production of eagerly awaited SARS-CoV-2 monoclonal antibodies for passive immunization, which is meant to manage the disease.

In this emergency scenario, our topic aims to gather all relevant information on plant sciences to fight the COVID-19 pandemic by stimulating a "plant scientific hub" that builds on the enormous potential of plant science to contribute effectively to fighting present and future pandemics.

The current Research Topic includes six reviews, one mini review, one perspective, two opinion articles, and four original research studies focusing on (i) natural plant products, covering all the steps from production to extraction of bioactive molecules from medicinal plants, and on (ii) recombinant expression dissecting the use of plant molecular pharming for the production of viral recombinant proteins as diagnostic reagents, vaccines, and antibodies.

The former aspect is investigated in the four reviews, one mini-review, and two opinion articles.

In their review, Garcia provides a historical perspective on the use of plants in coping with pandemics, focusing on therapeutic use.

Another review by Pandey et al. focuses on the strategic use of phyto-inibitors to cope with the current pandemic in a way that could develop remedies for COVID-19 in a time-effective way.

In the mini-review of Matveeva et al., the authors summarize information on methods and

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approaches to searching for plant compounds including cheminformatics, bioinformatics, genetic engineering of viral targets, and others. As the authors discuss, this summary serves as an inspiration for experimental designs and to provide guidance for newcomers in the field.

In their review, Khan et al. review the potential of different *in vitro* plant cultures to produce medicinally important secondary metabolites to fight COVID-19.

Javed et al., Lucas et al., and Clark and Taylor-Robinson, respectively, in a review and in two opinion articles, focus on specific plant species and secondary metabolites, providing an overview of their potential for managing COVID-19 based on previous *in vitro* and *in vivo* studies. Specifically, carvacrol (Javed et al.), extracts of hops, Ceylon cinnamon (Lucas et al.), and pheophorbide *a* (Clark and Taylor-Robinson), a chlorophyll derivative, are considered in detail.

Plant molecular pharming is the main object of the remaining two reviews, perspective, and four original research articles.

In their review, Tusé et al. frame the use of plant molecular pharming from a perspective of feasibility by considering its potential from manufacturing to the current regulatory limitations and by comparing it to conventional manufacturing platforms based on mammalian cell cultures.

In this framework, Lico et al. in a perspective article, point out how molecular pharming could help in dealing with the current pandemic by focusing on Italy as a case study and the investment required in molecular pharming infrastructures. They define a roadmap for the development of diagnostic reagents and biopharmaceuticals to cope with COVID-19.

Poghossian et al., in their review, explore in detail the possibilities of plant virus-derived nanoparticles for the enhancement of virus biodetection based on field-effect devices (FEDs) used as biosensors. Previously, a brief perspective on recent advancements in the technology associated with FEDs has been provided.

In the four original research articles present in our Research Topic, some practical examples of the potential of plant molecular pharming to tackle SARS-Co V-2 are reported.

A complete and elegant description of the potential of plant molecular pharming is given by Diego-Martin et al., who describe production of milligram amounts of six different monoclonal antibodies and the receptor binding domain (RBD) of the Spike protein in *Nicotiana benthamiana* plants in the framework of an academic laboratory in just a few weeks, highlighting one major advantage of this approach, its speed.

Diagnostic reagents, meant to build up serological test for detecting the presence in sera of antibodies toward SARS-Co V-2, are the main focus of the research articles by Williams et al. and by Makatsa et al., respectively producing in plant systems the C-terminal half of SARS-CoV-2 N protein, and the S protein and RBD.

Plant-made therapeutic agents are described by Siriwattananon et al., that transiently produced Ace2 fused with the Fc region of human IgG1 assessing *in vitro* its potent neutralization capacity toward SARS-CoV-2, thus making the protein a therapeutic candidate for COVID-19.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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OPINION
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COVID-19 Therapy: Could a Chlorophyll Derivative Promote Cellular Accumulation of Zn^{2+} Ions to Inhibit SARS-CoV-2 RNA Synthesis?

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MEDICINAL USES OF CHLOROPHYLL DERIVATIVES

A range of tetrapyrrole derivatives are in development for their observed cytotoxicity (Kang et al., 2018; Singh et al., 2019). Non-toxic, water-soluble chlorophyll derivative compounds are a popular dietary supplement among health-conscious members of high-income countries. Many organometallic compounds derived from chlorophyll *a* or *b* are approved for human consumption. For instance, sodium copper chlorophyllin is promoted for both its anti-bacterial and anti-viral properties (Ulbricht et al., 2014; Solymosi and Mysliwa-Kurdziel, 2017). It contains replacement ions of Cu^{2+} instead of Mg^{2+}, which aids its water solubility and intestinal absorption (Ferruzzi and Blakeslee, 2007; Mishra et al., 2011; Ulbricht et al., 2014). While most derivatives are not deemed suitable for non-prescribed ingestion, their use in the medical field, particularly for the treatment of cancers, is considered a far safer alternative to most conventional therapies (Solymosi and Mysliwa-Kurdziel, 2017). Intramuscular injection and intravenous infusion are common modes of drug delivery for compounds derived from chlorophyll *a* in a clinical setting (Yoshida et al., 1980; Wang et al., 2001; Kochneva et al., 2010; Ulbricht et al., 2014).

CHLOROPHYLL DERIVATIVES AND FREE ZN^{2+}

Zinc pheophorbide *a* (ZnPh), a chlorophyll derivative for which a Zn^{2+} ion replaces the naturally occurring Mg^{2+}, is similar to non-metallated organic molecules in possessing photosensitiser properties. These are harnessed to produce a cytotoxic effect in the treatment of cancer cells (Ahn et al., 2012; Jakubowska et al., 2013; Ocakoglu et al., 2015; Ahn et al., 2017; Solymosi and Mysliwa-Kurdziel, 2017; Mokwena et al., 2018; Singh et al., 2019). While this is particularly relevant to human adenocarcinoma

cell lines, the most commonly used *in vitro* model to represent the alveoli of the lungs, a broad array of cell types has been shown to respond to ZnPh (Jakubowska et al., 2013; Ocakoglu et al., 2015). Zn^{2+} ions attached to this tetrapyrrole derivative can pass through cell membranes as it is water-soluble. Aggregation and accumulation by different organelles of pheophorbide *a* and other chlorophyll breakdown products, both with and without Zn^{2+}, are demonstrated in many studies (Tao et al., 1990; Szczygieł et al., 2008; Yoon et al., 2011; Ahn et al., 2012; Tamiaki et al., 2012; Xodo et al., 2012; Jakubowska et al., 2013; Ocakoglu et al., 2015; Ahn et al., 2017; Kang et al., 2018; Mokwena et al., 2018; Singh et al., 2019; Zhou et al., 2019; Tamiaki et al., 2020). Interestingly, no localization of ZnPh to mitochondria has been observed (Jakubowska et al., 2013; Ocakoglu et al., 2015). Therefore, ZnPh is unlikely to impair the function of mitochondria in healthy cells, highlighting a number of potential benefits to this treatment. Moreover, since the charge separation of Cu^{2+} and Zn^{2+} ions is identical, with the latter displaying only a slightly higher electronegativity, ion substitution of pheophorbides with Zn^{2+} aids the solubility of ZnPh. Hence, this improves the ability of ZnPh to be a carrier molecule for the free ionisation of Zn^{2+} (Ferruzzi and Blakeslee, 2007; Jakubowska et al., 2013; Ocakoglu et al., 2015; Martinez De Pinillos Bayona et al., 2017; Kang et al., 2018) and thus potentially to act as a drug delivery system.

It has been suggested that as a clinical treatment, ZnPh may be used in conjunction with photodynamic therapy (ultraviolet B irradiation) to induce singlet oxygen stress and ionisation of Zn^{2+} molecules, resulting in cytotoxicity towards carcinogenic cells lining the human lungs (Jakubowska et al., 2013). There is also a low overall cytotoxic effect in murine models, with animals generally healthy throughout treatment indicative of limited systemic side effects (Jakubowska et al., 2013; Ocakoglu et al., 2015). As with non-metallated organic molecules, above a threshold concentration ZnPh, is cytotoxic in the dark, a phenomenon that continues upon illumination; however, at low concentrations, no cytotoxic effect is observed regardless of the level of light intensity (Jakubowska et al., 2013; Ocakoglu et al., 2015). Exposure and singlet oxygen stress can be controlled according to light spectrum settings, i.e. red light versus blue light. Whether irradiated or not, light-induced cytotoxicity that is triggered by pheophorbides is dose-related and diminishes rapidly (Ahn et al., 2012; Jakubowska et al., 2013; Ocakoglu et al., 2015), and no systemic toxicity has been detected (Ahn et al., 2017).

ZnPh and similar non-metallated forms facilitate the successful transmembrane passage of non-polar components of a molecule, wherein irradiance alters the ionic concentration of free ions once it has entered into the cytoplasm (Martinez De Pinillos Bayona et al., 2017). Free Zn^{2+} appears to promote an antiviral effect, research *in vitro* demonstrating accumulation of Zn^{2+} in human lung tissue (Hagimori et al., 2019). Moreover, increasing the cellular concentration of Zn^{2+} through zinc supplementation and using ionophores such as pyrithione efficiently impairs RNA replication by human coronaviruses, leading to improved treatment outcomes (te Velthuis et al., 2010; Read et al., 2019; Derwand and Scholz, 2020; Zhang and Liu, 2020). Similarly, sucking zinc lozenges reduces symptoms of viral respiratory infection (Turner and Cetnarowski, 2000). Zinc ionophores are effective at increasing concentrations of Zn^{2+} because they allow more zinc to pass through the cell membrane (Zhang and Liu, 2020). Yet, as uptake of zinc is a less efficient and therefore slower metabolic process in eukaryotes (Krężel and Maret, 2006), very little is known about zinc ionophores as transporters (Gaither and Eide, 2001).

The primary function of an ionophore is to increase cell permeability to enable transport of ionic compounds across the cell membrane by endocytosis. Although this is a role fundamental to normal functioning of a healthy cell, the presence of ionophores can weaken the integrity of the cell membrane, thus paradoxically leading to decreased cell defence systems (Gaither and Eide, 2001; te Velthuis et al., 2010; Derwand and Scholz, 2020). However, it is not known if ZnPh also weakens cell membranes nor whether the rate and/or extent of ionisation of free zinc is increased during this uptake. Despite ZnPh not being a recognized ionophore, as it is highly soluble when irradiated, uptake of Zn^{2+} is likely to be improved by this process (Szczygieł et al., 2008; Tamiaki et al., 2012; Martinez De Pinillos Bayona et al., 2017; Hagimori et al., 2019). Studies using *in vitro* cultured cells point to the capacity of ZnPh to elevate free ion concentrations of Zn^{2+} (Jakubowska et al., 2013; Ocakoglu et al., 2015). If this effect translates successfully from *in vitro* to *in vivo*, then such a mechanism would reduce the therapeutic demand for the use of toxic or dose-dependent ionophores such as hydroxychloroquine.

CLINICAL CAPACITY AND THERAPEUTIC POTENTIAL OF ZINC PHEOPHORBIDE *A*

While our understanding of the metabolic processes of zinc transporters remains limited, it is plausible to speculate that ZnPh combined with zinc supplementation could reach deep inside human lungs (Jakubowska et al., 2013). This would increase the cellular concentration of Zn^{2+} to target cells such as alveolar epithelia (Hagimori et al., 2019), corroborating *in vitro* studies on the free ionisation of Zn^{2+} (Szczygieł et al., 2008; Tamiaki et al., 2012; Hagimori et al., 2019; Zhou et al., 2019). Additionally, such raised intracellular levels may be achieved independently of the need for a cytotoxic effect experienced both in the dark and upon illumination, eliminating associated risks of singlet oxygen stress, ionisation and phosphorylated nuclear cell damage. Therefore, since ZnPh is water-soluble, it is reasonable to consider that ionic uptake of Zn^{2+} is increased, potentially producing a similar effect as observed with other ionophores such as pyrithione and hydroxychloroquine (Derwand and Scholz, 2020). Together with zinc supplementation, this may significantly impair replication of human coronaviruses, notably SARS-CoV-2, under experimental conditions. Hence, we propose that this novel therapy merits further evaluation to develop long-term as a possible clinical treatment of symptomatic COVID-19 patients.

The drug delivery capacity of chlorophyll derivatives depends on the properties of each compound (Ulbricht et al., 2014; Kang et al., 2018), as some display non-polar behaviour in a dose-dependent ratio (Jakubowska et al., 2013; Ocakoglu et al., 2015; Martinez De Pinillos Bayona et al., 2017), thus emphasising the need for clinical trials (Tang et al., 2006; Ferruzzi and Blakeslee,

2007; Mishra et al., 2011; Ulbricht et al., 2014; Solymosi and Mysliwa-Kurdziel, 2017). It is important to appreciate that replacement of the central ion in pheophorbides can yield varied results *in vitro* and *in vivo*, with some studies suggesting that ionic inclusion may reduce toxicity (Szczygieł et al., 2008; Tamiaki et al., 2012; Martinez De Pinillos Bayona et al., 2017; Tamiaki et al., 2020). Accidental ingestion of non-dietary levels of pheophorbide *a*, a compound that lacks a central ion, induces a rare but mild phototoxic effect at low doses; however, this was still deemed safe for human consumption (Hwang et al., 2005).

Chlorophyll is generally considered not harmful to humans (Ulbricht et al., 2014), but information is scarce on any possible toxicity posed by chlorophyll derivatives, especially those compounds, both with and without ion inclusion, e.g. Zn^{2+}, which have not been subject to clinical trials. Pre-clinical and clinical safety tests on ZnPh are required. However, it is generally accepted that toxicity is dependent on a photo-reactive response (Tao et al., 1990; Hwang et al., 2005; Szczygieł et al., 2008; Tamiaki et al., 2012; Jakubowska et al., 2013; Ocakoglu et al., 2015; Martinez De Pinillos Bayona et al., 2017; Zhou et al., 2019; Tamiaki et al., 2020), given that phototoxicity induced by ZnPh is short-lived (Ahn et al., 2012; Jakubowska et al., 2013; Ocakoglu et al., 2015; Solymosi and Mysliwa-Kurdziel, 2017). Rapid clearance of zinc chlorophyll compounds is observed in human cell lines, with low doses invoking no or limited cytotoxic effect (Tao et al., 1990; Tang et al., 2006; Jakubowska et al., 2013; Ocakoglu et al., 2015; Martinez De Pinillos Bayona et al., 2017; Diogo et al., 2018).

CONCLUSION

Here, we provide an insight into the properties of ZnPh that make this chlorophyll derivative a potential therapeutic agent for treating COVID-19. Our understanding of ZnPh and similar compounds relates to the reported success of *in vitro* and *in vivo* cancer studies, so this recognised drug delivery system has not been used yet as a treatment for respiratory illness. However, the possibility that this may translate to an anti-viral property merits investigation.

Unless a pronounced phototoxic effect is induced — highly unlikely under physiological conditions — ZnPh is non-toxic to humans. Therefore, there is a strong likelihood that this compound offers great potential to act as a carrier molecule for Zn^{2+} to trigger an anti-viral response that impairs SARS-CoV-2 replication. Hence, Zn^{2+} ionophores provide a novel therapeutic option that may be of benefit to the development of an effective treatment for the severe clinical manifestations of COVID-19.

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Both authors (NC and AT-R) made substantial contributions to the conception of the work and to literature search, contributed significantly to writing the manuscript, revised it critically for important intellectual content, approved its final version and agreed to its submission.

REFERENCES

Ahn, M.-Y., Kwon, S.-M., Kim, Y.-C., Ahn, S.-G., and Yoon, J.-H. (2012). Pheophorbide *a*-mediated photodynamic therapy induces apoptotic cell death in murine oral squamous cell carcinoma *in vitro* and *in vivo*. *Oncol. Rep.* 27, 1772–1778. doi: 10.3892/or.2012.1748

Ahn, M.-Y., Yoon, H.-E., Moon, S.-Y., Kim, Y.-C., and Yoon, J.-H. (2017). Intratumoral photodynamic therapy with newly synthesized pheophorbide a in murine oral cancer. *Oncol. Res.* 25, 295–304. doi: 10.3727/096504016X 14732527645922

Derwand, R., and Scholz, M. (2020). Does zinc supplementation enhance the clinical efficacy of chloroquine/hydroxychloroquine to win todays battle against COVID-19? *Med. Hypotheses* 142, 109815. doi: 10.1016/j.mehy.2020.109815

Diogo, P., Mota, M., Fernandes, C., Sequeira, D., Palma, P., Caramelo, F., et al. (2018). Is the chlorophyll derivative Zn(II)e_6Me a good photosensitizer to be used in root canal disinfection? *Photodiagn. Photodyn. Ther.* 22, 205–211. doi: 10.1016/j.pdpdt.2018.04.009

Ferruzzi, M. G., and Blakeslee, J. (2007). Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives. *Nutr. Res.* 27, 1–2. doi: 10.1016/j.nutres.2006.12.003

Gaither, L. A., and Eide, D. J. (2001). Eukaryotic zinc transporters and their regulation. *Biometals* 14, 251–270. doi: 10.1023/A:1012988914300

Hagimori, M., Taniura, M., Mizuyama, N., Karimine, Y., Kawakami, S., Saji, H., et al. (2019). Synthesis of a novel pyrazine–pyridone biheteroaryl-based fluorescence sensor and detection of endogenous labile zinc ions in lung cancer cells. *Sensors* 19, 2049. doi: 10.3390/s19092049

Hwang, D. F., Tsai, Y. S., Chou, S. S., Liu, S. M., Wu, J. T., Lin, S. J., et al. (2005). HPLC determination of pheophorbide *a* and pyropheophorbide *a* in dried laver product implicated in food poisoning. *Shokuhin Eiseigaku Zasshi: J. Food Hyg. Soc. Jpn.* 46, 45–48. doi: 10.3358/shokueishi.46.45

Jakubowska, M., Szczygieł, M., Michalczyk-Wetula, D., Susz, A., Stochel, G., Elas, M., et al. (2013). Zinc-pheophorbide *a* — highly efficient low-cost photosensitizer against human adenocarcinoma in cellular and animal models. *Photodiagn. Photodyn. Ther.* 10, 266–277. doi: 10.1016/j.pdpdt.2012.12.004

Kang, Y.-R., Park, J., Jung, S. K., and Chang, Y. H. (2018). Synthesis, characterization, and functional properties of chlorophylls, pheophytins, and Zn-pheophytins. *Food Chem.* 245, 943–950. doi: 10.1016/j.foodchem.2017.11.079

Kochneva, E. V., Filonenko, E. V., Vakulovskaya, E. G., Scherbakova, E. G., Seliverstov, O. V., Markichev, N. A., et al. (2010). Photosensitizer Radachlorin®: Skin cancer PDT phase II clinical trials. *Photodiagn. Photodyn. Ther.* 7, 258–267. doi: 10.1016/j.pdpdt.2010.07.006

Krężel, A., and Maret, W. (2006). Zinc-buffering capacity of a eukaryotic cell at physiological pZn. *J. Biol. Inorg. Chem.* 11, 1049–1062. doi: 10.1007/s00775-006-0150-5

Martinez De Pinillos Bayona, A., Mroz, P., Thunshelle, C., and Hamblin, M. R. (2017). Design features for optimization of tetrapyrrole macrocycles as antimicrobial and anticancer photosensitizers. *Chem. Biol. Drug Des.* 89, 192–206. doi: 10.1111/cbdd.12792

Mishra, V. K., Bacheti, R. K., and Husen, A. (2011). "Medicinal uses of chlorophyll: a critical overview," in *Chlorophyll: Structure, Function and Medicinal Uses*. Eds. H. Le and E. Salcedo (Hauppauge, NY: Nova Scientific), 177–196.

Mokwena, M. G., Kruger, C. A., Ivan, M.-T., and Heidi, A. (2018). A review of nanoparticle photosensitizer drug delivery uptake systems for photodynamic treatment of lung cancer. *Photodiagn. Photodyn. Ther.* 22, 147–154. doi: 10.1016/j.pdpdt.2018.03.006

Ocakoglu, K., Er, O., Kiyak, G., Lambrecht, F. Y., Gunduz, C., and Kayabasi, C. (2015). 131I–Zn–chlorophyll derivative photosensitizer for tumor imaging and photodynamic therapy. *Int. J. Pharm.* 493, 96–101. doi: 10.1016/j.ijpharm.2015.07.047

Read, S. A., Obeid, S., Ahlenstiel, C., and Ahlenstiel, G. (2019). The role of zinc in antiviral immunity. *Adv. Nutr.* 10, 696–710. doi: 10.1093/advances/nmz013

Singh, K., Chaturvedi, D., and Singh, V. K. (2019). Review on different aspects of the photodynamic product pheophorbide. *Spec. J. Biol. Sci.* 5, 6–13.

Solymosi, K., and Mysliwa-Kurdziel, B. (2017). Chlorophylls and their derivatives used in food industry and medicine. *Mini Rev. Med. Chem.* 17, 1194–1222. doi: 10.2174/1389557516666161004161411

Szczygieł, M., Urbańska, K., Jurecka, P., Stawoska, I., Stochel, G., and Fiedor, L. (2008). Central metal determines pharmacokinetics of chlorophyll-derived xenobiotics. *J. Med. Chem.* 51, 4412–4418. doi: 10.1021/jm7016368

Tamiaki, H., Okamoto, Y., Mikata, Y., and Shoji, S. (2012). Photooxidative cleavage of zinc 20-substituted chlorophyll derivatives: conformationally *P*-helix-favored formation of regioselectively 19–20 opened linear tetrapyrroles. *Photochem. Photobiol. Sci.* 11, 898–907. doi: 10.1039/C1PP05301A

Tamiaki, H., Fukai, K., and Nakamura, S. (2020). Intramolecular interaction of synthetic chlorophyll heterodyads with different π-skeletons. *Photochem. Photobiol. Sci.* 19, 332–340. doi: 10.1039/C9PP00373H

Tang, P. M.-K., Chan, J. Y.-W., Au, S. W.-N., Kong, S.-K., Tsui, S. K.-W., Waye, M. M.-Y., et al. (2006). Pheophorbide *a*, an active compound isolated from *Scutellaria barbata*, possesses photodynamic activities by inducing apoptosis in human hepatocellular carcinoma. *Cancer Biol. Ther.* 5, 1111–1116. doi: 10.4161/cbt.5.9.2950

Tao, H., Sun, Z., Liu, M., and Wen, X. (1990). Synthesis of zinc chlorophyllin *a* and its preliminary clinical application. *Hua Xi Yi Ke Da Xue Xue Bao* 21, 341–343.

te Velthuis, A. J. W., van den Worm, S. H., Sims, A. C., Baric, R. S., Snijder, E. J., and van Hemert, M. J. (2010). Zn^{2+} inhibits coronavirus and arterivirus RNA polymerase activity *in vitro* and zinc ionophores block the replication of these viruses in cell culture. *PLoS Pathog.* 6, e1001176. doi: 10.1371/journal.ppat.1001176

Turner, R. B., and Cetnarowski, W. E. (2000). Effect of treatment with zinc gluconate or zinc acetate on experimental and natural colds. *Clin. Infect. Dis.* 31, 1202–1208. doi: 10.1086/317437

Ulbricht, C., Bramwell, R., Catapang, M., Giese, N., Isaac, R., Le, T. D., et al. (2014). An evidence-based systematic review of chlorophyll by the Natural Standard Research Collaboration. *J. Diet. Suppl.* 11, 198–239. doi: 10.3109/19390211.2013.859853

Wang, H., Tan, Y., Wang, X., and Xie, J. (2001). Identification of pelvic lymph nodes with chlorophyllin after injection into the uterine cervix: an experimental and clinical study. *Lymphology* 34, 69–76.

Xodo, L. E., Rapozzi, V., Zacchigna, M., Drioli, S., and Zorzet, S. (2012). The chlorophyll catabolite pheophorbide *a* as a photosensitizer for the photodynamic therapy. *Curr. Med. Chem.* 19, 799–807. doi: 10.2174/092986712799034879

Yoon, I., Park, H.-S., Cui, B. C., Li, J. Z., Kim, J.-H., Lkhagvadulam, B., et al. (2011). Photodynamic and antioxidant activities of divalent transition metal complexes of methyl pheophorbide-*a*. *Bull. Korean Chem. Soc* 32, 2981–2987. doi: 10.5012/bkcs.2011.32.8.2981

Yoshida, A., Yokono, O., and Oda, T. (1980). Therapeutic effect of chlorophyll-*a* in the treatment of patients with chronic pancreatitis. *Gastroenterol. Jpn.* 15, 49–61. doi: 10.1007/BF02773704

Zhang, L., and Liu, Y. (2020). Potential interventions for novel coronavirus in China: a systemic review. *J. Med. Virol.* 92, 479–490. doi: 10.1002/jmv.25707

Zhou, H., Xia, L., Zhong, J., Xiong, S., Yi, X., Chen, L., et al. (2019). Plant-derived chlorophyll derivative loaded liposomes for tri-model imaging guided photodynamic therapy. *Nanoscale* 11, 19823–19831. doi: 10.1039/C9NR06941K

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REVIEW
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Pandemics and Traditional Plant-Based Remedies. A Historical-Botanical Review in the Era of COVID19

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Pandemics are as old as humanity and since ancient times we have turned to plants to find solutions to health-related problems. Traditional medicines based mostly on plants are still the only therapeutic possibility in many developing countries, but even in the richest ones, herbal formulation currently receives increased attention. Plants are natural laboratories whose complex secondary metabolism produces a wealth of chemical compounds, leading to drug discovery – 25% of widespread use drugs are indeed of plant origin. Their therapeutic potential is even bigger: although many plant-based compounds show inhibitory effects against a myriad of pathogens, few reach the stage of clinical trials. Their mechanism of action is often unknown, yet traditional plant-based remedies have the advantage of a long-term experience in their use, usually of hundreds to thousands of years, and thus a precious experience on their safety and effects. Here I am providing a non-systematic historical-botanical review of some of the most devastating pandemics that humanity has faced, with a focus on plant therapeutic uses. I will revisit the Middle Ages black death, in which a plant-based lotion (the four thieves vinegar) showed some effectiveness; the smallpox, a viral disease that lead to the discovery of vaccination but for which the native Americans had a plant ally, an interesting carnivorous plant species; tuberculosis and the use of garlic; the Spanish flu and the widespread recommendation of eating onions, among other plant-based treatments; and malaria, whose first effective treatment, quinine, came from the bark of a Peruvian tree, properties already known by the Quechua people. Synthetic analogues of quinine such as chloroquine or hydroxychloroquine are now being revisited for the treatment of COVID19 symptoms, as they are artemisinin and derivatives, other plant-based compounds effective against malaria. Finally, I will give some hints on another facet of plants to aid us in the prevention of infectious diseases: the production of biotechnological plant-based vaccines. Altogether, my aim is to stress the significant role of plants in global health (past, present and future) and the need of enhancing and protecting the botanical knowledge, from systematics to conservation, from ecology to ethnobotany.

Keywords: traditional medicine, plant-based alternatives, pandemics, antiviral, traditional knowledge

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INTRODUCTION

Pandemics have shaped the history of mankind, and plants were usually the first available therapeutic choice. There is evidence of herbal preparations by Egyptians around 1500 BC, later improved by Greeks and Romans, and widely documented in official drug books known as Pharmacopoeias. Still in our days, traditional medicines based mostly on plants are the only therapeutic possibility for many people in developing countries (Akerele, 1993). But, also in the first world, with wide access to the most modern drugs, the use of plant-based traditional medicine is experiencing a revival, as it is seen as safer and healthier than synthetic drugs. Indeed, one advantage of traditional remedies over modern drugs is that their effects and margin of safety have been known for long. There is also a renewed scientific interest on plant-derived drug discovery, according to the current increasing publication trend on the topic (Atanasov et al., 2015). The rich secondary metabolism that characterises plants make them a source of compounds that may have a yet unknown therapeutic potential, only limited by the availability of resources to perform clinical trials. It is claimed that natural products (mostly from plant origin) will be the most important source of new drugs in the future (Atanasov et al., 2015).

A recent editorial (Nature Plants, 2020) highlighted the need of funding and understanding botanical knowledge in the context of the current, and possibly future, pandemics. It is urgent to develop therapeutic tools to protect from high risk of infection (Mitjà and Clotet, 2020) and plant-based remedies with proven safety profiles could be one of the faster solutions. Here I present a non-systematic review with a historical-botanical perspective on some of the most important pandemics that humanity has faced, and in some cases is still facing, and how certain plants or plant-based remedies have been used, and may continue being used, to treat these diseases, possibly including COVID19.

THE BLACK DEATH

The Black Death or Black Plague took place in the Middle Ages (1347–1351) in Eurasia, and still is the deadliest pandemic ever, with an estimated loss of 200 million of human lives wiping out 30 to 50% of European population in roughly four years (DeLeo and Hinnebusch, 2005). Although this is the most know outbreak of the bacterium *Yersinia pestis*, the much earlier -and longer- Plague of Justinian (from 541 to 750 AD) was also caused by the same pathogen (Harbeck et al., 2013), killing about 25–50 million people during two centuries. There have been other less spectacular, but still important plague outbreaks, arriving to the most recent ones in Madagascar during the present decade. Originated in China, the plague was usually spread by trade boats, whose rats carried fleas with the bacterium, which was transmitted to humans directly by the bite of the flea, and then between humans by contact or aerosol inhalation. There are several forms of the disease, the most common being the bubonic plague, which provokes the inflammation of the lymph nodes (buboes) as its most recognizable sign; a second form is the pneumonic plague which affects the respiratory system and is more deadly; the third form, the septicaemic plague, is the least common but has a mortality ca. 100% (Byrne, 2004). The antibiotic treatment, starting in early XXth century, reduced the death rate to about 1%–5% which previously was between 40%–60%; however, little is known on the remedies used before antibiotics were a reality and the major plague outbreaks occurred much earlier. In the Middle Ages, some preventive measures included, among others, carrying sweet smelling herbs to clear "the evil air" (which was believed to carry the pathogen) around the person (Jones, 2000), garlic for cleaning kidneys and liver, and lavender or chamomile teas to calm the stomach bile (Khaytin, 2019). A remedy named "the four thieves vinegar" was very popular: it consisted in several herbs, such as angelica (*Angelica archangelica*), camphor (*Cinnamomum camphora*), cloves (*Syzygium aromaticum*), garlic (*Allium sativum*), marjoram (*Origanum majorana*), meadowsweet (*Filipendula ulmaria*), wormwood (*Artemisia absinthium*), and sage (*Salvia officinalis*), brewed in vinegar (Gattefosse, 1937). Before going out, people should apply it on hands and face for avoiding to contract the plague. Some of these plants are well known flea repellents, so this may be one of the reasons for its efficacy. Other herbs such as meadowsweet might have been included to release pain (as it contains salicylic acid, a precursor of aspirin) and to mask odours, a very helpful property considering that decomposing bodies were usually encountered – the legend states that the name of the remedy might refer to thieves using it to rob the plague dead or sick (Lucas, 1969). Another treatment coming from ancient Greeks also gained popularity: the King Mithridates antidote (Totelin, 2004), an extract of about fifty plants in a mixture with opium (*Papaver somniferum*) paste, which if any, at least eased the pain or promoted a peaceful death. Other prescriptions included lavender or rosewater baths, probably due to their antimicrobial and buboes healing properties. Willow bark (another source of salicylates) was also given as a painkiller (Khaytin, 2019). A curious "prophylactic" plant-based remedy was recommended by the Napolitan doctor Angelerio during a plague outbreak in Alguero in the XVI century (1582–1583) (**Figure 1**): "any person going out from home must carry a cane (note: probably from the species *Arundo donax*) six spans long, and as long as the cane is, one must not approach other people" (Bianucci et al., 2013).

SMALLPOX

The origin of smallpox, a viral infectious disease caused by Variola virus (two variants: *V. major* and *V. minor*) is unknown, but it dates back at least to the ancient Egypt (third century BC), since some mummies showed smallpox-like eruption, the characteristic macules of the disease. As with the plague and other pandemics, the disease has occurred in several outbreaks around the world, the most recent in the late 60s. This virus has killed between 300 and 500 million people during the 20th century (Koplow, 2003) until the global eradication campaign by the World Health Organisation

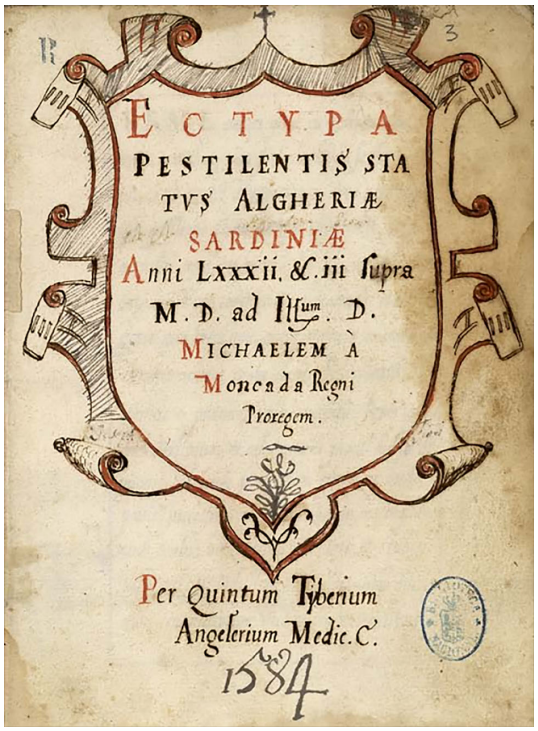

FIGURE 1 | Cover of the manuscript "*Ectypa pestilentis status Algheriae Sardiniae anni LXXXII et III supra MD*" by Quintum Tyberio Angelerio, a physician in Alghero that faced the epidemy of black plague in 1582–1583 in Alghero (Sardinia). The text is written both in Latin and in Catalan. Image from the repository of the Biblioteca Nacional de España (BNE), under the CC-BY-NC-SA International Creative Commons License.

(WHO) in 1967. Smallpox was the first infectious disease to have been eradicated (1980), the (only) second one being rinderpest, a viral illness of cattle. The smallpox vaccine (the first ever) was based on Edward Jenner's demonstration, by the end of the XVIIIth century, that inoculation with cowpox (a variant of the smallpox virus infecting cows) protected against the disease. Actually, Jenner's contribution popularized the practice of vaccination, a word coined by himself coming from the latin word *vaccinus* (i.e., or/from the cow) for the prevention of several other infectious diseases.

However, before vaccination was discovered, how did people deal with the illness? Particularly interesting was the approach of the Native Americans, which were deeply affected by the disease. By the end of XIXth century several surgeons and practitioners related to the US army, as well as the prestigious botanist Charles F. Millspaugh (1892), described the use of poultices and infusions from the Indigenous medical flora based on the plant *Sarracenia purpurea* (family Sarraceniaceae) to be effective for treating smallpox, in a likely case of medical appropriation of the Indigenous therapeutic knowledge (Lawrence-Mackey, 2019). Known by Native Americans (Mi'kmaq people) as Mqo'oqewi'k, also named purple pitcher plant, it belongs to a genus of carnivorous species that use modified pitcher-shaped leaves to trap insects. Possibly, the spotted appearance of the plant (**Figure 2**), resembling one of the main clinical signs of the disease (Clarke, 1996), inspired its use to the Indigenous people. This may be another example of the doctrine of signatures, an ancient concept by which God somehow indicated to men what plants would be useful for, by certain signs (Coles, 1657), a pseudoscience which has caused more harm than good in general, although exceptions appear. Compelling descriptions of their effectiveness were recorded, such as "the greatest remedy known for the dreadful scourge" or "'it seemed to arrest the development of the pustules, killing, as it were, the virus from within" (Clarke, 1996). The advent of vaccination put forward the botanical remedy, but the antiviral properties of *Sarracenia purpurea* have been later demonstrated *in vitro* (Arndt et al., 2012). The authors showed that the plant extract was not only active against smallpox, but also against other poxviruses, papovirus SV-40 and various herpes viruses, including papillomavirus and Epstein-Barr virus-associated carcinomas, usually by inhibiting the virus replication at the level of early transcription (Moore and Langland, 2018).

TUBERCULOSIS

Another global and persistent pandemic is tuberculosis, caused by *Mycobacterium tuberculosis*. Together with smallpox, it is one of the oldest known diseases, since molecular data and archaeological evidence support that it coexisted with humans from the Neolithic (Gutierrez et al., 2005; Nicklisch et al., 2012). Relevant figures in the history of medicine such as Hippocrates or Avicenna identified the disease, which involved coughing blood and fever and which was often lethal. Actually, Avicenna detected the infectious nature of the illness and based on tuberculosis, was probably the first to come up with the idea of quarantine to stop the spread of infectious diseases (Shephard, 2015). Tuberculosis infected 10 million people only in 2019, of which 1.5 million died (UNAIDS, 2020). It is found in every country, being the first infectious cause of death worldwide.

FIGURE 2 | The carnivorous purple pitcher plant, *Sarracenia purpurea*, a folk remedy by the Native Americans to treat smallpox. The image shows the "pitchers" which are traps to little invertebrates. Image taken at the John Bryan State Park (Ohio, USA) courtesy of Elizabeth McGee from Curious Plant (https://curiousplant.com/carnivorous-plants-ohio/).

From the HIV/AIDS outbreak, the combination of both is usually fatal where tuberculosis is endemic (mainly developing countries), as the immune weakening caused by HIV facilitates the onset of tuberculosis. This disease is indeed the final cause of death of many HIV infected people; paradoxically, while many could live now with AIDS they are dying from tuberculosis. The main reason is that the bacterium has developed resistance to most antibiotics, which usually have to be taken during long and tedious treatments. This is why researchers have turned to the search of effective alternatives also among medicinal plants, as some of them have already demonstrated anti-tuberculosis activity. Besides, an effective plant-based treatment would be more affordable in poor countries which are those more affected by the disease. Among the plants that are being investigated, garlic (*Allium sativum*, family Alliaceae), a former remedy already used to treat the plague (see above) stands out for its renowned properties, although it still far from being an alternative. The benefits of garlic (of which there are about 300 varieties) are well-known already from the Ayurvedic and Unani medicine systems (Raghunandana et al., 1946) as well as from the Chinese traditional medicine, but also ancient Greeks, Romans and even Egyptians used it to treat illnesses. It has been used as a food and folk medicine for centuries by many cultures. Garlic has a variety of pharmacological virtues, including antimicrobial, anticancer, antioxidant (Dini et al., 2011), fungicidal and as a cure for heart diseases, among others (Majewski, 2014). The antitubercular and other antimicrobial activities of garlic, however, have been demonstrated *in vitro* but still, seldom *in vivo*. Although garlic has more than 2000 biologically active compounds, allicin is the most relevant, albeit highly unstable; therefore, depending on the preparation of the garlic-based remedy the efficacy may not be as high as expected (Majewski, 2014). The wide antimicrobial and even antifungal spectrum of allicin is explained by its inhibitory effects on sulfhydryl metabolic enzymes. By interacting with these enzymes, allicin induces thiol stress in bacteria, which, among others, inhibits the growth of the microorganisms (Müller et al., 2016).

MALARIA

Malaria, caused by protozoan species of the *Plasmodium* group, is an infectious disease coming from the bite of a mosquito, usually an infected *Anopheles* sp. female. The current name of the illness was given by the Italians around XIXth century, as a contraction of the words "mal aria" (i.e., bad air) from the belief that the disease was transmitted by the "miasma" coming from marshes (Macip, 2020). The most typical symptom is fever, together with nausea and vomiting, tiredness, headache, occasionally yellow skin and in severe cases it can lead to seizures, coma and death. It is another ancient disease, spanning from the Neolithic to our days, and currently found in all intertropical continents. Recent studies detected the parasite in African monkeys, probably being the source of the disease, although it is still debated how it spread worldwide (Molina-Cruz and Barillas-Mury, 2014). It is known that malaria arrived to Europe by the first century AD, probably coming from the African rainforests and travelling by the Nile to the Mediterranean, where it spread to the Middle East and from there to Greece, Italy - historians hypothesize on the triggering role of malaria in the fall of the Roman Empire - and the rest of Europe, even as far as England and Denmark (Karlen, 1996). Between the XVIth - XIXth centuries the disease crossed the Atlantic Ocean probably on slave ships to reach the American continent (Yalcindag et al., 2012). It was suffered by presidents of North America such as G. Washington or A. Lincoln and it raged specially with Native Americans, taking thousands of their lives. The Centers for Disease Control and Prevention, the leading national public health institute of the United States, was founded because of malaria in 1946. In the last century, probably 150–300 million people have died from the disease, accounting for 2%–5% of deaths (Carter and Mendis, 2002). At present malaria is most-worrying in sub-Saharan Africa, accounting for ca. 90% of current cases, although there is also a resurge in southern Asia (Arrow, 2004).

The most well-known and one of the most effective historical treatments against malaria is quinine, an alkaloid extracted from the bark of the cinchona tree (*Cinchona officinalis*) belonging to family Rubiaceae, the same of coffee. It is original from Peru (where it is the national tree, although currently it is considered an endangered species) and the Quechua traditionally used the ground bark of these trees to stop shivering because of cold, not for malaria treatment *per se*. Most likely, Spanish Jesuits missionaries brought cinchona to Europe for the first time, having observed how the Quechuas used it to threat shivering, by the end of XVIth century - a second case of medical appropriation in this story. The tree was named (by C. Linnaeus) after the Spanish Countess of Chinchon, who was treated with its bark in Peru back in the early XVIth – Linneaus misspelled the name of the countess, omitting the first "h" in the name (Meshnick, 1998). It is also said that the Countess may have introduced the curative bark to Europe when she returned to Spain, but it is currently considered that this a legend rather than what actually happened (Haggis, 1941). Quinine is effective on the "cessation of febrile paroxysms" (Stephens et al., 1917), one of the main symptoms of malaria, and which has given its popular name to the species (fever tree). Malaria outbreaks, however, continued to appear during centuries with no alternative to quinine. During World War I the German army was strongly affected by the parasite in the troops of East Africa, as the Allies controlled Java, the main worldwide quinine producer. In an interesting historical moment that impelled science, the German government commissioned a search for a substitute to quinine, determined not to suffer again from its shortage. In 1934 chloroquine, a synthetic compound similar to quinine was synthesized at the Bayer laboratories. Much later (1955) another very similar derivative, hydroxychloroquine, was produced in the US. Both chloroquine and hydroxychloroquine are used to prevent and treat malaria, being some of the antimalarial drugs of choice in areas where the disease is not resistant to them (a recurrent problem in this disease); they were preferred over quinine because of much less severe adverse effects, although at present there are many other even safer alternative drugs for the treatment of malaria. In recent years, these antimalarials have shown several

immunomodulatory effects and they currently treat, mostly, diseases such as lupus erythematosus or rheumatoid arthritis.

As with tuberculosis, the parasite tended to develop resistance to these treatments sooner or later, and researchers were urged to look for alternatives. A very popular one was found in the plant *Artemisia annua* (sweet wormwood, from family Asteraceae) (**Figure 3**) a remedy known in Chinese traditional medicine as qing-hao for more than 2000 years (Klayman, 1985). Chinese herbalists had been using it for treating haemorrhoids, chills, and fevers (Trigg and Kondrachine, 1998). The species, as other members of genus *Artemisia* such as absinthe or tarragon, is aromatic and bitter. One of the compounds responsible for its bitterness, a sesquiterpene lactone extracted from the glandular trichomes named artemisinin, is the active compound against malaria. The discovery of artemisinin is also very remarkable from the historical point of view. In 1967, during the Chinese Cultural Revolution under Mao Zedong's mandate, the secret "Project 523" was a plant screening research program to find an alternative treatment for malaria, which was ravaging Vietnamese army during Vietnam war. In 1972, Dr. Youyou Tu a researcher of that program, isolated artemisinin, "rediscovering" the ancient remedy qing-hao. The drug started to be used in 1979, a relatively short-period to establish a new medicine in the market, but it was also based on thousands of years of experience by the Chinese traditional practitioners. Nowadays artemisinin and its synthetic derivatives are one of the main defences against drug-resistant malaria in the Asiatic southeast. However, WHO recommends it in combined therapy with other drugs, in part to avoid the development of resistance and in part to counteract the short half-life of artemisinin in plasma, leading to the Artemisinin Combination Therapies (ACTs) which include companion drugs such as some cloroquine derivatives (e.g., mefloquine). In 2015 Tu was awarded the Nobel Prize for the discovery of artemisinin, which represents an important contribution of China to the global health, as well as the first and awaited Nobel prize in the sciences for China. It is considered the most significant milestone of tropical medicine of the last century, contributing to a better health and saving tens of thousands of lives every year in tropical developing countries of South Asia, South America and Africa.

FIGURE 3 | Synflorescences of the sweet wormwood, *Artemisia annua*. Image by Kristian Peters, under the CC BY-SA 3.0 International Creative Commons License.

SPANISH FLU

In the spring of 1918 started one of the deadliest pandemics in recent history, caused by one aggressive strain of the H1N1 influenza virus (the same virus that caused the 2009 swine flu pandemic). It was popularly known as the Spanish flu because in the context of World War I censorship minimized the effects of the pandemic to keep people's morale in the countries involved in the conflict, while in the neutral Spain newspapers were free to report its effects, giving the impression that this country had been particularly devastated by the disease. During 15 months, until summer 1919, there were three waves of the pandemic, being the second the worst. A fourth, much fainter wave, took place in the spring of 1920 and after this one, the virus disappeared as it had arrived. It infected about half a billion people (ca. 1/3 of the world's population) and killed about 50 million (with some estimates as high as 100 million); for a reference World War I estimates range from 15 to 22 million deaths. The origin of the virus is unclear but it is thought that it started as a zoonosis from birds to humans which later was transmitted from humans to swine. The symptoms were an amplified version of those of normal flu, but typically deaths were caused by complications derived from a secondary pneumonia. Contrary to other H1N1 flu strains, this one was unusually lethal among young people, and almost one century later its high-virulence is only partly understood (Tumpey et al., 2005). Since at that time there was neither vaccine, nor antibiotics to treat secondary pneumonia, the main prophylactic options were, as with the COVID19 pandemic, to avoid contact through lockdown and quarantines, to increase personal hygiene and to use disinfectants widely. Also, people turned to folk remedies and some recommendations got popular, such as the widespread advice "Eat more onions!" (**Figure 4**) (Arnold, 2018). As with garlic, onion (*Allium cepa*) has certain compounds (particularly a polyphenol named quercetin) which have demonstrated antiviral properties (Lee et al., 2012; Sharma, 2019) but still more research and clinical trials are needed. Besides, in the USA a group of doctors known as "The Eclectics" got positive results by treating the flu symptoms with plant remedies, together with other measures that included exercise. They reported a fatality rate ca. 0.6% for their patients while the average in that pandemic was ca. 3% (Abascal, 2006). By selecting the herbs to match the symptoms, they used a wide variety of species. The most remarkable among them, and that have later proved therapeutic, were: *Gelsemium sempervirens* (known as yellow yasmine, with antipyretic properties), *Eupatorium perfoliatum* (boneset, already known by native Americans to treat cold-related symptoms), *Actea racemosa* (black cohosh, also used by Native Americans as a painkiller, probably due to the content in salicylic acids of its roots) and *Asclepias tuberosa* (pleurisy root, used to treat respiratory problems and with expectorating properties) (Abascal, 2006). However, despite their long history of use, again there is little applied research on these plants. Currently, flu is partly under

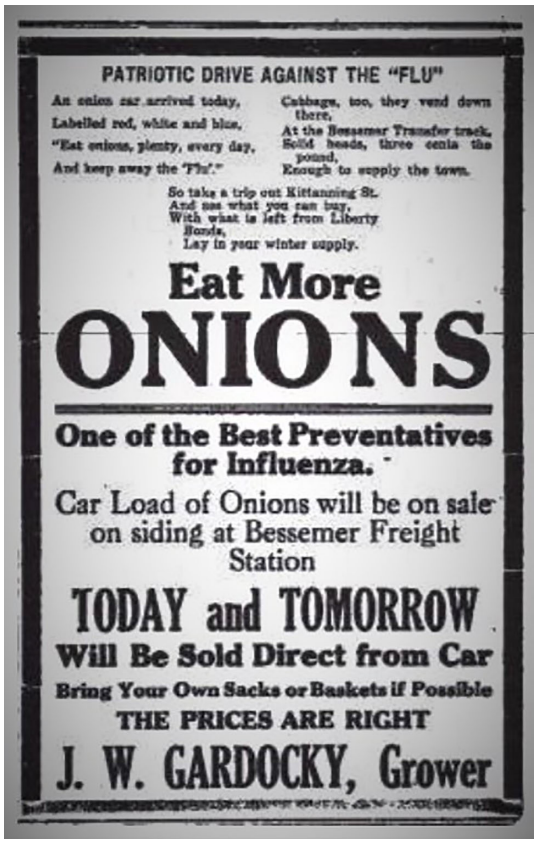

FIGURE 4 | Propaganda poster that got popular during the 1918 flu pandemic in the USA. From reddit.com, image under the CC-BY-NC-SA International Creative Commons License.

control by the release of annual vaccination campaigns with newly synthesized vaccines that collect most of the virus' seasonal variability. In the latter most important flu pandemic (2009) besides the vaccine, oseltamivir (Tamiflu®) a drug derived from the species *Ilicium verum* (star anise, from family Schisandraceae) was also crucial to treat most severe cases, although the production of this compound is limited by the low productivity of the tree, and synthetic derivatives are being developed (Macip, 2020). Finding adequate treatments for flu is still and urgent task as the fear of a pandemic similar to the one in 1918 is a still a sword of Damocles in the concerns of most epidemiologists.

THE POSSIBILITY OF PLANT-BASED VACCINES

From the 1980s, the science of "molecular farming" gives another potential role to plants on the prevention of infectious diseases, involving plants or plant cell cultures to produce recombinant proteins (Rybicki, 2014). The first steps of this approach were the "manufacturing" of the human growth hormone, monoclonal antibodies or human serum albumin in transgenic tobacco or sunflower plants (Barta et al., 1986; Hiatt et al., 1989; Sijmons et al., 1990). Other recombinant proteins more recently produced in plants -shifted from bacterial, mammalian or fungal cell to plants and plant cell cultures- and commercialized, include human type collagen I manufactured in tobacco, bovine trypsin in maize or human lysozyme and lactoferrin in rice (Yang et al., 2003; Hennegan et al., 2005; Shoseyov et al., 2014; Takeyama et al., 2015). As with the mentioned proteins, the vaccine production would follow similar steps: isolation of a specific antigen protein, the one that triggers an immune response from the virus; the gene(s) encoding that protein is transferred to bacteria and these bacteria are used to infect plants, so the plant will in turn produce the antigen protein – the vaccine. Plants would provide a flexible, cheap and easily scalable method to manufacture vaccines. They would also be safer than traditional vaccines, because of the absence of pathogens of animal origin. Plant-based vaccines for humans are not yet in the market, although some candidates have entered clinical trials (Rosales-Mendoza et al., 2020). It is likely, however, that they will start being approved in the mid-term, at least in the cases of Ebola or rabies, or in a longer term for seasonal influenza (Rybicki, 2014).

WHAT PLANT-BASED AND PLANT-RELATED TREATMENTS CAN OFFER TO COVID19 THERAPY?

The outbreak of COVID19 caused by the coronavirus SARS-CoV-2, originated in China (province of Wuhan) in December 2019 and has caused 7,238,484 infections and 409,644 deaths worldwide (updated 9th June 2020). Even earlier than the pandemic status was declared by WHO (11th March 2020) researchers across the world engaged in hundreds of clinical trials, in an unprecedented quest for a cure to the disease, vaccine, drug or both. Given the long time-frames that usually imply finding a good candidate, many research groups have turned to repurpose other drugs. The reasons are that the effects (including adverse or side effects) of these drugs are well known and have been used in broad population groups with different ages and idiosyncrasies, so the security margin is increased, allowing to save precious time in long trials. In this regard, antimalarials are potential candidates (Schlagenhauf et al., 2020) and both chloroquine but particularly hydroxychloroquine (as explained above, synthetic derivatives of quinine, the antimalarial alkaloid coming from the bark of the fever tree) are being studied to fight COVID19, although it is perhaps too soon to draw conclusions on their efficacy. Several studies have reported their utility for some patients and some national guidelines have recommended both drugs for treatment of COVID19 (see Colson et al., 2020 and Singh et al., 2020) despite there has been certain controversy. The WHO halted studies on these drugs by the end of May 2020 prompted by an observational study reporting that hydroxychloroquine produced a higher mortality rate in hospitalised patients, but the study was soon retracted on the basis of questionable veracity of data and analysis (Mehra et al., 2020) and trials on the drug have been resumed shortly after. The effectiveness of hydroxycloroquine taken at initial stages of the disease was recently tested in a multicentre randomised

controlled trial based on previous experiences of Post Exposure Prophylaxis (PEP) drugs to prevent infections (Mitjà and Clotet, 2020), but no benefit was observed beyond the usual care (Mitjà et al., 2020). Other plant-based antimalarials, artemisinin and derivatives, are also being tested against SARS-CoV-2, again not without controversy. In many African countries, an elixir based on *Artemisia annua* extract, "covid-organics" is being distributed as a cure against COVID19. However, there is little scientific evidence of the effectiveness of such elixir and its extended consumption can have associated problems, the most important the development of resistance to the drug by the malaria parasites in a continent particularly sensitive to the disease. Nevertheless, there is evidence that the extract of *Artemisia annua* has antiviral properties, being active against SARS-CoV-1 (Li et al., 2005), herpes simplex (Karamoddini et al., 2011), hepatitis A (Seo et al., 2017), hepatitis B, bovine viral diarrhoea, and Epstein-Barr (Haq et al., 2020). This has stimulated the research of the potential use of artemisinin and derivatives (such as artesunate) to treat COVID19, which is now being conducted by several biotech companies (e.g., Mateon Therapeutics, ArtemiLife) as well as by public research institutions (e.g., the Liverpool School of Tropical Medicine, the Max Planck Institute of Colloids and Interfaces). Traditional Chinese Medicine has also had a say in the cure of COVID19: the National Administration of Traditional Chinese Medicine (NATCM) organized a study in late January 2020 to identify potential treatments, and the lung cleansing and detoxifying decoction (LCDD) was widely used and studied through clinical trials; its apparently high effectiveness made that the NATCM officially recommended LCDD as a treatment for COVID 19 (Weng, 2020). Among the LCDD 21 ingredients, there were species such as *Ephedra sinica* (well known as decongestant and bronchodilator through the active compound ephedrine), *Atractylodes macrocephala* (showing antiviral activity against influenza viruses in experimental assays) or *Scutellaria baicalensis* (containing anti-inflammatory flavonoids), and the combination of these effects and others from the remaining ingredients likely counteracts COVID19 by their synergistic activities. However, as Weng (2020) points out, it is difficult to transfer the success of the LCDD treatment to other countries, both because the cultural acceptance of TCM is not present outsides China, and due to the lack of knowledge on the precise chemical composition and mechanism of action, which are required in modern therapy.

Once the first vaccine for COVID19 is finally developed (with estimates ranging from September 2020 to several years) another plant may also play an important role in order to produce it in large amounts: *Nicotiana benthamiana*, a close relative to tobacco. This species is the focus of the EU project NEWCOTIANA, coordinated by researcher Dr. Diego Orzáez at the CSIC, the leading public research body in Spain. In this project, genome editing practises (e.g., CRISPR) will be used to transform the plant in a biofactory for the large-scale production of the vaccine once it is available. Moreover, one company may be currently developing a COVID19 vaccine based on the expression of a SARS-CoV-2 protein in tobacco - Kentuchy Bioprocessing, a biotechnological branch of British American Tobacco (Capell et al., 2020). As Rosales-Mendoza et al. (2020) stated in a recent review, the production of a plant-based vaccine in the context of the current pandemic would have the advantages of low cost, fast and escalable production, easy administration, and safety.

Beyond plant-based vaccines, molecular farming through plants, usually by transient expression of target proteins, can also be deployed to produce diagnostic reagents, as well as antibodies and antiviral proteins for therapeutic use. The Italian biotechnology company Diamante is generating antigens, to use in ELISA tests (serological), based on a SARS-CoV-2 protein also in tobacco plants (Capell et al., 2020). Another EU consortium, Pharma-Factory is also developing plant-based platforms to produce medical, veterinary and diagnostic products, for dealing with COVID19 and also other diseases.

CONCLUSION

I hope that the reader finds this review useful to call for the important role that plants have played and still play in human health. As Schlagenhauf et al. (2020) commented, plant-based remedies are, more than an "alternative medicine", the organisms to which we owe some of the most useful therapeutic tools. Still in the era of wide implementation of (synthetic) drug treatments, we turn to plants in many cases when resistances appear, as shown. Paradoxically, there is a human tendency to ignore plants, a form of cognitive bias known as "plant blindness" (Allen, 2003) that should be opposed, perhaps by enhancing and implementing more widely the botanical education. In this context, it is also essential not only to maintain but to increase societal funding into basic sciences such as botany, as well as to foster collaboration between scientists from different disciplines, whose interaction may open new therapeutic possibilities. Finally, I would like that this review serves as a little recognition to the usually ignored ethnobotanical traditional knowledge of many indigenous peoples across the world of which the so-called Western culture has in most occasions, illegitimately appropriated.

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The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

Abascal, K. (2006). *Herbs and influenza: how herbs used in the 1918 flu pandemic can be effective in the next* (Vashon, WA: Tigana Press).

Akerele, O. (1993). "Nature's medicinal bounty: don't throw it away," in *World health forum 1993*, vol. 14, 390–395.

Allen, W. (2003). Plant blindness. *BioScience* 53, 926–926. doi: 10.1641/0006-3568(2003)053[0926:PB]2.0.CO;2

Arndt, W., Mitnik, C., Denzler, K. L., White, S., Waters, R., Jacobs, B. L., et al. (2012). In vitro characterization of a nineteenth-century therapy for smallpox. *PloS One* 7, e32610. doi: 10.1371/journal.pone.0032610

Arnold, C. (2018). *Pandemic 1918: The story of the deadliest influenza in history* (London: Michael O'Mara Books).

Arrow, K. J. (2004). The locus of decisions about aids/hiv, malaria treatment: What does welfare economics say? a question. *Econom. Public Econom.* 12, 9–11.

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E.-M., Linder, T., Wawrosch, C., Uhrin, P., et al. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotech. Adv.* 33, 1582–1614. doi: 10.1016/j.biotechadv.2015.08.001

Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M. A., and Matzke, A. J. (1986). The expression of a nopaline synthase - human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. *Plant Mol. Biol.* 6, 347–357. doi: 10.1007/BF00034942

Bianucci, R., Benedictow, O. J., Fornaciari, G., and Giuffra, V. (2013). Quinto Tiberio Angelerio and new measures for controlling plague in 16th-century Alghero, Sardinia. *Emerg. Infect. Dis.* 19, 1478–1483. doi: 10.3201/eid1909.130311

Byrne, J. P. (2004). *The black death* (Westport: Greenwood Publishing Group, Inc).

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Pl. Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Carter, R., and Mendis, K. N. (2002). Evolutionary and historical aspects of the burden of malaria. *Clin. Microbiol. Rev.* 15, 564–594. doi: 10.1128/CMR.15.4.564-594.2002

Clarke, J. A. (1996). *Dictionary of practical materia medica in three volumes* (Delhi, India: Jain Publishers).

Coles, W. (1657). *Adam in Eden, or Nature's Paradise* (London: J. Streater).

Colson, P., Rolain, J. M., Lagier, J. C., Brouqui, P., and Raoult, D. (2020). Chloroquine and hydroxychloroquine as available weapons to fight COVID-19. *Int. J. Antimicrob. Agents* 55, 105932. doi: 10.1016/j.ijantimicag.2020.105932

DeLeo, F. R., and Hinnebusch, B. J. (2005). A plague upon the phagocytes. *Nat. Med.* 11, 927–928. doi: 10.1038/nm0905-927

Dini, C., Fabbri, A., and Geraci, A. (2011). The potential role of garlic (Allium sativum) against the multi-drug resistant tuberculosis pandemic: a review. *Ann. I. Super Sanita* 47, 465–473. doi: 10.4415/ANN_11_04_18

Gattefosse, R. M. (1937). *Aromathérapie – les huiles essentielles hormones végétales* (Paris, France: Librarie des sciencies Girardot).

Gutierrez, M. C., Brisse, S., Brosch, R., Fabre, M., Omaïs, B., Marmiesse, M., et al. (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PloS Pathog.* 1, e5. doi: 10.1371/journal.ppat.0010005

Haggis, A. W. (1941). Fundamental Errors in the Early History of Cinchona. *Bullet. History Med.* 10, 568–592.

Haq, F. U., Roman, M., Ahmad, K., Rahman, S. U., Shah, S. M. A., Suleman, N., et al. (2020). *Artemisia annua:* trials are needed for COVID-19. *Phytother. Res.* doi: 10.1002/ptr.6733

Harbeck, M., Seifert, L., Hänsch, S., Wagner, D. M., Birdsell, D., Parise, K. L., et al. (2013). Yersinia pestis DNA from skeletal remains from the 6th century AD reveals insights into Justinianic Plague. *PloS Pathog.* 9, e1003349. doi: 10.1371/journal.ppat.1003349

Hennegan, K., Yang, D., Nguyen, D., Wu, L., Goding, J., Huang, J., et al. (2005). Improvement of human lysozyme expression in transgenic rice grain by combining wheat (*Triticum aestivum*) puroindoline b and rice (*Oryza sativa*) Gt1 promoters and signal peptides. *Transgenic Res.* 14, 583–592. doi: 10.1007/s11248-004-6702-y

Hiatt, A., Cafferkey, R., and Bowdish, K. (1989). Production of antibodies in transgenic plants. *Nature* 342, 76–78. doi: 10.1038/342076a0

Jones, M. (2000). *The New Cambridge Medieval History: C. 1300-c. 1415*. (Cambridge University Press).

Karamoddini, M. K., Emami, S. A., Ghannad, M. S., Sani, E. A., and Sahebkar, A. (2011). Antiviral activities of aerial subsets of Artemisia species against Herpes Simplex virus type 1 (HSV1) in vitro. *Asian Biomed.* 5, 63–68. doi: 10.5372/1905-7415.0501.007

Karlen, A. (1996). *Man and microbes: disease and plagues in history and modern times* (New York: Simon and Schuster Paperbacks).

Khaytin (2019). *ArcGIS StoryMaps. Herbal Medicine in the Black Plague*. Available at: https://storymaps.arcgis.com/stories/4a9ae6b9dcc1460b8e97775b285592e9 (Accessed April 10, 2020).

Klayman, D. L. (1985). Qinghaosu (artemisinin): an antimalarial drug from China. *Science* 228, 1049–1055. doi: 10.1126/science.3887571

Koplow, D. A. (2003). *Smallpox: the fight to eradicate a global scourge* (Berkeley: University of California Press).

Lawrence-Mackey, F. (2019). *Medical Appropriation in the 'Red'Atlantic: Translating a Mi'kmaq smallpox cure in the mid-nineteenth century*. Doctoral dissertation (London, UK: University College London (UCL)).

Lee, J. B., Miyake, S., Umetsu, R., Hayashi, K., Chijimatsu, T., and Hayashi, T. (2012). Anti-influenza A virus effects of fructan from Welsh onion (*Allium fistulosum* L.). *Food Chem.* 134, 2164–2168. doi: 10.1016/j.foodchem.2012.04.016

Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., et al. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antivir. Res.* 67, 18–23. doi: 10.1016/j.antiviral.2005.02.007

Lucas, R. M. (1969). *Nature's medicines; the folklore, romance, and value of herbal remedies* (West Nyack NY: Parker Pub. Co).

Macip, S. (2020). *Les grans epidèmies modernes (edició actualitzada): La lluita de l'home contra els enemics invisibles* (Barcelona: La Campana edicions).

Majewski, M. (2014). *Allium sativum*: facts and myths regarding human health. *Rocz Panstw. Zakl. Hig.* 65, 1–8.

Mehra, M. R., Ruschitzka, F., and Patel, A. N. (2020). Retraction—Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: a multinational registry analysis. *Lancet*. doi: 10.1016/S0140-6736(20)31324-6

Meshnick, S. (1998). "From Quinine to qinghaosu: Historical perspectives," in *Malaria: Parasite Biology, Pathogenesis, and Protection*. Ed. I. W. Sherman (Washington: ASM Press), 341–353.

Millspaugh, C. F. (1892). *Medicinal Plants: an illustrated and descriptive guide to plants indigenous to and naturalized in the united states which are used in medicine, their description, origin, history, preparation, chemistry and physiological effects fully described (Vol. 1)* (The New York Public Library: J.C. Yorston and Company).

Mitjà, O., and Clotet, B. (2020). Use of antiviral drugs to reduce COVID-19 transmission. *Lancet Glob. Health* 8, e639–e640. doi: 10.1016/S2214-109X(20)30114-5

Mitjà, O., Corbacho-Monné, M., Ubals, M., Tebe, C., Peñafiel, J., Tobias, A., et al. (2020). Hydroxychloroquine for Early Treatment of Adults with Mild Covid-19: A Randomized-Controlled Trial. *Clin. Infect. Dis.* doi: 10.1093/cid/ciaa1009 ciaa1009.

Molina-Cruz, A., and Barillas-Mury, C. (2014). The remarkable journey of adaptation of the *Plasmodium falciparum* malaria parasite to New World anopheline mosquitoes. *Mem. I Oswaldo Cruz* 109, 662–667. doi: 10.1590/0074-0276130553

Moore, M., and Langland, J. O. (2018). *Sarracenia purpurea:* a botanical extract with anti-papilloma virus and oncolytic activity. *Integr. Med.* 17, 61–61.

Müller, A., Eller, J., Albrecht, F., Prochnow, P., Kuhlmann, K., Bandow, J. E., et al. (2016). Allicin induces thiol stress in bacteria through S-allylmercapto modification of protein cysteines. *J. Biol. Chem.* 291, 11477–11490. doi: 10.1074/jbc.M115.702308

Nicklisch, N., Maixner, F., Ganslmeier, R., Friederich, S., Dresely, V., Meller, H., et al. (2012). Rib lesions in skeletons from early neolithic sites in Central Germany: on the trail of tuberculosis at the onset of agriculture. *Am. J. Phys. Anthropol.* 149, 391–404. doi: 10.1002/ajpa.22137

Plants (Editorial) (2020). Redeploying plant defences. *Nat. Plants* 6, 177. doi: 10.1038/s41477-020-0628-0

Raghunandana, R. R., Srinivasa, S. R., and Venkataraman, P. R. (1946). Investigations on plant antibiotics; studies on allicin, the antibacterial principle of Allium sativum (garlic). *J. Sci. Ind. Res. (New Delhi India: 1942)* 5, 31–35.

Rosales-Mendoza, S., Márquez-Escobar, V. A., González-Ortega, O., Nieto-Gómez, R., and Arévalo-Villalobos, J. II (2020). What does plant-based

vaccine technology offer to the fight against COVID-19? *Vaccines* 8, 183. doi: 10.3390/vaccines8020183

Rybicki, E. P. (2014). Plant-based vaccines against viruses. *Virol. J.* 11, 205. doi: 10.1186/s12985-014-0205-0

Schlagenhauf, P., Grobusch, M. P., Maier, J. D., and Gautret, P. (2020). Repurposing antimalarials and other drugs for COVID-19. *Travel Med. Infect. Dis.* 34, 101658. doi: 10.1016/j.tmaid.2020.101658

Seo, D. J., Lee, M., Jeon, S. B., Park, H., Jeong, S., Lee, B. H., et al. (2017). Antiviral activity of herbal extracts against the hepatitis A virus. *Food Control* 72, 9–13. doi: 10.1016/j.foodcont.2016.07.028

Sharma, N. (2019). Efficacy of garlic and onion against virus. *Int. J. Res. Pharm. Sci.* 10, 3578–3586. doi: 10.26452/ijrps.v10i4.1738

Shephard, R. J. (2015). "The Middle-Ages: monasteries, medical schools and the dawn of state health care," in *An Illustrated History of Health and Fitness, from Pre-History to our Post-Modern World*. Ed. R. J. Shephard (Switzerland: Springer International Publishing Switzerland), 241–346.

Shoseyov, O., Posen, Y., and Grynspan, F. (2014). Human collagen produced in plants: more than just another molecule. *Bioengineered* 5, 49–52. doi: 10.4161/bioe.26002

Sijmons, P. C., Dekker, B. M., Schrammeijer, B., Verwoerd, T. C., van den Elzen, P. J., and Hoekema, A. (1990). Production of correctly processed human serum albumin in transgenic plants. *Biotechnology* 8, 217–221.

Singh, B., Ryan, H., Kredo, T., Chaplin, M., and Fletcher, T. (2020). Chloroquine or hydroxychloroquine for prevention and treatment of COVID-19 (Protocol). *Cochrane Db. Syst. Rev.* 4, CD013587. doi: 10.1002/14651858.CD013587

Stephens, J. W. W., Yorke, W., Blacklock, B., Macfie, J. W. S., Cooper, C. F., and Carter, H. F. (1917). Studies in the treatment of malaria: v. intramuscular injections of quinine alkaloid in simple tertian malaria. *Ann. Trop. Med. Parasitol.* 11, 173–182. doi: 10.1080/00034983.1917.11684134

Takeyama, N., Kiyono, H., and Yuki, Y. (2015). Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. *Ther. Adv. Vacc.* 3, 139–154. doi: 10.1177/2051013615613272

Totelin, L. (2004). Mithradates' Antidote–A Pharmacological Ghost. *Early Sci. Med.* 9, 1–19. doi: 10.1163/1573382041153179

Trigg, P. I., and Kondrachine, A. V. (1998). Commentary: malaria control in the 1990s. *B. World Health Organ.* 76, 11–16.

Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solórzano, A., Swayne, D. E., et al. (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310, 77–80. doi: 10.1126/science.1119392

UNAIDS (2020). *Tuberculosis—good progress, but not enough*. Available at: https://www.unaids.org/en/resources/presscentre/featurestories/2020/march/20200323_tb#:~:text=The%20bad%20news%20is%20that,000%20people%20living%20with%20HIV (Accessed March 23, 2020).

Weng, J. K. (2020). Plant Solutions for the COVID-19 Pandemic and Beyond: Historical Reflections and Future Perspectives. *Mol. Plant* 13, 803–807. doi: 10.1016/j.molp.2020.05.014

Yalcindag, E., Elguero, E., Arnathau, C., Durand, P., Akiana, J., Anderson, T. J., et al. (2012). Multiple independent introductions of Plasmodium falciparum in South America. *P. Natl. Acad. Sci. U.S.A.* 109, 511–516. doi: 10.1073/pnas.1119058109

Yang, D., Guo, F., Liu, B., Huang, N., and Watkins, S. C. (2003). Expression and localization of human lysozyme in the endosperm of transgenic rice. *Planta* 216, 597–603. doi: 10.1007/s00425-002-0919-x

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frontiers in Plant Science

REVIEW
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Natural Plant Products: A Less Focused Aspect for the COVID-19 Viral Outbreak

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The sudden emergence of COVID-19 caused by a novel coronavirus (nCoV) led the entire world to search for relevant solutions to fight the pandemic. Although continuous trials are being conducted to develop precise vaccines and therapeutic antibodies, a potential remedy is yet to be developed. Plants have largely contributed to the treatment of several human diseases and different phytoconstituents have been previously described to impede the replication of numerous viruses. Despite the previous positive reports of plant-based medications, no successful clinical trials of phyto-anti-COVID drugs could be conducted to date. In this article, we discuss varying perspectives on why phyto-anti-viral drug clinical trials were not successful in the case of COVID-19. The issue has been discussed in light of the usage of plant-based therapeutics in previous coronavirus outbreaks. Through this article, we aim to identify the disadvantages in this research area and suggest some measures to ensure that phytoconstituents can efficiently contribute to future random viral outbreaks. It is emphasized that if used strategically phyto-inhibitors with pre-established clinical data for other diseases can save the time required for long clinical trials. The scientific community should competently tap into phytoconstituents and take their research up to the final stage of clinical trials so that potential phyto-anti-COVID drugs can be developed.

Keywords: clinical trials, coronavirus, coronavirus disease of 2019, natural compounds, molecular docking, phytoconstituents, severe acute respiratory syndrome coronavirus 2

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INTRODUCTION

Nobody knew that the coronavirus disease of 2019 (COVID-19) was going to be a greater disaster when compared to the former coronavirus outbreaks, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS). The novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that caused COVID-19 is alarming to the scientific world as it has resulted in the unexpected viral pandemic. However, was COVID-19 an unexpected viral pandemic? The answer is no.

The previous two corona outbreaks, SARS-CoV, and MERS-CoV, were first reported in Guangdong, China in November 2002 (Zhong et al., 2003) and Saudi Arabia in April 2012, respectively (de Wit et al., 2016; Cui et al., 2019). After the identification of SARS-CoV (Ksiazek et al., 2003), several strategies were adopted to eradicate the disease, however, infection control was proven to be more effective than medical intervention leading to the termination of the SARS

pandemic. Although the end of the SARS pandemic was announced in July 2003, different types of SARS re-occurred in different years either by zoonotic or human-to-human transmission in several countries including Canada, Vietnam, the Middle East, Hong Kong, South Korea, and Jordan (Wang et al., 2005; Zaki et al., 2012; Hijawi et al., 2013). Later, evidence proved that intermediate hosts may not be required for straight human infection as Chinese horseshoe bats are the main reservoirs of SARS-CoV (Ge et al., 2013). Besides, it was suggested that there was a higher risk of recurrence of SARS-CoV from circulating viruses in bat populations (Menachery et al., 2015).

The emergence of SARS-CoV-2 is just a confirmation of the previous predictions. After previous outbreaks, several studies determined the poor efficacy of therapeutics and both monoclonal antibodies and vaccines against CoV infection (Menachery et al., 2015). In this scenario, new measures should have been created to fight the problem. The fact that more than 619,150 deaths around the world have been reported, as of July 23 2020, has proven that we, as a scientific world, were less prepared for such an abrupt viral outbreak (WHO, 2020, Siuation Report, 185).

Though plants and their extracts have been recognized as effective anti-viral agents for several decades (Chantrill et al., 1952), plant-based medications have been largely ignored. Despite the vast research conducted in several directions after the previous CoV pandemics, plant-based therapeutics could not achieve a satisfactory level in clinical trials against the disease. Medicinal plant extracts have been reported to impede the replication of several viruses including human immunodeficiency virus (HIV), hepatitis B virus (HBV), poxvirus, severe acute respiratory syndrome (SARS) virus, and herpes simplex virus type 2 (HSV-2) (Vermani and Garg, 2002; Kotwal et al., 2005; Huang et al., 2006). Despite that, there are no reports of plant-based medicines that have been successful in preventing the spread of COVID-19 or curing COVID-19. Thus, further study is required to affirm why plant-based medications could not work in the case of COVID-19. In this article, we discuss different aspects of the utilization of plant-based therapeutics in controlling viral outbreaks like COVID-19. Future research for such viral pandemics should be designed in light of the success stories of previous plant-based medications. The disadvantages in this field of research have been deliberated so that lessons can be learned and the scientific community can prepare for future outbreaks.

MEDICINAL PLANTS AND THEIR EXTRACTS AS AN ANTI-VIRAL AGENT

Medicinal plants can be used as anti-viral agents either as first-generation drugs where plant crudes are used in their natural forms or as second-generation drugs where the active metabolites of plants which are responsible for the anti-viral activity are employed (Jassim and Naji, 2003). However, to be successful, plant-based herbal medicines have to address the issue of genetic variability of viruses, competent replication of DNA and RNA viruses within the host cells, and their capability to survive in the host cells (Wagner and Hewlett, 1999; Jassim and Naji, 2003). In contrast to synthetic drugs, some of the plant-based metabolites hinder the replication of viruses without disturbing the host metabolism; which consequently have restricted side effects as drugs (Hussain et al., 2017).

The interest in anti-viral plant research development started with the suppression of the amplification of the influenza A virus by 12 plant extracts (Chantrill et al., 1952). Afterward, continuous efforts have been made to screen different plant sources *via in silico, in vitro*, and *in vivo* assays for anti-viral activity towards several viruses such as parainfluenza virus type 3, respiratory syncytial virus, poliovirus type 1, herpes simplex virus (HSV), enteric coronavirus, and rotavirus (RV). (Ahmad et al., 1996; Rajbhandari et al., 2001; Jassim and Naji, 2003).

Plant crudes contain several metabolites and it is extremely crucial to identify which component makes it a potential candidate for an effective anti-viral drug. Different anti-viral compounds of plants including peptides, lignans, terpenoids, polysaccharides, flavonoids, polyacetylenes, and alkaloids are effective against different targets of viruses such as DNA, RNA genomes, membranes, the replication process, and ribosomal activity (Jassim and Naji, 2003; Ireland et al., 2008; Sencanski et al., 2015; Vilas Boas et al., 2019). As an example, the strong *in vitro* activity of extracts of *Macaranga barteri* against E7 and E19 echoviruses suggests that it can be an effective therapeutic agent for enteroviral infections such as encephalitis (Ogbole et al., 2018). However, among all the components, three stilbenoids (especially vedelianin) isolated from *M. barteri* extracts are largely responsible for the anti-viral activity against echoviruses (Segun et al., 2019). Similarly, other stilbenes such as Resveratrol and trans-arachidin isolated from grapes and the hairy root culture of peanut are capable of reducing the replication rate of the African swine fever virus (ASFV) and RVs, respectively (Abba et al., 2015; Ball et al., 2015).

UNDERSTANDING THE CORONAVIRUS OUTBREAKS

Coronaviruses, which have the largest genomes among the RNA viruses, consist of a long positive-sense RNA that behaves like mRNA encoding the synthesis of two replicase polyproteins (pp), i.e., pp1a and pp1ab. These polyproteins are processed by a main protease, i.e., chymotrypsin-like (3CLPro) protease and papain like protease (PLP). While the main protease cleaves at 11 sites, PLP cleaves at two or more than two sites on the polyproteins. Due to the essential role of these proteases in proteolytic processing during viral replication, these proteases are considered as the main targets for the development of therapeutic drugs (Deng et al., 2014). CoVs are grouped into four categories: alpha, beta, gamma, and delta types; among which only alpha and beta types are known to infect humans. Coronaviruses are difficult to handle because of the higher

mutation rates of their nucleotides as compared to other single-stranded RNA viruses.

Before the present SARS-CoV-2, six different types of human coronaviruses (HCoV) had been discovered including HCoV-HKU1, HCoV-OC43, HCoV-NL63, HCoV-229E, MERS-CoV, and SARS-CoV (Friedman et al., 2018). The enveloped HCoV viruses fit in to Coronaviridae family and are known to develop respiratory diseases (Geller et al., 2012). SARS-CoV and MERS-CoV were reported as the most deadly viral corona outbreaks causing an epidemic in different countries with a fatality rate of 9% (during 2002 and 2003) and 35.4% (up to December 2016), respectively (De Wit et al., 2016).

THE SUCCESS STORIES OF PHYTOCONSTITUENTS AGAINST SARS-COV AND MERS-COV

The first outbreak of SARS-CoV in China led to a sprint of screening of Chinese medicinal herbs against the disease and some of them came out as potential anti-viral agents. During the SARS outbreak in the absence of effective therapies, ribavirin, a licensed drug for the respiratory syncytial virus (RSV) was commonly suggested as a treatment (Van Vonderen et al., 2003). However, it was later found to cause the death of SARS patients by inducing anemia and hemolysis (Booth et al., 2003; Chiou et al., 2005). Glycyrrhizin (triterpene glycoside glycyrrhizic acid), a phytoconstituent extracted from liquorice roots (*Glycyrrhiza radix*) proved to be a more efficient anti-viral agent for SARS-CoV when compared to ribavirin (Cinatl et al., 2003). Glycyrrhizin was tried against the isolates of SARS-CoV replicated in Vero cells (kidney epithelial cells isolated from African green monkeys) and was proven to be the most compelling interceptor of replication of SARS-CoV when compared to other anti-viral agents such as mycophenolic acid, pyrazofurin, 6-azauridine, and ribavirin (Cinatl et al., 2003). Besides it also hinders the entry of the virus which is a main step in the replication cycle. The concentration of glycyrrhizin required to impede the cytopathic effect of a virus in a Vero cell culture to 50% of the control value, i.e., EC50, was 300 mg/L. EC50 stands for the effective concentration of a drug that gives a half-maximal response. Although the complete effect of glycyrrhizin activity towards SARS-CoV is not clear, it increases the production of nitrous oxide by the overexpression of nitrous oxide synthase that inhibits the viral replication (Jeong and Kim, 2002; Cinatl et al., 2003). A concentration of 1000 mg/L of glycyrrhizin was found to be effective in lowering the expression of SARS-CoV antigens (extracted from patient's serum) in a Vero cell culture. Further analysis of glycyrrhizin derivatives against SARS-CoV showed that the addition of different compounds to functional groups may lead to a 10-70 fold increase in the anti-SARS activity; however, they may also increase the cytotoxic effects (Hoever et al., 2005). Although cytotoxic effects need to be discussed, such studies open the platform for the modification of glycyrrhizin for the production of novel anti-SARS-CoV drugs with enhanced activity.

Several plants and their extracts have been reported to have a remedial approach against SARS-CoV and MERS-CoV by modulating the immune response. Based on a virus-induced cytopathic effect (CPE) assay and an MTS [(5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium] cell proliferation assay, extracts of *Linder aggregata*, *Pyrrosia lingua*, *Artemisia annua*, and *Lycoris radiata* with EC50 values ranging from 2.4 ± 0.2 to 88.2 ± 7.7 mg/L showed much better anti-SARS-CoV activity in a Vero cell culture when compared to glycyrrhizin (Li et al., 2005). However, among these four extracts, *Lycoris radiata* with an EC50 value of 2.4 ± 0.2 mg/L was found to be the most effective candidate for anti-viral medicine against SARS-CoV. Lycorine, one of the phytoconstituents fractionated from the extract of *Lycoris radiata* is mainly responsible for its anti-SARS-CoV activity showing a lower EC50 value than its original extract (± 0.0012 μM). Lycorine is also recognized for its potential inhibition activity against herpes simplex virus (type I) and the poliomyelitis virus. Thus, it is crucial to explore the broad anti-viral feature of lycorine in detail using real-time PCR to assess its capacity to inhibit viral RNA replication and its interface with viral antigens. Aescin, an extensively utilized drug in Europe, which is extracted from horse chestnut trees and reserpine which is extracted from the Rauwolfia species have shown EC50 values of 3.4 μM and 6.0 μM against SARS-CoV in a Vero cell culture, respectively (Wu et al., 2004). In addition, *Radix ginseng*, eucalyptus, and *Lonicera japonica* extracts have shown anti-viral activity towards SARS-CoV at 100 μM. Later, Ginsenoside-Rb1 isolated from the traditional Chinese herb, *Radix ginseng*, was reported to lessen acute lung injury in rats by inhibiting the inflammatory signaling pathway (Yuan et al., 2014).

Many research experiments have been conducted to determine the inhibition capacity of phytoconstituents against the main protease (3CLpro) and papain-like protease (PLpro) of SARS and MERS coronaviruses. Strangely, even the constituents in tea can be potentially effective against a deadly virus like SARS-CoV. It is one of the positive points that can be counted on while considering the negative effects of tannic acid in the tea. The 3-isotheaflavin-3-gallate (TF2B), theaflavin-3,3'-digallate (TF3), and tannic acid compounds that are abundant in black tea extracts are potent inhibitors of the main protease of SARS-CoV, 3CLpro at IC50 < 10 μM as determined by an HPLC proteolytic assay (Chen et al., 2005). IC50 stands for the concentration of an inhibitor where the response is reduced by half. Quercetin, a plant flavonoid, and its derivative quercetin-3-β-galactoside show potent inhibition of viral replication in SARS-CoV 3CLpro where sugar moiety is crucial for inhibitory action (Yi et al., 2004; Lin et al., 2005; Chen et al., 2006). An extract of *Houttuynia cordata*, a traditional Chinese medicine, at 200μg/ml had been reported to have an inhibitory effect on the RNA-dependent RNA polymerase (RdRp) and 3C-like protease (3CLpro) of SARS-CoV which was non-toxic to mice at an oral dosage of 16 g/kg (Lau et al., 2008). Scutellarein extracted from *Scutettaria baicalensis* can be a potential SARS-CoV inhibitor as it hinders the ATPase activity of the helicase protein of SARS-

CoV *in vitro* (Yu et al., 2012). Few compounds such as dihydrotanshinone isolated from the root of *Salvia miltiorrhiza* showed anti-viral activity against both SARS and MERS CoV by the inhibition of proteases and hindering of the viral entry, respectively (Park et al., 2012; Kim et al., 2018). A phlorotannin, dieckol, extracted from the edible brown algae *Ecklonia cava* showed potential inhibitory effects on the 3CLpro of SARS-CoV at IC50 = 2.7 μM. It is more repressive on the cell-based 3CLpro cis-cleavage when compared to the other natural CoV protease inhibitors such as quinone-methide triterpene extracted from *Tripterygium regelii* (Ryu et al., 2010; Park et al., 2013).

Not only are these extracts potent against 3CLpro but also several natural compounds have been reported to have a greater inhibitory action against PLpro. A polyphenol compound, papyriflavonol A, derived from *Broussonetia papyrifera* which hinders the SARS-CoV PLpro with an IC50 value of 3.7 μM can be utilized for the development of anti-CoV agents (Park et al., 2017). A cinnamic amide with an infrequent carbinolamide motif derived from the methanol extract of *T. terrestris* fruit showed effective inhibitory action towards SARS-CoV PLpro with IC50 = 15.8 μM (Song et al., 2014).

Resveratrol (trans-3, 5, 4′-trihydroxystilbene), a natural stilbene derivative, can be extracted from different plants including cranberry (*Vaccinium macrocarpon*), grape (*Vitis vinifera*), and Huzhang (*Polygonum cuspidatum*) is efficient in inhibiting MERS-CoV replication *in vitro* by reducing cell death and alleviating the expression of nucleocapsid protein that is required for viral replication (Lin et al., 2017). The inhibition of the NF-κB pathway by resveratrol in signal transduction shows its potential capacity to be an effective broad-spectrum anti-viral agent and thus, its functioning against CoV should be investigated *in vivo*.

The anti-viral activity of several medicinal herbal extracts such as *Sophora subprostrata radix*, *Phellodendron cortex*, *Coptidis rhizoma*, *Meliae cortex*, and *Cimicifuga rhizome* was identified against mouse hepatitis virus (MHV) which is widely considered as a prototype of coronavirus. The EC50 values of these compounds is in the range of 2.0 to 27.5 μg/ml suggesting that these can be potent candidates for developing anti-viral therapeutics (Kim et al., 2008).

PHYTOCONSTITUENTS WITH BROAD-SPECTRUM ACTIVITY AGAINST COVS

The accidental outbreaks of SARS and MERS coronaviruses pointed towards the chances of the emergence of novel CoVs in the future. Until 2020, there was a dearth of approved drugs for SARS-CoV and MERS-CoV and it emphasized the significance of broad-spectrum viral inhibitors. The extent of conservation in crucial active domains of different human coronaviruses such as RNA helicase and 3CLpro can be utilized as a target when developing potential broad-spectrum anti-CoV drugs. Silvestrol, extracted from *Aglaia* sp., inhibits the cap-dependent mRNA translation of HCoV-229E and MERS-CoV in human embryonic lung fibroblast (MRC-5) cells with EC50 values of less than 0.003 μM and 0.0013 μM, respectively (Muller et al., 2018). Several phytocompounds including mycophenolate mofetil, emetine, and lycorine were identified as the potential broad-spectrum inhibitors that hindered the *in vitro* replication of four CoVs; MERS-CoV, MHV-A59, HCoV-NL63, and HCoV-OC43-WT with EC50 values less than 5 μM (Shen et al., 2019). Among these, the inhibition capacity of lycorine against HCoV-OC43 by reducing the viral lethality in the central nervous system of mice was reported *in vivo via* bioluminescence imaging (Shen et al., 2019). The effectiveness of most of these phytocompounds as broad-spectrum inhibitors has been confirmed in *in vitro* infection models and was tested in a particular cell line that may be influenced by specific host cell types. The identification of broad-spectrum inhibitors and determining their inhibition capacity in an *in vivo* system may lead to the production of potential drugs.

WHERE CAN WE FIND PHYTOCONSTITUENTS IN THE RACE OF DEVELOPING DRUGS FOR PANDEMICS LIKE COVID-19?

The emergence of the COVID-19 outbreak and its spread around the world led to an urgent search for a solution against the disease. While expected methods such as the combined medication of systematic corticosteroids and anti-viral treatment along with interferons are being tried for the speedy recovery of patients, plant-based treatment regimes are also potentially being explored in the race. The release of the gene sequence of SARS-CoV-2, the crystallization of its main protease, and its availability in the Protein Data Bank (PDB) showed that the main proteins of SARS-CoV-2 shares great similarities with those of SARS-CoV and MERS-CoV (Zhang L. et al., 2020; Zhou et al., 2020). It should be considered that despite the reported mutations in the novel coronavirus as compared to SARS-CoV and MERS-CoV, the effectiveness of potential plants and their phytoconstituents that were operative against SARS-CoV and MERS-CoV could have been employed for SARS-CoV-2. SARS-CoV and SARS-CoV-2 belong to beta coronaviruses with high homology in the genomic sequence at the nucleotide level; however, there are six regions of differences in their genome sequence. These regions can be supportive to developing new drugs for SARS-CoV-2 (Xu et al., 2020). In addition, proteins of SARS-CoV-2 share 95% - 100% homology with SARS-CoV with only two non-homologous proteins, orf8 and orf10. The amino acid sequence of orf8 is different in both the viruses (Chan et al., 2020). A Blastp comparison of SARS-CoV and SARS-CoV-2 showed a more than 95% similarity in helicase, nsp7,8,9,10, 3C-like proteinase, 3'-to-5' exonuclease, and RNA-dependent RNA polymerase (Xu et al., 2020). The antibodies active towards the N protein of SARS-CoV may have increased the probability of binding to the N protein of SARS-CoV-2 due to approximately 90% identity in the N protein amino acids of both viruses (Gralinski and Menachery, 2020). These similarities and dissimilarities between the two viruses should be considered

while utilizing the phyto-inhibitors active against SARS-CoV for the novel coronavirus (nCoV), SARS-CoV-2. However, not taking potential plants or their phytoconstituents to an effective therapeutic anti-viral drug stage during previous corona outbreaks is one of the major disadvantages of the present scenario. The availability of potent plant-based drugs against SARS-CoV and MERS-CoV could have opened new treatment pathways for sudden outbreaks such as COVID-19.

Given this disadvantage, most of the plant-based investigations directed towards COVID-19 either focus on bioinformatic tools such as *in silico* processing, molecular docking, or concentrate on molecular farming such as the production of recombinant proteins involving vaccines and antibodies (Islam et al., 2020; Rosales-Mendoza et al., 2020). Several molecules of known herbal medicines, when docked with the proteins of SARS-CoV-2, have been reported to inhibit 3CLpro, PLpro, spike proteins, and viral replication by binding in different domains (Islam et al., 2020). This binding hinders the substrate from going to the enzyme's active sites, prevents dimer formation, or averts viral entry (Park et al., 2013; Zhang D. H. et al., 2020). The traditional herbs which contain these potential anti-viral compounds and are regularly used in handling viral respiratory infections might be employed to provide immediate support in the treatment of COVID-19. However, clinical manifestations are required for the routine implementation of these phytoconstituents as drugs.

Additionally, it should also be kept in mind that molecular docking is a crucial process in the identification of potential anti-viral compounds and is based on the available genome information of the novel coronavirus. In the case of mutation in the existing SARS-CoV-2, the suggested compounds may not be effective and new investigations will be required (**Figure 1**). Ul Qamar et al. (2020) created a 3D homology model of the 3CLpro sequence of SARS-CoV-2 and highlighted its conserved nature comparable with the main protease sequence of SARS-CoV which shared a 99.02% sequence similarity. However, 12 point-mutations have been reported in SARS-CoV-2 which dislocate crucial hydrogen bonds, change the receptor binding site of its main protease, and thus, SARS-CoV-2 may behave differently towards some phyto-inhibitors that were effective towards SARS-CoV and need to be tested. A detailed molecular docking-based screening of more than 32,000 phytoconstituents resulted in nine potential compounds (including myricitrin, licoleafol, and amaranthin) that may hinder the activity of the SARS-CoV-2 3CLpro (Ul Qamar et al., 2020). Another molecular docking-based study using lopinavir and nelfinavir as standards revealed that epicatechin-gallate, catechin, curcumin, oleuropein, apigenin-7-glucoside,

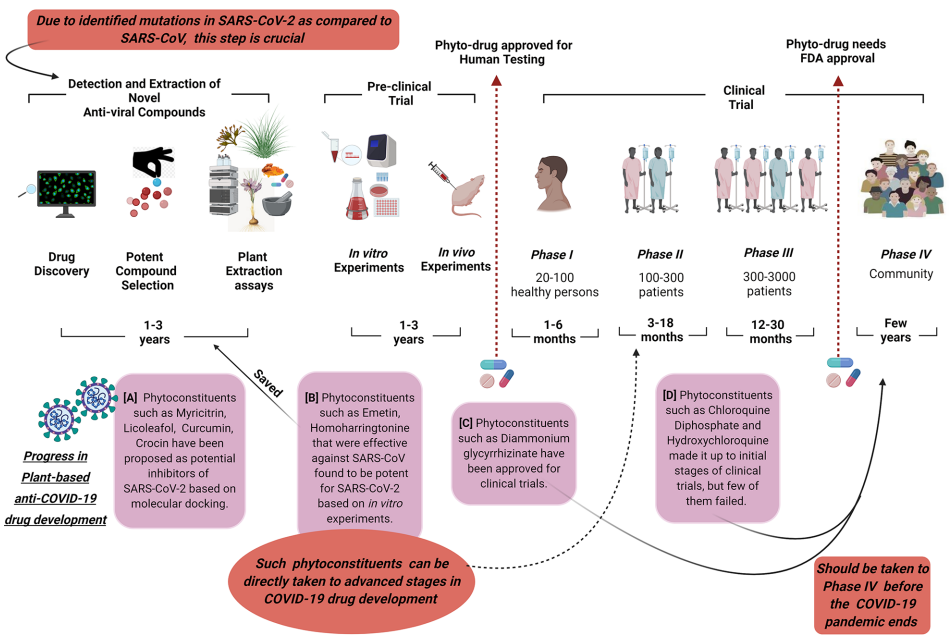

FIGURE 1 | This picture shows the current status of phytoconstituents in the development process of anti-COVID-19 drugs. Due to the identified mutations in SARS-CoV-2 as compared to SARS-CoV, the detection of potent anti-viral compounds is necessary. Molecular docking can largely contribute to this process. The phytocompounds (A) that are identified as potential inhibitors of SARS-CoV-2 should be immediately forwarded to pre-clinical and clinical trials (Aanouz et al., 2020; Khaerunnisa et al., 2020; Ul Qamar et al., 2020). The phytocompounds (B) that were effective against SARS-CoV and are potent for SARS-CoV-2 based on *in vitro* experiments saved the time of target compound selection and extraction assays (Nemunaitis et al., 2013; Choy et al., 2020). As their efficacy and safety has already been proven in previous the SARS-CoV outbreak, these can be directly taken to advanced stages in COVID-19 clinical trials. The phytocompounds (C) that are approved for clinical trials or are currently in the process of clinical trials (D) should be taken forward to phase IV as soon as possible so that the wide effect of the developed drugs can be observed before the COVID-19 pandemic ends (Borba et al., 2020; Gautret et al., 2020; Qiu et al., 2020). This is extremely critical for future random viral corona outbreaks.

naringenin, demethoxycurcumin, luteolin-7-glucoside, quercetin, and kaempferol compounds extracted from medicinal plants may act as potential inhibitors of the main protease of COVID-19 (Khaerunnisa et al., 2020). Three phytocompounds, β-Eudesmol, digitoxigenin, and Ccrocin isolated from *Lauris nobilis* L, *Nerium oleander*, and *Crocus sativus* L, respectively, have been proposed as potential inhibitors of the spike protein of SARS-CoV-2 based on the molecular docking study (Aanouz et al., 2020) (**Figure 1**).

Another important aspect while dealing with phyto-inhibitors against CoVs is that the results obtained from *in vitro* experiments may be different from the clinical efficacy in *in vivo* experiments. This may be due to the fact that the oral intake of these phyto-drugs may not reach the expected blood serum concentration as observed in *in vitro* experiments. Emetine, an alkaloid extracted from the root of the plant *Psychotria ipecacuanha* (ipecac root), that was determined to be a broad-spectrum inhibitor regulating different CoVs *in vitro* was found to inhibit SARS-CoV-2 replication at 0.5 μM. However, as a therapeutic its plasma concentration stretches up to 0.156 μM that is much lower than its toxic plasma concentration of 1.04 μM and its EC50 value towards SARS-CoV-2 (Regenthal et al., 1999; Choy et al., 2020). Homoharringtonine, isolated from *Cephalotoxus fortunei*, is a broad spectrum anti-viral drug effective against murine hepatitis and porcine epidemic diarrhea coronaviruses and inhibits SARS-CoV-2 at EC50 = 2.10 μM. However, a semi-synthetic type of homoharringtonine, omacetaxine, is reported to have a therapeutic plasma concentration of 0.066 μM after 11 days of treatment that was much lower than its EC50 value against SARS-CoV-2 *in vitro* (Nemunaitis et al., 2013; Choy et al., 2020). These results of emetine and homoharringtonine confirmed that plant-based therapeutics which are potentially effective against SARS-CoV-2 should be immediately considered for dose optimization. Isolated from liquorice roots, diammonium glycyrrhizinate combined with Vitamin C tablets had been prescribed for common COVID-19 symptoms and had been approved for randomized clinical trials (Chen et al., 2020; Qiu et al., 2020). These results also give us hope that broad-spectrum phyto-anti-virals can be effective for sudden viral outbreaks in the future.

WHY WERE SUCCESSFUL CLINICAL TRIALS NOT POSSIBLE FOR SARS-COV PHYTO-INHIBITORS?

Different researchers are investigating diverse plant forms based on ethnopharmacological data to find effective anti-CoV drugs with novel action mechanisms especially targeting viral replication. In this crucial situation, it is required to discuss why phyto-inhibitors could not reach an effective drug level in previous corona outbreaks so that proper strategies could be developed for future viral epidemics.

The development of drugs is a costly and long-term process. As the screening of plant-based anti-virals is very similar to the testing procedure of synthetic drugs, a better correlation of their *in vitro* and *in vivo* (IVIVC) results may hasten their approval process (Babar, 2013; Bose et al., 2020) (**Figure 1**). The clinical trials of plants-based anti-virals pass through five phases including the pre-clinical phase (unrestricted dose on animals or *in vitro* experiments), phase I, II, III, and IV (**Figure 1**). Phase I includes the testing of the drug and its doses on healthy people, while phase II and phase III are comprised of screening in patients to check the efficacy, side-effects, and safety issues associated with the drug. The number of participants increases in each phase with approximately 300-3000 patients in phase III. Phase IV is one of the most crucial steps including post-marketing surveillance to observe the safety and long-term effects of the drug when used in public. Several potential plant-based anti-virals against different viral diseases have entered into the market of licensed products as they are effective against particular cellular responses without an added destruction of the cell. *Echinacea purpurea* has reached Phase IV of non-randomized clinical trials in Spain to illustrate the interaction between the anti-retroviral drug, darunavir, and *Echinacea purpurea* in HIV-1 infected patients (Mólto et al., 2010; Kurapati et al., 2016). *Triptolide woldifiion* in China made it up to phase III in randomized clinical trials and its impact on the HIV-1 reservoir was estimated (Li, 2014). (+)-Calanolide A extracted from *Calophyllum lanigerum* hinders HIV-1 reverse transcriptase and was one of the few initial anti-HIV agents that went into a clinical trial. It successfully passed the Phase I clinical trial which was performed on healthy people (Creagh et al., 2001); however, it was not further evaluated for efficacy and safety (Usach et al., 2013).

Phyllanthus urinaria and *Phyllanthus niruri* were found to block endogenous DNA polymerase enzyme necessary for hepatitis B virus (HBV) replication and made it up to clinical trial (Jassim and Naji, 2003). However, a randomized controlled trial on 47 patients suffering from chronic HBV showed that 12 months of the intervention of 250 mg capsule of *Phyllanthus niruri* twice in a day did not reduce the virus load and could not clear the hepatitis B antigens. Accordingly, *Phyllanthus niruri* was not recommended as a standard drug for chronic hepatitis B patients as its efficiency was found to be wide-ranging according to the variations in the treated populations (Baiguera et al., 2018). Similarly, in the case of COVID-19, two forms of cinchona bark-based antimalarials, chloroquine diphosphate and hydroxychloroquine have also been the center of interest due to the reported inhibitory effects of these compounds on SARS-CoV. Positive reports of recovery in SARS-CoV-2 affected patients after hydroxychloroquine treatment are available where a daily dose of 600mg of hydroxychloroquine along with azithromycin significantly reduced virus load after six days of inclusion (Gautret et al., 2020). However, the main drawback of this study was the small sample size and limited time for a long-term follow-up of the patients. Another study on chloroquine diphosphate reported a lethality rate of 15% and 39% in low-dosage (450 mg twice a day on the 1st day and once a day for 4 days) and high-dosage (600 mg twice a day for 10 days) groups in critical SARS-CoV-2 patients after 13 days of treatment (Borba et al., 2020). Thus, a higher dosage of chloroquine diphosphate along with azithromycin in critically ill patients especially suffering from cardio disorders has been reported to be unsafe. Thus, evaluation of chloroquine as a drug through randomized clinical trials is

required. The chances of the effective use of similar phytocompounds in the COVID-19 outbreak would have been higher if experiments focusing on these phyto-inhibitors had been performed for and after SARS-CoV.

One of the possible reasons for the failure of such natural products could be the differences in the prepared drug due to the ecological and seasonal variations in the plant growth, genotypic variation, timing of harvest, variations in the storage, and manufacturing conditions (Islam et al., 2020). Changes in these factors may influence the production of the main component and contaminants (Shimanovskii, 2020). Studies such as that of Borba et al. (2020) and Gautret et al. (2020) reported a daily requirement of a minimum 600 mg of chloroquine compounds per SARS-CoV-2 patient. Though plants produce enough of these natural products for their own use, it is not sufficient to fulfill the commercial manufacturing needs of pharmaceutical companies. Thus, sustainable and reproducible large-scale production of these natural products is another challenge in their successful utilization for the treatment of SARS-CoV-2. Plant cell and tissue culture approaches can be effective for the extraction and multiplication of many of these natural constituents (Hussain et al., 2012; Shimanovskii, 2020). Extraction of phytocompounds from the tissue culture is quick and efficient when compared to the isolation from whole plants. Moreover, plant tissue culture techniques facilitate the production of phytocompounds in completely controlled conditions following the regulations of good manufacturing practices (GMP). Phytocompounds extracted from tissue cultures can be free of microbes and other compounds found in soil-grown plants and are protected from climatic changes (Hussain et al., 2012). Other than plant tissue culture methods, vertical farming units (VFUs) can be promising for the controlled and monitored exponential production of the target crop that prevents the cross-pollination of the target crop with genetically compatible species (Ma et al., 2005; Buyel et al., 2017; Buyel, 2018). Not only for growing and harvesting the plants, GMPs must be followed for the extraction and purification of the pure and homogenous phytocompound from the harvested biomass. This downstream processing of phytocompounds may account for approximately 80% of the total production cost depending on the removal of the contaminants and purity of the extracted compound (Ma et al., 2005; Fischer et al., 2012). Moreover, the chances of success of natural anti-viral products may largely increase if they are prepared in line with ethnopharmacological guidelines. The proper application of in silico and *in vitro* methods followed by *in vivo* experiments may smooth the way for clinical trials of phytocompounds. More than a hundred phytocompounds have been found to inhibit different types of coronaviruses either by inhibiting the interaction of the SARS-CoV (S) protein and the ACE2 receptor or by inhibiting the viral replication, cell division, 3CL protease, papain-like protease (PL pro) or by hindering the viral entry (Islam et al., 2020). It should be noted that for SARS-CoV that emerged in 2003, it was not until 2017 when *in vitro* studies confirming the action mechanism of natural products were conducted (Kim et al., 2014; Schwarz et al., 2014; Park et al., 2017); however, the natural products with the same mechanism against SARS-CoV and even more effective mechanisms were already identified in the initial years of the SARS-CoV epidemic. If more efforts had been given to those phyto-resources from the start of the SARS-CoV epidemic, they could have reached successful clinical trials. Moreover, the inability of SARS-CoV phyto-inhibitors-based research to reach an extensive level of *in vivo* studies reduced the chances of phyto-anti-CoV drugs being developed and passing successfully through clinical trials.

CONCLUSION

The emergency SARS-CoV-2 outbreak led to the utilization of several phytocompounds for the treatment of patients. The direct benefit of most of these compounds is that their effects on the human body and their safety are already established through clinical trials for other diseases. However, utmost care is required while prescribing the dose of these phytocompounds as their uncontrolled use can have long term side-effects on the patient's body. Though numerous phytoconstituents were found to be effective against SARS and MERS CoVs, they fell behind in the drug development process as most of them could not reach the clinical trial stage. One of the possible reasons for incomplete clinical trials could be the intermittent nature of the SARS and MERS epidemics. The absence of patients for the advanced phase trials (phase II, III, IV) of phytoconstituents may have contributed to their failure as licensed drugs. Thus, one of the potential suggestions for COVID-19 recovery could be to identify the potential phytoconstituents based on *in vitro* results and with minimum side effects in phase I trials and take them up to advanced phase trials (phase II, III, IV) as soon as possible (**Figure 1**). So that, in case the COVID-19 pandemic ends abruptly by chance like SARS-CoV, then we can have at least a few efficient phyto-anti-COVID drugs that would have completed randomized clinical trials. Such drugs may not only be effective on re-emergence of SARS-CoV-2 in the coming years but can also be potent against similar viral respiratory outbreaks. Moreover, creating an effective phyto-anti-COVID drug during this pandemic may provide an idea on the duration and the strategy required for the development of potent plant-based therapeutics in case of such random viral outbreaks (**Figure 1**). We as plant biologists need to be more concerned and vigilant about the status of the plants, their extracts, and phytoconstituents in developing phyto-anti-viral drugs and controlling pandemics like COVID-19. It is as crucial as the production of important cereals in the world.

AUTHOR CONTRIBUTIONS

AP and MKK conceived, wrote, and edited the manuscript. MH and SG made intellectual contributions to the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

Aanouz, I., Belhassan, A., El Khatabi, K., Lakhlifi, T., El Idrissi, M., and Bouachrine, M. (2020). Moroccan Medicinal plants as inhibitors of COVID-19: Computational investigations. *J. Biomol. Struct. Dynamics* 38, 1–12. doi: 10.1080/07391102.2020.1758790

Abba, Y., Hassim, H., Hamzah, H., and Noordin, M. M. (2015). Antiviral activity of resveratrol against human and animal viruses. *Adv. Virol.* 2015. doi: 10.1155/2015/184241

Ahmad, A., Davies, J., Randall, S., and Skinner, G. (1996). Antiviral properties of extract of Opuntia streptacantha. *Antiviral Res.* 30, 75–85. doi: 10.1016/0166-3542(95)00839-X

Babar, M. (2013). Antiviral Drug Therapy- Exploiting Medicinal Plants. *J. Antivirals Antiretrovirals* 05, 28–36. doi: 10.4172/jaa.1000060

Baiguera, C., Boschetti, A., Raffetti, E., Zanini, B., Puoti, M., and Donato, F. (2018). Phyllanthus niruri versus Placebo for Chronic Hepatitis B Virus Infection: A Randomized Controlled Trial. *Complement. Med. Res.* 25, 376–382. doi: 10.1159/000484927

Ball, J. M., Medina-Bolivar, F., Defrates, K., Hambleton, E., Hurlburt, M. E., Fang, L., et al. (2015). Investigation of stilbenoids as potential therapeutic agents for rotavirus gastroenteritis. *Adv. Virol.* 2015. doi: 10.1155/2015/293524

Booth, C. M., Matukas, L. M., Tomlinson, G. A., Rachlis, A. R., Rose, D. B., Dwosh, H. A., et al. (2003). Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. *Jama* 289, 2801–2809. doi: 10.1001/jama.289.21.JOC30885

Borba, M. G. S., Val, F. F. A., Sampaio, V. S., Alexandre, M. A. A., Melo, G. C., Brito, M., et al. (2020). Effect of High vs Low Doses of Chloroquine Diphosphate as Adjunctive Therapy for Patients Hospitalized With Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection. *JAMA Netw. Open* 3, e208857. doi: 10.1001/jamanetworkopen.2020.8857

Bose, S., Malik, J., and Mandal, S. C. (2020). "Application of Phytochemicals in Pharmaceuticals," in *Advances in Pharmaceutical Biotechnology*, vol. 55-68. (Singapore: Springer).

Buyel, J. F., Twyman, R. M., and Fischer, R. (2017). Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnol. Adv.* 35, 458–465. doi: 10.1016/j.biotechadv.2017.03.011

Buyel, J. F. (2018). Plants as sources of natural and recombinant anti-cancer agents. *Biotechnol. Adv.* 36, 506–520. doi: 10.1016/j.biotechadv.2018.02.002

Chan, J. F.-W., Kok, K.-H., Zhu, Z., Chu, H., To, K. K.-W., Yuan, S., et al. (2020). Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg. Microbes Infect.* 9, 221–236. doi: 10.1080/22221751.2020.1719902

Chantrill, B., Coulthard, C., Dickinson, L., Inkley, G., Morris, W., and Pyle, A. (1952). The action of plant extracts on a bacteriophage of Pseudomonas pyocyanea and on influenza A virus. *Microbiology* 6, 74–84. doi: 10.1099/00221287-6-1-2-74

Chen, C. N., Lin, C. P., Huang, K. K., Chen, W. C., Hsieh, H. P., Liang, P. H., et al. (2005). Inhibition of SARS-CoV 3C-like Protease Activity by Theaflavin-3,3'-digallate (TF3). *Evid. Based. Complement Alternat. Med.* 2, 209–215. doi: 10.1093/ecam/neh081

Chen, L., Li, J., Luo, C., Liu, H., Xu, W., Chen, G., et al. (2006). Binding interaction of quercetin-3-β-galactoside and its synthetic derivatives with SARS-CoV 3CLpro: Structure–activity relationship studies reveal salient pharmacophore features. *Bioorg. Med. Chem.* 14, 8295–8306. doi: 10.1016/j.bmc.2006.09.014

Chen, L., Hu, C., Hood, M., Zhang, X., Zhang, L., Kan, J., et al. (2020). A Novel Combination of Vitamin C, Curcumin and Glycyrrhizic Acid Potentially Regulates Immune and Inflammatory Response Associated with Coronavirus Infections: A Perspective from System Biology Analysis. *Nutrients* 12, 1193. doi: 10.3390/nu12041193

Chiou, H. E., Liu, C. L., Buttrey, M. J., Kuo, H. P., Liu, H. W., Kuo, H. T., et al. (2005). Adverse effects of ribavirin and outcome in severe acute respiratory syndrome: experience in two medical centers. *Chest* 128, 263–272. doi: 10.1378/chest.128.1.263

Choy, K. T., Wong, A. Y., Kaewpreedee, P., Sia, S. F., Chen, D., Hui, K. P. Y., et al. (2020). Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication in vitro. *Antiviral Res.* 178, 104786. doi: 10.1016/j.antiviral.2020.104786

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H., and Doerr, H. W. (2003). Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. *Lancet* 361, 2045–2046. doi: 10.1016/S0140-6736(03)13615-X

Creagh, T., Ruckle, J. L., Tolbert, D. T., Giltner, J., Eiznhamer, D. A., Dutta, B., et al. (2001). Safety and pharmacokinetics of single doses of (+)-calanolide a, a novel, naturally occurring nonnucleoside reverse transcriptase inhibitor, in healthy, human immunodeficiency virus-negative human subjects. *Antimicrobial Agents Chemother.* 45, 1379–1386. doi: 10.1128/AAC.45.5.1379-1386.2001

Cui, J., Li, F., and Shi, Z.-L. (2019). Origin and evolution of pathogenic coronaviruses. *Nat. Rev. Microbiol.* 17, 181–192. doi: 10.1038/s41579-018-0118-9

De Wit, E., Van Doremalen, N., Falzarano, D., and Munster, V. J. (2016). SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* 14, 523–534. doi: 10.1038/nrmicro.2016.81

Deng, X., Stjohn, S. E., Osswald, H. L., O'brien, A., Banach, B. S., Sleeman, K., et al. (2014). Coronaviruses resistant to a 3C-like protease inhibitor are attenuated for replication and pathogenesis, revealing a low genetic barrier but high fitness cost of resistance. *J. Virol.* 88, 11886–11898. doi: 10.1128/JVI.01528-14

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R. M., and Drossard, J. (2012). GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnol. Adv.* 30, 434–439. doi: 10.1016/j.biotechadv.2011.08.007

Friedman, N., Alter, H., Hindiyeh, M., Mendelson, E., Shemer Avni, Y., and Mandelboim, M. (2018). Human Coronavirus Infections in Israel: Epidemiology, Clinical Symptoms and Summer Seasonality of HCoV-HKU1. *Viruses* 10, 515 doi: 10.3390/v10100515

Gautret, P., Lagier, J. C., Parola, P., Hoang, V. T., Meddeb, L., Mailhe, M., et al. (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int. J. Antimicrob. Agents* 56. doi: 10.1016/j.ijantimicag.2020.105949

Ge, X.-Y., Li, J.-L., Yang, X.-L., Chmura, A. A., Zhu, G., Epstein, J. H., et al. (2013). Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* 503, 535–538. doi: 10.1038/nature12711

Geller, C., Varbanov, M., and Duval, R. E. (2012). Human coronaviruses: insights into environmental resistance and its influence on the development of new antiseptic strategies. *Viruses* 4, 3044–3068. doi: 10.3390/v4113044

Gralinski, L. E., and Menachery, V. D. (2020). Return of the Coronavirus: 2019-nCoV. *Viruses* 12, 135. doi: 10.3390/v12020135

Hijawi, B., Abdallat, M., Sayaydeh, A., Alqasrawi, S., Haddadin, A., Jaarour, N., et al. (2013). Novel coronavirus infections in Jordan, April 2012: epidemiological findings from a retrospective investigation. *EMHJ-Eastern Mediterranean Health J. 19 (Supp. 1) S12-S18 2013.* 19, 12–18. doi: 10.26719/2013.19.supp1.S12

Hoever, G., Baltina, L., Michaelis, M., Kondratenko, R., Baltina, L., Tolstikov, G. A., et al. (2005). Antiviral Activity of Glycyrrhizic Acid Derivatives against SARS– Coronavirus. *J. Med. Chem.* 48, 1256–1259. doi: 10.1021/jm0493008

Huang, K.-L., Lai, Y.-K., Lin, C.-C., and Chang, J.-M. (2006). Inhibition of hepatitis B virus production by Boehmeria nivea root extract in HepG2 2.2.15 cells. *World J. Gastroenterol.: WJG* 12, 5721–5725. doi: 10.3748/wjg.v12.i35.5721

Hussain, M. S., Fareed, S., Saba Ansari, M., Rahman, A., Ahmad, I. Z., and Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. *J. Pharm. Bioallied Sci.* 4, 10. doi: 10.4103/0975-7406.92725

Hussain, W., Haleem, K. S., Khan, I., Tauseef, I., Qayyum, S., Ahmed, B., et al. (2017). Medicinal plants: a repository of antiviral metabolites. *Future Virol.* 12, 299–308. doi: 10.2217/fvl-2016-0110

Ireland, D. C., Wang, C. K., Wilson, J. A., Gustafson, K. R., and Craik, D. J. (2008). Cyclotides as natural anti-HIV agents. *Pept. Sci.* 90, 51–60. doi: 10.1002/bip.20886

Islam, M. T., Sarkar, C., El-Kersh, D. M., Jamaddar, S., Uddin, S. J., Shilpi, J. A., et al. (2020). Natural products and their derivatives against coronavirus: A review of the non-clinical and pre-clinical data. *Phytother. Res.* 2020, 1–22. doi: 10.1002/ptr.6700

Jassim, S. A., and Naji, M. A. (2003). Novel antiviral agents: a medicinal plant perspective. *J. Appl. Microbiol.* 95, 412–427. doi: 10.1046/j.1365-2672.2003.02026.x

Jeong, H. G., and Kim, J. Y. (2002). Induction of inducible nitric oxide synthase expression by 18β-glycyrrhetinic acid in macrophages. *FEBS Lett.* 513, 208–212. doi: 10.1016/S0014-5793(02)02311-6

Khaerunnisa, S., Kurniawan, H., Awaluddin, R., Suhartati, S., and Soetjipto, S. (2020). Potential Inhibitor of COVID-19 Main Protease (M^{pro}) From Several

Medicinal Plant Compounds by Molecular Docking Study. *Preprints*, 2020030226. doi: 10.20944/preprints202003.0226.v1

Kim, H. Y., Shin, H. S., Park, H., Kim, Y. C., Yun, Y. G., Park, S., et al. (2008). In vitro inhibition of coronavirus replications by the traditionally used medicinal herbal extracts, Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, and Phellodendron cortex. *J. Clin. Virol.* 41, 122–128. doi: 10.1016/j.jcv.2007.10.011

Kim, D. W., Seo, K. H., Curtis-Long, M. J., Oh, K. Y., Oh, J.-W., Cho, J. K., et al. (2014). Phenolic phytochemical displaying SARS-CoV papain-like protease inhibition from the seeds of Psoralea corylifolia. *J. Enzyme Inhibition Med. Chem.* 29, 59–63. doi: 10.3109/14756366.2012.753591

Kim, J. Y., Kim, Y. I., Park, S. J., Kim, I. K., Choi, Y. K., and Kim, S.-H. (2018). Safe, high-throughput screening of natural compounds of MERS-CoV entry inhibitors using a pseudovirus expressing MERS-CoV spike protein. *Int. J. Antimicrobial Agents* 52, 730–732. doi: 10.1016/j.ijantimicag.2018.05.003

Kotwal, G. J., Kaczmarek, J. N., Leivers, S., Ghebremariam, Y. T., Kulkarni, A. P., Bauer, G., et al. (2005). Anti-HIV, Anti-Poxvirus, and Anti-SARS Activity of a Nontoxic, Acidic Plant Extract from the Trifollium Species Secomet-V/anti-Vac Suggests That It Contains a Novel Broad-Spectrum Antiviral. *Ann. New Y. Acad. Sci.* 1056, 293. doi: 10.1196/annals.1352.014

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., et al. (2003). A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. *New Engl. J. Med.* 348, 1953–1966. doi: 10.1056/NEJMoa030781

Kurapati, K. R. V., Atluri, V. S., Samikkannu, T., Garcia, G., and Nair, M. P. N. (2016). Natural Products as Anti-HIV Agents and Role in HIV-Associated Neurocognitive Disorders (HAND): A Brief Overview. *Front. Microbiol.* 6, 1444. doi: 10.3389/fmicb.2015.01444

Lau, K. M., Lee, K. M., Koon, C. M., Cheung, C. S., Lau, C. P., Ho, H. M., et al. (2008). Immunomodulatory and anti-SARS activities of Houttuynia cordata. *J. Ethnopharmacol.* 118, 79–85. doi: 10.1016/j.jep.2008.03.018

Li, T. (2014). Clinicaltrials.gov, Study on the Impact of Triptolide Woldifiion on HIV-1 Reservoir In Acute HIV-1 Infection. Available at: Clinicaltrials.gov.

Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., et al. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Res.* 67, 18–23. doi: 10.1016/j.antiviral.2005.02.007

Lin, C.-W., Tsai, F.-J., Tsai, C.-H., Lai, C.-C., Wan, L., Ho, T.-Y., et al. (2005). Anti-SARS coronavirus 3C-like protease effects of Isatis indigotica root and plant-derived phenolic compounds. *Antiviral Res.* 68, 36–42. doi: 10.1016/j.antiviral.2005.07.002

Lin, S. C., Ho, C. T., Chuo, W. H., Li, S., Wang, T. T., and Lin, C. C. (2017). Effective inhibition of MERS-CoV infection by resveratrol. *BMC Infect. Dis.* 17, 144. doi: 10.1186/s12879-017-2253-8

Ma, J. K. C., Chikwamba, R., Sparrow, P., Fischer, R., Mahoney, R., and Twyman, R. M. (2005). Plant-derived pharmaceuticals – the road forward. *Trends Plant Sci.* 10, 580–585. doi: 10.1016/j.tplants.2005.10.009

Menachery, V. D., Yount, B. L., Debbink, K., Agnihothram, S., Gralinski, L. E., Plante, J. A., et al. (2015). A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat. Med.* 21, 1508–1513. doi: 10.1038/nm.3985

Moltó, J., Valle, M, Miranda, C., Cedeño, S., Negredo, E., Barbanoj, MJ., et al. (2011). Herb-Drug Interaction between *Echinacea purpurea* and Darunavir-Ritonavir in HIV-Infected Patients. *Antimicrob. Agents Chemother.* 55, 326.

Muller, C., Schulte, F. W., Lange-Grunweller, K., Obermann, W., Madhugiri, R., Pleschka, S., et al. (2018). Broad-spectrum antiviral activity of the eIF4A inhibitor silvestrol against corona- and picornaviruses. *Antiviral Res.* 150, 123–129. doi: 10.1016/j.antiviral.2017.12.010

Nemunaitis, J., Mita, A., Stephenson, J., Mita, M. M., Sarantopoulos, J., Padmanabhan-Iyer, S., et al. (2013). Pharmacokinetic study of omacetaxine mepesuccinate administered subcutaneously to patients with advanced solid and hematologic tumors. *Cancer Chemother. Pharmacol.* 71, 35–41. doi: 10.1007/s00280-012-1963-2

Ogbole, O. O., Akinleye, T. E., Segun, P. A., Faleye, T. C., and Adeniji, A. J. (2018). In vitro antiviral activity of twenty-seven medicinal plant extracts from Southwest Nigeria against three serotypes of echoviruses. *Virol. J.* 15, 110. doi: 10.1186/s12985-018-1022-7

Park, J.-Y., Kim, J. H., Kim, Y. M., Jeong, H. J., Kim, D. W., Park, K. H., et al. (2012). Tanshinones as selective and slow-binding inhibitors for SARS-CoV cysteine proteases. *Bioorg. Med. Chem.* 20, 5928–5935. doi: 10.1016/j.bmc.2012.07.038

Park, J. Y., Kim, J. H., Kwon, J. M., Kwon, H. J., Jeong, H. J., Kim, Y. M., et al. (2013). Dieckol, a SARS-CoV 3CL(pro) inhibitor, isolated from the edible brown algae Ecklonia cava. *Bioorg. Med. Chem.* 21, 3730–3737. doi: 10.1016/j.bmc.2013.04.026

Park, J.-Y., Yuk, H. J., Ryu, H. W., Lim, S. H., Kim, K. S., Park, K. H., et al. (2017). Evaluation of polyphenols from Broussonetia papyrifera as coronavirus protease inhibitors. *J. Enzyme Inhibition Med. Chem.* 32, 504–512. doi: 10.1080/14756366.2016.1265519

Qiu, R., Wei, X., Zhao, M., Zhong, C., Zhao, C., Hu, J., et al. (2020). Outcome reporting from protocols of clinical trials of Coronavirus Disease 2019 (COVID-19): a review. *medRxiv* 2020. doi: 10.1101/2020.03.04.20031401

Rajbhandari, M., Wegner, U., Jülich, M., Schoepke, T., and Mentel, R. (2001). Screening of Nepalese medicinal plants for antiviral activity. *J. Ethnopharmacol.* 74, 251–255. doi: 10.1016/S0378-8741(00)00374-3

Regenthal, R., Krueger, M., Koeppel, C., and Preiss, R. (1999). Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. *J. Clin. Monitor. Comput.* 15, 529–544. doi: 10.1023/A:1009935116877

Rosales-Mendoza, S., Márquez-Escobar, V. A., González-Ortega, O., Nieto-Gómez, R., and Arévalo-Villalobos, J. I. (2020). What Does Plant-Based Vaccine Technology Offer to the Fight against COVID-19? *Vaccines* 8, 183. doi: 10.3390/vaccines8020183

Ryu, Y. B., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, J.-Y., Kim, D., et al. (2010). Biflavonoids from Torreya nucifera displaying SARS-CoV 3CLpro inhibition. *Bioorg. Med. Chem.* 18, 7940–7947. doi: 10.1016/j.bmc.2010.09.035

Schwarz, S., Sauter, D., Wang, K., Zhang, R., Sun, B., Karioti, A., et al. (2014). Kaempferol derivatives as antiviral drugs against the 3a channel protein of coronavirus. *Planta Med.* 80, 177–182. doi: 10.1055/s-0033-1360277

Segun, P. A., Ogbole, O. O., Akinleye, T. E., Faleye, T. O. C., and Adeniji, A. J. (2019). In vitro anti-enteroviral activity of stilbenoids isolated from the leaves of Macaranga barteri. *Nat. Prod. Res.* 37, 1–5. doi: 10.1080/14786419.2019.1644505

Sencanski, M., Radosevic, D., Perovic, V., Gemovic, B., Stanojevic, M., Veljkovic, N., et al. (2015). Natural products as promising therapeutics for treatment of influenza disease. *Curr. Pharm. Design* 21, 5573–5588. doi: 10.2174/1381612821666151002113426

Shen, L., Niu, J., Wang, C., Huang, B., Wang, W., Zhu, N., et al. (2019). High-throughput screening and identification of potent broad-spectrum inhibitors of coronaviruses. *J. Virol.* 93, e00023–e00019. doi: 10.1128/JVI.00023-19

Shimanovskii, N. (2020). The Role of Phytoengineering in the Preparation and Production of Herbal Medicines. *Pharm. Chem. J.* 53, 961–963. doi: 10.1007/s11094-020-02105-1

Song, Y. H., Kim, D. W., Curtis-Long, M. J., Yuk, H. J., Wang, Y., Zhuang, N., et al. (2014). Papain-like protease (PLpro) inhibitory effects of cinnamic amides from Tribulus terrestris fruits. *Biol. Pharm. Bull.* 37, 1021–1028. doi: 10.1248/bpb.b14-00026

Ul Qamar, M. T., Alqahtani, S. M., Alamri, M. A., and Chen, L. L. (2020). Structural basis of SARS-CoV-2 3CL(pro) and anti-COVID-19 drug discovery from medicinal plants. *J. Pharm. Anal.* 10, 313–319. doi: 10.1016/j.jpha.2020.03.009

Usach, I., Melis, V., and Peris, J. E. (2013). Non-nucleoside reverse transcriptase inhibitors: a review on pharmacokinetics, pharmacodynamics, safety and tolerability. *J. Int. AIDS Soc.* 16, 1–14. doi: 10.7448/IAS.16.1.18567

Van Vonderen, M. G., Bos, J. C., Prins, J. M., Wertheim-Van Dillen, P., and Speelman, P. (2003). Ribavirin in the treatment of severe acute respiratory syndrome (SARS). *Neth J. Med.* 61, 238–241.

Vermani, K., and Garg, S. (2002). Herbal medicines for sexually transmitted diseases and AIDS. *J. Ethnopharmacol.* 80, 49–66. doi: 10.1016/S0378-8741(02)00009-0

Vilas Boas, L. C. P., Campos, M. L., Berlanda, R. L. A., De Carvalho Neves, N., and Franco, O. L. (2019). Antiviral peptides as promising therapeutic drugs. *Cell Mol. Life Sci.* 76, 3525–3542. doi: 10.1007/s00018-019-03138-w

Wagner, E., and Hewlett, M. (1999). Basic virology (p. 656). *Malden MA: Blackwell Sci.*

Wang, M., Yan, M., Xu, H., Liang, W., Kan, B., Zheng, B., et al. (2005). SARS-CoV infection in a restaurant from palm civet. *Emerg. Infect. Dis.* 11, 1860. doi: 10.3201/eid1112.041293

World Health Organization (2020). *Coronavirus disease 2019 (COVID-19): situation report, 185*. World Health Organization. Available at: https://apps.who.int/iris/handle/10665/333573

Wu, C. Y., Jan, J. T., Ma, S. H., Kuo, C. J., Juan, H. F., Cheng, Y. S., et al. (2004). Small molecules targeting severe acute respiratory syndrome human coronavirus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10012–10017. doi: 10.1073/pnas.0403596101

Xu, J., Zhao, S., Teng, T., Abdalla, A. E., Zhu, W., Xie, L., et al. (2020). Systematic Comparison of Two Animal-to-Human Transmitted Human Coronaviruses: SARS-CoV-2 and SARS-CoV. *Viruses* 12, 244. doi: 10.3390/v12020244

Yi, L., Li, Z., Yuan, K., Qu, X., Chen, J., Wang, G., et al. (2004). Small molecules blocking the entry of severe acute respiratory syndrome coronavirus into host cells. *J. Virol.* 78, 11334–11339. doi: 10.1128/JVI.78.20.11334-11339.2004

Yu, M.-S., Lee, J., Lee, J. M., Kim, Y., Chin, Y.-W., Jee, J.-G., et al. (2012). Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13. *Bioorg. Med. Chem. Lett.* 22, 4049–4054. doi: 10.1016/j.bmcl.2012.04.081

Yuan, Q., Jiang, Y. W., Ma, T. T., Fang, Q. H., and Pan, L. (2014). Attenuating effect of Ginsenoside Rb1 on LPS-induced lung injury in rats. *J. Inflammation (Lond)* 11, 40. doi: 10.1186/s12950-014-0040-5

Zaki, A. M., Van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D., and Fouchier, R. A. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *New Engl. J. Med.* 367, 1814–1820. doi: 10.1056/NEJMoa1211721

Zhang, D. H., Wu, K. L., Zhang, X., Deng, S. Q., and Peng, B. (2020). In silico screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. *J. Integr. Med.* 18, 152–158. doi: 10.1016/j.joim.2020.02.005

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020). Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science* 368, 409–412. doi: 10.1126/science.abb3405

Zhong, N. S., Zheng, B. J., Li, Y. M., Poon, L. L. M., Xie, Z. H., Chan, K. H., et al. (2003). Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February 2003. *Lancet* 362, 1353–1358. doi: 10.1016/S0140-6736(03)14630-2

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

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REVIEW
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The Emergency Response Capacity of Plant-Based Biopharmaceutical Manufacturing-What It Is and What It Could Be

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Several epidemic and pandemic diseases have emerged over the last 20 years with increasing reach and severity. The current COVID-19 pandemic has affected most of the world's population, causing millions of infections, hundreds of thousands of deaths, and economic disruption on a vast scale. The increasing number of casualties underlines an urgent need for the rapid delivery of therapeutics, prophylactics such as vaccines, and diagnostic reagents. Here, we review the potential of molecular farming in plants from a manufacturing perspective, focusing on the speed, capacity, safety, and potential costs of transient expression systems. We highlight current limitations in terms of the regulatory framework, as well as future opportunities to establish plant molecular farming as a global, de-centralized emergency response platform for the rapid production of biopharmaceuticals. The implications of public health emergencies on process design and costs, regulatory approval, and production speed and scale compared to conventional manufacturing platforms based on mammalian cell culture are discussed as a forward-looking strategy for future pandemic responses.

Keywords: plant molecular farming, severe acute respiratory syndrome coronavirus 2, rapid scalability, regulatory approval, transient expression

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INTRODUCTION

The impact of the COVID-19 pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was foreshadowed by earlier epidemics of new or re-emerging diseases such as SARS (2002/2003), influenza (2009), Middle East Respiratory Syndrome (MERS, 2012), Ebola (2014/2015), and Zika (2016/2017) affecting localized regions (Bradley and Bryan, 2019; Kobres et al., 2019; Park et al., 2019). These events showed that novel and well-known viral diseases alike can pose a threat to global health. In 2014, an article published in *Nature Medicine* stated that the Ebola outbreak should have been "a wake-up call to the research and pharmaceutical communities, and to federal governments, of the continuing need to invest resources in the study and cure of emerging infectious diseases" (Anonymous, 2014). Recommendations and even new regulations have been implemented to reduce the risk of

zoonotic viral infections (Li et al., 2019), but the extent to which these recommendations are applied and enforced on a regional and, more importantly, local level remains unclear. Furthermore, most vaccine programs for SARS, MERS, and Zika are still awaiting the fulfillment of clinical trials, sometimes more than 5 years after their initiation, due to the lack of patients (Pregelj et al., 2020). In light of this situation, and despite the call to action, the SARS-CoV-2 pandemic has resulted in nearly 20 million infections and more than 700,000 deaths at the time of writing (August 2020) based on the Johns Hopkins University Hospital global database.[1] The economic impact of the pandemic is difficult to assess, but support programs are likely to cost more than €4 trillion (US$4.7 trillion) in the United States and EU alone. Given the immense impact at both the personal and economic levels, this review considers how the plant-based production of recombinant proteins (e.g., vaccines, therapeutics, diagnostics, and laboratory reagents) can contribute to a global response in such an emergency scenario. Several recent publications describe in broad terms how plant-made countermeasures against SARS-CoV-2 can contribute to the global COVID-19 response (Capell et al., 2020; McDonald and Holtz, 2020; Rosales-Mendoza, 2020). This review will focus primarily on process development, manufacturing considerations, and evolving regulations to identify gaps and research needs, as well as regulatory processes and/or infrastructure investments that can help to build a more resilient pandemic response system. We first highlight the technical capabilities of plants, such as the speed of transient expression, making them attractive as a first-line response to counter pandemics, and then we discuss the regulatory pathway for plant-made pharmaceuticals (PMPs) in more detail. Next, we briefly present the types of plant-derived proteins that are relevant for the prevention, treatment, or diagnosis of disease. This sets the stage for our assessment of the requirements in terms of production costs and capacity to mount a coherent response to a pandemic, given currently available infrastructure and the intellectual property (IP) landscape. We conclude by comparing plant-based expression with conventional cell culture and highlight where investments are needed to adequately respond to pandemic diseases in the future. Due to the quickly evolving information about the pandemic, our statements are supported in some instances by data obtained from web sites (e.g., governmental publications). Accordingly, the scientific reliability has to be treated with caution in these cases.

TECHNICAL ASPECTS OF PLANT-BASED PRODUCTION SYSTEMS

Screening of Product Candidates

The development of a protein-based vaccine, therapeutic, or diagnostic reagent for a novel disease requires the screening of numerous expression cassettes, for example, to identify suitable regulatory elements (e.g., promoters) that achieve high levels of product accumulation, a sub-cellular compartment that ensures product integrity, as well as different product candidates to identify the most active and most amenable to manufacturing in plants (Buyel et al., 2013a; Kohli et al., 2015; DiCara et al., 2018; Spiegel et al., 2019; Kerwin et al., 2020). A major advantage of plants in this respect is the ability to test multiple product candidates and expression cassettes in parallel by the simple injection or infiltration of leaves or leaf sections with a panel of *Agrobacterium tumefaciens* clones carrying each variant cassette as part of the transferred DNA (T-DNA) in a binary transformation vector (Piotrzkowski et al., 2012; Norkunas et al., 2018; Rademacher et al., 2019). This procedure does not require sterile conditions, transfection reagents, or skilled staff, and can, therefore, be conducted in standard biosafety level 1 (BSL 1) laboratories all over the world. The method can produce samples of even complex proteins such as glycosylated monoclonal antibodies (mAbs) for analysis ~14 days after the protein sequence is available. With product accumulation in the range of 0.1–4.0 g kg^{-1} biomass (Sainsbury and Lomonossoff, 2008; Zischewski et al., 2015; Yamamoto et al., 2018), larger-scale quantities (several grams) can be supplied after 4–8 weeks (Shoji et al., 2012), making this approach ideal for emergency responses to sudden disease outbreaks. Potential bottlenecks include the preparation of sufficiently large candidate libraries, ideally in an automated manner as described for conventional expression systems, and the infiltration of plants with a large number (>100) of candidates. Also, leaf-based expression can result in a coefficient of variation (CV) >20% in terms of recombinant protein accumulation, which reduces the reliability of expression data (Buyel and Fischer, 2014a). The variability issue has been addressed to some extent by a parallelized leaf-disc assay at the cost of a further reduction in sample throughput (Piotrzkowski et al., 2012).

The reproducibility of screening was improved in 2018 by the development of plant cell pack technology, in which plant cell suspension cultures deprived of medium are used to form a plant tissue surrogate that can be infiltrated with *A. tumefaciens* in a 96-well microtiter plate format to produce milligram quantities of protein in an automated, high-throughput manner. The costs (without analysis) can be as low as €0.50 (US$0.60) per 60-mg sample with a product accumulation of ~100 mg kg^{-1} and can typically result in a CV of <5% (Gengenbach et al., 2020). These costs include the fermenter-based upstream production of plant cells as well as all materials and labor. The system can be integrated with the cloning of large candidate libraries, allowing a throughput of >1,000 samples per week, and protein is produced 3 days after infiltration. The translatability of cell pack data to intact plants was successfully demonstrated for three mAbs and several other proteins, including a toxin (Gengenbach et al., 2019; Rademacher et al., 2019). Therefore, cell packs allow the rapid and automated screening of product candidates such as vaccines and diagnostic reagents. In addition to recombinant proteins, the technology can, in principle, also be used to produce virus-like particles (VLPs) based on plant viruses, which further broadens its applicability for screening and product evaluation but, to our knowledge, according results had not been published as of September 2020. In the future, plant cell packs could be combined with a recently developed

[1]https://coronavirus.jhu.edu/map.html

method for rapid gene transfer to plant cells using carbon nanotubes (Demirer et al., 2019). Such a combination would not be dependent on bacteria for cloning (*Escherichia coli*) or gene transfer to plant cells (*A. tumefaciens*), thereby reducing the overall duration of the process by an additional 2–3 days (Demirer et al., 2019).

For the rapid screening of even larger numbers of candidates, cost-efficient cell-free lysates based on plant cells have been developed and are commercially available in a ready-to-use kit format. Proteins can be synthesized in ~24 h, potentially in 384-well plates, and the yields expressed as recombinant protein mass per volume of cell lysate can reach 3 mg ml^{-1} (Buntru et al., 2015). Given costs of ~€1,160 (US$1,363) ml^{-1} according to the manufacturer LenioBio (Germany), this translates to ~€400 ($470) mg^{-1} protein, an order of magnitude less expensive than the SP6 system (Promega, United States), which achieves 0.1 mg ml^{-1} at a cost of ~€360 ($423) ml^{-1} (€3,600 or $4,230 mg^{-1}) based on the company's claims. Protocol duration and necessary labor are comparable between the two systems and so are the proteins used to demonstrate high expression, e.g., luciferase. However, the scalability of the plant-cell lysates is currently limited to several hundred milliliters, and transferability to intact plants has yet to be demonstrated, i.e., information about how well product accumulation in lysates correlates with that in plant tissues. Such correlations can then form the basis to scale-up lysate-based production to good manufacturing practice (GMP)-compliant manufacturing in plants using existing facilities. Therefore, the cell packs are currently the most appealing screening system due to their favorable balance of speed, throughput, and translatability to whole plants for large-scale production.

In any pandemic, the pathogen genome has to be sequenced, made publically available, and freely disseminated in the global scientific community (for which there are currently no well-defined workflows) to accelerate therapeutic and vaccine development. Once sequence information is available, a high priority is the rapid development, synthesis, and distribution of DNA sequences coding for individual viral open reading frames. These reagents are not only important for screening subunit vaccine targets but also as enabling tools for research into the structure, function, stability, and detection of the virus (Khailany et al., 2020). Because many viral pathogens (including SARS-CoV-2) mutate over time, the sequencing of clinical virus samples is equally important to enable the development of countermeasures to keep pace with virus evolution (Kupferschmidt, 2020). To ensure the broadest impact, the gene constructs must be codon optimized for expression in a variety of hosts (Hanson and Coller, 2018); cloned into plasmids with appropriate promoters, purification tags, and watermark sequences to identify them as synthetic and so that their origin can be verified (Liss et al., 2012); and made widely available at minimal cost to researchers around the world. Not-for-profit plasmid repositories, such as Addgene and DNASU, in cooperation with global academic and industry contributors, play an important role in providing and sharing these reagents. However, the availability of codon-optimized genes for plants and the corresponding expression systems is often limited (Webster et al., 2017). For example, there were 41,247 mammalian, 16,560 bacterial, and 4,721 yeast expression vectors in the Addgene collection as of August 2020, but only 1,821 for plants, none of which contained SARS-CoV-2 proteins. Sharing plant-optimized SARS-CoV-2 synthetic biology resources among the academic and industry research community working on PMPs would further accelerate the response to this pandemic disease.

Screening and process development can also be expedited by using modeling tools to identify relevant parameter combinations for experimental testing. For example, initial attempts have been made to establish correlations between genetic elements or protein structures and product accumulation in plants (Buyel et al., 2013a; Jansing and Buyel, 2019). Similarly, heuristic and model-based predictions can be used to optimize downstream processing (DSP) unit operations including chromatography (Buyel et al., 2013b; Buyel and Fischer, 2014c; Alam et al., 2018). Because protein accumulation often depends on multiple parameters, it is typically more challenging to model than chromatography and probably needs to rely on data-driven rather than mechanistic models. Based on results obtained for antibody production, a combination of descriptive and mechanistic models can reduce the number of experiments and thus the development time by 75% (Möller et al., 2019), which is a substantial gain when trying to counteract a global pandemic such as COVID-19. These models are particularly useful if combined with the high-throughput experiments described above. Techno-economic assessment (TEA) computer-aided design tools, based on engineering process models, can be used to design and size process equipment, solve material and energy balances, generate process flowsheets, establish scheduling, and identify process bottlenecks. TEA models have been developed and are publicly available for a variety of plant-based biomanufacturing facilities, including whole plant and plant cell bioreactor processes for production of mAbs (Nandi et al., 2016), antiviral lectins (Alam et al., 2018), therapeutics (Tusé et al., 2014; Corbin et al., 2020), and antimicrobial peptides (McNulty et al., 2020). These tools are particularly useful for the development of new processes because they can indicate which areas would benefit most from focused research and development (R&D) efforts to increase throughput, reduce process mass intensity, and minimize overall production costs.

Transient Protein Expression in Plants

The rapid production of protein-based countermeasures for SARS-CoV-2 will most likely, at least initially, require biomanufacturing processes based on transient expression rather than stable transgenic lines. Options include the transient transfection of mammalian cells (Gutiérrez-Granados et al., 2018), baculovirus-infected insect cell expression systems (Contreras-Gomez et al., 2014), cell-free expression systems for *in vitro* transcription and translation (Zemella et al., 2015), and transient expression in plants (Sainsbury, 2020). The longer-term production of these countermeasures may rely on mammalian or plant cell lines and/or transgenic plants, in which the expression cassette has been stably integrated into

the host genome, but these will take months or even years to develop, optimize, and scale-up. Among the available transient expression systems, only plants can be scaled-up to meet the demand for COVID-19 countermeasures without the need for extensive supply chains and/or complex and expensive infrastructure, thus ensuring low production costs (Nandi et al., 2016). These manufacturing processes typically use *Nicotiana benthamiana* (a relative of tobacco) as the production host and each plant can be regarded as a biodegradable, single-use bioreactor (Buyel, 2018). The plants are grown either in greenhouses or indoors, either hydroponically or in a growth substrate, often in multiple layers to minimize the facility footprint, and under artificial lighting such as LEDs. In North America, large-scale commercial PMP facilities have been built in Bryan, TX (Caliber Biotherapeutics, acquired by iBio), Owensboro, KY (Kentucky Bioprocessing), Durham, NC (Medicago), and Quebec, Canada (Medicago; Pogue et al., 2010; Holtz et al., 2015; Lomonossoff and D'Aoust, 2016). The plants are grown from seed until they reach 4–6 weeks of age before transient expression, which is typically achieved by infiltration using recombinant *A. tumefaciens* carrying the expression cassette (as described above for screening) or by the introduction of a viral expression vector such as tobacco mosaic virus (TMV), for example, the GENEWARE platform (Pogue et al., 2010). For transient expression by infiltration with *A. tumefaciens*, the plants are turned upside down and the aerial portions are submerged in the bacterial suspension. A moderate vacuum is applied for a few minutes, and when it is released, the bacteria are drawn into the interstitial spaces within the leaves. The plants are removed from the suspension and moved to an incubation room/chamber for 5–7 days for recombinant protein production. A recent adaptation of this process replaces vacuum infiltration with the aerial application of the *A. tumefaciens* suspension mixed with a surfactant. The reduced surface tension of the carrier solution allows the bacteria to enter the stomata, achieving a similar effect to agroinfiltration (Hahn et al., 2015). This agrospray strategy can be applied anywhere, thus removing the need for vacuum infiltrators and associated equipment (and transfer to and from the infiltration units). For transient expression using viral vectors, the viral suspension is mixed with an abrasive for application to the leaves using a pressurized spray, and the plants are incubated for 6–12 days as the recombinant protein is produced. Large-scale production facilities have an inventory of plants at various stages of growth and they are processed (by infiltration or inoculation) in batches. Depending on the batch size (the number of plants per batch), the vacuum infiltration throughput, and the target protein production kinetics, the infiltration/incubation process time is 5–8 days. The inoculation/incubation process is slightly longer at 6–13 days.

The overall batch time from seeding to harvest is 33–55 days depending on the optimal plant age, transient expression method, and target protein production kinetics (Pogue et al., 2010; Holtz et al., 2015; Lomonossoff and D'Aoust, 2016). Importantly, plant growth can be de-coupled from infiltration, so that the plants are kept at the ready for instant use, which reduces the effective first-reaction batch time from gene to product to ~10–15 days if a platform downstream process is available (e.g., Protein A purification for mAbs). The time between batches can be reduced even further to match the longest unit operation in the upstream or downstream process. The number of plants available under normal operational scenarios is limited to avoid expenditure, but more plants can be seeded and made available in the event of a pandemic emergency. This would allow various urgent manufacturing scenarios to be realized, for example, the provision of a vaccine candidate or other prophylactic to first-line response staff.

Processing of Plant Biomass

The speed of transient expression in plants allows the rapid adaptation of a product even when the process has already reached manufacturing scale. For example, decisions about the nature of the recombinant protein product can be made as little as 2 weeks before harvest because the cultivation of bacteria (including a seed train) takes less than 7 days (Houdelet et al., 2017) and the post-infiltration incubation of plants takes ~5–7 days. By using large-scale cryo-stocks of ready-to-use *A. tumefaciens*, the decision can be delayed until the day of infiltration and thus 5–7 days before harvesting the biomass (Spiegel et al., 2019). This flexibility is desirable in an early pandemic scenario because the latest information on improved drug properties can be channeled directly into production, for example, to produce gram quantities of protein that are required for safety assessment, pre-clinical and clinical testing, or even compassionate use if the fatality rate of a disease is high (see section "European guidance for COVID-19 medicine developers and companies" below).

Although infiltration is typically a discontinuous process requiring stainless-steel equipment due to the vacuum that must be applied to plants submerged in the bacterial suspension, most other steps in the production of PMPs can be designed for continuous operation, incorporating single-use equipment and thus complying with the proposed concept for biofacilities of the future (Klutz et al., 2015). Accordingly, continuous harvesting and extraction can be carried out using appropriate equipment such as screw presses (Buyel and Fischer, 2015), whereas continuous filtration and chromatography can take advantage of the same equipment successfully used with microbial and mammalian cell cultures (David et al., 2020). Therefore, plant-based production platforms can benefit from the same >4-fold increase in space-time yield (e.g., measured in g L^{-1} d^{-1} or g m^{-2} d^{-1}) that can be achieved by continuous processing with conventional cell-based systems (Arnold et al., 2019). As a consequence, a larger amount of product can be delivered earlier, which can help to prevent the disease from spreading once a vaccine becomes available.

In addition to conventional chromatography, several generic purification strategies have been developed to rapidly isolate products from crude plant extracts in a cost-effective manner (Rosenberg et al., 2015; Buyel et al., 2016). Due to their generic nature, these strategies typically require little optimization and can immediately be applied to products meeting the necessary requirements, which reduces the time needed to respond to a new disease. For example, purification by ultrafiltration/

diafiltration is attractive for both small (<30 kDa) and large (>500 kDa) molecules because they can be separated from plant host cell proteins (HCPs), which are typically 100–450 kDa in size, under gentle conditions such as neutral pH to ensure efficient recovery (Opdensteinen et al., 2018). This technique can also be used for simultaneous volume reduction and optional buffer exchange, reducing the overall process time and ensuring compatibility with subsequent chromatography steps. HCP removal triggered by increasing the temperature (~65°C) and/or reducing the pH (pH < 4.5) is mostly limited to stable proteins such as antibodies, and especially, the former method may require extended product characterization to ensure the function of products, such as vaccine candidates, is not compromised (Beiss et al., 2015; Menzel et al., 2018). The fusion of purification tags to a protein product can be tempting to accelerate process development when time is pressing during an ongoing pandemic. These tags can stabilize target proteins *in planta* while also facilitating purification by affinity chromatography or non-chromatographic methods such as aqueous two-phase systems (Bornhorst and Falke, 2010; Reuter et al., 2014). On the downside, such tags may trigger unwanted aggregation or immune responses that can reduce product activity or even safety (Khan et al., 2012). Some tags may be approved in certain circumstances (Jin et al., 2017), but their immunogenicity may depend on the context of the fusion protein.

The substantial toolkit available for rapid plant biomass processing and the adaptation of even large-scale plant-based production processes to new protein products ensure that plants can be used to respond to pandemic diseases with at least an equivalent development time and, in most cases, a much shorter one than conventional cell-based platforms. Although genetic vaccines for SARS-CoV-2 have been produced quickly (e.g., mRNA vaccines by Pfizer/BioNTech and Moderna/NIAID), they have never been manufactured at the scale needed to address a pandemic and their stability during transport and deployment to developing world regions remains to be shown.

REGULATORY CONSIDERATIONS FOR PRODUCT APPROVAL AND DEPLOYMENT DURING PUBLIC HEALTH EMERGENCIES

Regulatory Oversight During Non-emergency Situations

Regulatory oversight is a major and time-consuming component of any drug development program, and regulatory agencies have needed to revise internal and external procedures in order to adapt normal schedules for the rapid decision-making necessary during emergency situations. Just as important as rapid methods to express, prototype, optimize, produce, and scale new products are the streamlining of regulatory procedures to maximize the technical advantages offered by the speed and flexibility of plants and other high-performance manufacturing systems. Guidelines issued by regulatory agencies for the development of new products, or the repurposing of existing products for new indications, include criteria for product manufacturing and characterization, containment and mitigation of environmental risks, stage-wise safety determination, clinical demonstration of safety and efficacy, and various mechanisms for product licensure or approval to deploy the products and achieve the desired public health benefit.

Regardless of which manufacturing platform is employed, the complexity of product development requires that continuous scrutiny is applied from preclinical research to drug approval and post-market surveillance, thus ensuring that the public does not incur an undue safety risk and that products ultimately reaching the market consistently conform to their label claims. These goals are common to regulatory agencies worldwide, and higher convergence exists in regions that have adopted the harmonization of standards (e.g., the United States, EU, and Japan) as defined by the International Council for Harmonization (ICH),[2] in key product areas including quality, safety, and efficacy.

Summary of the United States and European Regulatory Approval Processes

Both the United States and the EU have stringent pharmaceutical product quality and clinical development requirements, as well as regulatory mechanisms to ensure product quality and public safety. Differences and similarities between regional systems have been discussed elsewhere (Downing et al., 2012; Sparrow et al., 2013; van Norman, 2016; Chiodin et al., 2019; Detela and Lodge, 2019) and are only summarized here.

Stated simply, the United States, EU, and other jurisdictions follow generally a two-stage regulatory process, comprising (a) clinical research authorization and monitoring and (b) result's review and marketing approval. The first stage involves the initiation of clinical research *via* submission of an Investigational New Drug (IND) application in the United States or its analogous Clinical Trial Application (CTA) in Europe. At the preclinical-clinical translational interphase of product development, a sponsor (applicant) must formally inform a regulatory agency of its intention to develop a new product and the methods and endpoints it will use to assess clinical safety and preliminary pharmacologic activity (e.g., a Phase I clinical study). Because the EU is a collective of independent Member States, the CTA can be submitted to a country-specific (national) regulatory agency that will oversee development of the new product.

The regulatory systems of the EU and the United States both allow pre-submission consultation on the proposed development programs *via* discussions with regulatory agencies or expert national bodies. These are known as pre-IND (PIND) meetings in the United States (FDA, 2017, 2020) and Investigational Medicinal Product Dossier (IMPD)[3] discussions in the EU. These meetings serve to guide the structure of the

[2]International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) website. Accessed July 14, 2020. https://www.ich.org/

[3]European Investigational Medicinal Product Dossiers. Accessed July 14, 2020. http://www.imp-dossier.eu/

clinical programs and can substantially reduce the risk of regulatory delays as the programs begin. PIND meetings are common albeit not required, whereas IMPD discussions are often necessary prior to CTA submission. At intermediate stages of clinical development (e.g., Phase II dose and schedule optimization studies), pauses for regulatory review must be added between clinical study phases. Such End of Phase (EOP) review times may range from one to several months depending on the technology and disease indication. In advanced stages of product development after pivotal, placebo-controlled randomized Phase III studies are complete, drug approval requests that typically require extensive time (see below) for review and decision-making on the part of the regulatory agencies.

In the United States, the Food and Drug Administration (FDA) controls the centralized marketing approval/authorization/licensing (depending on product class and indication) of a new product, a process that requires in-depth review and acceptance of a New Drug Application (NDA) for chemical entities, or a Biologics License Application (BLA) for biologics, the latter including PMP proteins. The EU follows both decentralized (national) processes as well as centralized procedures covering all Member States. The Committee for Medicinal Products for Human Use (CHMP), part of the European Medicines Agency (EMA), has responsibilities similar to those of the FDA and plays a key role in the provision of scientific advice, evaluation of medicines at the national level for conformance with harmonized positions across the EU, and the centralized approval of new products for market entry in all Member States.

Regulatory Approval Is a Slow and Meticulous Process by Design

The statute-conformance review procedures practiced by the regulatory agencies require considerable time because the laws were established to focus on patient safety, product quality, verification of efficacy, and truth in labeling. The median times required by the FDA, EMA, and Health Canada for full review of NDA applications were reported to be 322, 366, and 352 days, respectively (Downing et al., 2012; van Norman, 2016). Collectively, typical interactions with regulatory agencies will add more than 1 year to a drug development program. Although these regulatory timelines are the *status quo* during normal times, they are clearly incongruous with the needs for rapid review, approval, and deployment of new products in emergency use scenarios, such as emerging pandemics.

Regulation of PMP Products in the United States and Europe

Plant-made intermediates, including reagents for diagnostics, antigens for vaccines, and bioactive proteins for prophylactic and therapeutic medical interventions, as well as the final products containing them, are subject to the same regulatory oversight and marketing approval pathways as other pharmaceutical products. However, the manufacturing environment as well as the peculiarities of the plant-made active pharmaceutical ingredient (API) can affect the nature and extent of requirements for compliance with various statutes, which in turn will influence the speed of development and approval. In general, the more contained the manufacturing process and the higher the quality and safety of the API, the easier it has been to move products along the development pipeline. Guidance documents on quality requirements for plant-made biomedical products exist and have provided a framework for development and marketing approval (FDA, 2002; EMA, 2006).

Upstream processes that use whole plants grown indoors under controlled conditions, including plant cell culture methods, followed by controlled and contained downstream purification, have fared best under regulatory scrutiny. This is especially true for processes that use non-food plants such as *Nicotiana* species as expression hosts. The backlash over the Prodigene incident of 2002 in the United States has refocused subsequent development efforts on contained environments (Ellstrand, 2003). In the United States, field-based production is possible and even practiced, but such processes require additional permits and scrutiny by the United States Department of Agriculture (USDA). In May 2020, to encourage innovation and reduce the regulatory burden on the industry, the USDA's Agricultural Plant Health Inspection Service (APHIS) revised legislation covering the interstate movement or release of genetically modified organisms (GMOs) into the environment in an effort to regulate such practices with higher precision [SECURE Rule revision of 7 Code of Federal Regulations (CFR) 340].[4] The revision will be implemented in steps (final implementation is scheduled for October 2021) and could facilitate the field-based production of PMPs.

In contrast, the production of PMPs using GMOs or transient expression in the field comes under heavy regulatory scrutiny in the EU, and several statutes have been developed to minimize environmental, food, and public risk. Many of these regulations focus on the use of food species as hosts. The major perceived risks of open-field cultivation are the contamination of the food/feed chain, and gene transfer between GM and non-GM plants. This is true today even though containment and mitigation technologies have evolved substantially since those statutes were first conceived, with the advent and implementation of transient and selective expression methods; new plant breeding technologies; use of non-food species; and physical, spatial, and temporal confinement (Passmore, 2012; Sparrow et al., 2013; Menary et al., 2020).

The United States and the EU differ in their philosophy and practice for the regulation of PMP products. In the United States, regulatory scrutiny is at the product level, with less focus on how the product is manufactured. In the EU, much more focus is placed on assessing how well a manufacturing process conforms to existing statutes. Therefore, in the United States, PMP products and reagents are regulated under pre-existing sections of the United States CFR, principally under various parts of Title 21 (Food and Drugs), which also apply

[4]USDA/APHIS SECURE Rule revision to 7 CFR 340. Accessed July 17, 2020. https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/biotech-rule-revision/secure-rule/secure-about/340_2017_perdue_biotechreg

to conventionally sourced products. These include current good manufacturing practice (cGMP) covered by 21 CFR Parts 210 and 211, good laboratory practice (GLP) toxicology (21 CFR 58), and a collection of good clinical practice (CGP) requirements specified by the ICH and accepted by the FDA (especially ICH E6 R1, R2 and draft R3). In the United States, upstream plant cultivation in containment can be practiced using qualified methods to ensure consistency of vector, raw materials, and cultivation procedures and/or, depending on the product, under good agricultural and collection practices (GACP). For PMP products, cGMP requirements do not come into play until the biomass is disrupted in a fluid vehicle to create a process stream. All process operations from that point forward, from crude hydrolysate to bulk drug substance and final drug product, are guided by 21 CFR 210/211 (cGMP).

In Europe, biopharmaceuticals regardless of manufacturing platform are regulated by the EMA, and the Medicines and Healthcare products Regulatory Agency (MHRA) in the United Kingdom. Pharmaceuticals from GM plants must adhere to the same regulations as all other biotechnology-derived drugs. These guidelines are largely specified by the European Commission (EC) in Directive 2001/83/EC and Regulation (EC) No 726/2004. However, upstream production in plants must also comply with additional statutes. Cultivation of GM plants in the field constitutes an environmental release and has been regulated by the EC under Directive 2001/18/EC and 1829/2003/EC if the crop can be used as food/feed (Passmore, 2012). The production of PMPs using whole plants in greenhouses or cell cultures in bioreactors is regulated by the "Contained Use" Directive 2009/41/EC, which are far less stringent than an environmental release and do not necessitate a fully-fledged environmental risk assessment. Essentially, the manufacturing site is licensed for contained use and production proceeds in a similar manner as a conventional facility using microbial or mammalian cells as the production platform.

With respect to GMP compliance, the major differentiator between the regulation of PMP products and the same or similar products manufactured using other platforms is the upstream production process. This is because many of the DSP techniques are product-dependent and, therefore, similar regardless of the platform, including most of the DSP equipment, with which regulatory agencies are already familiar. Of course, the APIs themselves must be fully characterized and shown to meet designated criteria in their specification, but this applies to all products regardless of source.

Regulatory Oversight During Public Health Emergency Situations

During a health emergency, such as the COVID-19 pandemic, regulatory agencies worldwide have re-assessed guidelines and restructured their requirements to enable the accelerated review of clinical study proposals, to facilitate clinical studies of safety and efficacy, and to expedite the manufacturing and deployment of re-purposed approved drugs as well as novel products (**Tables 1** and **2**). These revised regulatory procedures could be implemented again in future emergency situations. It is also possible that some of the streamlined procedures that can expedite product development and regulatory review and approval will remain in place even in the absence of a health emergency, permanently eliminating certain redundancies and bureaucratic requirements. Changes in the United States and European regulatory processes are highlighted, with a cautionary note that these modified procedures are subject to constant review and revision to reflect an evolving public health situation.

TABLE 1 | United States Food and Drug Administration (FDA) Coronavirus Treatment Acceleration Program (CTAP) emergency response timelines.

Task or function	Response time to sponsor's request	
	Typical	Emergency
Providing information on regulatory processes to develop or evaluate new drug and biologic therapies	<30 days	1 day
Providing rapid, interactive input on most development plans (e.g., PIND summary documents)	<60 days	<72 h
Providing ultra-rapid review and comments on proposed clinical protocols	Variable (case specific)	<24 h (case specific)
Completing review of single-patient expanded access requests	Variable (case specific)	<3 h
Working closely with applicants and other regulatory agencies to expedite quality assessments for products to treat COVID-19 patients and to transfer manufacturing to alternative or new sites to avoid supply disruption	N/A	Variable but expedited (case specific)

Adapted from: FDA (2020) and FDA Press Announcement of March 31, 2020.

United States FDA Coronavirus Treatment Acceleration Program

In the spring of 2020, the FDA established a special emergency program for candidate diagnostics, vaccines, and therapies for SARS-CoV-2 and COVID-19. The Coronavirus Treatment Acceleration Program (CTAP)[5] aims to utilize every available method to move new treatments to patients in need as quickly as possible, while simultaneously assessing the safety and efficacy of new modes of intervention. As of September 2020, CTAP was overseeing more than 300 active clinical trials for new treatments (>30 antivirals, >30 cell and gene therapies, >100 immunomodulators, >40 neutralizing antibodies, and >70 combination products and other categories) and was reviewing nearly 600 preclinical-stage programs for new medical interventions.

Responding to pressure for procedural streamlining and rapid response, the FDA refocused staff priorities, modified its guidelines to fit emergency situations, and achieved a remarkable set of benchmarks (**Table 1**). In comparison to the review and response timelines described in the previous section, the FDA's emergency response structure within CTAP is exemplary and, as noted, these changes have successfully

[5]FDA (2020). Coronavirus Treatment Acceleration Program (CTAP). Accessed July 17, 2020. https://www.fda.gov/drugs/coronavirus-covid-19-drugs/coronavirus-treatment-acceleration-program-ctap

TABLE 2 | European Medicines Agency (EMA) COVID-19 Pandemic Emergency Task Force response timelines.

Task or function	Response time to sponsor's request	
	Typical	**Emergency**
Rapid scientific advice, at no cost to sponsors, without pre-established submission deadlines, more flexible requirements for scientific dossiers (i.e., IMPD)	40–70 days	20 days
Rapid agreement of pediatric investigation plans and rapid compliance check	120 days from first contact, 10 days for EMA decision following review	20 days (minimum), 2 days
Rolling review, which is an *ad hoc* procedure used in emergency contexts to allow the EMA to continuously assess the data for an upcoming highly promising application as they become available (i.e., preceding the formal submission of a complete application for a NMA).	N/A	Variable and case-specific (accelerated from normal cycle times)
Marketing authorization is expected to benefit from rolling review to minimize the common practice of stopping and re-starting the review clocks. Should an applicant not wish to use rolling review, or in case the application has not been accepted for such review, the applicant may still apply for accelerated assessment. In such case, the review of the application is started only after validation of a complete application.	210 days active review time	The maximum active review time is reduced to 150 days, which in practice may even be shorter, according to the EMA
Extension of indication and extension of marketing authorization. The abovementioned support measures are also available for already authorized products being repurposed for COVID-19	Variable (case specific)	Variable (case specific)
Compassionate use: certain unauthorized medicinal products may be made available at a national level through compassionate use programs during a health emergency to facilitate the availability of new experimental treatments that are still under development	Variable (case specific)	Variable (case specific)

Adapted from: EMA (2020b).

enabled the rapid evaluation of hundreds of new diagnostics and candidate vaccine and therapeutic products.

European Guidance for COVID-19 Medicine Developers and Companies

The European Medicines Agency has established initiatives for the provision of accelerated development support and evaluation procedures for COVID-19 treatments and vaccines. These initiatives generally follow the EMA Emergent Health Threats Plan published at the end of 2018 (EMA, 2018). Similar to FDA's CTAP, EMA's COVID-19 Pandemic Emergency Task Force (EMA, 2020b) aims to coordinate and enable fast regulatory action during the development, authorization, and safety monitoring of products or procedures intended for the treatment and prevention of COVID-19 (EMA, 2020a). Collectively, this task force and its accessory committees are empowered to rapidly address emergency use requests (**Table 2**). Although perhaps not as dramatic as the aspirational time reductions established by the FDA's CTAP, the EMA's refocusing of resources and shorter response times to accelerate the development and approval of emergency use products are nevertheless laudable. In the United Kingdom, the MHRA[6] has also revised customary regulatory procedures to conform with COVID-19 emergency requirements by creating flexible regulations spanning early consultation, accelerated clinical development and review, and alternatives to facility inspection.

Implications of Streamlined Regulations for the Development of PMP Emergency Response Diagnostics, Vaccines, Prophylactics, and Therapeutics

During a public health emergency, one can envision the preferential utilization of existing indoor (contained) manufacturing capacity, at least in the near term. Processes making use of indoor cultivation (whole plants or cell culture) and conventional purification can be scrutinized more quickly by regulatory agencies due to their familiarity, resulting in shorter time-to-clinic and time-to-deployment periods. Although many, perhaps most, process operations will be familiar to regulators, there are some peculiarities of plant-based systems that differentiate them from conventional processes and, hence, require the satisfaction of additional criteria. Meeting these criteria is in no way insurmountable, as evidenced by the rapid planning and implementation of PMP programs for SARS-CoV-2/COVID-19 by PMP companies such as Medicago, iBio, and Kentucky Bioprocessing.[7]

[6]MHRA regulatory flexibilities resulting from coronavirus (COVID-19). Accessed July 17, 2020. https://www.gov.uk/guidance/mhra-regulatory-flexibilities-resulting-from-coronavirus-covid-19

[7]Medicago. Accessed July 17, 2020, https://www.medicago.com/en/pipeline/; iBio Inc. Accessed July 17, 2020, https://www.ibioinc.com/therapeutics-and-vaccines; and Kentucky BioProcessing Inc. Accessed July 17, 2020; https://www.kentuckybioprocessing.com/

Rationale for the Choice of Expression Platform

During emergency situations when speed is critical, transient expression systems (Gleba et al., 2014; Hahn et al., 2015) are more likely to be used than stable transgenic hosts, unless GM lines were developed in advance and can be activated on the basis of demand (e.g., lines expressing interferons, broad-spectrum antiviral lectins, or anti-inflammatory proteins). The vectors used for transient expression in plants are non-pathogenic in mammalian hosts and environmentally containable if applied indoors, and by now they are well known to the regulatory agencies. Accordingly, transient expression systems have been deployed rapidly for the development of COVID-19 interventions.

The vaccine space has shown great innovation and the World Health Organization (WHO) has maintained a database of COVID-19 vaccines in development,[8] including current efforts involving PMPs. For example, Medicago announced the development of its VLP-based vaccine against COVID-19 in March 2020, within 20 days of receiving the virus genome sequence, and initiated a Phase I safety and immunogenicity study in July.[9] If successful, the company expects to commence Phase II/III pivotal trials by late 2020. Medicago is also developing therapeutic antibodies for patients infected with SARS-CoV-2, and this program is currently in preclinical development. Furthermore, iBio has announced the preclinical development of two SARS-CoV-2 vaccine candidates, one VLP and one subunit vaccine.[10] Kentucky Bioprocessing has announced the production and preclinical evaluation of a conjugate TMV-based vaccine and has requested regulatory authorization for a first-in-human clinical study.[11] These efforts required only a few months to reach these stages of development and are a testament to the rapid expression, prototyping, and production advantages offered by transient expression.

Regulatory Bias: Process vs. Product

The PMP vaccine candidates described above are all being developed by companies in North America. The rapid translation of PMPs from bench to clinic reflects the conformance of chemistry, manufacturing, and control (CMC) procedures on one hand, and environmental safety and containment practices on the other, with existing regulatory statutes. This legislative system has distinct advantages over the European model, by offering a more flexible platform for discovery, optimization, and manufacturing. New products are not evaluated for compliance with GM legislation as they are in the EU and the United States (Sparrow et al., 2013) but are judged on their own merits. In contrast, development programs in the EU face additional hurdles even when using well-known techniques and even additional scrutiny if new plant breeding technologies are used, such as the CRISPR/Cas9 system or zinc finger nucleases (Menary et al., 2020).

Manufacturing Process and Facility Validation

Process validation in manufacturing is a necessary but resource-intensive measure required for marketing authorization. Following the publication of the Guidance for Industry "Process Validation: General Principles and Practices," and the EU's revision of Annex 15 to Directive 2003/94/EC for medicinal products for human use and Directive 91/412/EEC for veterinary use, validation became a life-cycle process with three principal stages: (1) process design, (2) process qualification, and (3) continuous process verification (FDA, 2011; EMA, 2015, 2020c). During emergency situations, the regulatory agencies have authorized the *concurrent validation* of manufacturing processes, including design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). Although new facility construction or repurposing/re-qualification may not immediately help with the current pandemic, given that only existing and qualified facilities will be used in the near term, it will position the industry for the rapid scale-up of countermeasures that may be applied over the next several years. An example is the April 2020 announcement by the Bill & Melinda Gates Foundation of its intention to fund "at-risk" development of vaccine manufacturing facilities to accommodate pandemic-relevant volumes of vaccines, before knowing which vaccines will succeed in clinical trials. Manufacturing at-risk with existing facilities is also being implemented on a global scale. The Serum Institute of India, the world's largest vaccine manufacturer, is producing at-risk hundreds of millions of doses of the Oxford University COVID-19 vaccine, while the product is still undergoing clinical studies.[12] Operation Warp Speed (OWS)[13] in the United States is also an at-risk multi-agency program that aims to expand resources to deliver 300 million doses of safe and effective but "yet-to-be-identified" vaccines for COVID-19 by January 2021, as part of a broader strategy to accelerate the development, manufacturing, and distribution of COVID-19 countermeasures, including vaccines, therapeutics, and diagnostics. The program had access to US$10 billion initially and can be readily expanded. As of August 2020, OWS had invested more than US$8 billion in various companies to accelerate manufacturing, clinical evaluation, and enhanced distribution channels for critical products.[14] For example, over a period of approximately 6 months, OWS helped to accelerate development, clinical evaluation (including Phase III pivotal studies), and at-risk manufacturing of two mRNA-based COVID-19 vaccines, with at least three more vaccines (including adenovirus-based and recombinant/baculovirus-based candidates) heading into advanced clinical development and large-scale manufacturing by September/October 2020.

[8]WHO 2020. DRAFT landscape of COVID-19 candidate vaccines. Accessed July 20, 2020. https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines

[9]Press release March 12, 2020. https://www.medicago.com/en/newsroom/medicago-announces-production-of-a-viable-vaccine-candidate-for-covid-19/

Press release July 14, 2020. https://www.medicago.com/en/newsroom/medicago-begins-phase-i-clinical-trials-for-its-covid-19-vaccine-candidate/

[10]iBio Inc. Press release June 4, 2020. https://www.ibioinc.com/news/ibio-announces-second-covid-19-vaccine-program

[11]British American Tobacco. Press release April 1, 2020. https://www.bat.com/group/sites/UK__9D9KCY.nsf/vwPagesWebLive/DOBN8QNL

[12]https://www.nytimes.com/2020/08/01/world/asia/coronavirus-vaccine-india.html

[13]Fact Sheet: Explaining Operation Warp Speed. Accessed July 18, 2020. https://www.hhs.gov/about/news/2020/06/16/fact-sheet-explaining-operation-warp-speed.html

[14]https://www.nytimes.com/2020/07/31/health/covid-19-vaccine-sanofi-gsk.html

At the time of writing, no PMP companies had received support from OWS. However, in March 2020, Medicago received CAD$7 million from the Government of Quebec (Medicago 2020c) and part of the Government of Canada CAD$192 million investment in expansion programs (Medicago, 2020d), both of which were applied to PMP vaccine and antibody programs within the company.[15]

Product Quality Attributes

Once manufactured, PMP products must pass quality criteria meeting a defined specification before they reach the clinic. These criteria apply to properties such as identity, uniformity, batch-to-batch consistency, potency, purity, stability (including API and the formation of aggregates, truncations, and low-molecular-weight species over time), residual DNA, absence of vector, low levels of plant metabolites such as pyridine alkaloids, and other criteria as specified in guidance documents (FDA, 2002; EMA, 2006). Host and process-related impurities in PMPs, such as residual HCP, residual vector, pyridine alkaloids from solanaceous hosts (e.g., nicotine, anabasine, and related alkaloids), phenolics, heavy metals (some of which can bioaccumulate in transfected plants), and other impurities that could introduce a health risk to consumers, have been successfully managed by upstream process controls and/or state-of-the-art purification methods and have not impeded the development of PMP products (Tusé, 2011; Ma et al., 2015).

The theoretical risk posed by non-mammalian glycans, once seen as the Achilles heel of PMPs, has not materialized in practice. Plant-derived vaccine antigens carrying plant-type glycans have not induced adverse events in clinical studies, where immune responses were directed primarily to the polypeptide portion of glycoproteins (McCormick et al., 2008; Tusé, 2011; Tusé et al., 2015). One solution for products intended for systemic administration, where glycan differences could introduce a pharmacokinetic and/or safety risk (such as mAbs or therapeutic enzymes), is the engineering of plant hosts to express glycoproteins with mammalian-compatible glycan structures (Strasser et al., 2004, 2014; Chen, 2016). For example, ZMapp (an antibody cocktail for the treatment of Ebola patients) was manufactured using the transgenic *N. benthamiana* line ΔXT/FT, expressing RNA interference constructs to knock down the expression of the enzymes XylT and FucT responsible for plant-specific glycans, as a chassis for transient expression of the mAbs (Hiatt et al., 2015).

In addition to meeting molecular identity and physicochemical quality attributes, PMP products must also be safe for use at the doses intended and efficacious in model systems *in vitro*, *in vivo*, and *ex vivo*, following the guidance documents listed above. Once proven efficacious and safe in clinical studies, successful biologic candidates can be approved *via* a BLA in the United States and a new marketing authorization (NMA) in the EU.

Deployment in Emergency Situations

In emergency situations, diagnostic reagents, vaccine antigens, and prophylactic and therapeutic proteins may be deployed prior to normal marketing authorization *via* fast-track procedures such as the FDA's emergency use authorization (EUA).[16] This applies to products approved for marketing in other indications that may be effective in a new emergency indication (repurposing), and new products that may have preclinical data but little or no clinical safety and efficacy data. Such pathways enable controlled emergency administration of a novel product to patients simultaneously with traditional regulatory procedures required for subsequent marketing approval.

In the United States, the FDA has granted EUAs for several diagnostic devices, personal protective devices, and certain other medical devices, and continuously monitors EUAs for drugs. For example, the EUA for chloroquine and hydroxychloroquine to treat COVID-19 patients was short-lived, whereas remdesivir remains under EUA evaluation for severe COVID-19 cases. The mRNA-based SARS-CoV-2 vaccines currently undergoing Phase III clinical evaluation by Pfizer/BioNTech and Moderna/NIAID, and other vaccines reaching advanced stages of development, are prime candidates for rapid deployment *via* the EUA process. No PMPs have yet been granted EUA, but plant-made antibodies and other prophylactic and therapeutic APIs may be evaluated and deployed *via* this route. One example of such a PMP candidate is griffithsin, a broad-spectrum antiviral lectin that could be administered as a prophylactic and/or therapeutic for viral infections, as discussed later.

The FDA's EUA is a temporary authorization subject to constant review and can be rescinded or extended at any time based on empirical results and the overall emergency environment. Similarly, the EU has granted *conditional marketing authorisation* (12-month duration) to rapidly deploy drugs such as remdesivir for COVID-19 in parallel with the standard marketing approval process for the new indication.

Accelerated Product Development *via* the Animal Rule

The regulations commonly known as the *animal rule* (US 21 CFR 314.600-650 for drugs; 21 CFR 601.90-95 for biologics; first effective on 1 July 2002)[17] allow for the approval of drugs and licensure of biologic products when human efficacy studies are not ethical and field trials to study the effectiveness of drugs or biologic products are not feasible. The animal rule is intended for drugs and biologics developed to reduce or prevent serious or life-threatening conditions caused by exposure to lethal or permanently disabling toxic chemical, biological, radiological, or nuclear substances. Under the animal rule, efficacy is established based on adequate and well-controlled studies in animal models of the human disease or condition of interest, and safety is evaluated under the pre-existing requirements for drugs and biologic products.

[15]Press release March 21, 2020. https://www.medicago.com/en/newsroom/government-of-quebec-providing-7-million-towards-medicagou2019s-covid-19-vaccine-development/

News release by Government of Canada March 23, 2020. https://pm.gc.ca/en/news/news-releases/2020/03/23/canadas-plan-mobilize-science-fight-covid-19

[16]Emergency Use Authorization. Accessed July 20, 2020. https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#covid19euas

[17]Animal Rule. Accessed July 20, 2020. https://www.fda.gov/drugs/nda-and-bla-approvals/animal-rule-approvals

As an example, the plant-derived mAb cocktail ZMapp for Ebola virus disease, manufactured by Kentucky Bioprocessing for Mapp Biopharmaceutical (San Diego, CA, United States)[18] and other partners, and deployed during the Ebola outbreak in West Africa in 2014, was evaluated only in primates infected with the Congolese variant of the virus (EBOV-K), with no randomized controlled clinical trial before administration to infected patients under a compassionate use protocol (Qiu et al., 2014). A conventional NIH-supported clinical study was conducted subsequent to first deployment (Davey et al., 2016).

Accelerated Product Development *via* Human Challenge Clinical Studies

Although the fast-track and streamlined review and authorization procedures described above can reduce time-to-deployment and time-to-approval for new or repurposed products, current clinical studies to demonstrate safety and efficacy generally follow traditional sequential designs. Products are licensed or approved for marketing based on statistically significant performance differences compared to controls, including placebo or standards of care, typically generated in large Phase III pivotal trials. One controversial proposal, described in a draft WHO report (World Health Organization Advisory Group, 2020), is to accelerate the assessment of safety and efficacy for emergency vaccines by administering the medical intervention with deliberate exposure of subjects to the threat agent in a challenge study.

Although the focus of the WHO draft report was on vaccines, the concept could conceivably be extended to non-vaccine prophylactics and therapeutics. Results could be generated quickly as the proportion of treated and control subjects would be known, as would the times of infection and challenge. Challenge studies in humans, also known as controlled human infection models or controlled human infection studies (CHIMs or CHIs, respectively), are fraught with ethical challenges but have already been used to assess vaccines for cholera, malaria, and typhoid (Cohen et al., 2002; Njue et al., 2018; Raymond et al., 2019). The dilemma for a pathogen like SARS-CoV-2 is that there is no rescue medication yet available for those who might contract the disease during the challenge, as there was for the other diseases, putting either study participants (due to current lack of effective treatment) or emergency staff (due to increased exposure) at risk (Shah et al., 2020).

Perspective for PMP Regulatory Approval

In the EU, the current regulatory environment is a substantial barrier to the rapid expansion of PMP resources to accelerate the approval and deployment of products and reagents at relevant scales in emergency situations. A recent survey of the opinions of key stakeholders in two EU Horizon 2020 programs (Pharma-Factory and Newcotiana), discussing the barriers and facilitators of PMPs and new plant breeding techniques in Europe, indicated that the current (EU and United Kingdom) regulatory environment was seen as one of the main barriers to the further development and scale-up of PMP programs (Menary et al., 2020). In contrast, regulations have not presented a major barrier to PMP development in the United States or Canada, other than the lengthy timescales required for regulatory review and product approval in normal times.

Realizing current national and global needs, regulatory agencies in the United States, Canada, the EU, and the United Kingdom have drastically reduced the timelines for product review, conditional approval, and deployment. In turn, the multiple unmet needs for rapidly available medical interventions have created opportunities for PMP companies to address such needs with gene expression tools and manufacturing resources that they already possess. This has enabled the ultra-rapid translation of product concepts to clinical development in record times – weeks to months instead of months to years – in keeping with other high-performance biomanufacturing platforms. The current pandemic situation, plus the tangible possibility of global recurrences of similar threats, may provide an impetus for new investments in PMPs for the development and deployment of products that are urgently needed.

PLANT-DERIVED PRODUCTS TO COUNTERACT PANDEMICS

Considerations for SARS-CoV-2 Vaccines

An effective vaccine is the best long-term solution to COVID-19 and other pandemics. Worldwide, governments are trying to expedite the process of vaccine development by investing in research, testing, production, and distribution programs, and streamlining regulatory requirements to facilitate product approval and deployment and are doing so with highly aggressive timelines (**Tables 1** and **2**). A key question that has societal implications beyond vaccine development is whether the antibody response to SARS-CoV-2 will confer immunity against re-infection and, if so, for how long? Will humans who recover from this infection be protected against a future exposure to the same virus months or years later? Knowing the duration of the antibody response to SARS-CoV-2 vaccines will also help to determine whether, and how often, booster immunizations will be needed if the initial response exceeds the protection threshold (Moore and Klasse, 2020). It is clear that some candidate vaccines will have low efficacy (e.g., protection in <50% of individuals), some vaccines will have high efficacy (e.g., protection in 70–80% of individuals or more), and some will decline over time and will need booster doses.

An updated list of the vaccines in development can be found in the WHO draft landscape of COVID-19 candidate vaccines.[19] As of August 2020, among the ~25 COVID vaccines in advanced development, five had entered Phase III clinical studies, led by Moderna/NIAID, Oxford University/Astra Zeneca, Pfizer/BioNTech, Sinopharm, and Sinova Biotech.[20] Most of these candidates are intended to induce antibody responses that

[18]Mapp Biopharmaceutical website. Accessed July 15, 2020. https://mappbio.com/

[19]https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines

[20]https://www.forbes.com/sites/stevensalzberg/2020/08/02/start-vaccinating-now/#49550ee6cf6e

neutralize SARS-CoV-2, thereby preventing the virus from entering target cells and infecting the host. In some cases, the vaccines may also induce antibody and/or cellular immune responses that eliminate infected cells, thereby limiting the replication of the virus within the infected host (Moore and Klasse, 2020). The induction of neutralizing antibodies directed against the SARS-CoV-2 spike (S) glycoprotein (see **Figures 1A,B** in Moore and Klasse, 2020) is considered a priority. The immunogens used to elicit neutralizing antibodies are various forms of the S protein, including the isolated receptor-binding domain (RBD; Callaway, 2020; Quinlan et al., 2020). The S protein variants can be expressed *in vivo* from DNA or mRNA constructs or recombinant adenovirus or vaccinia virus vectors, among others. Alternatively, they can be delivered directly as recombinant proteins with or without an adjuvant or as a constituent of a killed virus vaccine (see **Table 1** in Moore and Klasse, 2020). Many of these approaches are included among the hundreds of vaccine candidates now at the pre-clinical and animal model stages of development.

Antibody responses in COVID-19 patients vary greatly. Nearly all infected people develop IgM, IgG, and IgA antibodies against the SARS-CoV-2 nucleocapsid (N) and S proteins 1–2 weeks after symptoms become apparent, and the antibody titers (sometimes including neutralizing antibodies) remain elevated for at least several weeks after the virus is no longer detected in the convalescent patient (Huang et al., 2020; Long et al., 2020; Ma et al., 2020; Okba et al., 2020). The nature and longevity of the antibody response to coronaviruses are relevant to the potency and duration of vaccine-induced immunity.

By far the most immunogenic vaccine candidates for antibody responses are recombinant proteins (Moore and Klasse, 2020). The most straightforward approach to vaccine development would be based on inactivated or attenuated strains of SARS-CoV-2, but the production of sufficient material generally takes longer than is the case for subunit vaccines, high-level containment would be necessary to grow the virus before attenuation/inactivation, and the candidates would carry a risk of reacquired virulence (Regla-Nava et al., 2015). For subunit vaccines, target antigens must be selected carefully. Research on the original SARS-CoV strain indicated that the N protein is highly conserved among coronavirus families, including strains responsible for mild respiratory tract infections, thus suggesting the possibility of developing a universal vaccine. However, antibodies induced by N proteins did not provide protective immunity; likewise, the M and E proteins elicited only weak protective responses (Gralinski and Menachery, 2020). These studies helped to confirm the S protein as the most suitable target for eliciting a neutralizing humoral response.

Potential for Plant-Produced Vaccines

The entry of coronaviruses into host cells is facilitated by the S protein, which assembles into homotrimers on the virus surface (Tortorici and Veesler, 2019). The S protein comprises two functional subunits: S1, which binds to the host cell receptor, and S2, which facilitates the fusion of the viral and host cell membranes. For many coronaviruses, the S protein is cleaved at the boundary between the S1 and S2 subunits and mostly remains non-covalently bound in the pre-fusion conformation (Kirchdoerfer et al., 2018). Hence, the uptake of coronaviruses into host cells is a complex process that requires receptor binding and proteolytic processing of the S protein to stimulate membrane fusion and viral uptake (Walls et al., 2020).

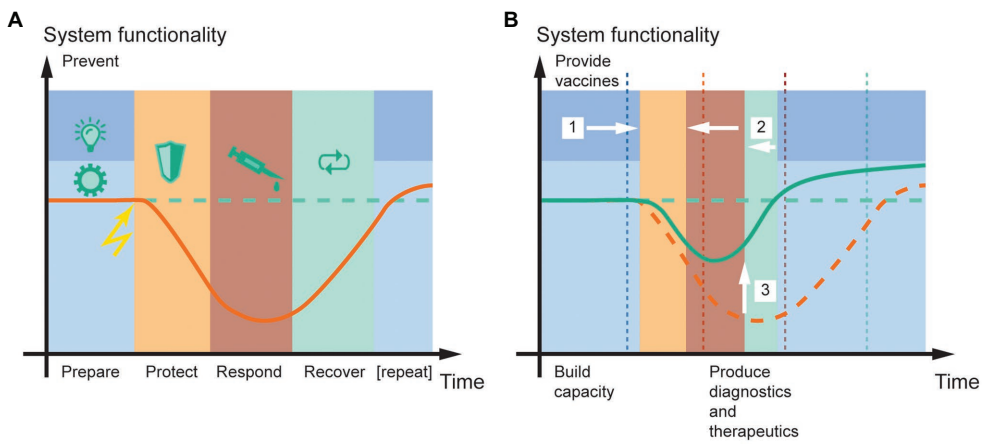

FIGURE 1 | System functionality in the face of a pandemic, and the potential for resilience engineering based on molecular farming in plants. **(A)** The resilience cycle typically consists of five phases [*prevent* (dark blue), *prepare* (light blue), *protect* (orange), *respond* (red), and *recover* (green); Thoma et al., 2016]. Upon encountering a negative event (lightning symbol), the system loses functionality (orange line) compared to the pre-event state (dashed green line) until *protect* and *response* measures stabilize it at a certain level and *recover* measures can begin. **(B)** Plant molecular farming can improve public health resilience to pandemic disease outbreaks by (1) enabling the large-scale production of vaccines that reduce virus spreading and the likelihood of recurrent outbreaks, (2) facilitating faster response and recovery by rapidly providing diagnostics, emergency vaccines, and therapeutics, and (3) thereby minimizing the loss of system functionality (green line). A prerequisite to deliver these benefits is that sufficient production capacity is built before the event, during the *prepare* phase. For comparison, the original time points of phase transitions in **(A)** are shown as dashed vertical lines in **(B)**. The time and functionality scales are in arbitrary units but drawn to scale between panels **(A,B)**. The curves illustrate typical scenarios but are not quantitative.

Companies currently developing COVID-19 vaccines are mainly expressing variants of the SARS-CoV-2 S1 protein or RBD. The S1 proteins of SARS-CoV and SARS-CoV-2 are heavily glycosylated, with an approximately equal mixture of complex and high-mannose glycans (Shajahan et al., 2020; Watanabe et al., 2020). It is unclear whether plant-type complex glycans would affect the efficacy of a recombinant SARS-CoV-2 S-protein vaccine expressed in plants. High-mannose glycans are generally conserved across higher eukaryotes, so it could be expected that at least some high-mannose glycans will be added during the expression of the antigen in plants. Furthermore, it is not clear whether sialic acid plays a role in host-receptor interactions. This is not generally present on native or recombinant plant glycoproteins, although engineered plant varieties that produce sialylated proteins have been described (Kallolimath et al., 2016).

Virus-like particles displaying SARS-CoV-2 antigens are larger than subunit vaccines, promoting recognition and internalization by antigen-presenting cells and thus triggering an adaptive immune response. Furthermore, the regular array of epitopes acts as pathogen-associated molecular patterns to induce strong cellular and humoral responses (Lua et al., 2014). VLPs are readily produced at scale in plants by molecular farming (Rybicki, 2017). The Medicago VLP platform is a prime example and has previously been used to produce millions of doses of seasonal influenza vaccines (D'Aoust et al., 2010; Wu, 2020). Furthermore, iBio is also using a proprietary system to develop VLP-based vaccines in *N. benthamiana* plants.

Production of Therapeutic Proteins in Plants

Given the time required to develop and test a COVID-19 vaccine, the possibility that a vaccine may not be effective in all populations due to the variability of immune responses, and the likelihood that SARS-CoV-2 will mutate, we foresee an ongoing demand for therapeutic proteins, such as mAbs, immunoadhesins, interferons, and antivirals, to either target the virus itself or reduce the severity of the associated acute respiratory syndrome (Capell et al., 2020; Rosales-Mendoza, 2020).

Monoclonal Antibodies

Several recombinant mAbs and antibody cocktails against COVID-19 are currently undergoing clinical development for therapeutic and prophylactic applications, including REGN-CoV-2 (Regeneron Therapeutics, Phase III), CSL312 (CSL Behring, Phase II), LY-CoV555 (Eli Lilly/AbCellera, Phase III), and TYO27 (Tychan, Phase I; Marovich et al., 2020). Many of the mAbs in development target the S-protein, aiming to block interactions with its receptor, angiotensin-converting enzyme 2 (ACE2). Efforts to exploit convalescent sera from patients who recovered from COVID-19 have helped identify antibodies with neutralizing potential. For example, Eli Lilly/AbCellera identified such an antibody in a blood sample from one of the first United States patients who recovered from the disease. The mAb was developed into LY-CoV555, a potent, neutralizing IgG1 that binds the S protein. In collaboration with NIAID, the product began Phase III clinical evaluation in high-risk assisted living facilities in August 2020.[21]

Most COVID-19 antibody products in development are produced in mammalian cells, but antibodies were among the first products of molecular farming in plants (Hiatt et al., 1989) and many different mAb products have been expressed, including complex secretory IgA (Wycoff, 2005). The dose of a mAb or mAb cocktail needed for the prevention or treatment of COVID-19 is currently unclear. About 9 g of the ZMapp cocktail was needed per treatment against Ebola virus and in a subsequent clinical study (Davey et al., 2016), but that dose level was selected from the outcome of studies in non-human primates (animal rule), which enabled rapid deployment under the compassionate use protocol and did not benefit from dose optimization studies in humans. Assuming similar doses, manufacturing scalability is likely to be a key challenge in the production of COVID-19 antibodies. The scaling up of conventional bioreactors is particularly challenging due to changes in mixing, mass transfer, and heat exchange, whereas transient expression in plants can be scaled in a linear manner because each plant is effectively an independent bioreactor, equating to a process of numbering up by increasing the plant inventory and throughput of the facility. Similarly, cost will be an important consideration. In 2013, total sales of mAbs produced in mammalian cell bioreactors amounted to ~€48.5 (US$57) billion for 8,182 kg of product, with an average sales price of ~€5,957 ($7,000) g^{-1} (Ecker et al., 2015). Production costs and capital expenses for the transient expression of mAbs in plants are estimated to be at least 50% lower than mammalian cell culture production facilities (Nandi et al., 2016), allowing manufacturers to reduce sales prices while still making some profit or providing these therapeutics at cost, and saving taxpayer resources.

Immunoadhesins

Another promising therapeutic approach is the use of plants to produce immunoadhesins (Wycoff et al., 2015). Such molecules combine the virus-binding region of a receptor, in this case ACE2, with the immunoglobulin Fc domain (Kruse, 2020; Qian and Hu, 2020). The ACE2 component acts as a decoy to bind SARS-CoV-2 *via* the S protein, preventing it from engaging with native ACE2 on the surface of human cells, while the Fc region confers a longer circulatory half-life and provides effector functions that promote viral clearance, as well as facilitating product purification by Protein A affinity chromatography during manufacturing. Immunoadhesins form dimers *via* disulfide linkages between Fc domains, increasing their avidity when binding the S protein. One advantage of this strategy is that if the coronavirus mutates to escape binding to the immunoadhesins, it would similarly lose affinity for native ACE2, reducing its infectivity. Likewise, the SARS virus that re-emerged in 2003–2004 had a lower affinity for ACE2 than the original isolate, resulting in less severe infections and no secondary transmission (Li et al., 2005). An additional

[21]https://investor.lilly.com/news-releases/news-release-details/lilly-initiates-phase-3-trial-ly-cov555-prevention-covid-19-long

advantage of this strategy is that exogenous ACE2 would compensate for lower ACE2 levels in the lungs during infection, thereby contributing to the treatment of acute respiratory distress. Several companies in the United States and the EU have developed recombinant ACE2 and ACE2-Fc fusion proteins for preclinical and clinical testing, although all these products are currently produced in mammalian cell lines (Qian and Hu, 2020). The impact of plant-specific complex glycans on the ability of ACE2-Fc to bind the RBD has been studied using molecular dynamic simulations and illustrates the important role that glycosylation may play in the interaction between the S protein and ACE2 (Bernardi et al., 2020).

Broad-Spectrum Antiviral Griffithsin

Griffithsin is a lectin that binds high-mannose glycans, and is currently undergoing clinical development as an antiviral against HIV-1. However, it also binds many other viruses that are pathogenic in humans, including HSV (Nixon et al., 2013), HCV (Meuleman et al., 2011), Nipah virus (Lo et al., 2020), Ebola virus, and coronaviruses including SARS-CoV and MERS (O'Keefe et al., 2010), and as recently determined, also SARS-CoV-2. A clinical product in development by University of Louisville is currently manufactured in *N. benthamiana* by Kentucky Bioprocessing using a TMV vector. The API is also undergoing preclinical development as a nasal spray for use as a non-vaccine prophylactic against coronaviruses, with clinical evaluation planned for 2020 (University of Pittsburgh, 2020). This candidate PMP antiviral could be deployed under the EUA pathway if found effective in controlled clinical studies.

Griffithsin is an interesting example of a product that is ideally matched to plant-based manufacturing because it is naturally produced by a marine alga. Griffithsin has been expressed with limited success in *E. coli* and tobacco chloroplasts, but better results have been achieved by transient expression in *N. benthamiana* using *A. tumefaciens* infiltration or TMV vectors, with expression levels of up to 1 g kg^{-1} fresh mass and recoveries of up to 90% (Vafaee et al., 2014; Fuqua et al., 2015a,b; Hahn et al., 2015). A TEA model of griffithsin manufactured in plants at initial commercial launch volumes (20 kg) for use in HIV microbicides revealed that process was readily scalable and (subject to efficiency improvements) could provide the needed market volumes of the lectin within an acceptable range of costs, even for cost-constrained markets (Alam et al., 2018). The manufacturing process was also assessed for environmental, health, and safety impact and found to have a highly favorable environmental output index with negligible risks to health and safety.

Production of Diagnostic Reagents in Plants

In addition to COVID-19 PCR tests, which detect the presence of SARS-CoV-2 RNA, there is a critical need for protein-based diagnostic reagents that test for the presence of viral proteins and thus report a current infection, as well as serological testing for SARS-CoV-2 antibodies that would indicate prior exposure, recovery, and possibly protection from subsequent infection. The most common formats for these tests are the ELISA and lateral flow assay. The design and quality of the binding reagents (antibodies to SARS-CoV-2 proteins for the viral antigen tests, or full-length/truncated SARS-CoV-2 proteins for the serological tests), along with other test conditions such as sample quality, play a key role in establishing the test specificity and selectivity, which determine the proportion of false positive and false negative results. Although the recombinant protein mass needed for diagnostic testing is relatively small (0.3–1.0 μg per test), the number of tests needed for the global population is massive, given that many individuals will need multiple and/or frequent tests. For example, 8 billion tests would require a total of ~2.5 kg purified recombinant protein, which is not an insurmountable target. However, although the production of soluble trimeric full-length S protein (as a diagnostic reagent for the serological test) by transient transfection in HEK293 cells has been improved by process optimization, current titers are only ~5 mg L^{-1} after 92 h (Esposito et al., 2020). Given a theoretical recovery of 50% during purification, a fermentation volume of 1,000 m^3 would be required to meet the demand for 2.5 kg of this product. Furthermore, to our knowledge, the transient transfection of mammalian cells has only been scaled up to ~0.1 m^3 (Girard et al., 2002). The transient expression of such protein-based diagnostic reagents in plants could increase productivity while offering lower costs and more flexibility to meet fluctuating demands or the need for variant products. Furthermore, diagnostic reagents can include purification tags with no safety restrictions, and quality criteria are less stringent compared to an injectable vaccine or therapeutic. Several companies have risen to the challenge of producing such reagents in plants, including Diamante (Verona, Italy), Leaf Expression Systems (Norwich, United Kingdom), and a collaborative venture between PlantForm, Cape Bio Pharms, Inno-3B, and Microbix.

Targeting the Resilience Cycle With Plant Molecular Farming

Resilience is the state of preparedness of a system, defining its ability to withstand unexpected, disastrous events (such as outbreaks of pandemic disease), and to preserve critical functionality while responding quickly so that normal functionality can be restored (Thoma et al., 2016). The concept was popularized by the 2011 Fukushima nuclear accident (Hollnagel and Fujita, 2013) but received little attention in the pharmaceutical sector until COVID-19. Of the 277 publications retrieved from the National Library of Medicine[22] on July 9th 2020 using the search terms "resilience" and "pandemic," 82 were evenly distributed between 2002 and 2019 (~5 per year) and 195 were published between January and July 2020.

Resilience can be analyzed by defining up to five stages of a resilient system under stress, namely *prevent* (optional), *prepare*, *protect*, *respond*, and *recover* (**Figure 1A**; Thoma et al., 2016). Here, *prevent* includes all measures to avoid the problem all together. In the context of COVID-19, this may have involved the banning of bush meat from markets in densely populated areas (Li et al., 2019). The *prepare* stage summarizes activities that build capacities to protect a system and pre-empt a disruptive event. In a pandemic scenario, this can include stockpiling personal protective equipment but also ensuring the availability

[22]https://pubmed.ncbi.nlm.nih.gov/

of rapid-response biopharmaceutical manufacturing capacity. The *protect* and *respond* stages involve measures that limit the loss of system functionality (e.g., emergency hospitalization capacity or gross domestic product) and minimize the time until it starts to recover, respectively. In terms of a disease outbreak, the former can consist of quarantining infected persons, especially in the healthcare sector, to avoid super-spreaders and maintain healthcare system operability (Steiner et al., 2020). The *response* measures may include passive strategies such as the adjustment of legislation, including social distancing and public testing regimes, or active steps such as the development of vaccines and therapeutics (Grein et al., 2020). Finally, the *recover* phase is characterized by regained functionality, for example by reducing the *protect* and *response* measures that limit system functionality, such as production lockdown. Ultimately, this can result in an increased overall system functionality at the end of a resilience cycle and before the start of the next "iteration" (**Figure 1B**). For example, a system such as society can be better prepared for a pandemic situation due to increased pharmaceutical production capacity or platforms like plants.

From our perspective, the production of recombinant proteins in plants could support the engineering of increased resilience primarily during the *prepare* and *respond* stages and, to a lesser extent, during the *prevent* and *recover* stages (**Figure 1B**). During the *prepare* stage, it is important to build sufficient global production capacity for recombinant proteins to mount a rapid and scalable response to a pandemic. These capacities can then be used during the *response* stage to produce appropriate quantities of recombinant protein for diagnostic (antigens and mAbs), prophylactic (vaccines or lectins), or therapeutic (mAbs) purposes as discussed above. The speed of the plant system will reduce the time taken to launch the *response* and *recovery* stages, and the higher the production capacity, the more system functionality can be maintained. The same capacities can also be used for the large-scale production of vaccines in transgenic plants if the corresponding pathogen has conserved antigens. This would support the *prevent* stage by ensuring a large portion of the global population can be supplied with safe and low-cost vaccines, for example, to avoid recurrent outbreaks of the disease. Similarly, existing agricultural capacities may be re-directed to pharmaceutical production as recently discussed (Webb et al., 2020). There will be indirect benefits during the *recover* phase because the speed of plant-based production systems will allow the earlier implementation of measures that bring system functionality back to normal, or at least to a "new or next normal." Therefore, we conclude that plant-based production systems can contribute substantially to the resilience of public healthcare systems in the context of an emergency pandemic.

PRODUCTION COST AND GLOBAL CAPACITY OF PLANT-BASED SYSTEMS

Product-Dependent and Process-Dependent Costs

The cost of pharmaceuticals is increasing in the United States at the global rate of inflation, and a large part of the world's population cannot afford the cost of medicines produced in developed nations[23] (Wineinger et al., 2019). Technical advances that reduce the costs of production and help to ensure that medicines remain accessible, especially to developing nations, are, therefore, welcome. Healthcare in the developing world is tied directly to social and political will, or the extent of government engagement in the execution of healthcare agendas and policies (Hefferon, 2014). Specifically, community-based bodies are the primary enforcers of government programs and policies to improve the health of the local population (Langridge, 2012; Tsekoa et al., 2020).

Planning for the expansion of a biopharmaceutical manufacturing program to ensure that sufficient product will be available to satisfy the projected market demand should ideally begin during the early stages of product development. Efficient planning facilitates reductions in the cost and time of the overall development process to shorten the time to market, enabling faster recouping of the R&D investment and subsequent profitability. In addition to the cost of the API, the final product form (e.g., injectable vs. oral formulation), the length and complexity of the clinical program for any given indication (e.g., infectious disease vs. oncology), and the course of therapy (e.g., vaccination vs. chronic care) have a major impact on cost. The cost of a pharmaceutical product, therefore, depends on multiple economic factors that ultimately shape how a product's sales price is determined (Azhakanandam et al., 2015). Product-dependent costs and pricing are common to all products regardless of platform.

Plant-based systems offer several options in terms of equipment and the scheduling of upstream production and DSP, including their integration and synchronization (Spiegel et al., 2019). Early process analysis is necessary to translate R&D methods into manufacturing processes (Nandi et al., 2005). The efficiency of this translation has a substantial impact on costs, particularly if processes are frozen during early clinical development and must be changed at a subsequent stage. Process-dependent costs begin with production of the API. The manufacturing costs for PMPs are determined by upstream (biomass) production and downstream recovery and purification costs. The cost of biopharmaceutical manufacturing depends mostly on protein accumulation levels, the overall process yield, and the production scale.

Techno-economic assessment models for the manufacture of biopharmaceuticals are rarely presented in detail, but analysis of the small number of available PMP studies (Nandi et al., 2005, 2016; Buyel and Fischer, 2012; Tusé et al., 2014; Walwyn et al., 2015; Alam et al., 2018; Corbin et al., 2020) has shown that the production of biopharmaceuticals in plants can be economically more attractive than in other platforms (Nandi et al., 2016; Gengenbach et al., 2019; Corbin et al., 2020). A simplified TEA model was recently proposed for the manufacture of mAbs using different systems, and this can be applied to any production platform, at least in principle, by focusing on the universal factors that determine the cost and efficiency of bulk drug manufacturing (Mir-Artigues et al., 2019).

[23]https://www.who.int/publications/10-year-review/chapter-medicines.pdf?ua=1

Minimal processing may be sufficient for oral vaccines and some environmental detection applications and can thus help to limit process development time and production costs (Rosenberg et al., 2015). However, most APIs produced in plants are subject to the same stringent regulation as other biologics, even in an emergency pandemic scenario (see section "Regulatory considerations for product approval and deployment during public health emergency situations"). It is, therefore, important to balance production costs with potential delays in approval that can result from the use of certain process steps or techniques. For example, flocculants can reduce consumables costs during clarification by 50% (Buyel and Fischer, 2014b), but the flocculants that have been tested are not yet approved for use in pharmaceutical manufacturing. Similarly, elastin-like peptides and other fusion tags can reduce the number of unit operations in a purification process, streamlining development and production, but only a few are approved for clinical applications (Jin et al., 2017). At an early pandemic response stage, speed is likely to be more important than cost, and production will, therefore, rely on well-characterized unit operations that avoid the need for process additives such as flocculants. Single-use equipment is also likely to be favored under these circumstances, because although more expensive than permanent stainless-steel equipment, it is also more flexible (modules of different sizes can be integrated as required) and there is no need for cleaning or cleaning validation between batches or campaigns, allowing rapid switching to new product variants if required. As the situation matures (and the production scale increases), a shift toward cost-saving operations and multi-use equipment would be more beneficial.

Capacity Requirements for an Effective Global Response to SARS-CoV-2

An important question is whether current countermeasure production capacity is sufficient to meet the needs for COVID-19 therapeutics, vaccines, and diagnostics. For example, a recent report from the Duke Margolis Center for Health Policy[24] estimated that ~22 million doses of therapeutic mAbs would be required to meet demand in the United States alone (including non-hospitalized symptomatic patients, hospitalized patients, and people in the same household as those who contract COVID-19), assuming one dose per patient and using rates of infection estimated in June 2020. The current demand for non-COVID-19 mAbs in the United States is >50 million doses per year[27], so COVID-19 has triggered a 44% increase in demand in terms of doses. Although the mAb doses required for pre-exposure and post-exposure COVID-19 treatment will not be known until the completion of clinical trials, it is likely to be 1–10 g per patient based on the dose ranges being tested and experience from other disease outbreaks such as Ebola (Davey et al., 2016). Accordingly, 22–222 tons of mAb would be needed per year, just in the United States. The population of the United States represents ~4.25% of the world's population, suggesting that 500–5,200 tons of mAb would be needed to meet global demand. The combined capacity of mammalian cell bioreactors (mainly in North America, Europe, and Asia) is ~6 million liters[27], and even assuming mAb titers of 2.2 g L^{-1}, which is the mean titer for well-optimized large-scale commercial bioreactors (Budzinski et al., 2019), a 13-day fed-batch culture cycle (28 batches per year), and a 30% loss in downstream recovery, the entirety of global mammalian cell bioreactor capacity could only provide ~259 tons of mAb per year. In other words, if the mammalian cell bioreactors all over the world were repurposed for COVID-19 mAb production, it would be enough to provide treatments for 50% of the global population if low doses (1 g or lower) were effective but only 5% if high doses (~10 g) were required. This illustrates the importance of identifying mAbs that are effective at the lowest dose possible, production systems that can achieve high titers and efficient downstream recovery, and the need for additional production platforms that can be mobilized quickly and that *do not rely* on bioreactor capacity. Furthermore, it is not clear how much of the existing bioreactor capacity can be repurposed quickly to satisfy pandemic needs, considering that ~78% of that capacity is dedicated to in-house products, many to treat cancer and other life-threatening diseases (Rader and Langer, 2018). The demand-on-capacity for vaccines will fare better, given the amount of protein per dose is 1×10^4 to 1×10^6 times lower than a therapeutic mAb. Even so, most of the global population (~7.8 billon people) may need to be vaccinated against SARS-CoV-2 over the next 2–3 years to eradicate the disease, and it is unclear whether sufficient quantities of vaccine can be made available, even if using adjuvants to reduce immunogen dose levels and/or the number of administrations required to induce protection. Even if an effective vaccine or therapeutic is identified, it may be challenging to manufacture and distribute this product at the scale required to immunize or treat most of the world's population (Hosangadi et al., 2020; Zerhouni et al., 2020). In addition, booster immunizations, viral antigen drift necessitating immunogen revision/optimization, adjuvant availability, and standard losses during storage, transport, and deployment may still make it difficult to close the supply gap.

Regardless of the product, the supply of recombinant proteins is challenging during emergency situations due to the simultaneous requirements for rapid manufacturing and extremely high numbers of doses. The realities we must address include: (1) the projected demand exceeds the entire manufacturing capacity of today's pharmaceutical industry (even if the production of all other biologics is paused); (2) there is a shortage of delivery devices (syringes) and the means to fill them; (3) there is insufficient lyophilization capacity to produce dry powder for distribution; and (4) distribution, including transportation and vaccination itself, will be problematic on such a large scale without radical changes in the public health systems of most countries. Vaccines developed by a given country will almost certainly be distributed within that country and to its allies/neighbors first and, thereafter, to countries willing to pay for priority. One solution to the product access challenge is to decentralize the production of countermeasures, and in fact one of the advantages of plant-based manufacturing

[24]https://healthpolicy.duke.edu/publications/covid-19-manufacturing-monoclonal-antibodies

is that it decouples developing countries from their reliance on the pharmaceutical infrastructure. Hence, local production facilities could be set up based on greenhouses linked to portable clean rooms housing disposable DSP equipment. In this scenario, the availability of multiple technology platforms, including plant-based production, can only be beneficial.

Impact of IP on Freedom to Operate for Rapid Manufacturing of Critical Supplies

Several approaches can be used to manage potential IP conflicts in public health emergencies that require the rapid production of urgently needed products. Licensing (including cross-licensing) of key IP to ensure freedom to operate (FTO) is preferred because such agreements are cooperative rather than competitive. Likewise, cooperative agreements to jointly develop products with mutually beneficial exit points offer another avenue for productive exploitation. These arrangements allow collaborating institutions to work toward a greater good.

Licensing has been practiced in past emergencies when PMP products were developed and produced using technologies owned by multiple parties. In the authors' experience, the ZMapp cocktail (deployed in the 2014 outbreak of Ebola virus) was subject to IP ownership by multiple parties covering the compositions, the gene expression system, manufacturing process technology/knowhow, and product end-use. Stakeholders included the Public Health Agency of Canada's National Microbiology Laboratory, the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Mapp Biopharmaceutical, Icon Genetics, and Kentucky Bioprocessing, among others. Kentucky Bioprocessing is also involved in a more recent collaboration to develop a SARS-CoV-2 vaccine candidate, aiming to produce 1–3 million doses of the antigen, with other stakeholders invited to take on the tasks of large-scale antigen conjugation to the viral delivery vector, product fill, and clinical development.[25]

Collaboration and pooling of resources and knowhow among big pharma/biopharma companies raises concerns over antitrust violations, which could lead to price fixing and other unfair business practices. With assistance from the United States Department of Justice (DOJ), this hurdle has been temporarily overcome by permitting several biopharma companies to share knowhow around manufacturing facilities and other information that could accelerate the manufacturing of COVID-19 mAb products.[26] Genentech (United States subsidiary of Roche), Amgen, AstraZeneca, Eli Lilly, GlaxoSmithKline, and AbCellera Biologics will share information about manufacturing facilities, capacity, raw materials, and supplies in order to accelerate the production of mAbs even before the products gain regulatory approval. This is driven by the realization that none of these companies can satisfy more than a small fraction of projected demands by acting alone. Under the terms imposed by the DOJ, the companies are not allowed to exchange information about manufacturing cost of goods or sales prices of their drugs, and the duration of the collaboration is limited to the current pandemic.

Yet another approach is a government-led strategy in which government bodies define a time-critical national security need that can only be addressed by sequestering critical technology (including IP, reagents, materials, software, facilities, knowhow, and existing stockpiles) controlled by the private sector. In the United States, for example, the Defense Production Act was first implemented in 1950 but has been reauthorized more than 50 times since then (FEMA, 2009). Similar national security directives exist in Canada and the EU. In the United States, the Defense Production Act gives the executive branch substantial powers, allowing the president, largely through executive order, to direct private companies to prioritize orders from the federal government. The president is also empowered to "allocate materials, services, and facilities" for national defense purposes. The Defense Production Act has been implemented during the COVID-19 crisis to accelerate manufacturing and the provision of medical devices and personal protective equipment, as well as drug intermediates.

Therefore, a two-tiered mechanism exists to create FTO and secure critical supplies: the first and more preferable involving cooperative licensing/cross-licensing agreements and manufacturing alliances, and alternatively (or if the first should fail), a second mechanism involving legislative directives.

CONCLUSION: ADVANTAGES OF PLANT MOLECULAR FARMING AS A FIRST RESPONSE TO GLOBAL PANDEMICS

Many companies have modified their production processes to manufacture urgently-required products in response to COVID-19, including distillers and perfume makers switching to sanitizing gels, textiles companies making medical gowns and face masks, and electronics companies making respirators.[27] Although this involves some challenges, such as production safety and quality requirements, it is far easier than the production of APIs, where the strict regulations discussed earlier in this article must be followed. The development of a mammalian cell line achieving titers in the 5 g L^{-1} range often takes 10–12 months or at least 5–6 months during a pandemic (Kelley, 2020). These titers can often be achieved for mAbs due to the similar properties of different mAb products and the standardized DSP unit operations (Gottschalk, 2016), but the titers of other biologics are often lower due to product toxicity or the need for bespoke purification strategies. Even if developmental obstacles are overcome, pharmaceutical companies may not be able to switch rapidly to new products because existing capacity is devoted to the manufacture of other important biopharmaceuticals. The capacity of mammalian cell culture facilities currently exceeds market demand by ~30% (Ecker and Seymour, 2020). Furthermore, contract manufacturing organizations (CMOs), which can respond most quickly to a demand for new products due to their flexible

[25]Press release April 1, 2020. https://www.bat.com/group/sites/UK__9D9KCY.nsf/vwPagesWebLive/DOBN8QNL

[26]https://www.bizjournals.com/sanfrancisco/news/2020/07/23/covid-coronavirus-doj-genentech-antibody-amgen.html

[27]Deutsche Welle, accessed July 21, 2020, https://www.dw.com/en/the-coronavirus-economy-switching-production-for-the-greater-good/a-52852712

business model, control only ~19% of that capacity. From our experience, this CMO capacity is often booked in advance for several months if not years, and little is available for short-term campaigns. Furthermore, even if capacity is available, the staff and consumables must be available too. Finally, there is a substantial imbalance in the global distribution of mammalian cell culture capacity, favoring North America and Europe. This concentration is risky from a global response perspective because these regions were the most severely affected during the early and middle stages of the COVID-19 pandemic, and it is, therefore, possible that this capacity would become unusable following the outbreak of a more destructive virus.

Patents covering several technologies related to transient expression in plants will end during or shortly after 2020, facilitating the broader commercial adoption of the technology. This could accelerate the development of new PMP products in a pandemic situation (see section "Plant-derived products to counteract pandemics"). However, PMP production capacity is currently limited. There are less than five large scale PMP facilities in operation, and we estimate that these facilities could manufacture ~2,200 kg of product per year, assuming a combined annual biomass output of ~1,100 tons as well as similar recombinant protein production (~2 g kg^{-1}) and DSP losses (30%) as for mammalian cells. Therefore, plant-based production certainly does currently not meet the anticipated demand for pandemic countermeasures. We have estimated a global demand of 500–5,200 tons per year for mAbs, depending on the dose, but only ~259 tons per year can be produced by using the current global capacity provided by mammalian cell bioreactors (at least based on publicly-available data) and plant-based systems currently represent less than 1% of the global production capacity of mammalian cell bioreactors. Furthermore, the number of plant molecular farming companies decreased from 37 to 23 between 2005 and 2020, including many large industry players that would be most able to fund further technology development (Fischer and Buyel, 2020). Nevertheless, the current plant molecular farming landscape has three advantages in terms of a global first-line response compared to mammalian cells. First, almost two thirds of global production capacity is held by CMOs or hybrid companies (working as CMOs while pursuing their own product pipeline), which can make their facilities available for production campaigns on short notice, as shown by their rapid response to COVID-19 allowing most to produce initial product batches by March 2020. In contrast, only ~20% of fermentation facilities are operated by CMOs (Seymour and Ecker, 2017). Second, despite the small number of plant molecular farming facilities, they are distributed around the globe with sites in the United States, Canada, United Kingdom, Germany, Japan, Korea, and South Africa, with more planned or under construction in Brazil and China (the largest facilities are currently located in North America and Europe). Finally, transient expression in plants is much faster than any other eukaryotic system with a comparable production scale, moving from gene to product within 20 days and allowing the production of up to 7,000 kg biomass per batch with product accumulation of up to 2 g kg^{-1} (Holtz et al., 2015; Zischewski et al., 2015). Even if the time required for protein production in mammalian cells can be reduced to 6 months as recently proposed (Kelley, 2020), Medicago has shown that transient expression in plants can achieve the same goals in less than 3 months (**Figure 2**). Therefore, the production of vaccines, therapeutics, and diagnostics in plants has the potential to function as a first

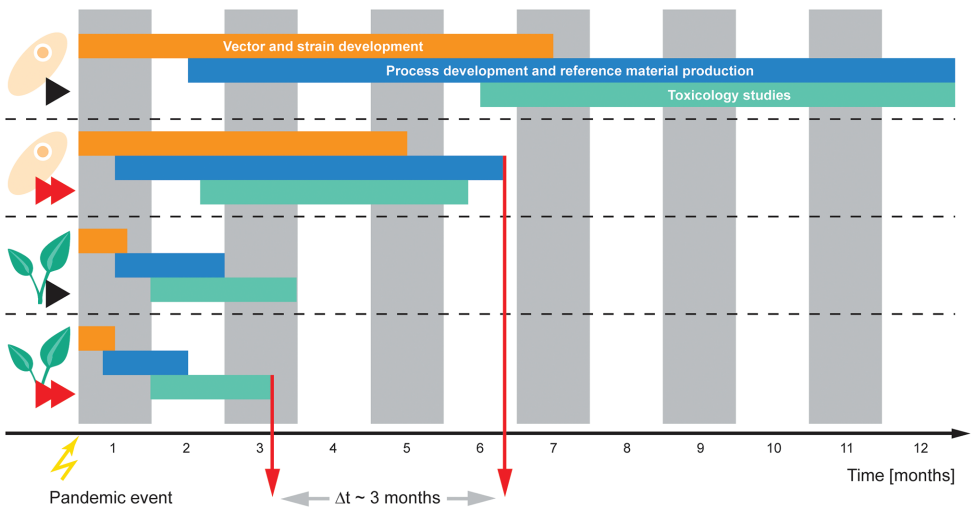

FIGURE 2 | Comparison of mammalian cell culture and transient expression in plants for the production of emergency biopharmaceuticals. Timelines for conventional scheduling (black arrows) and accelerated procedures (double red arrows) are based on recent publications and announcements, as well as the authors' experience (Shoji et al., 2011, 2012; Kelley, 2020). Transient expression allows much quicker vector development, process development, and reference material production, whereas the duration of toxicity studies is not reduced to the same degree because the time needed to run the studies remains the same regardless of the platform. Even so, transient expression in plants has the potential to reduce the emergency response time from gene sequence to clinical trial by at least 50% from ~6 months to <3 months.

line of defense against pandemics. Given the limited number and size of plant molecular farming facilities, we believe that the substantial investments currently being allocated to the building of biopharmaceutical production capacity should be shared with PMP production sites, allowing this technology to be developed as another strategy to improve our response to future pandemics.

AUTHOR CONTRIBUTIONS

DT, SN, KAM, and JB jointly wrote the manuscript. JB combined the contributions, revised the text, and prepared the figures. All authors contributed to the article and approved the submitted version.

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REFERENCES

Alam, A., Jiang, L., Kittleson, G. A., Steadman, K. D., Nandi, S., Fuqua, J. L., et al. (2018). Technoeconomic modeling of plant-based griffithsin manufacturing. *Front. Bioeng. Biotechnol.* 6:102. doi: 10.3389/fbioe.2018.00102

Anonymous (2014). Ebola: a call to action. *Nat. Med.* 20:967. doi: 10.1038/nm.3689

Arnold, L., Lee, K., Rucker-Pezzini, J., and Lee, J. H. (2019). Implementation of fully integrated continuous antibody processing: effects on productivity and COGm. *Biotechnol. J.* 14:e1800061. doi: 10.1002/biot.201800061

Azhakanandam, K., Silverstone, A., Daniell, H., and Davey, M. R. (2015). *Recent advancements in gene expression and enabling technologies in crop plants*. New York, NY: Springer.

Beiss, V., Spiegel, H., Boes, A., Kapelski, S., Scheuermayer, M., Edgue, G., et al. (2015). Heat-precipitation allows the efficient purification of a functional plant-derived malaria transmission-blocking vaccine candidate fusion protein. *Biotechnol. Bioeng.* 112, 1297–1305. doi: 10.1002/bit.25548

Bernardi, A., Huang, Y., Harris, B., Xiong, Y., Nandi, S., McDonald, K. A., et al. (2020). Development and simulation of fully glycosylated molecular models of ACE2-Fc fusion proteins and their interaction with the SARS-CoV-2 spike protein binding domain. *PLoS One* 15:e0237295. doi: 10.1371/journal.pone.0237295

Bornhorst, J. A., and Falke, J. J. (2010). Purification of proteins using polyhistidine affinity tags. *Methods Enzymol.* 326, 245–254. doi: 10.1016/s0076-6879(00)26058-8

Bradley, B. T., and Bryan, A. (2019). Emerging respiratory infections: the infectious disease pathology of SARS, MERS, pandemic influenza, and *Legionella*. *Semin. Diagn. Pathol.* 36, 152–159. doi: 10.1053/j.semdp.2019.04.006

Budzinski, K., Blewis, M., Dahlin, P., D'Aquila, D., Esparza, J., Gavin, J., et al. (2019). Introduction of a process mass intensity metric for biologics. *New Biotechnol.* 49, 37–42. doi: 10.1016/j.nbt.2018.07.005

Buntru, M., Vogel, S., Stoff, K., Spiegel, H., and Schillberg, S. (2015). A versatile coupled cell-free transcription-translation system based on tobacco BY-2 cell lysates. *Biotechnol. Bioeng.* 112, 867–878. doi: 10.1002/bit.25502

Buyel, J. F. (2018). Plant molecular farming—integration and exploitation of side streams to achieve sustainable biomanufacturing. *Front. Plant Sci.* 9:1893. doi: 10.3389/fpls.2018.01893

Buyel, J. F., and Fischer, R. (2012). Predictive models for transient protein expression in tobacco (*Nicotiana tabacum* L.) can optimize process time, yield, and downstream costs. *Biotechnol. Bioeng.* 109, 2575–2588. doi: 10.1002/bit.24523

Buyel, J. F., and Fischer, R. (2014a). Characterization of complex systems using the design of experiments approach: transient protein expression in tobacco as a case study. *J. Vis. Exp.* 1:e51216. doi: 10.3791/51216

Buyel, J. F., and Fischer, R. (2014b). Flocculation increases the efficacy of depth filtration during the downstream processing of recombinant pharmaceutical proteins produced in tobacco. *Plant Biotechnol. J.* 12, 240–252. doi: 10.1111/pbi.12132

Buyel, J. F., and Fischer, R. (2014c). Generic chromatography-based purification strategies accelerate the development of downstream processes for biopharmaceutical proteins produced in plants. *Biotechnol. J.* 9, 566–577. doi: 10.1002/biot.201300548

Buyel, J. F., and Fischer, R. (2015). A juice extractor can simplify the downstream processing of plant-derived biopharmaceutical proteins compared to blade-based homogenizers. *Process Biochem.* 50, 859–866. doi: 10.1016/j.procbio.2015.02.017

Buyel, J. F., Hubbuch, J., and Fischer, R. (2016). Comparison of tobacco host cell protein removal methods by blanching intact plants or by heat treatment of extracts. *J. Vis. Exp.* e54343. doi: 10.3791/54343

Buyel, J. F., Kaever, T., Buyel, J. J., and Fischer, R. (2013a). Predictive models for the accumulation of a fluorescent marker protein in tobacco leaves according to the promoter/5′UTR combination. *Biotechnol. Bioeng.* 110, 471–482. doi: 10.1002/bit.24715

Buyel, J. F., Woo, J. A., Cramer, S. M., and Fischer, R. (2013b). The use of quantitative structure-activity relationship models to develop optimized processes for the removal of tobacco host cell proteins during biopharmaceutical production. *J. Chromatogr. A* 1322, 18–28. doi: 10.1016/j.chroma.2013.10.076

Callaway, E. (2020). The race for coronavirus vaccines: a graphical guide. *Nature* 580, 576–577. doi: 10.1038/d41586-020-01221-y

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. -C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Chen, Q. (2016). Glycoengineering of plants yields glycoproteins with polysialylation and other defined N-glycoforms. *Proc. Natl. Acad. Sci. U. S. A.* 113, 9404–9406. doi: 10.1073/pnas.1610803113

Chiodin, D., Cox, E. M., Edmund, A. V., Kratz, E., and Lockwood, S. H. (2019). Regulatory affairs 101: introduction to investigational new drug applications and clinical trial applications. *Clin. Transl. Sci.* 12, 334–342. doi: 10.1111/cts.12635

Cohen, M. B., Giannella, R. A., Bean, J., Taylor, D. N., Parker, S., Hoeper, A., et al. (2002). Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. *Infect. Immun.* 70, 1965–1970. doi: 10.1128/iai.70.4.1965-1970.2002

Contreras-Gomez, A., Sanchez-Miron, A., Garcia-Camacho, F., Molina-Grima, E., and Chisti, Y. (2014). Protein production using the baculovirus-insect cell expression system. *Biotechnol. Prog.* 30, 1–18. doi: 10.1002/btpr.1842

Corbin, J. M., McNulty, M. J., Macharoen, K., McDonald, K. A., and Nandi, S. (2020). Technoeconomic analysis of semicontinuous bioreactor production of biopharmaceuticals in transgenic rice cell suspension cultures. *Biotechnol. Bioeng.* doi: 10.1002/bit.27475 [Epub ahead of print]

D'Aoust, M. A., Couture, M. M., Charland, N., Trepanier, S., Landry, N., Ors, F., et al. (2010). The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. *Plant Biotechnol. J.* 8, 607–619. doi: 10.1111/j.1467-7652.2009.00496.x

Davey, R. T., Dodd, L., Proschan, M. A., Neaton, J., Neuhaus Nordwall, J., Koopmeiners, J. S., et al. (2016). A randomized, controlled trial of ZMapp

for Ebola virus infection. *N. Engl. J. Med.* 375, 1448–1456. doi: 10.1056/NEJMoa1604330

David, L., Schwan, P., Lobedann, M., Borchert, S. -O., Budde, B., Temming, M., et al. (2020). Side-by-side comparability of batch and continuous downstream for the production of monoclonal antibodies. *Biotechnol. Bioeng.* 117, 1024–1036. doi: 10.1002/bit.27267

Demirer, G. S., Zhang, H., Goh, N. S., González-Grandío, E., and Landry, M. P. (2019). Carbon nanotube-mediated DNA delivery without transgene integration in intact plants. *Nat. Protoc.* 14, 2954–2971. doi: 10.1038/s41596-019-0208-9

Detela, G., and Lodge, A. (2019). EU regulatory pathways for ATMPs: standard, accelerated and adaptive pathways to marketing authorisation. *Mol. Ther. Methods Clin. Dev.* 13, 205–232. doi: 10.1016/j.omtm.2019.01.010

DiCara, D. M., Andersen, N., Chan, R., Ernst, J. A., Ayalon, G., Lazar, G. A., et al. (2018). High-throughput screening of antibody variants for chemical stability: identification of deamidation-resistant mutants. *MAbs* 10, 1073–1083. doi: 10.1080/19420862.2018.1504726

Downing, N. S., Aminawung, J. A., Shah, N. D., Braunstein, J. B., Krumholz, H. M., and Ross, J. S. (2012). Regulatory review of novel therapeutics—comparison of three regulatory agencies. *N. Engl. J. Med.* 366, 2284–2293. doi: 10.1056/NEJMsa1200223

Ecker, D. M., Jones, S. D., and Levine, H. L. (2015). The therapeutic monoclonal antibody market. *MAbs* 7, 9–14. doi: 10.4161/19420862.2015.989042

Ecker, D. M., and Seymour, P. (2020). Supply and demand trends: mammalian biomanufacturing industry overview. *Bioprocess Int.* 18, 10–14.

Ellstrand, N. C. (2003). Going to "great lengths" to prevent the escape of genes that produce specialty chemicals. *Plant Physiol.* 132, 1770–1774. doi: 10.1104/pp.103.025908020

EMA (2006). Guideline on quality apects for active substances produced by stable transgene expression in higher plants.

EMA (2015). EU guidelines for good manufacturing practice for medicinal products for human and veterinary use.

EMA (2018). EMA health threats plan—final version for website publication.

EMA (2020a). EMA initiatives for acceleration of procedures for COVID-19 treatments and vaccines.

EMA (2020b). Mandate, objectives and rules of procedure of the COVID-19 EMA pandemic task force (COVID-ETF).

EMA (2020c). Questions and answers regulatory expectations for medicinal products for human use during the Covid-19 pandemic.

Esposito, D., Mehalko, J., Drew, M., Snead, K., Wall, V., Taylor, T., et al. (2020). Optimizing high-yield production of SARS-CoV-2 soluble spike trimers for serology assays. *Protein Expr. Purif.* 174:105686. doi: 10.1016/j.pep.2020.105686

FDA (2002). Draft guidance for industry: Drugs, biologics and medical devices derived from bioengineered plants for use in humans and animals.

FDA (2011). Process validation: general principles and practices.

FDA (2017). Formal meetings between the FDA and sponsors or applicants of PDUFA products guidance for industry.

FDA (2020). COVID-19 public health emergency: general considerations for pre IND meeting requests for COVID 19 related drugs and biological products.

FEMA (2009). The Defense Production Act of 1950, as amended: 50 U.S.C. App. § 2061.

Fischer, R., and Buyel, J. F. (2020). Molecular farming—the slope of enlightenment. *Biotechnol. Adv.* 40:107519. doi: 10.1016/j.biotechadv.2020.107519

Fuqua, J. L., Hamorsky, K., Khalsa, G., Matoba, N., and Palmer, K. E. (2015a). Bulk production of the antiviral lectin griffithsin. *Plant Biotechnol. J.* 13, 1160–1168. doi: 10.1111/pbi.12433

Fuqua, J. L., Wanga, V., and Palmer, K. E. (2015b). Improving the large scale purification of the HIV microbicide, griffithsin. *BMC Biotechnol.* 15:12. doi: 10.1186/s12896-015-0120-5

Gengenbach, B. B., Keil, L. L., Opdensteinen, P., Muschen, C. R., Melmer, G., Lentzen, H., et al. (2019). Comparison of microbial and transient expression (tobacco plants and plant-cell packs) for the production and purification of the anticancer mistletoe lectin viscumin. *Biotechnol. Bioeng.* 116, 2236–2249. doi: 10.1002/bit.27076

Gengenbach, B. B., Opdensteinen, P., and Buyel, J. F. (2020). Robot cookies—plant cell packs as an automated high-throughput screening platform based on transient expression. *Front. Bioeng. Biotechnol.* 8:393. doi: 10.3389/fbioe.2020.00393

Girard, P., Derouazi, M., Baumgartner, G., Bourgeois, M., Jordan, M., Jacko, B., et al. (2002). 100-liter transient transfection. *Cytotechnology* 38, 15–21. doi: 10.1023/A:1021173124640

Gleba, Y. Y., Tusé, D., and Giritch, A. (2014). Plant viral vectors for delivery by *Agrobacterium*. *Curr. Top. Microbiol. Immunol.* 375, 155–192. doi: 10.1007/82_2013_352

Gottschalk, U. (2016). *Process scale purification of antibodies*. *2nd Edn*. New York: Wiley-Interscience.

Gralinski, L. E., and Menachery, V. D. (2020). Return of the coronavirus: 2019-nCoV. *Viruses* 12:135. doi: 10.3390/v12020135

Grein, J., Ohmagari, N., Shin, D., Diaz, G., Asperges, E., Castagna, A., et al. (2020). Compassionate use of remdesivir for patients with severe Covid-19. *N. Engl. J. Med.* 382, 2327–2336. doi: 10.1056/NEJMoa2007016

Gutiérrez-Granados, S., Cervera, L., Kamen, A. A., and Gòdia, F. (2018). Advancements in mammalian cell transient gene expression (TGE) technology for accelerated production of biologics. *Crit. Rev. Biotechnol.* 38, 918–940. doi: 10.1080/07388551.2017.1419459

Hahn, S., Giritch, A., Bartels, D., Bortesi, L., and Gleba, Y. (2015). A novel and fully scalable *Agrobacterium* spray-based process for manufacturing cellulases and other cost-sensitive proteins in plants. *Plant Biotechnol. J.* 13, 708–716. doi: 10.1111/pbi.12299

Hanson, G., and Coller, J. (2018). Codon optimality, bias and usage in translation and mRNA decay. *Nat. Rev. Mol. Cell Biol.* 19, 20–30. doi: 10.1038/nrm.2017.91

Hefferon, K. L. (2014). *Plant-derived pharmaceuticals: Principles and applications for developing countries*. Wallingford, Oxfordshire, UK, Boston, MA: CABI.

Hiatt, A., Cafferkey, R., and Bowdish, K. (1989). Production of antibodies in transgenic plants. *Nature* 342, 76–78. doi: 10.1038/342076a0

Hiatt, A., Pauly, M., Whaley, K., Qiu, X., Kobinger, G., and Zeitlin, L. (2015). The emergence of antibody therapies for Ebola. *Hum. Antibodies* 23, 49–56. doi: 10.3233/HAB-150284

Hollnagel, E., and Fujita, Y. (2013). The Fukushima disaster—systematic failures as the lack of resilience. *Nucl. Eng. Technol.* 45, 13–20. doi: 10.5516/NET.03.2011.078

Holtz, B. R., Berquist, B. R., Bennett, L. D., Kommineni, V. J., Munigunti, R. K., White, E. L., et al. (2015). Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol. J.* 13, 1180–1190. doi: 10.1111/pbi.12469

Hosangadi, D., Warmbrod, K. L., Martin, E. K., Adalja, A., Cicero, A., Inglesby, T., et al. (2020). Enabling emergency mass vaccination: innovations in manufacturing and administration during a pandemic. *Vaccine* 38, 4167–4169. doi: 10.1016/j.vaccine.2020.04.037

Houdelet, M., Galinski, A., Holland, T., Wenzel, K., Schillberg, S., and Buyel, J. F. (2017). Animal component-free *Agrobacterium* tumefaciens cultivation media for better GMP-compliance increases biomass yield and pharmaceutical protein expression in *Nicotiana benthamiana*. *Biotechnol. J.* 12, 1–10. doi: 10.1002/biot.201600721

Huang, A. T., Garcia-Carreras, B., Hitchings, M. D. T., Yang, B., Katzelnick, L. C., Rattigan, S. M., et al. (2020). A systematic review of antibody mediated immunity to coronaviruses: antibody kinetics, correlates of protection, and association of antibody responses with severity of disease. medRxiv [Preprint]. doi: 10.1101/2020.04.14.20065771

Jansing, J., and Buyel, J. F. (2019). The correlation between DsRed mRNA levels and transient DsRed protein expression in plants depends on leaf age and the 5' untranslated region. *Biotechnol. J.* 14:e1800075. doi: 10.1002/biot.201800075

Jin, J., Hjerrild, K. A., Silk, S. E., Brown, R. E., Labbé, G. M., Marshall, J. M., et al. (2017). Accelerating the clinical development of protein-based vaccines for malaria by efficient purification using a four amino acid C-terminal 'C-tag'. *Int. J. Parasitol.* 47, 435–446. doi: 10.1016/j.ijpara.2016.12.001

Kallolimath, S., Castilho, A., Strasser, R., Grünwald-Gruber, C., Altmann, F., Strubl, S., et al. (2016). Engineering of complex protein sialylation in plants. *Proc. Natl. Acad. Sci. U. S. A.* 113, 9498–9503. doi: 10.1073/pnas.1604371113

Kelley, B. (2020). Developing therapeutic monoclonal antibodies at pandemic pace. *Nat. Biotechnol.* 38, 540–545. doi: 10.1038/s41587-020-0512-5

Kerwin, B. A., Bennett, C., Brodsky, Y., Clark, R., Floyd, J. A., Gillespie, A., et al. (2020). Framework mutations of the 10-1074 bnAb increase conformational stability, manufacturability, and stability while preserving full neutralization activity. *J. Pharm. Sci.* 109, 233–246. doi: 10.1016/j.xphs.2019.07.009

Khailany, R. A., Safdar, M., and Ozaslan, M. (2020). Genomic characterization of a novel SARS-CoV-2. *Gene Rep.* 19:100682. doi: 10.1016/j.genrep.2020.100682

Khan, F., Legler, P. M., Mease, R. M., Duncan, E. H., Bergmann-Leitner, E. S., and Angov, E. (2012). Histidine affinity tags affect MSP1(42) structural

stability and immunodominance in mice. *Biotechnol. J.* 7, 133–147. doi: 10.1002/biot.201100331

Kirchdoerfer, R. N., Wang, N., Pallesen, J., Wrapp, D., Turner, H. L., Cottrell, C. A., et al. (2018). Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. *Sci. Rep.* 8:15701. doi: 10.1038/s41598-018-34171-7

Klutz, S., Magnus, J., Lobedann, M., Schwan, P., Maiser, B., Niklas, J., et al. (2015). Developing the biofacility of the future based on continuous processing and single-use technology. *J. Biotechnol.* 213, 120–130. doi: 10.1016/j.jbiotec.2015.06.388

Kobres, P. -Y., Chretien, J. -P., Johansson, M. A., Morgan, J. J., Whung, P. -Y., Mukundan, H., et al. (2019). A systematic review and evaluation of Zika virus forecasting and prediction research during a public health emergency of international concern. *PLoS Negl. Trop. Dis.* 13:e0007451. doi: 10.1371/journal.pntd.0007451

Kohli, N., Jain, N., Geddie, M. L., Razlog, M., Xu, L., and Lugovskoy, A. A. (2015). A novel screening method to assess developability of antibody-like molecules. *MAbs* 7, 752–758. doi: 10.1080/19420862.2015.1048410

Kruse, R. L. (2020). Therapeutic strategies in an outbreak scenario to treat the novel coronavirus originating in Wuhan, China. *F1000Res.* 9:72. doi: 10.12688/f1000research.22211.2

Kupferschmidt, K. (2020). Genome analyses help track coronavirus' moves. *Science* 367, 1176–1177. doi: 10.1126/science.367.6483.1176

Langridge, W. (2012). Mucosal vaccination against enteric pathogens in the developing world. *Br. J. Med. Med. Res.* 2, 260–291. doi: 10.9734/BJMMR/2012/882

Li, Y. -T., Linster, M., Mendenhall, I. H., Su, Y. C. F., and Smith, G. J. D. (2019). Avian influenza viruses in humans: lessons from past outbreaks. *Br. Med. Bull.* 132, 81–95. doi: 10.1093/bmb/ldz036

Li, W., Zhang, C., Sui, J., Kuhn, J. H., Moore, M. J., Luo, S., et al. (2005). Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. *EMBO J.* 24, 1634–1643. doi: 10.1038/sj.emboj.7600640

Liss, M., Daubert, D., Brunner, K., Kliche, K., Hammes, U., Leiherer, A., et al. (2012). Embedding permanent watermarks in synthetic genes. *PLoS One* 7:e42465. doi: 10.1371/journal.pone.0042465

Lo, M. K., Spengler, J. R., Krumpe, L. R. H., Welch, S. R., Chattopadhyay, A., Harmon, J. R., et al. (2020). Griffithsin inhibits Nipah virus entry and fusion and can protect Syrian golden hamsters from lethal Nipah virus challenge. *J. Infect. Dis.* 221(Suppl. 4), S480–S492. doi: 10.1093/infdis/jiz630

Lomonossoff, G. P., and D'Aoust, M. -A. (2016). Plant-produced biopharmaceuticals: a case of technical developments driving clinical deployment. *Science* 353, 1237–1240. doi: 10.1126/science.aaf6638

Long, Q. -X., Tang, X. -J., Shi, Q. -L., Li, Q., Deng, H. -J., Yuan, J., et al. (2020). Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. *Nat. Med.* 26, 1200–1204. doi: 10.1038/s41591-020-0965-6

Lua, L. H. L., Connors, N. K., Sainsbury, F., Chuan, Y. P., Wibowo, N., and Middelberg, A. P. J. (2014). Bioengineering virus-like particles as vaccines. *Biotechnol. Bioeng.* 111, 425–440. doi: 10.1002/bit.25159

Ma, J. K., Drossard, J., Lewis, D., Altmann, F., Boyle, J., Christou, P., et al. (2015). Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnol. J.* 13, 1106–1120. doi: 10.1111/pbi.12416

Ma, C., Su, S., Wang, J., Wei, L., Du, L., and Jiang, S. (2020). From SARS-CoV to SARS-CoV-2: safety and broad-spectrum are important for coronavirus vaccine development. *Microbes Infect.* 22, 245–253. doi: 10.1016/j.micinf.2020.05.004

Marovich, M., Mascola, J. R., and Cohen, M. S. (2020). Monoclonal antibodies for prevention and treatment of COVID-19. *JAMA* 324, 131–132. doi: 10.1001/jama.2020.10245

McCormick, A. A., Reddy, S., Reinl, S. J., Cameron, T. I., Czerwinkski, D. K., Vojdani, F., et al. (2008). Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10131–10136. doi: 10.1073/pnas.0803636105

McDonald, K. A., and Holtz, R. B. (2020). From farm to finger prick—a perspective on how plants can help in the fight against COVID-19. *Front. Bioeng. Biotechnol.* 8:782. doi: 10.3389/fbioe.2020.00782

McNulty, M. J., Gleba, Y., Tusé, D., Hahn-Löbmann, S., Giritch, A., Nandi, S., et al. (2020). Techno-economic analysis of a plant-based platform for manufacturing antimicrobial proteins for food safety. *Biotechnol. Prog.* 36:e2896. doi: 10.1002/btpr.2896

Menary, J., Hobbs, M., Mesquita de Albuquerque, S., Pacho, A., Drake, P. M. W., Prendiville, A., et al. (2020). Shotguns vs. lasers: identifying barriers and facilitators to scaling-up plant molecular farming for high-value health products. *PLoS One* 15:e0229952. doi: 10.1371/journal.pone.0229952

Menzel, S., Holland, T., Boes, A., Spiegel, H., Fischer, R., and Buyel, J. F. (2018). Downstream processing of a plant-derived malaria transmission-blocking vaccine candidate. *Protein Expr. Purif.* 152, 122–130. doi: 10.1016/j.pep.2018.07.012

Meuleman, P., Albecka, A., Belouzard, S., Vercauteren, K., Verhoye, L., Wychowski, C., et al. (2011). Griffithsin has antiviral activity against hepatitis C virus. *Antimicrob. Agents Chemother.* 55, 5159–5167. doi: 10.1128/AAC.00633-11

Mir-Artigues, P., Twyman, R. M., Alvarez, D., Cerda Bennasser, P., Balcells, M., Christou, P., et al. (2019). A simplified techno-economic model for the molecular pharming of antibodies. *Biotechnol. Bioeng.* 116, 2526–2539. doi: 10.1002/bit.27093

Möller, J., Kuchemüller, K. B., Steinmetz, T., Koopmann, K. S., and Pörtner, R. (2019). Model-assisted design of experiments as a concept for knowledge-based bioprocess development. *Bioprocess Biosyst. Eng.* 42, 867–882. doi: 10.1007/s00449-019-02089-7

Moore, J. P., and Klasse, P. J. (2020). SARS-CoV-2 vaccines: 'Warp Speed' needs mind melds not warped minds. *J. Virol.* 94, e01083–e01020. doi: 10.1128/JVI.01083-20

Nandi, S., Kwong, A. T., Holtz, B. R., Erwin, R. L., Marcel, S., and McDonald, K. A. (2016). Techno-economic analysis of a transient plant-based platform for monoclonal antibody production. *MAbs* 8, 1456–1466. doi: 10.1080/19420862.2016.1227901

Nandi, S., Yalda, D., Lu, S., Nikolov, Z., Misaki, R., Fujiyama, K., et al. (2005). Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. *Transgenic Res.* 14, 237–249. doi: 10.1007/s11248-004-8120-6

Nixon, B., Stefanidou, M., Mesquita, P. M. M., Fakioglu, E., Segarra, T., Rohan, L., et al. (2013). Griffithsin protects mice from genital herpes by preventing cell-to-cell spread. *J. Virol.* 87, 6257–6269. doi: 10.1128/JVI.00012-13

Njue, M., Njuguna, P., Kapulu, M. C., Sanga, G., Bejon, P., Marsh, V., et al. (2018). Ethical considerations in controlled human malaria infection studies in low resource settings: experiences and perceptions of study participants in a malaria challenge study in Kenya. *Wellcome Open Res.* 3:39. doi: 10.12688/wellcomeopenres.14439.2

Norkunas, K., Harding, R., Dale, J., and Dugdale, B. (2018). Improving agroinfiltration-based transient gene expression in *Nicotiana benthamiana*. *Plant Methods* 14:71. doi: 10.1186/s13007-018-0343-2

Okba, N. M. A., Müller, M. A., Li, W., Wang, C., GeurtsvanKessel, C. H., Corman, V. M., et al. (2020). Severe acute respiratory syndrome coronavirus 2-specific antibody responses in coronavirus disease patients. *Emerg. Infect. Dis.* 26, 1478–1488. doi: 10.3201/eid2607.200841

O'Keefe, B. R., Giomarelli, B., Barnard, D. L., Shenoy, S. R., Chan, P. K. S., McMahon, J. B., et al. (2010). Broad-spectrum in vitro activity and in vivo efficacy of the antiviral protein griffithsin against emerging viruses of the family Coronaviridae. *J. Virol.* 84, 2511–2521. doi: 10.1128/JVI.02322-09

Opdensteinen, P., Clodt, J. I., Müschen, C. R., Filiz, V., and Buyel, J. F. (2018). A combined ultrafiltration/diafiltration step facilitates the purification of cyanovirin-N from transgenic tobacco extracts. *Front. Bioeng. Biotechnol.* 6:206. doi: 10.3389/fbioe.2018.00206

Park, S., Park, J. Y., Song, Y., How, S. H., and Jung, K. -S. (2019). Emerging respiratory infections threatening public health in the Asia-Pacific region: a position paper of the Asian Pacific Society of Respirology. *Respirology* 24, 590–597. doi: 10.1111/resp.13558

Passmore, I. (2012). Plant made pharmaceuticals: The Parliamentary Office of Science and Technology, 424.

Piotrzkowski, N., Schillberg, S., and Rasche, S. (2012). Tackling heterogeneity: a leaf disc-based assay for the high-throughput screening of transient gene expression in tobacco. *PLoS One* 7:e45803. doi: 10.1371/journal.pone.0045803

Pogue, G. P., Vojdani, F., Palmer, K. E., Hiatt, E., Hume, S., Phelps, J., et al. (2010). Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnol. J.* 8, 638–654. doi: 10.1111/j.1467-7652.2009.00495.x

Pregelj, L., Hine, D. C., Oyola-Lozada, M. G., and Munro, T. P. (2020). Working hard or hardly working? Regulatory bottlenecks in developing a COVID-19 vaccine. *Trends Biotechnol.* 38, 943–947. doi: 10.1016/j.tibtech.2020.06.004

Qian, K., and Hu, S. (2020). Ig-like ACE2 protein therapeutics: a revival in development during the COVID-19 pandemic. *MAbs* 12:e1782600. doi: 10.1080/19420862.2020.1782600

Qiu, X. G., Wong, G., Audet, J., Bello, A., Fernando, L., Alimonti, J. B., et al. (2014). Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature* 514, 47–53. doi: 10.1038/nature13777

Quinlan, B. D., Mou, H., Zhang, L., Guo, Y., He, W., Ojha, A., et al. (2020). The SARS-CoV-2 receptor-binding domain elicits a potent neutralizing response without antibody-dependent enhancement. bioRxiv [Preprint]. doi: 10.1101/2020.04.10.036418

Rademacher, T., Sack, M., Blessing, D., Fischer, R., Holland, T., and Buyel, J. F. (2019). Plant cell packs: a scalable platform for recombinant protein production and metabolic engineering. *Plant Biotechnol. J.* 17, 1560–1566. doi: 10.1111/pbi.13081

Rader, R. A., and Langer, E. S. (2018). Worldwide biopharmaceutical manufacturing capacity analysis: growth continues across the board. *Bioprocess Int.* 19, 20–59.

Raymond, M., Gibani, M. M., Day, N. P. J., and Cheah, P. Y. (2019). Typhoidal Salmonella human challenge studies: ethical and practical challenges and considerations for low-resource settings. *Trials* 20(Suppl. 2), 704. doi: 10.1186/s13063-019-3844-z

Regla-Nava, J. A., Nieto-Torres, J. L., Jimenez-Guardeño, J. M., Fernandez-Delgado, R., Fett, C., Castaño-Rodríguez, C., et al. (2015). Severe acute respiratory syndrome coronaviruses with mutations in the E protein are attenuated and promising vaccine candidates. *J. Virol.* 89, 3870–3887. doi: 10.1128/JVI.03566-14

Reuter, L. J., Bailey, M. J., Joensuu, J. J., and Ritala, A. (2014). Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. *Plant Biotechnol. J.* 12, 402–410. doi: 10.1111/pbi.12147

Rosales-Mendoza, S. (2020). Will plant-made biopharmaceuticals play a role in the fight against COVID-19? *Expert. Opin. Biol. Ther.* 20, 545–548. doi: 10.1080/14712598.2020.1752177

Rosenberg, Y. J., Walker, J., Jiang, X., Donahue, S., Robosky, J., Sack, M., et al. (2015). A highly stable minimally processed plant-derived recombinant acetylcholinesterase for nerve agent detection in adverse conditions. *Sci. Rep.* 5:13247. doi: 10.1038/srep13247

Rybicki, E. P. (2017). Plant-made vaccines and reagents for the One Health initiative. *Hum. Vaccin. Immunother.* 13, 2912–2917. doi: 10.1080/21645515.2017.1356497

Sainsbury, F. (2020). Innovation in plant-based transient protein expression for infectious disease prevention and preparedness. *Curr. Opin. Biotechnol.* 61, 110–115. doi: 10.1016/j.copbio.2019.11.002

Sainsbury, F., and Lomonossoff, G. P. (2008). Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* 148, 1212–1218. doi: 10.1104/pp.108.126284

Seymour, P., and Ecker, D. M. (2017). Global biomanufacturing trends, capacity, and technology drivers: industry biomanufacturing capacity overview. *Am. Pharm. Rev.* 19.

Shah, S. K., Miller, F. G., Darton, T. C., Duenas, D., Emerson, C., Lynch, H. F., et al. (2020). Ethics of controlled human infection to address COVID-19. *Science* 368, 832–834. doi: 10.1126/science.abc1076

Shajahan, A., Supekar, N. T., Gleinich, A. S., and Azadi, P. (2020). Deducing the N- and O-glycosylation profile of the spike protein of novel coronavirus SARS-CoV-2. *Glycobiology*. doi: 10.1093/glycob/cwaa042 [Epub ahead of print]

Shoji, Y., Chichester, J. A., Jones, M., Manceva, S. D., Damon, E., Mett, V., et al. (2011). Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. *Hum. Vaccin.* 7, 41–50. doi: 10.4161/hv.7.0.14561

Shoji, Y., Farrance, C. E., Bautista, J., Bi, H., Musiychuk, K., Horsey, A., et al. (2012). A plant-based system for rapid production of influenza vaccine antigens. *Influenza Other Respir. Viruses* 6, 204–210. doi: 10.1111/j.1750-2659.2011.00295.x

Sparrow, P., Broer, I., Hood, E., Eversole, K., Hartung, F., and Schiemann, J. (2013). Risk assessment and regulation of molecular farming—a comparison between Europe and US. *Curr. Pharm. Design* 19, 5513–5530. doi: 10.2174/1381612811319310007

Spiegel, H., Boes, A., Perales Morales, C., Rademacher, T., and Buyel, J. F. (2019). Ready to use stocks of *Agrobacterium tumefaciens* can simplify process development for the production of recombinant proteins by transient expression in plants. *Biotechnol. J.* 14:e1900113. doi: 10.1002/biot.201900113

Steiner, G., Zenk, L., and Schernhammer, E. (2020). Preparing for the next wave of COVID-19: resilience in the face of a spreading pandemic. *Int. J. Environ. Res. Public Health* 17:4098. doi: 10.3390/ijerph17114098

Strasser, R., Altmann, F., Mach, L., Glössl, J., and Steinkellner, H. (2004). Generation of *Arabidopsis thaliana* plants with complex N-glycans lacking β1,2-linked xylose and core α1,3-linked fucose. *FEBS Lett.* 561, 132–136. doi: 10.1016/S0014-5793(04)00150-4

Strasser, R., Altmann, F., and Steinkellner, H. (2014). Controlled glycosylation of plant-produced recombinant proteins. *Curr. Opin. Biotechnol.* 30, 95–100. doi: 10.1016/j.copbio.2014.06.008

Thoma, K., Scharte, B., Hiller, D., and Leismann, T. (2016). Resilience engineering as part of security research: definitions, concepts and science approaches. *Eur. J. Secur. Res.* 1, 3–19. doi: 10.1007/s41125-016-0002-4

Tortorici, M. A., and Veesler, D. (2019). Structural insights into coronavirus entry. *Adv. Virus Res.* 105, 93–116. doi: 10.1016/bs.aivir.2019.08.002

Tsekoa, T. L., Singh, A. A., and Buthelezi, S. G. (2020). Molecular farming for therapies and vaccines in Africa. *Curr. Opin. Biotechnol.* 61, 89–95. doi: 10.1016/j.copbio.2019.11.005

Tusé, D. (2011). Safety of plant-made pharmaceuticals product development and regulatory considerations based on case studies of two autologous human cancer vaccines. *Hum. Vaccin.* 7, 322–330. doi: 10.4161/hv.7.3.14213

Tusé, D., Ku, N., Bendandi, M., Becerra, C., Collins, R., Langford, N., et al. (2015). Clinical safety and immunogenicity of tumor-targeted, plant-made Id-KLH conjugate vaccines for follicular lymphoma. *Biomed. Res. Int.* 2015:648143. doi: 10.1155/2015/648143

Tusé, D., Tu, T., and McDonald, K. A. (2014). Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed. Res. Int.* 2014:256135. doi: 10.1155/2014/256135

Vafaee, Y., Staniek, A., Mancheno-Solano, M., and Warzecha, H. (2014). A modular cloning toolbox for the generation of chloroplast transformation vectors. *PLoS One* 9:e110222. doi: 10.1371/journal.pone.0110222

van Norman, G. A. (2016). Drugs and devices: comparison of European and U.S. approval processes. *JACC Basic Transl. Sci.* 1, 399–412. doi: 10.1016/j.jacbts.2016.06.003

Walls, A. C., Park, Y. -J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281.e6–292.e6. doi: 10.1016/j.cell.2020.02.058

Walwyn, D. R., Huddy, S. M., and Rybicki, E. P. (2015). Techno-economic analysis of horseradish peroxidase production using a transient expression system in *Nicotiana benthamiana*. *Appl. Biochem. Biotechnol.* 175, 841–854. doi: 10.1007/s12010-014-1320-5

Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S., and Crispin, M. (2020). Site-specific glycan analysis of the SARS-CoV-2 spike. *Science* 369, 330–333. doi: 10.1126/science.abb9983

Webb, S. R., Twyman, R. M., and Moloney, M. (2020). Agtech infrastructure for pandemic preparedness. *Nat. Biotechnol.* 38, 1025–1027. doi: 10.1038/s41587-020-0654-5

Webster, G. R., Teh, A. Y. -H., and Ma, J. K. -C. (2017). Synthetic gene design—the rationale for codon optimization and implications for molecular pharming in plants. *Biotechnol. Bioeng.* 114, 492–502. doi: 10.1002/bit.26183

Wineinger, N. E., Zhang, Y., and Topol, E. J. (2019). Trends in prices of popular brand-name prescription drugs in the United States. *JAMA Netw. Open* 2:e194791. doi: 10.1001/jamanetworkopen.2019.4791

World Health Organization Advisory Group (2020). Feasibility, potential value and limitations of establishing a closely monitored challenge model of experimental COVID-19 infection and illness in healthy young adult volunteers. New York: WHO.

Wu, S. -C. (2020). Progress and concept for COVID-19 vaccine development. *Biotechnol. J.* 15:e2000147. doi: 10.1002/biot.202000147

Wycoff, K. L. (2005). Secretory IgA antibodies from plants. *Curr. Pharm. Design* 11, 2429–2437. doi: 10.2174/1381612054367508

Wycoff, K., Maclean, J., Belle, A., Yu, L., Tran, Y., Roy, C., et al. (2015). Anti-infective immunoadhesins from plants. *Plant Biotechnol. J.* 13, 1078–1093. doi: 10.1111/pbi.12441

Yamamoto, T., Hoshikawa, K., Ezura, K., Okazawa, R., Fujita, S., Takaoka, M., et al. (2018). Improvement of the transient expression system for production of recombinant proteins in plants. *Sci. Rep.* 8:4755. doi: 10.1038/s41598-018-23024-y

Zemella, A., Thoring, L., Hoffmeister, C., and Kubick, S. (2015). Cell-free protein synthesis: pros and cons of prokaryotic and eukaryotic systems. *Chembiochem* 16, 2420–2431. doi: 10.1002/cbic.201500340

Zerhouni, W., Nabel, G. J., and Zerhouni, E. (2020). Patents, economics, and pandemics. *Science* 368:1035. doi: 10.1126/science.abc7472

Zischewski, J., Sack, M., and Fischer, R. (2015). Overcoming low yields of plant-made antibodies by a protein engineering approach. *Biotechnol. J.* 11, 107–116. doi: 10.1002/biot.201500255

Disclaimer: The literature on COVID-19 accumulates daily, and we have cited not only peer-reviewed publications but also manuscripts deposited on preprint servers and reliable online sources. We acknowledge that some information in such sources may be inaccurate or outdated by the time this manuscript is published. As such, we urge readers to inspect key papers themselves and also recommend the use of additional resources to reach conclusions. We have no doubt that we may have overlooked some key papers in this rapidly evolving area of research. In addition, some information described herein was based on the authors' personal participation in R&D projects or their direct knowledge of events, and as such no citable references existed at the time of writing. We have noted such instances in the text as "*authors' experience*."

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GLOSSARY

Term	Definition
API	Active pharmaceutical ingredient
BLA	Biologics License Application
BSL	Biosafety level
CHMP	Committee for Medicinal Products for Human Use
cGMP	Current good manufacturing practice
CTA	Clinical Trial Application
CV	Coefficient of variation
EMA	European Medicines Agency
EOP	End of phase
FDA	Food and Drug Administration
EUA	Emergency use authorization
GMO	Genetically modified organisms
HCP	Host cell proteins
ICH	International Council for Harmonization
IMPD	Investigational Medicinal Product Dossier
IND	Investigational New Drug
IP	Intellectual property
mAbs	Monoclonal antibodies
MERS	Middle East Respiratory Syndrome
NDA	New Drug Application
NMA	New marketing authorization
PMP	Plant-made pharmaceuticals
R&D	Research and development
SARS	Severe acute respiratory syndrome
T-DNA	Transferred DNA
TEA	Techno-economic assessment
TMV	Tobacco mosaic virus
VLP	Virus-like particles

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In Search of Herbal Anti-SARS-Cov2 Compounds

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On March 11, 2020, the World Health Organization (WHO) announced that the spread of the new coronavirus had reached the stage of a pandemic. To date (23.10.2020), there are more than 40 million confirmed cases of the disease in the world, at the same time there is still no effective treatment for the disease. For management and treatment of SARS-Cov2, the development of an antiviral drug is needed. Since the representatives of all human cultures have used medicinal plants to treat viral diseases throughout their history, plants can be considered as sources of new antiviral drug compounds against emerging viruses. The huge metabolic potential of plants allows us to expect discovery of plant compounds for the prevention and treatment of coronavirus infection. This idea is supported by number of papers on the anti-SARS-Cov2 activity of plant extracts and specific compounds in the experiments *in silico*, *in vitro*, and *in vivo*. Here, we summarize information on methods and approaches aimed to search for anti-SARS-Cov2 compounds including cheminformatics, bioinformatics, genetic engineering of viral targets, interacting with drugs, biochemical approaches etc. Our mini-review may be useful for better planning future experiments (including rapid methods for screening compounds for antiviral activity, the initial assessment of the antiviral potential of various plant species in relation to certain pathogens, etc.) and giving a hand to those who are making first steps in this field.

Keywords: genetic engineering, cheminformatics, secondary metabolites, antiviral drugs, coronavirus, medicinal plants (herbal drugs), biochemical methods

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INTRODUCTION

A novel coronavirus strain causing fatal respiratory syndrome was reported in late 2019. In January 2020, it was revealed that it belongs to the beta-coronaviruses, sharing similarity to SARS-coronaviruses, and that its spike protein interacts strongly with the human angiotensin-converting enzyme 2 (ACE2) receptor (Dhama et al., 2020; Xu et al., 2020). On March 11, the World Health Organization (WHO, 2020) announced that the spread of the new coronavirus had reached a pandemic stage. To date (23.10.2020), there are more than 40 million confirmed cases of the disease in the world, while there is still no effective treatment for the disease. In this regard, the search for cures for this disease is undoubtedly relevant and significant. Plant-based medicine is attracting a lot of attention today, since medicinal plants are enriched with variety of secondary metabolites including those with antiviral properties (Gurib-Fakim, 2006; Adedeji and Sarafianos, 2014; Dhama et al., 2018; Divya et al., 2020; Vellingiri et al., 2020).

About, a third of FDA-approved drugs over the past 20 years are based on natural products or their derivatives (Carter, 2011).

Natural products can offer safe and inexpensive platforms for discovery of efficient and novel agents for treatment of SARS-CoV-2 with minimizing side effects (Ghildiyal et al., 2020; Huang et al., 2020; Mani et al., 2020). Although structures for 200,000 natural products are known, only 15% of the estimated 350,000 plant species have been investigated for their chemical constituents (Cragg and Newman, 2013). Thus, plants hold great potential as a material for drug development.

POTENTIAL TARGETS FOR ANTI-SARS-Cov2 DRUG DESIGN

Coronaviruses are single-stranded (+) RNA viruses, having four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The other 25 nonstructural proteins (NSPs) regulate assembling of copies of virus particles and their passing through the host's immune system (Xu et al., 2020).

Inside the host cell, two polyproteins, pp1a and pp1ab, are directly translated from the viral RNA and then cleaved by two viral proteases, main protease (M^{pro}, also called 3-chymotrypsin-like cysteine protease, or $3CL^{pro}$) and papain-like protease (PL^{pro}). PL^{pro} cleaves junctions of NSP 1, 2, and 3 while M^{pro} cleaves polyprotein at 11 distinct sites downstream from the NSP4 to generate various NSPs that are important for viral replication (Perlman and Netland, 2009). The M^{pro} is one of the best-characterized drug targets among the coronaviruses (Anand et al., 2003; Hilgenfeld, 2014). Since no human proteases with similar cleavage specificity are known, the inhibitors of M^{pro} are unlikely to be toxic (Zhang et al., 2020b). The PL^{pro}, is also attractive antiviral drug target, because it affects not only coronaviral replication, but also has the additional function of deubiquitination of host cell proteins and ISG15 removal, finally leading to the immune suppression of host cells (Báez-Santos et al., 2015; Lin et al., 2018; Clemente et al., 2020).

The viral S protein is essential for viral attachment, fusion, and entry. It uses host angiotensin-converting enzyme 2 (ACE2) as a receptor to get into the host cells. Since, the structure of S protein of SARS-CoV-2 is revealed (Coutard et al., 2020; Huang et al., 2020; Wrapp et al., 2020) and receptor-binding domain was identified, it can serve as a target for development of inhibitors of S protein and ACE2 interaction (Tai et al., 2020).

Approaches to search for substances interacting with these targets will be described below together with successful examples of their application.

DRUG SEARCH STRATEGIES IN PHYTOMEDICINE

Previously, new drugs were discovered mostly by a "trial and error" approach (Butcher et al., 2004). As methods and knowledge in chemistry advance, researchers began to purify the active compounds in herbal extracts known to have medicinal properties and determine their structures (Drews, 2000). The emergence of genomics and proteomics and the development of bioinformatics and cheminformatics made a breakthrough into the drug design, bringing the concept and techniques for large-scale screening. Newly emerging diseases require faster pace of drug development. Worldwide transmission of COVID-19 (van Dorp et al., 2020) and high infectivity (Zhang and Holmes, 2020) of the virus demands rapid development of suitable drugs.

In phytomedicine, there are several schemes used for this purpose. Let us focus on each of them.

Classical Approach

Classical approach represents either the screening based on previously purified natural compounds or on activity-guided screening of crude extract mixture for active compounds. Further, active compounds are purified by activity-guided purification (Szajdak, 2016). This way of identification of antiviral compounds was implemented by Li et al. (2005). More than 200 Chinese medicinal herb extracts were screened for anti-SARS-CoV activities by assay for virus-induced cytopathic effect. The crude extracts of *Lycoris radiata*, *Artemisia annua*, *Pyrrosia lingua*, and *Lindera aggregate* showed antiviral activity. The ethanolic extract of *L. radiata* was the most active. The majority of bioactive components of *L. radiata* belong to alkaloids that were confirmed by reversed phase high performance liquid chromatography (LC). Finally, lycorine was identified by LC-MS/MS as an effective anti-SARS-CoV component. Now, anti-SARS-CoV2 effect of the lycorine is also shown (Murck, 2020).

Often activity-guided screening indicates that plant fractions have higher antiviral activity than pure substances, because medicinal plants usually contain several biologically active compounds. For example, *Kickxia elatine*, contain flavonoid pectolinarin (Yuldashev et al., 1996) and iridoid glycosides with anti-inflammatory and antiviral activities (Handjieva et al., 1995), and therefore, provide some synergetic. Besides, some compounds are able to prevent disease by various means. For example, pectolinarin efficiently blocks the enzymatic activity of M^{pro} and PL^{pro} (Jo et al., 2020) and demonstrates anti-inflammatory activities (Ho et al., 2020).

Structure-Activity-Relationship Approach

Structure-activity-relationship is an approach for finding the relationships between the chemical structure (structural-related properties) and the biological activity of studied compounds. The need to streamline the drug development process has spawned the development of such strategies as "rational drug design," where large-scale screening can be done through a database of potential candidate molecules to find those of interest (https://www.ebi.ac.uk/chembl, https://pubchem.ncbi.nlm.nih.gov/, Santos et al., 2016; Mumtaz et al., 2017). Medicinal plants contain wide range of secondary metabolites of different activity spectrums. Some of them are known to have antiviral and other properties, for others only structural data is available. The properties of many plants-derived substances remain unclear. These unknown properties can be predicted by computer modeling methods.

Topological indices are able to predict different activities and physicochemical characteristics such as boiling point, entropy, enthalpy, etc. Similarly, based on the topological properties of some chemical structures with antiviral activity, (for example remdesivir, chloroquine, hydroxychloroquine, and theaflavine), the antiviral activity of the new compounds can be predicted (Mondal et al., 2020).

Antiviral features of compounds can be determined by matching them as a ligand to known targets using molecular dynamics and docking simulations (Zhang et al., 2020a). Tallei et al. (2020) made a list of natural components with M^{pro}-inhibitor effect; among them are hesperidin, morine, rhoifolin, pectolinarin, and nabiximols. Binding interaction and ligand affinity of these compounds to M^{pro} was the same as of nelfinavir, and even better than chloroquine and hydroxychloroquine sulfate, – recommended by the FDA as emergency anti-COVID-treatment anti-malarial drugs (Tallei et al., 2020). Quercetin-3-β-galactoside showed inhibitory activity against SARS-CoV M^{pro} *in silico*, *via* docking simulation, and also in enzymatic inhibition assays. Molecular modeling strongly suggested that the residue Q189 plays a key role, and it was confirmed by site-directed mutagenesis of the M^{pro} (Chen et al., 2006). Molecular docking analysis of M^{pro} and compounds of medicinal plants revealed such inhibiting substances as beta-eudesmol from *Lauris nobilis*, digitoxigenin from *Nerium oleander*, crocin from *Crocus sativus* (Aanouz et al., 2020), pavetannin-C1 and tenuifolin from cinnamon (Prasanth et al., 2020), catechins/polyphenols from green tea (Ghosh et al., 2020), withanoside V from *Withania somnifera* (Tripathi et al., 2020), and tinosponone from *Tinospora cordifolia* (Krupanidhi et al., 2020). Inhibitory effects of alkylated chalcones isolated from *Angelica keiskei* against the SARS-CoV proteases M^{pro} and PL^{pro} were found by Park et al. (2016).

Quercetin showed inhibitory activity against SARS-CoV PL^{pro}, although Papyriflavonol A was the most effective inhibitor of PL^{pro} among those studied by Park et al. (2017). Virtual structure-based screening revealed that withanolide A, isocodonocarpine, and calonysterone bind to PL^{pro} (Alamri et al., 2020).

Molecular docking analysis applied on the binding positions with S protein indicated that cannabinoids along with epigallocatechin gallate, herbacetin, hesperidine, pectolinarin, curcumin, and withanoside X hold remarkable binding sites, which could support them to be excellent S protein inhibitors, preventing viral attachment to host cells (Chikhale et al., 2020; Jena et al., 2020; Tallei et al., 2020).

Unfortunately, molecular docking does not consider the effect of selected substances on other key points of the disease and the cumulative effect of several biologically active compounds. However, concept of large-scale screening can be applied to identify new candidates for further analysis, shortening the search process and making it more powerful (Chen et al., 2006; Theerawatanasirikul et al., 2020).

The Data-Driven Approach

Repurposing of known drugs could significantly accelerate the deployment of novel therapies for COVID-19. The main difference of data-driven approach from the structure-activity-relationship approach is in use of databases of drugs, including phytochemical ones (https://www.drugbank.ca, http://drugcentral.org etc.). The identification of new areas of application for already known drugs saves time for testing their biosafety, but does not provide an opportunity to find fundamentally new substances that can be more effective than previously known drugs. The approach includes three steps:

1. Selection of drugs aimed to treat "Warm diseases," "Pestilence," or "Epidemic diseases."
2. Identification of the active substances of plant origin, responsible for these pharmacology effects.
3. *In silico* assessment of the activities of selected drugs in relation to key proteins (Mpro, PLpro S protein, and ACE2) involved in the development of the disease.

According to this scheme, among the 96,606 formulations of Traditional Chinese Medicine, the 574 drugs were selected, and only 26 ones remained after the third stage of analysis. These biologicaly activity substances from the licorice (*Glycyrrhizae radix*) and skullcaps (*Scutellariae baicalensis*) could also interact with the targets involving in immune and inflammation diseases (Ren et al., 2020).

Similar approach based on the ReFRAME (Repurposing, Focused Rescue, and Accelerated Medchem) library was utilized by Riva et al. (2020).

Immunomodulatory Effects of Herbs

As we can see from the examples mentioned above, extracts of many plants, and even specific compounds, often inhibit several SARS-CoV2 protein targets. Besides, identified compounds can possess a wide range of other pharmacological activities, including immunomodulatory effects. For example, Curcumin inhibits S protein (Jena et al., 2020) and also participates in regulation of immune and inflammatory response associated with coronavirus infections (Chen et al., 2020).

Moreover, plants often contain a number of compounds, with different activities that can complement each other. Thus, *Cirsium japonicum*, popular in traditional Chinese medicine, contains pectolinarin and pectolinarigenin, which have antiviral and immunomodulatory effects, respectively (Cheriet et al., 2020). Pectolinarigenin shows high suppressive immunomodulatory potency, including inhibitory activity of neutrophil phagocytes respiratory burst as well as T-cells proliferation (Erukainure et al., 2017).

Withania somnifera, a key plant of Ayurveda, contains compounds inhibiting M^{pro} and S protein, (Chikhale et al., 2020; Tripathi et al., 2020) and the extract of the plant provides anti-viral immunity by increasing interferon gamma responses and anti-inflammatory activities by decreasing the quantity of interleukin-1, interleukin-6 and Tumor necrosis factor related to COVID-19 (Niraj and Varsha, 2020).

Tinosponone from *Tinospora cordifolia* inhibits M^{pro} (Krupanidhi et al., 2020), and the aqueous extracts of the plant affects the cytokine production and activation of immune effector cells (Niraj and Varsha, 2020).

The immune response against coronavirus is vital to control and get rid of the infection. In the ideal situation, the antiviral

drug should check the infection while the immune system prepares to destroy the last virus particles (Mukherjee, 2019) From other hand, maladjusted immune responses may lead to the immunopathology of the disease, resulting in impairment of pulmonary gas exchange (Dhama et al., 2020). Thus, a fine selection of herbal immunomodulators is required for the treatment of different stages of the diseases and different degrees of its severity.

GENETIC ENGINEERING FOR ANALYSIS OF ANTI-SARS-Cov2 ACTIVITY

Genetic engineering methods allow to optimize the evaluation of anti-SARS activity of compounds at different stages of research. First of all, they include obtaining of recombinant target proteins for searching for antiviral drugs.

The recombinant proteases M^{pro} and PL^{pro} can be easily expressed in *Escherichia coli* or other organism, purified by routine biochemical methods (Lin et al., 2004, 2005) and used for cell-free cleavage assay (Lin et al., 2005; Chou et al., 2008; Chen et al., 2009; Jo et al., 2020) or cell-based cleavage assay (Lin et al., 2005), as well as for study of x-ray structures of the unliganded SARS-CoV-2 proteins and their complexes with potential drugs (Zhang et al., 2020b).

Cell-free cleavage assay involves the use of a purified enzyme and a substrate, modified at the C and N terminus. Depending on the type of modification (protein tag or fluorescent group), further Enzyme Linked Immunosorbent Assay (ELISA) or protein based fluorogenic assay are used to assess cell-free proteolytic activity and its inhibition by different compounds (Kuo et al., 2004; Lin et al., 2005).

The cell-based cleavage assay does not require purification of the active protease, and represents closely the natural physiological state. Investigating the *Isatis indigotica* phenolic compounds as potential anti-SARS drugs, Lin et al. (2005) have made in-frame construction, containing the M^{pro}, the substrate, and the luciferase, and transformed it into Vero cells. Since a more than 30 kDa protein fused at the N-terminus of the luciferase resulted in a dramatic decrease of luciferase activity, the detection of activity of luciferase was considered by authors as a measure for the cis-cleavage by M^{pro}. Epigallocatechin gallate abundant in green tea (*Camellia sinensis*), inhibits the proteolytic activity of SARS-CoV M^{pro}, expressed in *Pichia pastoris* (Nguyen et al., 2012).

In addition, approaches described above, demonstrated that tanshinones of *Salvia miltiorrhiza* (Park et al., 2012b), diarylheptanoids from *Alnus japonica* (Park et al., 2012a), and geranylated flavonoids from the *Paulownia tomentosa* tree (Cho et al., 2013) are inhibitors of SARS-CoV PL^{pro} activity. M^{pro} proteases of SARS-CoV and SARS-CoV-2 are inhibited by such compounds as herbacetin, rhoifolin, pectolinarin (Jo et al., 2020) hesperetin, sinigrin (Lin et al., 2005) quercetin-3-β-galactoside (Chen et al., 2006) etc.

Thus, at the initial stages, the assessment of the antiviral activity of drugs does not require the use of the virus itself. This makes it possible to significantly expand the number of laboratories where it is permissible to conduct such studies and select the best compounds for the next stages of testing.

Genetic engineering technologies are also applicable at the stage of evaluating the therapeutic effect of drugs using preclinical animal models. Several animal models, from mice to and non-human primates, have been shown to be susceptible to SARS-CoV infection. From one hand, primates are closest to humans; the clinical picture of their disease is almost the same as in humans. From other hand, they are much more expensive than mice. Therefore, drugs are initially tested on mice (Bevinakoppamath et al., 2020; Lutz et al., 2020). Unfortunately, due to structural differences in mouse ACE2 compared to human ACE2 proteins, the SARS coronaviruses exhibit poor tropism characteristics for mouse tissues. So, the wild-type lines are not optimal for studying infections of the newly discovered coronavirus. Several transgenic mice strains, carrying hACE2 under control of different promoters, including human cytokeratin 18 (K18) promoter, composite CAG promoter consisting of the cytomegalovirus immediate early enhancer, the chicken β-actin promoter and rabbit globulin splicing and polyadenylation sites, HFH4 lung ciliated epithelial cell-specific promoter, were developed in 2006–2016 to study SARS-CoV infection (McCray et al., 2007; Tseng et al., 2007; Menachery et al., 2016). It was shown that the hACE2 transgenic mice infected with a human SARS-CoV strain *via* intranasal inoculation demonstrate the symptoms observed in infected human patients. These hACE2 transgenic mice can provide significant findings to support the development of COVID-19 therapeutics (Lutz et al., 2020).

TAXONOMIC STUDIES AND GENOME SEQUENCING SEARCHING FOR BETTER PRODUCERS OF PHYTOCHEMICAL DRUGS

For a more efficient search for better plant producers of specific secondary metabolites and their combinations, it makes sense to use the concept of plant chemotaxonomy – a branch of the science of taxonomy, were plants are classified depending on the similarities and differences in the spectrum of their secondary metabolites. Since some chemicals can be found in nature only in organisms of certain genera, families, or orders, this can be used both for their classification and to search for certain metabolites in the related species (Zidorn, 2019).

Whole genome data in its turn led to insights into biosynthesis pathways (Denoeud et al., 2014). The list of species with sequenced genomes is growing, collaborations are emerging to join efforts and collect more data. The Medicinal Plant Genomic Resource (MPRG)[1] is a domain-specific database created by consortium efforts to collect deep DNA sequencing, RNA sequencing, and metabolomics data. In general, genome sequencing is used to discover new candidates and to elucidate biosynthesis pathways to prepare data sets of new molecules for further analysis. One can predict the presence of a compound

[1]http://medicinalplantgenomics.msu.edu

among plant metabolites if gene involved in the biosynthesis of the compound in question was discovered in the plant genome. For example, cardenolides discovered in the *Calotropis gigantea* genome were historically used to cure pneumonia and as an anti-inflammatory, anticancer, and antimalaria (Hoopes et al., 2018; Boone et al., 2020), and they also can be easily converted to mappicine ketone, an antiviral led compound (Das et al., 1998). Genes to biosynthesize curcumin were found in *Oryza sativa* genome (Katsuyama et al., 2007). Recently conducted chromosome-scale genome assembly of *I. indigotica* assisted to reveal new candidate genes for the biosynthesis of several groups of active compounds in this medicinal plant (Kang et al., 2020). Phenolic compounds of *I. indigotica* have already shown activity against the M^{pro} (Lin et al., 2005), and indole alkaloids in this plant demonstrated inhibition of HSV-2 reproduction (Sun et al., 2010). Besides, cultivars of the same species can differ in the amount of the medicinal compounds (Hisashi and Saito, 2013; Kajikawa et al., 2017). Complete genome data allow to identify gene clusters for secondary metabolism, which opens the way to metabolic engineering. For example, whole-genome sequencing can reveal huge number of "silent" gene clusters which can be learned to activate to run currently non-working biosynthetic pathways (Osbourn, 2010).

Thus, in the nearest future chemotaxonomy and genomic data will allow to improve the procedure for obtaining phytochemical drugs.

CONCLUSION

In this short article, we have tried to present various methods of searching for plant compounds with anti-coronavirus activities, as well as approaches that make it possible to accelerate this search. Chemi-informatics methods provide opportunities for primary screening of large amounts of data to find candidate compounds. Genetic engineering methods make it possible to assess the interaction of candidate compounds with their targets in cell-free systems and in cell culture without resorting to the use of viruses, which significantly expands the list of laboratories for such research. Combining these approaches allows for more accurate study of targeting of potential drugs for subsequent trials. Acceleration of the preclinical phase of new drugs testing can be achieved through the use of transgenic animal models.

Analysis of pharmacological databases allows quickly selection of "candidates," already having assessments of their biosafety and stability, but it is unlikely that a "breakthrough" drug will be obtained at the exit. However, these approaches buy time to treat people while longer studies are still pending.

The "hunt" for new substances makes it possible to find substances with new mechanisms of action, reveal new functional groups, etc. The most effective drugs may be among them. However, this path is the longest, since it will require the most complete study of its pharmacological properties, including toxicity, side effects, stability, etc. Therefore, rapid screening systems for target activities are extremely important.

The analysis of a mixture of compounds in plant extracts seems to us the most promising. The development of metabolomics methods based on a small amount of plant material allows to obtain data on the entire spectrum of substances in the sample under study, containing several compounds with antiviral activity.

In the future, our knowledge of the structure of plant genomes will allow to obtaining the most effective producers of anti-coronavirus compounds by metabolic engineering methods.

AUTHOR CONTRIBUTIONS

TM wrote the basic structure of the paper. GK and SS participated in writing and correcting the paper. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aanouz, I., Belhassan, A., El-Khatabi, K., Lakhlifi, T., El-Ldrissi, M., and Bouachrine, M. (2020). Moroccan medicinal plants as inhibitors against SARS-CoV-2 main protease: computational investigations. *J. Biomol. Struct. Dyn.* 1-9. doi: 10.1080/07391102.2020.1758790 [Epub ahead of print]

Adedeji, A. O., and Sarafianos, S. (2014). Antiviral drugs specific for coronaviruses in preclinical development. *Curr. Opin. Virol.* 8, 45–53. doi: 10.1016/j.coviro.2014.06.002

Alamri, M. A., Altharawi, A., Alabbas, A. B., Alossaimi, M. A., and Alqahtani, S. M. (2020). Structure-based virtual screening and molecular dynamics of phytochemicals derived from Saudi medicinal plants to identify potential COVID-19 therapeutics. ChemRxiv [Preprint]. doi: 10.26434/chemrxiv.12336635.v1

Anand, K., Ziebuhr, J., Wadhwani, P., Mesters, J. R., and Hilgenfeld, R. (2003). Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. *Science* 300, 1763–1767. doi: 10.1126/science.1085658

Báez-Santos, Y. M., St John, S. E., and Mesecar, A. D. (2015). The SARS-coronavirus papain-like protease: structure, function and inhibition by designed antiviral compounds. *Antivir. Res.* 115, 21–38. doi: 10.1016/j.antiviral.2014.12.015

Bevinakoppamath, S., Ramachandra, S. C., and Akila, P. (2020). An insight into the use of transgenic animal models for conducting research on coronavirus. *Int. J. Health Allied Sci.* 9, 18–23. doi: 10.4103/ijhas.IJHAS_86_20

Boone, H. A., Medunjanin, D., and Sijerčić, A. (2020). Review on potential of phytotherapeutics in fight against COVID-19. *Int. J. Innov. Sci. Res. Technol.* 5, 481–491.

Butcher, E. C., Berg, E. L., and Kunkel, E. J. (2004). Systems biology in drug discovery. *Nat. Biotechnol.* 22, 1253–1259. doi: 10.1038/nbt1017

Carter, G. T. (2011). Natural products and Pharma 2011: strategic changes spur new opportunities. *Nat. Prod. Rep.* 28, 1783–1789. doi: 10.1039/c1np00033k

Chen, X., Chou, C. Y., and Chang, G. G. (2009). Thiopurine analogue inhibitors of severe acute respiratory syndrome-coronavirus papain-like protease, a deubiquitinating and deISGylating enzyme. *Antivir. Chem. Chemother.* 19, 151–156. doi: 10.1177/095632020901900402

Chen, L., Hu, C., Hood, M., Zhang, X., Zhang, L., Kan, J., et al. (2020). Novel combination of vitamin C, curcumin and glycyrrhizic acid potentially regulates immune and inflammatory response associated with coronavirus infections: a perspective from system biology analysis. *Nutrients* 12:1193. doi: 10.3390/nu12041193

Chen, L., Li, J., Luo, C., Liu, H., Xu, W., Chen, G., et al. (2006). Binding interaction of quercetin-3-β-galactoside and its synthetic derivatives with SARS-CoV $3CL^{pro}$: structure–activity relationship studies reveal salient pharmacophore features. *Bioorg. Med. Chem.* 14, 8295–8306. doi: 10.1016/j.bmc.2006.09.014

Cheriet, T., Ben-Bachir, B., Thamri, O., Seghiri, R., and Mancini, I. (2020). Isolation and biological properties of the natural flavonoids pectolinarin and pectolinarigenin—a review. *Antibiotics* 9:417. doi: 10.3390/antibiotics9070417

Chikhale, R. V., Gurav, S. S., Patil, R. B., Sinha, S. K., Prasad, S. K., Shakya, A., et al. (2020). SARS-Cov-2 host entry and replication inhibitors from Indian ginseng: an in-silico approach. *J. Biomol. Struct. Dyn.* 1–12. doi: 10.1080/07391102.2020.1778539 [Epub ahead of print]

Cho, J. K., Curtis-Long, M. J., Lee, K. H., Kim, D. W., Ryu, H. W., Yuk, H. J., et al. (2013). Geranylated flavonoids displaying SARS-CoV papain-like protease inhibition from the fruits of *Paulownia tomentosa*. *Bioorg. Med. Chem.* 21, 3051–3057. doi: 10.1016/j.bmc.2013.03.027

Chou, C. Y., Chien, C. H., Han, Y. S., Prebanda, M. T., Hsieh, H. P., Turk, B., et al. (2008). Thiopurine analogues inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biochem. Pharmacol.* 75, 1601–1609. doi: 10.1016/j.bcp.2008.01.005

Clemente, V., D'Arcy, P., and Bazzaro, M. (2020). Deubiquitinating enzymes in coronaviruses and possible therapeutic opportunities for COVID-19. *Int. J. Mol. Sci.* 21:3492. doi: 10.3390/ijms21103492

Coutard, B., Valle, C., De Lamballerie, X., Canard, B., Seidah, N., and Decroly, E. (2020). The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antivir. Res.* 176:104742. doi: 10.1016/j.antiviral.2020.104742

Cragg, G. M., and Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochim. Biophys. Acta* 1830, 3670–3695. doi: 10.1016/j.bbagen.2013.02.008

Das, B., Madhusudhan, P., and Kashinatham, A. (1998). Two efficient methods for the conversion of camptothecin to mappicine ketone, an antiviral lead compound. *Tetrahedron Lett.* 39, 431–432. doi: 10.1016/S0040-4039(97)10539-1

Denoeud, F., Carretero-Paulet, L., Dereeper, A., Droc, G., Guyot, R., Pietrella, M., et al. (2014). The coffee genome provides insight into the convergent evolution of caffeine biosynthesis. *Science* 345, 1181–1184. doi: 10.1126/science.1255274

Dhama, K., Karthik, K., Khandia, R., Munjal, A., Tiwari, R., Rana, R., et al. (2018). Medicinal and therapeutic potential of herbs and plant metabolites/extracts countering viral pathogens—current knowledge and future prospects. *Curr. Drug Metab.* 19, 236–263. doi: 10.2174/1389200219666180129145252

Dhama, K., Khan, S., Tiwari, R., Sircar, S., Bhat, S., Malik, Y. S., et al. (2020). Coronavirus disease 2019-COVID-19. *Clin. Microbiol. Rev.* 33, e00028–e00020. doi: 10.1128/CMR.00028-20

Divya, M., Vijayakumar, S., Chen, J., Vaseeharan, B., and Durán-Lara, E. F. (2020). A review of south Indian medicinal plant has the ability to combat against deadly viruses along with COVID-19? *Microb. Pathog.* 104277. doi:10.1016/j.micpath.2020.104277 [Epub ahead of print]

Drews, J. (2000). Drug discovery: a historical perspective. *Science* 287, 1960–1964. doi: 10.1126/science.287.5460.1960

Erukainure, O. L., Mesaik, M. A., Atolani, O., Muhammad, A., Chukwuma, C. I., and Islam, M. S. (2017). Pectolinarigenin from the leaves of Clerodendrum volubile shows potent immunomodulatory activity by inhibiting T-cell proliferation and modulating respiratory oxidative burst in phagocytes. *Biomed. Pharmacother.* 93, 529–535. doi: 10.1016/j.biopha.2017.06.060

Ghildiyal, R., Prakash, V., Chaudhary, V. K., Gupta, V., and Gabrani, R. (2020). "Phytochemicals as antiviral agents: recent updates" in *Plant-derived bioactives*. ed. M. Swamy (Singapore: Springer).

Ghosh, R., Chakraborty, A., Biswas, A., and Chowdhuri, S. (2020). Evaluation of green tea polyphenols as novel corona virus (SARS CoV-2) main protease (Mpro) inhibitors—an in silico docking and molecular dynamics simulation study. *J. Biomol. Struct. Dyn.* doi: 10.1080/07391102.2020.1779818 [Epub ahead of print]

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Mol. Asp. Med.* 27, 1–93. doi: 10.1016/j.mam.2005.07.008

Handjieva, N., Tersieva, L., Popov, S., and Evstatieva, L. (1995). Two iridoid glucosides, 5-O-Menthiafoloylkickxioside and kickxin, from *Kickxia* Dum. Species. *Phytochemistry* 39, 925–927. doi: 10.1016/0031-9422(95)00019-4

Hilgenfeld, R. (2014). From SARS to MERS: crystallographic studies on coronaviral proteases enable antiviral drug design. *FEBS J.* 281, 4085–4096. doi: 10.1111/febs.12936

Hisashi, Y., and Saito, K. (2013). Network analysis for gene discovery in plant-specialized metabolism. *Plant Cell Environ.* 36, 1597–1606. doi: 10.1111/pce.12069

Ho, K. V., Schreiber, K. L., Park, J., Vo, P. H., Lei, Z., Sumner, L. W., et al. (2020). Identification and quantification of bioactive molecules inhibiting pro-inflammatory cytokine production in spent coffee grounds using metabolomics analyses. *Front. Pharmacol.* 11:229. doi: 10.3389/fphar.2020.00229

Hoopes, G. M., Hamilton, J. P., Kim, J., Zhao, D., Wiegert-Riningeer, K., Crisovan, E., et al. (2018). Genome assembly and annotation of the medicinal plant *Calotropis gigantea*, a producer of anticancer and antimalarial cardenolides. *G3: GENES, GENOMES, GENETICS* 8, 385–391. doi: 10.1534/g3.117.300331

Huang, J., Song, W., Huang, H., and Sun, Q. (2020). Pharmacological therapeutics targeting RNA-dependent RNA polymerase, proteinase and spike protein: from mechanistic studies to clinical trials for COVID-19. *J. Clin. Med.* 9:1131. doi: 10.3390/jcm9041131

Jena, A. B., Kanungo, N., Nayak, V., Chainy, G. B. N., and Dandapat, J. (2020). Catechin and Curcumin interact with corona (2019-nCoV/SARS-CoV2) viral S protein and ACE2 of human cell membrane: insights from computational study and implication for intervention. Research square [Preprint]. doi: 10.21203/rs.3.rs-22057/v1

Jo, S., Kim, S., Shin, D. H., and Kim, M. S. (2020). Inhibition of SARS-CoV 3CL protease by flavonoids. *J. Enzyme Inhib. Med. Chem.* 35, 145–151. doi: 10.1080/14756366.2019.1690480

Kajikawa, M., Sierro, N., Kawaguchi, H., Bakaher, N., Ivanov, N. V., Hashimoto, T., et al. (2017). Genomic insights into the evolution of the nicotine biosynthesis pathway in tobacco. *Plant Physiol.* 174, 999–1011. doi: 10.1104/pp.17.00070

Kang, M., Wu, H., Yang, Q., Huang, L., Hu, Q., Ma, T., et al. (2020). A chromosome-scale genome assembly of *Isatis indigotica*, an important medicinal plant used in traditional Chinese medicine. *Hortic. Res.* 7:18. doi: 10.1038/s41438-020-0240-5

Katsuyama, Y., Matsuzawa, M., Funa, N., and Horinouchi, S. (2007). In vitro synthesis of curcuminoids by type III polyketide synthase from *Oryza sativa*. *J. Biol. Chem.* 282, 37702–37709. doi: 10.1074/jbc.M707569200

Krupanidhi, S., Abraham Peele, K., Venkateswarulu, T. C., Ayyagari, V. S., Nazneen Bobby, M., John Babu, D., et al. (2020). Screening of phytochemical compounds of *Tinospora cordifolia* for their inhibitory activity on SARS-CoV-2: an *in silico* study. *J. Biomol. Struct. Dyn.* 1–5. doi: 10.1080/07391102.2020.1787226 [Epub ahead of print]

Kuo, C. J., Chi, Y. H., Hsu, J. T., and Liang, P. H. (2004). Characterization of SARS main protease and inhibitor assay using a fluorogenic substrate. *Biochem. Biophys. Res. Commun.* 318, 862–867. doi: 10.1016/j.bbrc.2004.04.098

Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., et al. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antivir. Res.* 67, 18–23. doi: 10.1016/j.antiviral.2005.02.007

Lin, M. H., Moses, D. C., Hsieh, C. H., Cheng, S. C., Chen, Y. H., Sun, C. Y., et al. (2018). Disulfiram can inhibit MERS and SARS coronavirus papain-like proteases via different modes. *Antivir. Res.* 150, 155–163. doi: 10.1016/j.antiviral.2017.12.015

Lin, C. -W., Tsai, C. -H., Tsai, F. -J., Chen, P. J., Lai, C. -C., Wan, L., et al. (2004). Characterization of trans- and cis-cleavage activity of the SARS coronavirus 3CLpro protease: basis for the *in vitro* screening of anti-SARS drugs. *FEBS Lett.* 574, 131–137. doi: 10.1016/j.febslet.2004.08.017

Lin, C. -W., Tsai, F. -J., Tsai, C. -H., Lai, C. -C., Wan, L., Ho, T. -Y., et al. (2005). Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds. *Antivir. Res.* 68, 36–42. doi: 10.1016/j.antiviral.2005.07.002

Lutz, C., Maher, L., Lee, C., and Kang, W. (2020). COVID-19 preclinical models: human angiotensin-converting enzyme 2 transgenic mice. *Hum. Genomics* 14:20. doi: 10.1186/s40246-020-00272-6

Mani, J. S., Johnson, J. B., Steel, J. C., Broszczak, D. A., Neilsen, P. M., Walsh, K. B., et al. (2020). Natural product-derived phytochemicals as potential agents against coronaviruses: a review. *Virus Res.* 284:197989. doi: 10.1016/j.virusres.2020.197989

McCray, P. B. Jr., Pewe, L., Wohlford-Lenane, C., Hickey, M., Manzel, L., Shi, L., et al. (2007). Lethal infection of K18-hACE2 mice infected with

severe acute respiratory syndrome coronavirus. *J. Virol.* 81, 813–821. doi: 10.1128/JVI.02012-06

Menachery, V. D., Yount, B. L. Jr., Sims, A. C., Debbink, K., Agnihothram, S. S., Gralinski, L. E., et al. (2016). SARS-like WIV1-CoV poised for human emergence. *Proc. Natl. Acad. Sci. U. S. A.* 113, 3048–3053. doi: 10.1073/pnas.1517719113

Mondal, S., De, N., and Pal, A. (2020). Topological indices of some chemical structures applied for the treatment of COVID-19 patients. *Polycycl. Aromat. Compd.* 1–15. doi: 10.1080/10406638.2020.1770306

Mukherjee, P. K. (2019). "Antiviral evaluation of herbal drugs" in *Quality control and evaluation of herbal drugs*. ed. K. Morrissey (Cambridge, MA, USA: Elsevier), 599–628.

Mumtaz, A., Ashfaq, U. A., ul Qamar, M. T., Anwar, F., Gulzar, F., Ali, M. A., et al. (2017). MPD3: a useful medicinal plants database for drug designing. *Nat. Prod. Res.* 31, 1228–1236. doi: 10.1080/14786419.2016.1233409

Murck, H. (2020). Symptomatic protective action of glycyrrhizin (Licorice) in COVID-19 infection? *Front. Immunol.* 11:1239. doi: 10.3389/fimmu.2020.01239

Nguyen, T. T. H., Woo, H. -J., Kang, H. -K., Nguyen, V. D., Kim, D. -W., Ahn, S. -A., et al. (2012). Flavonoid-mediated inhibition of SARS coronavirus 3C-like protease expressed in *Pichia pastoris*. *Biotechnol. Lett.* 34, 831–838. doi: 10.1007/s10529-011-0845-8

Niraj, S., and Varsha, S. (2020). A review on scope of immuno-modulatory drugs in Ayurveda for prevention and treatment of Covid-19. *Plant Sci. Today* 7, 417–423. doi: 10.14719/pst.2020.7.3.831

Osbourn, A. (2010). Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends Genet.* 26, 449–457. doi: 10.1016/j.tig.2010.07.001

Park, J. Y., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, S. J., Kim, D., et al. (2012a). Diarylheptanoids from *Alnus japonica* inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biol. Pharm. Bull.* 35, 2036–2042. doi: 10.1248/bpb.b12-00623

Park, J. Y., Kim, J. H., Kim, Y. M., Jeong, H. J., Kim, D. W., Park, K. H., et al. (2012b). Tanshinones as selective and slow-binding inhibitors for SARS-CoV cysteine proteases. *Bioorg. Med. Chem.* 20, 5928–5935. doi: 10.1016/j.bmc.2012.07.038

Park, J. -Y., Ko, J. -A., Kim, D. W., Kim, Y. M., Kwon, H. -J., Jeong, H. J., et al. (2016). Chalcones isolated from *Angelica keiskei* inhibit cysteine proteases of SARS-CoV. *J. Enzyme Inhib. Med. Chem.* 31, 23–30. doi: 10.3109/14756366.2014.1003215

Park, J. Y., Yuk, H. J., Ryu, H. W., Lim, S. H., Kim, K. S., Park, K. H., et al. (2017). Evaluation of polyphenols from *Broussonetia papyrifera* as coronavirus protease inhibitors. *J. Enzyme Inhib. Med. Chem.* 32, 504–512. doi: 10.1080/14756366.2016.1265519

Perlman, S., and Netland, J. (2009). Coronaviruses post-SARS: update on replication and pathogenesis. *Nat. Rev. Microbiol.* 7, 439–450. doi: 10.1038/nrmicro2147

Prasanth, D. S. N. B. K., Murahari, M., Chandramohan, V., Panda, S. P., Atmakuri, L. R., and Guntupalli, C. (2020). In silico identification of potential inhibitors from *Cinnamon* against main protease and spike glycoprotein of SARS CoV-2. *J. Biomol. Struct. Dyn.* doi: 10.1080/07391102.2020.1779129 [Epub ahead of print]

Ren, X., Shao, X. X., Li, X. X., Jia, X. H., Song, T., Zhou, W. Y., et al. (2020). Identifying potential treatments of COVID-19 from traditional Chinese medicine (TCM) by using a data-driven approach. *J. Ethnopharmacol.* 258:112932. doi: 10.1016/j.jep.2020.112932

Riva, L., Yuan, S., Yin, X., Martin-Sancho, L., Matsunaga, N., Pache, L., et al. (2020). Discovery of SARS-CoV-2 antiviral drugs through large-scale compound repurposing. *Nature* 586, 113–119. doi: 10.1038/s41586-020-2577-1

Santos, R., Ursu, O., Gaulton, A., Bento, A. P., Donadi, R. S., Bologa, C. G., et al. (2016). A comprehensive map of molecular drug targets. *Nat. Rev. Drug Discov.* 16, 19–34. doi: 10.1038/nrd.2016.230

Sun, D. -D., Dong, W., Li, X., and Zhang, H. -Q. (2010). Indole alkaloids from the roots of Isatis ingigotica and their antiherpes simplex virus type 2 (HSV-2) activity in vitro. *Chem. Nat. Compd.* 46, 763–766. doi: 10.1007/s10600-010-9735-x

Szajdak, L. W. (ed.) (2016). "Introduction: biologically active compounds" in *Bioactive compounds in agricultural soils*. (Cham: Springer).

Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., et al. (2020). Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell. Mol. Immunol.* 17, 613–620. doi: 10.1038/s41423-020-0400-4

Tallei, T. E., Tumilaar, S. G., Niode, N. J., Fatimawali, F., Kepel, B. J., Idroes, R., et al. (2020). Potential of plant bioactive compounds as SARS-CoV-2 main protease (Mpro) and spike (S) glycoprotein inhibitors: a molecular docking study. Preprints [Preprint], 2020040102. doi: 10.20944/preprints202004.0102.v1

Theerawatanasirikul, S., Kuo, C. J., Phetcharat, N., and Lekcharoensuk, P. (2020). *In silico* and *in vitro* analysis of small molecules and natural compounds targeting the 3CL protease of feline infectious peritonitis virus. *Antivir. Res.* 174:104697. doi: 10.1016/j.antiviral.2019.104697

Tripathi, M. K., Singh, P., Sharma, S., Singh, T. P., Ethayathulla, A. S., and Kaur, P. (2020). Identification of bioactive molecule from *Withania somnifera* (Ashwagandha) as SARS-CoV-2 main protease inhibitor. *J. Biomol. Struct. Dyn.* 1–14. doi: 10.1080/07391102.2020.1790425. [Epub ahead of print]

Tseng, C. T., Huang, C., Newman, P., Wang, N., Narayanan, K., Watts, D. M., et al. (2007). Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human angiotensin-converting enzyme 2 virus receptor. *J. Virol.* 81, 1162–1173. doi: 10.1128/JVI.01702-06

van Dorp, L., Acman, M., Richard, D., Shaw, L. P., Ford, C. E., Ormond, L., et al. (2020). Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. *Infect. Genet. Evol.* 83:104351. doi: 10.1016/j.meegid.2020.104351

Vellingiri, B., Jayaramayya, K., Iyer, M., Narayanasamy, A., Govindasamy, V., Giridharan, B., et al. (2020). COVID-19: a promising cure for the global panic. *Sci. Total Environ.* 725:138277. doi: 10.1016/j.scitotenv.2020.138277

World Health Organization (WHO) (2020). Coronavirus disease (COVID-2019) situation reports. Available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (Accessed October 19, 2020).

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. -L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507

Xu, X., Chen, P., Wang, J., Feng, J., Zhou, H., Li, X., et al. (2020). Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Sci. China Life Sci.* 63, 457–460. doi: 10.1007/s11427-020-1637-5

Yuldashev, M. P., Malikov, V. M., and Batirov, É. K. (1996). Flavonoids of the epigeal part of *Kickxia elatine*. *Chem. Nat. Compd.* 32, 30–32. doi: 10.1007/BF01373784

Zhang, Y. -Z., and Holmes, E. C. (2020). Commentary a genomic perspective on the origin and emergence of SARS-CoV-2. *Cell* 181, 223–227. doi: 10.1016/j.cell.2020.03.035

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020b). Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors. *Science* 368, 409–412. doi: 10.1126/science.abb3405

Zhang, D. H., Wu, K. L., Zhang, X., Deng, S. Q., and Peng, B. (2020a). *In silico* screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. *J. Integr. Med.* 18, 152–158. doi: 10.1016/j.joim.2020.02.005

Zidorn, C. (2019). Plant chemophenetics—a new term for plant chemosystematics/plant chemotaxonomy in the macro-molecular era. *Phytochemistry* 163, 147–148. doi: 10.1016/j.phytochem.2019.02.013

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frontiers in Plant Science

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Field-Effect Sensors for Virus Detection: From Ebola to SARS-CoV-2 and Plant Viral Enhancers

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Coronavirus disease 2019 (COVID-19) is a novel human infectious disease provoked by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Currently, no specific vaccines or drugs against COVID-19 are available. Therefore, early diagnosis and treatment are essential in order to slow the virus spread and to contain the disease outbreak. Hence, new diagnostic tests and devices for virus detection in clinical samples that are faster, more accurate and reliable, easier and cost-efficient than existing ones are needed. Due to the small sizes, fast response time, label-free operation without the need for expensive and time-consuming labeling steps, the possibility of real-time and multiplexed measurements, robustness and portability (point-of-care and on-site testing), biosensors based on semiconductor field-effect devices (FEDs) are one of the most attractive platforms for an electrical detection of charged biomolecules and bioparticles by their intrinsic charge. In this review, recent advances and key developments in the field of label-free detection of viruses (including plant viruses) with various types of FEDs are presented. In recent years, however, certain plant viruses have also attracted additional interest for biosensor layouts: Their repetitive protein subunits arranged at nanometric spacing can be employed for coupling functional molecules. If used as adapters on sensor chip surfaces, they allow an efficient immobilization of analyte-specific recognition and detector elements such as antibodies and enzymes at highest surface densities. The display on plant viral bionanoparticles may also lead to long-time stabilization of sensor molecules upon repeated uses and has the potential to increase sensor performance substantially, compared to conventional layouts. This has been demonstrated in different proof-of-concept biosensor devices. Therefore, richly available plant viral particles, non-pathogenic for animals or humans, might gain novel importance if applied in receptor layers of FEDs. These perspectives are explained and discussed with regard to future detection strategies for COVID-19 and related viral diseases.

Keywords: COVID-19, plant VLP tool, label-free detection, virus, field effect, biosensor, charged biomolecules, plant virus nanoparticle

INTRODUCTION

Virus outbreaks remain one of the global problems of our time. Due to the increased mobility of populations as well as the sustained growth in international travel accelerated by globalization, a large number of viruses are spreading rapidly around the globe causing infectious disease outbreaks. Recent well-known examples are severe acute respiratory syndrome coronavirus (SARS-CoV, 2002–2003), H1N1 influenza A virus (swine flu, 2009–2010), Middle East respiratory syndrome coronavirus (MERS-CoV, 2012), Ebola virus (2014–2016), or Zika virus (2015–2016) (Choi et al., 2017; Lee et al., 2018). Moreover, viruses present a growing concern as potential agents for biological warfare and terrorism.

Coronavirus disease 2019 (COVID-19) is a novel human infectious disease provoked by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Paraskevis et al., 2020; Seo et al., 2020; Weiss et al., 2020; Zhang and Holmes, 2020; Zhou et al., 2020). SARS-CoV-2 is highly contagious and has been widely spread worldwide, provoking public health crisis and an unprecedented socio-economic burden in most countries (Morales-Narváez and Dincer, 2020). Due to the rapid increase in the rate of human-to-human infection transmission, the World Health Organization (WHO) has classified the COVID-19 outbreak as a pandemic [as on March 11, 2020 World Health Organization [WHO], 2020 Situation Report-52]. As of November 02, 2020, the total number of confirmed COVID-19 cases around the world was more than 46 millions, resulting in more than 1.2 million deaths (WorldOmeter, 2020; COVID-19).

Currently, no United States Food and Drug Administration (FDA)-approved specific vaccines or curative drugs for the treatment of COVID-19 patients are available. Therefore, one of the key challenges in the effective fight against COVID-19 is the rapid and accurate identification of virus-infected patients (including asymptomatic patients), in order to apply appropriate protective measures (e.g., quarantine, isolation of patients in an early stage, and lockdown) and to slow the rate of transmission of the infection. This is crucial for hospitals to provide sufficient rooms, supplies, doctors, and medical personnel for successful treatment of all patients who need care. In this context, diagnostic tests play an essential role in control and surveillance of the novel COVID-19 outbreak. Moreover, timely and broad application of testing can lead to lower mortality rates, as, for instance, in Germany or South Korea (Morales-Narváez and Dincer, 2020).

Standard methods for emerging virus identification have been reviewed recently (Nguyen et al., 2020; Udugama et al., 2020; Younes et al., 2020) and are primarily based on real-time reverse transcription polymerase chain reaction (RT-PCR). Once the RNA (ribonucleic acid) sequence of SARS-CoV-2 was identified in January 2020 (Zhou et al., 2020), the WHO recommended the nucleic acid-based RT-PCR molecular diagnosis technique for SARS-CoV-2 detection from patients' nasopharyngeal and/or oropharyngeal swab samples (WHO/COVID-19/laboratory/2020.5, 2020). The RT-PCR test is highly sensitive and detects even a tiny viral load in patients. However, the test is labor-intensive, requires skilled personnel, bulky and expensive equipment, is not suitable as a first-line screening tool or for on-site applications, and time-consuming (takes from 3 h up to 2–3 days including preparation of the viral RNA to give results) (Morales-Narváez and Dincer, 2020; Ozer et al., 2020; Ravina et al., 2020). For an effective outbreak containment, this time span is too long.

In order to overcome the limitations of RT-PCR-based systems and to facilitate massive diagnostic testing to counteract the increasing number of undetected cases, test manufacturers around the world have recently developed various portable/handheld, rapid, easy-to-use, point-of-care immunodiagnostic devices for on-site SARS-CoV2 detection in low-resource settings (e.g., in doctors' practices or directly at home), each of which with its pros and cons (Morales-Narváez and Dincer, 2020; Nguyen et al., 2020; Ozer et al., 2020; Ravina et al., 2020; Udugama et al., 2020; Younes et al., 2020). These simple test kits are mostly based either on the detection of virus proteins in respiratory samples (e.g., sputum and throat swab), or of antibodies in human blood/serum, generated by the immune system in response to infection. However, based on current data, the WHO recommends the use of these new immunodiagnostic tests only in research settings and not yet for clinical decision-making, until evidence supporting their use for specific indications is available (WHO Scientific Brief, 2020). Therefore, there is an urgent need for new diagnostic tests and biosensors for virus detection, which are faster, more sensitive, accurate and reliable, easier, and more cost-efficient than existing ones (Bhalla et al., 2020). Such devices should also be capable of label-free, real-time detection/identification of viruses in clinical samples without or with minimal sample preparation steps, making on-site and in-field testing of a larger number of people possible within a shorter time period. Due to the small size, fast response time, label-free operation without need for expensive and time-consuming labeling steps, the possibility of real-time and multiplexed measurements, robustness and compatibility with advanced micro- and nanofabrication technology, biosensors based on semiconductor field-effect devices (BioFEDs) are one of the most fascinating platforms for an electrical detection of charged biomolecules and bioparticles by their intrinsic charge (Poghossian et al., 2013, 2015; Poghossian and Schöning, 2014; Yang and Zhang, 2014; Veigas et al., 2015; Syu et al., 2018). In this review, recent advances and key developments in the field of label-free detection of viruses (including plant viruses) with various types of BioFEDs are presented. Plant viruses are additionally introduced as promising bionanotools and building blocks of smart materials (e.g., Mao et al., 2009; Culver et al., 2015; Khudyakov and Pumpens, 2016; Koch et al., 2016; Wen and Steinmetz, 2016; Dragnea, 2017; Steele et al., 2017; Chu et al., 2018a; Lomonossoff, 2018; Lomonossoff and Wege, 2018; Wege and Lomonossoff, 2018; Chen et al., 2019; Eiben et al., 2019; Wege and Koch, 2020; Wen et al., 2020) that may bring about novel options for biosensor technology if applied as model particles, signal-amplifying colloids or, most importantly, multivalent adapter templates for the high surface-density presentation of detector components.

FUNCTIONING PRINCIPLE OF BioFEDs

Although, at present, numerous BioFEDs based on an electrolyte-insulator-semiconductor (EIS) system have been developed using different sensor configurations, sensitive materials and fabrication technologies, the transducer principle of using an electric field to create regions of excess charge in a semiconductor is common to all of them. In this context, ion-sensitive field-effect transistors (ISFET) (Moser et al., 2016; Syu et al., 2018), extended-gate ISFETs (Pullano et al., 2018), capacitive EIS sensors (Poghossian et al., 2011; Bronder et al., 2015, 2019), light-addressable potentiometric sensors (Yoshinobu et al., 2001, 2017; Wu et al., 2016), silicon nanowire FETs (SiNW-FET) (Patolsky et al., 2006; Wang et al., 2016; Ambhorkar et al., 2018), graphene-based FETs (G-FET) (Choi et al., 2017; Syu et al., 2018), and carbon nanotube-based FETs (CNT-FET) (Choi et al., 2017; Alabsi et al., 2020) modified with biological recognition elements or receptors [e.g., enzymes, antibodies, antigens, peptides, DNA (deoxyribonucleic acid), and living cells] are typical examples of BioFEDs. During the last few years, label-free sensing of molecules by their intrinsic charge has become one of the most reported applications for BioFEDs (Poghossian and Schöning, 2014; Wu et al., 2015; de Moraes and Kubota, 2016; Kaisti, 2017; Bronder et al., 2018). Since FEDs are surface-charge-sensitive devices and because the vast majority of biomolecules are charged under physiological conditions, BioFEDs represent a universal platform for label-free electrostatic detection of a large variety of biomolecules and bioparticles including viruses. In the following, functioning of BioFEDs is briefly explained using the example of SiNW-FETs, which currently receive tremendous interest in biosensor design.

The typical structure of a SiNW-FET biosensor is illustrated in **Figure 1A**, where the channel region in a top Si nanowire between source and drain electrodes serves as the active sensing component. A gate voltage (V_G) is applied via the third capacitively coupled electrode (reference electrode) to regulate the channel conductivity, working point and sensitive characteristics of the SiNW-FET. In order to selectively recognize target biomolecules or bioparticles in solution, the gate insulator surface of the SiNW-FET is functionalized with respective receptors [e.g., antibodies or single-stranded (ss) DNA probes]. The electric potential or charge changes at the SiNW-FET surface induced via the adsorption or binding of charged target biomolecules will alter the density of charge carriers in the channel and will, thus, modulate the conductivity of the channel and current between source and drain terminals. For a p-type SiNW-FET, by binding of positively charged biomolecules or bioparticles on the sensing gate surface, a depletion of charge carriers (in this case, holes) occurs in the nanowire channel. This will decrease the SiNW conductance and current in the nanowire channel for a fixed voltage between drain and source. Conversely, binding of negatively charged biomolecules induces an accumulation of holes, thus increasing the SiNW conductance and current. The opposite changes will be observed for n-type SiNW-FETs. For more detailed information concerning the operation principle and applications of BioFEDs, see reviews (Schöning and Poghossian, 2006; Poghossian and Schöning, 2014; Wang et al., 2016; Choi et al., 2017; Kaisti, 2017; Syu et al., 2018).

LABEL-FREE DETECTION OF VIRUSES WITH BioFEDs

The strategies for label-free electrical detection/identification of viruses with BioFEDs can be subdivided into four categories: (1) direct identification of intact virus particles (virions) via the complete particle charge; (2) detection of viral antigens including non-virion proteins; (3) detection of viral nucleic acids (RNA or DNA); and (4) detection of antibodies produced by the immune system to identify and counteract or neutralize substances foreign to the body. The detection mechanism is always based on the direct measurement of changes in the electrical characteristics of BioFEDs caused from the binding events. The methods based on detection of intact virus particles, certain viral antigens and nucleic acids are more suitable for diagnosing new infected cases, while antibody detection techniques (serological tests) are better suited to determine whether an individual has previously been infected.

To date, different kinds of BioFEDs functionalized with various recognition elements have been applied successfully for the detection of numerous dangerous viruses. Some recent results reported in the literature are summarized in **Table 1**, which also includes the virus type, target, transducer, measurement range and lower detection limit. Selected key developments, including current results on detection of SARS-CoV-2 with BioFEDs, are discussed below.

Intact Virus Particle Detection

The viral load depends on the days after illness onset. Therefore, detection of intact virus particles can provide information to clinicians about the phase of the infection or therapy response. Viral particle concentration is often determined by way of plaque-based assays, by inoculating patient samples to cultivated cell lines and looking for cell death indicated by countable plaques in the confluent cell layer (Ozer et al., 2020). This method is, however, slow and is not applicable for point-of-care or on-site testing.

Since virus particles are generally charged in a wide pH range, BioFEDs are capable for the label-free electrostatic detection of adsorption or binding of charged intact virus particles onto their gate surface. To achieve specificity and to selectively capture the whole virus, the BioFED surface is usually functionalized with antibodies against specific surface proteins of the virus particle. To our best knowledge, the first direct and real-time detection of individual influenza A virus particles using antibody-functionalized SiNW biosensors was demonstrated in 2004 (Patolsky et al., 2004). The SiNWs were able to detect virus particles from a solution containing as low as 5×10^4 particles/mL (Patolsky et al., 2004, 2006). In addition, a multiplexed simultaneous detection of different viruses (influenza A and adenovirus) using an array of individually addressable SiNWs was carried out. Thereafter, a lot of BioFED types have been designed and proven for

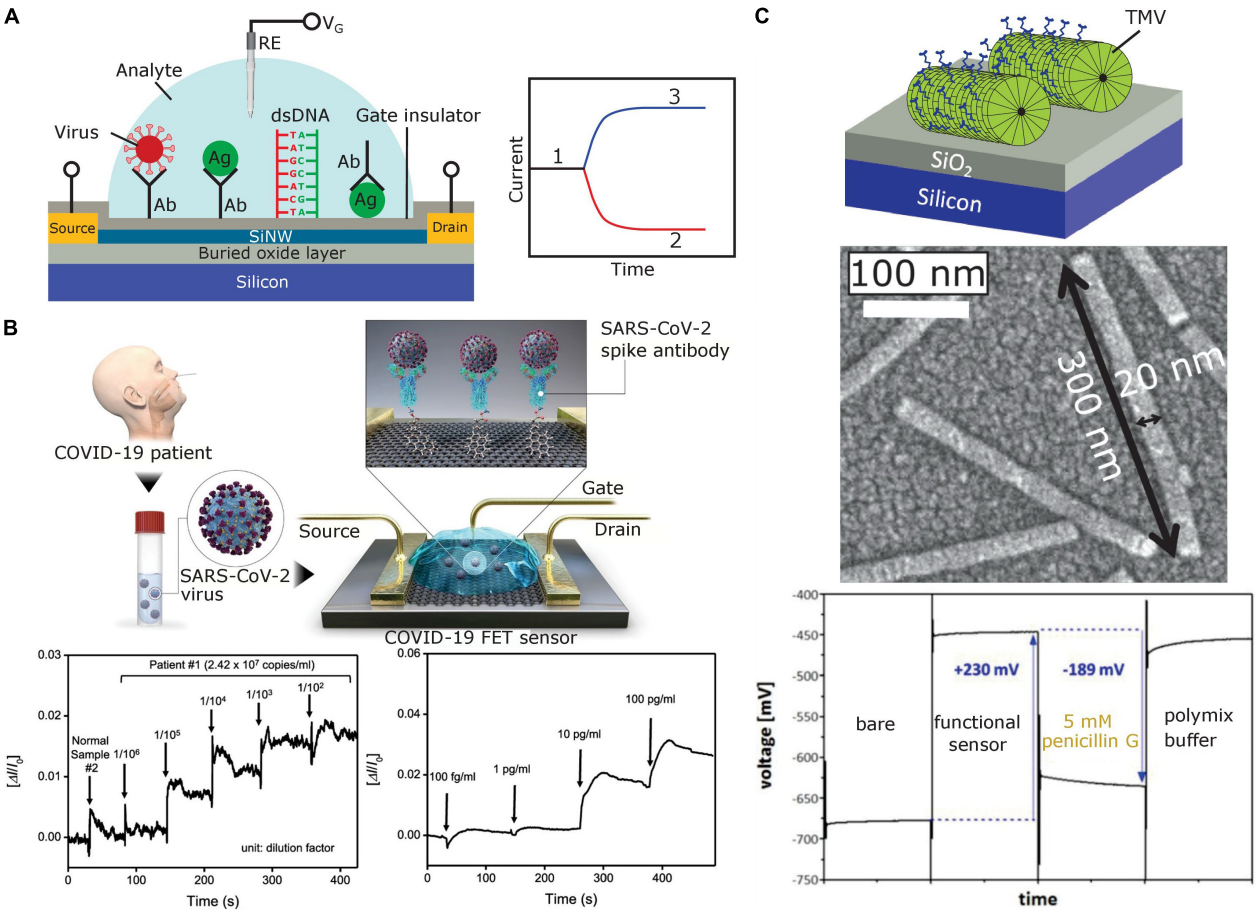

FIGURE 1 | (A) Typical structure of a SiNW-FET prepared on a silicon-on-insulator wafer (left) and expected shift of original sensor signal (1) for a p-type SiNW-FET after binding of positively (2) or negatively charged (3) biomolecules (right). The SiNW-FET is composed of an active top thin Si layer covered with a gate insulator, source and drain electrodes, a thick buried SiO_2 layer, and a bulk Si substrate. To selectively recognize target biomolecules or bioparticles, the gate insulator surface is functionalized with respective receptors (e.g., antibodies, antigens or ssDNA). **(B)** Schematic diagram of the COVID-19 FET sensor operation procedure (top), real-time response of COVID-19 FET toward SARS-CoV-2 cultured virus (bottom left), and SARS-CoV-2 antigen protein (bottom right). Adapted from Seo et al. (2020) with permission of the American Chemical Society. **(C)** Capacitive field-effect EIS sensor modified with TMV particles (top), scanning electron microscopy image of TMV particles on the sensor surface [middle, adapted from Poghossian et al. (2018) with permission from Elsevier], and constant-capacitance response of the EIS sensor [bottom, adapted from Koch et al. (2018a) with Creative Commons Attribution License]. Ab, antibody; Ag, antigen; RE, reference electrode; V_G, gate voltage.

the electrostatic detection of various intact virus particles, including several subtypes of influenza A [H1N1 (Hideshima et al., 2019; Park et al., 2019), H3N2 (Shen et al., 2012), H5N1 (Hideshima et al., 2019), H5N2 (Chiang et al., 2012)], human immunodeficiency virus (HIV) (Kim et al., 2019), rotavirus (Liu et al., 2013), Ebola (Jin et al., 2019), and SARS-CoV-2 (Seo et al., 2020). For instance, a SiNW biosensor for the rapid (within minutes) and low-cost diagnosis of seasonal flu that could detect H3N2 viruses in clinical, exhaled breath condensate samples down to $\sim 3 \times 10^4$ particles/mL, was developed by Shen et al. (2012). An ultrasensitive detection of H5N2 avian influenza virus with a detection limit of 10^4 viruses/mL (ca. 16 aM) has been demonstrated by a reusable SiNW-FET with reversible surface functionalization strategy (Chiang et al., 2012). More recently, a highly sensitive silicon-nanonet FET for the detection of H1N1 influenza A, which is one of the most virulent human pathogens among various types of influenza, was realized (Park et al., 2019). The nanonet FETs were able to detect H1N1 virus particles with a limit of detection down to 10 pg/mL (~0.167 pM). Moreover, the detection and discrimination of human H1N1 and avian H5N1 influenza A viruses in nasal mucus samples by means of glycan-immobilized dual-channel FETs was discussed (Hideshima et al., 2019). To assess the feasibility of remote biosensing and to enable rapid information sharing, the biosensor system was connected to the smartphone via a Bluetooth connection.

In addition to SiNW-FET biosensors, G-FETs have been extensively studied for whole virus particle detection. For example, a G-FET based on micropatterned reduced graphene oxide was applied for real-time rotavirus detection (Liu et al., 2013). The lowest detection limit for rotavirus was determined as 10^2 pfu/mL (plaque-forming units/mL), which is superior to conventional ELISA (enzyme-linked immunosorbent assay) tests. The biosensor was applied in fecal samples spiked with different concentrations (10–10^4 pfu/mL) of rotavirus solution.

TABLE 1 | Selected examples of virus detection with different kinds of BioFEDs.

Virus, subtype	Target	Transducer	Measurement range	Lower detection limit	References
Influenza A, H1N1	Virus particle	Dual-channel FET	$10^{0.5}$–$10^{8.5}$ $TCID_{50}$/mL	$10^{0.5}$ $TCID_{50}$/mL	Hideshima et al., 2019
		Nanonet FET	0.01–100 ng/mL	10 pg/mL	Park et al., 2019
		SiNW-FET	n.s.	∼3 × 10^4 particles/mL	Shen et al., 2012
	Nucleic acid	SiNW-FET	2–102 pM	40 pM	Karnaushenko et al., 2015
Influenza A, H3N2	Virus particle	SiNW-FET	n.s.	∼3 × 10^4 particles/mL	Shen et al., 2012
Influenza A, H5N2	Virus particle	SiNW-FET	10^4–10^7 particles/mL	10^4 particles/mL	Chiang et al., 2012
Influenza A, H5N1	Virus particle	FET	$10^{0.5}$–$10^{8.5}$ $TCID_{50}$/mL	$10^{0.5}$ $TCID_{50}$/mL	Hideshima et al., 2019
	Viral antigen	FET	10 pM–10 nM	5.9 pM	Kwon et al., 2020
	Nucleic acid	CNT-FET	1 pM–100 nM	1.25 pM	Thu et al., 2013
Influenza A	Virus particle	SiNW array	n.s.	5 × 10^4 particles/mL	Patolsky et al., 2004
	Nucleic acid	SiNW-FET	1 fM–10 pM	n.s.	Lin et al., 2009
		SiNW-FET	n.s.	100 pM	Lin et al., 2012
		CNT-FET	1 pM–10 nM	1 pM	Tran et al., 2017
	Antibody	SiNW-FET	0.4–4 μg/mL	∼1 nM	Kim et al., 2014
		SiNW-FET	n.s.	20 μg/mL	Ahn et al., 2015
		FET	50 ng/mL–10 μg/mL	n.s.	Gu et al., 2009
Dengue	Nucleic acid	SiNW-FET	n.s.	2 fM	Nuzaihan et al., 2016
		SiNW-FET	n.s.	10 fM	Nuzaihan et al., 2018
		SiNW-FET	1–100 fM	∼10 fM	Zhang et al., 2010
	Viral antigen	FET	0.25–5 μg/mL	0.25 μg/mL	Vieira et al., 2014
HIV-1	Virus particle	G-FET	47.8 aM–4.78 fM	47.8 aM	Kim et al., 2019
HIV-2	Antibody	SiNW-FET	n.s.	4 μg/mL	Kim et al., 2014
Rotavirus	Virus particle	G-FET	10–10^4 pfu/mL (fecal samples)	10^2 pfu/mL	Liu et al., 2013
Ebola	Virus particle	G-FET	2.4 pg/mL–1.2 μg/mL	2.4 pg/mL	Jin et al., 2019
	Viral antigen	G-FET	1–444 ng/mL	1 ng/mL	Chen et al., 2017
SARS-CoV-2	Virus particle	G-FET	16–1.6 × 10^4 pfu/mL (cultured virus) 242–24 × 10^4 particles/mL (clinical samples)	16 pfu/mL 242 particles/mL	Seo et al., 2020
	Viral antigen	G-FET	1 fg/mL–10 pg/mL	1 fg/mL in buffer, 100 fg/mL in CTM	Seo et al., 2020
Hepatitis B	Nucleic acid	SiNW-FET	1 fM–1 pM	3.2 fM	Wu et al., 2014
Hepatitis C	Nucleic acid	CNT-FET	0.5 pM–5 nM	0.5 pM	Dastagir et al., 2007
Zika	Viral antigen	G-FET	n.s.	450 pM	Afsahi et al., 2018
Plum Pox (plant virus)	Virus particle	Organic FET	5 ng/mL–50 μg/mL	180 pg/mL (theoretical)	Berto et al., 2019

TCID, 50% tissue culture infectious dose; n.s., not specified; pfu, plaque-forming unit; CTM, clinical transport medium for nasopharyngeal swabs.

A FET modified with reduced graphene oxide for the detection of inactivated Ebola virus particles from a spiked buffer solution with a detection limit of a 2.4 pg/mL was reported (Jin et al., 2019). Ebola virus is a highly pathogenic virus that invades most major organs and causes multisystem failure in humans with a case fatality rate of up to 90% (Jin et al., 2019). The biosensor with immobilized antibodies against the virus spike glycoprotein was successfully applied for the quantitation of inactivated Ebola viruses diluted in human serum, with a high specificity and a low detection limit of 12 pg/mL. An attomolar (47.8 aM) detection of HIV by coplanar-gate G-FETs prepared on flexible plastic substrates was demonstrated by Kim et al. (2019). Finally, quite recently, highly sensitive G-FETs have been engineered to determine the SARS-CoV-2 viral load in clinical nasopharyngeal swab samples from COVID-19 patients by way of graphene sheets coated with an antibody specific for the viral spike protein (Seo et al., 2020; see **Figure 1B**). The novel SARS-CoV-2 virus is highly infectious with a particle diameter of 60–140 nm (Scheller et al., 2020). The G-FET was successfully applied for label-free electrostatic and rapid detection of SARS-CoV-2 in culture medium and clinical samples (without sample pre-treatment) with a detection limit of 16 pfu/mL and 242 particles/mL, respectively (Seo et al., 2020).

Detection of Viral Antigens

Biosensors for the detection of viral proteins represent immunologically sensitive FEDs (ImmunoFEDs), which detect affinity binding of viral antigens in a sample (e.g., from the respiratory tract of a person) to specific antibodies, antibody fragments or fusion proteins, affibodies or aptamers immobilized

onto the gate surface, generating a detectable electrical signal (Poghossian and Schöning, 2014; de Moraes and Kubota, 2016). ImmunoFEDs for the label-free viral antigen detection were used to identify influenza A (Hideshima et al., 2013; Uhm et al., 2019; Kwon et al., 2020), Ebola (Chen et al., 2017; Generalov et al., 2019), dengue (Vieira et al., 2014), Zika (Afsahi et al., 2018), and SARS-CoV-2 (Seo et al., 2020) viruses. For example, a SiNW-FET functionalized with respective antibodies as receptor molecules was utilized for the detection of Ebola virus VP40 matrix proteins (Generalov et al., 2019). Extended-gate FET immunosensors were applied for the label-free detection of dengue virus non-structural proteins (Vieira et al., 2014) as well as of hemagglutinin glycoproteins of the highly pathogenic avian influenza virus H5N1 with a detection limit of 5.9 pM (Kwon et al., 2020). Moreover, attomolar detection of influenza A virus antigens with a glycan-modified FET was demonstrated (Hideshima et al., 2013). Real-time, quantitative detection of Zika viral antigens with a detection limit of 450 pM in buffer solution using commercially available graphene biosensor chips was reported (Afsahi et al., 2018). The potential of the biosensor for diagnostic applications was demonstrated by measuring the Zika antigen in diluted human serum samples. A G-FET modified with Au nanoparticles functionalized with anti-Ebola antibodies for real-time, highly sensitive and specific detection of the Ebola virus glycoprotein with a detection limit down to 1 ng/mL was developed as well (Chen et al., 2017). The applicability of this G-FET for point-of-care applications was evaluated in diluted buffer, human serum, and plasma spiked with Ebola glycoproteins. More recently, a G-FET biosensor was developed for the detection of SARS-CoV-2 spike proteins (**Figure 1B**; Seo et al., 2020). As receptor layer, specific antibodies against the SARS-CoV-2 spike glycoprotein were immobilized on graphene sheets [i.e., two-dimensional (2D) sheets of hexagonally arranged carbon atoms]. SARS-CoV-2 encodes four structural proteins (spike, envelope, membrane, and nucleocapsid), 16 non-structural proteins and nine accessory factors (Fehr and Perlman, 2015; Gordon et al., 2020). Among those, the spike proteins exposed on the virion surface are highly immunogenic and elicit specific antibodies best suited as reliable diagnostic markers for the immunodetection of a productive virus infection (Meyer et al., 2014; Mavrikou et al., 2020). The G-FET could detect the SARS-CoV-2 spike proteins with a detection limit of 1 fg/mL in phosphate-buffered saline and 100 fg/mL in clinical transport medium used for nasopharyngeal swabs. Moreover, the biosensor could distinguish the SARS-CoV-2 antigen protein from that of MERS-CoV (Seo et al., 2020). The authors claim that their biosensor can detect viral antigens in clinical samples without any preparation steps.

BioFEDs for the detection of viral antigens including virion and non-virion proteins could potentially be used for the rapid identification of infected patients, reducing or eliminating the need for expensive molecular confirmatory testing for viral nucleic acids. Antigen tests may thus be one way to scale up testing capacities to much greater levels. On the other hand, antigen tests are reliable only if the target viral proteins expressed by the virus are present in a sample in sufficient concentrations (i.e., when the respective gene products accumulate to detectable titers upon active virus replication). Swabs of patients (especially, for asymptomatic patients) infected with respiratory viruses often lack enough antigen material to be detectable. If, however, suitable antigens have been determined, their immunology-based detection is an excellent method allowing the identification of acute or early infection (WHO Scientific Brief, 2020).

Detection of Virus Nucleic Acids

Most DNA BioFEDs are based on the detection of DNA-hybridization events and are constructed by immobilizing ssDNA capture probes onto the gate surface of the FED (Poghossian and Schöning, 2014; Bronder et al., 2015; Mu et al., 2015). During the DNA hybridization process, target DNA or RNA, respectively, within a sample is identified by a probe ssDNA that forms a double-stranded (ds) DNA or DNA/RNA helix with two reverse-complementary strands. Since nucleic acids are negatively charged in near-neutral aqueous solution, the additional charge associated with the hybridization-captured target molecule will effectively alter the gate surface charge, modulating the output signal of the BioFED.

Viral particles include either an RNA or a DNA genome of ss or ds nucleic acids. Single-stranded viral nucleic acids may exist in positive (+) sense, i.e., directly translatable, or negative (−) sense, i.e., complementary polarity. Therefore, a variety of BioFEDs (mostly based on SiNW-FETs or CNT-FETs) have been developed for detecting nucleic acid sequences of different viruses directly or after reverse transcription, including the genomic RNAs of influenza A virus [(−)ssRNA] (Lin et al., 2009, 2012; Kao et al., 2011; Thu et al., 2013; Karnaushenko et al., 2015; Tran et al., 2017), dengue virus [(+)ssRNA] (Zhang et al., 2010; Nuzaihan et al., 2016, 2018), or (+)ssRNA of hepatitis C virus (Dastagir et al., 2007), and the partially ds DNA of hepatitis B viruses (Wu et al., 2014; Lu et al., 2016). These BioFEDs are highly sensitive with detection limits often in the pM range, although a detection limit in the fM range was reported for ultrasensitive SiNW-FETs as well (Lin et al., 2009; Wu et al., 2014; Nuzaihan et al., 2016). However, most of the DNA probe-based BioFEDs have been tested in buffer solutions using short synthetic DNA sequences as model targets.

In spite of the ultrahigh sensitivity of SiNW-FET DNA biosensors reported in the literature, direct detection of unamplified nucleic acid in clinical specimens is still very difficult due to several reasons. To detect nucleic acids from real samples, the virus particles need to be disrupted (via heating or chemical treatment) in order to release the nucleic acid, which adds additional sample preparation procedures. Moreover, the amount of virus genomes present in clinical samples is in many cases far below the lower detection limit of reported DNA BioFEDs. Therefore, viral low-titer nucleic acids often demand for pre-amplification, e.g., by PCR using suitable primers. To enable a reliable detection of virus RNA [including the (+)RNA of SARS-CoV-2] from clinical samples without additional amplification, the detection limit of nucleic acid biosensors should be below ∼100 aM (Ozer et al., 2020). Otherwise, RT-PCR techniques may be used for reverse transcription of virus RNA extracted from patient samples into complementary cDNA, and amplification of target sequences

from the resulting cDNA template. One example of such concept is a silicon-based microfluidic system combining a chip-based PCR module for amplification of nucleic acid targets, and a multiplexed SiNW sensing module developed for detection and differentiation of influenza A strains (swine-originated H1N1 and seasonal Flu A) according to sequence variations in the viral (−)RNA as identified through the corresponding cDNAs (Kao et al., 2011). Highly appealing due to their convincing sensitivity, speed and easy use even without pricey laboratory equipment are isothermal amplification techniques such as reverse-transcription-loop-mediated isothermal amplification (RT-LAMP; Russo et al., 2020) and recombinase polymerase amplification (RPA; Esbin et al., 2020).

Taken together and due to the low sample consumption, high sensitivity and specificity, such chip-based PCR or isothermal amplification modules in combination with FED sensor systems could be attractive alternatives for point-of-care applications.

Detection of Host Antibodies

Antibody tests are typically used to detect the presence of virus-specific antibodies (immunoglobulins) in the blood of virus hosts, when the immune system is responding to a particular infection (Younes et al., 2020). Immunoglobulin M (IgM) antibodies are usually produced during the onset of the infectious disease (between 4 and 10 days after virus uptake), whereas immunoglobulin G (IgG) responses occur later (around 2 weeks post inoculation) (Morales-Narváez and Dincer, 2020). Therefore, antibody detection tests can be useful to understand how many people have been exposed to a virus and underwent a symptomatic or asymptomatic infection (which is of primary importance for a better understanding of the SARS-CoV-2 epidemiology), as well as to support the development of vaccines.

Immuno-FEDs for the detection of specific host antibodies against viruses are prepared by an immobilization of viral capture antigens serving as receptors on the gate surface. Such biosensors detect charge changes, induced by affinity binding of host target antibodies to viral antigens. Immuno-FEDs for the detection of antibodies against viruses have been rarely studied (mostly as proof-of-concept experiments). For example, a nanogap FET (Gu et al., 2009), an underlap channel-embedded FET (Lee et al., 2010), and a SiNW-FET (Ahn et al., 2015) were realized to detect specific antibodies directed against avian influenza viruses. Moreover, the multiplexed detection of antibodies against avian influenza and human immunodeficiency viruses (HIV) by means of an underlap-embedded SiNW-FET is demonstrated (Kim et al., 2014). In another approach, a SiNW biosensor integrated with a microfluidic channel was applied for the detection of antibodies against Aleutian disease virus in serum samples from infected minks (Svendsen et al., 2011). Finally, an extended-gate FET was developed and tested for the detection of antibodies against bovine herpes virus-1 in both commercially available antiserum and real serum samples from cattle (Tarasov et al., 2016).

Summarizing this section, it should be noted that the stability of the reference electrode and the level of leakage current are crucial factors for a correct functioning of BioFEDs; they will essentially impact accuracy, reproducibility and reliability of measurements. In spite of this fact, in many papers discussed in the literature, information on type or stability of (quasi-) reference electrodes used, as well as on the leakage current level is missing.

DETECTION OF PLANT VIRUSES AS PATHOGENS AND POTENTIAL MODEL PARTICLES

Plant viruses are among the major contributors to economic losses in agriculture [more than 50 billion €/year worldwide (Pallás et al., 2018)]. Therefore, there is great interest in sensitive, rapid and easy-to-use portable devices for an early detection of viruses in infected plants by in-field or on-site application (Khater et al., 2017; Cassedy et al., 2020).

Notwithstanding, we have found only two cases of electrostatic detection of plant virus particles with FEDs. The usability of capacitive field-effect EIS sensors for label-free electrical detection of plant virus particles was initially demonstrated by Koch et al. (2018a) and Poghossian et al. (2018) for tobacco mosaic virus (TMV). Here, EIS structures with adsorbed TMV particles were used for designing a penicillin biosensor, where the TMV particles served as nanocarriers for enzymes installed at high surface densities on the viral coat protein (CP) subunits. TMV has a nanotube-like structure with a single RNA molecule and 2,130 CPs helically assembled into full-length particles of 300 nm, outer diameter of 18 nm and a longitudinal internal channel of 4 nm diameter (Klug, 1999; Scholthof et al., 1999; Culver, 2002; Lomonossoff and Wege, 2018; Wege and Koch, 2020). TMV is harmless for mammals (Nikitin et al., 2016) and lacks a membrane envelope; it and related tobamoviruses infect numerous plant species in several families through mechanical transmission fast and efficiently (Zaitlin, 2000; Adams et al., 2017). Tobacco (family *Solanaceae*) leaves systemically infected with wildtype TMV develop characteristic mosaic-like patterns, but symptoms in other plants and with TMV mutants may be less distinctive (Culver, 2002). Frequent outbreaks of TMV and related viruses in cultivated plants, namely in greenhouse crops such as tomato, pepper, cucurbits, and ornamentals thus demand for rapid identification to avoid substantial economic losses (Scholthof et al., 2011; Moriones and Verdin, 2020). This seems possible by way of FEDs: A single loading of TMV particles onto a Ta_2O_5-gate EIS sensor surface resulted in a large signal change of 230 mV (see **Figure 1C**; Koch et al., 2018a), which is associated with the negative charge of the TMV particles. The model study used biotinylated TMV, but should be valid also for native TMV particles exhibiting a similar charge. Recently, an electrolyte-gated organic FET biosensor for the quantification of plum pox virus (PPV) in plant extracts was realized (Berto et al., 2019). PPV is highly infectious, causes the devastating Sharka disease and thereby affects stone fruit trees in most parts of the world (Hajizadeh et al., 2019). Early PPV recognition is crucial to eliminate infected trees from orchards before the virus has been spread by its insect vectors (aphids) further. Anti-PPV polyclonal antibodies were immobilized by Berto et al. (2019) on the separated Au gate electrode. The biosensor shows great

promise for in-field applications as it was able to detect specific binding of PPV particles to anti-PPV antibodies in plant extracts with a sub ng/mL detection limit.

These two incidences of FED-based plant virus detection do not only point to agronomically relevant perspectives for monitoring such viruses by label-free biosensors, they also demonstrate a huge potential of plant-harvested viruses as harmless model and calibration particles for the electrical detection of animal and human viral diseases. For a plenitude of differently shaped and charged plant-borne viruses, purification and storage protocols have been optimized during the last decades (Dijkstra and de Jager, 1998; Wege and Lomonossoff, 2018). Furthermore, several robust plant viruses are employed already commercially for the production of recombinant virus-like particles (VLPs) displaying domains of non-plant viral proteins on their outer CP surfaces, including SARS-CoV-2 epitopes (Capell et al., 2020; Rosales-Mendoza, 2020). Such preparations could be of high value for the development of FED formats suitable to pre-select or identify COVID-19-infected samples from patients, by help of plant-derived mimics of SARS-CoV-2 that serve as model particles to determine appropriate FED setups and detection conditions.

PLANT VIRUS-BASED BUILDING BLOCKS ENHANCING BIOSENSOR PERFORMANCE

Since more than two decades, several plant viruses attract increasing attention also from a different point of view: Their precise and robust nanostructures with repetitively organized, multivalent protein surfaces lend these viruses and derivatives thereof to uses in medical and technical environments, as carrier particles for the delivery and/or display of functional units enclosed and/or exposed at high densities (Bittner et al., 2013; Lin and Ratna, 2014; Culver et al., 2015; Khudyakov and Pumpens, 2016; Koch et al., 2016; Wen and Steinmetz, 2016; Dragnea, 2017; Steele et al., 2017; Lomonossoff and Wege, 2018; Wege and Lomonossoff, 2018; Balke and Zeltins, 2019; Chen et al., 2019; Eiben et al., 2019; Roeder et al., 2019; Chung et al., 2020; Wege and Koch, 2020; Wen et al., 2020). The respective plant viruses and VLPs are richly and sustainably available by farming (Marsian and Lomonossoff, 2016; Gowtham and Sathishkumar, 2019; Rybicki, 2020), and despite a remarkable durability biodegradable after use. Certain plant viral CPs are amenable to modifications facilitating the selective coupling of functional molecules, and to *in vivo* or *in vitro* assembly into VLPs even in the absence of viral nucleic acids (Wege and Koch, 2020). This allows the fabrication of artificial, bioinstructive carrier particles of adapted shapes and surface chemistries. These benefits of plant virus-based immobilization templates might offer novel options for improving SARS-CoV-2 biosensors, in analogy to promising results with previously developed virus nanoparticle-assisted detection systems.

Applicability of Plant Viral Nanoscaffolds

On account of the properties sketched above, many biomedical uses of plant viral nanoparticles are emerging and have been reviewed in detail (Franzen and Lommel, 2009; Czapar and Steinmetz, 2017; Steele et al., 2017; Aljabali et al., 2018; Eiben et al., 2019; Hema et al., 2019; Benjamin et al., 2020; Chung et al., 2020; Silva et al., 2020). They include, among others, the directed delivery of imaging agents and therapeutics to target sites, e.g., via the blood stream to tumors or atherosclerotic lesions. Cell culture and tissue engineering were shown to profit from cell adhesion and differentiation-mediating peptides presented on plant viral scaffolds in 2D and three-dimensional (3D) layouts. The largest and most advanced area of medical uses are plant VLP-based self-adjuvanting vaccines (Chackerian, 2007; Crisci et al., 2012; Matić and Noris, 2015; Hefferon, 2018; Balke and Zeltins, 2020; Rybicki, 2020; Santoni et al., 2020) with candidates against COVID-19 in the developmental pipelines of at least two companies (Rosales-Mendoza, 2020), as specified also in this research topic. Similarly, plant viral particles are being evaluated on various technological platforms that gain enhanced or even novel functionality through an integration of multivalent, selectively addressable bionanostructures (Fan et al., 2013; Culver et al., 2015; Koch et al., 2016; Dragnea, 2017; Narayanan and Han, 2017; Chu et al., 2018a; Chen et al., 2019; Wege and Koch, 2020). Uses as templates for inorganic and synthetic compounds have led to biohybrid materials of convincing properties (Douglas and Young, 1998; Bittner et al., 2013; Vilona et al., 2015; Tiu et al., 2016; Wen and Steinmetz, 2016; Lee et al., 2017; Zhang et al., 2018; Eiben et al., 2019), such as high-capacity battery electrodes or spatially ordered dye ensembles for light-harvesting. If employed as immobilization scaffolds for biomolecules, from peptides and antibodies up to enzymes, plant VLPs exhibit special advantages (Sapsford et al., 2006; Werner et al., 2006; Comellas-Aragones et al., 2007; Minten et al., 2011; Aljabali et al., 2012; Pille et al., 2013; Uhde-Holzem et al., 2016; Roeder et al., 2017; Dickmeis et al., 2018; Koch et al., 2018b; Tian et al., 2018; Yuste-Calvo et al., 2019a; Aves et al., 2020; Park et al., 2020). This has laid the foundation for novel plant virus-supported biocatalytic nanomaterials (Carette et al., 2007; Cardinale et al., 2012; Koch et al., 2015; Besong-Ndika et al., 2016; Cuenca et al., 2016; Brasch et al., 2017; Schwarz et al., 2017; Aumiller et al., 2018; Chakraborti et al., 2019), and for biodetection formats that may serve as blueprints for novel SARS-CoV-2 sensor layouts, as outlined in the following.

Plant Virus-Enhanced Biosensors: State-of-the Arts and Perspectives

Plant viral soft-matter nanoparticles with hundreds up to thousands CP subunits offer one-of-a-kind opportunities for enhancing the performance of miniaturized biosensors including BioFEDs for a fast, reliable, durable and economically reasonable on-site detection of many targets (Mao et al., 2009; Koch et al., 2016; Lomonossoff and Wege, 2018; Eiben et al., 2019; Benjamin et al., 2020; Wege and Koch, 2020). Natural and engineered viral CPs allow selective coupling of biorecognition elements by direct or linker-mediated chemical conjugation and/or affinity docking, and in some cases genetic or (auto-)catalytic fusion (see references above). Thereby, capture units such as antibodies, trappable peptides or target-specific single-type or cooperating enzymes can be installed at down to nanometer

distances in monolayers or staggered arrangements on the viral backbones. In turn, plant viral adapter scaffolds may be immobilized efficiently on different types of sensor surfaces. Various deposition techniques for viruses and VLPs to bare and pre-treated technical surfaces have been optimized, including adsorption, spin- and convective coating, intermediate self-organization at liquid/liquid interfaces (Wege and Lomonossoff, 2018; Zhang et al., 2018), electrokinetics such as electrophoresis and dielectrophoresis (Lapizco-Encinas and Rito-Palomares, 2007; Bittner et al., 2013), electrowetting (Chu et al., 2018b) or microfluidics (Zang et al., 2017). They can be applied to viral nanoparticles before or after their loading with target recognition elements to yield receptor layers of high surface densities, which may increase sensor sensitivity substantially in comparison to conventional layouts. Such "ultradense" presentation of efficiently immobilized capture units by way of plant VLP adapter templates has been demonstrated in several biosensor layouts for distinct systems with both indirect and label-free read-out (e.g., Szuchmacher Blum et al., 2011; Zang et al., 2014, 2017; Fan et al., 2015; Koch et al., 2015, 2018a; Tinazzi et al., 2015; Bäcker et al., 2017; González-Gamboa et al., 2017; Poghossian et al., 2018; Yuste-Calvo et al., 2019b), as also detailed in the respective sections of recent reviews (Koch et al., 2016; Eiben et al., 2019; Benjamin et al., 2020).

For COVID-19 diagnostics by point-of-care devices, highest detection sensitivities through SARS-CoV-2 enrichment on densely antibody- or aptamer-equipped sensors will be crucial to minimize false-negative results in swab samples from early or late infection stages, and in diluted gargle lavages ("mouthwashes") (Malecki et al., 2020) increasingly utilized for convenient high-throughput testing. Plant virus interlayers on sensor chips may be of high practical value in this context. Furthermore, robust plant VLPs can also serve as additional signal-amplifying colloids if applied post target trapping on a sensor surface. In this case, bifunctional VLPs displaying both capture and signal-generating elements are suitable for indirect sensor layouts, like ELISA or fluorescent microchip arrays (Soto et al., 2006, 2008, 2009), whereas direct, label-free sensors including BioFEDs can make use of VLPs equipped with target capture units only.

Last but not least, different sensor systems with biorecognition elements exposed on plant viral carriers were shown to harbor unexpectedly enhanced reusability and long-term stability over weeks up to months, in comparison to their plant virus-free counterparts (Koch et al., 2015, 2018a). Enzyme-based, TMV-assisted capacitive field-effect EIS sensors for antibiotics retained full sensitivity over at least one year of repeated uses (Poghossian et al., 2018). They were also compatible with "real-world" samples, i.e., diluted milk. Amperometric glucose sensors with TMV nanocarriers for glucose oxidase did not only exhibit higher sensitivity than the sensors devoid of TMV, they also had faster response time and extended linear detection range (Bäcker et al., 2017). However, an electrical detection of coronaviruses in combination with plant VLP-immobilized SARS-CoV-2-specific antibodies, synthetic recognition elements or virus nucleic acid-directed probes, respectively, yet remains to be evaluated, preferably in a BioFED layout. Against the background of the above and several other examples, advantageous sensor properties conveyed by the plant viral carrier templates are likely, with respect to shelf-life, sensor robustness, reusability and overall performance. To evaluate and establish options for routine applications in commercially available devices, it seems crucial to define globally harmonized regulatory prerequisites and standardized rules of good manufacturing practice (GMP).

CONCLUSION

Viral diseases are one of the major threats to health and life of the world population. Early diagnosis and treatment are essential in order to slow down the virus spread and to contain the disease outbreak. The SARS-CoV-2 pandemic has dramatically highlighted the critical role of diagnostic technologies in the control of infectious diseases. Hence, development of new rapid, highly sensitive, accurate and reliable, easy-to-use and cost-efficient, portable point-of-care diagnostic tests, and devices for virus detection in low-resource settings has tremendous importance for medical healthcare. In this review, recent advances and key developments in the field of a label-free detection of various dangerous viruses by means of different types of BioFEDs, which represent one of the most promising transducer platforms for miniaturized biosensors, are presented.

The study of the current state of BioFEDs for virus detection reveals that BioFEDs, especially SiNW-FETs and G-FETs, enable an ultrasensitive label-free electrical detection of intact virus particles (including plant viruses), viral antigens and nucleic acids as well as antibodies against viruses by their intrinsic charge. For some BioFEDs, a detection limit down to the fM concentration range has been reported. In addition, multiplexed detection and discrimination of viruses was demonstrated. Other advantages of BioFEDs are small sizes, fast response time and the possibility of real-time detection on the one hand, and the possibility of integration with on-chip microfluidics and compatibility with complementary metal-oxide-semiconductor (CMOS) technology allowing the fabrication of large volumes of reproducible devices with lower costs on the other hand. Moreover, the optional detection of more than one virus-related parameter (intact virus particle, viral antigen, viral nucleic acids, and antibodies generated against virus) with an array of BioFEDs on the same chip could offer more accurate and reliable disease diagnosis.

Despite remarkable progress in BioFEDs for label-free virus detection, it should be noted that BioFEDs are often studied under rather ideal experimental conditions. There are still some limitations (e.g., screening the charge of biomolecules or virus particles by counter ions in the solution, or possible non-specific binding of further biomolecules present in samples on the sensor surface) for BioFED applications with real biological samples (whole blood, plasma, serum, urine, saliva, nasopharyngeal swabs, or gargle lavage) that must be overcome, before their transfer from scientific laboratories to real life will appear. Biological samples contain a large number of charged chemical species, which are able to non-specifically adsorb on the gate surface of the FEDs, generating a false-positive signal or masking the useful signal from the target of interest. This could substantially hamper the sensitivity,

specificity and reliability of FEDs. Therefore, recently, several different strategies have been proposed to reduce the influence of the counter-ion screening effects (e.g., the use of desalted/filtered samples or short receptors) or non-specific adsorption (the use of blocking agents, pre-filtering/purifying the biological liquids or on-chip separation and pre-concentration). Thus, the sensitivity and detection limit of FEDs can be distinctly enhanced. Another task is the development of a stable and reliable, miniaturized reference electrode integrated onto the FED chip. In addition, the reproducibility of surface modification and receptor immobilization procedures, specificity, stability, time-to-result and reliability of FEDs represent further key parameters, which need to be improved for "real life" measurements. Generally, a real-time label-free electrostatic detection of charged molecules and biological particles in untreated biological samples still remains challenging. The success in implementation and widespread application of FEDs for virus detection will depend on how advanced they are compared with the current gold standards in terms of simplicity, rapidity, sensitivity, specificity and reliability.

In this context, plant viruses and VLPs applied as nanocarriers for target recognition elements offer exciting perspectives for enhancing the performance of miniaturized on-site biosensors with regard to stability, functionality in complex sample mixtures, sensitivity, and further detection parameters. Their endurance may obviate the need for frequent re-calibration of handheld devices, which would be of particular importance for COVID-19 and other viral diseases' early detection in developing countries and remote regions. Sustainably produced by farming, and by increasing the operating life of sensor chips, they also avoid wastage of energy and resources and may thus give new impetus to the development of powerful "biologized" smart mini-tools. Plant virus-assisted BioFED sensors could therefore be among the high-priority developments in the multi-toolbox currently worked out to disarm SARS-CoV-2. Time seems high to harmonize international regulations for the use of plant viral building blocks in technical devices (see also Eiben et al., 2019), including both natural types and genetically engineered variants that enable simplified technical applications as carrier/adapter platforms due to, e.g., increased numbers of easily addressable docking sites for analyte-specific receptors displayed on the plant viral particles.

In summary, we believe that BioFEDs, plant viruses and combinations thereof can play significant roles in point-of-care and on-site testing for an early diagnosis and treatment of infectious diseases in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Adams, M. J., Adkins, S., Bragard, C., Gilmer, D., Li, D., MacFarlane, S. A., et al. (2017). ICTV virus taxonomy profile: virgaviridae. *J. Gen. Virol.* 98, 1999–2000. doi: 10.1099/jgv.0.000884

Afsahi, S., Lerner, M. B., Goldstein, J. M., Lee, J., Tang, X., Bagarozzi, D. A., et al. (2018). Novel graphene-based biosensor for early detection of Zika virus infection. *Biosens. Bioelectron.* 100, 85–88. doi: 10.1016/j.bios.2017.08.051

Ahn, J.-H., Im, M., Park, T. J., Lee, S. Y., and Choi, Y.-K. (2015). Label-free and real-time detection of avian influenza using nanowire field effect transistors. *J. Biomed. Nanotechnol.* 11, 1640–1643. doi: 10.1166/jbn.2015.1501

Alabsi, S. S., Ahmed, A. Y., Dennis, J. O., Md Khir, M. H., and Algamili, A. S. (2020). A review of carbon nanotube field effect-based biosensors. *IEEE Access* 8, 69509–69521. doi: 10.1109/ACCESS.2020.2987204

Aljabali, A. A., Barclay, J. E., Steinmetz, N. F., Lomonossoff, G. P., and Evans, D. J. (2012). Controlled immobilisation of active enzymes on the cowpea mosaic virus capsid. *Nanoscale* 4, 5640–5645. doi: 10.1039/C2NR31485A

Aljabali, A. A. A., Berardi, A., and Evans, D. J. (2018). "Chapter two - nature's nanoparticles: using viruses as nanomedicines and for bioimaging," in *Fundamentals of Nanoparticles*, eds A. Barhoum and A. S. Hamdy Makhlouf (Amsterdam: Elsevier), 29–50. doi: 10.1016/b978-0-323-51255-8.00002-1

Ambhorkar, P., Wang, Z., Ko, H., Lee, S., Koo, K., Kim, K., et al. (2018). Nanowire-based biosensors: from growth to applications. *Micromachines* 9:679. doi: 10.3390/mi9120679

Aumiller, W. M., Uchida, M., and Douglas, T. (2018). Protein cage assembly across multiple length scales. *Chem. Soc. Rev.* 47, 3433–3469. doi: 10.1039/C7CS00818J

Aves, K.-L., Goksøyr, L., and Sander, A. F. (2020). Advantages and prospects of tag/catcher mediated antigen display on capsid-like particle-based vaccines. *Viruses* 12:185. doi: 10.3390/v12020185

Bäcker, M., Koch, C., Eiben, S., Geiger, F., Eber, F., Gliemann, H., et al. (2017). Tobacco mosaic virus as enzyme nanocarrier for electrochemical biosensors. *Sens. Actuators B Chem.* 238, 716–722. doi: 10.1016/j.snb.2016.07.096

Balke, I., and Zeltins, A. (2019). Use of plant viruses and virus-like particles for the creation of novel vaccines. *Adv. Drug Deliv. Rev.* 145, 119–129. doi: 10.1016/j.addr.2018.08.007

Balke, I., and Zeltins, A. (2020). Recent advances in the use of plant virus-like particles as vaccines. *Viruses* 12:270. doi: 10.3390/v12030270

Benjamin, C., Brohlin, O., Shahrivarkevishahi, A., and Gassensmith, J. J. (2020). "Chapter eleven - virus like particles: fundamental concepts, biological interactions, and clinical applications," in *Nanoparticles for Biomedical Applications*, eds E. J. Chung, L. Leon, and C. Rinaldi (Amsterdam: Elsevier), 153–174. doi: 10.1016/b978-0-12-816662-8.00011-4

Berto, M., Vecchi, E., Baiamonte, L., Condò, C., Sensi, M., Di Lauro, M., et al. (2019). Label free detection of plant viruses with organic transistor biosensors. *Sens. Actuators B Chem.* 281, 150–156. doi: 10.1016/j.snb.2018.10.080

Besong-Ndika, J., Wahlsten, M., Cardinale, D., Pille, J., Walter, J., Michon, T., et al. (2016). Towards the reconstitution of a two-enzyme cascade for resveratrol synthesis on potyvirus particles. *Front. Plant Sci.* 7:89. doi: 10.3389/fpls.2016.00089

Bhalla, N., Pan, Y., Yang, Z., and Payam, A. F. (2020). Opportunities and challenges for biosensors and nanoscale analytical tools for pandemics: COVID-19. *ACS Nano* 14, 7783–7807. doi: 10.1021/acsnano.0c04421

Bittner, A. M., Alonso, J. M., Górzny, M. L., and Wege, C. (2013). "Nanoscale science and technology with plant viruses and bacteriophages," in *Structure and Physics of Viruses: an Integrated Textbook*, ed. M. G. Mateu (Dordrecht: Springer Science+Business Media), 667–702. doi: 10.1007/978-94-007-6552-8_22

Brasch, M., Putri, R. M., de Ruiter, M. V., Luque, D., Koay, M. S. T., Castón, J. R., et al. (2017). Assembling enzymatic cascade pathways inside virus-based nanocages using dual-tasking nucleic acid tags. *J. Am. Chem. Soc.* 139, 1512–1519. doi: 10.1021/jacs.6b10948

Bronder, T. S., Jessing, M. P., Poghossian, A., Keusgen, M., and Schöning, M. J. (2018). Detection of PCR-amplified tuberculosis DNA fragments with polyelectrolyte-modified field-effect sensors. *Anal. Chem.* 90, 7747–7753. doi: 10.1021/acs.analchem.8b01807

Bronder, T. S., Poghossian, A., Jessing, M. P., Keusgen, M., and Schöning, M. J. (2019). Surface regeneration and reusability of label-free DNA biosensors based

on weak polyelectrolyte-modified capacitive field-effect structures. *Biosens. Bioelectron.* 126, 510–517. doi: 10.1016/j.bios.2018.11.019

Bronder, T. S., Poghossian, A., Scheja, S., Wu, C., Keusgen, M., Mewes, D., et al. (2015). DNA immobilization and hybridization detection by the intrinsic molecular charge using capacitive field-effect sensors modified with a charged weak polyelectrolyte layer. *ACS Appl. Mater. Interfaces* 7, 20068–20075. doi: 10.1021/acsami.5b05146

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Cardinale, D., Carette, N., and Michon, T. (2012). Virus scaffolds as enzyme nano-carriers. *Trends Biotechnol.* 30, 369–376. doi: 10.1016/j.tibtech.2012.04.001

Carette, N., Engelkamp, H., Akpa, E., Pierre, S. J., Cameron, N. R., Christianen, P. C. M., et al. (2007). A virus-based biocatalyst. *Nat. Nanotechnol.* 2, 226–229. doi: 10.1038/nnano.2007.76

Cassedy, A., Mullins, E., and O'Kennedy, R. (2020). Sowing seeds for the future: the need for on-site plant diagnostics. *Biotechnol. Adv.* 39:107358. doi: 10.1016/j.biotechadv.2019.02.014

Chackerian, B. (2007). Virus-like particles: flexible platforms for vaccine development. *Expert Rev. Vaccines* 6, 381–390. doi: 10.1586/14760584.6.3.381

Chakraborti, S., Korpi, A., Kumar, M., Stępień, P., Kostiainen, M. A., and Heddle, J. G. (2019). Three-dimensional protein cage array capable of active enzyme capture and artificial chaperone activity. *Nano Lett.* 19, 3918–3924. doi: 10.1021/acs.nanolett.9b01148

Chen, M. Y., Butler, S. S., Chen, W., and Suh, J. (2019). Physical, chemical, and synthetic virology: reprogramming viruses as controllable nanodevices. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 11:e1545. doi: 10.1002/wnan.1545

Chen, Y., Ren, R., Pu, H., Guo, X., Chang, J., Zhou, G., et al. (2017). Field-effect transistor biosensor for rapid detection of Ebola antigen. *Sci. Rep.* 7:10974. doi: 10.1038/s41598-017-11387-7

Chiang, P.-L., Chou, T.-C., Wu, T.-H., Li, C.-C., Liao, C.-D., Lin, J.-Y., et al. (2012). Nanowire transistor-based ultrasensitive virus detection with reversible surface functionalization. *Chem. Asian J.* 7, 2073–2079. doi: 10.1002/asia.201200222

Choi, J., Seong, T. W., Jeun, M., and Lee, K. H. (2017). Field-effect biosensors for on-site detection: recent advances and promising targets. *Adv. Healthc. Mater.* 6:1700796. doi: 10.1002/adhm.201700796

Chu, S., Brown, A. D., Culver, J. N., and Ghodssi, R. (2018a). Tobacco mosaic virus as a versatile platform for molecular assembly and device fabrication. *Biotechnol. J.* 13:1800147. doi: 10.1002/biot.201800147

Chu, S., Winkler, T. E., Brown, A. D., Culver, J. N., and Ghodssi, R. (2018b). Localized three-dimensional functionalization of bionanoreceptors on high-density micropillar arrays via electrowetting. *Langmuir* 34, 1725–1732. doi: 10.1021/acs.langmuir.7b02920

Chung, Y. H., Cai, H., and Steinmetz, N. F. (2020). Viral nanoparticles for drug delivery, imaging, immunotherapy, and theranostic applications. *Adv. Drug Deliv. Rev.* doi: 10.1016/j.addr.2020.06.024 [Epub ahead of print].

Comellas-Aragones, M., Engelkamp, H., Claessen, V. I., Sommerdijk, N. A. J. M., Rowan, A. E., Christianen, P. C. M., et al. (2007). A virus-based single-enzyme nanoreactor. *Nat. Nanotechnol.* 2, 635–639. doi: 10.1038/nnano.2007.299

Crisci, E., Barcena, J., and Montoya, M. (2012). Virus-like particles: the new frontier of vaccines for animal viral infections. *Vet. Immunol. Immunopathol.* 148, 211–225. doi: 10.1016/j.vetimm.2012.04.026

Cuenca, S., Mansilla, C., Aguado, M., Yuste-Calvo, C., Sánchez, F., Sánchez-Montero, J. M., et al. (2016). Nanonets derived from turnip mosaic virus as scaffolds for increased enzymatic activity of immobilized *Candida antarctica* lipase B. *Front. Plant Sci.* 7:464. doi: 10.3389/fpls.2016.00464

Culver, J. N. (2002). Tobacco mosaic virus assembly and disassembly: determinants in pathogenicity and resistance. *Annu. Rev. Phytopathol.* 40, 287–308.

Culver, J. N., Brown, A. D., Zang, F., Gnerlich, M., Gerasopoulos, K., and Ghodssi, R. (2015). Plant virus directed fabrication of nanoscale materials and devices. *Virology* 479–480, 200–212. doi: 10.1016/j.virol.2015.03.008

Czapar, A. E., and Steinmetz, N. F. (2017). Plant viruses and bacteriophages for drug delivery in medicine and biotechnology. *Curr. Opin. Chem. Biol.* 38, 108–116. doi: 10.1016/j.cbpa.2017.03.013

Dastagir, T., Forzani, E. S., Zhang, R., Amlani, I., Nagahara, L. A., Tsui, R., et al. (2007). Electrical detection of hepatitis C virus RNA on single wall carbon nanotube-field effect transistors. *Analyst* 132, 738–740. doi: 10.1039/b707025j

de Moraes, A. C. M., and Kubota, L. T. (2016). Recent trends in field-effect transistors-based immunosensors. *Chemosensors* 4:20. doi: 10.3390/chemosensors4040020

Dickmeis, C., Altintoprak, K., van Rijn, P., Wege, C., and Commandeur, U. (2018). "Bioinspired silica mineralization on viral templates," in *Virus-Derived Nanoparticles for Advanced Technologies*, eds C. Wege and G. P. Lomonossoff (New York, NY: Humana Press), 337–362. doi: 10.1007/978-1-4939-7808-3_23

Dijkstra, J., and de Jager, C. P. (1998). *Practical Plant Virology: Protocols and Exercises*. New York, NY: Springer.

Douglas, T., and Young, M. (1998). Virus particles as templates for materials synthesis. *Adv. Mater.* 11, 679–681. doi: 10.1002/(sici)1521-4095(199906)11:8<679::aid-adma679>3.0.co;2-j

Dragnea, B. (2017). Virus-based devices: prospects for allopoiesis. *ACS Nano* 11, 3433–3437. doi: 10.1021/acsnano.7b01761

Eiben, S., Koch, S., Altintoprak, K., Southan, A., Tovar, G., Laschat, S., et al. (2019). Plant virus-based materials for biomedical applications: trends and prospects. *Adv. Drug Deliv. Rev.* 145, 96–118. doi: 10.1016/j.addr.2018.08.011

Esbin, M. N., Whitney, O. N., Chong, S., Maurer, A., Darzacq, X., and Tjian, R. (2020). Overcoming the bottleneck to widespread testing: a rapid review of nucleic acid testing approaches for COVID-19 detection. *RNA* 26, 771–783. doi: 10.1261/rna.076232.120

Fan, X. Z., Naves, L., Siwak, N. P., Brown, A., Culver, J., and Ghodssi, R. (2015). Integration of genetically modified virus-like-particles with an optical resonator for selective bio-detection. *Nanotechnology* 26:205501. doi: 10.1088/0957-4484/26/20/205501

Fan, X. Z., Pomerantseva, E., Gnerlich, M., Brown, A., Gerasopoulos, K., McCarthy, M., et al. (2013). Tobacco mosaic virus: a biological building block for micro/nano/biosystems. *J. Vac. Sci. Technol. A* 31:050815. doi: 10.1116/1.4816584

Fehr, A. R., and Perlman, S. (2015). "Coronaviruses: an overview of their replication and pathogenesis," in *Coronaviruses: Methods and Protocols*, eds H. J. Maier, E. Bickerton, and P. Britton (New York, NY: Springer), 1–23. doi: 10.1007/978-1-4939-2438-7_1

Franzen, S., and Lommel, S. A. (2009). Targeting cancer with 'smart bombs': equipping plant virus nanoparticles for a 'seek and destroy' mission. *Nanomedicine* 4, 575–588. doi: 10.2217/nnm.09.23

Generalov, V. M., Naumova, O. V., Fomin, B. I., P'yankov, S. A., Khlistun, I. V., Safatov, A. S., et al. (2019). Detection of Ebola virus VP40 protein using a nanowire SOI biosensor. *Optoelectron. Instrum. Data Process.* 55, 618–622. doi: 10.3103/S875669901906013X

González-Gamboa, I., Manrique, P., Sánchez, F., and Ponz, F. (2017). Plant-made potyvirus-like particles used for log-increasing antibody sensing capacity. *J. Biotechnol.* 254, 17–24. doi: 10.1016/j.jbiotec.2017.06.014

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583, 459–468. doi: 10.1038/s41586-020-2286-9

Gowtham, I., and Sathishkumar, R. (2019). "Advances in plant based biologics," in *Next Generation Biomanufacturing Technologies*, eds R. K. Sani and R. N. Krishnaraj (Washington, DC: American Chemical Society), 57–79. doi: 10.1021/bk-2019-1329.ch004

Gu, B., Park, T. J., Ahn, J.-H., Huang, X.-J., Lee, S. Y., and Choi, Y.-K. (2009). Nanogap field-effect transistor biosensors for electrical detection of avian influenza. *Small* 5, 2407–2412. doi: 10.1002/smll.200900450

Hajizadeh, M., Gibbs, A. J., Amirnia, F., and Glasa, M. (2019). The global phylogeny of plum pox virus is emerging. *J. Gen. Virol.* 100, 1457–1468. doi: 10.1099/jgv.0.001308

Hefferon, L. K. (2018). Repurposing plant virus nanoparticles. *Vaccines* 6:11. doi: 10.3390/vaccines6010011

Hema, M., Vishnu Vardhan, G. P., Savithri, H. S., and Murthy, M. R. N. (2019). "Chapter six - emerging trends in the development of plant virus-based nanoparticles and their biomedical applications," in *Recent Developments in Applied Microbiology and Biochemistry*, ed. V. Buddolla (Cambridge, MA: Academic Press), 61–82. doi: 10.1016/b978-0-12-816328-3.00006-4

Hideshima, S., Hayashi, H., Hinou, H., Nambuya, S., Kuroiwa, S., Nakanishi, T., et al. (2019). Glycan-immobilized dual-channel field effect transistor biosensor for the rapid identification of pandemic influenza viral particles. *Sci. Rep.* 9:11616. doi: 10.1038/s41598-019-48076-6

Hideshima, S., Hinou, H., Ebihara, D., Sato, R., Kuroiwa, S., Nakanishi, T., et al. (2013). Attomolar detection of influenza A virus hemagglutinin human H1 and avian H5 using glycan-blotted field effect transistor biosensor. *Anal. Chem.* 85, 5641–5644. doi: 10.1021/ac401085c

Jin, X., Zhang, H., Li, Y.-T., Xiao, M.-M., Zhang, Z.-L., Pang, D.-W., et al. (2019). A field effect transistor modified with reduced graphene oxide for immunodetection of Ebola virus. *Microchim. Acta* 186:223. doi: 10.1007/s00604-019-3256-5

Kaisti, M. (2017). Detection principles of biological and chemical FET sensors. *Biosens. Bioelectron.* 98, 437–448. doi: 10.1016/j.bios.2017.07.010

Kao, L. T.-H., Shankar, L., Kang, T. G., Zhang, G., Tay, G. K. I., Rafeah, S., et al. (2011). Multiplexed detection and differentiation of the DNA strains for influenza A (H1N1 2009) using a silicon-based microfluidic system. *Biosens. Bioelectron.* 26, 2006–2011. doi: 10.1016/j.bios.2010.08.076

Karnaushenko, D., Ibarlucea, B., Lee, S., Lin, G., Baraban, L., Pregl, S., et al. (2015). Light weight and flexible high-performance diagnostic platform. *Adv. Healthc. Mater.* 4, 1517–1525. doi: 10.1002/adhm.201500128

Khater, M., de la Escosura-Muñiz, A., and Merkoçi, A. (2017). Biosensors for plant pathogen detection. *Biosens. Bioelectron.* 93, 72–86. doi: 10.1016/j.bios.2016.09.091

Khudyakov, Y., and Pumpens, P. (2016). *Viral Nanotechnology*. New York, NY: CRC Press.

Kim, J. W., Kim, S., Jang, Y.-H., Lim, K.-I., and Lee, W. H. (2019). Attomolar detection of virus by liquid coplanar-gate graphene transistor on plastic. *Nanotechnology* 30:345502. doi: 10.1088/1361-6528/ab0f52

Kim, J.-Y., Ahn, J.-H., Moon, D.-I., Park, T. J., Lee, S. Y., and Choi, Y.-K. (2014). Multiplex electrical detection of avian influenza and human immunodeficiency virus with an underlap-embedded silicon nanowire field-effect transistor. *Biosens. Bioelectron.* 55, 162–167. doi: 10.1016/j.bios.2013.12.014

Klug, A. (1999). The tobacco mosaic virus particle: structure and assembly. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354, 531–535. doi: 10.1098/rstb.1999.0404

Koch, C., Eber, F. J., Azucena, C., Foerste, A., Walheim, S., Schimmel, T., et al. (2016). Novel roles for well-known players: from tobacco mosaic virus pests to enzymatically active assemblies. *Beilstein J. Nanotechnol.* 7, 613–629. doi: 10.3762/bjnano.7.54

Koch, C., Poghossian, A., Schöning, M. J., and Wege, C. (2018a). Penicillin detection by tobacco mosaic virus-assisted colorimetric biosensors. *Nanotheranostics* 2, 184–196. doi: 10.7150/ntno.22114

Koch, C., Poghossian, A., Wege, C., and Schöning, M. J. (2018b). "TMV-based adapter templates for enhanced enzyme loading in biosensor applications," in *Virus-Derived Nanoparticles for Advanced Technologies*, eds C. Wege and G. P. Lomonossoff (New York, NY: Humana Press), 553–568. doi: 10.1007/978-1-4939-7808-3_35

Koch, C., Wabbel, K., Eber, F. J., Krolla-Sidenstein, P., Azucena, C., Gliemann, H., et al. (2015). Modified TMV particles as beneficial scaffolds to present sensor enzymes. *Front. Plant Sci.* 6:1137. doi: 10.3389/fpls.2015.01137

Kwon, J., Lee, Y., Lee, T., and Ahn, J.-H. (2020). Aptamer-based field-effect transistor for detection of avian influenza virus in chicken serum. *Anal. Chem.* 92, 5524–5531. doi: 10.1021/acs.analchem.0c00348

Lapizco-Encinas, B. H., and Rito-Palomares, M. (2007). Dielectrophoresis for the manipulation of nanobioparticles. *Electrophoresis* 28, 4521–4538. doi: 10.1002/elps.200700303

Lee, K.-W., Choi, S.-J., Ahn, J.-H., Moon, D.-I., Park, T. J., Lee, S. Y., et al. (2010). An underlap field-effect transistor for electrical detection of influenza. *Appl. Phys. Lett.* 96:033703. doi: 10.1063/1.3291617

Lee, P. W., Shukla, S., Wallat, J. D., Danda, C., Steinmetz, N. F., Maia, J., et al. (2017). Biodegradable viral nanoparticle/polymer implants prepared via melt-processing. *ACS Nano* 11, 8777–8789. doi: 10.1021/acsnano.7b02786

Lee, T., Ahn, J.-H., Park, S. Y., Kim, G.-H., Kim, J., Kim, T.-H., et al. (2018). Recent advances in AIV biosensors composed of nanobio hybrid material. *Micromachines* 9:651. doi: 10.3390/mi9120651

Lin, B., and Ratna, B. (2014). *Virus Hybrids as Nanomaterial: Methods and Protocols*. London: Humana Press. doi: 10.1007/978-1-62703-751-8

Lin, C.-H., Chu, C.-J., Teng, K.-N., Su, Y.-J., Chen, C.-D., Tsai, L.-C., et al. (2012). Recovery based nanowire field-effect transistor detection of pathogenic avian influenza DNA. *Jpn. J. Appl. Phys.* 51(2 Part 2):02BL02. doi: 10.1143/JJAP.51.02BL02

Lin, C.-H., Hung, C.-H., Hsiao, C.-Y., Lin, H.-C., Ko, F.-H., and Yang, Y.-S. (2009). Poly-silicon nanowire field-effect transistor for ultrasensitive and label-free detection of pathogenic avian influenza DNA. *Biosens. Bioelectron.* 24, 3019–3024. doi: 10.1016/j.bios.2009.03.014

Liu, F., Kim, Y. H., Cheon, D. S., and Seo, T. S. (2013). Micropatterned reduced graphene oxide based field-effect transistor for real-time virus detection. *Sens. Actuators B Chem.* 186, 252–257. doi: 10.1016/j.snb.2013.05.097

Lomonossoff, G. P. (2018). "Chapter seven - so what have plant viruses ever done for virology and molecular biology?," in *Advances in Virus Research*, Vol. 100, eds M. Kielian, T. C. Mettenleiter, and M. J. Roossinck (Cambridge, MA: Academic Press), 145–162. doi: 10.1016/bs.aivir.2017.12.001

Lomonossoff, G. P., and Wege, C. (2018). "Chapter six - TMV particles: the journey from fundamental studies to bionanotechnology applications," in *Advances in Virus Research*, Vol. 102, eds P. Palukaitis and M. J. Roossinck (Cambridge, MA: Academic Press), 149–176. doi: 10.1016/bs.aivir.2018.06.003

Lu, N., Gao, A., Zhou, H., Wang, Y., Yang, X., Wang, Y., et al. (2016). Progress in silicon nanowire-based field-effect transistor biosensors for label-free detection of DNA. *Chin. J. Chem.* 34, 308–316. doi: 10.1002/cjoc.201500857

Malecki, M., Lüsebrink, J., Teves, S., and Wendel, A. F. (2020). Pharynx gargle samples are suitable for SARS-CoV-2 diagnostic use and save personal protective equipment and swabs. *Infect. Control Hosp. Epidemiol.* doi: 10.1017/ice.2020.229 [Epub ahead of print].

Mao, C., Liu, A., and Cao, B. (2009). Virus-based chemical and biological sensing. *Angew. Chem. Int. Ed.* 48, 6790–6810. doi: 10.1002/anie.200900231

Marsian, J., and Lomonossoff, G. P. (2016). Molecular pharming - VLPs made in plants. *Curr. Opin. Biotechnol.* 37, 201–206. doi: 10.1016/j.copbio.2015.12.007

Matić, S., and Noris, E. (2015). "Chapter five - in planta produced virus-like particles as candidate vaccines," in *Applied Plant Genomics and Biotechnology*, eds Y. Hong and P. Poltronieri (Oxford: Woodhead Publishing), 73–85. doi: 10.1016/b978-0-08-100068-7.00005-7

Mavrikou, S., Moschopoulou, G., Tsekouras, V., and Kintzios, S. (2020). Development of a portable, ultra-rapid and ultra-sensitive cell-based biosensor for the direct detection of the SARS-CoV-2 S1 spike protein antigen. *Sensors* 20:3121. doi: 10.3390/s20113121

Meyer, B., Drosten, C., and Müller, M. A. (2014). Serological assays for emerging coronaviruses: challenges and pitfalls. *Virus Res.* 194, 175–183. doi: 10.1016/j.virusres.2014.03.018

Minten, I. J., Claessen, V. I., Blank, K., Rowan, A. E., Nolte, R. J. M., and Cornelissen, J. J. L. M. (2011). Catalytic capsids: the art of confinement. *Chem. Sci.* 2, 358–362. doi: 10.1039/c0sc00407c

Morales-Narváez, E., and Dincer, C. (2020). The impact of biosensing in a pandemic outbreak: COVID-19. *Biosens. Bioelectron.* 163:112274. doi: 10.1016/j.bios.2020.112274

Moriones, E., and Verdin, E. (2020). "Viral diseases," in *Integrated Pest and Disease Management in Greenhouse Crops*, eds M. L. Gullino, R. Albajes, and P. C. Nicot (Cham: Springer International Publishing), 3–31. doi: 10.1016/b978-0-12-800964-2.00001-x

Moser, N., Lande, T. S., Toumazou, C., and Georgiou, P. (2016). ISFETs in CMOS and emergent trends in instrumentation: a review. *IEEE Sens. J.* 16, 6496–6514. doi: 10.1109/JSEN.2016.2585920

Mu, L., Chang, Y., Sawtelle, S. D., Wipf, M., Duan, X., and Reed, M. A. (2015). Silicon nanowire field-effect transistors - a versatile class of potentiometric nanobiosensors. *IEEE Access* 3, 287–302. doi: 10.1109/ACCESS.2015.2422842

Narayanan, K. B., and Han, S. S. (2017). Helical plant viral nanoparticles - bioinspired synthesis of nanomaterials and nanostructures. *Bioinspir. Biomim.* 12:031001. doi: 10.1088/1748-3190/aa6bfd

Nguyen, T., Duong Bang, D., and Wolff, A. (2020). 2019 Novel coronavirus disease (COVID-19): paving the road for rapid detection and point-of-care diagnostics. *Micromachines* 11:306. doi: 10.3390/mi11030306

Nikitin, N. A., Trifonova, E. A., Karpova, O. V., and Atabekov, J. G. (2016). Biosafety of plant viruses for human and animals. *Moscow Univ. Biol. Sci. Bull.* 71, 128–134. doi: 10.3103/S0096392516030081

Nuzaihan, M., Mazlan, M. I., Zulkiffli, M. N. F., Hazri, S. M., Fathil, M. F. M., Ibau, C., et al. (2018). Electrical responses of dengue virus (DENV) using poly-Si nanowire array biosensor. *AIP Conf. Proc.* 2045:020034. doi: 10.1063/1.5080847

Nuzaihan, M. N., Hashim, U., Md Arshad, M. K., Kasjoo, S. R., Rahman, S. F. A., Ruslinda, A. R., et al. (2016). Electrical detection of dengue virus (DENV) DNA oligomer using silicon nanowire biosensor with novel molecular gate control. *Biosens. Bioelectron.* 83, 106–114. doi: 10.1016/j.bios.2016.04.033

Ozer, T., Geiss, B. J., and Henry, C. S. (2020). Review-chemical and biological sensors for viral detection. *J. Electrochem. Soc.* 167:037523. doi: 10.1149/2.0232003JES

Pallás, V., Sánchez-Navarro, J. A., and James, D. (2018). Recent advances on the multiplex molecular detection of plant viruses and viroids. *Front. Microbiol.* 9:2087. doi: 10.3389/fmicb.2018.02087

Paraskevis, D., Kostaki, E. G., Magiorkinis, G., Panayiotakopoulos, G., Sourvinos, G., and Tsiodras, S. (2020). Full-genome evolutionary analysis of the novel corona virus (2019-nCoV) rejects the hypothesis of emergence as a result of a recent recombination event. *Infect. Genet. Evol.* 79:104212. doi: 10.1016/j.meegid.2020.104212

Park, C., Choi, W., Kim, D., Jin, B., and Lee, J.-S. (2019). Highly sensitive detection of influenza A (H1N1) virus with silicon nanonet BioFETs. *IEEE Sens. J.* 19, 10985–10990. doi: 10.1109/JSEN.2019.2936216

Park, J., Chariou, P. L., and Steinmetz, N. F. (2020). Site-specific antibody conjugation strategy to functionalize virus-based nanoparticles. *Bioconjug. Chem.* 31, 1408–1416. doi: 10.1021/acs.bioconjchem.0c00118

Patolsky, F., Zheng, G., Hayden, O., Lakadamyali, M., Zhuang, X., and Lieber, C. M. (2004). Electrical detection of single viruses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14017–14022. doi: 10.1073/pnas.0406159101

Patolsky, F., Zheng, G., and Lieber, C. M. (2006). Nanowire sensors for medicine and life sciences. *Nanomedicine* 1, 51–65. doi: 10.2217/17435889.1.1.51

Pille, J., Cardinale, D., Carette, N., Di Primo, C., Besong-Ndika, J., Walter, J., et al. (2013). General strategy for ordered noncovalent protein assembly on well-defined nanoscaffolds. *Biomacromolecules* 14, 4351–4359. doi: 10.1021/bm401291u

Poghossian, A., Bäcker, M., Mayer, D., and Schöning, M. J. (2015). Gating capacitive field-effect sensors by the charge of nanoparticle/molecule hybrids. *Nanoscale* 7, 1023–1031. doi: 10.1039/C4NR05987E

Poghossian, A., Jablonski, M., Koch, C., Bronder, T. S., Rolka, D., Wege, C., et al. (2018). Field-effect biosensor using virus particles as scaffolds for enzyme immobilization. *Biosens. Bioelectron.* 110, 168–174. doi: 10.1016/j.bios.2018.03.036

Poghossian, A., Malzahn, K., Abouzar, M. H., Mehndiratta, P., Katz, E., and Schöning, M. J. (2011). Integration of biomolecular logic gates with field-effect transducers. *Electrochim. Acta* 56, 9661–9665. doi: 10.1016/j.electacta.2011.01.102

Poghossian, A., and Schöning, M. J. (2014). Label-free sensing of biomolecules with field-effect devices for clinical applications. *Electroanalysis* 26, 1197–1213. doi: 10.1002/elan.201400073

Poghossian, A., Weil, M., Cherstvy, A. G., and Schöning, M. J. (2013). Electrical monitoring of polyelectrolyte multilayer formation by means of capacitive field-effect devices. *Anal. Bioanal. Chem.* 405, 6425–6436. doi: 10.1007/s00216-013-6951-9

Pullano, S. A., Critello, C. D., Mahbub, I., Tasneem, N. T., Shamsir, S., Islam, S. K., et al. (2018). EGFET-based sensors for bioanalytical applications: a review. *Sensors* 18:4042. doi: 10.3390/s18114042

Ravina, R., Dalal, A., Mohan, H., Prasad, M., and Pundir, C. S. (2020). Detection methods for influenza A H1N1 virus with special reference to biosensors: a review. *Biosci. Rep.* 40:BSR20193852. doi: 10.1042/BSR20193852

Roeder, J., Dickmeis, C., and Commandeur, U. (2019). Small, smaller, nano: new applications for potato virus X in nanotechnology. *Front. Plant Sci.* 10:158. doi: 10.3389/fpls.2019.00158

Roeder, J., Fischer, R., and Commandeur, U. (2017). Engineering potato virus X particles for a covalent protein based attachment of enzymes. *Small* 13:1702151. doi: 10.1002/smll.201702151

Rosales-Mendoza, S. (2020). Will plant-made biopharmaceuticals play a role in the fight against COVID-19? *Expert Opin. Biol. Ther.* 20, 545–548. doi: 10.1080/14712598.2020.1752177

Russo, A., Minichini, C., Starace, M., Astorri, R., Calò, F., Coppola, N., et al. (2020). Current status of laboratory diagnosis for COVID-19: a narrative review. *Infect. Drug Resist.* 13, 2657–2665. doi: 10.2147/IDR.S264020

Rybicki, E. P. (2020). Plant molecular farming of virus-like nanoparticles as vaccines and reagents. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 12:e1587. doi: 10.1002/wnan.1587

Santoni, M., Zampieri, R., and Avesani, L. (2020). Plant virus nanoparticles for vaccine applications. *Curr. Protein Pept. Sci.* 21, 344–356. doi: 10.2174/1389203721666200212100255

Sapsford, K. E., Soto, C. M., Blum, A. S., Chatterji, A., Lin, T., Johnson, J. E., et al. (2006). A cowpea mosaic virus nanoscaffold for multiplexed antibody conjugation: application as an immunoassay tracer. *Biosens. Bioelectron.* 21, 1668–1673. doi: 10.1016/j.bios.2005.09.003

Scheller, C., Krebs, F., Minkner, R., Astner, I., Gil-Moles, M., and Wätzig, H. (2020). Physicochemical properties of SARS-CoV-2 for drug targeting, virus inactivation and attenuation, vaccine formulation and quality control. *Electrophoresis* 41, 1137–1151. doi: 10.1002/elps.202000121

Scholthof, K. B., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., et al. (2011). Top 10 plant viruses in molecular plant pathology. *Mol. Plant Pathol.* 12, 938–954. doi: 10.1111/j.1364-3703.2011.00752.x

Scholthof, K.-B. G., Shaw, J. G., and Zaitlin, M. (eds). (1999). *Tobacco Mosaic Virus: One Hundred Years of Contributions to Virology*. St Paul, MN: APS Press.

Schöning, M. J., and Poghossian, A. (2006). Bio FEDs (field-effect devices): state-of-the-art and new directions. *Electroanalysis* 18, 1893–1900. doi: 10.1002/elan.200603609

Schwarz, B., Uchida, M., and Douglas, T. (2017). "Chapter one - biomedical and catalytic opportunities of virus-like particles in nanotechnology," in *Advances in Virus Research*, eds M. Kielian, T. C. Mettenleiter, and M. J. Roossinck (Cambridge, MA: Academic Press), 1–60. doi: 10.1016/bs.aivir.2016.09.002

Seo, G., Lee, G., Kim, M. J., Baek, S.-H., Choi, M., Ku, K. B., et al. (2020). Rapid detection of COVID-19 causative virus (SARS-CoV-2) in human nasopharyngeal swab specimens using field-effect transistor-based biosensor. *ACS Nano* 14, 5135–5142. doi: 10.1021/acsnano.0c02823

Shen, F., Wang, J., Xu, Z., Wu, Y., Chen, Q., Li, X., et al. (2012). Rapid flu diagnosis using silicon nanowire sensor. *Nano Lett.* 12, 3722–3730. doi: 10.1021/nl301516z

Silva, L. P., da Rocha Vaz, G. M., Pupe, J. M., Chafran, L. S., Neto, L. A. A., Santiago, T. R., et al. (2020). "Green nanoparticles for biomedical and bioengineering applications," in *Nanoparticles and their Biomedical Applications*, ed. A. K. Shukla (Singapore: Springer), 225–262. doi: 10.1007/978-981-15-0391-7_9

Soto, C. M., Blaney, K. M., Dar, M., Khan, M., Lin, B., Malanoski, A. P., et al. (2009). Cowpea mosaic virus nanoscaffold as signal enhancement for DNA microarrays. *Biosens. Bioelectron.* 25, 48–54. doi: 10.1016/j.bios.2009.06.009

Soto, C. M., Blum, A. S., Vora, G. J., Lebedev, N., Meador, C. E., Won, A. P., et al. (2006). Fluorescent signal amplification of carbocyanine dyes using engineered viral nanoparticles. *J. Am. Chem. Soc.* 128, 5184–5189. doi: 10.1021/ja058574x

Soto, C. M., Martin, B. D., Sapsford, K. E., Blum, A. S., and Ratna, B. R. (2008). Toward single molecule detection of staphylococcal enterotoxin B: mobile sandwich immunoassay on gliding microtubules. *Anal. Chem.* 80, 5433–5440. doi: 10.1021/ac800541x

Steele, J. F. C., Peyret, H., Saunders, K., Castells-Graells, R., Marsian, J., Meshcheriakova, Y., et al. (2017). Synthetic plant virology for nanobiotechnology and nanomedicine. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 9:e1447. doi: 10.1002/wnan.1447

Svendsen, W. E., Jørgensen, M., Andresen, L., Andersen, K. B., Larsen, M. B. B., Skov, S., et al. (2011). Silicon nanowire as virus sensor in a total analysis system. *Procedia Eng.* 25, 288–291. doi: 10.1016/j.proeng.2011.12.071

Syu, Y.-C., Hsu, W.-E., and Lin, C.-T. (2018). Field-effect transistor biosensing: devices and clinical applications. *ECS J. Solid State Sci. Technol.* 7, Q3196–Q3207. doi: 10.1149/2.0291807jss

Szuchmacher Blum, A., Soto, C. M., Sapsford, K. E., Wilson, C. D., Moore, M. H., and Ratna, B. R. (2011). Molecular electronics based nanosensors on a viral scaffold. *Biosens. Bioelectron.* 26, 2852–2857. doi: 10.1016/j.bios.2010.11.021

Tarasov, A., Gray, D. W., Tsai, M.-Y., Shields, N., Montrose, A., Creedon, N., et al. (2016). A potentiometric biosensor for rapid on-site disease diagnostics. *Biosens. Bioelectron.* 79, 669–678. doi: 10.1016/j.bios.2015.12.086

Thu, V. V., Tam, P. D., and Dung, P. T. (2013). Rapid and label-free detection of H5N1 virus using carbon nanotube network field effect transistor. *Curr. Appl. Phys.* 13, 1311–1315. doi: 10.1016/j.cap.2013.04.001

Tian, Y., Zhou, M., Shi, H., Gao, S., Xie, G., Zhu, M., et al. (2018). Integration of cell-penetrating peptides with rod-like bionanoparticles: virus-inspired gene-silencing technology. *Nano Lett.* 18, 5453–5460. doi: 10.1021/acs.nanolett.8b01805

Tinazzi, E., Merlin, M., Bason, C., Beri, R., Zampieri, R., Lico, C., et al. (2015). Plant-derived Chimeric virus particles for the diagnosis of primary Sjögren syndrome. *Front. Plant Sci.* 6:1080. doi: 10.3389/fpls.2015.01080

Tiu, B. D. B., Tiu, S. B., Wen, A. M., Lam, P., Steinmetz, N. F., and Advincula, R. C. (2016). Free-standing, nanopatterned Janus membranes of conducting polymer-virus nanoparticle arrays. *Langmuir* 32, 6185–6193. doi: 10.1021/acs.langmuir.6b00808

Tran, T. L., Nguyen, T. T., Huyen Tran, T. T., van Chu, T., Thinh Tran, Q., and Tuan Mai, A. (2017). Detection of influenza A virus using carbon nanotubes field effect transistor based DNA sensor. *Physica E Low Dimens. Syst. Nanostruct.* 93, 83–86. doi: 10.1016/j.physe.2017.05.019

Udugama, B., Kadhiresan, P., Kozlowski, H. N., Malekjahani, A., Osborne, M., Li, V. Y. C., et al. (2020). Diagnosing COVID-19: the disease and tools for detection. *ACS Nano* 14, 3822–3835. doi: 10.1021/acsnano.0c02624

Uhde-Holzem, K., McBurney, M., Tiu, B. D. B., Advincula, R. C., Fischer, R., Commandeur, U., et al. (2016). Production of immunoabsorbent nanoparticles by displaying single-domain protein A on potato virus X. *Macromol. Biosci.* 16, 231–241. doi: 10.1002/mabi.201500280

Uhm, M., Lee, J.-M., Lee, J., Lee, J. H., Choi, S., Park, B.-G., et al. (2019). Ultrasensitive electrical detection of hemagglutinin for point-of-care detection of influenza virus based on a CMP-NANA probe and top-down processed silicon nanowire field-effect transistors. *Sensors* 19:4502. doi: 10.3390/s19204502

Veigas, B., Fortunato, E., and Baptista, P. V. (2015). Field effect sensors for nucleic acid detection: recent advances and future perspectives. *Sensors* 15, 10380–10398. doi: 10.3390/s150510380

Vieira, N. C. S., Figueiredo, A., dos Santos, J. F., Aoki, S. M., Guimarães, F. E. G., and Zucolotto, V. (2014). Label-free electrical recognition of a dengue virus protein using the SEGFET simplified measurement system. *Anal. Methods* 6, 8882–8885. doi: 10.1039/C4AY01803F

Vilona, D., Di Lorenzo, R., Carraro, M., Licini, G., Trainotti, L., and Bonchio, M. (2015). Viral nano-hybrids for innovative energy conversion and storage schemes. *J. Mater. Chem. B* 3, 6718–6730. doi: 10.1039/c5tb00924c

Wang, Z., Lee, S., Koo, K., and Kim, K. (2016). Nanowire-based sensors for biological and medical applications. *IEEE Trans. Nanobiosci.* 15, 186–199. doi: 10.1109/TNB.2016.2528258

Wege, C., and Koch, C. (2020). From stars to stripes: RNA-directed shaping of plant viral protein templates - structural synthetic virology for smart biohybrid nanostructures. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 12:e1591. doi: 10.1002/wnan.1591

Wege, C., and Lomonossoff, G. P. (2018). *Virus-Derived Nanoparticles for Advanced Technologies*. New York, NY: Humana Press.

Weiss, C., Carriere, M., Fusco, L., Capua, I., Regla-Nava, J. A., Pasquali, M., et al. (2020). Toward nanotechnology-enabled approaches against the COVID-19 pandemic. *ACS Nano* 14, 6383–6406. doi: 10.1021/acsnano.0c03697

Wen, A. M., Lee, K. L., and Steinmetz, N. F. (2020). "Plant virus-based nanotechnologies," in *Women in Nanotechnology: Contributions from the Atomic Level and Up*, eds P. M. Norris and L. E. Friedersdorf (Cham: Springer International Publishing), 57–69. doi: 10.1007/978-3-030-19951-7_5

Wen, A. M., and Steinmetz, N. F. (2016). Design of virus-based nanomaterials for medicine, biotechnology, and energy. *Chem. Soc. Rev.* 45, 4074–4126. doi: 10.1039/C5CS00287G

Werner, S., Marillonnet, S., Hause, G., Klimyuk, V., and Gleba, Y. (2006). Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17678–17683. doi: 10.1073/pnas.0608869103

WHO Scientific Brief (2020). *Advice on the Use of Point-of-Care Immunodiagnostic Tests for COVID-19*. Available online at: https://www.who.int/publications/i/item/advice-on-the-use-of-point-of-care-immunodiagnostic-tests-for-covid-19-scientific-brief (accessed August 23, 2020).

WHO/COVID-19/laboratory/2020.5 (2020). *Laboratory Testing for Coronavirus Disease (COVID-19) in Suspected Human Cases. Interim Guidance, 19 March 2020*. Available online at: https://apps.who.int/iris/handle/10665/331329 (accessed August 23, 2020).

World Health Organization [WHO] (2020). *Coronavirus Disease 2019 (COVID-19): Situation Report-52*. Available online at: https://apps.who.int/iris/handle/10665/331476 (accessed August 23, 2020).

WorldOmeter (2020). *COVID-19*. Available online at: https://www.worldometers.info/coronavirus (accessed November 02, 2020).

Wu, C., Bronder, T. S., Poghossian, A., Werner, C. F., and Schöning, M. J. (2015). Label-free detection of DNA using a light-addressable potentiometric sensor modified with a positively charged polyelectrolyte layer. *Nanoscale* 7, 6143–6150. doi: 10.1039/C4NR07225A

Wu, C., Poghossian, A., Bronder, T. S., and Schöning, M. J. (2016). Sensing of double-stranded DNA molecules by their intrinsic molecular charge using the light-addressable potentiometric sensor. *Sens. Actuators B Chem.* 229, 506–512. doi: 10.1016/j.snb.2016.02.004

Wu, C.-Y., Cheng, H.-Y., Ou, K.-L., and Wu, C.-C. (2014). Real-time sensing of hepatitis B virus X gene using an ultrasensitive nanowire field effect transistor. *J. Polym. Eng.* 34, 273–277. doi: 10.1515/polyeng-2013-0216

Yang, F., and Zhang, G.-J. (2014). Silicon nanowire-transistor biosensor for study of molecule-molecule interactions. *Rev. Anal. Chem.* 33, 95–110. doi: 10.1515/revac-2014-0010

Yoshinobu, T., Ecken, H., Poghossian, A., Lüth, H., Iwasaki, H., and Schöning, M. J. (2001). Alternative sensor materials for light-addressable potentiometric sensors. *Sens. Actuators B Chem.* 76, 388–392. doi: 10.1016/S0925-4005(01)00646-3

Yoshinobu, T., Miyamoto, K., Werner, C. F., Poghossian, A., Wagner, T., and Schöning, M. J. (2017). Light-addressable potentiometric sensors for quantitative spatial imaging of chemical species. *Annu. Rev. Anal. Chem.* 10, 225–246. doi: 10.1146/annurev-anchem-061516-045158

Younes, N., Al-Sadeq, D. W., Al-Jighefee, H., Younes, S., Al-Jamal, O., Daas, H. I., et al. (2020). Challenges in laboratory diagnosis of the novel coronavirus SARS-CoV-2. *Viruses* 12:582. doi: 10.3390/v12060582

Yuste-Calvo, C., González-Gamboa, I., Pacios, L. F., Sánchez, F., and Ponz, F. (2019a). Structure-based multifunctionalization of flexuous elongated viral nanoparticles. *ACS Omega* 4, 5019–5028. doi: 10.1021/acsomega.8b02760

Yuste-Calvo, C., López-Santalla, M., Zurita, L., Cruz-Fernández, C. F., Sánchez, F., Garín, M. I., et al. (2019b). Elongated flexuous plant virus-derived nanoparticles functionalized for autoantibody detection. *Nanomaterials* 9:1438. doi: 10.3390/nano9101438

Zaitlin, M. (2000). *Tobacco Mosaic Virus. AAB Descriptions of Plant Viruses, No. 370*. Available online at: http://www.dpvweb.net/dpv/showdpv.php?dpvno=370 (accessed March 2000).

Zang, F., Gerasopoulos, K., Brown, A. D., Culver, J. N., and Ghodssi, R. (2017). Capillary microfluidics-assembled virus-like particle bionanoreceptor interfaces for label-free biosensing. *ACS Appl. Mater. Interfaces* 9, 8471–8479. doi: 10.1021/acsami.6b14045

Zang, F., Gerasopoulos, K., Fan, X. Z., Brown, A. D., Culver, J. N., and Ghodssi, R. (2014). An electrochemical sensor for selective TNT sensing based on tobacco mosaic virus-like particle binding agents. *Chem. Commun.* 50, 12977–12980. doi: 10.1039/C4CC06735E

Zhang, G.-J., Zhang, L., Huang, M. J., Luo, Z. H. H., Tay, G. K. I., Lim, E.-J. A., et al. (2010). Silicon nanowire biosensor for highly sensitive and rapid detection of Dengue virus. *Sens. Actuators B Chem.* 146, 138–144. doi: 10.1016/j.snb.2010.02.021

Zhang, Y., Dong, Y., Zhou, J., Li, X., and Wang, F. (2018). Application of plant viruses as a biotemplate for nanomaterial fabrication. *Molecules* 23:2311. doi: 10.3390/molecules23092311

Zhang, Y.-Z., and Holmes, E. C. (2020). A genomic perspective on the origin and emergence of SARS-CoV-2. *Cell* 181, 223–227. doi: 10.1016/j.cell.2020.03.035

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

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Plant Molecular Farming as a Strategy Against COVID-19 – The Italian Perspective

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has killed more than 37,000 people in Italy and has caused widespread socioeconomic disruption. Urgent measures are needed to contain and control the virus, particularly diagnostic kits for detection and surveillance, therapeutics to reduce mortality among the severely affected, and vaccines to protect the remaining population. Here we discuss the potential role of plant molecular farming in the rapid and scalable supply of protein antigens as reagents and vaccine candidates, antibodies for virus detection and passive immunotherapy, other therapeutic proteins, and virus-like particles as novel vaccine platforms. We calculate the amount of infrastructure and production capacity needed to deal with predictable subsequent waves of COVID-19 in Italy by pooling expertise in plant molecular farming, epidemiology and the Italian health system. We calculate the investment required in molecular farming infrastructure that would enable us to capitalize on this technology, and provide a roadmap for the development of diagnostic reagents and biopharmaceuticals using molecular farming in plants to complement production methods based on the cultivation of microbes and mammalian cells.

Keywords: therapeutics, vaccines, antibodies, diagnostics, recombinant proteins, transient expression, molecular farming, COVID-19

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INTRODUCTION

Severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) is a new virus responsible for the COVID-19 pandemic, which is the worst public health crisis of this century[1]. The emergence of SARS-CoV-2 in late 2019 and its rapid spread in 2020 has posed several global challenges that demand new solutions in public healthcare and the biomedical research ecosystem (Webb et al., 2020). The former is facing acute pressure on hospital beds and frontline medical staff, whereas the latter is experiencing an explosion in new research, leading to a mountain of rapidly-disseminated literature often in the form of fast-tracked preprint articles. COVID-19 also dramatically highlighted the need for preparedness and long-term investments in platforms suitable for the rapid, flexible and sustainable production of medical countermeasures (diagnostics, vaccines, and therapeutics) against emerging, re-emerging, and bioterrorism-related infectious diseases (Franconi et al., 2018; Capell et al., 2020).

Italy was the first country in Europe to face a large-scale COVID-19 outbreak and it is one of the hardest-hit countries in the EU (Remuzzi and Remuzzi, 2020). Considering the situation with the resurgence of the disease after summer 2020, our national system must be prepared to react promptly by finding and exploiting new and flexible solutions. This requires an extraordinary effort aiming to capitalize on existing national expertise at different levels and will also provide assurance in the face of future pandemics. A major effort should also be done to build and potentiate the network with other countries in order to guide decisions in a global perspective always considering national peculiarities.

In this article, we discuss how plant molecular farming could provide practical solutions to address the outbreak of COVID-19 in Italy. Once effective vaccines and therapeutics are identified, it will be necessary to ramp up manufacturing to produce the massive numbers of doses needed to protect the entire population. The current global uneven distribution of manufacturing capacity is one of the current drawbacks of plant-based molecular farming as a realistic alternative to conventional expression systems (Capell et al., 2020; Tusé et al., 2020). Given this perspective, we have defined the product categories that would benefit most from molecular farming (diagnostics, vaccines, and therapeutics) and have calculated the quantities for each product category that would be needed to meet national demand, based on official Italian epidemiological reports in the February–June 2020 period.

Plant molecular farming encompasses a variety of different expression technologies, ranging from stable nuclear transformation (transgenic plants) or plastid transformation (transplastomic plants) to transient expression without stable transgene integration (Fischer and Buyel, 2020). In the latter case, this is achieved by the infiltration of adult wild-type plants—usually tobacco (*Nicotiana tabacum*) or its relative *Nicotiana benthamiana*—with strains of *Agrobacterium tumefaciens* or recombinant plant viral vectors carrying the appropriate transgene cassette (McDonald and Holtz, 2020). Given the urgent need for diagnostics, vaccines, and therapeutics for a rapidly-spreading novel or re-emerging disease, only transient expression systems provide the necessary speed and scalability, and we therefore focus on such systems in this article (Tusé et al., 2020).

[1]https://coronavirus.jhu.edu/

DIAGNOSTIC REAGENTS

The effective management of COVID-19 requires an increase in diagnostic capacity, particularly the development, manufacture, and stockpiling of assays to detect the SARS-CoV-2 genome and/or antigens itself or the antibodies it elicits. The former assays are used to confirm infections (thus ensuring effective quarantine measures and priority medical treatment) whereas antigenic tests or Rapid Tests, in point-of-care (PoC) format, have emerged as a valid approach in large screening of schools and vulnerable communities. On the other hand, antibody tests are used to assess prior infection and immunity status as the basis for epidemiological surveillance and vaccine studies. The number of different tests has increased rapidly and many are being marketed for point-of-care use. However, their accuracy has not been formally evaluated, and risks of bias, heterogeneity and limited generalizability have been reported for point-of-care testing (Bastos et al., 2020). In spite of this, frequent rapid tests are considered, in the moment of revising this paper, a "game change" tool before vaccines become available (Rubin, 2020).

Accurate antibody tests for COVID-19 require high-quality reagents, although differences between analytical and clinical sensitivity has not yet been defined for any test. The huge demand for diagnostic kits has highlighted not only the critical shortage of reagents (recombinant antigens and antibodies) but also the means to produce them.

Plants have already been shown to produce SARS-CoV antigens (Demurtas et al., 2016). The nucleoprotein (N), transiently expressed in *N. benthamiana*, was recognized by sera from Chinese SARS-convalescent patients around the time of the 2003 outbreak. Furthermore, the full-length membrane (M) protein was produced in plants but not in bacteria due to unanticipated toxicity (Carattoli et al., 2005). This provided proof of principle that plants could be used as a robust, rapid and flexible production system for SARS diagnostic reagents, potentially allowing the development of immunological assays for stockpiling in case of recurring SARS outbreaks (De Martinis et al., 2016).

Many other antigens have been produced in plants for diagnostic use or the preclinical/clinical evaluation of vaccine candidates, mostly by transient expression in *N. benthamiana*. The yields vary widely, as shown for a selection of antigens in **Table 1**. Antigens were selected among those that have been produced transiently in *N. benthamiana* plants and for which final purification yields were reported. In all these cases, the products were purified by affinity chromatography due to the presence of N-terminal or C-terminal affinity tags. Such tags are suitable for diagnostic reagents as long as they do not interfere with immunoreactivity, which is indeed the case

TABLE 1 | Yields of selected purified molecules transiently expressed in plant systems.

Diagnostic reagent	Yields (μg purified protein/g LFW)	References
Bet V 1	23.4	Santoni et al., 2019
Dul51	4.9	Margolin et al., 2019
CAP256 SU	6.2	Margolin et al., 2019
NS1-ELP-ER	220	Marques et al., 2020
rHAO	200	Kanagarajan et al., 2012
SARS N protein	10	Demurtas et al., 2016
Antibodies		
mAb 2G12 (anti-HIV)	100	Sainsbury et al., 2010
mAb 6D8 (anti-Ebola)	500	Huang et al., 2010
mAb 2A10G6 (anti-Zika)	1,500	Diamos et al., 2020
mAb 4E10 (anti-HIV)	250	Zischewski et al., 2016
mAb M12 (tumor-specific)	2,000	Zischewski et al., 2016
Other therapeutics		
Griffithsin	1,000	O'Keefe et al., 2009
Griffithsin	519	Fuqua et al., 2015
Subunit vaccine		
HAC1	90	Shoji et al., 2011
HAI-05	50	Shoji et al., 2011
VLP-vaccine		
HBcAgD176	1,000	Peyret et al., 2015
HPV8 L1DC22	240	Matić et al., 2011
BPV1 LI	180	Love et al., 2012
HA Influenza Virus H5N1	50	D'Aoust et al., 2008
BTV	70	Thuenemann et al., 2013
HBcAg	2,380	Huang et al., 2006
CVPs-Flu epitope	1,100	Lico et al., 2009
CVPs-HIV epitope	600	Marusic et al., 2001

for all the reported examples. The wide range of yields (1–220 μg/g fresh leaf mass, average 77.4 μg/g) shows that the feasibility of molecular farming for antigen manufacturing is exquisitely sensitive to the intrinsic nature of the product candidate (and also the quantification method). It is not yet possible to accurately predict yields based on a given candidate protein sequence, and empirical evaluation is therefore necessary, including the testing of multiple expression strategies – which is also facilitated by the scalability of transient expression systems (Gengenbach et al., 2020).

Most immunological diagnostic tests proposed for the detection of SARS-CoV-2 antibodies are based on the full-length viral spike (S) glycoprotein, the shorter external S1 segment, its receptor binding domain (RBD), or the N protein (Freeman et al., 2020; Klumpp-Thomas et al., 2020; Rosendal et al., 2020). For example, Amanat et al. (2020) reported a serological test based on the RBD in a classic direct ELISA design, in which the plate is coated with the recombinant RBD produced in mammalian or insect cells. Nevertheless, the surface-located S protein is under continuous selective pressure by the immune system, and new reagents would be needed whenever a new coronavirus subtype arises in the human population (Ou et al., 2020). In contrast, the N protein is highly conserved among coronaviruses and is abundantly expressed during the early stages of infection, triggering a strong antibody response. This makes it a suitable diagnostic reagent, in combination with other antigens such as RBD or the more conserved M protein, to develop pan-reactive coronavirus tests. The S and N proteins are typically produced in mammalian cells (HEK293) or insect cells infected with baculovirus vectors. The N protein has also been expressed in *Escherichia coli* (Carattoli et al., 2005; Pei et al., 2005), but differences between the bacterial and eukaryotic cytoplasmic compartments and the inability of bacteria to carry out eukaryotic-type post-translational modifications can reduce the affinity of such recombinant antigens for antibodies present in serum (Vankadari and Wilce, 2020).

The amount of coronavirus antigen required to detect IgG and IgM in patient serum ranged from 50–200 ng/well in a standard 96-well plate assay (Amanat et al., 2020; Freeman et al., 2020; Klumpp-Thomas et al., 2020; Rosendal et al., 2020). Based on this, we calculated the quantity of diagnostic reagent required to meet the demand in Italy, assuming a mid-range value for sensitive detection (100 ng/well), with two technical replicates for each individual and accounting for the fact that immunological tests would be required in quantities at least equivalent to the molecular assays currently used for COVID-19 diagnosis. This is because reliable serological tests are fundamental requirements for long-term follow-up studies in order to confirm the stability of (neutralizing) antibody responses and to define the threshold of neutralizing antibodies that prevents re-infection (Seow et al., 2020). In our calculation for the number of serological tests required in Italy, we considered the number of molecular tests performed in Italy between March and June 2020 as reported by the Italian Health Ministry[2]. Based on these criteria, and assuming average yields from **Table 1** (77.4 μg/g), two technical repetitions for each patient, an average biomass of 10 g of infiltrated leaves per *N. benthamiana* plant during transient expression, and an average density of plants in a greenhouse of 75 plants per sq. m., we predict that approximately up to 6.5 kg of plant biomass would be required per month, corresponding to 8.7 sq. m. of greenhouse space, to produce enough reagents for all serological assays in Italy (**Table 2**).

THERAPEUTICS

Neutralizing Antibodies

Several trials suggest that passive immunotherapy based on the use of neutralizing antibodies from COVID-19 patients may be an important weapon in the fight against SARS-CoV-2 (Tortorici et al., 2020). Several pharmaceutical companies have already announced phase I trials of monoclonal neutralizing antibodies that protected animal models against SARS-CoV-2 (Ren et al., 2020). One of these studies is evaluating REGN-COV2, a cocktail of two antibodies that bind non-overlapping regions of the

[2]http://opendatadpc.maps.arcgis.com/apps/opsdashboard/index.html#/b0c68bce2cce478eaac82fe38d4138b1

TABLE 2 | Estimates of the surface area of greenhouse necessary to produce the monthly amount of diagnostic or therapeutic reagents or vaccine molecules potentially required to fight SARS-CoV-2 in the March-June pandemic in Italy.

				Therapeutics					Vaccine				
	Diagnostic Reagent[a]				Antibody[b]		Other[c]			Soluble Antigen[d]		VLP[e]	
	Number of tests	Kg LFW	m^2	Number of persons treated	Kg LFW	m^2	Kg LFW	m^2	Number of doses	Kg LFW	m^2	Kg LFW	m^2
Mar	483,623	1.25	1.67	10,376	153334	204445	40983	54644	$3{,}6 \times 10^7$	20696	27592	9388	12518
Apr	847,933	2.19	2.92	9,967	147297	196396	39370	52493					
May	2,523,838	6.52	8.69	2,756	40723	54298	10885	14513					
Jun	1,435,880	3.71	4.95	729	107775	14367	2880	3840					

[a]*Amount per test: 100 ng × 2,* [b]*9 g per dose,* [c]*3 g per dose,* [d]*40 μg per dose,* [e]*120 μg per dose.*

RBD (Baum et al., 2020; Hansen et al., 2020). Recently, Eli/Lilly disclosed the preliminary findings of randomized, double-blind, placebo-controlled Phase 2 study BLAZE-1 evaluating the combination of LY-CoV555 and LY-CoV016, two SARS-CoV-2 neutralizing antibodies, for the treatment of symptomatic COVID-19 in the outpatient setting[3]. The combination cohort enrolled recently diagnosed patients with mild-to-moderate COVID-19, who were assigned to 2,800 mg of each antibody or placebo. The combination therapy significantly reduced viral load at days 3, 7, and 11. Combination therapy has been generally well tolerated with no drug-related serious adverse events. These studies provide a first indication of an effective therapeutic dose of monoclonal antibodies against SARS-CoV-2 in the range of 5.6 g per patient.

Plants could provide an alternative system for the production of recombinant anti-COVID-19 antibodies given the efficient production of antibodies reported in plants, particularly by transient expression using viral vectors (Donini and Marusic, 2019). Recent examples of plant-derived neutralizing antibodies and their yields are listed in **Table 1**. The average yield of about 870 μg/g suggests that molecular farming could provide a useful complement for traditional manufacturing systems, especially when antibodies are required rapidly and on a large scale.

In a worst-case scenario, based on the Italian situation in the middle of March 2020, the proportion of COVID-19 patients admitted to an intensive care unit (ICU) was 9–11% (Remuzzi and Remuzzi, 2020). We calculated the amount of antibody required to meet the demand in Italy by assuming that all ICU patients would benefit from passive immunotherapy and that presumably the dose would be similar to that of the plant-derived cocktail ZMapp for the treatment of Ebola during the 2014 outbreak in West Africa – 9 g per patient (Mulangu et al., 2019). Based on these criteria, and again assuming average yields from **Table 1** (870 μg/g), a final 70% recovery of highly purified mAb necessary for intra-venous administration route and the same values stated above for biomass and plant density in a greenhouse, we predict that approximately up to 153,000 kg of biomass would be required per month, corresponding to approximately 204,000 sq. m. of greenhouse space (**Table 2**).

[3]https://clinicaltrials.gov/ct2/show/NCT04427501

Other Proteins

In addition to passive and active immunization, antiviral proteins could provide an additional weapon against the virus during the infection phase, and other biologics could help to address the symptoms of COVID-19 such as the cytokine storm provoked by the initial wave of infection.

Among virus inhibitors, lectins are particularly interesting from the perspective of plant molecular farming because they are naturally produced in many higher plants and bind reversibly to carbohydrates. Given the frequent presence of glycans on the surface of viruses, lectins have been explored as antivirals and have been shown to block the infection cycle of HIV, cytomegalovirus, respiratory syncytial virus, influenza A, and also several coronaviruses. Indeed, more than 20 different plant lectins have already been shown to block infections by SARS-CoV, probably via selective binding to glycans on the S protein (Keyaerts et al., 2007). One of the best candidates is the algal lectin griffithsin (**Table 1**), which shows potent activity against SARS-CoV but limited toxicity toward human cells, and has been expressed at high levels in tobacco with average yields of 760 μg/g (O'Keefe et al., 2009; Fuqua et al., 2015; Hoelscher et al., 2018).

There is preliminary evidence that natural or artificial peptides can also inhibit viruses (Struck et al., 2011; Mookherjee et al., 2020) and short heterologous peptides have been successfully expressed in plants either independently or displayed on the surface of plant virus nanoparticles or virus-like particles (VLPs) by genetic fusion to the viral coat protein gene (Lico et al., 2012b). Furthermore, several approved drugs have been repurposed for COVID-19 including existing biologics used for anticoagulation therapy (Barrett et al., 2020) and immunomodulators used to block inflammatory cytokine signaling pathways (Rizk et al., 2020). Molecular farming could significantly reduce the manufacturing time and costs for such products, given that functional thrombin inhibitors and fibrinolytics (Abdoli Nasab et al., 2016; Pitek et al., 2018) as well as antibody-based immunomodulators (Jantan et al., 2015) have already been expressed successfully in plants.

Using the same strategy described above for passive immunotherapy, we considered the amount of griffithsin required in Italy for the treatment of 10% of COVID-19 patients admitted to ICUs, basing the dosing schedule on preclinical

studies involving two intranasal doses of 5 mg/kg body mass daily for 4 days (O'Keefe et al., 2010). We came to a dose of 3 g, assuming an average patient mass of 74 kg (Pierlorenzi, 2010). Based on these criteria, and again assuming average yields from **Table 1** (759.5 μg/g) and the same values stated above for biomass and plant density in a greenhouse, we predict that 41,000 kg of plant biomass would be required per month, corresponding to 55,000 sq. m. of greenhouse space (**Table 2**).

VACCINES

The race to produce a COVID-19 vaccine began as soon as the SARS-CoV-2 genome sequence was published, involving hundreds of academic and industry research groups around the world using the most innovative biotechnology-based approaches. Thus far, 232 COVID-19 vaccine clinical trials have been registered and in some cases phase I safety data are already available[4].

Conventional inactivated or live-attenuated vaccines against COVID-19 are likely to be efficacious but also difficult to manufacture and distribute widely, as well as presenting a risk of reverting to virulence. Subunit vaccines based on recombinant antigens, such as the S-protein/RBD (or VLPs displaying said antigens), are more attractive in terms of safety and manufacturing, and can also be produced by molecular farming in plants. **Table 1** provides some examples of subunit and VLP vaccines produced in plants, and indicates average yields of 70 and 702.5 μg/g, respectively.

To determine the amount of vaccine antigen needed for a primary prevention approach in Italy (**Table 2**), we assumed that the dose of a soluble subunit vaccine would be the same as that recommended for the approved quadrivalent influenza vaccine, i.e., one shot of 40 μg of each antigen per individual, and that the dose of a VLP vaccine would be the same as that recommended for the VLP-based hepatitis B vaccine, i.e., one shot of 120 μg per individual (Mulangu et al., 2019). Such calculation is consistent with recent Phase 1 trials with the recombinant SARS-CoV-2 Spike protein adjuvated vaccine NVX-CoV2373, which showed promising immunogenic response and tolerability with an antigen dose of 50 μg, split in two administrations of 25 μg each 21 days apart (Keech et al., 2020). Also the ongoing Phase 2 clinical trial NCT04466085 with another vaccine based on recombinant RBD domain of the Spike protein, is testing doses ranging from 50 to 150 μg of antigen per individual. We also assumed that 60% of the Italian population (36,000,000) would need to be vaccinated in order to achieve herd immunity. Based on these criteria, and again assuming average yields from **Table 1** (70 μg/g for soluble antigens, 703 μg/g for VLPs) and the same values stated above for biomass and plant density in a greenhouse, we calculate that approximately 21,000 kg of plant biomass and 28,000 sq. m. of greenhouse space would be required to satisfy the demand for a soluble antigen, whereas 9,400 kg of plant biomass and 12,500 sq. m. of greenhouse space would be required to produce sufficient quantities of the VLP vaccine (**Table 2**).

[4]https://clinicaltrials.gov/ (search term = vaccine and COVID-19)

Antigen-Based Vaccines

Neutralizing antibodies against coronaviruses often block interactions between the S protein and its receptor. In the case of SARS-CoV-2, this is angiotensin converting enzyme 2 (ACE2) primarily found on the surface of lung epithelial cells, although also in other tissues (Liu et al., 2020; Long et al., 2020). The S protein or parts thereof are therefore the favored as vaccine candidates, although the N and M proteins have also been considered. Previous coronavirus vaccines (against SARS-CoV and MERS-CoV) induced Th2-mediated immunopathology in animal models, and researchers are focusing on protein engineering strategies to minimize this effect (Koirala et al., 2020). One promising approach is to express the S1 or RBD parts of the S protein, focusing the immune response on the parts of the S protein that interact directly with ACE2 and thus increasing the likelihood of eliciting neutralizing antibodies. The S1/RBD surface is heavily glycosylated and these proteins are therefore more effective as antigens when expressed in eukaryotic cells (Walls et al., 2020). The highest yield of recombinant RBD has been achieved in yeast, reaching 400 mg/L in the fermentation supernatant at a production scale of up to 60 L (Chen et al., 2020). Another promising approach is the fusion of the S-protein or its components to the Fc region of a human IgG1 antibody, which prolongs its exposure to the immune system. An S-IgG1 fusion protein produced in CHO-K1 cells elicited promising immune responses in primates (Ren et al., 2020). According to the authors, following the theoretical vaccination schedule of the anti-VZV Shringrix vaccine (two doses of 50 μg) and considering a yield of 50 mg/L in CHO-K1 cells, 3 million doses of vaccine could be produced every 14 days in a 3000-L bioreactor. In the Italian scenario, with a herd immunity threshold of 60% (36,000,000 people), the required 72,000,000 doses could be produced in a single 3000-L bioreactor in approximately 1 year.

The S protein of several avian, swine and murine coronaviruses, as well as the N-terminal fragment of the SARS-CoV S protein, have been produced successfully in transgenic maize, potato, tomato, or tobacco plants by classic *Agrobacterium*-mediated transformation, or by display on the surface of plant viruses, and in all cases the products induced an immune response following oral delivery (Tuboly et al., 2000; Bae et al., 2003; Lamphear et al., 2004; Zhou et al., 2004) or nasal delivery (Koo et al., 1999). However, transient expression is more suitable for the speed and scale of production needed to address a rapidly-spreading disease live COVID-19. Two companies are known to be developing a plant-based subunit vaccine against COVID-19: Kentucky BioProcessing (Owensboro, KY, United States)[5] and iBio (Bryan, TX, United States)[6].

VLPs and Chimeric VLPs

VLPs are nanoparticles formed from virus structural proteins – they resemble the authentic virion but lack the virus genome. Chimeric VLPs include components of more than one virus, often because one virus provides the structural components of

[5]https://www.bat.com/group/sites/UK__9D9KCY.nsf/vwPagesWebLive/DOBN8QNL

[6]https://www.ibioinc.com/therapeutics-and-vaccines/ibio-201

the VLP and another provides epitopes to display on the surface. VLP-based vaccines are already approved for immunization against hepatitis B virus, papillomaviruses, bluetongue virus, and Norwalk virus (Balke and Zeltins, 2019; Syomin and Ilyin, 2019) and more than 100 VLP-based candidate vaccines are currently undergoing clinical trials[7]. Several chimeric VLPs displaying coronavirus peptides identified by *in silico* analysis or docking are also in development (Kalitaa et al., 2020; Wang et al., 2020). VLPs based on plant viruses can be produced on a large scale by molecular farming (Lico et al., 2011, 2012b; Thuenemann et al., 2013). The examples listed in **Table 1** range from simple VLPs based on a single viral protein to more complex structures containing up to four proteins (Marsian and Lomonossoff, 2016).

The strong potential of VLP vaccines made in plants is demonstrated by the VLPs developed by Medicago Inc. (QC, Canada) for vaccination against seasonal and pandemic influenza, which have reached phase III and II clinical trials, respectively. These vaccines are based on combinations of hemagglutinin proteins from different viral strains, which naturally assemble to form VLPs even in the absence of the other influenza virus structural proteins (D'Aoust et al., 2010). During the 2009 H1N1 pandemic, the company produced the first batch of research-grade vaccine only 3 weeks after receiving the A/H1N1 sequence (D'Aoust et al., 2010).

Although plant-derived SARS-CoV-2 VLPs have yet to be reported, the feasibility of this approach has been demonstrated by the successful production of other coronavirus VLPs in insect and mammalian cells (Lu et al., 2007, 2010; Bai et al., 2008; Lokugamage et al., 2008). This suggests that SARS-CoV2 VLPs could be assembled in plants by co-expressing the M, E, and S proteins. Medicago announced a program to develop a VLP-based COVID-19 vaccine candidate in July 2020, combining their recombinant coronavirus virus-like particle (CoVLP) technology with adjuvants from GlaxoSmithKline and Dynavax Technologies for the phase I trial[8]. A VLP-based COVID-19 vaccine program has also been announced by iBio Inc.[6] This company was established with funding from the United States Defense Advanced Research Projects Agency (DARPA) and was part of the Blue Angel initiative to establish centers for the rapid delivery of medical countermeasures in response to emerging diseases, as demonstrated by the production of ∼10 million doses of influenza vaccine in only 1 month using its plant-based[9].

In addition to SARS-CoV-2 VLPs, another potential route to a VLP-based COVID-19 vaccine is the production of chimeric VLPs displaying SARS-CoV-2 epitopes, as previously shown for human papillomavirus particles displaying influenza virus antigens (Matić et al., 2011) and hepatitis B virus particles displaying foot and mouth disease virus antigens (Huang et al., 2005). In the context of coronaviruses, this principle has been demonstrated by fusing the S1 domain of SARS-CoV to the transmembrane domain and C-terminus of avian influenzavirus hemagglutinin and expressing this with the avian influenzavirus matrix 1 protein in insect cells (Liu et al., 2011). Another successful strategy involves the RBD of the MERS-CoV S protein fused to the canine parvovirus structural protein VP2, resulting in VLPs that conferred protection and elicited neutralizing antibodies (Wang et al., 2017). Transient expression in plants should be able to produce similar VLPs on a larger scale, and more rapidly than any system based on cells cultivated in bioreactors. Plant viruses could also be used to display multiple immunogenic and immunomodulatory peptide epitopes (Lico et al., 2012a; Santoni et al., 2020), providing an effective strategy to induce simultaneous immune responses against different targets and to stimulate different components of the immune system – innate and adaptive, humoral and cell-mediated (Lico et al., 2013; Balke and Zeltins, 2019).

DISCUSSION

In many countries, schools have begun and workplaces reopened in order to restart the economy after the devastating effects of the COVID-19 lockdown, while several countries are already experiencing a second epidemic wave (Ali, 2020). In this context, we have shown how plant molecular farming could contribute to an effective response strategy in a country like Italy, where expertise in this field is mainly restricted to the research sector. National experts were gathered to envisage the scenario of a second epidemic wave, with a distribution and infection rate similar to the primary wave, in order to quantify the potential of plant molecular farming as a manufacturing platform for the production of diagnostic reagents, therapeutics and vaccines.

In all our forecasts, we restricted the platform technology to transient expression, because this allows the rapid initiation and scale-up of production, generating the first batches in only a few weeks (Hefferon, 2014). Furthermore, the large-scale production of recombinant proteins by transient expression is already considered consistent with good manufacturing practice in several countries, which is a prerequisite for the manufacture of pharmaceutical products (Fischer et al., 2012). Plant molecular farming would require only minimal investment compared to the expansion of fermenter infrastructure for microbes or animal cells, and would be much more flexible in the face of emergency scenarios as seen with COVID-19.

Recent commercial investments in large-scale automated vertical farming facilities for the production of biopharmaceuticals have shown that it is possible to process up to 7.58 kg of biomass per month per sq. m. (Holtz et al., 2015) and more recently Buyel et al. (2017) reported for a vertical farming unit yields of 68.25 kg of biomass per month per sq. m. Therefore, two such facilities would be able to process enough biomass in one week to meet Italy's entire demand for a VLP-based vaccine (sufficient to achieve herd immunity) and for diagnostic reagents (sufficient to test the entire population) with only 10% of the capital costs required for fermenter-based infrastructure. For the production of neutralizing antibodies

[7]https://clinicaltrials.gov/

[8]https://www.medicago.com/en/newsroom/medicago-begins-phase-i-clinical-trials-for-its-covid-19-vaccine-candidate/

[9]https://www.globenewswire.com/Tracker?data=_2Yc5Uv_N4cIurClgphsjzVbq4NvRaSxtXk1OPx9fG_oeiLQR7BDxDPNyC1SS5rtmVNyZIhMkgfgshOzS1F8nQ_-XWg65SAx7NHQATzShrRT2s0NubFL7PbzaOYg-QR6

and antivirals such as griffithsin, our projection suggests that plant molecular farming could provide additional capacity, thus complementing other biopharmaceutical production platforms to ramp up the speed and scale of manufacturing at the time of greatest need. The footprint of such production facilities could also be reduced by advances that improve the yield of recombinant protein in plant tissues (Barbante et al., 2008; Rigano et al., 2009; Avesani et al., 2010) thus lowering unit costs (Nandi et al., 2016). Our projections for Italy also suggest that investments in molecular farming infrastructure could provide a valuable approach for any country looking to improve its preparedness for a second wave of COVID-19 and future epidemic or pandemic diseases. Furthermore, this technology has the main advantage of easy-scalability that enables to set rapidly the platform on the basis of the ongoing needs; in a rapidly changing scenario, such as the one that we are experiencing, this aspect may help in directing national decisions also on the basis of global experiences, taking advantage from the global network that has been recently established.

In this regard, to help consolidate molecular farming in Europe, the network could benefit from the service offered by the EU Research Infrastructures (RIs) that are being established in Europe according to the ESFRI roadmap (program H2020-EU.1.4.1.2) and will be fully operational in the coming years. RIs are facilities, resources or services, identified by European research communities to conduct and to support top-level research activities in their domains. In particular, IBISBA (Industrial Biotechnology Innovation and Synthetic Biology Accelerator[10]) is an Engineering Biology Research Infrastructure that brings together research organizations providing experimental and *in silico* services for industry and academia, to accelerate bio-based manufacturing processes.

Furthermore, we believe that joint investments from public and private funding may help to reach this ambitious goal, having a major impact on the complex health national eco-system and possibly contributing in a global perspective to adopt effective countermeasures in emergency situations as the current one.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LA coordinated the single parts and assembled the final version. All authors wrote the article.

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[10] http://www.ibisba.eu

REFERENCES

Abdoli Nasab, M., Jalali Javaran, M., Cusido, R. M., and Palazon, J. (2016). Purification of recombinant tissue plasminogen activator (rtPA) protein from transplastomic tobacco plants. *Plant Physiol. Biochem.* 108, 139–144. doi: 10.1016/j.plaphy.2016.06.029

Ali, I. (2020). COVID-19: are we ready for the second wave? *Disaster Med. Public Health Prep.* 7, 1–3. doi: 10.1017/dmp.2020.149

Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T. H. O., Chromikova, V., McMahon, M., et al. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat. Med.* 26, 1033–1036. doi: 10.1038/s41591-020-0913-5

Avesani, L., Vitale, A., Pedrazzini, E., Devirgilio, M., Pompa, A., Barbante, A., et al. (2010). Recombinant human GAD65 accumulates to high levels in transgenic tobacco plants when expressed as an enzymatically inactive mutant. *Plant Biotech. J.* 8, 1–11. doi: 10.1111/j.1467-7652.2010.00514.x

Bae, J.-L., Lee, J.-G., Kang, T.-J., Jang, H.-S., Jang, Y.-S., and Yang, M.-S. (2003). Induction of antigen-specific systemic and mucosal immune responses by feeding animals transgenic plants expressing the antigen. *Vaccine* 21, 4052–4058. doi: 10.1016/s0264-410x(03)00360-8

Bai, B., Hu, Q., Hu, H., Zhou, P., Shi, Z., Meng, J., et al. (2008). Virus-like particles of SARS-like coronavirus formed by membrane proteins from different origins demonstrate stimulating activity in human dendritic cells. *PLoS One* 3:e2685. doi: 10.1371/journal.pone.0002685

Balke, I., and Zeltins, A. (2019). Use of plant viruses and virus-like particles for the creation of novel vaccines. *Adv. Drug Deliv. Rev.* 145, 119–129. doi: 10.1016/j.addr.2018.08.007

Barbante, A., Irons, S., Hawes, C., Frigerio, L., Vitale, A., and Pedrazzini, E. (2008). Anchorage to the cytosolic face of the ER membrane: a new strategy to stabilize a cytosolic recombinant antigen in plants. *Plant Biotech. J.* 6, 560–575. doi: 10.1111/j.1467-7652.2008.00342.x

Barrett, C. D., Moore, H. B., Moore, E. E., McIntyre, R. C., Moore, P. K., Burke, J., et al. (2020). Fibrinolytic therapy for refractory COVID-19 acute respiratory distress syndrome: scientific rationale and review. *Res. Pract. Thromb Haemost.* 4, 524–531. doi: 10.1002/rth2.12357

Bastos, L. M., Tavaziva, G., Abidi, S. K., Campbell, J. R., Haraoui, L. P., Johnston, J. C., et al. (2020). Diagnostic accuracy of serological tests for COVID-19: systematic review and meta-analysis. *BMJ* 370:m2516. doi: 10.1136/bmj.m2516

Baum, A., Fulton, B. O., Wloga, E., Copin, R., Pascal, K. E., Russo, V., et al. (2020). Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science* 369:eabd0831. doi: 10.1126/science.abd0831

Buyel, J. F., Twyman, R. M., and Fischer, R. (2017). Very large-scale production of antibodies in plants: the biologization of manufacturing. *Biotechnol. Adv.* 35, 458–465. doi: 10.1016/j.biotechadv.2017.03.011

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K.-C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Carattoli, A., Di Bonito, P., Grasso, F., Giorgi, C., Blasi, F., Niedrig, M., et al. (2005). Recombinant protein-based ELISA and immuno-cytochemical assay for the diagnosis of SARS. *J. Med. Virol.* 76, 137–142. doi: 10.1002/jmv.20338

Chen, W.-H., Hotez, P. J., and Bottazzi, M. E. (2020). Potential for developing a SARS-CoV receptor-binding domain (RBD) recombinant protein as a heterologous human vaccine against coronavirus infectious disease (COVID)-19. *Hum. Vaccines Immunother.* 16, 1239–1242. doi: 10.1080/21645515.2020.1740560

D'Aoust, M.-A., Couture, M. M.-J., Charland, N., Trépanier, S., Landry, N., Ors, F., et al. (2010). The production of hemagglutinin-based virus-like particles

in plants: a rapid, efficient and safe response to pandemic influenza. *Plant Biotechnol. J.* 8, 607–619. doi: 10.1111/j.1467-7652.2009.00496.x

D'Aoust, M. A., Lavoie, P. O., Couture, M. M., Trepanier, S., Guay, J. M., Dargis, M., et al. (2008). Influenza virus−like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. *Plant Biotechnol. J.* 6, 930–940. doi: 10.1111/j.1467-7652.2008.00384.x

De Martinis, D., Rybicki, E. P., Fujiyama, K., Franconi, R., and Benvenuto, E. (2016). Molecular farming: fast, scalable, cheap, sustainable. *Front. Plant Sci.* 7:1148. doi: 10.3389/fpls.2016.01148

Demurtas, O. C., Massa, S., Illiano, E., De Martinis, D., Chan, P. K. S., Di Bonito, P., et al. (2016). Antigen production in plant to tackle infectious diseases flare up: the case of SARS. *Front. Plant Sci.* 7:54. doi: 10.3389/fpls.2016.00054

Diamos, A. G., Hunter, J. G. L., Pardhe, M. D., Rosenthal, S. H., Sun, H., Foster, B. C., et al. (2020). High level production of monoclonal antibodies using an optimized plant expression system. *Front. Bioeng. Biotechnol.* 17:472. doi: 10.3389/fbioe.2019.00472

Donini, M., and Marusic, C. (2019). Current state-of-the-art in plant-based antibody production systems. *Biotechnol. Lett.* 41, 335–346. doi: 10.1007/s10529-019-02651-z

Fischer, R., and Buyel, J. F. (2020). Molecular farming–the slope of enlightenment. *Biotechnol. Adv.* 40:107519. doi: 10.1016/j.biotechadv.2020.107519

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R. M., and Drossard, J. (2012). GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnol. Adv.* 30, 434–439. doi: 10.1016/j.biotechadv.2011.08.007

Franconi, R., Illiano, E., Paolini, F., Massa, S., Venuti, A., and Demurtas, O. C. (2018). "Rapid and low-cost tools derived from plants to face emerging/re-emerging infectious diseases and bioterrorism agents," in *Defence against Bioterrorism. NATO Science for Peace and Security Series A: Chemistry and Biology*, eds V. Radosavljevic, I. Banjari, and G. Belojevic (Dordrecht: Springer). doi: 10.1007/978-94-024-1263-5_10

Freeman, B., Lester, S., Mils, L., Moye, M., Abiona, O., Hutchinson, G. B., et al. (2020). Validation of a SARS-CoV-2 spike protein ELISA for use in contact investigations and sero-surveillance. *bioRxiv* [Preprint]. doi: 10.1101/2020.04.24.057323

Fuqua, J. L., Wanga, V., and Palmer, K. E. (2015). Improving the large scale purification of the HIV microbicide, griffithsin. *BMC Biotechnol.* 15:12. doi: 10.1186/s12896-015-0120-5

Gengenbach, B. B., Opdensteinen, P., and Buyel, J. F. (2020). Robot cookies–plant cell packs as an automated high-throughput screening platform based on transient expression. *Front. Bioeng. Biotechnol.* 8:393.

Hansen, J., Baum, A., Pascal, K. E., Russo, V., Giordano, S., Wloga, E., et al. (2020). Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody cocktail. *Science* 369:eabd0827. doi: 10.1126/science.abd0827

Hefferon, K. (2014). Plant virus expression vector development: new perspectives. *Biomed. Res. Int.* 2014:785382. doi: 10.1155/2014/785382

Hoelscher, M., Tiller, N., Teh, A. Y., Wu, G. Z., Ma, J. K., and Bock, R. (2018). High-level expression of the HIV entry inhibitor griffithsin from the plastid genome and retention of biological activity in dried tobacco leaves. *Plant Mol. Biol.* 97, 357–370. doi: 10.1007/s11103-018-0744-7

Holtz, B. R., Berquist, B. R., Bennett, L. D., Kommineni, V. J. M., Munigunti, R. K., White, E. L., et al. (2015). Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol. J.* 13, 1180–1190. doi: 10.1111/pbi.12469

Huang, Y., Liang, W., Wang, Y., Zhou, Z., Pan, A., Yang, X., et al. (2005). Immunogenicity of the epitope of the foot-and-mouth disease virus fused with a hepatitis B core protein as expressed in transgenic tobacco. *Viral Immunol.* 18, 668–677. doi: 10.1089/vim.2005.18.668

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., et al. (2010). High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnol. Bioeng.* 106, 9–17. doi: 10.1002/bit.22652

Huang, Z., Santi, L., LePore, K., Kilbourne, J., Arntzen, C. J., and Mason, H. S. (2006). Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. *Vaccine* 24, 2506–2513. doi: 10.1016/j.vaccine.2005.12.024

Jantan, I., Ahmad, W., and Bukhari, S. N. A. (2015). Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials. *Front. Plant Sci.* 6:655. doi: 10.3389/fpls.2015.00655

Kalitaa, P., Padhic, A. K., Zhang, K. Y. J., and Tripathi, T. (2020). Design of a peptide-based subunit vaccine against novel coronavirus SARS-CoV-2. *Microbial Pathogen.* 145:104236. doi: 10.1016/j.micpath.2020.104236

Kanagarajan, S., Tolf, C., Lundgren, A., Waldenström, J., and Brodelius, P. E. (2012). Transient expression of hemagglutinin antigen from low pathogenic avian influenza A (H7N7) in nicotiana benthamiana. *PLoS One* 7:e33010. doi: 10.1371/journal.pone.0033010

Keech, C., Albert, G., Cho, I., Robertson, A., Reed, P., Neal, S., et al. (2020). Phase 1-2 trial of a SARS-CoV-2 recombinant spike protein nanoparticle vaccine. *N. Engl. J. Med.* 2020:NEJMoa2026920. doi: 10.1056/NEJMoa2026920.)

Keyaerts, E., Vijgen, L., Pannecouque, C., Van Damme, E., Peumans, W., Egberink, H., et al. (2007). Plant lectins are potent inhibitors of coronaviruses by interfering with two targets in the viral replication cycle. *Antiviral Res.* 75, 179–187. doi: 10.1016/j.antiviral.2007.03.003

Klumpp-Thomas, C., Drew, M., Husberger, S., Snead, K., Fay, M. P., Mehalko, J., et al. (2020). Standardization of enzyme-linked immunosorbent assays for serosurveys of the SARS-CoV-2 pandemic using clinical and at-home blood sampling. *medRxiv* [Preprint]. doi: 10.1101/2020.05.21.20109280

Koirala, A., Joo, Y. J., Khatami, A., Chiu, C., and Britton, P. N. (2020). Vaccines for COVID-19: the current state of play. *Paediatric Respir. Rev.* 35, 43–49. doi: 10.1016/j.prrv.2020.06.010

Koo, M., Bendahmane, M., Lettieri, G. A., Paoletti, A. D., Lane, T. E., Fitchen, J. H., et al. (1999). Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7774–7779. doi: 10.1073/pnas.96.14.7774

Lamphear, B. J., Jilka, J. M., Kesl, L., Welter, M., Howard, J. A., and Streatfield, S. J. (2004). A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. *Vaccine* 22, 2420–2424. doi: 10.1016/j.vaccine.2003.11.066

Lico, C., Buriani, G., Capuano, F., Benvenuto, E., and Baschieri, S. (2011). *Influenza Vaccines: New Perspectives from Plants. Plant-derived Vaccines: Technologies & Applications*. London: Future Science Group, 104–115. doi: 10.2217/EBO.11.73

Lico, C., Mancini, C., Italiani, P., Betti, C., Boraschi, D., Benvenuto, E., et al. (2009). Plant-produced potato virus X chimeric particles displaying an influenza virus-derived peptide activate specific CD8+ T cells in mice. *Vaccine* 27, 5069–5076. doi: 10.1016/j.vaccine.2009.06.045

Lico, C., Marusic, C., Capuano, F., Buriani, G., Benvenuto, E., and Baschieri, S. (2012a). *Plant-based Vaccine Delivery Strategies Innovation in Vaccinology: from Design, through to Delivery and Testing*. Dordrecht: Springer Science+Business Media.

Lico, C., Santi, L., Twyman, R. M., Pezzotti, M., and Avesani, L. (2012b). The use of plants for the production of therapeutic human peptides. *Plant Cell Rep.* 31, 439–451. doi: 10.1007/s00299-011-1215-7

Lico, C., Schoubben, A., Baschieri, S., Blasi, P., and Santi, L. (2013). Nanoparticles in biomedicine: new insights from plant viruses. *Curr. Med. Chem.* 20, 3471–3487. doi: 10.2174/09298673113209990035

Liu, A., Li, Y., Peng, J., Huang, Y., and Xu, D. (2020). Antibody responses against SARS-CoV-2 in COVID-19 patients. *J. Med. Virol.* 30, 1–5. doi: 10.1002/jmv.26241

Liu, Y. V., Massare, M. J., Barnard, D. L., Kort, T., Nathan, M., Wang, L., et al. (2011). Chimeric severe acute respiratory syndrome coronavirus (SARS-CoV) S glycoprotein and influenza matrix 1 efficiently form virus-like particles (VLPs) that protect mice against challenge with SARS-CoV. *Vaccine* 29, 6606–6613. doi: 10.1016/j.vaccine.2011.06.111

Lokugamage, K. G., Yoshikawa-Iwata, N., Ito, N., Watts, D. M., Wyde, P. R., Wang, N., et al. (2008). Chimeric coronavirus-like particles carrying severe acute respiratory syndrome coronavirus (SCoV) S protein protect mice against challenge with SCoV. *Vaccine* 26, 797–808. doi: 10.1016/j.vaccine.2007.11.092

Long, Q. X., Liu, B. Z., Deng, H. J., Wu, G. C., Deng, K., Chen, Y. K., et al. (2020). Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat. Med.* 26, 845–848. doi: 10.1093/cid/ciaa344

Love, A. J., Chapman, S. N., Matic, S., Noris, E., Lomonossoff, G., and Taliansky, M. (2012). In planta production of a candidate vaccine against bovine papillomavirus type 1. *Planta* 236, 1305–1313. doi: 10.1007/s00425-012-1692-0

Lu, B., Huang, Y., Huang, L., Li, B., Zheng, Z., Chen, Z., et al. (2010). Effect of mucosal and systemic immunization with virus-like particles of severe acute respiratory syndrome coronavirus in mice. *Immunology* 130, 254–261. doi: 10.1111/j.1365-2567.2010.03231.x

Lu, X., Chen, Y., Bai, B., Hu, H., Tao, L., Yang, J., et al. (2007). Immune responses against severe acute respiratory syndrome coronavirus induced by virus-like particles in mice. *Immunology* 122, 496–502. doi: 10.1111/j.1365-2567.2007.02676.x

Margolin, E., Chapman, R., Meyers, A. E., van Diepen, M. T., Ximba, P., Hermanus, T., et al. (2019). Production and immunogenicity of soluble plant-produced HIV-1 subtype C envelope gp140 immunogens. *Front. Plant Sci.* 10:1378. doi: 10.3389/fpls.2019.01378

Marques, L. E. C., Silva, B. B., Fireman Dutra, R., Tramontina Florean, E. O. P., Menassa, R., and Guedes, M. I. F. (2020). Transient expression of dengue virus NS1 antigen in *Nicotiana benthamiana* for use as a diagnostic antigen. *Front. Plant Sci.* 16:1674. doi: 10.3389/fpls.2019.01674

Marsian, J., and Lomonossoff, G. P. (2016). Molecular pharming-VLPs made in plants. *Curr. Opin. Biotech.* 37, 201–206. doi: 10.1016/j.copbio.2015.12.007

Marusic, C., Rizza, P., Lattanzi, L., Mancini, C., Spada, M., Belardelli, F., et al. (2001). Chimeric plant virus particles as immunogens for inducing murine and human immune responses against human immunodeficiency virus type 1. *J. Virol.* 75, 8434–8439. doi: 10.1128/JVI.75.18.8434-8439.2001

Matić, S., Rinaldi, R., Masenga, V., and Noris, E. (2011). Efficient production of chimeric Human papillomavirus 16 L1 protein bearing the M2e influenza epitope in *Nicotiana benthamiana* plants. *BMC Biotechnol.* 11:106. doi: 10.1186/1472-6750-11-106

McDonald, K. A., and Holtz, R. B. (2020). From farm to finger prick—a perspective on how plants can help in the fight against COVID-19 front. *Bioeng. Biotechnol.* 8:782. doi: 10.3389/fbioe.2020.00782

Mookherjee, N., Anderson, M. A., Haagsman, H. P., and Davidson, D. J. (2020). Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug Discovery* 19, 311–332. doi: 10.1038/s41573-019-0058-8

Mulangu, S., Dodd, L. E., Davey, R. T. Jr., Mbaya, O. T., Proschan, M., Mukadi, D., et al. (2019). A randomized, controlled trial of ebola virus disease therapeutics. *N. Engl. J. Med.* 381, 2293–2303. doi: 10.1056/NEJMoa1910993

Nandi, S., Kwong, A. T., Holtz, B. R., Erwin, B. R., Marcel, S., and McDonald, K. A. (2016). Techno-economic analysis of a transient plant-based platform for monoclonal antibody production. *MABS* 8, 1456–1466. doi: 10.1080/19420862.2016.1227901

O'Keefe, B. R., Giomarelli, B., Bernard, D. L., Shenoy, S. R., Chan, P. K. S., McMahon, J. B., et al. (2010). Broad-spectrum in vitro activity and in vivo efficacy of the antiviral protein griffithsin against emerging viruses of the family coronaviridae. *J. Virol.* 84, 2511–2521. doi: 10.1128/JVI.02322-09

O'Keefe, B. R., Vojdani, F., Buffa, V., Shattock, R. J., Montefiori, D. C., Bakke, J., et al. (2009). Scalable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6099–6104. doi: 10.1073/pnas.0901506106

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., et al. (2020). Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat. Commun.* 11:1620. doi: 10.1038/s41467-020-15562-9

Pei, H., Liu, J., Sun, C., Wang, C., Lu, Y., Ding, J., et al. (2005). Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination. *Appl. Microbiol. Biotechnol.* 68, 220–227. doi: 10.1007/s00253-004-1869-y

Peyret, H., Gehin, A., Thuenemann, E. C., Blond, D., El Turabi, A., Beales, L., et al. (2015). Tandem fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants with enhanced capacity to accommodate foreign proteins. *PLoS One* 10:e0120751. doi: 10.1371/journal.pone.0120751

Pierlorenzi, G. (2010). *L'italia si Misura*. Rome: Aracne Editrice.

Pitek, A. S., Park, J., Wang, Y., Gao, H., Hu, H., Simon, D. I., et al. (2018). Delivery of thrombolytic therapy using rod-shaped plant viral nanoparticles decreases the risk of hemorrhage. *Nanoscale* 10, 16547–16555. doi: 10.1039/c8nr02861c

Remuzzi, A., and Remuzzi, G. (2020). COVID-19 and Italy: what next? *Lancet* 395, 1225–1228. doi: 10.1016/s0140-6736(20)30627-9

Ren, W., Sun, H., Gao, G., Chen, J., Sun, S., Zhao, R., et al. (2020). Recombinant SARS-CoV-2 spike S1-Fc fusion protein induced high levels of neutralizing responses in nonhuman primates. *Vaccine* 38, 5653–5658. doi: 10.1016/j.vaccine.2020.06.066

Rigano, M. M., Manna, C., Giulini, A., Pedrazzini, E., Capobianchi, M., Castilletti, C., et al. (2009). Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cells. *Plant Biotech. J.* 6, 577–591. doi: 10.1111/j.1467-7652.2009.00425.x

Rizk, J. G., Kalantar-Zadeh, K., Mehra, M. R., Lavie, C. J., Rizk, Y., and Forthal, D. N. (2020). Pharmaco-immunomodulatory therapy in COVID-19. *Drugs* 80, 1267–1292. doi: 10.1007/s40265-020-01367-z

Rosendal, E., Wigren, J., Groening, R., Yongdae, K., Nilsson, E., Sharma, A., et al. (2020). Detection of asymptomatic SARS-CoV-2 exposed individuals by a sensitive S-based ELISA. *medRxiv* [Preprint]. doi: 10.1101/2020.06.02.20120477

Rubin, R. (2020). The challenges of expanding rapid tests to curb COVID-19. *JAMA* 324, 1813–1815. doi: 10.1001/jama.2020.21106

Sainsbury, F., Sack, M., Stadlmann, J., Quendler, H., Fischer, R., and Lomonossoff, G. P. (2010). Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. *PLoS One* 5:e13976. doi: 10.1371/journal.pone.0013976

Santoni, M., Ciardiello, M. A., Zampieri, R., Pezzotti, M., Giangrieco, L., Rafaiani, C., et al. (2019). Plant-made Bet V1 for molecular diagnosis. *Front. Plant Sci.* 10:1273. doi: 10.3389/fpls.2019.01273

Santoni, M., Zampieri, R., and Avesani, L. (2020). Plant virus nanoparticles for vaccine applications. *Curr. Protein Pept. Sci.* 21, 344–356. doi: 10.2174/1389203721666200212100255

Seow, J., Graham, C., Merrick, B., Acors, S., Steel, K. J. A., Hemmings, O., et al. (2020). Longitudinal evaluation and decline of antibody responses in SARS-CoV-2 infection. *medRxiv* [Preprint]. doi: 10.1101/2020.07.09.20148429

Shoji, Y., Chichester, J. A., Jones, M., Manceva, S. D., Damon, E., Mett, V., et al. (2011). Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. *Human Vaccines* 7(Suppl.), 41–50. doi: 10.4161/hv.7.0.14561

Struck, A. W., Axmann, M., Pfefferle, S., Drosten, C., and Meyer, B. (2011). A hexapeptide of the receptor-binding domain of SARS corona virus spike protein blocks viral entry into host cells via the human receptor ACE2. *Antiviral Res.* 94, 288–296. doi: 10.1016/j.antiviral.2011.12.012

Syomin, B., and Ilyin, Y. V. (2019). Virus-Like particles as an instrument for Vaccine production. *Mol. Biol.* 53, 323–334.doi: 10.1134/S0026893319030154

Thuenemann, E. C., Lenzi, P., Love, A. J., Taliansky, M., Becares, M., Zuniga, S., et al. (2013). The use of transient expression systems for the rapid production of virus-like particles in plants. *Curr. Pharm. Des.* 19, 5564–5573. doi: 10.2174/1381612811319310011

Tortorici, M. A., Beltramello, M., Lempp, F. A., Pinto, D., Dang, H. V., Rosen, L. E., et al. (2020). Ultrapotent human antibodies protect against SARS-CoV-2 challenge via multiple mechanisms. *Science* 370, 950–957. doi: 10.1126/science.abe3354

Tuboly, T., Yu, W., Bailey, A., Degrandis, S., Du, S., Erickson, L., et al. (2000). Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. *Vaccine* 18, 2023–2028. doi: 10.1016/S0264-410X(99)00525-3

Tusé, D., Nandi, S., McDonald, K. A., and Buyel, J. F. (2020). The emergency response capacity of plant-based biopharmaceutical manufacturing – what it is and what it could be. *Front. Plant Sci.* 11:594019. doi: 10.3389/fpls.2020.594019

Vankadari, N., and Wilce, J. A. (2020). Emerging WuHan (COVID-19) coronavirus: glycan shield and structure prediction of spike glycoprotein and its interaction with human CD26. *Emer. Microb. Infect.* 9, 601–604. doi: 10.1080/22221751.2020.1739565

Walls, A. C., Park, Y.-J., Tortorici, A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292. doi: 10.1016/j.cell.2020.02.058

Wang, C., Zheng, X., Gai, W., Wong, G., Wang, H., Jin, H., et al. (2017). Novel chimeric virus-like particles vaccine displaying MERS-CoV receptor-binding domain induce specific humoral and cellular immune response in mice. *Antiviral Res.* 140, 55–61. doi: 10.1016/j.antiviral.2016.12.019

Wang, N., Shang, J., Jiang, S., and Du, L. (2020). Subunit vaccines against emerging pathogenic human coronaviruses. *Front. Microbiol.* 11:298. doi: 10.3389/fmicb.2020.00298

Webb, S. R., Twyman, R. M., and Moloney, M. (2020). Agtech infrastructure for pandemic preparedness. *Nat. Biotech.* 38, 1025–1027. doi: 10.1038/s41587-020-0654-5

Zhou, T., Wang, H., Luo, D., Rowe, T., Wang, Z., Hogan, R. J., et al. (2004). An exposed domain in the severe acute respiratory syndrome coronavirus spike protein induces neutralizing antibodies. *J. Virol.* 78, 7217–7226. doi: 10.1128/JVI.78.13.7217-7226.2004

Zischewski, J., Sack, M., and Fischer, R. (2016). Overcoming low yields of plant-made antibodies by a protein engineering approach. *Biotechnol. J.* 11, 107–116. doi: 10.1002/biot.201500255

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Pilot Production of SARS-CoV-2 Related Proteins in Plants: A Proof of Concept for Rapid Repurposing of Indoor Farms Into Biomanufacturing Facilities

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The current CoVid-19 crisis is revealing the strengths and the weaknesses of the world's capacity to respond to a global health crisis. A critical weakness has resulted from the excessive centralization of the current biomanufacturing capacities, a matter of great concern, if not a source of nationalistic tensions. On the positive side, scientific data and information have been shared at an unprecedented speed fuelled by the preprint phenomena, and this has considerably strengthened our ability to develop new technology-based solutions. In this work, we explore how, in a context of rapid exchange of scientific information, plant biofactories can serve as a rapid and easily adaptable solution for local manufacturing of bioreagents, more specifically recombinant antibodies. For this purpose, we tested our ability to produce, in the framework of an academic lab and in a matter of weeks, milligram amounts of six different recombinant monoclonal antibodies against SARS-CoV-2 in *Nicotiana benthamiana*. For the design of the antibodies, we took advantage, among other data sources, of the DNA sequence information made rapidly available by other groups in preprint publications. mAbs were engineered as single-chain fragments fused to a human gamma Fc and transiently expressed using a viral vector. In parallel, we also produced the recombinant SARS-CoV-2 N protein and the receptor binding domain (RBD) of the Spike protein *in planta* and used them to test the binding specificity of the recombinant mAbs. Finally, for two of the antibodies, we assayed a simple scale-up production protocol based on the extraction of apoplastic fluid. Our results indicate that gram amounts of anti-SARS-CoV-2 antibodies could be easily produced in little more than 6 weeks in repurposed greenhouses with little infrastructure requirements using *N. benthamiana* as production platform. Similar procedures could be easily deployed to produce diagnostic reagents and, eventually, could be adapted for rapid therapeutic responses.

Keywords: COVID-19, SARS-CoV-2, *Nicotiana benthamiana*, plant-made antibody, plant-made antigen, biofactories, molecular farming

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INTRODUCTION

The current pandemic is evidencing several weaknesses in our ability to respond to a global crisis, one of which is the insufficient and heavily centralized distribution of the world manufacturing capacity of bioproducts such as antibodies, vaccines, and other biological reagents, especially proteins. Since it is economically impracticable to ensure readiness by maintaining inactive infrastructures during large periods of normality, the development of dual-use systems has been proposed, which would serve regular production needs in normal times but could be rapidly repurposed to strategic manufacturing requirements in times of crisis. Ideally, such adaptable infrastructures should be widespread to serve local demand in case of emergency.

Recombinant protein production in plants is a technologically mature bioengineering discipline, with most current plant-based bioproduction platforms making use of non-food crops, mainly the *Nicotiana* species tabacum and *Nicotiana benthamiana* as biomanufacturing chassis (Moon et al., 2019; Capell et al., 2020). *N. benthamiana* is most frequently used in association with *Agrobacterium*-mediated transient expression, also known as agroinfiltration, a technology that dramatically reduces the time required for product development. Briefly, agroinfiltration consists in the massive delivery of an *Agrobacterium* suspension culture to the intercellular space of plant leaves, either by pressure, using a syringe (small-scale), or applying vacuum to plants whose aerial parts have been submerged in a diluted *Agrobacterium* culture (large-scale). *Agrobacterium* transfers its T-DNA to the cell nucleus, therefore, massively reprograming the plant cell machinery toward the synthesis of the T-DNA-encoded protein(s)-of-interest. Transient expression of the transgene is often assisted by self-replicating deconstructed virus vectors that amplify the transgene dose, thus boosting protein production by several orders of magnitude (Gleba et al., 2004). Other systems, such as the pEAQ system, rely on viral genetic elements for boosting expression without recurring to viral replication (Sainsbury et al., 2009). Transient expression in *N. benthamiana* has become the standard in plant-based recombinant protein production due to a unique combination of advantages, with speed and high yield as the most obvious ones. Maximum production levels in the g/Kg fresh weight (FW) range for certain highly stable proteins such as antibodies have been reported (Marillonnet et al., 2005). Regarding speed, the *in-planta* incubation times required to obtain maximum yield of recombinant protein are no more than 2 weeks.

An important, often insufficiently highlighted feature of *N. benthamiana* transient expression is its relatively small infrastructure requirements, partially overlapping with those employed in more conventional, medium/high-tech indoors agriculture, such as hydroponics, vertical farming, etc. (Buyel, 2019). In this context, when confronted with the CoVid-19 crisis, we decided to exercise a partial reorientation of the activities in our academic lab, which is equipped with a multipurpose glass greenhouse facility, toward the production of SARS-CoV-2 antigens and antibodies against the virus. Here, we describe the recombinant production, purification, and analysis of six anti-SARS-CoV-2 monoclonal antibodies at laboratory scale, plus a pilot upscaling of two of those six antibodies. Next to production scale, a critical parameter to assess was the response time. The process described here started in mid-April 2020 with the selection of literature-available antibody variable sequences and finalized 9 weeks later with approximately 0.2 g of anti-SARS-CoV-2 antibody (Ab) produced in modular batches of 56 *N. benthamiana* plants and formulated as 1 L of Ab-enriched plant apoplastic fluid. Based on this experience, we estimate that the same process can be reduced up to 6–7 weeks with small pre-adaptations, a remarkably short-reaction time for a *de novo* antibody production system. Absolutely key for this fast reaction is the immediate availability of scientific data including antibody sequences in pre-print repositories. This is, in our opinion, one of the most positive lessons that can be extracted from the CoVid-19 crisis. We discuss here the possible applications of the fast plant-produced antigens and antibodies in diagnostics and therapy and propose the repurpose of high-tech agricultural facilities as an alternative for decentralized biomanufacturing in times of crisis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Both *N. benthamiana* wild type plants and 1,2-xylosyltransferase/alpha1,3-fucosyltransferase (ΔXT/FT) RNAi knock down lines (Strasser et al., 2008) were grown in the greenhouse. Growing conditions were 24°C (light)/20°C (darkness) with a 16-h-light/8-h-dark photoperiod.

Cloning of Anti-SARS-CoV-2 Antibodies and SARS-CoV-2 Antigens

All sequences were cloned and assembled using the GoldenBraid (GB) assembly system[1] (Sarrion-Perdigones et al., 2013). Antibody sequences were obtained from literature (see **Table 1**). All antibodies were cloned as single chain antibodies fused to the human IgG1 Fc domain. Those antibodies derived from synthetic or camelid single domain VHH libraries (sybody 3, sybody 17, and nanobody 72) were designed as direct fusions. CR3009, CR3018, and CR3022 human monoclonal antibodies were redesigned as single chain variable fragment (ScFv) by connecting the variable light (VL) and heavy (VH) chains with a GGGGSGGGGSGGGGSSGGGS peptide linker. Antibody sequences were codon optimized for *N. benthamiana* with the IDT optimization tool at http://eu.idtdna.com/CodonOpt. Here, the optimization is based on the frequencies of each codon usage for each organism. In this direction, the tool algorithm eliminates codons with less than 10% frequency and re-normalizes the remaining frequencies to 100%. Moreover, it reduces complexities that can interfere with manufacturing and downstream expression, such as repeats, hairpins, and extreme GC content.

The SARS-CoV-2 antigen sequences used (N protein, YP_009724397.2; and S-protein RBD domain, YP_009724390.1,

[1]https://gbcloning.upv.es

TABLE 1 | Detailed information of six different antibodies selected for recombinant expression in plants.

Name	Type	Size (with SP)	Target	Neutralizing activity	References
CR3022	ScFv-Fc IgG1 (VL-Linker-VH)	56.2 kDa	RBD (SARS-CoV and SARS-CoV-2)	No	Tian et al., 2020
sybody3	VHH-Fc IgG1	42.4 kDa	RBD (SARS-CoV-2)	Unknown	Walter et al., 2020
sybody17	VHH-Fc IgG1	42.2 kDa	RBD (SARS-CoV-2)	Unknown	Walter et al., 2020
nanobody72	VHH-Fc IgG1	43.3 kDa	RBD (SARS-CoV and SARS-CoV-2)	Yes	Wrapp et al., 2020
CR3009	ScFv-Fc IgG1 (VH-Linker-VL)	55.5 kDa	Protein N (SARS-CoV)	Unknown	van den Brink et al., 2005
CR3018	ScFv-Fc IgG1 (VH-Linker-VL)	54.9 kDa	Protein N (SARS-CoV)	Unknown	van den Brink et al., 2005

aa 319-541) derive from the Wuhan strain NC_045512. Four different versions of RBD were designed corresponding to (i) the native sequence with a C-terminal 6xHis-Tag (nRBD:His) or (ii) an N-terminal 6xHis-Tag and a C-terminal KDEL sequence for ER retention (His:nRBD:KDEL), and (iii and iv) their corresponding *N. benthamiana* codon optimized counterparts (bRBD:His and His:bRBD:KDEL), using the same tool as above. For N protein, a 6xHis tag was fused to the N-terminus of an *N. benthamiana* codon optimized sequence (His:bN).

DNA sequences were domesticated as level 0 phytobricks for GB cloning and ordered for synthesis as double-stranded DNA fragments (gBlocks, Integrated DNA Technologies). gBlocks were first cloned into the domestication vector pUPD2 (Vazquez-Vilar et al., 2017) in a BsmBI Golden Gate restriction/ligation reaction [37°C – 10 min, 50x (37°C – 3 min/16°C – 4 min), 50°C – 10 min, 80°C – 10 min]. The ligation product was transformed into *E. coli* Top 10 electrocompetent cells and positive clones were verified by restriction digestion analysis and sequencing. pUPD2 level 0 phytobricks were then cloned into the expression vectors pGreen SP-hIgG1 (antibody sequences), pCambiaV1 (RBD sequences), or pCambiaV2 (N sequences). pGreen SP-hIgG1 is a pGreen vector-based adaptation of the MagnICON® 3' provector pICH7410 (ICON Genetics; Giritch et al., 2006) that is designed for BsaI cloning of GB (B4-B5) standard parts as in-frame fusions with the tobacco (1–3)-beta-glucanase signal peptide and the human IgG1 Fc domain. Similarly, pCambiaV1 and pCambiaV2 are pCambia based adaptations of the MagnICON® 3' provector pICH7410 that are designed for BsaI cloning of GB standard parts as in-frame fusions with tobacco (1–3)-beta-glucanase signal peptide (pCambiaV1, for expression of secreted proteins) or without any subcellular localization signal (pCambiaV2, for expression of cytoplasmic proteins). Assembly reactions were performed as above, and the ligation reactions were transformed into *E. coli* Top 10 electrocompetent cells. Positive clones were verified by restriction digestion analysis. All level 0 parts and expression vectors used in this work are listed in **Supplementary Table 1**.

Transient Expression in *Nicotiana benthamiana*

For transient expression in *N. benthamiana*, the plasmids were transformed into *Agrobacterium tumefaciens* strain *GV3101 C58C1* by electroporation. The same strain but carrying the pSoup helper plasmid was employed to allow the replication of the pGreen vectors, which encode the antibodies. Overnight grown exponential cultures were collected by centrifugation and the bacterial pellets were resuspended in agroinfiltration solution (10 mM MES, 20 mM MgCl2, 200 μM acetosyringone, pH 5.6) and incubated for 2 h at RT in a horizontal rolling mixer. For small-scale agroinfiltration, culture optical density at 600 nm was adjusted to 0.1 with agroinfiltration solution and the bacterial suspensions harboring the 3' antibody or antigen modules, the Integrase (pICH14011), and the 5' module (pICH17388) were mixed in equal volumes. Negative control samples were agroinfiltrated with pICH11599_DsRed and Integrase module. Agroinfiltration of 5–6-week-old *N. benthamiana* plants was carried out through the abaxial leaf surface using a 1 ml needle-free syringe (Becton Dickinson S.A.). For pilot-scale production, the bacterial suspensions were prepared as above except that a lower OD_{600} was used (0.005 for sybody17 agroinfiltration and 0.01 for nanobody72 agroinfiltration). Additionally, for sybody17 agroinfiltration, a bacterial suspension of pICH11599_DsRed, a MagnICON® 3'module encoding the fluorescent protein DsRed, was added to the final *Agrobacterium* infiltration solution in a ratio 1:1:0.9:0.1 (pICH17388:pICH14011:pGreenSP-Sybody17-hIgG1:pICH11599_DsRed). Delivery of *Agrobacterium* to the plant cells was carried out by vacuum infiltration in a vacuum degassing chamber (model DP118, Applied Vacuum Engineering) provided with a 30 L infiltration tank. The aerial part of whole plants (seven plants at a time) was immersed into the *Agrobacterium* infiltration solution; vacuum was applied for 1 min at a vacuum pressure of 0.8 bar and then slowly released.

Apoplast Fluid Extraction

Fourteen days post-vacuum agroinfiltration leaves were excised and then infiltrated with 20 mM phosphate buffer (7.4 mM NaH_2PO_4, 12.6 mM $Na_2HPO_4.7H_2O$, pH 7), without (sybody17) or with (nanobody72) 0.5 mM PMSF (Sigma-Aldrich, #78830), following the same procedure as the vacuum agroinfiltration. After eliminating the buffer excess with tissue paper, the leaves were introduced into mesh zipped bags and then centrifuged at 2800 rpm for 5 min (twice) using a portable cloth dryer (*Orbegozo SC4500*). Thus, the apoplastic fluid was obtained from the drain tube. The apoplastic fluid was centrifuged (10 min, 11,000 x *g*, at 4°C) to remove any cell debris and *Agrobacterium*, the supernatant was collected and then four fractions of 4 ml were concentrated eight times using 10 kDa *Amicon Ultra-4 10K Centrifugal filters* (Millipore) after centrifugation (20 min, 3,700 x *g*, at 4°C). The concentration of proteins in the apoplastic fluid was quantified using the

Bio-Rad Protein Assay following the manufacturer's instructions and using BSA for standard curve preparation. The concentration of intact antibodies was estimated by densitometry of Coomassie stained-gel using the software ImageJ. For this, the peak area corresponding to the full-size antibody band was integrated, and its abundance in relation with the remaining bands was estimated.

Antibody Extraction and Purification

The *N. benthamiana* leaves infiltrated with the different anti-SARS-CoV-2 recombinant antibodies were collected 7 days post infiltration (dpi). Leaves were frozen in liquid nitrogen and stored at −80°C until used. Protein crude extracts were obtained by homogenizing ground frozen leaf tissue with cold PBS buffer (20 mM NaH_2PO_4, 80 mM $Na_2HPO_4.7H_2O$, 100 mM NaCl, pH 7.4) in a 1:3 (w/v) and were centrifuged at 13,000 rpm for 15 min at 4°C. For antibody purification, 4 g of ground agroinfiltrated tissue were extracted in 12 ml of cold 20 mM phosphate buffer. Samples were centrifuged at 10,000 x *g* for 15 min and the supernatant was transferred to a clean tube and further clarified by filtration through a 0.22 µm membrane filter. The recombinant antibodies were purified by affinity chromatography with Protein A agarose resin (ABT Technology) following a gravity-flow procedure according to the manufacturer's instructions. Hundred millimolar citrate buffer pH 3 was used for elution and 1 M Tris-HCl pH 9 was used for neutralization of the eluted sample (37.5 µl for each 250 µl elution fraction). Purified antibody preparations were quantified using the Bio-Rad Protein Assay following the manufacturer's instructions and using BSA for standard curve preparation. The concentration of intact antibody was estimated by densitometry as described in the previous section.

Antigen Extraction and Purification

The *N. benthamiana* leaves infiltrated with the different SARS-CoV-2 proteins were collected 5 (RBD) or 7 (N protein) dpi. Leaves were frozen in liquid nitrogen and stored at −80°C until used. Protein extraction was performed using 3–6 g of ground frozen tissue in 1:3 (w/v) extraction buffer. Three different buffers were tested as a first approach in order to optimize the purification yields. Buffer A: PBS buffer with 10 mM imidazole, pH 8. Buffer B: Buffer A supplemented with 1% Triton X-100, and Buffer C: Buffer B supplemented with 20% glycerol, 10% sucrose, and 0.05% 2-β-mercaptoethanol. Samples were vigorously vortexed and centrifuged at 10,000 x *g* for 15 min at 4°C. The supernatant was carefully transferred to a clean tube and filtered through a 0.22 µm syringe filter. Protein purification was carried out by Ni-NTA affinity chromatography as described in (Fernandez-del-Carmen et al., 2013). Purified proteins were quantified using the Bio-Rad Protein Assay following the manufacturer's instructions and using BSA for standard curve preparation.

SDS-PAGE and Western Blot Analysis

Proteins were separated by SDS-PAGE electrophoresis on NuPAGE 10% Bis-Tris Gels (Invitrogen) using MES-SDS running buffer (50 mM MES, 50 mM Tris-base, 3.5 mM SDS, 1 mM EDTA, pH 7.3) under reducing conditions. Gels were visualized by Coomassie blue staining. For Western blot analysis, proteins were transferred to PVDF membranes (Amersham Hybond™-P, GE Healthcare) by semi-wet blotting (XCell II™ Blot Module, Invitrogen, Life Technologies) following the manufacturer's instructions. Blots were blocked with 2% ECL Prime blocking agent (GE Healthcare) in PBS-T [PBS buffer supplemented with 0.1% (v/v) Tween-20]. For anti-SARS-CoV-2 antibody detection, the blots were incubated with 1:20,000 HRP-conjugated rabbit anti-human IgG (Sigma-Aldrich, #A8792). For SARS-CoV-2 antigen detection, the blots were incubated with 1:2,000 Anti-His mouse monoclonal primary antibody (Qiagen, #34660) and then incubated with 1:10,000 peroxidase labeled anti-mouse IgG secondary antibody (GE Healthcare). Blots were developed with ECL Prime Western blotting Detection Reagent (GE Healthcare) and visualized using a Fujifilm LAS-3000 imager.

Antigen ELISA

The overnight coating of Costar 96 Well EIA/RIA plates (Corning) was carried out at 4°C with 100 µl of 4 µg/ml RBD (RayBiotech, #230-30162), His:bN or BSA (used as control) in Coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). After four washes with 300 µl of PBS, the plate was blocked with 200 µl of a 2% (w/v) ECL Advance Blocking Reagent (GE Healthcare) solution in PBS-T [PBS supplemented with 0.1% (v/v) Tween-20] for 2 h at RT. The plate was washed four times with PBS, and then starting at 2 µg of the purified antibody preparation per well, or 1 µg of the full-size antibody from apoplastic fluid per well (both 100 µl), 1:5 serial dilutions in blocking solution were incubated for 1 h 30 min at RT. After four washing steps with PBS-T (PBS buffer supplemented with 0.05% Tween-20), 1:2,000 HRP-labeled rabbit anti-human IgG (Sigma-Aldrich, #A8792) in blocking solution was added. After 1 h, the plate was washed with PBS and the substrate o-phenylenediamine dihydrochloride SIGMAFAST™ OPD tablet (Sigma-Aldrich, #P9187) was added (following manufacturer's instructions). Reactions were stopped with 50 µl 3 M HCl per well and absorbance was measured at 492 nm. The endpoint dilution titer was determined as the last concentration of each purified antibody showing an absorbance value higher than the value defined as cutoff (mean blank +3SD). Blank is defined as the values from each ELISA test against BSA (Zrein et al., 1986; Armbruster and Pry, 2008).

Sandwich ELISA

The sandwich ELISAs were performed as described in the antigen ELISA section with a few changes. The plates were coated with 100 µl of 4 µg/ml murine anti-His mAb (Qiagen, #34660), and after blocking, the plates were incubated with 100 µl of the crude extracts of the (nRBD:His/His:bN) antigen expressing leaves. WT crude extracts were used as negative control. The crude extracts were prepared by adding 1:3 (w/v) PBS buffer and then were subjected to sonication. The extract was centrifuged (13,000 rpm, 4°C, and 15 min) and the supernatant was used in the incubation step. The antigens were sandwiched with 1 µg of the corresponding purified antibody preparation (100 µl in blocking solution) per well (1 h 30 min

incubation, RT). The same procedure as in the antigen ELISA was followed for the incubation with the conjugated secondary antibody, colorimetric reaction, and measurement.

RESULTS

Cloning and Expression of Anti-SARS-CoV-2 Recombinant Antibodies

Six different antibody sequences were selected for recombinant production in *N. benthamiana*, following a plant deconstructed viral strategy based on Magnifection technology as described earlier (Marillonnet et al., 2005; see **Table 1**). Four of those were directed against the receptor binding domain (RBD) of the SARS-CoV-2 spike (S) protein, whereas the remaining two were directed against the N protein. All six antibodies were engineered as single polypeptide chains fused to the human Cγ2-Cγ3 constant immunoglobulin domains. Three of them, those derived from single chain camelid or synthetic VHH antibody libraries, were produced as direct fusions. The other three, derived from full-size human monoclonal antibodies, were redesigned as scFvs, using a linker peptide that connects VH and VL regions (see **Figure 1A**). The nucleotide sequences of the different variable regions were obtained from the literature, then chemically synthesized with appropriate extensions and cloned into a destination Magnifection-adapted vector using a type IIS restriction enzyme strategy. The cloning cassette was flanked by a β-endoglucanase signal peptide for apoplastic localization in N-terminal and the human Cγ2-Cγ3 domains of the human IgG1 in the C-terminal side. The resulting vectors were transferred to *Agrobacterium* cultures and agroinfiltrated in *N. benthamiana* leaves in combination with a 5' MagnICON® module, containing the RNA polymerase and movement protein, and with an integrase module (**Figure 1B**). For antibody production, we used wild-type and RNAi ΔXT/FT glycoengineered *N. benthamiana* plant lines, the latter with a downregulation of plant-specific xylose and fucose glycosylation (Strasser et al., 2008). Infiltrated leaves were examined daily, and only minimal damage was observed in the agroinfiltrated tissues during the incubation period. After 7 days, leaf samples were collected, ground, and crude extracts were analyzed in SDS-PAGE under reducing conditions. As can be observed in **Figures 2A,B** (upper panel), all samples produced Coomassie-detectable bands of the expected antibody

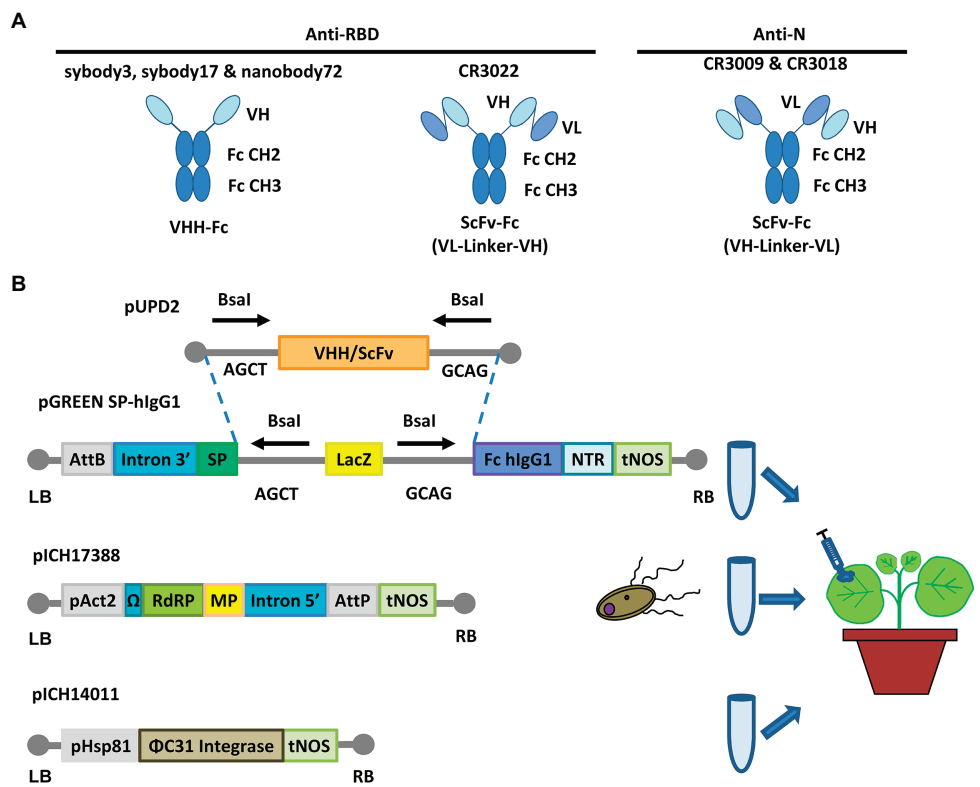

FIGURE 1 | Design for the expression of anti-SARS-CoV-2 recombinant antibodies. **(A)** Schematic representations of VHH-Fc and ScFv-Fc antibody versions. VHH, heavy chain only single domain antibody; ScFv, single chain variable fragment; Fc, human IgG1 Cγ2-Cγ3 domains. **(B)** Schematic representation of the cloning procedure for transient expression *via* MagnICON®. VHHs and ScFvs cloning into the pGreen SP-hIgG1 vector and coinfiltration together with the plasmids pICH17388 and pICH14011 for the *in planta* viral reconstruction (through ΦC31 mediated PB recombination) and antibody expression. AttB & AttP, site-specific recombination site; SP, signal peptide; NTR, nontranslated region; tNOS, terminator of nopaline synthase; pAct2, actine2 promoter of *Arabidopsis thaliana*; RdRP, RNA-dependent RNA polymerase; MP, viral movement protein; pHsp81 promoter of the heat shock protein 81.

size. scFv-IgG1 55–56 kDa antibodies migrated slightly above the 50–55 kDa Rubisco large subunit, partially masking its detection. VHH-Fc antibodies migrated at the expected 42–43 kDa size. The identity of the Coomassie bands was confirmed by Western blot using an anti-human IgG1 antibody for detection (**Figures 2A,B**, lower panel). As shown in **Figures 2A,B**, under reducing conditions lower molecular weight (MW) bands were also detected, probably as a result of partial proteolytic degradation. Small-scale affinity purification was carried out for all six antibodies produced in ΔXT/FT plants using protein A affinity chromatography (**Figures 2C,D**). The concentration of affinity-purified antibody preparations was used to estimate the yield of the final product, which ranged between 73.06 μg/g FW (CR3022 antibody) to 192.63 μg/g FW (nanobody72 antibody) at most (see **Table 2**). The percentage of the full-size antibody with respect to the total antibody preparation including cleavage fragments is also shown in **Table 2**.

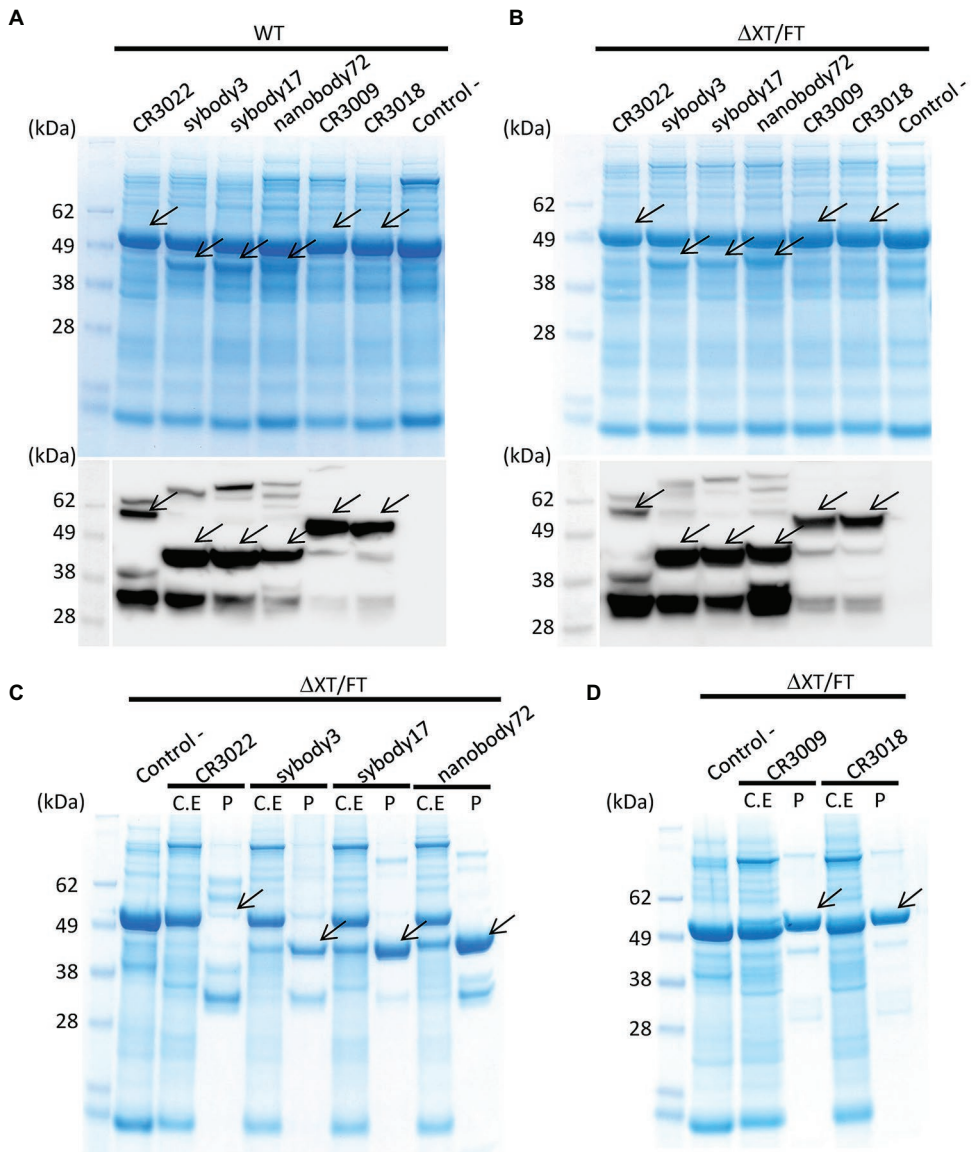

FIGURE 2 | Analysis of the expression of all six anti-SARS-CoV-2 recombinant antibodies produced in *N. benthamiana* leaves. Coomassie stained SDS gels (upper panel) and Western blots (down panel) of protein extracts from wild-type **(A)** and ΔXT/FT **(B)** *N. benthamiana* leaves agrotransformed all of them with six antibodies. Seventeen microliter of crude extract was loaded per well in SDS gels. The blots were incubated with an antibody against constant region of human IgG. **(C,D)** Coomassie stained SDS gels with purified (P) recombinant antibodies from ΔXT/FT plants compared to their corresponding protein crude extract (C.E) for each analyzed antibody. Twenty microgram of total protein from crude extract per well and 3 μg of purified protein per well were loaded in SDS gels. Control samples (–) were agroinfiltrated with Integrase module and pICH11599_DsRed vectors only, as indicated in section Materials and Methods. Arrows indicate the presence and position of the corresponding band protein for each antibody.

Cloning and Expression of SARS-CoV-2 Recombinant Antigens

The *in-planta* production of SARS-CoV-2 RBD and N protein antigens was also assayed in parallel using a similar strategy as described for antibody production. For this purpose, two versions of the expression vector were designed for RBD, one with the native viral sequence and the other with an *N. benthamiana* codon-optimized sequence. For the N protein, only the *N. benthamiana* codon-optimized sequence was employed. For RBD, native and codon optimized versions were targeted to the secretory pathway with the tobacco glucan endo-1,3-beta-glucosidase signal peptide (SP:nRBD:His and SP:bRBD:His) and versions containing a KDEL peptide for ER retention (SP:His:nRBD:KDEL and SP:His:bRBD:KDEL) were also generated. All nucleotide sequences were chemically synthesized with a small nucleotide extension coding for a histidine tag for detection (**Figure 3A**).

TABLE 2 | Yield estimation for all six purified recombinant antibodies, RBD and N proteins.

Purified protein	Yield (μg/g FW)	% Full-size Ab	% TSP
CR3022	73.06	5.07	1.96
sybody3	122.53	56.57	2.56
sybody17	153.36	83.91	3.31
nanobody72	192.63	75.95	3.18
CR3009	73.38	83.97	1.88
CR3018	81.24	90.19	1.79
nRBD:His (Buffer A)	4.31	ND	0.18
nRBD:His (Buffer B)	4.02	ND	0.051
bRBD:His (Buffer A)	2.94	ND	0.097
bRBD:His (Buffer B)	5.21	ND	0.051
His:bN (Buffer A)	30.98	ND	0.45

ND: non determined.

As described for antibody production, MagnICON®-derived 3' vector modules encoding RBD and N proteins were agroinfiltrated in combination with an integrase module and a 5'-module lacking any additional subcellular localization signal. Shorter incubation times were decided in antigen production as compared to antibodies because antigen constructs produced different degrees of necrotic lesions in the leaves, ranging from mild symptoms in N protein to severe necrosis after 4 days in native RBD. For those constructs producing more severe lesions, incubation time was reduced to 5 days, and for the rest the incubation period was extended to 7 days. RBD:His from SP:nRBD:His and SP:bRBD:His expressing leaves was extracted and purified using small-scale affinity-chromatography with Ni-NTA columns and the resulting Coomassie and a Western blot analysis, under reducing conditions, are shown in **Figure 3B**. RBD:His can be detected as an estimated 30 kDa band with the presence of higher MW bands that suggest multimerization and an 18 kDa band probably corresponding to a degradation product. ER retention did not improve expression levels of RBD for the native version, nor for the *N. benthamiana* optimized one (data not shown). Addition of 1% Triton

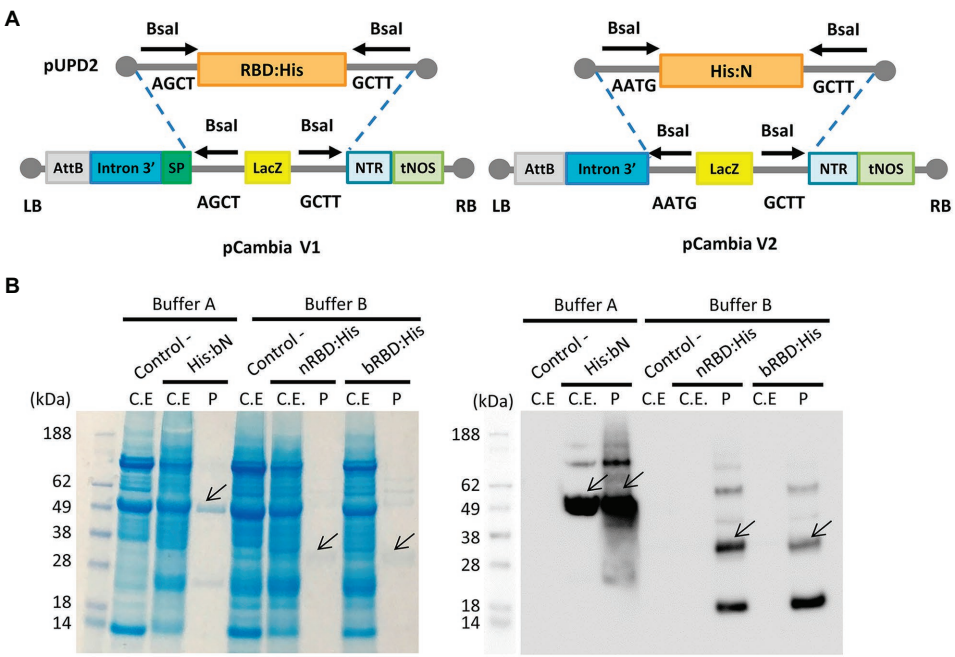

FIGURE 3 | SARS-CoV-2 RBD and N proteins expression in *N. benthamiana* agroinfiltrated leaves. **(A)** Schematic representation of the pUPD2 and 3' module vectors generated for antigen production. **(B)** Coomassie stained SDS-PAGE (left) and Western blot (right) of crude extracts from non-infiltrated *N. benthamiana* leaves (Control), leaves infiltrated with viral vectors for the expression of nRBD:His (n stands for native sequence), His:bN and bRBD:His (b stands for *N. benthamiana* codon optimized sequence) and the corresponding purified proteins.

X-100 to the standard extraction buffer (see Materials and Methods) did not improve the yield, which was estimated as 2–4 μg/g FW (**Table 2**). N protein was extracted from agroinfiltrated leaves and affinity purified following the same procedure described for RBD. A major 49 kDa band was detected both on the crude extract and upon purification (**Figure 3B**). Small-scale affinity-chromatography with Ni-NTA columns gave an estimated yield of 30 μg/g FW for N protein (**Table 2**).

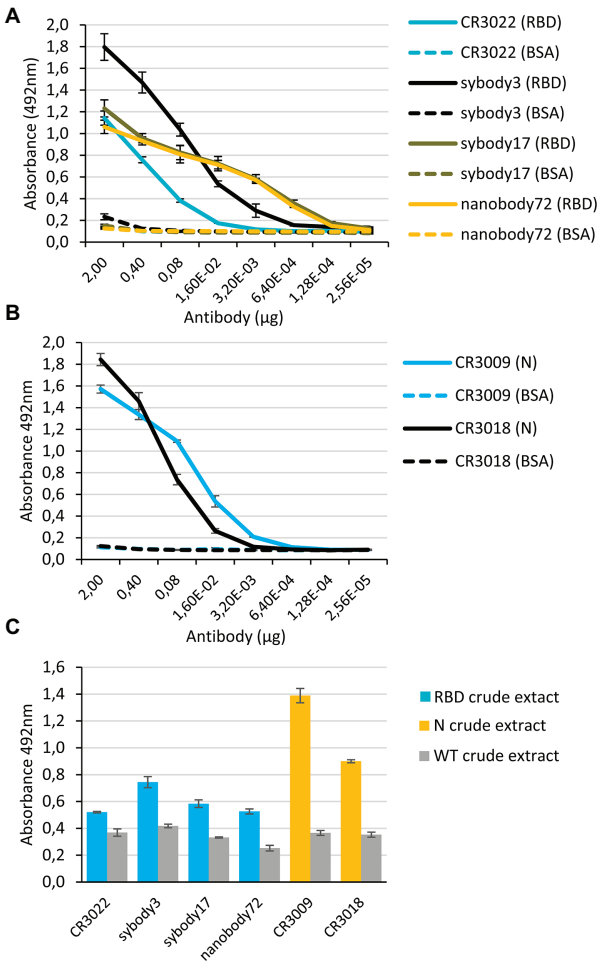

FIGURE 4 | Comparison of binding activities of anti-SARS-CoV-2 purified antibodies by ELISA tests. **(A,B)** Antigen ELISA titers graph of anti-RBD **(A)** or anti-N **(B)** purified antibody preparations. Binding activities were calculated by absorbance measurements at 492 nm. The X axis indicates the amount (μg) of antibody preparation per well. Solid lines indicate the ELISA test against RBD commercial or our purified His:bN protein, and dashed lines indicate the corresponding control ELISA tests against BSA. Bars represent mean ± SD, *n* = 3 independent replicates. **(C)** Sandwich ELISA plots obtained using anti-RBD and anti-N purified antibody preparations (1 μg per well). Plates were coated with commercial anti_His monoclonal antibody, incubated with crude extracts of plant-made antigens (nRBD:His or His:bN, respectively), and developed with the different plant-made antibody preparations as indicated. Wild type crude extracts samples were used as negative control. Bars represent mean ± SD, *n* = 3 independent replicates.

Characterization of Antigen-Antibody Binding Activities

Binding activities of affinity purified anti-RBD and anti-N antibodies were analyzed by antigen ELISA as shown in **Figures 4A,B**. As expected, all assayed antibodies were active in binding their respective antigen. Endpoint dilution titers of antibody preparations were calculated for anti-RBD and anti-N antibodies using the commercial RBD antigen and our purified His:bN antigen. Sybodies 3 and 17 and nanobody72, and CR3009 showed high dilution titers, (0.75, 0.03, 0.03, and 0.58 μM, respectively), but the performance of CR3022 and CR3018 was significantly lower (2.85 and 2.91 μM, respectively). In a parallel experiment, we tested the ability of plant-made antibodies to selectively detect our own plant-made antigens, including here also the N protein, using a sandwich ELISA approach. For this analysis, ELISA plates were coated with a murine anti-His mAb, incubated with crude plant extracts from antigen-producing plants and sandwiched with purified plant-made antibodies. As shown in **Figure 4C**, all antigen-producing plant extracts gave sandwich-ELISA signals significantly above the background when assayed using their cognate antibodies, thus evidencing the capacity of both, antibodies and antigens, to function as potent diagnostic tools. Background signals in this experiment are likely due to cross-reaction of the anti-human IgG secondary antibody with the murine anti-His mAb, and could be easily reduced for more potent diagnostic applications by employing recombinant antibody formats other than IgG.

Pilot Antibody Upscaling and Analysis of Apoplastic Fluid

In the design of a pilot upscaling experiment, we favored modularity and tried to maximize the affordability and adaptability of the process by reducing the requirements for highly specialized lab equipment. We carried out a final agroinfiltration for recombinant antibody production using a total of 112 plants, equivalent to approximately 2.5 kg of fresh plant material. The plants were divided in two batches of 56 plants each and used to produce sybody17 and nanobody72 respectively, as these antibodies showed the most promising binding activities and yields. To facilitate the upscaling of the agroinfiltration process, plant seedlings were transplanted in growth modules, each module comprising seven pots kept together in a double layer of disposable plastic-board hexagons as shown in **Figure 5A**. Each production batch consisted in eight hexagonal modules. When plants were 6 weeks old, they were agroinfiltrated by submerging each hexagon upside down into a 40 cm diameter cylindrical tank filled with 30 L of an *Agrobacterium* suspension, set inside a cylindrical vacuum degas chamber (**Figure 5A**). In this way, seven plants at a time were vacuum-agroinfiltrated by slowly releasing vacuum while leaves remained submerged in the solution. Next, plants were rinsed, brought back to the growth chamber and incubated for 14 days before harvest. Two different concentrations of the *Agrobacterium* suspension were used in this experiment. One of them (sybody17) consisted in an OD_{600} 0.005 final mix containing plasmids pICH14011, pICH17388, pGreenSybody17-IgG1, and

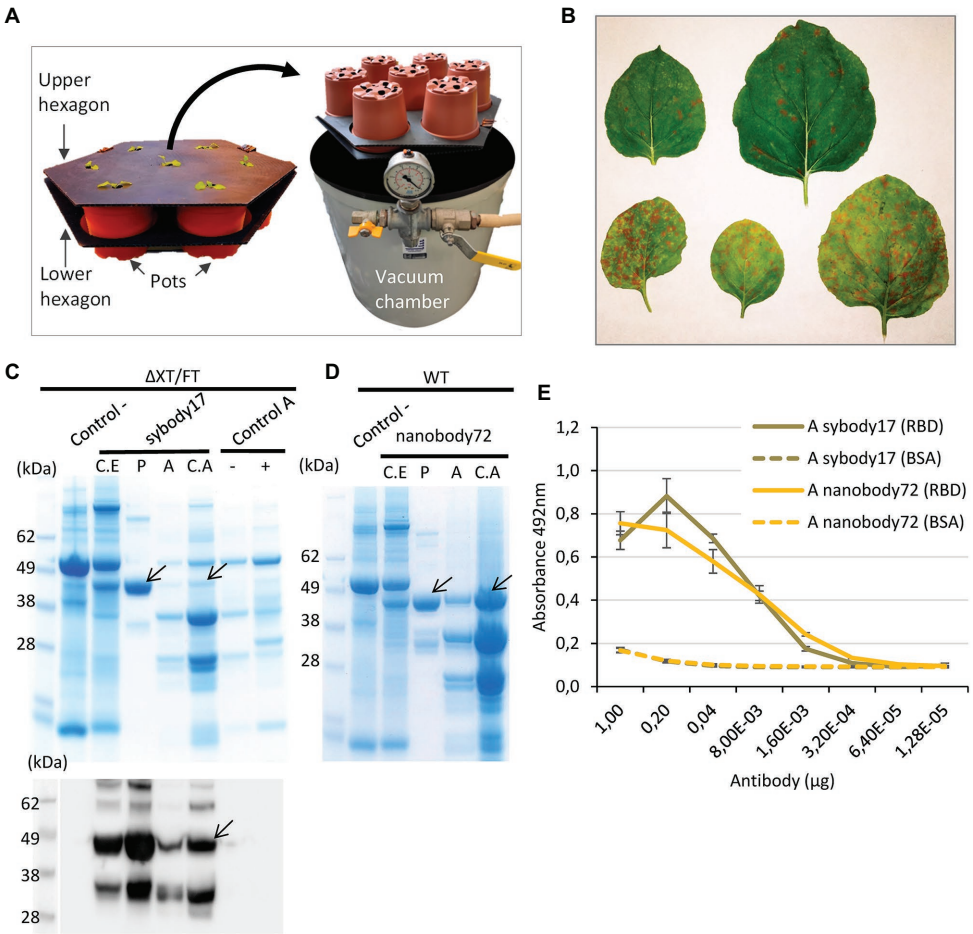

FIGURE 5 | Analysis of upscaling production of anti-RBD sybody17 and nanobody72 antibodies as an example for a pilot assay. **(A)** Hexagon growth modules used in the pilot assay with *N. benthamiana* plants and vacuum degas chamber used for vacuum agroinfiltration. **(B)** Different agroinfiltrated ΔXT/FT *N. benthamiana* leaves with DsRed tiles as indicator of the extension of the recombinant protein production. **(C)** Coomassie stained SDS gel and Western blot of protein extracts from ΔXT/FT plants expressing sybody17. **(D)** Coomassie stained SDS gel of protein extracts from WT plants expressing nanobody72. Total crude extract (C.E), purified extract (P), total apoplastic fluid **(A)** and concentrated apoplastic fluid (C.A). Wild type crude extracts (control) and apoplastic fluid with (+) or without (–) DsRed expression were used as control samples. Seventeen microliter of total protein extract per well and 3 µg of purified protein per well were loaded in SDS gels. Arrows show the position of each full-size antibody band. **(E)** Antigen ELISA titers graph of full-size antibody from apoplastic fluid of sybody17 and nanobody72-expressing plants. Binding activities were calculated by absorbance measurements at 492 nm. The X axis shows the amount (µg) of antibody per well. Solid lines indicate the ELISA test against commercial RBD, and dashed lines indicate the corresponding control ELISA tests against BSA. Bars represent mean ± SD, $n = 3$ independent replicates.

pICH11599_DsRed at 1:1:0.9:0.1 ratio, where pICH11599_DsRed is a MagnICON® 3'module encoding DsRed. The fluorescent marker was added to the infiltration mix to monitor the extension of the viral infection foci. As described elsewhere (Julve et al., 2013; Julve Parreño et al., 2018) superinfection exclusion among virial clones yields mosaic-like expression patterns of individual clones, therefore the tiles produced by red fluorescent proteins were used as an indication of the extension and distribution of the unlabeled foci producing the recombinant antibody. In parallel, nanobody72 up-scaled production was undertaken by agroinfiltration of an OD_{600} 0.01 *Agrobacterium* culture mix containing pICH14011, pICH17388, and pGreenNanobody72-IgG1 at 1:1:1 ratio. After 14 days, DsRed tiles in sybody17 experiment, clearly visible with the naked eye, finalized their expansion in most agroinfiltrated leaves, an indicator that the expression tiles had covered the whole leaf surface (**Figure 5B**). At this stage, leaves were harvested and submitted to an apoplastic fluid recovery assay, where >0.5 kg batches of detached leaves were vacuum-infiltrated in 20 mM phosphate buffer using the same vacuum device as described above. Once rinsed to remove the excess of buffer, leaves were packed in mesh zipped bags, spinned down in a spin portable cloth dryer, and the intercellular apoplastic fluid was recovered from the drain tube. With this simple procedure, between 940 and 1,200 ml of apoplastic fluid (sybody 17 and nanobody 72, respectively) was recovered from 1.2 kg of detached leaves. A fraction of the apoplastic fluid of both antibodies was concentrated eight times with

centrifugal filters (<10 kDa), and the rest was kept refrigerated for further analysis. **Figures 5C,D** show the Coomassie-staining and Western blot analysis, under reducing conditions, of crude extracts as well as apoplastic fluid preparations, and their corresponding purifications. Crude extracts in this pilot experiment showed a VHH-IgG1 band similar in intensity to that obtained in small-scale experiments (data not shown). Interestingly, apoplastic fluid consisted in a very simplified mix of proteins, with the recombinant antibody being among the most predominant ones. As shown, the different optical density of the *Agrobacterium* culture, together with the presence of a competing DsRed clone, clearly influenced the accumulation levels, with the yields of nanobody72 clearly outperforming those of its sybody counterpart. Unfortunately, the antibodies seemed partially degraded as indicated by the presence of two bands smaller than the expected VHH-Cɣ2-Cɣ3 size, which could be compatible with degradation fragments. Degradation was only partially solved with the addition of the protease inhibitor PMSF into the recovered phosphate buffer, as shown with nanobody72 production (**Figure 5D**). Despite degradation, in a densitometric analysis, we estimate that the recovered apoplastic fluid in this assay contained 159 mg per liter of intact mAb full-size (152 μg/g FW). Finally, we analyzed by antigen ELISA the binding activities of intact sybody17 and nanobody72 antibodies obtained from apoplastic fluid (**Figure 5E**). The high dilution titers observed (0.38 μM for sybody17 and 0.37 μM for nanobody72) showed that this simple antibody preparation can be directly employed in detection procedures without the need of additional purification steps.

DISCUSSION

Several *N. benthamiana*-dedicated bioproduction facilities are functioning worldwide, as those from Leaf Expression Systems in United Kingdom (Dobon, 2019), Icon Genetics (Giritch et al., 2006) and Fraunhofer in Germany (Wirz et al., 2012), iBio facility (Holtz et al., 2015) or Kentucky Bioprocessing in United States (Olinger et al., 2012), among others. Notably, Medicago recently announced the building a new 44,000 sqm facility with capacity for around 40–50 million of planned doses of flu vaccine per year. Such facilities usually involve separated modules for upstream processing, namely a wet-lab module for preparation of the bacterial inoculum, a regular plant growth chamber, and agroinfiltration room, and a post-infiltration growth chamber. In addition, downstream processing facilities are often situated next or to the production ones to minimize the handling time of fresh tissues. Whereas installed capacity of plant-dedicated biofactories is in continue growth, they are clearly insufficient to respond to global or even regional demands in times of crisis. We reasoned that, at least for upstream processes, the infrastructures required for medium-scale *N. benthamiana*-based production are not radically different to those employed in high-tech agriculture practices as hydroponics, aeroponics, or vertical farming, and thus high-tech agriculture facilities could be easily repurposed as biomanufacturing facilities in a matter of days or weeks (McDonald and Holtz, 2020). Also, the use of alternative plants and expression systems, in addition to *N. benthamiana*, could offer additional flexibility and speed. Interestingly in this context, *plant-based* platforms have important advantages in comparison to the use of more traditional production systems, especially in the case of diagnostic reagents in which the required production scale and the final purity are different from therapeutics. As an exercise to practically test the repurposing requirements, we describe here the partial adaptation of our research laboratory and greenhouse facilities to the production of SARS-CoV-2-related antigens and antibodies using *N. benthamiana* agroinfiltration as manufacturing platform.

In **Figure 6**, we show a chronogram of the activities undertaken by our team toward the production of SARS-CoV-2 antigens and antibodies, from the initial selection of the nucleotide sequences of the genes-of-interest to the production of 1 L of plant apoplastic fluid of recombinant sybody17 and nanobody72. In our hands, the whole process took a total of 9 weeks with non-exclusive personnel dedication and partially restricted access to our facilities. The process can be divided in three periods: the first step (DESIGN), taking approximately 10 days, was dedicated to construct design and gene synthesis. It was pivotal in this step to have open access to viral and antibody sequences deposited in pre-print repositories. Particularly remarkable was the openness of academic labs that immediately released primary sequence information of partially characterized anti-SARS-CoV-2 monoclonal antibodies, an exercise that should serve as an example in the future. Due to our limited testing capacity, the number of parallel designs per product was maintained relatively low, and several design decisions (e.g. codon optimization and purification tags) were taken based in a best-guess approach. Ideally, proper crisis preparedness should involve a centralized automated equipment such as a biofoundry (Hillson et al., 2019), with which the design space could be extended dramatically without causing delay. The second phase (BUILD) was dedicated to cloning and construct building and lasted less than 3 weeks. Adapted plasmids and cloning procedures from previous projects are available in our lab (Sarrion-Perdigones et al., 2011; Vazquez-Vilar et al., 2017), therefore no significant time lag occurred in this step. Importantly, this period also involved seeding a new plant batch at the scale required for pilot production in week seven (112 plants distributed in 16 hexagonal modules in this case). In a third phase (TEST), starting on week 5, all constructs were infiltrated at a small scale (three replicate leaves each), shortly incubated (5 or 7 days) and then tested functionally in parallel analyses. This small-scale assay took two additional weeks, summing a total of approximately 50 days for the complete process. Synthetic Biology-inspired Design-Build-Test (DBT) cycles are conceived as iterative processes. Here, we present it as a conceptual framework as no iterations are shown in this work. New DBT cycles can be run fuelled by the conclusion of previous cycles to generate new optimized versions of the product. Based on this experience, we estimate that the whole DBT cycle could be shortened to 30 days or less by optimizing the pipeline (e.g. introducing centralized, automatized design and build phases), and by improving

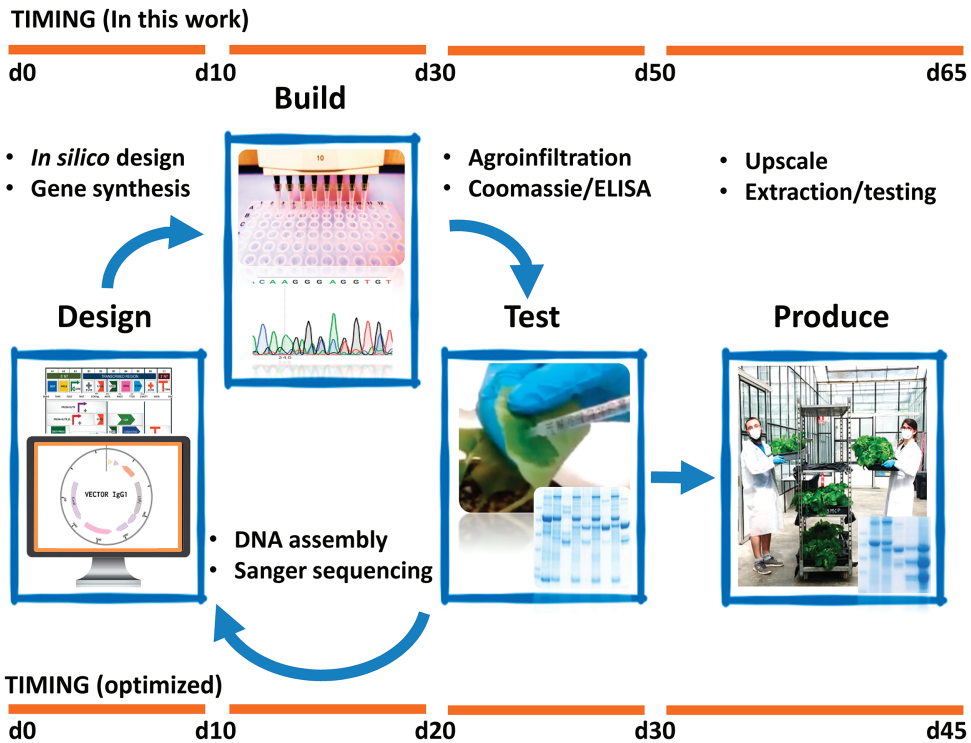

FIGURE 6 | Schematic view of the Design-Build-Test and Production pipelines employed in this work. The upper timeline represents the actual timespan of the experiments. Note that the time points represent approximately the days required to produce and initially characterize the designated products; notwithstanding, some of the results shown in previous figures correspond to extended analysis obtained at a later stage, during the preparation of this manuscript. The lower timeline describes the estimated minimal timespan that would result by introducing some of the optimizations described in the text.

preparation and anticipation in the facilities (**Figure 6**). For instance, note that moving from step 2 to step 3 without delay requires a small batch of plants be always maintained in the facility, as it was in our case to supply our research requirements. This only involves transplanting 10–15 seedlings every 3 weeks, and then disposing of them every other 3 weeks once they start flowering. If a continuous plant supply is not maintained, a minimum of three extra weeks needs to be considered to have plants ready for the first TEST iteration.

Whereas the first version of products shown here lack iterative optimization, it would serve eventually to respond to the most urgent demands. In our case, as the results of the first DBT process arose, the best performing version (V1.0) of two of the products were taken to PRODUCTION phase. In the exercise shown here, the upscaling was relatively small (112 plants, approximately 2.5 kg FW). Post-agroinfiltration incubation time was extended to 14 days to maximize yields. In the meantime, optimization of the purification/extraction methods were undertaken at small scale, so that the new knowledge acquired could be applied in the batch purification of the pilot experiment. In a crisis-scenario, and given the modularity of the proposed scheme, several medium-size production modules can be replicated in a farming facility and reproduced in several farms, allowing easy scalability. Successive iterations with small-scale agroinfiltration could be an effective way to maximize yields and reduce development times by comparing different small-scale strategies. It should be mentioned that the basic apoplast-based downstream processing proposed here could only be used, with the necessary adaptations, in a limited number of crisis-related applications, mainly related with detection and diagnosis. Other uses, certainly therapeutic ones, would involve additional regulatory considerations including GMP downstream facilities, which are beyond the scope of this exercise.

As a result of this experience, several improvements can be envisioned. We employed the MagnICON® vector system with few adaptations for all the attempted proteins. Although MagnICON® produces maximum yields for many products, some proteins, particularly viral antigens may express better with other (e.g., non-viral) systems. In our experiments, antigens showed rather low expression levels despite optimization attempts using codon optimization and different localization signals. In adapting to an emergency, it would be advisable to perform initial expression tests using different production platforms also involving non-replicative methods (Sainsbury and Lomonossoff, 2008) or DNA viruses (Diamos and Mason, 2018; Yamamoto et al., 2018; Diamos et al., 2020) and to incorporate them to the initial optimization test. As mentioned, this could be done in a centralized manner, later distributing expression clones to several repurposed production facilities. In contrast to

antigens, recombinant single-chain antibodies showed in general higher and more uniform expression levels, as could be expected from their more similar structure. We chose to adapt full human IgGs to a scFv-IgG1 format to facilitate cloning and expression procedures, since it has been earlier described in plants that single chain formats reproduce the binding activities of the original full-size antibodies from which they derive. Whereas full-size antibodies are likely more marketable for therapeutic uses, they require co-expression of heavy and light chains using non-interfering replicons, or the employ of non-replicative systems, which undergo chain shuffling and therefore are not suitable for the expression of antibody cocktails. We chose the single-chain antibody production first because this strategy facilitates the upstream process, making use of a single viral vector. Secondly, because our final goal is to produce antibody cocktails as described earlier (Julve Parreño et al., 2018), and this technology is currently optimized for single-chain antibody formats. Furthermore, the single chain format facilitated the comparisons with VHH antibodies, also produced as IgG1 fusions.

The plant-made SARS-CoV-2 products described here have several potential applications in the diagnosis area. Both RBD and N proteins can be used as reagents for serological assays (Amanat et al., 2020; Liu et al., 2020), although further yield optimizations should be required. For those assays, where antigen glycosylation is an important factor, glycoengineered plants (Strasser et al., 2008) can provide a competitive alternative to mammalian cells cultures (O'Flaherty et al., 2020). Regarding antibodies, they can serve as internal references for the quantification of serological responses. With small modifications, the same antibodies can be adapted for sandwich ELISA and employed in the detection and quantification of antigens or viral particles (e.g., using sandwich antibody pairs detecting different surface epitopes), a better proxy for infectiveness than RNA. We also show here that apoplastic fluid is an inexpensive antibody preparation suitable for certain applications that require low-cost preparations, e.g., the concentration of the virus from environmental samples. In terms of yields, the recovery of affinity-purified nanobody72 extracted from whole tissue (192 μg/g FW at most) is in the same range that the estimated concentration of the same antibody in the apoplastic fluid (approximately 160 μg/ml, equivalent to 152 μg/g FW at most). Therefore, we can conclude that in our hands recovery rates from intercellular fluid are similar to those obtained in affinity purified preparations. As shown here, the protein complexity in the apoplast is greatly reduced compared to whole extracts, therefore the apoplast could be regarded as a plant-equivalent of hybridoma supernatant or ascited fluid, although at much lower cost. Unfortunately, apoplastic preparations as well as whole tissue extracts are prone to partial antibody degradation probably due to endogenous proteases. It has been suggested that the length of CDR H3 could influence antibody stability, however in the case of the scFv-Fc reported here, CDR H3 lengths do not seem to be a major factor affecting proteolytic degradation (12 amino acids in CR3022, 14 amino acids in CR3009; Puchol Tarazona et al., 2020). As earlier reported, proteolytic degradation can be minimized using extraction buffers with appropriate protease inhibitors, as it was shown for nanobody72, or downregulating protease activity (Niemer et al., 2014). Other possibilities to solve degradation problems are to determine and redesign the cleavage sites, or ultimately to separate degradation bands during downstream processing *via* gel filtration or similar size exclusion technologies.

The current pandemic crisis has evidenced the power of new antibody selection procedures, either based on single-cell selection from human peripheral blood mononuclear cells, in the case of full-size antibodies, or based on ultra-high throughput selection of synthetic libraries (sybodies) in the case of camelid-derived nanobodies (Zimmermann et al., 2018; Walter et al., 2020). Large collections of anti-SARS-CoV-2 potentially neutralizing antibody sequences were made available to the scientific community in a question of weeks rather than months. It does not go unnoticed that the combination of rapid antibody selection procedures with fast, modular and scalable plant expression also has implications in the therapeutic arena as an ideal system for passive immunization. Intravenous polyclonal immunoglobulins (IVIG) from recovered patients have been shown a very effective CoVid-19 treatment in several studies (Montelongo-Jauregui et al., 2020, and references herein); however, the limited availability of patient sera hampers its application in practice. Interestingly, we showed in a recent work that large recombinant polyclonal antibody cocktails (pluribodies), mimicking a mammalian immune response can be produced in *N. benthamiana* with high batch-to-batch reproducibility (Julve Parreño et al., 2018). Passive immunization with recombinant antibody cocktails resembles a natural response more than a monoclonal therapy, requires shorter developmental times and is probably more robust against the development of resistances. Additionally, other antibody-based therapeutics, such as receptor decoys fused to Fc of IgG1 (such as ACE-Fc described recently; Glasgow et al., 2020), could be designed and produced rapidly in plants followed a similar scheme as described here.

In conclusion, based on the results of the exercise described here, we propose the repurposing of indoors farms into plant-based biomanufacturing facilities as a viable option to respond to local and global shortages of bioproducts such as diagnostics and therapeutic reagents in times of crisis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BD-M and BG equally contributed to this work (authors' position only responds to their alphabetical order). All authors designed and performed the experiments and analyzed the data. DO wrote this manuscript. All authors revised and edited the written manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.612781/full#supplementary-material

REFERENCES

Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T. H. O., Chromikova, V., McMahon, M., et al. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat. Med.* 26, 1033–1036. doi: 10.1038/s41591-020-0913-5

Armbruster, D. A., and Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *Clin. Biochem. Rev.* 29, S49–S52.

Buyel, J. F. (2019). Plant molecular farming—integration and exploitation of side streams to achieve sustainable biomanufacturing. *Front. Plant Sci.* 9:1893. doi: 10.3389/fpls.2018.01893

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. -C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Diamos, A. G., Hunter, J. G. L., Pardhe, M. D., Rosenthal, S. H., Sun, H., Foster, B. C., et al. (2020). High level production of monoclonal antibodies using an optimized plant expression system. *Front. Bioeng. Biotechnol.* 7:472. doi: 10.3389/fbioe.2019.00472

Diamos, A. G., and Mason, H. S. (2018). High-level expression and enrichment of norovirus virus-like particles in plants using modified geminiviral vectors. *Protein Expr. Purif.* 151, 86–92. doi: 10.1016/j.pep.2018.06.011

Dobon, A. (2019). Transient gene expression seeds plant-based bioproduction systems: Leaf Expression Systems' Hypertrans technology promises low costs and high yields. *GEN.* 39, 54–56. doi: 10.1089/gen.39.03.14

Fernandez-del-Carmen, A., Juárez, P., Presa, S., Granell, A., and Orzáez, D. (2013). Recombinant jacalin-like plant lectins are produced at high levels in *Nicotiana benthamiana* and retain agglutination activity and sugar specificity. *J. Biotechnol.* 163, 391–400. doi: 10.1016/j.jbiotec.2012.11.017

Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V., et al. (2006). Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc. Natl. Acad. Sci.* 103, 14701–14706. doi: 10.1073/pnas.0606631103

Glasgow, A., Glasgow, J., Limonta, D., Solomon, P., Lui, I., Zhang, Y., et al. (2020). Engineered ACE2 receptor traps potently neutralize SARS-CoV-2. *Proc. Natl. Acad. Sci. U. S. A.* 117, 28046–28055. doi: 10.1073/pnas.2016093117

Gleba, Y., Marillonnet, S., and Klimyuk, V. (2004). Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr. Opin. Plant Biol.* 7, 182–188. doi: 10.1016/j.pbi.2004.01.003

Hillson, N., Caddick, M., Cai, Y., Carrasco, J. A., Chang, M. W., Curach, N. C., et al. (2019). Building a global alliance of biofoundries. *Nat. Commun.* 10:2040. doi: 10.1038/s41467-019-10079-2

Holtz, B. R., Berquist, B. R., Bennett, L. D., Kommineni, V. J. M., Munigunti, R. K., White, E. L., et al. (2015). Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol. J.* 13, 1180–1190. doi: 10.1111/pbi.12469

Julve, J. M., Gandía, A., Fernández-del-Carmen, A., Sarrion-Perdigones, A., Castelijns, B., Granell, A., et al. (2013). A coat-independent superinfection exclusion rapidly imposed in *Nicotiana benthamiana* cells by tobacco mosaic virus is not prevented by depletion of the movement protein. *Plant Mol. Biol.* 81, 553–564. doi: 10.1007/s11103-013-0028-1

Julve Parreño, J. M., Huet, E., Fernández-del-Carmen, A., Segura, A., Venturi, M., Gandía, A., et al. (2018). A synthetic biology approach for consistent production of plant-made recombinant polyclonal antibodies against snake venom toxins. *Plant Biotechnol. J.* 16, 727–736. doi: 10.1111/pbi.12823

Liu, W., Liu, L., Kou, G., Zheng, Y., Ding, Y., Ni, W., et al. (2020). Evaluation of nucleocapsid and spike protein-based enzyme-linked immunosorbent assays for detecting antibodies against SARS-CoV-2. *J. Clin. Microbiol.* 58, e00461–e004620. doi: 10.1128/JCM.00461-20

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V., and Gleba, Y. (2005). Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* 23, 718–723. doi: 10.1038/nbt1094

McDonald, K. A., and Holtz, R. B. (2020). From farm to finger prick—a perspective on how plants can help in the fight against COVID-19. *Front. Bioeng. Biotechnol.* 8:782. doi: 10.3389/fbioe.2020.00782

Montelongo-Jauregui, D., Vila, T., Sultan, A. S., and Jabra-Rizk, M. A. (2020). Convalescent serum therapy for COVID-19: a 19th century remedy for a 21st century disease. *PLoS Pathog.* 16:e1008735. doi: 10.1371/journal.ppat.1008735

Moon, K. -B., Park, J. -S., Park, Y. -I., Song, I. -J., Lee, H. -J., Cho, H. S., et al. (2019). Development of systems for the production of plant-derived biopharmaceuticals. *Plants* 9:30. doi: 10.3390/plants9010030

Niemer, M., Mehofer, U., Torres Acosta, J. A., Verdianz, M., Henkel, T., Loos, A., et al. (2014). The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. *Biotechnol. J.* 9, 493–500. doi: 10.1002/biot.201300207

O'Flaherty, R., Bergin, A., Flampouri, E., Mota, L. M., Obaidi, I., Quigley, A., et al. (2020). Mammalian cell culture for production of recombinant proteins: a review of the critical steps in their biomanufacturing. *Biotechnol. Adv.* 43:107552. doi: 10.1016/j.biotechadv.2020.107552

Olinger, G. G., Pettitt, J., Kim, D., Working, C., Bohorov, O., Bratcher, B., et al. (2012). Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. *Proc. Natl. Acad. Sci.* 109, 18030–18035. doi: 10.1073/pnas.1213709109

Puchol Tarazona, A. A., Lobner, E., Taubenschmid, Y., Paireder, M., Torres Acosta, J. A., Göritzer, K., et al. (2020). Steric accessibility of the cleavage sites dictates the proteolytic vulnerability of the anti-HIV-1 antibodies 2F5, 2G12, and PG9 in plants. *Biotechnol. J.* 15:e1900308. doi: 10.1002/biot.201900308

Sainsbury, F., and Lomonossoff, G. P. (2008). Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* 148, 1212–1218. doi: 10.1104/pp.108.126284

Sainsbury, F., Thuenemann, E. C., and Lomonossoff, G. P. (2009). pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* 7, 682–693. doi: 10.1111/j.1467-7652.2009.00434.x

Sarrion-Perdigones, A., Falconi, E. E., Zandalinas, S. I., Juárez, P., Fernández-del-Carmen, A., Granell, A., et al. (2011). GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. *PLoS One* 6:e21622. doi: 10.1371/journal.pone.0021622

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palaci, J., Castelijns, B., Forment, J., Ziarsolo, P., et al. (2013). GoldenBraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. *Plant Physiol.* 162, 1618–1631. doi: 10.1104/pp.113.217661

Strasser, R., Stadlmann, J., Schähs, M., Stiegler, G., Quendler, H., Mach, L., et al. (2008). Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure: XylT and FucT down-regulation in *N. benthamiana*. *Plant Biotechnol. J.* 6, 392–402. doi: 10.1111/j.1467-7652.2008.00330.x

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., et al. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg. Microbes Infect.* 9, 382–385. doi: 10.1080/22221751.2020.1729069

van den Brink, E. N., ter Meulen, J., Cox, F., Jongeneelen, M. A. C., Thijsse, A., Throsby, M., et al. (2005). Molecular and biological characterization of human monoclonal antibodies binding to the spike and nucleocapsid proteins of severe acute respiratory syndrome coronavirus. *J. Virol.* 79, 1635–1644. doi: 10.1128/JVI.79.3.1635-1644.2005

Vazquez-Vilar, M., Quijano-Rubio, A., Fernandez-del-Carmen, A., Sarrion-Perdigones, A., Ochoa-Fernandez, R., Ziarsolo, P., et al. (2017). GB3.0: a platform for plant bio-design that connects functional DNA elements with associated biological data. *Nucleic Acids Res.* 45, 2196–2209. doi: 10.1093/nar/gkw1326

Walter, J. D., Hutter, C. A. J., Zimmermann, I., Wyss, M., Egloff, P., Sorgenfrei, M., et al. (2020). Sybodies targeting the SARS-CoV-2 receptor-binding domain. bioRxiv. [Preprint]. doi: 10.1101/2020.04.16.045419

Wirz, H., Sauer-Budge, A. F., Briggs, J., Sharpe, A., Shu, S., and Sharon, A. (2012). Automated production of plant-based vaccines and pharmaceuticals. *J. Lab. Autom.* 17, 449–457. doi: 10.1177/2211068212460037

Wrapp, D., De Vlieger, D., Corbett, K. S., Torres, G. M., Wang, N., Van Breedam, W., et al. (2020). Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. *Cell* 181, 1004–1015. e15. doi: 10.1016/j.cell.2020.04.031

Yamamoto, T., Hoshikawa, K., Ezura, K., Okazawa, R., Fujita, S., Takaoka, M., et al. (2018). Improvement of the transient expression system for production of recombinant proteins in plants. *Sci. Rep.* 8:4755. doi: 10.1038/s41598-018-23024-y

Zimmermann, I., Egloff, P., Hutter, C. A., Arnold, F. M., Stohler, P., Bocquet, N., et al. (2018). Synthetic single domain antibodies for the conformational trapping of membrane proteins. *eLife* 7:e34317. doi: 10.7554/eLife.34317

Zrein, M., De Marcillac, G., and Van Regenmortel, M. H. V. (1986). Quantitation of rheumatoid factors by enzyme immunoassay using biotinylated human IgG. *J. Immunol. Methods* 87, 229–237. doi: 10.1016/0022-1759(86)90536-3

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Development of Plant-Produced Recombinant ACE2-Fc Fusion Protein as a Potential Therapeutic Agent Against SARS-CoV-2

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease (COVID-19) which has recently emerged as a potential threat to global public health. SARS-CoV-2 is the third known human coronavirus that has huge impact on the human population after SARS-CoV and MERS-CoV. Although some vaccines and therapeutic drugs are currently in clinical trials, none of them are approved for commercial use yet. As with SARS-CoV, SARS-CoV-2 utilizes angiotensin-converting enzyme 2 (ACE2) as the cell entry receptor to enter into the host cell. In this study, we have transiently produced human ACE2 fused with the Fc region of human IgG1 in *Nicotiana benthamiana* and the *in vitro* neutralization efficacy of the plant-produced ACE2-Fc fusion protein was assessed. The recombinant ACE2-Fc fusion protein was expressed in *N. benthamiana* at 100 μg/g leaf fresh weight on day 6 post-infiltration. The recombinant fusion protein showed potent binding to receptor binding domain (RBD) of SARS-CoV-2. Importantly, the plant-produced fusion protein exhibited potent anti-SARS-CoV-2 activity *in vitro*. Treatment with ACE2-Fc fusion protein after viral infection dramatically inhibit SARS-CoV-2 infectivity in Vero cells with an IC_{50} value of 0.84 μg/ml. Moreover, treatment with ACE2-Fc fusion protein at the pre-entry stage suppressed SARS-CoV-2 infection with an IC_{50} of 94.66 μg/ml. These findings put a spotlight on the plant-produced ACE2-Fc fusion protein as a potential therapeutic candidate against SARS-CoV-2.

Keywords: COVID-19, SARS-CoV-2, ACE2-Fc fusion protein, molecular farming, *Nicotiana benthamiana*, plant-produced recombinant protein

INTRODUCTION

The coronavirus disease 2019 (COVID-19) outbreak originated in Wuhan, China in late December 2019 (Han et al., 2020; Li et al., 2020; Lupia et al., 2020). The outbreak has spread to more than 200 countries with more than 53.7 million confirmed cases and more than 1.3 million confirmed deaths as of 17 November 2020 (World Health Organization, 2020b). These numbers are still increasing with the ongoing pandemic which overwhelms national health care systems and has had major consequences on global economy. An effective vaccine and treatment are the main priorities to control the pandemic.

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to *Coronaviridae* family. Several members of the family *Coronaviridae* constantly circulate in the human population and usually cause mild respiratory disease (Ciotti et al., 2020; Han et al., 2020). In contrast, the closely related severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV) are initially transmitted from animals to humans and cause severe respiratory diseases (Fehr et al., 2017).

SARS-CoV-2 has a single-strand positive-sense RNA genome of approximately 30 kb. The virus comprises four structural proteins, spike (S), nucleocapsid (N), envelope (E), and membrane proteins (M) (Masters, 2006; Amanat and Krammer, 2020; Malik, 2020; Quinlan et al., 2020; Shanmugaraj et al., 2020b). The spike protein is responsible for viral entry into target cells. Entry depends on binding of the receptor binding domain (RBD) on the spike protein to its cellular receptor, which facilitates virus attachment to the receptor and fusion with cell membrane (Li et al., 2005; Masters, 2006; Lei et al., 2020; Quinlan et al., 2020). For SARS-CoV-2, the virus uses the RBD in spike protein to interact with human angiotensin-converting enzyme 2 (ACE2) as a critical initial step to enter into target cells, similar to SARS-CoV (Li et al., 2003; Wong et al., 2004; Hofmann et al., 2005; Shanmugaraj et al., 2020b; Zhang et al., 2020; Zhou et al., 2020b). Therefore, ACE2 has the potential to be used as therapeutic for SARS-CoV-2 infection (Kruse, 2020; Lei et al., 2020).

Our approach for developing SARS-CoV-2 therapeutics focus on transiently producing the human ACE2 protein in plants. Over the last few decades, plants have received considerable attention with advantages of low-cost production, scalability, speed and lack of animal and human pathogens (Phoolcharoen et al., 2011; Shanmugaraj and Ramalingam, 2014; Streatfield et al., 2015; Chan et al., 2016; Rosales-Mendoza et al., 2017). Several potential biologics have been expressed transiently in plants and this is likely to continue with the increasing demand for affordable vaccine (Komarova et al., 2010; Teh et al., 2014). Importantly, plants contain a post-translational modification mechanism which makes them suitable for production of complex proteins, such as antibodies and Fc fusion proteins as described here.

Fusion proteins based on the immunoglobulin Fc domain show the ability to facilitate protein expression and enable easy purification of recombinant protein by protein A chromatography (Carter, 2011; Rattanapisit et al., 2019c; Park et al., 2020). Additionally, the Fc domain can also prolong the half-life of the proteins (Cox et al., 2004; Suzuki et al., 2010; Czajkowsky et al., 2012; Kruse, 2020). Several types of Fc fusion proteins had been approved by the FDA (Peters et al., 2010; Powell et al., 2012; Lagassé et al., 2017). Therefore, we engineered ACE2 by fusing N-terminus of the Fc region of human immunoglobulin IgG1 (**Figure 1A**) and transiently expressed the construct in *Nicotiana benthamiana* using geminiviral vector. The plant produced ACE2-Fc fusion protein was used as a theraputic agent to prevent the attachment of virus to host cell by interacting with SARS-CoV-2 RBD (**Figure 1B**). Our results showed that the plant-produced ACE2-Fc fusion protein can bind to the RBD and inhibit SARS-CoV-2 infection *in vitro*.

MATERIALS AND METHODS

Plasmid Construction for Expression of ACE2-Fc

The human angiotensin converting enzyme 2 (hACE2) (GenBank accession number: NP_001358344.1) was designed to join with the Fc region of human IgG1 (Genbank accession number: 4CDH_A) by a peptide linker [$(GGGGS)_2$] at the C-terminus. The nucleotide sequence of ACE2-Fc construct was optimized for *N. benthamiana* and commercially synthesized by Genewiz, Suzhou, China. The ACE2-Fc sequences were ligated into a geminiviral vector pBYR2eK2Md (pBYR2e) using *Xba*I and *Sac*I restriction sites, at the N-terminus and C-terminus, respectively, to construct the expression vector pBYR2e-ACE2-Fc (**Figure 1A**).

Transient Expression of ACE2-Fc in *N. benthamiana* Leaves

The plant expression vector was transformed into *Agrobacterium tumefaciens* GV3101 by electroporation using a MicroPulser (Bio-Rad, United States). The transformants were confirmed by PCR. For ACE2-Fc expression, the *Agrobacterium* pellet containing pBYR2e-ACE2-Fc was resuspended and diluted in 1x infiltration buffer [10 mM 2-(N-morpholino] etanesulfonic acid (MES), 10 mM $MgSO_4$, at pH 5.5) to an OD_{600} of 0.2. The suspension was injected into the adaxial side of 6-week-old *N. benthamiana* leaves and maintained at 28°C. For optimization of plant-produced ACE2-Fc, the infiltrated leaves were collected from 3 individual plants on days 2, 4, 6, 8, and 10 after infiltration. Then, we used a vacuum infiltration for large-scale production of ACE2-Fc. The expression level of ACE2-Fc was measured by ELISA assay.

Protein Extraction and Quantification

The infiltrated leaves were extracted with 1xPBS buffer (phosphate-buffered saline: 137 mM NaCl, 2.68 mMKCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4) at pH 7.4. The suspensions were clarified by centrifugation at 26,000 g for 30 min at 4°C. The supernatants were collected and quantified by an indirect ELISA. 96-well plates (Greiner Bio-One GmbH, Austria) were coated with 50 μl of plant-produced

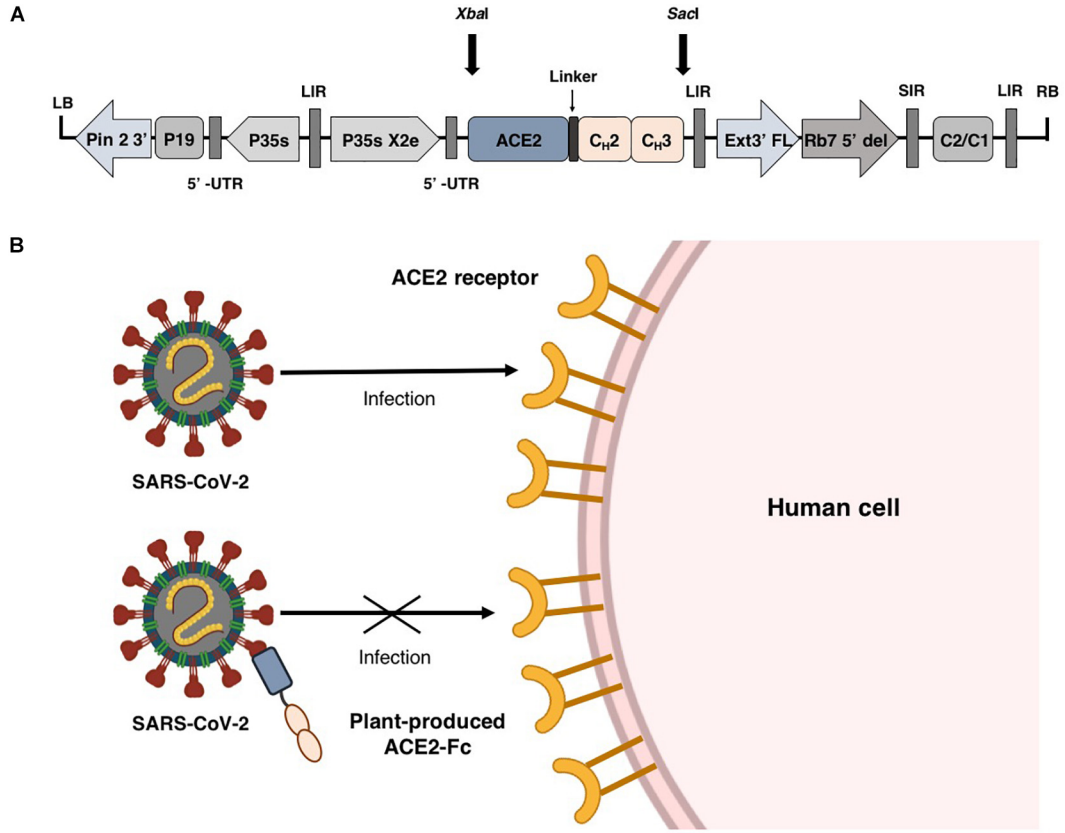

FIGURE 1 | Schematic representation of plant expression vector pBYR2e-ACE2-Fc used in the present study **(A)**. Diagrammatic representation showing the binding of plant-produced ACE2-Fc with SARS-CoV-2 thereby preventing the virus entry into the host cell **(B)**.

ACE2-Fc or commercial HEK293-produced ACE2-Fc (Abcam, United Kingdom) as a protein standard (diluted in 1xPBS) and plates were incubated overnight at 4°C. The plates were blocked with 5% skim milk powder in 1xPBS for 2 h at 37°C. After blocking, a 1:2,000 dilution of rabbit anti-ACE2 antibody (SinoBiological, United States) in 1xPBS was added into the wells and incubated for 2 h at 37°C. Then, goat anti-rabbit IgG-HRP fusion was added with the dilution of 1:2,000 in 1xPBS (BosterBio, United States) and incubated for 1 h at 37°C. The signal was developed by addition of 50 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) mixture (Promega, United States) followed by adding 1M H_2SO_4. The absorbance was measured at 450 nm using a 96-well plate reader (Molecular Devices, United States). Each sample was loaded in triplicates. Between each step, the plates were washed three times with 1xPBST (1xPBS plus 0.05% Tween-20).

Protein Purification and Characterization

The total soluble proteins from infiltrated leaves were extracted with 1xPBS pH 7.4 and clarified by centrifugation. The supernatant was filtered by 0.45 μm membrane filter (MilliporeSigma, United States) and loaded onto an affinity chromatography column containing protein-A beads. The column was washed with 1xPBS pH 7.4 followed by 0.1M glycine, pH 2.7 for ACE2-Fc elution. The elution sample was instantly neutralized with the addition of 1.5M Tris-HCl, pH 8.8. The purified plant-produced ACE2-Fc was analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting under non-reducing and reducing conditions using commercial HEK293-produced ACE2-Fc fusion protein as a positive control. The plant-produced ACE2-Fc samples were separated on 8% SDS-PAGE and the gel was stained with Coomassie brilliant blue. For western blot analysis, proteins were transferred to a nitrocellulose membrane (Biorad, United States). The membrane was blocked using 5% skim milk in 1xPBS and separately probed with ACE2-specific antibody using a rabbit anti-ACE2 antibody (SinoBiological, United States) followed by goat anti-rabbit-HRP fusion (BosterBio, United States) and Fc domain-specific antibody using an anti-human Gamma chain-HRP fusion (The Binding Sites, United Kingdom) with the dilution of 1:2,000 in 1xPBS. The membrane was washed with 1xPBST and the signal developed using an ECL reagent (Abcam, United Kingdom).

SARS-CoV-2 RBD Binding by ELISA

The binding activity of the purified plant-produced ACE2-Fc to SARS-CoV-2 RBD was analyzed by ELISA. 96-well plate (Greiner Bio-One GmbH, Austria) was coated with 100 ng

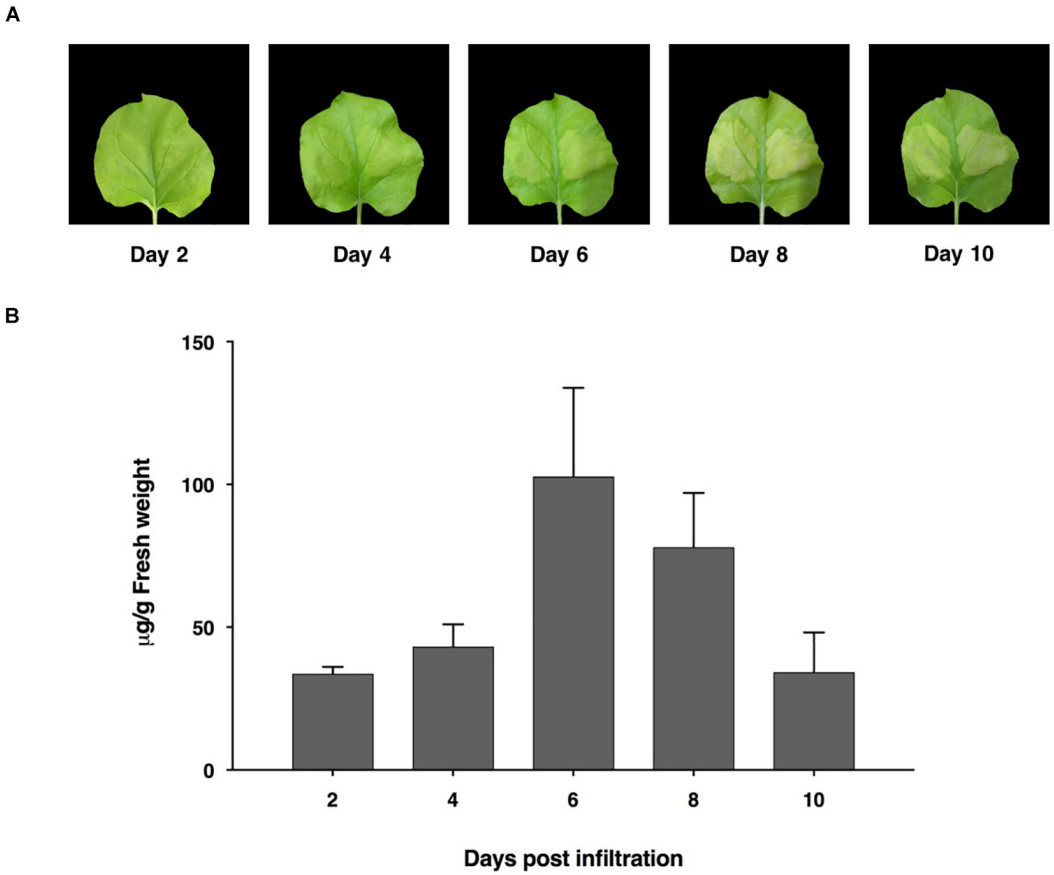

FIGURE 2 | Expression profiles of ACE2-Fc in *N. benthamiana* leaves on days 2, 4, 6, 8, and 10 after agroinfiltration. Leaf necrosis **(A)** Quantification of plant-produced ACE2-Fc **(B)**. The infiltrated leaves were collected from 3 individual plants in each day post infiltration. Data were analyzed by indirect ELISA assay using ACE2-specific antibody and presented as mean ± SD of triplicates.

of plant-produced ACE2-Fc and incubated overnight at 4°C. The wells were blocked using 5% skim milk in 1xPBS for 2 h at 37°C. The plate was washed three times with 1xPBST and incubated with various dilutions of proteins including the RBD of SARS-CoV-2 produced from Sf9 cells (Genscript Biotech, United States), S1 protein of porcine epidemic diarrhea virus (PEDV) (**Supplementary Figure S1**), and PBS as negative controls for 2 h at 37°C. After washing, an anti-6X His tag-HRP fusion (Abcam, UK) diluted in 1xPBS was added into the wells and incubated for 2 h at 37°C. For detection, a TMB solution (Promega, United States) was added into the plate. The enzymatic reactions were stopped by adding 1M H_2SO_4. The absorbance at 450 nm was measured using a 96-well microplate reader (Molecular Devices, United States).

In vitro Antiviral Assay

A total of 10,000 Vero E6 cells were cultured in a 96-well plate (Corning, United States) overnight at 37°C in a 5%CO_2 atmosphere. For the post-treatment condition, 25$TCID_{50}$ (50% tissue culture infective dose) of SARS-CoV-2 was adsorbed for 2 h at 37°C, after washing the cells with 1xPBS, fresh culture medium (DMEM with 2%FBS) was added. Various concentrations of ACE2-Fc were directly added to the culture medium and cells were maintained at 37°C in a 5%CO_2 incubator for 48 h. For pre-entry treatment, a mixture of ACE2-Fc and 25$TCID_{50}$ of SARS-CoV-2 was incubated at 37°C for 1 h before viral adsorption for 2 h. The cells were washed twice with 1xPBS followed by the addition of fresh culture medium (DMEM with 2%FBS) after which cells were maintained under standard conditions for an additional 48 h. Positive convalescent serum (heat-inactivated for 30 min at 56°C) of a COVID-19 patient and an anti-human IgG-FITC antibody (Santa Cruz Biotechnology, United States) were used as positive and negative controls, respectively. The experiment was performed in duplicates. A high-content imaging system was used for the detection of the SARS-CoV-2 nucleocapsid. The cells in the 96-well plate were fixed and permeabilized with 50% (v/v) acetone in methanol on ice for 20 min washed once with 1xPBST with 0.5% Tween detergent, followed by blocking in 1xPBST with 2% (w/v) BSA for 1 h at room temperature. After blocking, the cells were incubated with a 1:500 dilution of a rabbit monoclonal primary antibody specific for the SARS-CoV nucleoprotein (NP) (SinoBiological, United States) for 1 h at 37°C. The unbound antibody was removed by washing with 1xPBST thrice. Then,

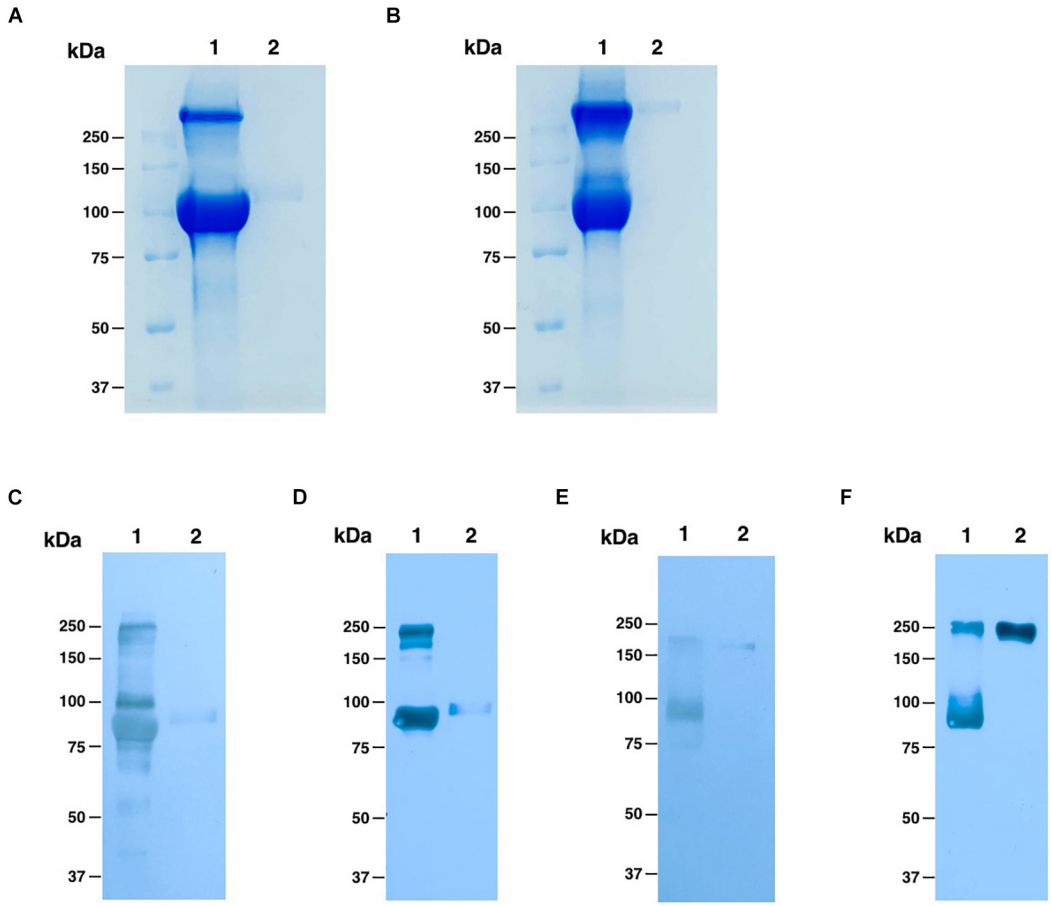

FIGURE 3 | Analysis of purified plant-produced ACE2-Fc (lane 1) and commercial HEK293-produced ACE2-Fc (lane 2). Coomassie-stained SDS-PAGE under reducing **(A)** and non-reducing conditions **(B)**. Western blotting analysis under reducing condition with detection using a rabbit anti-ACE2 antibody **(C)** and an anti-human gamma-HRP conjugated antibody **(D)**. Western blotting analysis under non-reducing condition probed with a rabbit anti-ACE2 antibody **(E)** and an anti-human gamma-HRP conjugated antibody **(F)**.

cells were incubated with a 1:500 dilution of an Alexa Fluor 488 conjugated goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody. Nuclei of the cells were stained with Hoechst dye (Thermo Fisher Scientific, United States). The fluorescent signals were detected and analyzed using a high-content imaging system (PerkinElmer, United Kingdom) at 40x magnitude. The percentage of infected cells in each well was automatically obtained randomly from 13 images per well using Harmony software (PerkinElmer, United Kingdom).

RESULTS

Transient Expression of ACE2-Fc Fusion Protein in *N. benthamiana* Plants

The ACE2-Fc fusion protein gene was designed using codon preferred for *N. benthamiana* and commercially synthesized. We generated the ACE2-Fc fusion protein by fusing the ACE2 protein to the N terminus of the Fc region. To produce the ACE2-Fc fusion protein in plants, the ACE2-Fc gene was incorporated into the pBYR2e geminiviral vector (Chen et al., 2011; Rattanapisit et al., 2019a,b) and subsequently introduced into *N. benthamiana* plants through agroinfiltration. The expression of the ACE2-Fc fusion protein induced mild necrosis in leaves (**Figure 2A**). The protein was expressed highest on day 6 post-infiltration, with up to 100 μg/g leaf fresh weight (**Figure 2B**).

Purification of ACE2-Fc Fusion Protein From *N. benthamiana* Leaves

To purify the ACE2-Fc fusion protein, we used one-step protein A affinity chromatography. We estimate that the plant-produced ACE2-Fc fusion protein was ∼90% pure based on visual inspection of a Coomassie blue stained gel, with a molecular weight of around 100 kDa under reducing condition, which had no different molecular weight comparing to commercial HEK293-prduced ACE2-Fc protein (**Figure 3A**), while we could observe the protein dimer of both ACE2-Fc fusion proteins under non-reducing condition, which showed the protein size at 250 kDa (**Figure 3B**). The folding of plant-produced ACE2-Fc fusion protein was confirmed by western blot analysis using

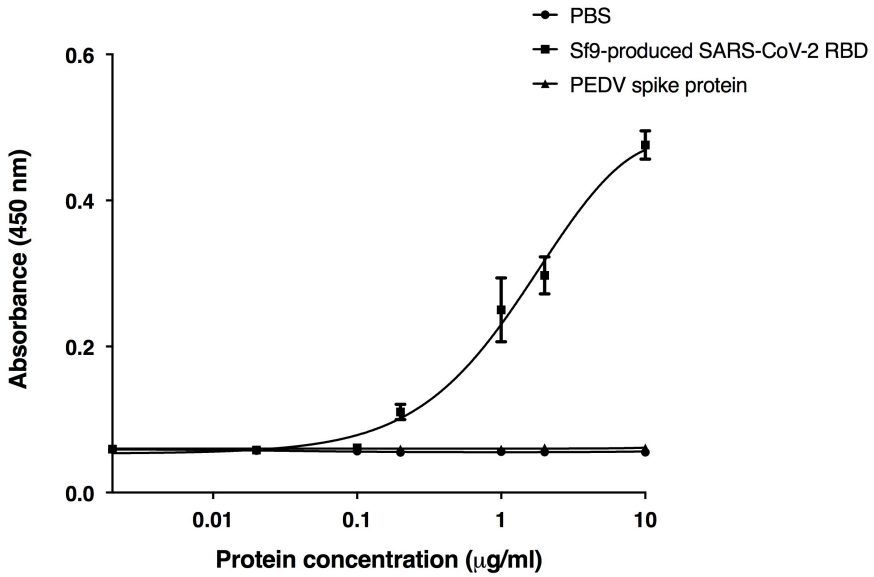

FIGURE 4 | Binding activity of the plant-produced ACE2-Fc with the commercial receptor binding domain of SARS-CoV-2 (SARS-CoV-2 RBD) from Sf9 cells was analyzed by ELISA. PBS buffer and S1 protein of PEDV were used as negative controls. Data are presented as mean ± SD of triplicates.

ACE2-specific and Fc domain-specific antibodies. The results indicated that purified plant-produced ACE2-Fc proteins showed two major bands at the molecular weight of approximately 100 and 250 kDa under reducing (**Figures 3C,D**) and non-reducing condition (**Figures 3E,F**), respectively, which were same as the profiles on Coomassie blue stained gel.

Binding of ACE2-Fc With RBD of SARS-CoV-2

The receptor binding domain (RBD) of SARS-CoV-2 was previously shown to bind to the human ACE2 receptor on host cells (Rabi et al., 2020; Shanmugaraj et al., 2020b; Yuki et al., 2020; Zhou et al., 2020a). We used RBD binding assay to confirm the *in vitro* binding activity of the plant-produced ACE2-Fc fusion protein. The purified ACE2-Fc fusion protein was immobilized in the wells of a microtiter plate. Eight different dilutions of the commercial Sf9-produced RBD protein of SARS-CoV-2, the S1 protein of the porcine epidemic diarrhea virus (PEDV), and PBS were added into the ELISA plate with triplicate wells. The results (**Figure 4**) showed that the plant-produced ACE2-Fc fusion protein produced substantially high OD signals with the RBD of SARS-CoV-2, compared to the negative PBS control and the PEDV S1 protein. Our data are consistent with the binding of the RBD of SARS-CoV-2 to the plant-produced ACE2-Fc fusion protein.

Anti-SARS-CoV-2 Activity of the Plant-Produced ACE2-Fc Fusion Protein

The plant-produced ACE2-Fc fusion protein was tested for anti-SARS-CoV-2 activity at the pre- and post-entry phases. For the pre-entry treatment, the protein was pre-incubated with SARS-CoV-2 at 37°C for 1 h before inoculation onto Vero cells. Viral adsorption was undertaken for 2 h in the presence of the protein before removing any unbound virus. The cells were cultured for 48 h before harvesting for analysis (**Figure 5A**). The ACE2-Fc fusion protein in pre-entry treatment showed lower efficiency of SARS-CoV-2 inhibition in Vero cells (**Figures 5B,C**). The IC_{50} of the plant-produced ACE2-Fc fusion protein for the pre-entry treatment was measured by the percentage of SARS-CoV-2 inhibition curve with 94.66 μg/ml (**Figure 5D**). For the post-entry treatment, Vero cells were inoculated with SARS-CoV-2 for 2 h. After washing, the protein was added and cells were incubated for 48 h before harvesting for analysis (**Figure 6A**). The results showed that the post-treatment inhibited SARS-CoV-2 infection at the concentration starting with 1 μg/ml (**Figures 6B,C**). The IC_{50} for post-entry treatment was 0.84 μg/ml (**Figure 6D**). The serum from COVID-19 patient and an anti-human IgG were used as the positive and negative controls, respectively, for both pre- and post-treatment experiments.

DISCUSSION

The COVID-19 outbreak caused by the novel betacoronavirus SARS-CoV-2 is responsible for an ongoing pandemic which is having an unprecedented impact on the human population with millions of infected cases. The virus has spread rapidly through human-to-human transmission and has affected numerous countries around the world and has emerged as a significant threat to public health, the global economy and society (Shanmugaraj et al., 2020b).

The World Health Organization (WHO) has declared COVID-19 as pandemic and a public health emergency of international concern (World Health Organization, 2020a).

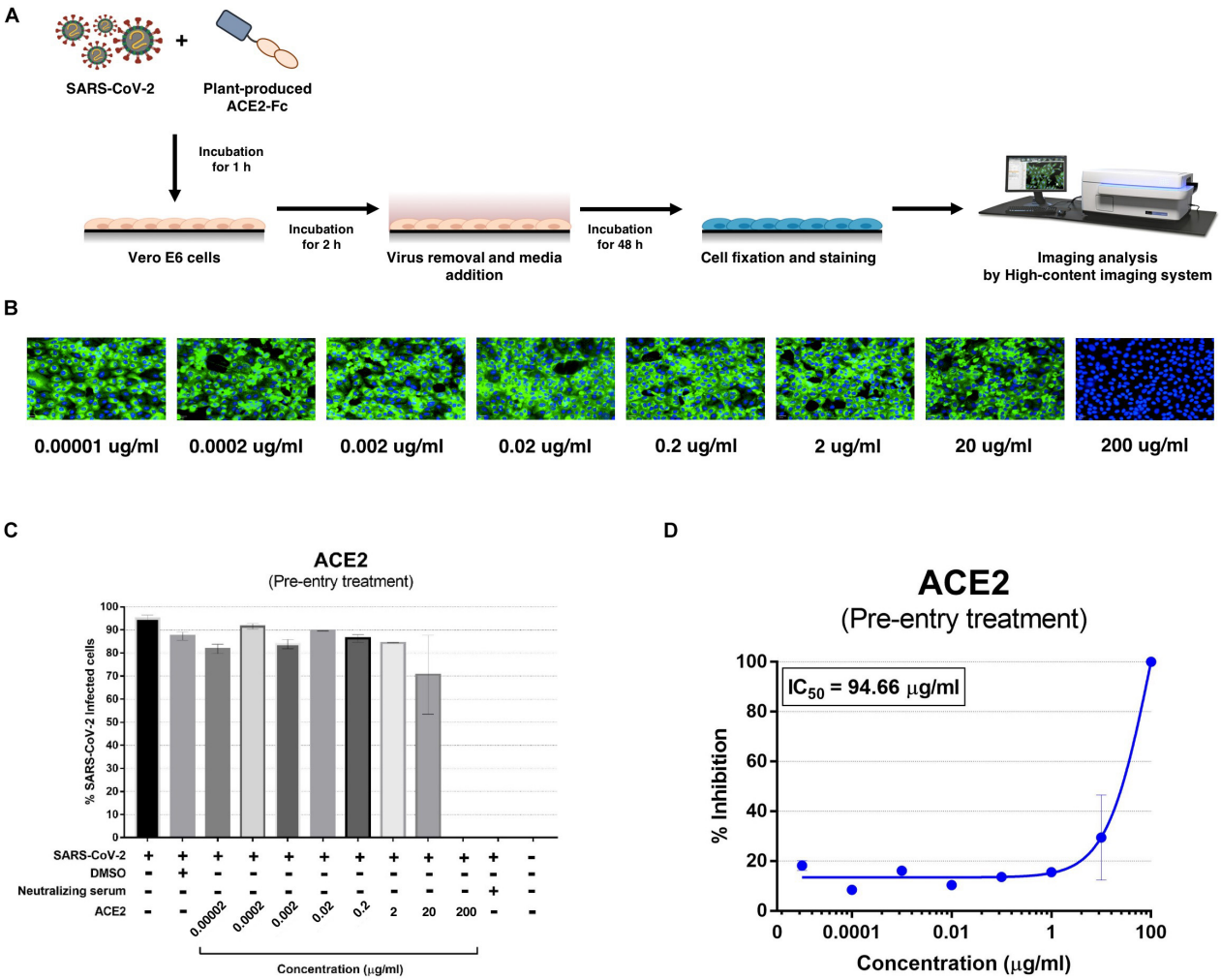

FIGURE 5 | Dose-dependent effect of plant-produced ACE2-Fc on SARS-CoV-2 inhibition and neutralization at the pre-infection phase. Experimental design of plant-produced ACE2-Fc and SARS-CoV-2 mixture added to Vero E6 cells (at 25$TCID_{50}$) **(A)**. SARS-CoV-2 infection profiles in Vero E6 cells which were treated with eight concentrations of plant-produced ACE2-Fc **(B)**. Percentage of SARS-CoV-2 inhibition in Vero E6 cells, which were treated with eight concentrations of plant-produced ACE2-Fc starting with 200 μg/ml **(C)**. Efficacy of SARS-CoV-2 inhibition in Vero E6 cells, which were treated by eight concentrations of plant-produced ACE2-Fc **(D)**. The data were showed as mean ± SD of triplicates in individual concentrations.

Although COVID-19 pathogenesis has still not been completely elucidated, early reports showed that SARS-CoV-2 binds to the host cell receptor angiotensin-converting enzyme-2 (ACE2) through the RBD domain in the spike (S) protein to infect human epithelial cells in alveoli, which can cause a cytokine storm resulting in respiratory failure and ultimately death. The virus can infect cells of the lungs, kidneys, heart and intestine, resulting in organ damage leading to multiple organ dysfunction syndrome (MODS) (Sun et al., 2020; Xie et al., 2020). Currently there are no approved antiviral drugs or vaccines for COVID-19. Reducing the mortality rate amongst COVID-19 infected patients is a primary goal, as is controlling the rapid spread of the infection by developing therapeutic and preventive strategies.

Recently, rapid progress has been made with diagnostic kits/reagents, drug repurposing, immunotherapeutic strategies and vaccine development in response to the COVID-19 pandemic. The scientific communities in almost all countries are in the race to develop an effective and safe vaccine against SARS-CoV-2. Many molecular targets are considered as potential candidates to combat COVID-19 (Le et al., 2020) including recombinant ACE2 as it has shown to have therapeutic potential for SARS-CoV, and is known to protect against severe acute lung injury (Huentelman et al., 2005; Imai et al., 2005; Kuba et al., 2005; Zou et al., 2014). ACE2 is the *in vivo* SARS-CoV-2 functional receptor expressed by epithelial cells of many organs such as the lung, intestine, kidney, and blood vessels (Hamming et al., 2004). Recombinant ACE2 (rACE2) was reported to have a short half-life and fast clearance rate in contrast to a rACE2 with a Fc fusion protein (rACE2-Fc) (Liu et al., 2018). Lei et al. (2020) showed that the ACE2-Fc fusion protein can able to neutralize both SARS-CoV and a SARS-CoV-2 pseudovirus *in vitro* (Lei et al., 2020).

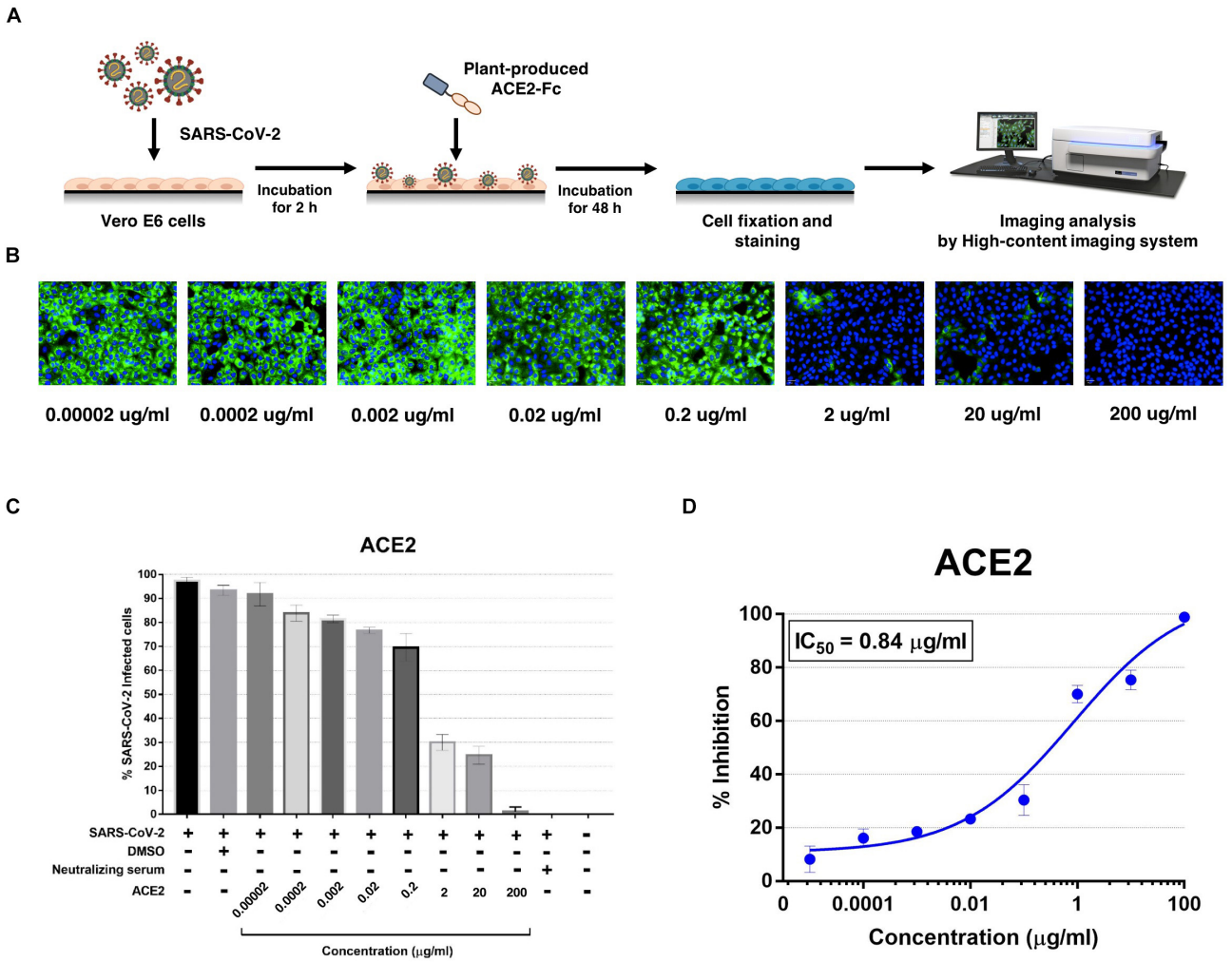

FIGURE 6 | Dose-dependent effect of plant-produced ACE2-Fc on SARS-CoV-2 inhibition and neutralization at the post-infection phase. Experimental design of plant-produced ACE2-Fc and SARS-CoV-2 mixture added to Vero E6 cells (at 25$TCID_{50}$) **(A)**. SARS-CoV-2 infection profiles in Vero E6 cells which were treated with eight concentrations of plant-produced ACE2-Fc **(B)**. Percentage of SARS-CoV-2 inhibition in Vero E6 cells, which were treated with eight concentrations of plant-produced ACE2-Fc starting with 200 μg/ml **(C)**. Efficacy of SARS-CoV-2 inhibition in Vero E6 cells, which were treated by eight concentrations of plant-produced ACE2-Fc **(D)**. The data were showed as mean ± SD of triplicates in individual concentrations.

In recent years, plants have been utilized for the production of recombinant biopharmaceuticals and vaccine candidates for several human and veterinary diseases. Many reports have shown the potential of plant expression systems for the rapid production of biopharmaceuticals and proven that plant produced recombinant proteins are as effective as the mammalian cell-produced counterparts in producing neutralizing antibodies against a particular pathogen or infection (Koya et al., 2005; Thanavala et al., 2005; Huang et al., 2008; Kanagarajan et al., 2012; Dolleweerd et al., 2014; Vamvaka et al., 2016; Shanmugaraj et al., 2020a). Moreover, plant biopharming provides the economical production of desirable biopharmaceuticals without high investment costs compared to other available industrial facilities using a fermentation system (Twyman et al., 2003; Ko and Koprowski, 2005; Basaran and Rodríguez-Cerezo, 2008; Shinmyo and Kato, 2010; Fischer et al., 2013; Stoger et al., 2014; Yao et al., 2015; Burnett and Burnett, 2019; Margolin et al., 2020). The production costs in plant biopharming processes are commonly 0.1% of mammalian cell-based technologies and 2–10% of bacterial expression systems (Yao et al., 2015). Due to the fact that plant-made biopharmaceuticals provide a cost-effective alternative to protect against emerging infectious diseases, in this study, we demonstrated the feasibility of using a plant expression system to transiently express an ACE2-Fc fusion protein that could be useful to develop affordable antiviral treatment against SARS-CoV-2. The biological activity of the plant produced ACE2-Fc fusion protein was characterized *in vitro*.

The ACE2-Fc fusion protein was codon optimized and cloned into the geminiviral vector pBYR2e for plant expression, and the recombinant fusion protein was transiently expressed in

N. benthamiana plants. Our results showed that the ACE2-Fc fusion protein can be produced in a large scale in *N. benthamiana* in less than 10 days after the construction of the plant expression vector. Recent advancements in plant protein production strategies through using deconstructed viral vector systems and transient expression has reduced the protein production timeline in contrast to stable expression systems which requires several months for recombinant protein production. The safety, scalability and robustness of the plant transient expression system have proved the commercial viability of the system (Sainsbury and Lomonossoff, 2014; Canto, 2016; Park and Wi, 2016). We showed that transiently expressing an ACE2-Fc fusion protein by using a geminiviral vector produced yields of up to 100 μg/g leaf fresh weight in *N. benthamiana* leaves with one-step protein A affinity chromatography. However, additional purification is required in order to meet the quality standards for commercial use. We then investigated the biological activity of the plant-derived ACE2-Fc fusion protein *in vitro*. The plant-produced ACE2-Fc fusion protein exhibits potent binding to the Sf9-produced RBD protein of SARS-CoV-2. The results from the antiviral assay demonstrated a potent inhibitory effect of the ACE2-Fc fusion protein against SARS-CoV-2. The IC_{50} of ACE2-Fc fusion protein was 94.66 and 0.84 μg/ml for the pre-entry and post-infection, respectively. The ACE2-Fc fusion protein showed better inhibition in the post-infection treatment (**Figure 6**), as compared to the pre-entry treatment (**Figure 5**).

ACE2 is found in many organs and ACE2 variants are reported to protect from acute respiratory distress syndrome (Imai et al., 2008) and kidney disease (Wysocki et al., 2010) by acting as a negative regulator of the renin angiotensin system (Kuba et al., 2010). Recently, ACE2 has been the focus of a rational therapeutic design against COVID pandemic. ACE2 was reported as the major receptor for SARS and SARS-CoV-2 wherein the virus binds to the ACE2 cell receptor and enters host cells resulting in severe lung injuries. Earlier reports have shown the therapeutic potential of rACE2 for SARS-CoV (Kuba et al., 2005) and its ability to protect from severe acute lung failure (Gu et al., 2016). Apeiron biologics is currently running a pilot human trial in China to investigate the potential of their drug candidate APN01, recombinant human ACE2 for use as a therapeutic agent for the treatment of COVID-19. Here we have shown for the first time that a plant-produced ACE2-Fc fusion protein has the potential to be developed as a therapeutic agent, alone or in combination with other therapeutic agents or with vaccines for the treatment of COVID-19.

CONCLUSION

The rapid global spread of SARS-CoV-2 emphasizes the urgent need for the development of effective vaccines and therapeutics that are affordable and scalable. Although studies have shown structural similarities between the RBD of SARS-CoV and SARS-CoV-2, known neutralizing monoclonal antibodies against SARS-CoV might not neutralize SARS-CoV-2 (Rattanapisit et al., 2020; Tian et al., 2020). Hence, it is necessary to develop specific vaccines or treatment strategies to treat SARS-CoV-2 infection. Here we showed that a plant expression system can rapidly and effectively produce a functional ACE2-Fc fusion protein on a large scale. Moreover, the plant produced ACE2-Fc fusion protein exhibits anti-SARS-CoV-2 activity in post-entry treatment *in vitro* suggesting it could be used as post-exposure therapeutic to treat COVID-19. However, further progress toward the goal of establishing an affordable therapeutic intervention program requires animal studies to confirm the efficacy and safety of the plant produced protein against SARS-CoV-2.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS, AT, and WP designed all experiments. KS, KR, and BS performed protein expression, protein purification, and ELISA. SM, PK, PB, and SB performed the anti-viral assay. All authors analyzed the data and contributed to manuscript preparation.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.604663/full#supplementary-material

REFERENCES

Amanat, F., and Krammer, F. (2020). SARS-CoV-2 Vaccines: status report. *Immunity* 52, 583–589. doi: 10.1016/j.immuni.2020.03.007

Basaran, P., and Rodríguez-Cerezo, E. (2008). Plant molecular farming: opportunities and challenges. *Crit. Rev. Biotechnol.* 28, 153–172. doi: 10.1080/07388550802046624

Burnett, M. J. B., and Burnett, A. C. (2019). Therapeutic recombinant protein production in plants: challenges and opportunities. *Plants People Planet* 2, 121–132. doi: 10.1002/ppp3.10073

Canto, T. (2016). Transient expression systems in plants: potentialities and constraints. *Adv. Exp. Med. Biol.* 896, 287–301. doi: 10.1007/978-3-319-27216-0_18

Carter, P. J. (2011). Introduction to current and future protein therapeutics: a protein engineering perspective. *Exp. Cell Res.* 317, 1261–1269. doi: 10.1016/j.yexcr.2011.02.013

Chan, H. T., Xiao, Y., Weldon, W. C., Oberste, S. M., Chumakov, K., and Daniell, H. (2016). Cold chain and virus-free chloroplast-made booster vaccine to confer immunity against different poliovirus serotypes. *Plant Biotechnol. J.* 14, 2190–2200. doi: 10.1111/pbi.12575

Chen, Q., He, J., Phoolcharoen, W., and Mason, H. S. (2011). Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Hum. Vaccin.* 7, 331–338. doi: 10.4161/hv.7.3.14262

Ciotti, M., Angeletti, S., Minieri, M., Giovannetti, M., Benvenuto, D., Pascarella, S., et al. (2020). COVID-19 Outbreak: an overview. *J. Chemother.* 64, 215–223.

Cox, G. N., Smith, D. J., Carlson, S. J., Bendele, A. M., Chlipala, E. A., and Doherty, D. H. (2004). Enhanced circulating half-life and hematopoietic properties of a human granulocyte colony-stimulating factor/immunoglobulin fusion protein. *Exp. Hematol.* 32, 441–449. doi: 10.1016/j.exphem.2004.01.012

Czajkowsky, D. M., Hu, J., Shao, Z., and Pleass, R. J. (2012). Fc-fusion proteins: new developments and future perspectives. *EMBO Mol. Med.* 4, 1015–1028. doi: 10.1002/emmm.201201379

Dolleweerd, C. J. V., Teh, A. Y.-H., Banyard, A. C., Both, L., Lotter-Stark, H. C. T., Tsekoa, T., et al. (2014). Engineering, expression in transgenic plants and characterisation of E559, a rabies virus-neutralising monoclonal antibody. *J. Infect. Dis.* 210, 200–208. doi: 10.1093/infdis/jiu085

Fehr, A. R., Channapannavar, R., and Perlman, S. (2017). Middle East respiratory syndrome (MERS): emergence of a pathogenic human Coronavirus. *Annu. Rev. Med.* 68, 387–399.

Fischer, R., Schillberg, S., Buyel, J. F., and Twyman, R. M. (2013). Commercial aspects of pharmaceutical protein production in plants. *Curr. Pharm. Des.* 19, 5471–5477. doi: 10.2174/1381612811319310002

Gu, H., Xie, Z., Li, T., Zhang, S., Lai, C., Zhu, P., et al. (2016). Angiotensin-converting enzyme 2 inhibits lung injury induced by respiratory syncytial virus. *Sci. Rep.* 6:19840.

Hamming, I., Timens, W., Bulthuis, M., Lely, A., Navis, G., and Goor, H. V. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. a first step in understanding SARS pathogenesis. *J. Pathol.* 203, 631–637. doi: 10.1002/path.1570

Han, Q., Lin, Q., Jin, S., and You, L. (2020). Coronavirus 2019-nCoV: a brief perspective from the front line. *J. Infect.* 80, 373–377. doi: 10.1016/j.jinf.2020.02.010

Hofmann, H., Pyrc, K., Hoek, L. V. D., Geier, M., Berkhout, B., and Hlmann, S. P. (2005). Human coronavirus NL63 employs the Severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc. Natl. Acad. Sci. U. S. A.* 102, 7988–7993. doi: 10.1073/pnas.0409465102

Huang, Z., LePore, K., Elkin, G., Thanavala, Y., and Mason, H. S. (2008). High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression system. *Plant Biotechnol. J.* 6, 202–209. doi: 10.1111/j.1467-7652.2007.00316.x

Huentelman, M. J., Grobe, J. L., Vazquez, J., Stewart, J. M., Mecca, A. P., Katovich, M. J., et al. (2005). Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. *Exp. Physiol.* 90, 783–790. doi: 10.1113/expphysiol.2005.031096

Imai, Y., Kuba, K., and Penninger, J. M. (2008). Lessons from SARS: a new potential therapy for acute respiratory distress syndrome (ARDS) with angiotensin converting enzyme 2 (ACE2). *Masui* 57:3.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., et al. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 436, 112–116. doi: 10.1038/nature03712

Kanagarajan, S., Tolf, C., Lundgren, A., Waldenström, J., and Brodelius, P. E. (2012). Transient expression of hemagglutinin antigen from low pathogenic avian influenza A (H7N7) in *Nicotiana benthamiana*. *PLoS One* 7:e33010. doi: 10.1371/journal.pone.0033010

Ko, K., and Koprowski, H. (2005). Plant biopharming of monoclonal antibodies. *Virus Res.* 111, 93–100. doi: 10.1016/j.virusres.2005.03.016

Komarova, T. V., Baschieri, S., Donini, M., Marusic, C., Benvenuto, E., and Dorokhov, Y. L. (2010). Transient expression systems for plant-derived biopharmaceuticals. *Expert. Rev. Vaccines* 9, 859–876. doi: 10.1586/erv.10.85

Koya, V., Moayeri, M., Leppla, S. H., and Daniell, H. (2005). Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. *Infect. Immun.* 73, 8266–8274. doi: 10.1128/iai.73.12.8266-8274.2005

Kruse, R. L. (2020). Therapeutic strategies in an outbreak scenario to treat the novel coronavirus originating in Wuhan, China. *F1000Res* 9:72. doi: 10.12688/f1000research.22211.2

Kuba, K., Imai, Y., Ohto-Nakanishi, T., and Penninger, J. M. (2010). Trilogy of ACE2: a peptidase in the renin-angiotensin system, a SARS receptor, and a partner for amino acid transporters. *Pharmaco. Ther.* 128, 119–128. doi: 10.1016/j.pharmthera.2010.06.003

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., et al. (2005). A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat. Med.* 11, 875–879. doi: 10.1038/nm1267

Lagassé, H. A. D., Alexaki, A., Simhadri, V. L., Katagiri, N. H., Jankowski, W., Sauna, Z. E., et al. (2017). Recent advances in (therapeutic protein) drug development. *F1000Res* 6:113. doi: 10.12688/f1000research.9970.1

Le, T. T., Andreadakis, Z., Kumar, A., Román, R. G., Tollefsen, S., Saville, M., et al. (2020). The COVID-19 vaccine development landscape. *Nat. Rev. Drug Discov.* 19, 305–306. doi: 10.1038/d41573-020-00073-5

Lei, C., Qian, K., Li, T., Zhang, S., Fu, W., Ding, M., et al. (2020). Neutralization of SARS-CoV-2 spike pseudotyped virus by recombinant ACE2-Ig. *Nat. Commun.* 11:2070. doi: 10.1038/s41467-020-16048-4

Li, F., Li, W., Farzan, M., and Harrison, S. C. (2005). Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 309, 1864–1868. doi: 10.1126/science.1116480

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., et al. (2020). Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. *N. Engl. J. Med.* 382, 1199–1207. doi: 10.1056/nejmoa2001316

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454.

Liu, P., Wysocki, J., Souma, T., Ye, M., Ramirez, V., Zhou, B., et al. (2018). Novel ACE2-Fc chimeric fusion provides long-lasting hypertension control and organ protection in mouse models of systemic renin angiotensin system activation. *Kidney Int.* 94, 114–125. doi: 10.1016/j.kint.2018.01.029

Lupia, T., Scabini, S., Pinna, S. M., Perri, G. D., Rosa, F. G. D., and Corcione, S. (2020). 2019 novel coronavirus (2019-nCoV) outbreak: a new challenge. *J. Glob. Antimicrob. Resist.* 21, 22–27. doi: 10.1016/j.jgar.2020.02.021

Malik, Y. A. (2020). Properties of coronavirus and SARS-CoV-2. *Malays. J. Pathol.* 42, 3–11.

Margolin, E. A., Strasser, R., Chapman, R., Williamson, A.-L., Rybicki, E. P., and Meyers, A. E. (2020). Engineering the plant secretory pathway for the production of next-generation pharmaceuticals. *Trends Biotechnol.* 38, 1034–1044. doi: 10.1016/j.tibtech.2020.03.004

Masters, P. S. (2006). The molecular biology of coronaviruses. *Adv. Virus Res.* 66, 193–292. doi: 10.1016/s0065-3527(06)66005-3

Park, K. Y., and Wi, S. J. (2016). Potential of plants to produce recombinant protein products. *J. Plant Biol.* 59, 559–568. doi: 10.1007/s12374-016-0482-9

Park, Y., Lee, S., Kang, H., Park, M., Min, K., Kim, N. H., et al. (2020). A classical swine fever virus E2 fusion protein produced in plants elicits a neutralizing humoral immune response in mice and pigs. *Biotechnol. Lett.* 42, 1247–1261. doi: 10.1007/s10529-020-02892-3

Peters, R. T., Low, S. C., Kamphaus, G. D., Dumont, J. A., Amari, J. V., Lu, Q., et al. (2010). Prolonged activity of factor IX as a monomeric Fc fusion protein. *Blood* 115, 2057–2064. doi: 10.1182/blood-2009-08-239665

Phoolcharoen, W., Bhoo, S. H., Lai, H., Ma, J., Arntzen, C. J., Chen, Q., et al. (2011). Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*. *Plant Biotechnol. J.* 9, 807–816. doi: 10.1111/j.1467-7652.2011.00593.x

Powell, J. S., Josephson, N. C., Quon, D., Ragni, M. V., Cheng, G., Li, E., et al. (2012). Safety and prolonged activity of recombinant factor VIII Fc fusion protein in hemophilia A patients. *Blood* 119, 3031–3037. doi: 10.1182/blood-2011-09-382846

Quinlan, B. D., Mou, H., Zhang, L., Guo, Y., He, W., Ojha, A., et al. (2020). The SARS-CoV-2 receptor-binding domain elicits a potent neutralizing response without antibody-dependent enhancement. *bioRxiv* [Preprint] doi: 10.1101/2020.04.10.036418

Rabi, F. A., Zoubi, M. S. A., Kasasbeh, G. A., Salameh, D. M., and Al-Nasser, A. D. (2020). SARS-CoV-2 and coronavirus disease 2019: what we know so far. *Pathogens* 9:231. doi: 10.3390/pathogens9030231

Rattanapisit, K., Chao, Z., Siriwattananon, K., Huang, Z., and Phoolcharoen, W. (2019a). Plant-produced anti-enterovirus 71 (EV71) monoclonal antibody efficiently protects mice against EV71 infection. *Plants (Basel)* 8:12. doi: 10.3390/plants8120560

Rattanapisit, K., Phakham, T., Buranapraditkun, S., Siriwattananon, K., Boonkrai, C., Pisitkun, T., et al. (2019b). Structural and *in vitro* functional analyses of novel plant-produced anti-Human PD1 antibody. *Sci. Rep.* 9:15205.

Rattanapisit, K., Shanmugaraj, B., Manopwisedjaroen, S., Purwono, P. B., Siriwattananon, K., Khorattanakulchai, N., et al. (2020). Rapid and scalable production of functional anti-coronavirus monoclonal antibody CR3022 in plants. *Sci. Rep.* 10:17698. doi: 10.21203/rs.3.rs-27160/v1

Rattanapisit, K., Srifa, S., Kaewpungsup, P., Pavasant, P., and Phoolcharoen, W. (2019c). Plant-produced recombinant Osteopontin-Fc fusion protein enhanced osteogenesis. *Biotechnol. Rep. (Amst)* 21:e00312. doi: 10.1016/j.btre.2019.e00312

Rosales-Mendoza, S., Nieto-Gómez, R., and Angulo, C. (2017). A perspective on the development of plant-made vaccines in the fight against Ebola virus. *Front. Immunol.* 8:252. doi: 10.3389/fimmu.2017.00252

Sainsbury, F., and Lomonossoff, G. P. (2014). Transient expressions of synthetic biology in plants. *Curr. Opin. Plant Biol.* 19, 1–7. doi: 10.1016/j.pbi.2014.02.003

Shanmugaraj, B., Malla, A., and Phoolcharoen, W. (2020a). Emergence of novel Coronavirus 2019-nCoV: need for rapid vaccine and biologics development. *Pathogens* 9:148. doi: 10.3390/pathogens9020148

Shanmugaraj, B., Siriwattananon, K., Wangkanont, K., and Phoolcharoen, W. (2020b). Perspectives on monoclonal antibody therapy as potential therapeutic intervention for Coronavirus disease-19 (COVID-19). *Asian Pac. J. Allergy Immunol.* 38, 10–18. doi: 10.12932/ap-200220-0773

Shanmugaraj, B. M., and Ramalingam, S. (2014). Plant expression platform for the production of recombinant pharmaceutical proteins. *Austin J. Biotechnol. Bioeng.* 1:4.

Shinmyo, A., and Kato, K. (2010). Molecular farming: production of drugs and vaccines in higher plants. *J. Antibiot. (Tokyo)* 63, 431–433. doi: 10.1038/ja.2010.63

Stoger, E., Fischer, R., Moloney, M., and Ma, J. K.-C. (2014). Plant molecular pharming for the treatment of chronic and infectious diseases. *Annu. Rev. Plant Biol.* 65, 743–768. doi: 10.1146/annurev-arplant-050213-035850

Streatfield, S. J., Kushnir, N., and Yusibov, V. (2015). Plant-produced candidate countermeasures against emerging and reemerging infections and bioterror agents. *Plant Biotechnol. J.* 13, 1136–1159. doi: 10.1111/pbi.12475

Sun, X., Wang, T., Cai, D., Hu, Z., Chen, J. A., Liao, H., et al. (2020). Cytokine storm intervention in the early stages of COVID-19 pneumonia. *Cytokine Growth Factor Rev.* 53, 38–42. doi: 10.1016/j.cytogfr.2020.04.002

Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, T., Kawanishi, T., et al. (2010). Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. *J. Immunol.* 184, 1968–1976. doi: 10.4049/jimmunol.0903296

Teh, A. Y. H., Maresch, D., Klein, K., and Ma, J. K. C. (2014). Characterization of VRC01, a potent and broadly neutralizing anti-HIV mAb, produced in transiently and stably transformed tobacco. *Plant Biotechnol. J.* 12, 300–311. doi: 10.1111/pbi.12137

Thanavala, Y., Mahoney, M., Pal, S., Scott, A., Richter, L., Natarajan, N., et al. (2005). Immunogenicity in humans of an edible vaccine for hepatitis B. *Proc. Natl. Acad. Sci. U. S. A.* 102, 3378–3382. doi: 10.1073/pnas.0409899102

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., et al. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg. Microbes Infect.* 9, 382–385. doi: 10.1080/22221751.2020.1729069

Twyman, R. M., Stoger, E., Schillberg, S., Christou, P., and Fischer, R. (2003). Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* 21, 570–578. doi: 10.1016/j.tibtech.2003.10.002

Vamvaka, E., Twyman, R. M., Murad, A. M., Melnik, S., Teh, A. Y.-H., Arcalis, E., et al. (2016). Rice endosperm produces an underglycosylated and potent form of the HIV-neutralizing monoclonal antibody 2G12. *Plant Biotechnol. J.* 14, 97–108. doi: 10.1111/pbi.12360

Wong, S. K., Li, W., Moore, M. J., Choe, H., and Farzan, M. (2004). A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J. Biol. Chem.* 279, 3197–3201. doi: 10.1074/jbc.c300520200

World Health Organization. (2020a). *Statement on The Second Meeting of The International Health Regulations Emergency Committee Regarding The Outbreak of Novel Coronavirus (2019-nCoV)* [Online]. Available online at: https://www.who.int/news-room/detail/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov) (accessed 10 February 2020)

World Health Organization. (2020b). *Weekly Epidemiological Update–17 November 2020* [Online]. Available online at: https://www.who.int/publications/m/item/weekly-epidemiological-update---17-november-2020 (accessed 18 Nov 2020)

Wysocki, J., Ye, M., Rodriguez, E., González-Pacheco, F. R., Barrios, C., Evora, K., et al. (2010). Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2: prevention of angiotensin II-dependent hypertension. *Hypertension* 55, 90–98. doi: 10.1161/hypertensionaha.109.138420

Xie, P., Ma, W., Tang, H., and Liu, D. (2020). Severe COVID-19: a review of recent progress with a look toward the future. *Front. Public Health* 8:189.

Yao, J., Weng, Y., Dickey, A., and Wang, K. Y. (2015). Plants as factories for human pharmaceuticals: applications and challenges. *Int. J. Mol. Sci.* 16, 28549–28565. doi: 10.3390/ijms161226122

Yuki, K., Fujiogi, M., and Koutsogiannaki, S. (2020). COVID-19 pathophysiology: a review. *Clin. Immunol.* 215:108427. doi: 10.1016/j.clim.2020.108427

Zhang, H., Penninger, J. M., Li, Y., Zhong, N., and Slutsky, A. S. (2020). Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intensive Care Med.* 46, 586–590. doi: 10.1007/s00134-020-05985-9

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020a). Discovery of a novel coronavirus associated with the recent pneumonia outbreak in humans and its potential bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020b). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273.

Zou, Z., Yan, Y., Shu, Y., Gao, R., Sun, Y., Li, X., et al. (2014). Angiotensin-converting enzyme 2 protects from lethal avian influenza A H5N1 infections. *Nat. Commun.* 5:3594. doi: 10.1038/ncomms4594

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REVIEW
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Carvacrol, a Plant Metabolite Targeting Viral Protease (M^{pro}) and ACE2 in Host Cells Can Be a Possible Candidate for COVID-19

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The recent outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) started in December 2019, resulting in the coronavirus disease-19 (COVID-19) pandemic. Coronaviruses are solely accountable for rising mortality and socioeconomic saddles. Presently, there are few repurposed drugs such as remdesivir or favipiravir approved for the treatment of COVID-19, although vaccines and plasma therapy is also subject to emergency approval. However, some potential natural treatments and cures have also been proposed. Molecules of natural origin showed therapeutic importance such as antiviral, anti-inflammatory, and antioxidant activity, and could be useful drug candidates for treating COVID-19. In recent years, essential oils have shown promising therapeutic effects against many viral diseases. Carvacrol is one of the monoterpene phenol with abundant presence in essential oils of many aromatic plants, including thyme and oregano. It is being used as food flavoring, additive, and preservatives. Carvacrol is also used as a fragrance in cosmetic products. A number of research studies have shown biological actions of carvacrol with its therapeutic potential is of clinical significance. The *in vitro* and *in vivo* studies have shown multiple pharmacological properties such as anticancer, anti-fungal, anti-bacterial, anti-oxidant, anti-inflammatory, vasorelaxant, hepatoprotective, and spasmolytic. This review highlights the various biological and pharmacological properties of carvacrol within the scope of COVID-19.

Keywords: carvacrol, terpenoid, M^{pro} binding, ACE2 receptor, SARS-CoV-2

INTRODUCTION

The novel virus strain, SARS-CoV-2 is responsible for the recent outbreak of respiratory infectious disease known as "coronavirus disease 19" (COVID 19) (Boopathi et al., 2020; Hemida and Ba Abduallah, 2020). Globally, the health and healthcare systems are under serious threat due to this major outbreak (Lu et al., 2020; Zhu et al., 2020). The pandemic has affected millions of individuals because of compulsory quarantine and isolation, and also devastated the healthcare facilities.

The pandemic have a severe impact on the world economy and will continue to impact if the spread of this novel virus is not stopped or a valuable treatment is not discovered. Coronaviruses are transmitted to humans through respiratory droplets and are single-stranded positive-sense RNA viruses. Patients infected with SARS-CoV-2 display many symptoms including fever, dry cough, diarrhea, loss of smell, and complications mainly beginning with acute respiratory distress following a rapid and robust rise in the levels of proinflammatory cytokines (Das et al., 2020; Jean et al., 2020; Zhang et al., 2020). The morphology of the coronavirus consists of transmembrane spike glycoproteins (S protein), which project outside from the surface of the virus (Tortorici and Veesler, 2019). SARS-CoV and SARS-CoV-2 both display structural morphology in their S proteins and preserved ectodomains. In light of previous studies, the binding inhibition of SARS-CoV to its host cell receptor angiotensin-converting enzyme-2 (ACE2) appears most relevant, as the cellular entry of SARS-CoV-2 also requires ACE2 (Hoffmann et al., 2020; Walls et al., 2020). The epithelial cells of the respiratory tract express ACE2, an exopeptidase, which may provide a potentially viable pharmacological approach to control the cell entry of SARS-CoV-2. Infections with SARS-CoV-2 may affect the gastrointestinal tract, central nervous system, kidney, liver, and heart (Tang et al., 2020). In comparison to SARS-CoV or MERS-CoV, SARS-CoV-2 is highly infectious and communicable (Baez-Santos et al., 2015).

In studies on the replication and infection processes of SARS-CoV-2, numerous mechanisms have been suggested that could be targeted on a pharmacological basis for prevention and treatment. The viral S protein is required by the mast cells of macrophages, pneumocytes, and pulmonary cells in infections. A diverse range of viruses require host cell proteases to activate the S glycoprotein for cellular entry (Bertram et al., 2012; Yamaya et al., 2015; Zhou et al., 2015). The membrane fusion and host cell entry require cleavage and activation of the spike protein (S protein) of SARS-CoV and are mediated by transmembrane protease/serine subfamily member 2 (TMPRSS2), an airway and alveolar cell serine protease (Matsuyama et al., 2010; Glowacka et al., 2011; Shulla et al., 2011). SARS-CoV-2 also involves TMPRSS2 for the priming of spike protein (S) driven cellular entry (Hoffmann et al., 2020).

A clinically established and commercially available, serine protease inhibitor, camostat mesylate, has partially inhibited the infection by HCoV-NL63 and SARS-CoV in HeLa cell lines, which express TMPRSS2 and ACE2 (Kawase et al., 2012). Camostat mesylate has also significantly inhibited the TMPRSS2 in human lung Calu-3 cells and lowered the infection with SARS-CoV-2 (Hoffmann et al., 2020). The viral host cell entry could be blocked by agents that inhibit TMPRSS2. Upon host cell entry, viral RNA (single-stranded) starts the replication process and subsequent translation of polyproteins which are eventually broken into full effecter proteins by the action of viral proteases (Baez-Santos et al., 2015). A viral infection is initiated by the interaction of S protein with ACE2 on the host cell cytoplasmic membrane.

Therapeutic strategies that disrupt the interaction of S protein with ACE2 could be of therapeutic importance. Recently, it was shown that the S protein of SARS-CoV-2 binding affinity to ACE2 is 10-20-times greater in comparison to the S protein of SARS-CoV indicating that the SARS-CoV-2 contagiousness is much higher than SARS-CoV (Tang et al., 2020). Recently, an *in vivo* study showed that multiple drugs including ritonavir/lopinavir and remdesivir targeted the MERS-CoV (Sheahan et al., 2020), and inhibited the RdRp of Ebola virus as well as the proteases of SARS-CoV-2 in humans. Moreover, these drugs are also recognized as potential drug candidates against SARS-CoV-2. The therapeutic efficacy of these drugs is now under investigation in two international clinical trials (SOLIDARITY Trial and DisCoVeRy Trial).

Numerous efforts are currently ongoing to accelerate the discovery and development of effective preventive and therapeutic candidate drugs against SARS-CoV-2 infections (Altay et al., 2020). In the past few months, since the emergence of COVID-19, several compounds have provided promising alternatives such as chloroquine, hydroxychloroquine, ritonavir, remdesivir, tocilizumab, interferon-β, ivermectin, lopinavir, ribavirin, and azithromycin (Wu et al., 2020). The repurposing of drugs has to date mainly concentrated on the pharmacological properties including antivirals, antibiotics, anti-inflammatory, and immunomodulators (Fan et al., 2020). The use of the above-mentioned drugs in COVID-19 patients is mostly empirical due to its lack of randomized controlled trials to demonstrate the efficacy and safety of these treatments. Taking into account COVID-19 related mortality, effective medications are required to improve the prognosis of patients and to curb the spread of the virus (Fan et al., 2020). Given the pharmacological perspective, all these drugs have the potential to either block the virus from entering host cells or prevent viral replication and/or attenuate the exacerbation of the host's immune response (Fan et al., 2020).

The pathogenesis and complications caused by SARS-CoV-2 are primarily based on an immune-inflammatory cascade. Taking this into account, therapeutic approaches should be focused on this cascade by attenuating inflammation and immune modulation (Allegra et al., 2020; Song et al., 2020). Numerous researches are currently in progress across different institutions around the world to identify novel drug candidates as well as vaccine for COVID-19. Learning from the discovery of Tamiflu®, phytochemicals, and natural products with antiviral, anti-inflammatory, and immunomodulatory properties should be investigated for the prevention and cure of SARS-CoV-2 infections. Among all these therapeutic approaches, natural products, mainly essential oils (EOs) have drawn much interest because of their robust use as anti-inflammatory, immunomodulator, and antioxidant as well as a source of novel antimicrobial, anti-inflammatory, and immunomodulator agents (Allegra et al., 2014; Sadgrove and Jones, 2019). EOs are well recognized for their strong antiviral, anti-inflammatory, and immunomodulatory activities (de Lavor et al., 2018; Asif et al., 2020; Gandhi et al., 2020). EO displays numerous beneficial effects in different diseases and produces systemic effects, consequently, it has been proposed as a possible candidate

for evaluation in prevention and treatment of COVID-19 (Agatonovic-Kustrin et al., 2019).

In viral infections, EO may have an important role as therapeutics in ameliorating the redox immune-inflammatory cascade by interfering with the pathways related to inflammatory processes in allergic and infectious airways. EO has shown time-tested safety and efficacy and because of that, it has been used in traditional medicine and food for a long time (Dosoky and Setzer, 2018). EOs are predominantly found in aromatic plants and are a complex mixture of lipophilic as well as volatile terpene compounds. They have been consumed via diets and recognized for potent antioxidant, anti-inflammatory, immunomodulatory, and antimicrobial properties (Aziz et al., 2018). Numerous experimental studies along with some clinical trials showed that EOs could be important therapeutic agents for immune system-related diseases (de Lavor et al., 2018; Gandhi et al., 2020). EOs supplementation along with other compounds are also very well recognized for their activity against bacteria and viruses that lead to respiratory infections. These have reported safe, and synergistic with potent antihistamine, and antioxidant properties (Boskabady et al., 2003; Leigh-de Rapper and van Vuuren, 2020).

Among many active principles of EOs, one of the compounds, carvacrol, received special attention due to recent reports of its specific binding with M^{pro}, a protease enzyme in the viral genome belonging to non-structural proteins showing a significant effect in the replication and maturation of SARS-CoV-2 (Kumar et al., 2020). In another recent study, carvacrol, a bioactive molecule in the EO of *Ammoides verticillata Briq*. was reported to inhibit ACE2 activity and suggested that it may block the host cell entry of SARS-CoV-2 (Abdelli et al., 2020). Both these studies demonstrate the potential of carvacrol on virus machinery as well as virus entry and the replication in the host cells. Moreover, Kulkarni et al. (2020) have reported that various monoterpenoid phenols including carvacrol have the potential to inhibit the binding of viral spike (S) glycoprotein to the host cell. Carvacrol has been docked against the S1 receptor binding domain of the spike protein, which is the key target for novel antiviral drugs, to ascertain their inhibitory effects based on their binding affinities.

Carvacrol ($C_{10}H_{14}O$), a monoterpenoid of the phenolic group [2-methyl-5-(1-methylethyl) phenol], found in an EO of ajwain (*Carum copticum* (L.) Benth. and Hook. f. ex C.B. Clarke*)*, oregano (*Origanum vulgare* L.), Shirazi thyme (*Zataria multiflora* Boiss.*)*, thyme (*Thymus vulgaris* L.), black cumin (*Nigella sativa* L.), wild bergamot [*Citrus aurantium bigaradia* (Loisel.) Brandis], pepperwort (*Lepidium flavum* Torr.), and other plants (Butt and Sultan, 2010; Tang et al., 2011; Fachini-Queiroz et al., 2012; Boskabady et al., 2014; Khazdair et al., 2018).

The biosynthetic pathway of carvacrol has been elucidated recently. Carvacrol is biosynthesized from isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), which are derived from the methylerythritol phosphate (MEP) pathway located in plastids (Rohmer et al., 1993; Dudareva et al., 2005). In the MEP pathway, which is involved in the biosynthesis of carvacrol, 1-deoxy-D-xylulose-5-phosphate (DXP) is irreversibly converted into 2-C-methyl-D-erythritol-4-phosphate (MEP) by a 1-deoxy-D-xylulose-5-phosphate reductoisomerase enzyme (DXR). This step has been described as the first committed step in the MEP pathway (Takahashi et al., 1998). Geranyl diphosphate synthase (GDS) is a key enzyme in this biosynthetic pathway, which catalyzes the head-to-tail condensation of IDP and DMADP to geranyl diphosphate (GDP) as the universal precursor of monoterpenes (Lichtenthaler, 1999). Subsequently, γ-terpinene synthase, which is a member of the monoterpene synthase family produces γ-terpinene through the cyclization of GDP. Furthermore, enzymes such as CYP71D180 and CYP71D181 belonging to the cytochrome P450 (CYP) monooxygenases, are also involved in further modification of γ-terpinene backbone to yield carvacrol (Crocoll et al., 2010).

Carvacrol is used as a microbicidal agent, and fragrance ingredient in cosmetic formulations (Andersen, 2006). Carvacrol is generally considered safe for consumption. It has been approved by the Federal Drug Administration for its use in food and is included by the Council of Europe in the list of chemical flavorings that can be found in alcoholic beverages, baked goods, chewing gum, condiment relish, frozen dairy, gelatin pudding, non-alcoholic beverages, and soft candy (Ultee et al., 2002; De Vincenzi et al., 2004). The structure and various pharmacological properties of carvacrol are represented in **Figure 1**.

In this article, it has been hypothesized that carvacrol could be a candidate for use as a preventive agent or therapeutic adjuvant in SARS-CoV-2 endowed with potent anti-inflammatory, antiviral, and immunomodulating properties to reduce the harshness and progression of the disease. In the present hypothesis the potential pharmacological and molecular mechanisms targeting oxidative stress, inflammation, immune system, and viremia or infectivity has been presented based on available literature that may provide logical speculation of its use in COVID-19. The proposed possible mechanisms of carvacrol on infection, immunity, and inflammation are presented in **Figure 2**.

CARVACROL AS AN IMMUNOMODULATOR AGENT

The first line of defense is the immune system of the body, which plays an important role in all types of infections including SARS-CoV-2. Immuno-modulation is a process that targets and augments immune response to prevent infections in immuno-deficiencies. In the case of allergies or autoimmune diseases immuno-modulation aims to inhibit the immune system, where the target is to lessen the immune system. At the initial stage of SARS-CoV-2 infection, M1 phenotypic macrophages show up and release ROS, IL-8, IL-6, nitric oxide, MCP-1, IL-1, TNF-α, and CXCL-10, which modulate the host defense against the virus, however, at the same time, it also enhances lung injury. Inflammation is primarily initiated by TNF-α. Moreover, TNF-α is also a key player in cell death mediator, differentiation, and immune modulation. Anti-inflammatory macrophages (M2) are activated once the pathogenic agent is

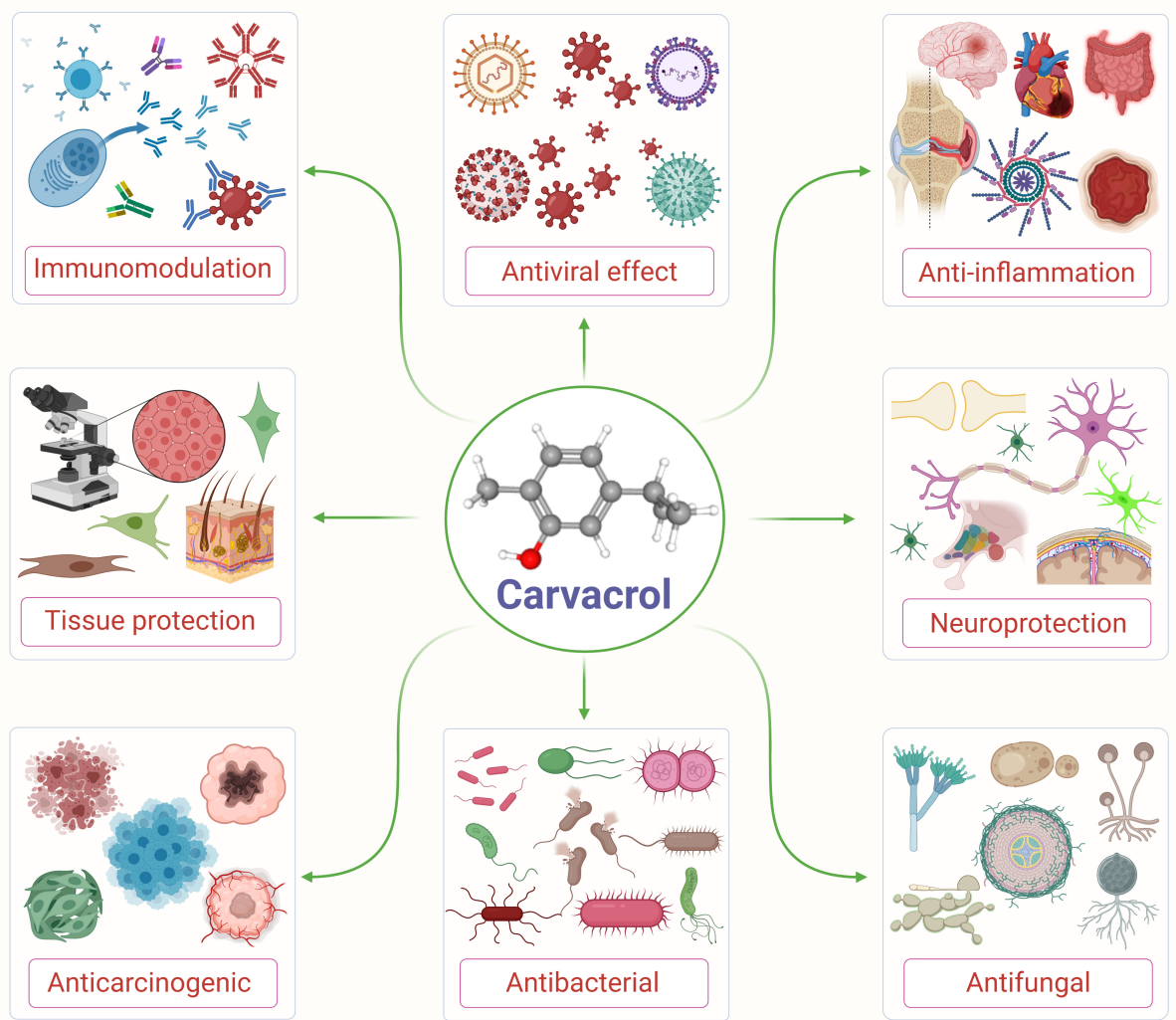

FIGURE 1 | An illustrative flow diagram showing the various pharmacological properties of carvacrol and its chemical structure (center). Adopted and modified from Suntres et al. (2015).

eliminated and eventually control the process of healing and restore the lung tissue. In addition, the innate immune system is significantly controlled by natural killer (NK) cells. At the time of infection, an innate immune response is initiated by eliminating the virally infected cells. Carvacrol administration to lipopolysaccharide (LPS) treated RAW 264.7 macrophages showed significant inhibition of LPS induced release of ROS, TNF-α, IL-1β, and NO. Moreover, carvacrol inhibited the nuclear translocation of the NF-κB (p65) subunit from the cytoplasm (Somensi et al., 2019).

Additionally, the virally infected cells are eliminated by the NK cells. Carvacrol treatment has been shown to attenuate the levels of pro- and anti- inflammatory cytokines in an animal model of experimental autoimmune encephalomyelitis. Carvacrol could be considered as a booster of autoimmunity as it ameliorated the levels of numerous cytokines such as IL-17, IFN-γ, TGF-β, IL-6, IL-10, and IL-4 (Mahmoodi et al., 2019). Carvacrol containing plant Zataria *multiflora* Boiss. also showed an immunomodulatory effect by augmenting the levels of IFN-γ and FOXP3 as well as IL-4, TGF-β, and IL-17 and could be useful in correcting immune dysregulation (Khazdair et al., 2018). In addition, carvacrol also showed strong anti-inflammatory and antioxidant properties, which could be beneficial in the cure of diseases associated with enhanced oxidative stress along with inflammatory and altered immune status (Khazdair et al., 2018). Adaptive immune responses (Giamarellos-Bourboulis et al., 2020; Kuri-Cervantes et al., 2020; Liao et al., 2020), mainly of T cells (Giamarellos-Bourboulis et al., 2020; Mathew et al., 2020), showed a significant role in the infection of SARS-CoV-2, similar to the infection of respiratory diseases. Carvacrol has been shown to reduce the transcription factors of T_H cell-related cytokines in ovalbumin immunized mice, thus suppressing the antigen-specific immune response, this indicates the potential of carvacrol to ameliorate the critical

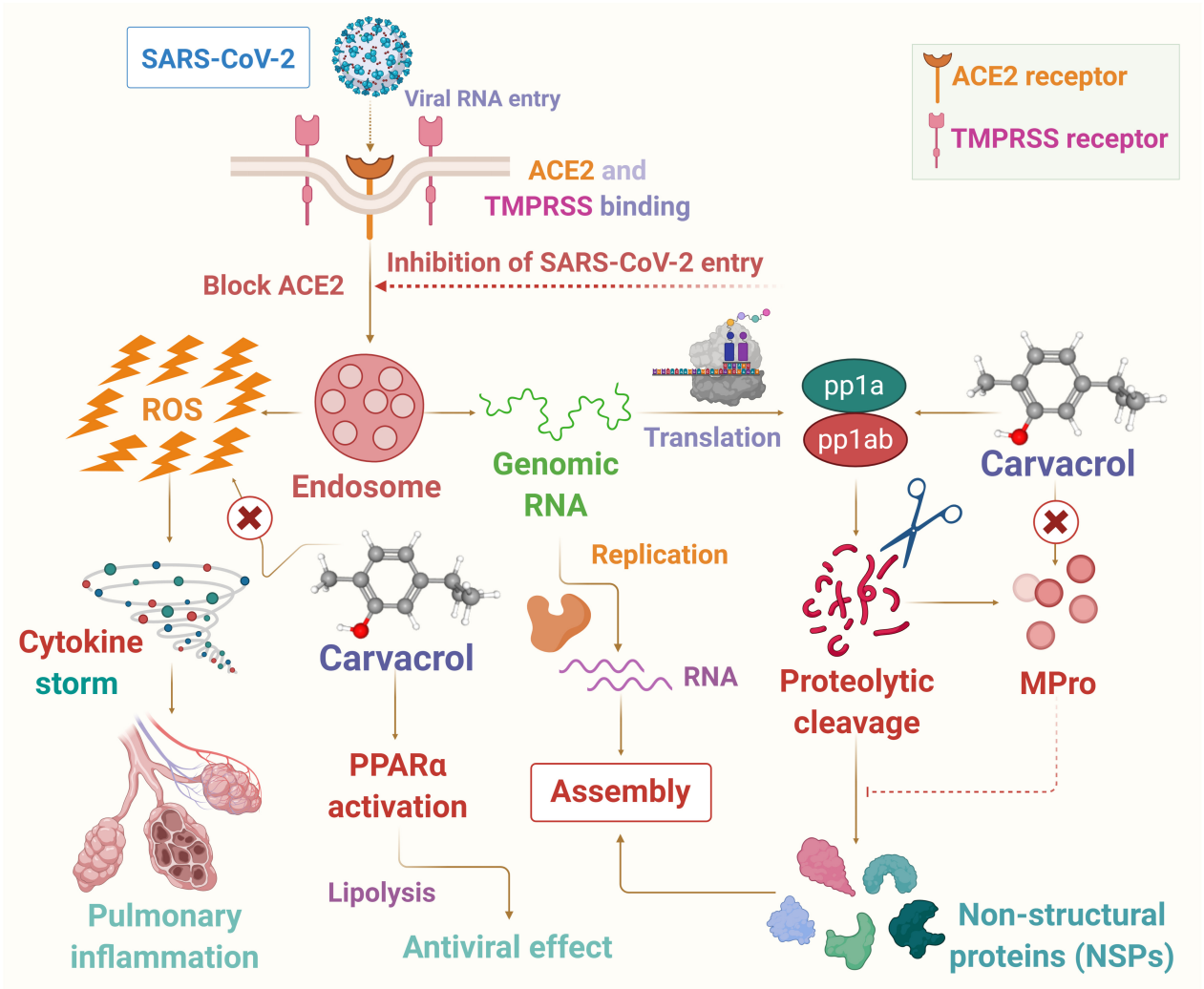

FIGURE 2 | The possible mechanism of carvacrol on infection, immunity and inflammation in context to COVID-19.

immune responses ascribed to the over activation of T-cells (Gholijani and Amirghofran, 2016).

CARVACROL AS AN ANTI-INFLAMMATORY AGENT

The inflammatory process is tackled through innate immune response and is consequently responsible for the release of inflammatory mediators (cytokines, ROS etc.) by the various cells of the immune system (neutrophils, macrophages, lymphocytes) that activates the adaptive immunity but enhances inflammation. T helper cells in turn result in the activation of various other types of cells (monocytes, B cells, etc.) by releasing cytokines, such as TNF-α, IL-1β, and IL-2, which institute the inflammatory cascade (Bradley, 2008).

Numerous studies have shown that the anti-inflammatory activity of carvacrol, derivatives of carvacrol, and plants containing a high amount of carvacrol in LPS-induced macrophages or monocytes or eosinophils, inhibit proinflammatory cytokines, inflammatory mediators, iNOS and COX-2, the production of NO and prostaglandin E2, and CD18 frequency on human lymphocytes (Lima Mda et al., 2013; Damasceno et al., 2014; Gholijani and Amirghofran, 2016; Li et al., 2018; Somensi et al., 2019). The hepatoprotective effect of carvacrol was observed in D-galactosamine-induced hepatotoxicity in rats. Carvacrol has been found to reduce CYP2E1 and enhance PPAR-α expression in alleviating liver damage (Aristatile et al., 2014). Carvacrol was also found as a COX-2 suppressor and PPAR-γ activator. Carvacrol has been shown to suppress the LPS induced mRNA and protein expression of COX-2 in human macrophage-like U937 cells, indicating that carvacrol ameliorates the expression of COX-2 via its agonistic effect on PPAR-γ (Hotta et al., 2010).

The role of activating PPAR-α and lipolysis has been shown to reduce the hepatitis C virus genotype-associated lipid

metabolic disorder in liver diseases (Patra et al., 2019). PPAR-α activation has also been shown to beneficially influence inflammatory alveolar epithelial cells and suggest the potential usefulness of PPAR-α in acute respiratory distress syndrome (Hecker et al., 2015). Carvacrol a natural dietary molecule has no adverse effects and could be beneficial for synthetic PPAR-α. Carvacrol has been also found to activate PPAR-α and reduce the expression of mRNA and the protein of inflammatory mediator COX-2 induced by LPS (Hotta et al., 2010). Overall, carvacrol's role as a PPAR-α agonist has also shown promising effects in regulating lipid metabolism in addition to its regulatory role on the immune system, cell proliferation, and differentiation, atherosclerosis vascular homeostasis, and inflammation (Lee et al., 2015; Gholijani and Amirghofran, 2016; Li et al., 2018). Moreover, *Nigella sativa* L., commonly known as black cumin, and its EO component carvacrol have shown strong pharmacological properties against dyslipidemia and respiratory disorders.

Carvacrol may also maintain the immune system balance through its role in immunomodulation (Butt and Sultan, 2010). Hence, carvacrol could be beneficial for limiting the orchestrated immune-inflammatory cascade in COVID-19.

Carvacrol as an Antiviral Agent

EOs and extracts of plant origin have been investigated for various pharmacological activities including anti-viral activities (Oltmans et al., 1986). Carvacrol was one of the compounds of the EOs that has an antiviral effect against herpes simplex virus types 1 (HSV-1). A study conducted by Pilau et al. (2011) on *Mentha pulegium* L. EOs showed that carvacrol is the main compound and its antiviral effect on humans and animals was investigated. They concluded that these EOs can inhibit different human and animal viruses such as rotavirus, bovine diarrhea virus, and respiratory syncytial virus *in vitro* (Pilau et al., 2011). The mechanism of action and anti-viral activities of carvacrol derived from oregano oil has been explored against murine norovirus (MNV), a non-enveloped virus. Carvacrol was found to help deactivate MNV, a human NoV surrogate, within 1 h of exposure, directly affecting the viral capsid and thereafter RNA (Gilling et al., 2014). Carvacrol inhibits MNV binding to host cells via hiding the capsid, however, there was no altered structural morphology of the virus reported (Gilling et al., 2014).

Carvacrol is a relatively safe agent of plant origin and classified as generally regarded as safe. Even though several antimicrobial agents are not preferred for safety reasons, in this situation, carvacrol would be a good choice as antimicrobials. For example, the application of antimicrobials on foods or food contact surfaces, or it could be used in place of corrosive compounds on surfaces. Carvacrol, isolated from oregano oil, has shown to possess strong antimicrobial activity against multiple pathogenic bacterial species (Ultee et al., 2002; Knowles et al., 2005; Cox and Markham, 2007; Ravishankar et al., 2009; Garcia-Garcia et al., 2011; Mild et al., 2011) and could be beneficial for possible use as a surface (fomite) sanitizer and natural food to manage norovirus (NoV). The mechanism of action of carvacrol on bacteria is quite different due to the complex components and structure of the bacterial cell wall. Further, there are also reports available that show a direct action of carvacrol on the bacterial membrane and cell wall (Ultee et al., 2002; Cox and Markham, 2007; Garcia-Garcia et al., 2011).

THE SAFETY PROFILE OF CARVACROL

There are numerous beneficial effects of EOs such as antioxidant, antimicrobial, and antimutagenic effects. However, apart from this pharmacological efficacy, EOs may have possible toxicity, for example, genotoxicity/mutagenicity (Llana-Ruiz-Cabello et al., 2015). Carvacrol has been used for a long time in diets as well as for medicinal purposes to support health and well-being. It is one of the vital constituents of numerous herbal formulations. It has been demonstrated that a high concentration of carvacrol (460 μM) may have mutagenicity and genotoxicity effects on the intestinal cell line Caco-2, as it caused DNA damage (Llana-Ruiz-Cabello et al., 2015). However, there were no adverse effects reported in human lymphocytes and hepatocytes as well as the lung fibroblast of Chinese hamsters (LLana-Ruiz-Cabello et al., 2014; Maisanaba et al., 2015).

PHARMACOKINETICS OF CARVACROL

Carvacrol possesses favorable pharmacokinetic and physicochemical properties to be developed as a drug, based on the popular rules including Lipinski's, Veber's, and Egan's (Egan et al., 2000; Lipinski et al., 2001; Veber et al., 2002) depict drug-like properties. In the intestine, carvacrol is gradually absorbed following oral administration (1.5 g) in rabbits. However, 30% remain in the GI tract and 25% excreted by urine after 22 h (Suntres et al., 2015).

In another study, multiple doses of sesame oil derived carvacrol were administered orally into rabbits (1,500 and 5,000 mg) and rats (500 mg). The results showed its distribution in the intestines, stomach, and urine, with little quantity in the muscle, liver, and lung. The intestinal delivery of carvacrol in animals was significantly enhanced by alginate–whey protein microcapsules with a diameter in the range of 250 and 800 μm, which contain 72 and 76 g/kg of carvacrol, respectively (Wang et al., 2009). Uncapsulated carvacrol has been shown to absorb or metabolize over 95% in duodenum and stomach. Even though microcapsules completely freed the compound in the intestinal tract, a better recovery has been observed in the small intestine than large intestine with larger microcapsules in particular (Wang et al., 2009). It was found that uncapsulated carvacrol greatly absorbed/metabolized in the upper GI tract of pigs upon oral ingestion, while alginate–microcapsules were found to reduce the absorption of carvacrol in the stomach and proximal intestine and enhanced the percentage of carvacrol reached to the distal small intestine (Zhang et al., 2016).

According to Austgulen et al. (1987), the metabolism of carvacrol occurs following two types of pathways. The primary metabolic pathway is the conjugation of the phenolic group with glucuronic acid ($C_6H_{10}O_7$) and sulfate (SO_4^{2-}), when administered at a low dose, the metabolism of carvacrol covers the terminal methyl groups' oxidation to primary alcohols (Austgulen et al., 1987). Carvacrol (1 mmol/kg) administration to albino rats showed its excretion in urine in their original form or the form of glucuronide and sulfate conjugates (Austgulen et al., 1987). Dong et al. (2012) revealed the cytochrome P450 role in carvacrol and its isomer thymol metabolism, in microsomes of the human liver. The isoform, CYP2A6 was observed in a primary drug-metabolizing enzyme and generated metabolites following oxidation of carvacrol (Dong et al., 2012).

LIMITATIONS

Among numerous compounds screened to date using *in silico* tools, carvacrol appears to target both, the viral protein as well as the viral entry factors in humans. Carvacrol has also been shown to possess favorable physicochemical properties and appears to be a druggable compound. There is a paucity of preclinical data on the potential of carvacrol on infection, inflammation, and immunity in the context of COVID-19. Thus, the therapeutic efficacy of carvacrol must be tested in currently available preclinical animal models for SARS-CoV-2 infection (Cleary et al., 2020; Munoz-Fontela et al., 2020; Yuan et al., 2020), to explore whether the candidate compounds can be used as a preventive agent or therapeutic adjuvant. To conclude the use in COVID-19, it should be investigated and validated in the preclinical models of COVID-19, despite strong evidence for their anti-inflammatory, immunomodulatory, and antiviral properties in other disease models. However, based on the potent immunomodulatory, anti-inflammatory, and antimicrobial properties and additional *in silico* observations, carvacrol seem to be a suitable candidate for further investigation.

CONCLUSION

In summary, based on the wide range of experimental studies, carvacrol, and its metabolites appear to exert protective effects against inflammation, immune dysfunction, and infection within the scope of COVID-19. The strong anti-inflammatory properties mediating multiple mechanisms such as the reduction in pro-inflammatory cytokines, chemokines, and adhesion molecules along with inhibition of macrophage infiltration, neutrophil-endothelial cell interaction, provide a plausible reason to inhibit the cytokine storm, a major player of severity, complications, and death in COVID-19.

Carvacrol as a potent antioxidant and immunomodulator may enhance the host cellular immunity against infections. Carvacrol's capability in interfering with ACE2 receptors in the host, along with its antiviral properties due to its interaction with viral protease and antibacterial properties further strengthen the candidature of carvacrol in viremia and secondary infections, which eventually lead to complications and fatal outcomes. This review highlight the pharmacological principles and observations outlined in published studies, which plausibly suggest possible anti-inflammatory, antiviral, and immunomodulatory properties in context to COVID-19.

When taken into account, the numerous properties of carvacrol such as immunomodulatory, anti-inflammatory, and antiviral effects, along with its molecular mechanisms, indicate that it could be an important therapeutic candidate for COVID-19. In addition, the pharmacological actions, negligible toxicity, drug likeliness properties of carvacrol indicate that it could be used as a nutraceutical or pharmacological agent and/or adjuvant against COVID-19. However, the use of carvacrol in COVID-19 remains inconclusive until preclinical and clinical data is available on its efficacy and safety and does not advice the use of carvacrol in any forms for COVID-19.

AUTHOR CONTRIBUTIONS

HJ drafted the manuscript and performed the correction. MFNM drew the scheme, edited the manuscript, and performed the literature survey. NKJ wrote the manuscript, and edited and improved the scheme artwork. SO conceptualized, wrote, and edited the manuscript, and performed the literature survey and ideation of scheme. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abdelli, I., Hassani, F., Bekkel Brikci, S., and Ghalem, S. (2020). In silico study the inhibition of angiotensin converting enzyme 2 receptor of COVID-19 by *Ammoides verticillata* components harvested from Western Algeria. *J. Biomol. Struct. Dynam.* 2, 1–14. doi: 10.1080/07391102.2020.1763199

Agatonovic-Kustrin, S., Kustrin, E., and Morton, D. W. (2019). Essential oils and functional herbs for healthy aging. *Neur. Regen. Res.* 14, 441–445. doi: 10.4103/1673-5374.245467

Allegra, A., Di Gioacchino, M., Tonacci, A., Musolino, C., and Gangemi, S. (2014). NF-κβ signaling and chronic inflammatory diseases: exploring the potential of natural products to drive new therapeutic opportunities. *Drug Discover. Today* 19, 373–378. doi: 10.1016/j.drudis.2013.11.002

Allegra, A., Di Gioacchino, M., Tonacci, A., Musolino, C., and Gangemi, S. (2020). Immunopathology of SARS-CoV-2 infection: immune cells and mediators, prognostic factors, and immune-therapeutic implications. *Int. J. Mol. Sci.* 21:4782. doi: 10.3390/ijms21134782

Altay, O., Mohammadi, E., Lam, S., Turkez, H., Boren, J., Nielsen, J., et al. (2020). Current status of COVID-19 therapies and drug repositioning applications. *iScience* 23:101303. doi: 10.1016/j.isci.2020.101303

Andersen, A. (2006). Final report on the safety assessment of sodium p-chloro-m-cresol, p-chloro-m-cresol, chlorothymol, mixed cresols, m-cresol, o-cresol, p-cresol, isopropyl cresols, thymol, o-cymen-5-ol, and

carvacrol. *Int. J. Toxicol.* 25(Suppl. 1), 29–127. doi: 10.1080/10915810600716653

Aristatile, B., Al-Assaf, A. H., and Pugalendi, K. V. (2014). Carvacrol ameliorates the PPAR-A and cytochrome P450 expression on D-galactosamine induced hepatotoxicity rats. *Afr. J. Tradit. Complemen. Alternat. Med.* 11, 118–123. doi: 10.4314/ajtcam.v11i3.18

Asif, M., Saleem, M., Saadullah, M., Yaseen, H. S., and Al Zarzour, R. (2020). COVID-19 and therapy with essential oils having antiviral, anti-inflammatory, and immunomodulatory properties. *Inflammopharmacology* 28, 1153–1161. doi: 10.1007/s10787-020-00744-0

Austgulen, L. T., Solheim, E., and Scheline, R. R. (1987). Metabolism in rats of p-cymene derivatives: carvacrol and thymol. *Pharmacol. Toxicol.* 61, 98–102. doi: 10.1111/j.1600-0773.1987.tb01783.x

Aziz, Z. A. A., Ahmad, A., Setapar, S. H. M., Karakucuk, A., Azim, M. M., and Lokhat, D. (2018). Essential oils: extraction techniques, pharmaceutical and therapeutic potential – a review. *Curr. Drug Metab.* 19, 1100–1110. doi: 10.2174/1389200219666180723144850

Baez-Santos, Y. M., St John, S. E., and Mesecar, A. D. (2015). The SARS-coronavirus papain-like protease: structure, function and inhibition by designed antiviral compounds. *Antiv. Res.* 115, 21–38. doi: 10.1016/j.antiviral.2014.12.015

Bertram, S., Heurich, A., Lavender, H., Gierer, S., Danisch, S., Perin, P., et al. (2012). Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. *PLoS one* 7:e35876. doi: 10.1371/journal.pone.0035876

Boopathi, S., Poma, A. B., and Kolandaivel, P. (2020). Novel 2019 coronavirus structure, mechanism of action, antiviral drug promises and rule out against its treatment. *J. Biomol. Struct. Dynam.* 8, 1–10. doi: 10.1080/07391102.2020.1758788

Boskabady, M. H., Alitaneh, S., and Alavinezhad, A. (2014). *Carum copticum* L.: a herbal medicine with various pharmacological effects. *BioMed Res. Int.* 2014:569087. doi: 10.1155/2014/569087

Boskabady, M. H., Ramazani, M., and Tabei, T. (2003). Relaxant effects of different fractions of essential oil from *Carum copticum* on guinea pig tracheal chains. *Phytother. Res.* 17, 1145–1149. doi: 10.1002/ptr.1238

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *J. Pathol.* 214, 149–160. doi: 10.1002/path.2287

Butt, M. S., and Sultan, M. T. (2010). Nigella sativa: reduces the risk of various maladies. *Crit. Rev. Food Sci. Nutr.* 50, 654–665. doi: 10.1080/10408390902768797

Cleary, S. J., Pitchford, S. C., Amison, R. T., Carrington, R., Robaina Cabrera, C. L., Magnen, M., et al. (2020). Animal models of mechanisms of SARS-CoV-2 infection and COVID-19 pathology. *Br. J. Pharmacol.* 177, 4851–4865. doi: 10.1111/bph.15143

Cox, S. D., and Markham, J. L. (2007). Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J. Appl. Microbiol.* 103, 930–936. doi: 10.1111/j.1365-2672.2007.03353.x

Crocoll, C., Asbach, J., Novak, J., Gershenzon, J., and Degenhardt, J. (2010). Terpene synthases of oregano (*Origanum vulgare* L.) and their roles in the pathway and regulation of terpene biosynthesis. *Plant Mol. Biol.* 73, 587–603. doi: 10.1007/s11103-010-9636-1

Damasceno, S. R., Oliveira, F. R., Carvalho, N. S., Brito, C. F., Silva, I. S., Sousa, F. B., et al. (2014). Carvacryl acetate, a derivative of carvacrol, reduces nociceptive and inflammatory response in mice. *Life Sci.* 94, 58–66. doi: 10.1016/j.lfs.2013.11.001

Das, S., Sarmah, S., Lyndem, S., and Singha Roy, A. (2020). An investigation into the identification of potential inhibitors of SARS-CoV-2 main protease using molecular docking study. *J. Biomol. Struct. Dynam.* 2020, 1–11. doi: 10.1080/07391102.2020.1763201

de Lavor, É. M., Fernandes, A. W. C., de Andrade Teles, R. B., Leal, A., de Oliveira Junior, R. G., Gama, E. S. M., et al. (2018). Essential oils and their major compounds in the treatment of chronic inflammation: a review of antioxidant potential in preclinical studies and molecular mechanisms. *Oxidat. Med. Cell. Longev.* 2018:6468593. doi: 10.1155/2018/6468593

De Vincenzi, M., Stammati, A., De Vincenzi, A., and Silano, M. (2004). Constituents of aromatic plants: carvacrol. *Fitoterapia* 75, 801–804. doi: 10.1016/j.fitote.2004.05.002

Dong, R. H., Fang, Z. Z., Zhu, L. L., Ge, G. B., Cao, Y. F., Li, X. B., et al. (2012). Identification of CYP isoforms involved in the metabolism of thymol and carvacrol in human liver microsomes (HLMs). *Die Pharmazie* 67, 1002–1006.

Dosoky, N. S., and Setzer, W. N. (2018). Chemical composition and biological activities of essential oils of curcuma species. *Nutrients* 10:1196. doi: 10.3390/nu10091196

Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., et al. (2005). The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 933–938. doi: 10.1073/pnas.0407360102

Egan, W. J., Merz, K. M. Jr., and Baldwin, J. J. (2000). Prediction of drug absorption using multivariate statistics. *J. Med. Chem.* 43, 3867–3877. doi: 10.1021/jm000292e

Fachini-Queiroz, F. C., Kummer, R., Estevao-Silva, C. F., Carvalho, M. D., Cunha, J. M., Grespan, R., et al. (2012). Effects of thymol and carvacrol, constituents of thymus vulgaris l. essential oil, on the inflammatory response. *Evid. Based Complemen. Alternat. Med.* 2012:657026. doi: 10.1155/2012/657026

Fan, S., Xiao, D., Wang, Y., Liu, L., Zhou, X., and Zhong, W. (2020). Research progress on repositioning drugs and specific therapeutic drugs for SARS-CoV-2. *Fut. Med. Chem.* 12, 1565–1578. doi: 10.4155/fmc-2020-0158

Gandhi, G. R., Vasconcelos, A. B. S., Haran, G. H., Calisto, V., Jothi, G., Quintans, J. S. S., et al. (2020). Essential oils and its bioactive compounds modulating cytokines: a systematic review on anti-asthmatic and immunomodulatory properties. *Phytomedicine* 73:152854. doi: 10.1016/j.phymed.2019.152854

Garcia-Garcia, R., Lopez-Malo, A., and Palou, E. (2011). Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against Listeria innocua. *J. Food Sci.* 76, M95–M100. doi: 10.1111/j.1750-3841.2010.02005.x

Gholijani, N., and Amirghofran, Z. (2016). Effects of thymol and carvacrol on T-helper cell subset cytokines and their main transcription factors in ovalbumin-immunized mice. *J. Immunotoxicol.* 13, 729–737. doi: 10.3109/1547691X.2016.1173134

Giamarellos-Bourboulis, E. J., Netea, M. G., Rovina, N., Akinosoglou, K., Antoniadou, A., Antonakos, N., et al. (2020). Complex immune dysregulation in COVID-19 patients with severe respiratory failure. *Cell Host Microbe* 27, 992–1000.e3. doi: 10.1016/j.chom.2020.04.009

Gilling, D. H., Kitajima, M., Torrey, J. R., and Bright, K. R. (2014). Antiviral efficacy and mechanisms of action of oregano essential oil and its primary component carvacrol against murine norovirus. *J. Appl. Microbiol.* 116, 1149–1163. doi: 10.1111/jam.12453

Glowacka, I., Bertram, S., Muller, M. A., Allen, P., Soilleux, E., Pfefferle, S., et al. (2011). Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J. Virol.* 85, 4122–4134. doi: 10.1128/JVI.02232-10

Hecker, M., Behnk, A., Morty, R. E., Sommer, N., Vadász, I., and Herold, S. (2015). PPAR-α activation reduced LPS-induced inflammation in alveolar epithelial cells. *Exp. Lung Res.* 41, 393–403. doi: 10.3109/01902148.2015.1046200

Hemida, M. G., and Ba Abduallah, M. M. (2020). The SARS-CoV-2 outbreak from a one health perspective. *One Health* 2020:100127. doi: 10.1016/j.onehlt.2020.100127

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280.e278. doi: 10.1016/j.cell.2020.02.052

Hotta, M., Nakata, R., Katsukawa, M., Hori, K., Takahashi, S., and Inoue, H. (2010). Carvacrol, a component of thyme oil, activates PPARalpha and gamma and suppresses COX-2 expression. *J. Lip. Res.* 51, 132–139. doi: 10.1194/jlr.M900255-JLR200

Jean, S. S., Lee, P. I., and Hsueh, P. R. (2020). Treatment options for COVID-19: the reality and challenges. *J. Microbiol. Immunol. Infect. Wei Mian Gan Ran Zhi* 53, 436–443. doi: 10.1016/j.jmii.2020.03.034

Kawase, M., Shirato, K., van der Hoek, L., Taguchi, F., and Matsuyama, S. (2012). Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *J. Virol.* 86, 6537–6545. doi: 10.1128/JVI.00094-12

Khazdair, M. R., Ghorani, V., Alavinezhad, A., and Boskabady, M. H. (2018). Pharmacological effects of *Zataria multiflora* Boiss L. and its constituents focus on their anti-inflammatory, antioxidant, and immunomodulatory effects. *Fund. Clin. Pharmacol.* 32, 26–50. doi: 10.1111/fcp.12331

Knowles, J. R., Roller, S., Murray, D. B., and Naidu, A. S. (2005). Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl. Environ. Microbiol.* 71, 797–803. doi: 10.1128/AEM.71.2.797-803.2005

Kulkarni, S. A., Nagarajan, S. K., Ramesh, V., Palaniyandi, V., Selvam, S. P., and Madhavan, T. (2020). Computational evaluation of major components from plant essential oils as potent inhibitors of SARS-CoV-2 spike protein. *J. Mol. Struct.* 1221:128823. doi: 10.1016/j.molstruc.2020.128823

Kumar, A., Choudhir, G., Shukla, S. K., Sharma, M., Tyagi, P., Bhushan, A., et al. (2020). Identification of phytochemical inhibitors against main protease of COVID-19 using molecular modeling approaches. *J. Biomol. Struct. Dynam.* 10, 1–11. doi: 10.1080/07391102.2020.1772112

Kuri-Cervantes, L., Pampena, M. B., Meng, W., Rosenfeld, A. M., Ittner, C. A. G., Weisman, A. R., et al. (2020). Comprehensive mapping of immune perturbations associated with severe COVID-19. *Sci. Immunol.* 5:eabd7114. doi: 10.1126/sciimmunol.abd7114

Lee, K. P., Sudjarwo, G. W., Jung, S. H., Lee, D., Lee, D. Y., Lee, G. B., et al. (2015). Carvacrol inhibits atherosclerotic neointima formation by downregulating reactive oxygen species production in vascular smooth muscle cells. *Atherosclerosis* 240, 367–373. doi: 10.1016/j.atherosclerosis.2015.03.038

Leigh-de Rapper, S., and van Vuuren, S. F. (2020). Odoriferous therapy: a review identifying essential oils against pathogens of the respiratory tract. *Chem. Biodiver.* 17:e2000062. doi: 10.1002/cbdv.202000062

Li, Y., Xu, J. Z., Gu, C. X., Liu, G. L., and Tian, K. (2018). Carvacrol suppresses inflammatory responses in rheumatoid arthritis fibroblast-like synoviocytes. *J. Cell. Biochem.* 120, 8169–8176. doi: 10.1002/jcb.28098

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., et al. (2020). Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat. Med.* 26, 842–844. doi: 10.1038/s41591-020-0901-9

Lichtenthaler, H. K. (1999). The 1-deoxy-D-Xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Ann. Rev. Plant Physiol. Mol. Biol.* 50, 47–65. doi: 10.1146/annurev.arplant.50.1.47

Lima Mda, S., Quintans-Junior, L. J., de Santana, W. A., Martins Kaneto, C., Pereira Soares, M. B., and Villarreal, C. F. (2013). Anti-inflammatory effects of carvacrol: evidence for a key role of interleukin-10. *Eur. J. Pharmacol.* 699, 112–117. doi: 10.1016/j.ejphar.2012.11.040

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 46, 3–26. doi: 10.1016/s0169-409x(00)00129-0

Llana-Ruiz-Cabello, M., Gutierrez-Praena, D., Puerto, M., Pichardo, S., Jos, A., and Camean, A. M. (2015). In vitro pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. *Toxicol. Vitro* 29, 647–656. doi: 10.1016/j.tiv.2015.02.006

LLana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A. I., Pichardo, S., Jos, A., et al. (2014). Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. *Food Chem. Toxicol.* 72, 122–128. doi: 10.1016/j.fct.2014.07.013

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/S0140-6736(20)30251-8

Mahmoodi, M., Amiri, H., Ayoobi, F., Rahmani, M., Taghipour, Z., Ghavamabadi, R. T., et al. (2019). Carvacrol ameliorates experimental autoimmune encephalomyelitis through modulating pro- and anti-inflammatory cytokines. *Life Sci.* 219, 257–263. doi: 10.1016/j.lfs.2018.11.051

Maisanaba, S., Prieto, A. I., Puerto, M., Gutierrez-Praena, D., Demir, E., Marcos, R., et al. (2015). In vitro genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 784–785, 37–44. doi: 10.1016/j.mrgentox.2015.05.005

Mathew, D., Giles, J. R., Baxter, A. E., Oldridge, D. A., Greenplate, A. R., Wu, J. E., et al. (2020). Deep immune profiling of COVID-19 patients reveals distinct immunotypes with therapeutic implications. *Science* 369:eabc8511. doi: 10.1126/science.abc8511

Matsuyama, S., Nagata, N., Shirato, K., Kawase, M., Takeda, M., and Taguchi, F. (2010). Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J. Virol.* 84, 12658–12664. doi: 10.1128/JVI.01542-10

Mild, R. M., Joens, L. A., Friedman, M., Olsen, C. W., McHugh, T. H., Law, B., et al. (2011). Antimicrobial edible apple films inactivate antibiotic resistant and susceptible *Campylobacter jejuni* strains on chicken breast. *J. Food Sci.* 76, M163–M168. doi: 10.1111/j.1750-3841.2011.02065.x

Munoz-Fontela, C., Dowling, W. E., Funnell, S. G. P., Gsell, P. S., Riveros-Balta, A. X., Albrecht, R. A., et al. (2020). Animal models for COVID-19. *Nature* 586, 509–515. doi: 10.1038/s41586-020-2787-6

Oltmans, G. A., Beales, M., and Lorden, J. F. (1986). Glutamic acid decarboxylase activity in micropunches of the deep cerebellar nuclei of the genetically dystonic (dt) rat. *Brain Res.* 385, 148–151. doi: 10.1016/0006-8993(86)91556-8

Patra, T., Sasaki, R., Meyer, K., Ray, R. B., and Ray, R. (2019). Transforming growth factor β acts as a regulatory molecule for lipogenic pathways among Hepatitis C Virus genotype-specific infections. *J. Virol.* 93:e00811-19. doi: 10.1128/JVI.00811-19

Pilau, M. R., Alves, S. H., Weiblen, R., Arenhart, S., Cueto, A. P., and Lovato, L. T. (2011). Antiviral activity of the *Lippia graveolens* (Mexican oregano) essential oil and its main compound carvacrol against human and animal viruses. *Braz. J. Microbiol.* 42, 1616–1624. doi: 10.1590/S1517-83822011000400049

Ravishankar, S., Zhu, L., Olsen, C. W., McHugh, T. H., and Friedman, M. (2009). Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens on meat and poultry products. *J. Food Sci.* 74, M440–M445. doi: 10.1111/j.1750-3841.2009.01320.x

Rohmer, M., Knani, M., Simonin, P., Sutter, B., and Sahm, H. (1993). Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem. J.* 295(Pt 2), 517–524. doi: 10.1042/bj2950517

Sadgrove, N. J., and Jones, G. L. (2019). From petri dish to patient: bioavailability estimation and mechanism of action for antimicrobial and immunomodulatory natural products. *Front. Microbiol.* 10:2470. doi: 10.3389/fmicb.2019.02470

Sheahan, T. P., Sims, A. C., Leist, S. R., Schafer, A., Won, J., Brown, A. J., et al. (2020). Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat. Commun.* 11:222. doi: 10.1038/s41467-019-13940-6

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., and Gallagher, T. (2011). A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J. Virol.* 85, 873–882. doi: 10.1128/JVI.02062-10

Somensi, N., Rabelo, T. K., Guimaraes, A. G., Quintans-Junior, L. J., de Souza Araujo, A. A., Moreira, J. C. F., et al. (2019). Carvacrol suppresses LPS-induced pro-inflammatory activation in RAW 264.7 macrophages through ERK1/2 and NF-kB pathway. *Int. Immunopharmacol.* 75:105743. doi: 10.1016/j.intimp.2019.105743

Song, J. W., Zhang, C., Fan, X., Meng, F. P., Xu, Z., Xia, P., et al. (2020). Immunological and inflammatory profiles in mild and severe cases of COVID-19. *Nat. Commun.* 11:3410. doi: 10.1038/s41467-020-17240-2

Suntres, Z. E., Coccimiglio, J., and Alipour, M. (2015). The bioactivity and toxicological actions of carvacrol. *Crit. Rev. Food Sci. Nutr.* 55, 304–318. doi: 10.1080/10408398.2011.653458

Takahashi, S., Kuzuyama, T., Watanabe, H., and Seto, H. (1998). A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9879–9884. doi: 10.1073/pnas.95.17.9879

Tang, B., Bragazzi, N. L., Li, Q., Tang, S., Xiao, Y., and Wu, J. (2020). An updated estimation of the risk of transmission of the novel coronavirus (2019-nCov). *Infect. Dis. Model.* 5, 248–255. doi: 10.1016/j.idm.2020.02.001

Tang, X., Chen, S., and Wang, L. (2011). Purification and identification of carvacrol from the root of Stellera chamaejasme and research on its insecticidal activity. *Nat. Product Res.* 25, 320–325. doi: 10.1080/14786419.2010.532796

Tortorici, M. A., and Veesler, D. (2019). Structural insights into coronavirus entry. *Adv. Virus Res.* 105, 93–116. doi: 10.1016/bs.aivir.2019.08.002

Ultee, A., Bennik, M. H., and Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus*

cereus. *Appl. Environ. Microbiol.* 68, 1561–1568. doi: 10.1128/aem.68.4.1561-1568.2002

Veber, D. F., Johnson, S. R., Cheng, H. Y., Smith, B. R., Ward, K. W., and Kopple, K. D. (2002). Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* 45, 2615–2623. doi: 10.1021/jm020017n

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292.e286. doi: 10.1016/j.cell.2020.02.058

Wang, Q., Gong, J., Huang, X., Yu, H., and Xue, F. (2009). In vitro evaluation of the activity of microencapsulated carvacrol against *Escherichia coli* with K88 pili. *J. Appl. Microbiol.* 107, 1781–1788. doi: 10.1111/j.1365-2672.2009.04374.x

Wu, R., Wang, L., Kuo, H.-C. D., Shannar, A., Peter, R., Chou, P. J., et al. (2020). An Update on current therapeutic drugs treating COVID-19. *Curr. Pharmacol. Rep.* 6, 56–70. doi: 10.1007/s40495-020-00216-7

Yamaya, M., Shimotai, Y., Hatachi, Y., Lusamba Kalonji, N., Tando, Y., Kitajima, Y., et al. (2015). The serine protease inhibitor camostat inhibits influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells. *Pulmon. Pharmacol. Therap.* 33, 66–74. doi: 10.1016/j.pupt.2015.07.001

Yuan, L., Tang, Q., Cheng, T., and Xia, N. (2020). Animal models for emerging coronavirus: progress and new insights. *Emerg. Microb. Infect.* 9, 949–961. doi: 10.1080/22221751.2020.1764871

Zhang, H., Penninger, J. M., Li, Y., Zhong, N., and Slutsky, A. S. (2020). Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. *Intens. Care Med.* 46, 586–590. doi: 10.1007/s00134-020-05985-9

Zhang, Y., Wang, Q. C., Yu, H., Zhu, J., de Lange, K., Yin, Y., et al. (2016). Evaluation of alginate-whey protein microcapsules for intestinal delivery of lipophilic compounds in pigs. *J. Sci. Food Agricult.* 96, 2674–2681. doi: 10.1002/jsfa.7385

Zhou, Y., Vedantham, P., Lu, K., Agudelo, J., Carrion, R. Jr., Nunneley, J. W., et al. (2015). Protease inhibitors targeting coronavirus and filovirus entry. *Antiv. Res.* 116, 76–84. doi: 10.1016/j.antiviral.2015.01.011

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *New Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

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OPINION
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Cinnamon and Hop Extracts as Potential Immunomodulators for Severe COVID-19 Cases

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INTRODUCTION

Despite intense focus, so far no effective treatment has been developed for severe cases of COVID-19. SARS-CoV-2 infection results in a multisystem hyperinflammatory syndrome with acute respiratory distress syndrome (ARDS), acute kidney failure, and cardiovascular and neurological complications (Wang et al., 2020). Severe cases of this condition are characterized by a "cytokine storm" and rampant inflammation (Renu et al., 2020; Ye et al., 2020). The hyperinflammation is associated with the generation and release of reactive oxygen and nitrogen species (ROS/RNS), which can further amplify inflammation (Lucas and Maes, 2013). Histopathological observation of COVID-19 has revealed diffuse alveolar damage with vascular endothelialitis, thrombosis, and intussusceptive angiogenesis (Ackermann et al., 2020). The angiocentric inflammation is not limited to the COVID-19-induced lung injury but also involves prolonged inflammation in other organs, such as the liver, brain, heart, or the gut (Ackermann et al., 2020; Wang et al., 2020). Any treatment that could limit the "cytokine storm," reduce ROS/RNS production, and counteract the formation of thrombosis would be highly attractive, and, in the best-case scenario, such a treatment would additionally interfere with viral replication. Recently, a preliminary study reported that the administration of dexamethasone, a corticosteroid with anti-inflammatory effects, could elicit a 30% reduction in mortality for patients receiving invasive mechanical ventilation (Horby et al., 2020).

In a previous study, we screened a panel of 99 ethanolic herbal extracts for their anti-inflammatory properties. Hop (*Humulus lupulus*, cones) and Ceylon cinnamon (*Cinnamomum verum* alias *C. zeylanicum*, bark) extracts were found to elicit particularly drastic reductions in activation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a key regulator of pro-inflammatory cytokines (Schink et al., 2018b).

The literature search was mainly performed in MedLine. In a first round, we looked for studies describing anti-inflammatory effects for Ceylon cinnamon, hops, and their major compounds. In a second approach, we then searched for clinical studies testing the efficacy of the plants in treating human disease.

HOPS

Hops, the seed cones of the plant *Humulus lupulus* from the family Cannabaceae, contain several pharmaceutically active compounds, such as humulone, lupulone, and xanthohumol (Gerhäuser, 2005; Knez Hrncic et al., 2019; Lin et al., 2019). Crude hop extracts as well as individual compounds have been described to exert anti-viral effects against several DNA and RNA viruses (Buckwold et al., 2004; Fuchimoto et al., 2013). For example, humulone, the most important bitter acid of hops,

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can suppress replication of the respiratory syncytial virus (RSV alias Human orthopneumovirus) in cell culture by disturbing the formation of viral filaments (Fuchimoto et al., 2013). Xanthohumol from hops showed synergistic effects with IFN-α in the treatment of bovine viral diarrhea virus (BVDV), and a combination of the two substances was more effective than IFN-α or xanthohumol alone (Zhang et al., 2010).

Scientific reports on the biological effects of hops and hop compounds are not limited to anti-viral properties; anti-bacterial activity, anti-fungal properties, and anti-malarial action have also been reported (Gerhäuser, 2005; Cermak et al., 2017; Weber et al., 2019). Lupulone and xanthohumol exhibit synergistic effects with selected clinically used antibiotics (Natarajan et al., 2008).

Hops also counteract inflammation; humulone can exert an anti-inflammatory effect on the TNF-induced expression of cyclooxygenase with effective doses that are in the same order of magnitude as dexamethasone (Yamamoto et al., 2000). Moreover, humulone can inhibit Toll-like receptor 4 (TLR4) and NF-κB signaling (Yamamoto et al., 2000; Fu et al., 2016; Schink et al., 2018b). In an animal model, topically applied humulone was shown to inhibit NF-κB, AP-1, and mitogen-activated protein kinases (MAPKs) (Lee et al., 2007). Xanthohumol from hops was found to reduce the expression of IL-1β, IL-6, IL-8, and TNF in virus-infected and LPS-stimulated porcine primary alveolar macrophages (Liu et al., 2019), and hop-derived humulone and lupulone were shown to mitigate the expression of IL-6 (Weber et al., 2019).

Hop extracts are further effective in counteracting oxidative and nitrosative stress; hop compounds were shown to mitigate neural nitric oxide synthase (nNOS) activity and 3-morpholinosydnonimine (SIN-1)-induced oxidation of LDL (Stevens et al., 2002). The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is a key regulator of the expression of antioxidant genes (Yamamoto et al., 2018). Hop compounds and especially xanthohumol were shown to activate NRF2 in different studies (Dietz et al., 2005; Lee et al., 2011; Yao et al., 2015). A small, placebo-controlled, clinical study showed that a low-intake dose of 12 mg/day xanthohumol decreased oxidative stress-induced DNA damage (Ferk et al., 2016).

Xanthohumol was also shown to be effective in preventing thrombosis in animal models (Xin et al., 2017). In contrast to what has been observed for blood thinners, no increased bleeding was seen when the substance was administrated orally in two doses of 10 mg/kg xanthohumol per day for 60 days (Xin et al., 2017). The antioxidant effects of xanthohumol were also confirmed in this same study (Xin et al., 2017). Further, hops exert anti-fibrogenic effects *in vitro*, and specifically xanthohumol was shown to possess this property *in vivo* (Dorn et al., 2012; Saugspier et al., 2012).

CEYLON CINNAMON

The genus *Cinnamomum* belongs to the family Lauraceae and comprises more than 100 species in the NCBI Taxonomy Database. Here, we review specifically Ceylon cinnamon (*Cinnamomum verum* alias *C. zeylanicum*, NCBI:txid128608), which we have used in our studies (Schink et al., 2018a,b; Ose et al., 2020). Generally, Chinese cassia or Chinese cinnamon (*Cinnamomum cassia* alias *Cinnamomum aromaticum*) is commercially sold as a spice for foods and does not represent a risk to human health *per se* (Oketch-Rabah et al., 2018). However, it can comprise high amounts of coumarin, which is highly hepatotoxic in higher doses. The German Federal Institute for Risk Assessment (BfR) therefore recommends that Cassia cinnamon with high coumarin content be consumed only at moderate levels. The differences between Ceylon cinnamon and Cassia have been reviewed recently (Oketch-Rabah et al., 2018).

Ethanolic extracts of Ceylon cinnamon possess anti-inflammatory activity and antagonize TLR2 and TLR4 activation in a dose-dependent manner with minimal effects on viability in cell culture (Kanuri et al., 2009; Schink et al., 2018b). We identified several active compounds in these extracts, amongst others, trans-cinnamaldehyde, cinnamic acid, cinnamyl alcohol, cinnamyl methyl ether, p-cymene, methyl salicylate, 1-tetradecanol (myristyl alcohol), and benzoic acid. We found synergy among the anti-inflammatory properties of the different compounds: the efficacy of the complex mixture was greater than those of the pure active compounds of cinnamon (Schink et al., 2018a).

Cinnamaldehyde is an effective NRF2 inducer (Long et al., 2015) and acts in this way to detoxify ROS/RNS (Wondrak et al., 2010). Further, cinnamaldehyde can inhibit angiogenesis and metastasis via mitigation of the PI3K/Akt pathway (Patra et al., 2019). A specific inhibition of VEGFR2 kinase and of angiogenesis was shown for a water-based extract from Ceylon cinnamon (Lu et al., 2010).

In an animal model, an extract of Ceylon cinnamon was shown to protect the aorta from dexamethasone-induced atherosclerosis and minimized the atherogenic risk (Nayak et al., 2017).

TABLE 1 | Active compounds from hops and Ceylon cinnamon.

Plant	Active compound	References
Hops	humulone	Palamand and Aldenhoff, 1973; Gerhäuser, 2005; Knez Hrncic et al., 2019; Lin et al., 2019
	lupulone	Palamand and Aldenhoff, 1973; Gerhäuser, 2005; Knez Hrncic et al., 2019; Lin et al., 2019
	xanthohumol	Palamand and Aldenhoff, 1973; Gerhäuser, 2005; Fu et al., 2016; Knez Hrncic et al., 2019; Lin et al., 2019
Ceylon cinnamon	trans-cinnamaldehyde	Schink et al., 2018a; Vasconcelos et al., 2018
	cinnamic acid	Schink et al., 2018a; Vasconcelos et al., 2018
	cinnamyl alcohol	Schink et al., 2018a

TABLE 2 | Selected clinical studies on Ceylon cinnamon.

Disease/condition or observed effect	Dosage form	References
Ceylon Cinnamon		
type 2 diabetes	encapsulated cinnamon power	Khan et al., 2003; Mang et al., 2006; Crawford, 2009; Akilen et al., 2010; Wainstein et al., 2011; Talaei et al., 2017; Mirmiran et al., 2019; Zare et al., 2019
	cinnamon extract	Lu et al., 2012; Ranasinghe et al., 2017a
	cinnamon in black tea	Azimi et al., 2016
polycystic ovary syndrome	encapsulated cinnamon power	Kort and Lobo, 2014; Borzoei et al., 2018; Hajimonfarednejad et al., 2018
dysmenorrhea (painful periods)		Jahangirifar et al., 2018
metabolic syndrome		Gupta Jain et al., 2017
migraine attacks and inflammatory markers		Zareie et al., 2020
pharmacodynamic properties and safety		Ranasinghe et al., 2017b
postmenopausal type 2 Diabetes		Vanschoonbeek et al., 2006
overweight or obese pre-diabetic subjects		Liu et al., 2015
non-alcoholic fatty liver disease		Askari et al., 2014
Ceylon cinnamon does not affect postprandial plasma glucose or insulin		Wickenberg et al., 2012
overweight or obese subjects	cinnamon extract	Roussel et al., 2009
effect on electrocardiographic parameters		Pender et al., 2018
perineal pain and healing of episiotomy		Mohammadi et al., 2014
Helicobacter pylori		Nir et al., 2000
type 1 diabetes	cinnamon pill	Altschuler et al., 2007
overactive bladder	cinnamon patch	Chen et al., 2021
postprandial (after meals) capillary blood glucose level	cinnamon tea	Bernardo et al., 2015

TABLE 3 | Selected clinical studies on hops.

Disease/condition or observed effect	Dosage form	References
Hops		
quality of sleep	standardized extracts of *Valeriana officinalis*, *Passiflora incarnate*, and *Humulus lupulus*	Maroo et al., 2013
	linolenic and linoleic acids in association with *Humulus lupulus* extract.	Cornu et al., 2010
	valerian/hop extract combination	Müller-Limmroth and Ehrenstein, 1977; Koetter et al., 2007; Dimpfel and Suter, 2008
Vigilance	tablets containing valerian and hops	Gerhard et al., 1996
menopausal symptoms	hop extract	Erkkola et al., 2010; van Breemen et al., 2020
	hop tablets	Aghamiri et al., 2016
appetite suppression	hop flower extract suspended in canola oil	Walker et al., 2019
body fat	matured hop extract	Morimoto-Kobayashi et al., 2016; Suzuki et al., 2018
dental plaque regrowth	hop bract polyphenols	Shinada et al., 2007
clinical safety and efficacy	combination of iso-alpha acids from hops, rosemary, and oleanolic acid	Minich et al., 2007
self-reported depression, anxiety, and stress levels	dry hop extract	Kyrou et al., 2017
overactive bladder	combination of seed oil from Uromedic pumpkin, Rhus aromatica (bark extract, and hop cone extract)	Gauruder-Burmester et al., 2019
intestinal conversion of isoxanthohumol in 8-prenylnaringenin	dose of isoxanthohumol	Possemiers et al., 2006
endothelial functions	isomerized hop extract	Tomita et al., 2017

RETRIEVED RESULTS FROM CLINICAL STUDIES

We searched for clinical studies on Cinnamon using the search term "Ceylon Cinnamon OR Cinnamaldehyde," while for clinical studies on hops we used the search term "Humulone OR Lupulone OR Xanthohumol OR hops" (**Tables 2**, **3**). For both search terms, we restricted the results to "Clinical Trial." For Cinnamon we obtained 123 hits, from which we excluded 57 hits, as cinnamon was not used to treat human disease in these studies. The ethanolic extract, as used in cell culture experiments, was not directly given to patients in any of the clinical studies. Rather, for the majority of clinical studies with Ceylon cinnamon, powder was administered in an encapsulated form; the clear advantage of administering Ceylon cinnamon in this way is that the formulation does not comprise ethanol. Since we cannot give here an appropriate dosage of cinnamon for the treatment of COVID-19, we refer to clinical studies that have used cinnamon to treat other conditions. Thirty of the clinical Cinnamon studies deal with diabetes, glucose levels, and insulin tolerance, five with polycystic ovary syndrome, and three with overweight and obesity. We could not find any clinical studies on "cytokine storms," but we hypothesize that the strong anti-inflammatory properties of Ceylon cinnamon may mitigate this complication.

Ranasinghe et al. evaluated the safety of Ceylon cinnamon in healthy adults, concluding that there were no significant side effects and toxicity of Ceylon cinnamon for the dosages applied (Ranasinghe et al., 2017b).

When searching for hops and compounds found in hops, we obtained 92 hits for clinical studies, from which we excluded 50 as non-matching. This is because many hits refer to the verb "to hop," i.e., "to jump." Eight studies are linked to sleep disorders, six to adiposity and metabolic syndrome, six to menopause, and two clinical studies are on anti-bacterial effects. Often hops are given orally as an extract (water, oil), which is frequently administered in combination with other drugs such as valerian. One study concerns specifically the use of *iso*-alpha-acids from hops, which include humulone, to dampen inflammation in knee osteoarthritis (Hall et al., 2008).

Taken together, among the so-far performed clinical studies on Ceylon cinnamon and hops, we could not find any that specifically described efficacy in preventing the "cytokine storm" or sepsis. However, results from diverse cell culture experiments make it likely that hops and Ceylon cinnamon may exert these effects.

DISCUSSION

Both hop and cinnamon extracts have been shown to exert several anti-inflammatory functions (Yamamoto et al., 2000; Schink et al., 2018a,b). For instance, both can dampen the release of pro-inflammatory cytokines (Lee et al., 2007; Schink et al., 2018b; Liu et al., 2019; Weber et al., 2019). Moreover, hop and cinnamon extracts can inhibit angiogenesis, thrombosis, and vascular endothelialitis (Dorn et al., 2012; Saugspier et al., 2012; Xin et al., 2017; Patra et al., 2019). Further, these herbal extracts can activate the key regulator of the antioxidant response, NRF2, which mitigates the ROS/RNS production generally associated with inflammation (Dietz et al., 2005, 2013; Wondrak et al., 2010; Lee et al., 2011; Pinto et al., 2014; Long et al., 2015; Yao et al., 2015). Taken together, we suggest that hop and Ceylon cinnamon extracts may ameliorate complications that are associated with severe cases of COVID-19 and that testing both extracts, either alone or in combination, and particularly as a supplemental treatment to other medications, might be a promising therapeutic approach. If the preliminary results for dexamethasone can be confirmed, this glucocorticoid may be widely used to treat cases of COVID-19 (Horby et al., 2020). Supplementation with Ceylon cinnamon extract could then ameliorate the potential side effects of dexamethasone such as atherosclerosis (Nayak et al., 2017). Hop extracts exert anti-viral effects against some virus strains, but whether these extracts are also effective against SARS-CoV-2 has not yet been experimentally verified (Buckwold et al., 2004; Zhang et al., 2010; Fuchimoto et al., 2013).

Pneumonia caused by bacteria is a frequent complication after artificial ventilation (Póvoa et al., 2020; Wu et al., 2020; Zhang et al., 2020). The anti-bacterial effects of hops (Gerhäuser, 2005; Natarajan et al., 2008; Cermak et al., 2017; Weber et al., 2019) and Ceylon cinnamon (Ranasinghe et al., 2013; Vasconcelos et al., 2018; Doyle and Stephens, 2019) could act preventatively in such cases. As they are derived from common foodstuffs, both hop and Ceylon cinnamon extracts can be regarded as safe. Of course, an allergy against a hop or cinnamon ingredient or alcohol intolerance would contraindicate their intake.

It has become clear that many COVID-19 patients suffer from inflammatory complications (Heneka et al., 2020; Portincasa et al., 2020). We suggest that treatment with the here-discussed extracts could also mitigate such complications. Should cinnamon and hops prove to exert positive effects in the treatment of COVID-19, they would be readily available at low cost and can be produced at multi-ton scales. In western medicine, it is common to use pure substances rather than less well-defined herbal extracts. Of course, the individual compounds as listed in **Table 1** can be used for treatment, but a certain loss of synergy may result (Schink et al., 2018a).

In conclusion, we recommend future experiments on hops and Ceylon cinnamon to evaluate their potential in limiting overshooting immune reactions in COVID-19. We work mainly with ethanolic extracts in cell culture. However, encapsulated cinnamon powder or water extracts of hops may be better suited for administration to patients. We suggest that appropriate doses for treatment of COVID-19 patients may be determined with reference to the clinical studies that have used hops and Ceylon cinnamon to treat other conditions.

AUTHOR CONTRIBUTIONS

KL and MA initiated the idea of the review and were involved in the manuscript writing. J-FN was involved in manuscript refinement. NO was involved in the literature search. All authors contributed to the article and approved the submitted version.

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REFERENCES

Ackermann, M., Verleden, S. E., Kuehnel, M., Haverich, A., Welte, T., Laenger, F., et al. (2020). Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. *N. Engl. J. Med.* 383, 120–128. doi: 10.1056/NEJMoa2015432

Aghamiri, V., Mirghafourvand, M., Mohammad-Alizadeh-Charandabi, S., and Nazemiyeh, H. (2016). The effect of Hop (*Humulus lupulus* L.) on early menopausal symptoms and hot flashes: a randomized placebo-controlled trial. *Complement. Ther. Clin. Pract.* 23, 130–135. doi: 10.1016/j.ctcp.2015.05.001

Akilen, R., Tsiami, A., Devendra, D., and Robinson, N. (2010). Glycated haemoglobin and blood pressure-lowering effect of cinnamon in multi-ethnic Type 2 diabetic patients in the UK: a randomized, placebo-controlled, double-blind clinical trial. *Diabet. Med.* 27, 1159–1167. doi: 10.1111/j.1464-5491.2010.03079.x

Altschuler, J. A., Casella, S. J., Mackenzie, T. A., and Curtis, K. M. (2007). The effect of cinnamon on A1C among adolescents with type 1 diabetes. *Diabetes Care* 30, 813–816. doi: 10.2337/dc06-1871

Askari, F., Rashidkhani, B., and Hekmatdoost, A. (2014). Cinnamon may have therapeutic benefits on lipid profile, liver enzymes, insulin resistance, and high-sensitivity C-reactive protein in nonalcoholic fatty liver disease patients. *Nutr. Res.* 34, 143–148. doi: 10.1016/j.nutres.2013.11.005

Azimi, P., Ghiasvand, R., Feizi, A., Hosseinzadeh, J., Bahreynian, M., Hariri, M., et al. (2016). Effect of cinnamon, cardamom, saffron, and ginger consumption on blood pressure and a marker of endothelial function in patients with type 2 diabetes mellitus: a randomized controlled clinical trial. *Blood Press.* 25, 133–140. doi: 10.3109/08037051.2015.1111020

Bernardo, M. A., Silva, M. L., Santos, E., Moncada, M. M., Brito, J., Proença, L., et al. (2015). Effect of cinnamon tea on postprandial glucose concentration. *J. Diabetes Res.* 2015:913651. doi: 10.1155/2015/913651

Borzoei, A., Rafraf, M., and Asghari-Jafarabadi, M. (2018). Cinnamon improves metabolic factors without detectable effects on adiponectin in women with polycystic ovary syndrome. *Asia Pac. J. Clin. Nutr.* 27, 556–563. doi: 10.6133/apjcn.062017.13

Buckwold, V. E., Wilson, R. J., Nalca, A., Beer, B. B., Voss, T. G., Turpin, J. A., et al. (2004). Antiviral activity of hop constituents against a series of DNA and RNA viruses. *Antiviral Res.* 61, 57–62. doi: 10.1016/S0166-3542(03)00155-4

Cermak, P., Olsovska, J., Mikyska, A., Dusek, M., Kadleckova, Z., Vanicek, J., et al. (2017). Strong antimicrobial activity of xanthohumol and other derivatives from hops (*Humulus lupulus* L.) on gut anaerobic bacteria. *Apmis* 125, 1033–1038. doi: 10.1111/apm.12747

Chen, L. L., Shen, Y. C., Ke, C. C., Imtiyaz, Z., Chen, H. I., Chang, C. H., et al. (2021). Efficacy of cinnamon patch treatment for alleviating symptoms of overactive bladder: a double-blind, randomized, placebo-controlled trial. *Phytomedicine* 80:153380. doi: 10.1016/j.phymed.2020.153380

Cornu, C., Remontet, L., Noel-Baron, F., Nicolas, A., Feugier-Favier, N., Roy, P., et al. (2010). A dietary supplement to improve the quality of sleep: a randomized placebo controlled trial. *BMC Complement. Altern. Med.* 10:29. doi: 10.1186/1472-6882-10-29

Crawford, P. (2009). Effectiveness of cinnamon for lowering hemoglobin A1C in patients with type 2 diabetes: a randomized, controlled trial. *J. Am. Board Fam. Med.* 22, 507–512. doi: 10.3122/jabfm.2009.05.080093

Dietz, B. M., Hagos, G. K., Eskra, J. N., Wijewickrama, G. T., Anderson, J. R., Nikolic, D., et al. (2013). Differential regulation of detoxification enzymes in hepatic and mammary tissue by hops *(Humulus lupulus*) *in vitro* and *in vivo*. *Mol. Nutr. Food Res.* 57, 1055–1066. doi: 10.1002/mnfr.201200534

Dietz, B. M., Kang, Y. H., Liu, G., Eggler, A. L., Yao, P., Chadwick, L. R., et al. (2005). Xanthohumol isolated from Humulus lupulus Inhibits menadione-induced DNA damage through induction of quinone reductase. *Chem. Res. Toxicol.* 18, 1296–1305. doi: 10.1021/tx050058x

Dimpfel, W., and Suter, A. (2008). Sleep improving effects of a single dose administration of a valerian/hops fluid extract–a double blind, randomized, placebo-controlled sleep-EEG study in a parallel design using electrohypnograms. *Eur. J. Med. Res.* 13, 200–204. doi: 10.1055/s-2008-1047853

Dorn, C., Heilmann, J., and Hellerbrand, C. (2012). Protective effect of xanthohumol on toxin-induced liver inflammation and fibrosis. *Int. J. Clin. Exp. Pathol.* 5, 29–36. doi: 10.1055/s-0031-1295738

Doyle, A. A., and Stephens, J. C. (2019). A review of cinnamaldehyde and its derivatives as antibacterial agents. *Fitoterapia* 139:104405. doi: 10.1016/j.fitote.2019.104405

Erkkola, R., Vervarcke, S., Vansteelandt, S., Rompotti, P., De Keukeleire, D., and Heyerick, A. (2010). A randomized, double-blind, placebo-controlled, cross-over pilot study on the use of a standardized hop extract to alleviate menopausal discomforts. *Phytomedicine* 17, 389–396. doi: 10.1016/j.phymed.2010.01.007

Ferk, F., Mišík, M., Nersesyan, A., Pichler, C., Jäger, W., Szekeres, T., et al. (2016). Impact of xanthohumol (a prenylated flavonoid from hops) on DNA stability and other health-related biochemical parameters: results of human intervention trials. *Mol. Nutr. Food Res.* 60, 773–786. doi: 10.1002/mnfr.201500355

Fu, W., Chen, L., Wang, Z., Zhao, C., Chen, G., Liu, X., et al. (2016). Determination of the binding mode for anti-inflammatory natural product xanthohumol with myeloid differentiation protein 2. *Drug Des. Dev. Ther.* 10, 455–463. doi: 10.2147/DDDT.S98466

Fuchimoto, J., Kojima, T., Okabayashi, T., Masaki, T., Ogasawara, N., Obata, K., et al. (2013). Humulone suppresses replication of respiratory syncytial virus and release of IL-8 and RANTES in normal human nasal epithelial cells. *Med. Mol. Morphol.* 46, 203–209. doi: 10.1007/s00795-013-0024-1

Gauruder-Burmester, A., Heim, S., Patz, B., and Seibt, S. (2019). *Cucurbita pepo-Rhus aromatica-Humulus lupulus* combination reduces overactive bladder symptoms in women–a noninterventional study. *Planta Med.* 85, 1044–1053. doi: 10.1055/a-0946-2280

Gerhard, U., Linnenbrink, N., Georghiadou, C., and Hobi, V. (1996). [Vigilance-decreasing effects of 2 plant-derived sedatives]. *Praxis (Bern 1994)* 85, 473–481.

Gerhäuser, C. (2005). Broad spectrum anti-infective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hop constituents and xanthohumol metabolites. *Mol. Nutr. Food Res.* 49, 827–831. doi: 10.1002/mnfr.200500091

Gupta Jain, S., Puri, S., Misra, A., Gulati, S., and Mani, K. (2017). Effect of oral cinnamon intervention on metabolic profile and body composition of Asian Indians with metabolic syndrome: a randomized double-blind control trial. *Lipids Health Dis.* 16:113. doi: 10.1186/s12944-017-0504-8

Hajimonfarednejad, M., Nimrouzi, M., Heydari, M., Zarshenas, M. M., Raee, M. J., and Jahromi, B. N. (2018). Insulin resistance improvement by cinnamon powder in polycystic ovary syndrome: a randomized double-blind placebo controlled clinical trial. *Phytother. Res.* 32, 276–283. doi: 10.1002/ptr.5970

Hall, A. J., Babish, J. G., Darland, G. K., Carroll, B. J., Konda, V. R., Lerman, R. H., et al. (2008). Safety, efficacy, and anti-inflammatory activity of rho iso-alpha-acids from hops. *Phytochemistry* 69, 1534–1547. doi: 10.1016/j.phytochem.2008.02.001

Heneka, M. T., Golenbock, D., Latz, E., Morgan, D., and Brown, R. (2020). Immediate and long-term consequences of COVID-19 infections for the development of neurological disease. *Alzheimers Res. Ther.* 12:69. doi: 10.1186/s13195-020-00640-3

Horby, P., Lim, W. S., Emberson, J. R., Mafham, M., Bell, J. L., Linsell, L., et al. (2020). Dexamethasone in hospitalized patients with Covid-19–preliminary report. *N. Engl. J. Med.* doi: 10.1101/2020.06.22.20137273

Jahangirifar, M., Taebi, M., and Dolatian, M. (2018). The effect of cinnamon on primary dysmenorrhea: a randomized, double-blind clinical trial. *Complement. Ther. Clin. Pract.* 33, 56–60. doi: 10.1016/j.ctcp.2018.08.001

Kanuri, G., Weber, S., Volynets, V., Spruss, A., Bischoff, S. C., and Bergheim, I. (2009). Cinnamon extract protects against acute alcohol-induced liver steatosis in mice. *J. Nutr.* 139, 482–487. doi: 10.3945/jn.108.100495

Khan, A., Safdar, M., Ali Khan, M. M., Khattak, K. N., and Anderson, R. A. (2003). Cinnamon improves glucose and lipids of people with type 2 diabetes. *Diabetes Care* 26, 3215–3218. doi: 10.2337/diacare.26.12.3215

Knez Hrncic, M., Spaninger, E., Kosir, I. J., Knez, Z., and Bren, U. (2019). Hop compounds: extraction techniques, chemical analyses, antioxidative, antimicrobial, and anticarcinogenic effects. *Nutrients* 11:257. doi: 10.3390/nu11020257

Koetter, U., Schrader, E., Käufeler, R., and Brattström, A. (2007). A randomized, double blind, placebo-controlled, prospective clinical study to demonstrate clinical efficacy of a fixed valerian hops extract combination (Ze 91019) in patients suffering from non-organic sleep disorder. *Phytother. Res.* 21, 847–851. doi: 10.1002/ptr.2167

Kort, D. H., and Lobo, R. A. (2014). Preliminary evidence that cinnamon improves menstrual cyclicity in women with polycystic ovary syndrome: a randomized controlled trial. *Am. J. Obstet. Gynecol.* 211, 487.e1–487.e6. doi: 10.1016/j.ajog.2014.05.009

Kyrou, I., Christou, A., Panagiotakos, D., Stefanaki, C., Skenderi, K., Katsana, K., et al. (2017). Effects of a hops (*Humulus lupulus* L.) dry extract supplement on self-reported depression, anxiety, and stress levels in apparently healthy young adults: a randomized, placebo-controlled, double-blind, crossover pilot study. *Hormones (Athens)* 16, 171–180. doi: 10.14310/horm.2002.1738

Lee, I. S., Lim, J., Gal, J., Kang, J. C., Kim, H. J., Kang, B. Y., et al. (2011). Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochem. Int.* 58, 153–160. doi: 10.1016/j.neuint.2010.11.008

Lee, J. C., Kundu, J. K., Hwang, D. M., Na, H. K., and Surh, Y. J. (2007). Humulone inhibits phorbol ester-induced COX-2 expression in mouse skin by blocking activation of NF-kappaB and AP-1: IkappaB kinase and c-Jun-N-terminal

kinase as respective potential upstream targets. *Carcinogenesis* 28, 1491–1498. doi: 10.1093/carcin/bgm054

Lin, M., Xiang, D., Chen, X., and Huo, H. (2019). Role of characteristic components of humulus lupulus in promoting human health. *J. Agric. Food Chem.* 67, 8291–8302. doi: 10.1021/acs.jafc.9b03780

Liu, X., Bai, J., Jiang, C., Song, Z., Zhao, Y., Nauwynck, H., et al. (2019). Therapeutic effect of Xanthohumol against highly pathogenic porcine reproductive and respiratory syndrome viruses. *Vet. Microbiol.* 238:108431. doi: 10.1016/j.vetmic.2019.108431

Liu, Y., Cotillard, A., Vatier, C., Bastard, J. P., Fellahi, S., Stévant, M., et al. (2015). A dietary supplement containing cinnamon, chromium, and carnosine decreases fasting plasma glucose and increases lean mass in overweight or obese pre-diabetic subjects: a randomized, placebo-controlled trial. *PLoS ONE* 10:e0138646. doi: 10.1145/2818302

Long, M., Tao, S., Rojo De La Vega, M., Jiang, T., Wen, Q., Park, S. L., et al. (2015). Nrf2-dependent suppression of azoxymethane/dextran sulfate sodium-induced colon carcinogenesis by the cinnamon-derived dietary factor cinnamaldehyde. *Cancer Prev. Res. (Phila)* 8, 444–454. doi: 10.1158/1940-6207.CAPR-14-0359

Lu, J., Zhang, K., Nam, S., Anderson, R. A., Jove, R., and Wen, W. (2010). Novel angiogenesis inhibitory activity in cinnamon extract blocks VEGFR2 kinase and downstream signaling. *Carcinogenesis* 31, 481–488. doi: 10.1093/carcin/bgp292

Lu, T., Sheng, H., Wu, J., Cheng, Y., Zhu, J., and Chen, Y. (2012). Cinnamon extract improves fasting blood glucose and glycosylated hemoglobin level in Chinese patients with type 2 diabetes. *Nutr. Res.* 32, 408–412. doi: 10.1016/j.nutres.2012.05.003

Lucas, K., and Maes, M. (2013). Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. *Mol. Neurobiol.* 48, 190–204. doi: 10.1007/s12035-013-8425-7

Mang, B., Wolters, M., Schmitt, B., Kelb, K., Lichtinghagen, R., Stichtenoth, D. O., et al. (2006). Effects of a cinnamon extract on plasma glucose, HbA, and serum lipids in diabetes mellitus type 2. *Eur. J. Clin. Invest.* 36, 340–344. doi: 10.1111/j.1365-2362.2006.01629.x

Maroo, N., Hazra, A., and Das, T. (2013). Efficacy and safety of a polyherbal sedative-hypnotic formulation NSF-3 in primary insomnia in comparison to zolpidem: a randomized controlled trial. *Indian J. Pharmacol.* 45, 34–39. doi: 10.4103/0253-7613.106432

Minich, D. M., Bland, J. S., Katke, J., Darland, G., Hall, A., Lerman, R. H., et al. (2007). Clinical safety and efficacy of NG440: a novel combination of rho iso-alpha acids from hops, rosemary, and oleanolic acid for inflammatory conditions. *Can. J. Physiol. Pharmacol.* 85, 872–883. doi: 10.1139/Y07-055

Mirmiran, P., Davari, M., Hashemi, R., Hedayati, M., Sahranavard, S., Bahreini, S., et al. (2019). A randomized controlled trial to determining the effect of cinnamon on the plasma levels of soluble forms of vascular adhesion molecules in type 2 diabetes mellitus. *Eur. J. Clin. Nutr.* 73, 1605–1612. doi: 10.1038/s41430-019-0523-9

Mohammadi, A., Mohammad-Alizadeh-Charandabi, S., Mirghafourvand, M., Javadzadeh, Y., Fardiazar, Z., and Effati-Daryani, F. (2014). Effects of cinnamon on perineal pain and healing of episiotomy: a randomized placebo-controlled trial. *J. Integr. Med.* 12, 359–366. doi: 10.1016/S2095-4964(14)60025-X

Morimoto-Kobayashi, Y., Ohara, K., Ashigai, H., Kanaya, T., Koizumi, K., Manabe, F., et al. (2016). Matured hop extract reduces body fat in healthy overweight humans: a randomized, double-blind, placebo-controlled parallel group study. *Nutr. J.* 15:25. doi: 10.1186/s12937-016-0144-2

Müller-Limmroth, W., and Ehrenstein, W. (1977). [Experimental studies of the effects of Seda-Kneipp on the sleep of sleep disturbed subjects; implications for the treatment of different sleep disturbances (author's transl)]. *Med. Klin.* 72, 1119–1125.

Natarajan, P., Katta, S., Andrei, I., Babu Rao Ambati, V., Leonida, M., and Haas, G. J. (2008). Positive antibacterial co-action between hop (*Humulus lupulus*) constituents and selected antibiotics. *Phytomedicine* 15, 194–201. doi: 10.1016/j.phymed.2007.10.008

Nayak, I. N., Chinta, R., and Jetti, R. (2017). Anti-atherosclerotic potential of aqueous extract of cinnamomum zeylanicum Bark against glucocorticoid induced atherosclerosis in wistar rats. *J. Clin. Diagn. Res.* 11, Fc19–fc23. doi: 10.7860/JCDR/2017/23910.9864

Nir, Y., Potasman, I., Stermer, E., Tabak, M., and Neeman, I. (2000). Controlled trial of the effect of cinnamon extract on Helicobacter pylori. *Helicobacter* 5, 94–97. doi: 10.1046/j.1523-5378.2000.00014.x

Oketch-Rabah, H. A., Marles, R. J., and Brinckmann, J. A. (2018). Cinnamon and cassia nomenclature confusion: a challenge to the applicability of clinical data. *Clin. Pharmacol. Ther.* 104, 435–445. doi: 10.1002/cpt.1162

Ose, R., Tu, J., Schink, A., Maxeiner, J., Schuster, P., Lucas, K., et al. (2020). Cinnamon extract inhibits allergen-specific immune responses in human and murine allergy models. *Clin. Exp. Allergy* 50, 41–50. doi: 10.1111/cea.13507

Palamand, S. R., and Aldenhoff, J. M. (1973). Bitter tasting compounds of beer. Chemistry and taste properties of some hop resin compounds. *J. Agric. Food Chem.* 21, 535–543. doi: 10.1021/jf60188a005

Patra, K., Jana, S., Sarkar, A., Mandal, D. P., and Bhattacharjee, S. (2019). The inhibition of hypoxia-induced angiogenesis and metastasis by cinnamaldehyde is mediated by decreasing HIF-1α protein synthesis via PI3K/Akt pathway. *Biofactors* 45, 401–415. doi: 10.1002/biof.1499

Pender, D. N., Crawford, P. F., Clark, J. M., Crawford, A. J., Prats, A. A., and Shah, S. A. (2018). Effect of water-soluble cinnamon extract on electrocardiographic parameters: an analysis of the CiNNaMON trial. *Complement. Ther. Med.* 41, 302–305. doi: 10.1016/j.ctim.2018.10.009

Pinto, C., Cestero, J. J., Rodríguez-Galdón, B., and Macías, P. (2014). Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus* L.), protects rat tissues against oxidative damage after acute ethanol administration. *Toxicol. Rep.* 1, 726–733. doi: 10.1016/j.toxrep.2014.09.004

Portincasa, P., Krawczyk, M., Machill, A., Lammert, F., and Di Ciaula, A. (2020). Hepatic consequences of COVID-19 infection. Lapping or biting? *Eur. J. Intern. Med.* 77, 18–24. doi: 10.1016/j.ejim.2020.05.035

Possemiers, S., Bolca, S., Grootaert, C., Heyerick, A., Decroos, K., Dhooge, W., et al. (2006). The prenylflavonoid isoxanthohumol from hops (*Humulus lupulus* L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. *J. Nutr.* 136, 1862–1867. doi: 10.1093/jn/136.7.1862

Póvoa, H. C. C., Chianca, G. C., and Iorio, N. (2020). COVID-19: an alert to ventilator-associated bacterial pneumonia. *Infect. Dis. Ther.* 9, 1–4. doi: 10.1007/s40121-020-00306-5

Ranasinghe, P., Galappaththy, P., Constantine, G. R., Jayawardena, R., Weeratunga, H. D., Premakumara, S., et al. (2017a). Cinnamomum zeylanicum (Ceylon cinnamon) as a potential pharmaceutical agent for type-2 diabetes mellitus: study protocol for a randomized controlled trial. *Trials* 18:446. doi: 10.1186/s13063-017-2192-0

Ranasinghe, P., Jayawardena, R., Pigera, S., Wathurapatha, W. S., Weeratunga, H. D., Premakumara, G., et al. (2017b). Evaluation of pharmacodynamic properties and safety of Cinnamomum zeylanicum (Ceylon cinnamon) in healthy adults: a phase I clinical trial. *BMC Complement. Altern. Med.* 17:550. doi: 10.1186/s12906-017-2067-7

Ranasinghe, P., Pigera, S., Premakumara, G. A., Galappaththy, P., Constantine, G. R., and Katulanda, P. (2013). Medicinal properties of 'true' cinnamon (Cinnamomum zeylanicum): a systematic review. *BMC Complement. Altern. Med.* 13:275. doi: 10.1186/1472-6882-13-275

Renu, K., Prasanna, P. L., and Valsala Gopalakrishnan, A. (2020). Coronaviruses pathogenesis, comorbidities, and multi-organ damage–a review. *Life Sci.* 255:117839. doi: 10.1016/j.lfs.2020.117839

Roussel, A. M., Hininger, I., Benaraba, R., Ziegenfuss, T. N., and Anderson, R. A. (2009). Antioxidant effects of a cinnamon extract in people with impaired fasting glucose that are overweight or obese. *J. Am. Coll. Nutr.* 28, 16–21. doi: 10.1080/07315724.2009.10719756

Saugspier, M., Dorn, C., Thasler, W. E., Gehrig, M., Heilmann, J., and Hellerbrand, C. (2012). Hop bitter acids exhibit anti-fibrogenic effects on hepatic stellate cells *in vitro*. *Exp. Mol. Pathol.* 92, 222–228. doi: 10.1016/j.yexmp.2011.11.005

Schink, A., Naumoska, K., Kitanovski, Z., Kampf, C. J., Fröhlich-Nowoisky, J., Thines, E., et al. (2018a). Anti-inflammatory effects of cinnamon extract and identification of active compounds influencing the TLR2 and TLR4 signaling pathways. *Food Funct.* 9, 5950–5964. doi: 10.1039/C8FO01286E

Schink, A., Neumann, J., Leifke, A. L., Ziegler, K., Fröhlich-Nowoisky, J., Cremer, C., et al. (2018b). Screening of herbal extracts for TLR2- and TLR4-dependent anti-inflammatory effects. *PLoS ONE* 13:e0203907. doi: 10.1371/journal.pone.0203907

Shinada, K., Tagashira, M., Watanabe, H., Sopapornamorn, P., Kanayama, A., Kanda, T., et al. (2007). Hop bract polyphenols reduced three-day dental plaque regrowth. *J. Dent. Res.* 86, 848–851. doi: 10.1177/154405910708600908

Stevens, J. F., Miranda, C. L., Wolthers, K. R., Schimerlik, M., Deinzer, M. L., and Buhler, D. R. (2002). Identification and *in vitro* biological activities of

hop proanthocyanidins: inhibition of nNOS activity and scavenging of reactive nitrogen species. *J. Agric. Food Chem.* 50, 3435–3443. doi: 10.1021/jf0116202

Suzuki, S., Yamazaki, T., Takahashi, C., Kaneko, Y., Morimoto-Kobayashi, Y., and Katayama, M. (2018). The relationship between the effect of matured hop extract and physical activity on reducing body fat: re-analysis of data from a randomized, double-blind, placebo-controlled parallel group study. *Nutr. J.* 17:98. doi: 10.1186/s12937-018-0405-3

Talaei, B., Amouzegar, A., Sahranavard, S., Hedayati, M., Mirmiran, P., and Azizi, F. (2017). Effects of cinnamon consumption on glycemic indicators, advanced glycation end products, and antioxidant status in type 2 diabetic patients. *Nutrients* 9:991. doi: 10.3390/nu9090991

Tomita, J., Mochizuki, S., Fujimoto, S., Kashihara, N., Akasaka, T., Tanimoto, M., et al. (2017). Acute improvement of endothelial functions after oral ingestion of isohumulones, bitter components of beer. *Biochem. Biophys. Res. Commun.* 484, 740–745. doi: 10.1016/j.bbrc.2017.01.133

van Breemen, R. B., Chen, L., Tonsing-Carter, A., Banuvar, S., Barengolts, E., Viana, M., et al. (2020). Pharmacokinetic interactions of a hop dietary supplement with drug metabolism in perimenopausal and postmenopausal women. *J. Agric. Food Chem.* 68, 5212–5220. doi: 10.1021/acs.jafc.0c01077

Vanschoonbeek, K., Thomassen, B. J., Senden, J. M., Wodzig, W. K., and Van Loon, L. J. (2006). Cinnamon supplementation does not improve glycemic control in postmenopausal type 2 diabetes patients. *J. Nutr.* 136, 977–980. doi: 10.1093/jn/136.4.977

Vasconcelos, N. G., Croda, J., and Simionatto, S. (2018). Antibacterial mechanisms of cinnamon and its constituents: a review. *Microb. Pathog.* 120, 198–203. doi: 10.1016/j.micpath.2018.04.036

Wainstein, J., Stern, N., Heller, S., and Boaz, M. (2011). Dietary cinnamon supplementation and changes in systolic blood pressure in subjects with type 2 diabetes. *J. Med. Food* 14, 1505–1510. doi: 10.1089/jmf.2010.0300

Walker, E., Lo, K., Tham, S., Pahl, M., Lomiwes, D., Cooney, J., et al. (2019). New Zealand bitter hops extract reduces hunger during a 24 h water only fast. *Nutrients* 11:2754. doi: 10.3390/nu11112754

Wang, T., Du, Z., Zhu, F., Cao, Z., An, Y., Gao, Y., et al. (2020). Comorbidities and multi-organ injuries in the treatment of COVID-19. *Lancet* 395:e52. doi: 10.1016/S0140-6736(20)30558-4

Weber, N., Biehler, K., Schwabe, K., Haarhaus, B., Quirin, K. W., Frank, U., et al. (2019). Hop extract acts as an antioxidant with antimicrobial effects against propionibacterium acnes and staphylococcus aureus. *Molecules* 24:223. doi: 10.3390/molecules24020223

Wickenberg, J., Lindstedt, S., Berntorp, K., Nilsson, J., and Hlebowicz, J. (2012). Ceylon cinnamon does not affect postprandial plasma glucose or insulin in subjects with impaired glucose tolerance. *Br. J. Nutr.* 107, 1845–1849. doi: 10.1017/S0007114511005113

Wondrak, G. T., Villeneuve, N. F., Lamore, S. D., Bause, A. S., Jiang, T., and Zhang, D. D. (2010). The cinnamon-derived dietary factor cinnamic aldehyde activates the Nrf2-dependent antioxidant response in human epithelial colon cells. *Molecules* 15, 3338–3355. doi: 10.3390/molecules15053338

Wu, C. P., Adhi, F., and Highland, K. (2020). Recognition and management of respiratory coinfection and secondary bacterial pneumonia in patients with COVID-19. *Cleve. Clin. J. Med.* 87, 659–663. doi: 10.3949/ccjm.87a.ccc015

Xin, G., Wei, Z., Ji, C., Zheng, H., Gu, J., Ma, L., et al. (2017). Xanthohumol isolated from *Humulus lupulus* prevents thrombosis without increased bleeding risk by inhibiting platelet activation and mtDNA release. *Free Radic. Biol. Med.* 108, 247–257. doi: 10.1016/j.freeradbiomed.2017.02.018

Yamamoto, K., Wang, J., Yamamoto, S., and Tobe, H. (2000). Suppression of cyclooxygenase-2 gene transcription by humulon of beer hop extract studied with reference to glucocorticoid. *FEBS Lett.* 465, 103–106. doi: 10.1016/S0014-5793(99)01727-5

Yamamoto, M., Kensler, T. W., and Motohashi, H. (2018). The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98, 1169–1203. doi: 10.1152/physrev.00023.2017

Yao, J., Zhang, B., Ge, C., Peng, S., and Fang, J. (2015). Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells. *J. Agric. Food Chem.* 63, 1521–1531. doi: 10.1021/jf505075n

Ye, Q., Wang, B., and Mao, J. (2020). The pathogenesis and treatment of the "Cytokine Storm" in COVID-19. *J. Infect.* 80, 607–613. doi: 10.1016/j.jinf.2020.03.037

Zare, R., Nadjarzadeh, A., Zarshenas, M. M., Shams, M., and Heydari, M. (2019). Efficacy of cinnamon in patients with type II diabetes mellitus: a randomized controlled clinical trial. *Clin. Nutr.* 38, 549–556. doi: 10.1016/j.clnu.2018.03.003

Zareie, A., Sahebkar, A., Khorvash, F., Bagherniya, M., Hasanzadeh, A., and Askari, G. (2020). Effect of cinnamon on migraine attacks and inflammatory markers: a randomized double-blind placebo-controlled trial. *Phytother. Res.* 34, 2945–2952. doi: 10.1002/ptr.6721

Zhang, H., Zhang, Y., Wu, J., Li, Y., Zhou, X., Li, X., et al. (2020). Risks and features of secondary infections in severe and critical ill COVID-19 patients. *Emerg. Microbes Infect.* 9, 1958–1964. doi: 10.1080/22221751.2020.1812437

Zhang, N., Liu, Z., Han, Q., Chen, J., and Lv, Y. (2010). Xanthohumol enhances antiviral effect of interferon α-2b against bovine viral diarrhea virus, a surrogate of hepatitis C virus. *Phytomedicine* 17, 310–316. doi: 10.1016/j.phymed.2009.08.005

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Plant *in vitro* Culture Technologies; A Promise Into Factories of Secondary Metabolites Against COVID-19

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The current pandemic has caused chaos throughout the world. While there are few vaccines available now, there is the need for better treatment alternatives in line with preventive measures against COVID-19. Along with synthetic chemical compounds, phytochemicals cannot be overlooked as candidates for drugs against severe respiratory coronavirus 2 (SARS-CoV-2). The important role of secondary metabolites or phytochemical compounds against coronaviruses has been confirmed by studies that reported the anti-coronavirus role of glycyrrhizin from the roots of *Glycyrrhiza glabra*. The study demonstrated that glycyrrhizin is a very promising phytochemical against SARS-CoV, which caused an outbreak in 2002–2003. Similarly, many phytochemical compounds (apigenin, betulonic acid, reserpine, emodin, etc.) were isolated from different plants such as *Isatis indigotica*, *Lindera aggregate*, and *Artemisia annua* and were employed against SARS-CoV. However, owing to the geographical and seasonal variation, the quality of standard medicinal compounds isolated from plants varies. Furthermore, many of the important medicinal plants are either threatened or on the verge of endangerment because of overharvesting for medicinal purposes. Therefore, plant biotechnology provides a better alternative in the form of *in vitro* culture technology, including plant cell cultures, adventitious roots cultures, and organ and tissue cultures. *In vitro* cultures can serve as factories of secondary metabolites/phytochemicals that can be produced in bulk and of uniform quality in the fight against COVID-19, once tested. Similarly, environmental and molecular manipulation of these *in vitro* cultures could provide engineered drug candidates for testing against COVID-19. The *in vitro* culture-based phytochemicals have an additional benefit of consistency in terms of yield as well as quality. Nonetheless, as the traditional plant-based compounds might prove toxic in some cases, engineered production of promising phytochemicals can bypass this barrier. Our article focuses on reviewing the potential of the different *in vitro plant* cultures to produce medicinally important secondary metabolites that could ultimately be helpful in the fight against COVID-19.

Keywords: SARS-CoV-2, COVID-19, *in vitro* cultures, Plants-medicinal, biotechnology, secondary metabolites

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become the deadliest virus in a century. SARS-CoV-2, which originated in Wuhan, China in December 2019, has killed more than two million people so far. This is the third time that a coronavirus has caused an outbreak during the 21st century, SARS and Middle East respiratory syndrome (MERS) being the previous ones. This virus has been termed the novel coronavirus (SARS-CoV-2) and causes a severe respiratory syndrome collectively called coronavirus disease 2019 (COVID-19). The disease, because of the ease of spread of its causative virus, became a pandemic very quickly. Owing to this, 2.37 million people have died because of COVID-19 and 108.33 million have tested positive for the virus as of February 13, 2021 (Worldometer, 2020).

Scientific advancements allowed researchers to advise governments across the world on quick prevention measures. Based on the rapid information coming out about the virus, including its transmission pattern, morphology, and deeper biological information, the World Health Organization (WHO) and other leading health organizations across the world advised on emergency containment and control measures. Control on a global scale allowed stakeholders from every sector to work toward mitigation measures more efficiently. Slowing down the spread and thus containment of the virus has also allowed scientists to work on many treatment options for COVID-19. Although the current option to treat COVID-19 patients is to alleviate symptoms and avoid co-infection with bacteria through medications, trials on different drug and vaccine candidates are underway (Thanh Le et al., 2020). However, the safety concerns regarding repurposed drugs and the fact that vaccines, when available, will only prevent infection, calls for additional avenues of drugs to treat patients. Plants provide one such avenue through the products of their secondary metabolism, i.e., phytochemicals. But these too are limited by safety concerns, seasonal and geographic dependence, and lesser uniformity in the metabolite profile of medicinal plants across the globe. The solution to these barriers in harnessing secondary metabolism running in plant cell factories is provided by plant biotechnology. Plant biotechnology is a very promising platform for providing uniform, safe to use, high-yield drugs against coronaviruses. This review article highlights the important potential role of plant cell factories to produce safe and high-yield medicinal compounds against COVID-19. The paper reviews the important biotechnological strategies that can be employed to make the best use of plants for providing secondary metabolites as candidates during anti-SARS-CoV-2 drug discovery.

TREATMENT OF COVID-19: A BRIEF INSIGHT

Treatment options currently explored include passive immunity (Abraham, 2020), repurposing of existing drugs, and vaccine candidates (Harrison, 2020). For instance, recently, the already available dexamethasone, an inexpensive steroidal drug has been shown to save the lives of COVID-19 patients in a trial, called RECOVERY (Ledford, 2020). Similarly, chloroquine and hydroxychloroquine, antimalarial drugs also showed impressive results when repurposed to treat COVID-19 patients (Keyaerts et al., 2004; Gautret et al., 2020; Wang et al., 2020). Vaccine trials are also underway and the Moderna biotech vaccine candidate mRNA-1273 (approved for use now by the Food and Drug Administration) which encodes the stabilized pre-fusion SARS-CoV-2 spike protein has provoked an immune response with no trial-limiting side effects (Jackson et al., 2020). However, vaccines, based on their very mechanism of action, only prevent a healthy individual from getting infected. Moreover, a successful vaccine is not thought, at least soon, to be available to the masses. Similarly, repurposing synthetic drugs also became controversial because of their safety concerns and adverse events (Ferner and Aronson, 2020).

The fight against COVID-19 has now become one of the greatest challenges of the current times. The pandemic has lasted for over a year now since its inception in December 2019. To date, over 90 vaccines are being developed for the COVID-19 virus by different research groups in universities and major companies. Currently, two vaccines (Pfizer-BioNTech COVID-19 vaccine and Moderna COVID-19 vaccine) have obtained emergency use authorization from the Food and Drug Administration in the United States. Pfizer and Moderna have developed messenger RNA-based vaccines that have been shown to be 90–95% effective when given at preventing doses 21 and 28 days apart, respectively (Levenson and Howard, 2020). Some of the groups are even testing the direct injection of viral proteins that will help in eliciting the immune system and developing resistance against the virus. One fascinating approach is the use of genetically modified viruses to develop coronavirus proteins in the human body. The carrier virus will act as a vector, carrying coronavirus protein sequences in its genome. Currently, measles or adenovirus (where the viruses are weakened) is used in this approach to make either replicating or non-replicating virus versions (Callaway, 2020). Another fascinating alternative is the plant-based vaccines developed by Medicago (PMI, 2020). The company is developing a *Nicotiana benthamiana*-based virus-like particle (VLP), to develop a potential vaccine against the coronavirus disease. The VLPs use genetic sequencing from the coronavirus to mimic it and produce an immune response in the body. Plant-based VLP technology offers a very safe alternative to the vaccines already approved or in the process of development. These vaccines are virus-free and do not rely on animal products.

PLANT SECONDARY METABOLITES AND THEIR ANTIVIRAL POTENTIAL

Plant metabolism as a factory to produce anti-SARS-CoV phytochemicals is an important area of consideration currently. It is important to highlight the antiviral potential of the main classes of plant secondary metabolites to understand the role of *in vitro plant* cultures and associated biotechnological manipulation in fighting SARS-CoV-2. Plants produce a diversity of organic compounds classified as primary and secondary metabolites

based on either being directly essential to the growth and development of plants (primary metabolites) or indirectly playing their role and not essential to growth (secondary metabolites). Secondary metabolites are produced in plants in situations of intracellular and/or extracellular stress and are used for interaction with the environment and protection from pathogens. This implies that there are thousands of secondary metabolites produced in plants, classified in different classes by their chemical structures. The four major classes of plant secondary metabolites are alkaloids, glycosides, phenolics, and terpenes. The purpose of highlighting the groups of these metabolites is to relate the role these classes of compounds could play against SARS-CoV-2. Several different plant-based compounds have been shown to be effective against the previous type of coronavirus, i.e., SARS-CoV. These compounds have been employed for their different mechanisms of actions against SARS-CoV (**Table 1**).

Alkaloids, for instance, are nitrogen-containing basic compounds and include compounds such as quinine, a bitter alkaloid isolated from the bark of the cinchona tree (Quina). A synthetic derivative of quinine, i.e., chloroquine has recently been tested and found to be a good drug candidate against SARS-CoV-2 because its DNA-intercalating properties prove potent in alleviating the symptoms of coronaviruses based on its biocompatibility (Devaux et al., 2020). Chloroquine, now being tested, has been found to result in side effects such as ventricular arrhythmias, serious cutaneous adverse reactions, and fulminant hepatic failure (Ferner and Aronson, 2020). Overall, despite the side effects, the experimentally proven efficacy of their analogs and derivatives mean that natural quinines could be effective in alleviating the symptoms based on their biocompatibility (Devaux et al., 2020). Similarly, reserpine, an alkaloid isolated from the dried root of *Rauvolfia serpentina* (Indian snakeroot) has been shown to inhibit the replication of SARS-CoV (the coronavirus that causes the first coronavirus-related epidemic of this century). Reserpine could thus prove to be an important candidate against SARS-CoV-2. Similarly, other important alkaloids, palmatine, and chelidonine were also reported as intercalating alkaloids and could be easily suggested as potential drug candidates against SARS-CoV-2 (Ho et al., 2007; Wink, 2020).

Similarly, flavone glycosides, phenolics, and polyphenolic compounds which are characterized by aromatic rings and hydroxyl (−OH) groups have also demonstrated important antiviral activity in many studies. For example, three flavone glycosides, quercetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside, and kaempferol 3-*O*-robinobioside have proven effective against herpes simplex virus and thus points toward their potential role in working against human viruses (Yarmolinsky et al., 2012). It has been suggested that the −OH groups inhibit the activity of viral proteins by forming hydrogen bonds with the positively charged amino groups of proteins. Additionally, polyphenols can intervene in the lipoprotein layers of the viral envelope and thus prevent viral entry in the host cells (Wink, 2020). For instance, the flavonoid chrysin, derived from genus *Rheum* and *Polygonum* were tested positive for their achrysinctivity against the S protein and inhibition of ACE2 interaction (Ho et al., 2007). Flavonoids and polyphenolic compounds like luteolin and quercetin have experimentally proven activity against SARS-CoV. They have significantly blocked the entry of the virus into the cells. This was shown through studies of Yi et al. (2004) wherein they reported that these small molecules showed promising results with half-maximal effective concentration (EC_{50}) of 83.4 and 10.6 μM, respectively.

Essential oils and terpenoids have an equally important role as antiviral plant secondary metabolites. Essential oils can enter non-specifically into the lipid bilayer of the viral envelope, altering the fluidity of the membrane and thus interfering with its pathogenicity even before the entry of the virus (Ben-Shabat et al., 2020). Terpenoids, comprised of isoprene units, terpenes, and their oxygenated derivatives, have also proved potent against many viruses including coronaviruses. For instance, α-cadinol, pinusolidic acid, and ferruginol, isolated from *Chamaecyparis obtuse*, betulonic acid, and cedrane-3β,12-diol, from *Juniperus formosana*, and cryptojaponol isolated from *Cryptomeria japonica* have been proven to be effective against SARS-CoV (Wen et al., 2007). The results of the study indicated that most of the terpenoids inhibited the replication of SARS-CoV at EC_{50} between 3.8 and 7.5 μM. Similarly, an important member of terpenoids, resveratrol has been shown to prevent the entry of MERS-CoV into the cell. Resveratrol fully prevented Vero E6 cell death at the concentration of 125–250 μM (Lin et al., 2017).

WHY PLANT BIOTECHNOLOGY?

While the search for anti-SARS-CoV-2 drugs is ongoing, according to Capell et al. (2020), one avenue for looking for anti-SARS-CoV-2 drugs is the plant kingdom. In the traditional setup, raw plants, as well as extracts from plants, were used to treat different diseases. The WHO has suggested that 80% of the world's population relies on plants for the treatment of many diseases (Bannerman et al., 1983). Plants have importantly been employed against human respiratory problems including respiratory viruses. Such is the importance of plant trials, that work is currently underway on dried fruit extracts of *Forsythiae fructus* as a part of the world's search for an effective treatment for COVID-19 (Maxmen, 2020).

However, plants face the threat of over-harvesting and thus endangerment when collected rigorously. Similarly, due to insufficient data on safety-related aspects of the use of phytomedicine, concerns are still there. There is an incorrect perception that herbal drugs are fully safe and free from any side effects. There are hundreds of toxic constituents in different plants. For this purpose, detailed insight into the pathways and products of the plant's secondary metabolism is important for drugs that are safe to use (Nature, 2020). Equally important is the fact that plants located in different regions of the world have different metabolite profiles and are highly dependent on geography and seasons. Plant biotechnology has the potential to overcome these barriers (Ramirez-Estrada et al., 2016). Plant *in vitro* cultures as an important pillar of plant biotechnology provides an option for making the best use of plant machinery to produce medicinally important secondary metabolites (**Figure 1**).

TABLE 1 | Compounds active against SARS-CoV along with their reported anti-SARS-CoV mechanism of action.

	Compound	Plant	Virus acting on	IC_{50} value	Reported antiviral mechanism	References
1	Aescin	*Aesculus hippocastanum*	SARS-CoV	3.4 μmol/L	–	Wu et al., 2004
2	Celastrol	*Tripterygium regelii*	SARS-CoV	10.3 μmol/L	Inhibits SARS-CoV 3CLpro	Ryu et al., 2010b
3	Cepharanthine	*Stephania japonica*	SARS-CoV-2	0.98 μmol/L	ACE inhibitor	Fan et al., 2020
4	Chalcones I–IX	*Angelica keiskei*	SARS–CoV	11.4–129.8 μmol/L	Competitively inhibits SARS-CoV 3CLpro	Park et al., 2016
5	Dihydrotanshinone	*Salvia miltiorrhiza*	MERS-CoV	1 μg/mL	–	Kim et al., 2018
6	Emodin	*Rheum palmatum*	SARS-CoV	200 μmol/L	Blocks the binding of S protein to ACE2	Ho et al., 2007
7	Ginsenoside-Rb1	*Panax ginseng*	SARS-CoV	100 μmol/L	Inhibits glycoprotein activity	Wu et al., 2004
8	Glycyrrhizin	*Licorice root*	SARS-CoV	300 mg/L	Upregulates nitrous oxide synthase and nitrous oxide production	Cinatl et al., 2003; Schoeman and Fielding, 2019
9	Hirsutenone	*Alnus japonica*	SARS-CoV	4.1 μmol/L	Inhibits PLpro activity	Park et al., 2012a,b
10	Iguesterin	*Tripterygium regelii*	SARS-CoV	2.6 μmol/L	Inhibits SARS-CoV 3CLpro	Ryu et al., 2010b
11	Leptodactylone	*Boenninghausenia sessilicarpa*	SARS-CoV	100 μg/mL	–	Yang et al., 2007
12	Lycorine	*Lycoris radiata*	SARS-CoV	15.7 ± 1.2 nmol/L	–	Li et al., 2005
13	Myricetin	*Myrica rubra*	SARS-CoV	2.71 ± 0.19 μmol/L	Inhibits ATPase activity	Yu et al., 2012
14	Pristimererin	*Tripterygium regelii*	SARS-CoV	5.5 μmol/L	Inhibits SARS-CoV 3CLpro	Ryu et al., 2010b
15	Quercetin-3-β-galactoside	*Ginkgo biloba*	SARS-CoV	42.79 ± 4.97 μmol/L	Competitively inhibits SARS-CoV 3CLpro	Chen et al., 2006
16	Reserpine	*Rauvolfia serpentine*	SARS-CoV	6.0 μmol/L	–	Wu et al., 2004
17	Resveratrol	*Polygonum cuspidatum*	MERS-CoV	–	–	Lin et al., 2017
18	Saikosaponin B_2	*Bupleurum chinense*	HCoV-229E	1.7 ± 0.1 μmol/L	Interferes with events of early viral attack	Li et al., 2005; Cheng et al., 2006
19	Scutellarein	*Scutellaria baicalensis*	SARS-CoV	0.86 ± 0.48 μmol/L	Inhibits ATPase activity	Yu et al., 2012
20	Tanshinones I–VII	*Salvia miltiorrhiza*	SARS–CoV	0.7–30 μmol/L	Inhibits PLpro activity	Park et al., 2012b
21	Tetrandrine	*Stephania tetrandra*	HCoV-OC43	0.33 ± 0.03 μmol/L	Inhibits p38 MAPK pathway	Kim et al., 2019
22	Theaflavin	Black tea	SARS-CoV-2	–	Inhibits RdRp activity	Lung et al., 2020

SARS-CoV, severe acute respiratory syndrome-coronavirus; 3CLPro, 3C-like protease; PLpro, papain-like protease; MERS, Middle East respiratory syndrome coronavirus; ACE2, angiotensin-converting enzyme 2; H-CoV-229E, human coronavirus 229E; H-CoV-OC43, human coronavirus OC43; p38 MAPK, p38 mitogen-activated protein kinases; RdRp, RNA-dependent RNA polymerases.

Plant cell suspension cultures, callus cultures, hairy root cultures, adventitious root cultures, and other organ cultures can serve as the best sources of uniform production of phytomedicine for COVID-19 (Verpoorte et al., 2002). The importance of plant *in vitro* cultures lies in the reason that these cultures can be manipulated to trigger their defense response through activating their secondary metabolism. These triggers include elicitation by biotic and abiotic stresses given *in vitro* to produce enhanced quantities of phytochemicals. For instance, Ramirez-Estrada et al. (2016) reviewed the potential of methyl jasmonate as an important biotic elicitor to trigger the production of a diversity of secondary metabolites in different plant cell cultures (Ramirez-Estrada et al., 2016). Similarly, metabolic engineering backed by genetic manipulation tools has been a very viable biotechnology method to obtain novel metabolites and enhance the yield of the existing metabolites of interest (Gandhi et al., 2015).

Micropropagation

Micropropagation is a robust and reliable technique used for the multiplication of plants through *in vitro* cultures; it produces many homogeneous plants in a short period. Besides, the production of bioactive secondary metabolites can be enhanced in medicinal plants with this technique (Khan I. et al., 2020). During micropropagation, tiny parts of the plants commonly called explants excised from different plant species can be micro-propagated under optimized growth conditions of culture media, temperature, and photoperiod (Abbasi et al., 2016).

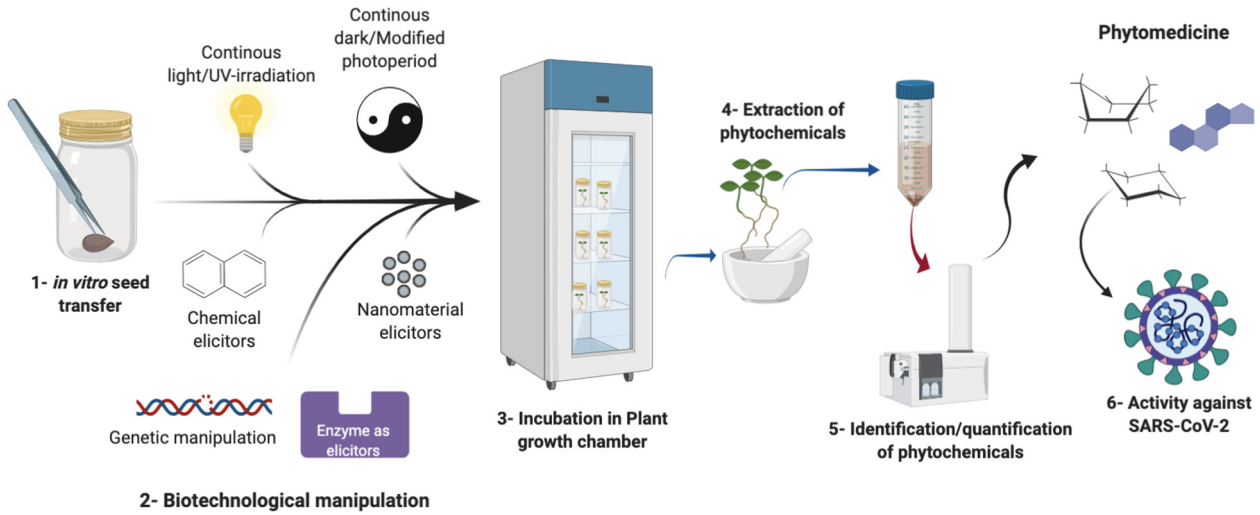

FIGURE 1 | A schematic representation of the potential plant biotechnology methods that lead to the enhanced production of phytomedicine through *in vitro* cultures against SARS-CoV-2.

As indicated in **Table 2**, several health-promoting metabolites, especially those which are reported for a multitude of antiviral potential, have been produced in many plants through micropropagation *in vitro*. For instance, Santoro et al. (2013) reported the production of higher quantities of pulegone and menthofuran in *Mentha piperita*, when micro-propagated *in vitro* under the effects of 4-indol-3-ylbutyric acid (IBA) and 6-benzylaminopurine (BAP).

In a recent study, Ali et al. (2018b) reported that *Ajuga bracteosa* (a high-valued medicinal plant) accumulated higher levels of monoterpene hydrocarbons, which could be potentially used as essential oil-based medicine against human viruses. These hydrocarbons included limonene (3.4%), α-pinene (5.3%), camphene (4.45%), α-thujone (9.4%), 1,8-cineole (14.3%), borneol (11.4%), camphor (12.2%), and nerol (9.2) in the shoots raised *in vitro* in response to the application of TDZ (Ali et al., 2018a). Similarly, the supplementation of TDZ into the MS media produced a substantial amount of monoterpenes and sesquiterpenes through shoot cultures in the medicinally potent plant *Lallemantia Iberica* (Pourebad et al., 2015). The higher production of the important terpene volatiles (candidate anti-SARS-CoV-2 metabolites) in the regenerated shoots can be attributed to the different attributes of shoot cultures, such as the juvenile stage of the differentiated shoots, as the monoterpenes biosynthesis is directly linked to the young and immature shoot with higher metabolic potential (Bassolino et al., 2015). Biosynthesis of terpene metabolites generally takes place in epidermal cells of shoot or leaf and is stored in special glandular structures called leaf trichomes (Ali et al., 2018a). In another study, compared with callus cultures, the *in vitro* raised shoot cultures in medicinally important plants *Lavandula angustifolia* and *Rosmarinus officinalis* were found to accumulate higher levels of monoterpenes hydrocarbons (Gounaris, 2010). As the growth and development during *in vitro* shoot cultures are highly influenced by the effects of different plant growth regulators, the biosynthesis of terpenes could be correlated to *in vitro* growth and development. The ontogenetic changes in the shoots as a result of plant cell growth and the accelerated but controlled secondary metabolism during *in vitro* cultures are other important reasons which influence and regulate the biosynthesis of secondary metabolites (Khan et al., 2019a,b; Khan M. A. et al., 2020). Apart from micro-propagated plantlets many other *in vitro* cultures are also serving as useful sources of different medicinally important secondary metabolites (**Figure 2**).

Callus and Cell Cultures

Plant cell cultures compared with wild plants and other types of cultures have the advantage of being (1) less prone to various environmental variations, (2) stable production platforms of homogeneous and uniform yield, (3) rapid growth, (4) reproducible, and (5) able to synthesize novel products that do not normally exist in the native plants (Khan et al., 2017; Khan M. A. et al., 2020). In addition to medicinal products, cell suspensions have been employed to produce compounds used as fragrances, food flavors, and additives, dyes, and coloring agents (Saeed et al., 2017). A lot of important medicinal plants have been exploited for the production of useful antiviral medicinal metabolites through callus and cell cultures (Ali et al., 2018a,b). For example, as listed in **Table 2**, considerable levels of diosgenin (an anti-SARS-CoV metabolite) were detected in the callus cultures of *Helicteres isora* L (Shaikh et al., 2020). Callus and cell cultures in *Gymnema sylvestre* have shown an optimal production of gymnemic acid (GA) which possesses the potential to work against SARS-CoV-2 (Veerashree et al., 2012). In some studies, the cell cultures were found to only accumulate the precursors of volatile medicinal compounds; while, cultures of other plants such as mentha have been recommended to produce high-valued medicinal monoterpenes compared to those found in the intact mentha plants. Likewise, callus cultures of *M. piperita* have been reported for the accumulation of monoterpenes in special

TABLE 2 | Production and enhancement of potential compounds against SARS-CoV-2 through plant biotechnological approaches.

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
1	Saponins	Javanese ginseng (*Talinum paniculatum)*	Adventitious root cultures	Temperature 25 ± 1°C in the dark	Indole-3-butyric acid (IBA) or NAA (1-napthaleneacetic acid)	Methyl jasmonate (MeJA) and salicylic acid (SA)	1.5-fold upon elicitation with MeJA and 1.3-fold upon SA	Faizal and Sari, 2019
2	Astragaloside (AG)	*Astragalus membranaceus*	Hairy root cultures	Orbital shaker (100 rpm); 28 ± 1°C in the dark	–	Methyl jasmonate (MJ)	MJ-elicited (2.1- and 2.0-folds greater)	Jiao et al., 2016
3	Diosgenin	*Helicteres isora L.*	Callus and suspension cultures	Rotatory shaker (50–60) rpm; 25 ± 2°C temperature; 40 lmol m^{-2} s^{-1} light intensity; 16/8-h light/dark cycle	2,4-dichlorophenoxyacetic acid (2,4-D); kinetin (Kin); and 6-Benzylaminopurine (BAP)	*Escherichia coli*; *Bacillus subtilis*; *Saccharomyces cerevisiae*; and *Aspergillus niger*	E. coli (1.5%) proved best with a 9.1-fold increase	Shaikh et al., 2020
4	Gymnemic acid (GA)	*Gymnema sylvestre R. Br.*	Cell suspension cultures	Rotatory shaker (110 rpm); incubator at 25°C in dark; pH 5.8	2,4-dichlorophenoxyacetic acid (2,4-D); naphthaleneacetic acid (NAA); 6-benzyladenine (BA); picloram	Methyl jasmonate (MJ); yeast extract; chitin; and pectin	Yeast extract (5.25-folds); MJ (2.8-folds); pectin (2.65-folds); while chitin (2.62-folds)	Veerashree et al., 2012
5	Flavonoid	*Isatis tinctoria L.*	Hairy root cultures	Temperature 30°C; pH 7.0; and time 72 h	–	*Aspergillus niger* and *Aspergillus oryzae*	6.83-fold increase	Jiao et al., 2018
6	Rosmarinic acid	Purple basil (*Ocimum basilicum L. var. purpurascen*)	Callus cultures	Temperature (25 ± 2°C); pH of 5.6–5.7; 16/8 h light/dark	Naphthaleneacetic acid (NAA)	Melatonin; and UV-C irradiations	Melatonin (1.4-fold); UV-C radiations (2.3-fold) elevation	Nazir et al., 2020
7	Glycyrrhizin	*Glycyrrhiza glabra L.*	Hairy root cultures	Temperature 28 ± 2°C; 60 μ mol photon m^{-2} s^{-1} light for 16 h day and 8 h dark	Indole-3-acetic acid (IAA)	Abiotic elicitors: polyethylene glycol (PEG); CdCl2 Biotic elicitor: cellulase; mannan	PEG enhanced the yield up to 5.4-folds; cellulase (8.6-folds); Mannan (7.8-folds)	Srivastava et al., 2019

(Continued)

TABLE 2 | Continued

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
8	Chicoric acid	Purple basil (*Ocimum basilicum L. var. purpurascens*)	Callus cultures	Temperature (25 ± 2°C; pH of 5.6–5.7; 16/8 h light/dark	Naphthaleneacetic acid (NAA)	Melatonin and UV-C irradiations	Melatonin (3.2-folds) and UV-C radiations (4.1-folds)	Nazir et al., 2020
9	Quercetin	*Abutilon indicum L.*	Callus cultures	Temperature 25°C; pH 5.75 under dark conditions	2,4-dichloro phenoxy acetic acid (2,4-D) with indole-3-acetic acid (IAA)	phenylalanine (PA)	Three-fold increase	Sajjalaguddam and Paladugu, 2015
10	Peonidin	Purple basil (*Ocimum basilicum L. var. purpurascens*)	Callus cultures	Temperature 25 ± 2°C; pH of 5.6–5.7; 16/8 h light/dark	Naphthaleneacetic acid (NAA)	Melatonin and UV-C radiations	Melatonin (2.0-fold); and UV-C radiations (2.7-fold)	Jiao et al., 2018
11	Kaempferol	*Dysosma pleiantha (Hance) Woodson*	Callus cultures	Temperature 25 ± 1°C; pH 5.6–5.8 16 H photoperiod from white fluorescent lamps at a light intensity of 43 μmol m^{-2} S^{-1}/8 h dark cycle	Medium (B5) 2,4-dichlorophenoxyacetic acid (2,4-D); kinetin	Casein hydro lysate; coconut water; and peptone extract	12.39-folds enhancement	Karuppaiya and Tsay, 2020
12	Ephedrine	*Ephedra alata*	Suspension cultures	Temperature 25 ± 2°C; pH 5.7–5.8; fluorescent light (2500–3000 lux); 16-h photoperiod	2,4-dichlorophenoxy acetic acid (2,4-D); and kinetin (Kn)	*Aspergillus niger* and yeast extract	Seven-fold increase	Hegazi et al., 2020
13	Caffeic acid	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	Naphthaleneacetic acid (NAA); benzyl aminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	1.5-folds increase	Skrzypczak-Pietraszek et al., 2018
14	Chlorogenic acid	*Cecropia obtusifolia*	Callus and cell suspension cultures	Rotatory shaker at 110 rpm; temperature 26°C; photoperiod of 16-h light with cool white fluorescent lamps at 50 lMm^{-2} s^{-1}	Naphthalene acetic acid (NAA); 2,4-dichlorophenoxyacetic acid (2,4-D); indole-3-butyric acid (IBA); and indole-3-acetic acid (IAA); 6-benzylaminopurine (BAP)	Nitrate deficiency (lacking ammonium)	7.7-fold increase	Del Pilar Nicasio-Torres et al., 2012

(Continued)

TABLE 2 | Continued

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
15	Glycyrrhetinic acid	*Taverniera cuneifolia (Roth) Arn*	Root cultures	Microbial: 150 rpm; temperature 26 ± 2°C; for 24 h Methyl jasmonate (MJ): 120 rpm; 26 ± 2°C; 18/6 photoperiod white fluorescent light (30 μmol m^{-2} S^{-1})	–	Microbial elicitation (fungal and bacterial); methyl jasmonate (MJ)	Microbial elicitation (three-folds); methyl jasmonate (2.5) enhancement	Awad et al., 2014
16	Matairesinol	*Forsythia × intermedia*	Cell suspension cultures	Gyratory shaker at 110 rpm; 25°C; in the dark	–	Methyl jasmonate and coniferyl alcohol	Seven-fold increase	Schmitt and Petersen, 2002
17	Lignans	*Linum ussitatsimum L*	Cell suspension cultures	Gyratory shaker at 100 rpm placed in optimum conditions	Naphthalene acetic acid (NAA)	Salicylic acid (SA)	2.7-fold increase	Nadeem et al., 2019
18	Neochlorogenic acid	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	α-naphthaleneacetic acid (NAA); benzylaminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	16-fold	Skrzypczak-Pietraszek et al., 2018
19	Neolignans (dehydrodiconiferyl)	*Linum ussitatsimum L*	Cell suspension cultures	Gyratory shaker at 100 rpm with optimum growth conditions	Naphthalene acetic acid (NAA)	Salicylic acid	3.88-fold	Nadeem et al., 2019
20	p-coumaric acid	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	α-naphthaleneacetic acid (NAA); benzylaminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	5.3-fold	Skrzypczak-Pietraszek et al., 2018
21	(–) Menthone	Peppermint (*Mentha piperita*)	Micro propagation	Temperature 22 ± 2°C; pH 5.6–5.8; relative humidity (~70%); light (16/8 h light/dark cycle)	Auxins 4-indole-3-ylbutyric acid (IBA); and the cytokinins 6-benzylaminopurine (BAP)	–	Two-fold increase	Santoro et al., 2013
22	Cynaroside	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	α-naphthaleneacetic acid (NAA); benzylaminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	1.5-fold	Skrzypczak-Pietraszek et al., 2018

(Continued)

TABLE 2 | Continued

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
23	(+)-Pulegone	Peppermint (*Mentha piperita*)	Micro propagation	Temperature 22 ± 2°C; pH 5.6–5.8; relative humidity (~70%); light (16/8 h light/dark cycle)	Auxins 4-indole-3-ylbutyric acid (IBA); and the cytokinins 6-benzylaminopurine (BAP)	–	Three-fold increase	Santoro et al., 2013
24	Limonene	Pennyroyal (*Mentha pulegium)*	Cell suspension cultures	Shaker with 100 round per minute in 25 ± 1°C	2,4-D	Yeast extract; salicylic acid	Limonene increased with increasing concentrations of yeast extract elicitor	Darvishi et al., 2016
25	(+)-menthofuran	Peppermint (*Mentha piperita*)	Micro propagation	Temperature 22°C ± 2°C; pH 5.6–5.8; relative humidity (~70%); light (16/8 h light/dark cycle)	Auxins 4-indole-3-ylbutyric acid (IBA); and the cytokinins 6-benzylaminopurine (BAP)	–	Two-fold enhancement	Santoro et al., 2013
26	Isoorientin (ISO)	*Cecropia obtusifolia*	Cell suspension cultures	Rotatory shaker at 110 rpm; temperature 26°C; photoperiod of 16-h light with cool white fluorescent lamps at 50 lMm^{-2} s^{-1}	Naphthalene acetic acid (NAA); 2,4-dichlorophenoxyacetic acid (2,4-D); indole-3-butyric acid (IBA); indole-3-acetic acid (IAA); 6-benzylaminopurine (BAP)	Nitrate deficiency (lacking ammonium)	ISO synthesis was induced earlier and for longer time period	Del Pilar Nicasio-Torres et al., 2012
27	Gallic acid	*Barringtonia racemosa L.*	Cell suspension cultures	25 ± 2°C under 18 h light and 6 h dark	2,4-D and kinetin	Biotic (chitosan); abiotic (silver nitrate)	2.64-fold (silver nitrate); 1.34-fold (chitosan) increase	Osman et al., 2018
28	Aloe-Emodin	*Cassia tora*	Root cultures	Shaking at 60 rpm; 25 ± 2°C; in dark conditions	1-naphthaleneacetic acid and kinetin	Chitosan; yeast extract	Chitosan (8.82 times); yeast extract (6.21 times)	Teptat et al., 2020
29	Rosin	*Rhodiola rosea* (rose root)	Compact callus aggregate cultures	Shaken at 14.14 rad s^{-1} (135 rpm)	MS-Rh media supplemented with 6-benzylaminopurine (BAP); naphthalene acetic acid (NAA); sucrose	Cinnamyl alcohol	3 to 6-fold increase	György et al., 2004

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TABLE 2 | Continued

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
30	Salidroside	*Rhodiola imbricata Edgew.*	Callus and suspension cultures	100 rpm shaker for 8 h and kept static for 16 h; pH 5.7	Indole-3-butyric acid (IBA); 6-Benzylaminopurine (BAP); gibberellic acid (GA3); kinetin (KN) and Thidiazuron (TDZ)	Chemical elicitors (growth hormones); physical elicitors (photosynthetic lights, ultraviolet light)	5.35-fold	Kad et al., 2018
31	Scopoletin	*Spilanthes acmella Murr.*	Cell Suspension cultures	Rotary shaker at 100 ± 10 rpm; 25 ± 2°C; 16–8 h light-dark regime, using fluorescent lamps at a light intensity of 35 μ mol m^2 s^{-1}	6-benzyladenine; 2,4-dichlorophenoxyacetic acid	Casein hydrolysate and L-phenylalanine	1.39-fold (casein hydrolysate); 3.43-fold (L-phenylalanine)	Abyari et al., 2016
32	Tyrosol	*Rhodiola crenulata*	Cell suspension cultures	Rotary shaker at 120 rpm; 25 ± 1°C; light intensity 24 lmo^l/m^2/s; 16 h light photoperiod	6-benzyaldenine (BA); naphthalene acetic acid (NAA) and thidiazuron (TDZ)	–	High level of tyrosol were detected	Shi et al., 2013
33	Wogonin	*Scutellaria lateriflora*	Hairy root cultures	Shaking (121 rpm) at 26 ± 1°C	Phytohormone-free MS medium having sucrose and supplemented with antibiotic ampicillin and cefotaxim	*YE and bacterial suspensions (A) A. rhizogenes A4, (B) Pectobacterium carotovorum 1043 (Pba 1043), (C) Pseudomonas syringae var. syringae 764 (Pss 764), (D) Klebsiella pneumoniae 3896, and (E) Enterobacter sakazakii*	4.4-fold increase	Wilczañska-Barska et al., 2012

(Continued)

TABLE 2 | Continued

Serial No.	Compound/class of compounds	Plant	*In vitro* culture type	*In vitro* culture conditions used	Plant growth regulator used	Elicitor used	Remarks (Results)	References
34	Rutin	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	α-naphthaleneacetic acid (NAA); benzylaminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	2.8-fold enhancement	Skrzypczak-Pietraszek et al., 2018
35	Anthocyanin	Purple basil (*Ocimum basilicum L. var. purpurascens)*	Callus cultures	Temperature 25 ± 2°C; pH 5.6–5.7; 16/8 h light/dark	NAA (2.5 mg/L)	Melatonin and UV-C radiations	Melatonin (3.7-fold) and UV-C radiations (4.1-fold) increase	Nazir et al., 2020
36	Cynaroside	*Vitex agnus castus L.*	Agitated shoot cultures	Rotary shaker at 140 rpm	α-naphthaleneacetic acid (NAA); benzylaminopurine (BAP); gibberellic acid (GA3)	L-phenylalanine	1.5-fold yield increase	Skrzypczak-Pietraszek et al., 2018
37	Luteolin	*Dracocephalum kotschyi* Boiss.	Seed germination	Temperature 28°C day/20°C night; 50% air relative humidity; photoperiod of 16 h light and 8 h dark	Melatonin (N-acetyl-5-methoxytryptamine); Calcium (Ca^{2+})	Salinity stress	Salinity stress alone (3.21-fold); salinity stress with melatonin and Ca^{2+} (2.83-fold increase)	Vafadar et al., 2020
38	Saikosaponins	*Bupleurum falcatum L.*	Root cultures	Gyratory shaker 105 rpm; 23 ± 2°C; 12:12 light-dark cycle (h)	Indole-3-butyric acid (IBA)	Two step sucrose concentration	Four-fold yield increase	Kusakari et al., 2000
39	Phenolic compounds	*Morinda coreia* Buck. And Ham.	Adventitious roots cultures	Agitated in dark on gyratory shaker at 100 rpm; 25 ± 2°C temperature for 8 days	Indole-3-butyric acid (IBA); BAP and Kin	Chitosan	1.21-fold more than IBA treated root suspension culture	Kannan et al., 2020
40	Essential oils (Thymol and p-cymene)	*Carum copticum L.*	Callus cultures	Temperature 25°C; pH 5.8; 16-h photoperiod supplied by white fluorescent lamps at 90 lmol m^{-2} s^{-1} in growth chamber	2,4-dichlorophenoxyacetic acid (2,4-D); benzyl amino purine (BAP)	Salt stress and chitosan	Thymol (from 14.5 to 25.1-fold); p-cymene (from 10 to 14.5-fold increase)	Razavizadeh et al., 2020

CdCl, cadmium chloride; MSRs, Murashige and skoog-Rhodiola rosea medium; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; MeJa, methyl jasmonate; SA, salicylic acid; 2, 4D, 2,4-dichlorophenoxyacetic acid; Kn, kinetin; BAP, 6-Benzylaminopurine; PEG, polyethylene glycol; GA, gibberellic acid; TDZ, thidiazuron; BA, 6-benzyaldenine.

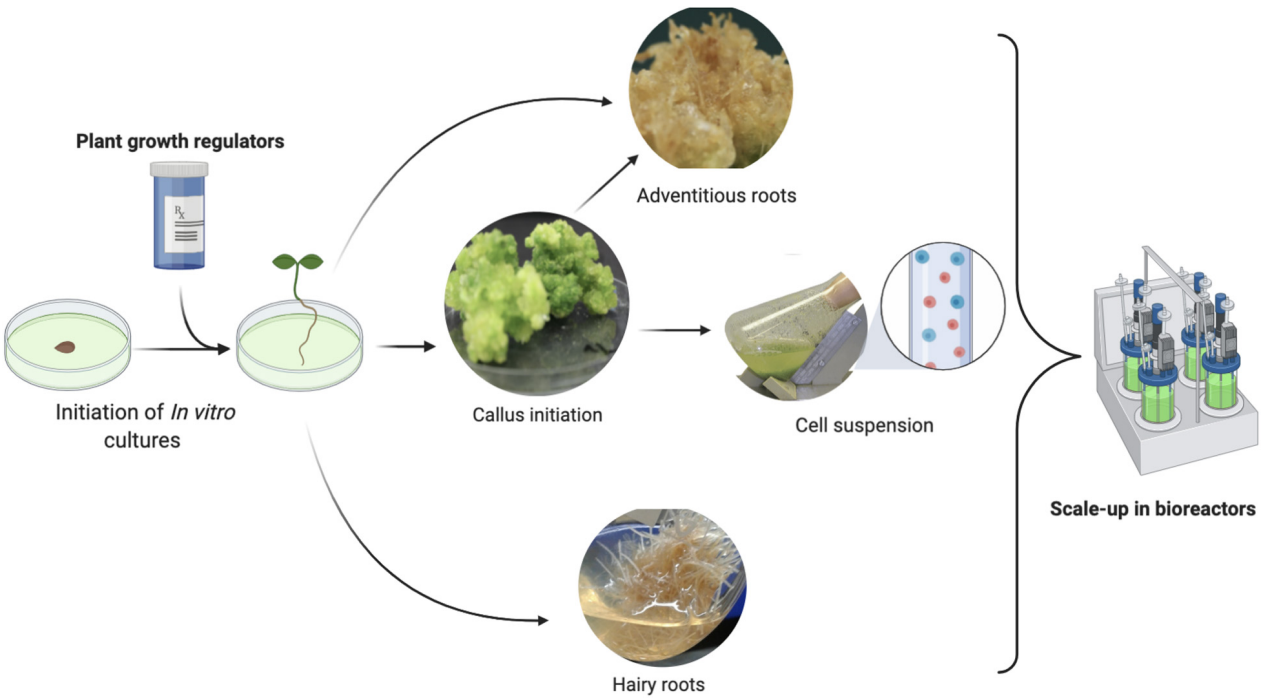

FIGURE 2 | A pictorial representation of the various *in vitro* cultures generated through plant biotechnology methods and their potential for the commercial scale production of phytomedicine.

secretory organs (Khan et al., 2019a). Similarly, results from another study have shown that callus cultures of *Carum copticum* also accumulated higher levels of thymol and p-cymene than their wild-grown respective plants (Razavizadeh et al., 2020).

Light intensity or quality during *in vitro* cultures can influence the plant cell's physiological and hormonal status through the initiation of distinct metabolic pathways that ultimately influence and regulate the biosynthesis of important essential oils (Giri and Zaheer, 2016; Ali et al., 2018b). In cell cultures of *Ocimum basilicum*, constant light illumination produced higher total essential oil yield including the potent volatile linalool than the cell cultures grown under complete darkness. The process of elicitation by application of chemical elicitors, e.g., phenylacetic acid and methyl jasmonate and under the effects of physical elicitors such as the absence of light illuminance in the cultures have positively influenced the production of medicinally potent metabolites in an *A. bracteosa* cell culture (Ali et al., 2018b). In another study, important monoterpenes such as limonene and terpinolene (potential anti-SARS-CoV-2 metabolites) were elicited by methyl jasmonate under dark in higher amounts in *Rosa damascene* cell cultures (Olgunsoy et al., 2017). The process of elicitation is directly linked with the biosynthesis of essential oils in the plant cell. Several factors are responsible for the regulation of volatile compound biosynthesis. These factors include the genetic makeup of the explant used in cell cultures, the type of culture media, and the *in vitro* developmental phase of plant cells (Holopainen, 2011). There are, however, many limitations to cell suspension culture technology including slow growth and scale-up hurdles, the instability of cell lines, and subsequent lower yield of some important metabolites (Abbasi et al., 2016). Nevertheless, optimization of cell cultures could result in the generation of very viable factories to produce medicinal compounds that could work against SARS-CoV-2 and other human viruses.

Hairy Roots

Generally, the potential of plant cell cultures to produce bioactive secondary metabolites can be enhanced by the induction of cell differentiation. Within the different cell culture approaches, hairy root cultures hold tremendous potential for the biosynthesis of volatile organic compounds besides other classes of potent secondary metabolites. When plant tissue is genetically transformed by *Agrobacterium rhizogenes* which inserts its T-DNA through the Ri plasmid, it results in the formation of hair-like small and fine roots. The advantage of hairy root culture technology is that it does not require further media supplementation with plant growth regulators because the inserted T-DNA carries the genes responsible for the endogenous biosynthesis of auxins. Lacking the property of geotropism, hairy roots are highly branched and can grow faster than normal roots. They not only produce the metabolites at levels like the normal roots but also generate metabolites that are produced in the aerial parts of the natural plants. Furthermore, hairy roots are physiologically and biochemically stable like any other cell culture technology (Gounaris, 2010). An excellent study has concluded the potential of hairy roots culture technology for the optimal production of antiviral flavonoids in *Isatis tinctoria* (Jiao et al., 2018). *Isatis tinctoria* (A.K.A. *Isatis indigotica*) has

been shown to possess potential against SARS-CoV through its root-derived phytochemicals (Li et al., 2005). Among the different plants, the hairy roots of *Pimpinella anisum* and *Achillea millefolium* resulted in the production of medicinally important essential oils (Santos et al., 1998). In certain cases, such as the hairy roots of *Daucus carota* and *Laburnum alpinum*, the essential oil profiles of the volatiles were found in elevated levels, compared with the respective callus and cell cultures. Further, the metabolic pathways for the biosynthesis of volatiles can be manipulated by using more effective transgenes that can be inserted into the T-DNA region.

ELICITATION OF *IN VITRO* CULTURES; A PROMISING AVENUE FOR ANTI-CORONAVIRUS MEDICINAL COMPOUNDS

Apart from the diversity of *in vitro* cultures, which can provide avenues for phytochemical compounds against SARS-CoV-2, enhanced production of these compounds is one area where plant biotechnology thrives. This enhancement is achieved through triggering or in other words eliciting the defense response of plant cultures, discussed in the previous section. To give a very brief overview of the mechanism of elicitation, the process starts at the cell membrane of the plant cell. Many different receptors are elicited to trigger the secondary metabolism for defense. For instance, the plasmalemma membrane-associated receptors attach the ligand or chemical compound (exogenous or endogenous). The signal reception is followed by transduction which includes steps like reversible phosphorylation and dephosphorylation of plasma membrane and cytosolic proteins, enhancement of Ca^{2+} in the cytosol, H^+ influx/Cl^- and K^+ efflux, extracellular alkalinization and cytoplasmic acidification, mitogen-activated protein kinase (MAPK) activation, NADPH oxidase activation and production of reactive oxygen and nitrogen species (ROS and RNS), early defense gene expression, jasmonate production, late defense response gene expression, and secondary metabolite accumulation (Ramirez-Estrada et al., 2016).

Being of biological origin (biotic) or non-biological origin (abiotic), the compounds or physical factors that stimulate the plant defense are termed elicitors. Biotic elicitors include compounds that are of the pathogenic origin or are produced by the plants after being exposed to pathogens. Abiotic elicitors include chemical compounds such as salts or physical factors such as environmental triggers (Devaux et al., 2020). The most relevant example of elicitation of important anti-SARS-CoV metabolites is that of glycyrrhizin from *Glycyrrhiza glabra* L. *G. glabra* L. has become an endangered medicinal plant due to the unabated extraction of glycyrrhizin (Srivastava et al., 2019). Glycyrrhizin is a triterpenoid saponin and has been shown to possess strong antiviral activity in killing SARS-CoV in a lancet study (Cinatl et al., 2003). Srivastava et al. (2019) have successfully elicited the yield of glycyrrhizin in hairy root cultures of *G. glabra* L. Through this study, it was proven that both biotic and abiotic elicitors are effective in eliciting higher yields of glycyrrhizin.

Biotic Elicitors Can Trigger the Production of Plant Secondary Metabolites Against SARS-CoV-2

Compounds of a biological origin that elicit plant defense response and thus produce higher quantities of secondary metabolites are produced in two ways. Biotic elicitors are either compounds coming from pathogens, i.e., exogenous in origin or are compounds/hormones produced as a response to the pathogen that in turn triggers the plant's defense response (endogenous elicitors). Plant *in vitro* cultures have been used as factories for the enhanced production of medicinally secondary metabolites through the application of many different exogenous and endogenous elicitors (Srivastava et al., 2019). Exogenous biotic elicitors include bacterial lysates, microbial enzymes, polysaccharides (chitin), and yeast extracts. For instance, cellulase, which directly serves bacteria and fungi and helps in attacking plant cell walls, has been shown to enhance the production of glycyrrhizin up to 8.6-fold through the application of cellulase to the hairy roots of *G. glabra* L. Besides, mannan oligosaccharides derived from the cell wall of yeasts (*Saccharomyces cerevisiae*) (De Oliveira et al., 2014) have also been reported to trigger the enhanced production of glycyrrhizin (7.8-fold compared to control) in hairy root cultures of *G. glabra* L. It should be reiterated here that glycyrrhizin possesses a demonstrated activity against the previously epidemic SARS-CoV. This saponin from licorice roots can inhibit the replication of SARS-associated coronavirus with an EC_{50} value ranging from 300 to 600 mg/L (Cinatl et al., 2003). Similarly, methyl jasmonate, a very important endogenous biotic elicitor has been shown to be effective in enhancing the production of glycyrrhizin up to almost 109 micrograms/g dry weight after 5 days of elicitation with 100 mM of methyl jasmonate. The study also demonstrated the role of other elicitors such as chitosan and yeast extract on the production of glycyrrhizin and demonstrated their effectiveness (Wongwicha et al., 2011). Other biotic elicitors ascorbic acid, eugenol, salicylic acid, and yeast extract have been employed for the enhancement of glycyrrhizin in cell cultures of *Abrus precatorius* (Karwasara et al., 2010).

Similarly, a higher yield of *trans*-resveratrol, previously shown to act strongly against MERS-CoV, has been reported in cell suspension cultures of *Vitis vinifera* through the application of 2, 3-dihydroxypropyl jasmonate (Shen et al., 2012). Chitosan is a polysaccharide that acts as a biotic elicitor and is used for high-yield production of medicinally important secondary metabolites (Hadwiger, 2013). Results from the study of Ferri et al. (2009) revealed that chitosan enhanced the production of important polyphenols, including stilbenes and flavonoids in cell cultures of *V. vinifera*. There are innumerable studies available on enhancing the yields of many important plant secondary metabolites that could be very effective in dealing with SARS-CoV-2. Plant secondary metabolites such as lycorine, reserpine, plant lectins, apigenin, luteolin, and quercetin (replication inhibitors of coronavirus) (Wu et al., 2004; Li et al., 2005; Keyaerts et al., 2007;

Ryu et al., 2010a) have been elicited through the use of biotic elicitors including methyl jasmonate, salicylic acid, and chitosan (Dyakov et al., 2007; Ptak et al., 2017; Chandran et al., 2020).

Enhanced Production of Anti-coronavirus Plant Secondary Metabolites Through Abiotic Elicitors

Just like biotic elicitors, chemical compounds of abiotic origin and physical factors such as environmental stimuli have also been proven effective in the elicitation of plant defense and thus increased production of phytochemicals (Halder et al., 2019). It is not possible to cover all abiotic elicitors in a single subsection, but for our purpose, abiotic elicitors compounds such as salts (e.g., $AgNO_3$, $CdCl_2$, etc.) and environmental stimuli such as continuous light/dark, different wavelengths of light, and osmotic stress, etc. have been employed to produce high-yield secondary metabolites in plant *in vitro* cultures.

Abiotic elicitation has been used for the enhanced production of important flavonoids such as hypericin and hyperforin in *in vitro* cultures of *Hypericum perforatum* (Shakya et al., 2019). For instance, Tirillini et al. (2006) reported that the application of chromium (0.01 mM) increased the production of hypericin by 38% in plantlets of *H. perforatum* (Tirillini et al., 2006). Compounds such as quercetin in *H. perforatum* have been shown to act as potent anti-SARS-CoV compounds and their enhanced production through elicitation promises an avenue for high-yield drug production (Ryu et al., 2010a; Shakya et al., 2019). A new addition to the lines of abiotic elicitors is the use of nanomaterials for triggering an intense plant defense response. For example, in one of our studies, we used silver nanoparticles (AgNPs) for the enhancement of medicinally important phenolics and flavonoids in callus cultures (Begum et al., 2020). Similarly, zinc nano-oxides and iron nano-oxides have been used to trigger the production of hypericin and hyperforin in cell suspension cultures of *H. perforatum* (Shakya et al., 2019). Apart from chemical compounds, environmental triggers have also proved to be valuable tools in plant biotechnology for the enhanced production of important secondary metabolites in plant *in vitro* cultures. For example, Khan et al. (2019c) showed that different spectral lights result in the enhanced production of phytochemicals such as myricetin and apigenin among many others. Myricetin is experimentally shown to inhibit the SARS-CoV helicase protein *in vitro* by affecting ATPase activity and thus could have potential against SARS-CoV-2 (Yu et al., 2012). Plants *in vitro* cultures can serve as factories for the elicitor-induced high-yield production of myricetin against SARS-CoV-2. In another study, Huang et al. (2016) used UV-B irradiation to cause flavonoid-related gene expression in hairy root cultures of *Fagopyrum tataricum*. The experiment resulted in enhanced biosynthesis of rutin and quercetin in the hairy root cultures of *F. tataricum*.

Conclusively, the application of biotic and abiotic elicitors during plant *in vitro* cultures is a promising avenue for the production of enhanced quantities of drug candidates against SARS-CoV-2.

GENETIC ENGINEERING OF PLANTS FOR ENHANCED METABOLITES BIOSYNTHESIS

Few reports are available on the genetic engineering of different plant species through a transformation with the candidate genes responsible for medicinal metabolites biosynthesis. Particularly, the metabolic pathways responsible for producing antiviral metabolites. In these studies, the cauliflower mosaic virus *promoter* (*CaMV 35S*) was used for the overexpression of the reductoisomerase DXR of the mevalonate MEP pathway in peppermint, and a significantly higher (50%) increase in total essential oil production was observed. The yields of cyclic monoterpenes were enhanced by the overexpression of the limonene synthase enzyme in the plastid. The overexpression of the rate-limiting factors significantly enhanced the specific yield of monoterpenes (Daviet and Schalk, 2010). It is crucial in some instances to enhance the yield of specific compounds of interest such as the monoterpenes α-pinene and d-limonene which are suitable alternatives to hazardous chemicals. Thus, the *in vitro* cultures through a genetic transformation in plants can boost the production of the desired compounds (Roberts, 2007). For instance, the production of monoterpene alcohols can be accelerated by the overexpression of linalool synthase, the enzyme responsible for the profound production of glycosylated forms than the free form. Likewise, overexpression of prenyltransferase has been found to increase the yields of the linear as well as some cyclic sesquiterpenes (Gounaris, 2010).

METABOLIC ENGINEERING OF PLANTS FOR PRODUCTION AND ENHANCEMENT OF ANTI-CORONAVIRUS COMPOUNDS

The metabolic machinery of plants could be targeted for engineering at different points that ultimately result in either novel compounds or the over-production of important medicinal metabolites (**Figure 3**). The metabolic engineering of plants is one area of plant biotechnology that possesses enormous potential for producing anti-SARS-CoV-2 compounds. Many of such pharmacological compounds have been extensively studied and reported in the literature (De Luca et al., 2012; Atanasov et al., 2015; Wurtzel and Kutchan, 2016; Buyel, 2018). Traditionally, people take these compounds orally as a whole plant, its decoction, or as a crude extract. However, it can be detrimental as most of the time unwanted compounds are also administered. Besides, these compounds are present at low native concentrations in plants and most of the time is not as effective as a pure compound. Commercial extraction of any such compound from any plant species may have detrimental effects on the plant population and can even push a plant species to the brink of extinction (Barone et al., 2019). For example, mass production of paclitaxel (source of Taxol®) led to the endangerment of not only its source plant (Pacific yew) but also other species of the same genus (Hawkins, 2007).

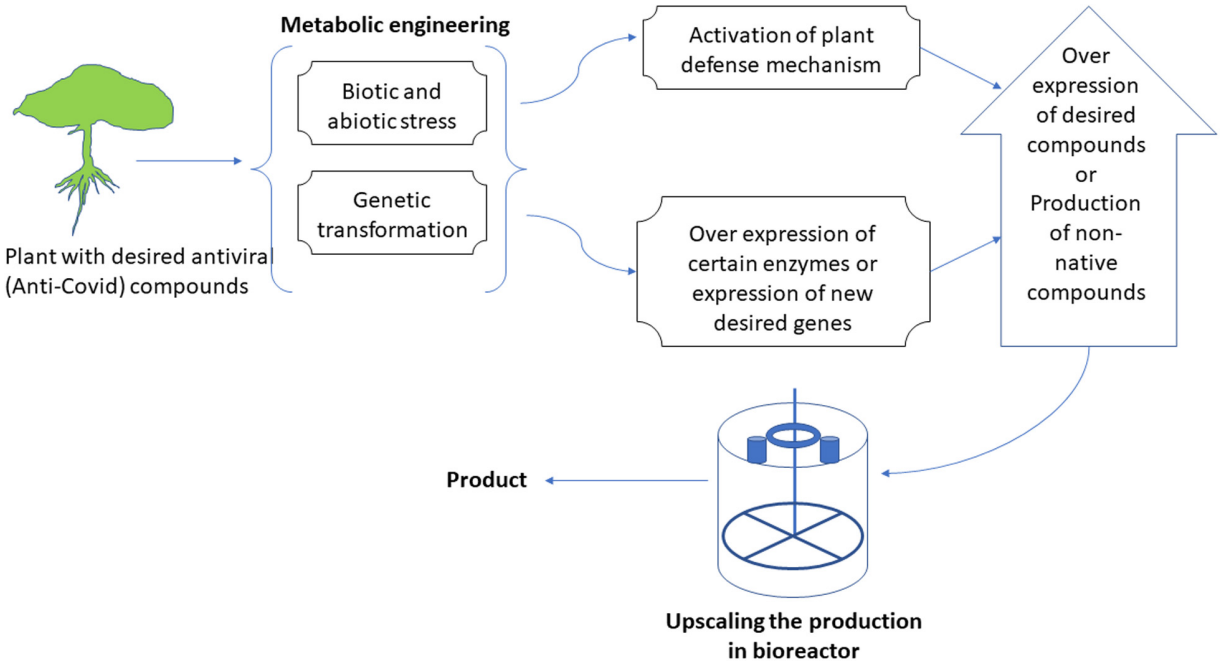

FIGURE 3 | A representative flow-chart for the process of metabolic engineering of in vitro cultures to produce novel compounds or to over-produce existing compounds.

Thus, an increasing interest has been observed focusing on *in-planta* production of important metabolites via genetic and metabolic engineering. For example, genistein and taxane (precursors of paclitaxel) have been successfully produced in plants other than their native species. However, alongside attempting the increased production of certain end-products or producing new products/compounds via genetic engineering, it has been understood that biosynthetic engineering is a highly complicated process that demands diverse knowledge in all fields of biochemistry, biotechnology, and molecular biology (Barone et al., 2019). Several *in vitro* cultures for growing plant tissues could be manipulated through metabolic engineering for secondary metabolite production and its quantity enhancement, for example; adventitious root culture, callus culture, somatic embryogenesis and regeneration, cell suspension culture, protoplast culture, and hairy root culture, etc. (Gantait et al., 2020).

Despite drawbacks, attempts to overproduce a single metabolite or end-product have progressed in recent years and several examples can be found in the literature in which metabolic engineering of plants has been applied in the field of medicine. For instance, the production of genistein, an isoflavone, and a known antiviral compound has been shown in non-leguminous species in which this compound is not native. It was made possible by introducing the isoflavone synthase (IFS) gene from soybean (glycine max) to non-leguminous species tobacco (*Nicotiana tabacum*), lettuce (*Lactuca sativa*), and petunia (*Petunia hybrida*) (Barone et al., 2019). Furthermore, to increase the quantity of genistein, anti-sense suppression of flavanone-3-hydroxylase (F3H) and overexpression of phenylalanine ammonia-lyase (PAL) has also been employed. It is important to highlight here that PAL is an enzyme of the phenylpropanoid pathway that feeds into flavonoid biosynthesis.

Artemisinin commercially known for its antimalarial activity is also reported for its antiviral activity (Lubbe et al., 2012). The low concentration of artemisinin in its native plant the sweet wormwood (*Artemisia annua*) and its high demand in the pharmaceutical industry have led researchers to investigate its *in-planta* production as well as in other culture systems. Although little success has been made in this direction, a potential bottleneck has been identified which may lead to its biosynthesis in the near future.

In another study, sweet wormwood has been successfully transformed using *Agrobacterium tumefaciens* to produce taxane (a paclitaxel precursor) (Li et al., 2015). It should be noted that paclitaxel is a famous anticancer compound and is also known for its anti-HIV activity (Ryang et al., 2019).

Glycyrrhizin, an active component of licorice roots, has been reported to show antiviral activity against SARS-CoV *in vitro* (Boccalone et al., 2020). This compound has been converted via biotransformation to several other compounds that are more stable, easily soluble, and having greater emulsifying properties than glycyrrhizin. Other advantages of biotransformation of glycyrrhizin include strong stereoselectivity and regioselectivity, low byproduct production, and increased activity (He et al., 2019). It has been found that cell suspension culture of *G. glabra* and *Eucalyptus perriniana* can transform glycyrrhetinic acid (a byproduct of glycyrrhizin) to several other important compounds. For instance, these include 3−*O*−[α−l−arabinopyranosyl−(1→2)−β−D−glucuronopyran

osyl]−24−hydroxy−18 β−glycyrrhetinic acid, 23,28−dihydroxy−18β−glycyrrhetinic acid−30β−glucopy ranosyl ester, and 28−hydroxy−18β−glycyrrhetinic acid−30β−glucopyranosyl ester which are reported to be important medicinal compounds (Hayashi et al., 1990; Orihara and Furuya, 1990).

Resveratrol is another example of a natural product that is reported for its anti-coronavirus activity (Lin et al., 2017) and has been successfully transformed via molecular engineering into plants that normally do not produce resveratrol (Giovinazzo et al., 2012). This compound has been reported to be found in grapes, berries, white tea, and passion fruit, etc. in a very low quantity which makes its extraction challenging (Shrestha et al., 2019). Metabolic engineering has been performed by several researchers to improve its quantity or to express it in new plants (Giovinazzo et al., 2012). For instance, the expression of grape genetic markers in tobacco leaves diverted the typical substrates of chalcone synthase to produce CHS up to 300 mg/g of resveratrol (Halls and Yu, 2008).

Plant metabolic engineering could prove to be an important tool in directing a plant's metabolic machinery to the synthesis of important natural compounds against coronaviruses. For instance, metabolically engineered soybean and canola produced a high level of monosaturated fatty acid which otherwise produces a high level of linolenic acid which is prone to oxidation (Dellapenna, 2001). In another example, the use of g-tocopherol methyltransferase (g-TMT) showed a 10-fold increase in vitamin E activity in engineered Arabidopsis seed oil (Shintani and Dellapenna, 1998). In an example of metabolic engineering, b-carotene (provitamin A) has been successfully engineered into rice endosperm (Ye et al., 2000). 3-*O*-glucosyl-resveratrol production in a *V. vinifera* cell culture was significantly stimulated by saliva, with a 7.0-fold increase compared to control (Cai et al., 2012). Methyl jasmonate elicitation is an effective strategy to induce and enhance the synthesis of the anticancer agent paclitaxel (Taxol®) in taxus cell suspension cultures (Patil et al., 2014).

In an interesting experiment, *N. tabacum* plants were transformed with the stilbene synthase gene from grapevine using *A. tumefaciens*-mediated gene transfer. The transformed plants not only expressed the gene but also showed resistance to the fungal pathogen *Botrytis cinerea* (Hain et al., 1993). Metabolic engineering has thus improved its composition as well as improved its level. Stilbene synthase gene (STS-encoding gene)-mediated transformation thus confirmed that plant molecular engineering with resveratrol may lead to novel functions such as resistance to stresses, fungal infection, or increased nutritional value. STS genes have been transferred to several other crops as well such as *Medicago sativa L.*, *Arabidopsis thaliana L*, *L. sativa L*, and *Solanum lycopersicum L.*, etc. (Giovinazzo et al., 2012). The gene expression is however controlled by the chosen promoter. Commonly used promoters to control the expression of STS-encoding genes include the well-characterized constitutive promoter pCaMV35S, fungus-inducible promoter pPR10.1, stress-responsive promoter pVst1, and the tissue-specific promoter p-nap (Stark-Lorenzen et al., 1997; Coutos-Thévenot et al., 2001; Fan et al., 2008). The increased nutritional values of several fruits and edible crops via transformation along with the higher yield of resveratrol can be exploited for use in SARS-CoV-2 management.

Ginsenoside (ginsenoside Rb1) occupies a unique place in the pharmaceutical industry around the globe as an active ingredient of *Panax ginseng*. It has recently been reported for its anti-SARS-CoV activity (Boccalone et al., 2020). While the traditional methods of isolation and purification of ginsenoside were challenging and time-consuming, the use of modern-day biotechnological approaches not only enhances its level but also makes the extraction process easier. These approaches include but are not limited to tissue culture, cell suspension culture, protoplast culture, polyploidy, *in vitro* mutagenesis, and hairy root culture (Gantait et al., 2020). For instance, Yu et al. (2016) have reported the enhanced production of ginsenoside by using a fungal strain *Alternaria panax* in cell suspension cultures. The cell wall exudates fungi that contain oligosaccharides and chitin that act as biotic elicitors (Yu et al., 2016).

Jasmonates have been reported to induce oxidative stress and downregulate many genes which lead to an augmentation of ginsenoside in the cell suspension culture. The mechanism behind the role of the elicitor mainly involve the activation of phenylalanine amino lyase which in turn elevates the level of defense compounds and hence ginsenoside biosynthesis (Yu et al., 2002; Kim et al., 2004; Wang et al., 2006).

Mutagenesis and *in vitro* cultures which incorporate genotypic changes in the culture is another popular method used for the enhanced production of ginsenoside. In this method, *P. ginseng* calli are exposed to varying doses of gamma radiation ranging from 10 to 100 Gy (Gray) and for various lengths of time to bring genetic changes and hence to increase the ginsenoside level in callus cultures (Kim et al., 2009, 2013). The increased production of primary ginsenosides was associated with the high expression of squalene epoxidase, dammarenediol synthase, and phytosterol synthase genes (Kim et al., 2013). Summing up, it can be concluded that engineering plant cell and tissue cultures, through *in vitro* mutagenesis, direct gene transfer, and *A. tumefaciens*-mediated transformation could play an important role in the production/enrichment of natural products that are easily repeatable in a short time and thus can be exploited in the fight against COVID-19.

COMMERCIALIZATION OF PLANT *IN VITRO* CULTURES FOR SECONDARY METABOLITE PRODUCTION

Advances in biotechnological approaches, particularly plant cell culturing methods, provides new means for commercially valuable, medicinally important plant secondary metabolites (Hussain et al., 2012). Different kinds of strategies have been used, for example, appropriate design of bioreactor systems, optimization of nutrient medium, highly productive line selection, elicitation, two-phase cultivation, and metabolic engineering (Marchev et al., 2020). The scaling-up of *in vitro*

plant culturing into large-scale, economically feasible bioreactors provides the sustainable and continuous production of high-valued plant secondary metabolites. Secondary metabolite production in bioreactors depends on pharmacological significance as well as human health benefits. The production of rosmarinic acid and saponins are the selected examples of the commercial production of secondary metabolites *in vitro* (Weathers et al., 2010). Different kinds of bioreactor systems are used to enhance the accumulation of rosmarinic acid such as hairy root cultures or shoot suspension cultures from different plants. Plant suspension culture technology offers a convenient way of upscaling plant *in-vitro* culture systems for the biosynthesis of secondary metabolites. The ease of its upscale is attributed to its shorter cycle of production and simpler methods for bioreactor construction (Marchev et al., 2020). The successful and rapid development of plant metabolic engineering offers an attractive opportunity to increase the content of secondary metabolites in cell and hairy root cultures from aromatic and medicinal plants at a feasible level. Moreover, plant metabolic engineering also makes it possible to understand the molecular biology of the biosynthesis of the secondary metabolites (Pavlov and Bley, 2018). Ginsenoside and taxole, examples of successfully commercialized plant cell suspension cultures, derived specialized metabolites. As reported earlier (**Table 1**), ginsenosides have proven to be effective against SARS-CoV in inhibiting glycoprotein activity (Wu et al., 2004). Although due to the limited understanding of the molecular basis of plant secondary metabolite biosynthesis, the widespread utilization of the plant suspension cultures platform has yet to be primarily realized (Arya et al., 2020). However, with much research effort, many secondary metabolites achieved a semi-commercial status (Weathers et al., 2010).

Regarding commercialization, the most promising fact associated with plant tissue cultures is that they offer an avenue for the production of these medicinally important phytochemicals in an appropriate bioreactor. The production of the plant *in vitro* cultures is indeed an important prerequisite for the large-scale yield and commercialization of phytomedicine. This is linked with the fact that once carefully selected and optimized, *in vitro* cultures of plants could yield ten times higher phytomedicine than plants grown naturally. However, the production of phytomedicine in bioreactors requires the selection of suitable cell lines/cultures, optimization of culture conditions, application of proper elicitation strategy, immobilization of cells, and efficient downstream processing (Marchev et al., 2020).

Commercial production of secondary metabolites largely depends either on higher market value or high demand which will undoubtedly be high if any of the phytochemicals tested proved effective against the current SARS-CoV virus. The continuous efforts in this field will lead to the controllable and successful production of specific, valuable, and yet unknown plant secondary metabolites (Hussain et al., 2012) against human viruses specifically coronaviruses. Such understanding will ultimately lead to the production of important phytochemicals that are active against SARS-CoV-2.

CONCLUSION

Plant biotechnology is a promising platform for exploring the unlimited potential of many diverse medicinal plants. Unfortunately, pandemics like COVID-19 are likely to occur again on a smaller or larger scale due to the array of known and unknown pathogens out there. Plant biotechnology tools and methods such as *in vitro* culture technology is an asset at our disposal to harness the diversity of secondary metabolites produced by different plants. *In vitro* culture technologies can potentially grow any plant anywhere and offer the added value of overproduction of plant cultures, enhanced production of secondary metabolites, and the generation of novel medicinal compounds.

AUTHOR CONTRIBUTIONS

TK and MK conceived the idea. TK, KK, MK, NU, and AN each drafted a different section of the manuscript. MK critically reviewed the manuscript. KK and Z-U-RM gathered the data and prepared and formatted the tables. TK, KK, and MK performed the revisions. TK constructed the figures and formatted the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abbasi, B. H., Ali, H., Yücesan, B., Saeed, S., Rehman, K., and Khan, M. A. (2016). Evaluation of biochemical markers during somatic embryogenesis in *Silybum marianum* L. *3 Biotech* 6:71. doi: 10.1007/s13205-016-0366-1

Abraham, J. (2020). Passive antibody therapy in COVID-19. *Nat. Rev. Immunol.* 20, 401–403. doi: 10.1038/s41577-020-0365-7

Abyari, M., Nasr, N., Soorni, J., and Sadhu, D. (2016). Enhanced accumulation of scopoletin in cell suspension culture of Spilanthes acmella Murr. using precursor feeding. *Braz. Arch. Biol. Technol.* 59:e16150533. doi: 10.1590/1678-4324-2016150533

Ali, H., Khan, M. A., Kayani, W. K., Khan, T., and Khan, R. S. (2018a). Thidiazuron regulated growth, secondary metabolism and essential oil profiles in shoot cultures of *Ajuga bracteosa*. *Ind. Crops Prod.* 121, 418–427. doi: 10.1016/j.indcrop.2018.05.043

Ali, H., Khan, M. A., Ullah, N., and Khan, R. S. (2018b). Impacts of hormonal elicitors and photoperiod regimes on elicitation of bioactive secondary volatiles in cell cultures of *Ajuga bracteosa*. *J. Photochem. Photobiol. B Biol.* 183, 242–250. doi: 10.1016/j.jphotobiol.2018.04.044

Arya, S. S., Rookes, J., Cahill, D., and Lenka, S. K. (2020). Next-generation metabolic engineering approaches towards development of plant cell suspension cultures as specialized metabolite producing biofactories. *Biotechnol. Adv.* 45:107635. doi: 10.1016/j.biotechadv.2020.107635

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E.-M., Linder, T., Wawrosch, C., Uhrin, P., et al. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* 33, 1582–1614. doi: 10.1016/j.biotechadv.2015.08.001

Awad, V., Kuvalekar, A., and Harsulkar, A. (2014). Microbial elicitation in root cultures of *Taverniera cuneifolia* (Roth) Arn. for elevated glycyrrhizic acid production. *Ind. Crops Prod.* 54, 13–16. doi: 10.1016/j.indcrop.2013.12.036

Bannerman, R. H. O., Burton, J., Bannerman, R. H., Ch'en, W. C., and World Health, O. (1983). *Traditional Medicine and Health Care Coverage: A Reader for Health Administrators and Practitioners*. Geneva: World Health Organization.

Barone, R. P., Knittel, D. K., Ooka, J. K., Porter, L. N., Smith, N. T., and Owens, D. K. (2019). The production of plant natural products beneficial to humanity by metabolic engineering. *Curr. Plant Biol.* 24:100121. doi: 10.1016/j.cpb.2019.100121

Bassolino, L., Giacomelli, E., Giovanelli, S., Pistelli, L., Cassetti, A., Damonte, G., et al. (2015). Tissue culture and aromatic profile in Salvia dolomitica Codd. *Plant Cell Tissue Organ Cult.* 121, 83–95. doi: 10.1007/s11240-014-0681-3

Begum, S., Zahid, A., Khan, T., Khan, N. Z., and Ali, W. (2020). Comparative analysis of the effects of chemically and biologically synthesized silver nanoparticles on biomass accumulation and secondary metabolism in callus cultures of *Fagonia indica*. *Physiol. Mol. Biol. Plants* 26, 1739–1750. doi: 10.1007/s12298-020-00851-w

Ben-Shabat, S., Yarmolinsky, L., Porat, D., and Dahan, A. (2020). Antiviral effect of phytochemicals from medicinal plants: applications and drug delivery strategies. *Drug Deliv. Transl. Res.* 10, 354–367. doi: 10.1007/s13346-019-00691-6

Boccalone, E., Lanni, V. M., and Magro, V. M. (2020). Role of natural drugs in severe acute respiratory syndrome coronavirus 2: potential beneficial effects in prevention or prior treatment in elderly and cancer patients. *Geriatr. Care* 6:9016. doi: 10.4081/gc.2020.9016

Buyel, J. (2018). Plants as sources of natural and recombinant anti-cancer agents. *Biotechnol. Adv.* 36, 506–520. doi: 10.1016/j.biotechadv.2018.02.002

Cai, Z., Knorr, D., and Smetanska, I. (2012). Enhanced anthocyanins and resveratrol production in *Vitis vinifera* cell suspension culture by indanoyl-isoleucine, N-linolenoyl-l-glutamine and insect saliva. *Enzyme Microb. Technol.* 50, 29–34. doi: 10.1016/j.enzmictec.2011.09.001

Callaway, E. (2020). The race for coronavirus vaccines: a graphical guide. *Nature* 580, 576–577. doi: 10.1038/d41586-020-01221-y

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. C., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Chandran, H., Meena, M., Barupal, T., and Sharma, K. (2020). Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. *Biotechnol. Rep.* 26:e00450. doi: 10.1016/j.btre.2020.e00450

Chen, L., Li, J., Luo, C., Liu, H., Xu, W., Chen, G., et al. (2006). Binding interaction of quercetin-3-β-galactoside and its synthetic derivatives with SARS-CoV 3CLpro: Structure–activity relationship studies reveal salient pharmacophore features. *Bioorg. Med. Chem.* 14, 8295–8306. doi: 10.1016/j.bmc.2006.09.014

Cheng, P. W., Ng, L. T., Chiang, L. C., and Lin, C. C. (2006). Antiviral effects of saikosaponins on human coronavirus 229E *in vitro*. *Clin. Exp. Pharmacol. Physiol.* 33, 612–616. doi: 10.1111/j.1440-1681.2006.04415.x

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H., and Doerr, H. W. (2003). Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. *Lancet* 361, 2045–2046. doi: 10.1016/S0140-6736(03)13615-X

Coutos-Thévenot, P., Poinssot, B., Bonomelli, A., Yean, H., Breda, C., Buffard, D., et al. (2001). In vitro tolerance to Botrytis cinerea of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase Vst 1 gene under the control of a pathogen-inducible PR 10 promoter. *J. Exp. Bot.* 52, 901–910. doi: 10.1093/jexbot/52.358.901

Darvishi, E., Kahrizi, D., Bahraminejad, S., and Mansouri, M. (2016). *In vitro* induction of α-pinene, pulegone, menthol, menthone and limonene in cell suspension culture of pennyroyal (*Mentha pulegium*). *Cell. Mol. Biol.* 62, 7–9.

Daviet, L., and Schalk, M. (2010). Biotechnology in plant essential oil production: progress and perspective in metabolic engineering of the terpene pathway. *Flavour Fragr. J.* 25, 123–127. doi: 10.1002/ffj.1981

De Luca, V., Salim, V., Atsumi, S. M., and Yu, F. (2012). Mining the biodiversity of plants: a revolution in the making. *Science* 336, 1658–1661. doi: 10.1126/science.1217410

De Oliveira, C. A. F., Corassin, C. H., Corrêa, B., and Oswald, I. P. (2014). "Animal health: Mycotoxins," in *Encyclopedia of Agriculture and Food Systems*, ed. N. K. Van Alfen (Oxford: Academic Press), 358–377. doi: 10.1016/B978-0-444-52512-3.00200-X

Del Pilar Nicasio-Torres, M., Meckes-Fischer, M., Aguilar-Santamaría, L., Garduño-Ramírez, M. L., Chávez-Ávila, V. M., and Cruz-Sosa, F. (2012). Production of chlorogenic acid and isoorientin hypoglycemic compounds in *Cecropia obtusifolia* calli and in cell suspension cultures with nitrate deficiency. *Acta Physiol. Plant.* 34, 307–316. doi: 10.1007/s11738-011-0830-9

Dellapenna, D. (2001). Plant metabolic engineering. *Plant Physiol.* 125, 160–163. doi: 10.1104/pp.125.1.160

Devaux, C. A., Rolain, J.-M., Colson, P., and Raoult, D. (2020). New insights on the antiviral effects of chloroquine against coronavirus: What to expect for COVID-19? *Int. J. Antimicrob. Agents* 55:105938. doi: 10.1016/j.ijantimicag.2020.105938

Dyakov, Y. T., Dzhavakhiya, V. G., and Korpela, T. (2007). "Chapter 15 - Molecular basis of plant immunization," in *Comprehensive and Molecular Phytopathology*, eds Y. T. Dyakov, V. G. Dzhavakhiya, and T. Korpela (Amsterdam: Elsevier), 423–438. doi: 10.1016/B978-044452132-3/50020-1

Faizal, A., and Sari, A. V. (2019). Enhancement of saponin accumulation in adventitious root culture of Javanese ginseng (*Talinum paniculatum* Gaertn.) through methyl jasmonate and salicylic acid elicitation. *Afr. J. Biotechnol.* 18, 130–135. doi: 10.5897/AJB2018.16736

Fan, C., Pu, N., Wang, X., Wang, Y., Fang, L., Xu, W., et al. (2008). Agrobacterium-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata*. *Plant Cell Tissue Organ Cult.* 92, 197–206. doi: 10.1007/s11240-007-9324-2

Fan, H.-H., Wang, L.-Q., Liu, W.-L., An, X.-P., Liu, Z.-D., He, X.-Q., et al. (2020). Repurposing of clinically approved drugs for treatment of coronavirus disease 2019 in a 2019-novel coronavirus-related coronavirus model. *Chin. Med. J.* 133, 1051–1056. doi: 10.1097/CM9.0000000000000797

Ferner, R. E., and Aronson, J. K. (2020). Chloroquine and hydroxychloroquine in covid-19. *BMJ* 369:m1432. doi: 10.1136/bmj.m1432

Ferri, M., Tassoni, A., Franceschetti, M., Righetti, L., Naldrett, M. J., and Bagni, N. (2009). Chitosan treatment induces changes of protein expression profile and stilbene distribution in *Vitis vinifera* cell suspensions. *Proteomics* 9, 610–624. doi: 10.1002/pmic.200800386

Gandhi, S. G., Mahajan, V., and Bedi, Y. S. (2015). Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants. *Planta* 241, 303–317. doi: 10.1007/s00425-014-2232-x

Gantait, S., Mitra, M., and Chen, J.-T. (2020). Biotechnological interventions for ginsenosides production. *Biomolecules* 10:538. doi: 10.3390/biom10040538

Gautret, P., Lagier, J.-C., Parola, P., Hoang, V. T., Meddeb, L., Mailhe, M., et al. (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int. J. Antimicrob. Agents* 56:105949. doi: 10.1016/j.ijantimicag.2020.105949

Giovinazzo, G., Ingrosso, I., Paradiso, A., De Gara, L., and Santino, A. (2012). Resveratrol biosynthesis: plant metabolic engineering for nutritional improvement of food. *Plant Foods Hum. Nutr.* 67, 191–199.

Giri, C. C., and Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production *in vitro* using Plant Cell, Tissue and Organ Cultures: recent trends and a sky eye view appraisal. *Plant Cell Tissue Organ Cult.* 126, 1–18. doi: 10.1007/S11240-016-0985-6

Gounaris, Y. (2010). Biotechnology for the production of essential oils, flavours and volatile isolates. A review. *Flavour Fragr. J.* 25, 367–386. doi: 10.1002/ffj.1996

György, Z., Tolonen, A., Pakonen, M., Neubauer, P., and Hohtola, A. (2004). Enhancing the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* by biotransformation of cinnamyl alcohol. *Plant Sci.* 166, 229–236. doi: 10.1016/j.plantsci.2003.09.011

Hadwiger, L. A. (2013). Multiple effects of chitosan on plant systems: solid science or hype. *Plant Sci.* 208, 42–49. doi: 10.1016/j.plantsci.2013.03.007

Hain, R., Reif, H.-J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., et al. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* 361, 153–156. doi: 10.1038/361153a0

Halder, M., Sarkar, S., and Jha, S. (2019). Elicitation: a biotechnological tool for enhanced production of secondary metabolites in hairy root cultures. *Eng. Life Sci.* 19, 880–895. doi: 10.1002/elsc.201900058

Halls, C., and Yu, O. (2008). Potential for metabolic engineering of resveratrol biosynthesis. *Trends Biotechnol.* 26, 77–81. doi: 10.1016/j.tibtech.2007.11.002

Harrison, C. (2020). Coronavirus puts drug repurposing on the fast track. *Nat. Biotechnol.* 38, 379–381. doi: 10.1038/d41587-020-00003-1

Hawkins, B. (2007). *Plants for Life Medicinal Plant Conservation and Botanic Gardens*. Wuhan: BGCI.

Hayashi, H., Fukui, H., and Tabata, M. (1990). Biotransformation of 18β-glycyrrhetinic acid by cell suspension cultures of Glycyrrhiza glabra. *Phytochemistry* 29, 2149–2152. doi: 10.1016/0031-9422(90)83026-W

He, Y., Hu, Z., Li, A., Zhu, Z., Yang, N., Ying, Z., et al. (2019). Recent advances in biotransformation of saponins. *Molecules* 24:2365. doi: 10.3390/molecules24132365

Hegazi, G., Ghareb, H., and Gabr, M. (2020). Ephedrine production from suspension cultures of *Ephedra alata* L. callus. *Biotechnologia* 101, 25–33. doi: 10.5114/bta.2020.92925

Ho, T.-Y., Wu, S.-L., Chen, J.-C., Li, C.-C., and Hsiang, C.-Y. (2007). Emodin blocks the SARS coronavirus spike protein and angiotensin-converting enzyme 2 interaction. *Antiviral Res.* 74, 92–101. doi: 10.1016/j.antiviral.2006.04.014

Holopainen, J. K. (2011). Can forest trees compensate for stress-generated growth losses by induced production of volatile compounds? *Tree Physiol.* 31, 1356–1377. doi: 10.1093/treephys/tpr111

Huang, X., Yao, J., Zhao, Y., Xie, D., Jiang, X., and Xu, Z. (2016). Efficient rutin and quercetin biosynthesis through flavonoids-related gene expression in *Fagopyrum tataricum* Gaertn. hairy root cultures with UV-B irradiation. *Front. Plant Sci.* 7:63. doi: 10.3389/fpls.2016.00063

Hussain, M. S., Fareed, S., Saba Ansari, M., Rahman, A., Ahmad, I. Z., and Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. *J. Pharm. Bioallied Sci.* 4, 10–20. doi: 10.4103/0975-7406.92725

Jackson, L. A., Anderson, E. J., Rouphael, N. G., Roberts, P. C., Makhene, M., Coler, R. N., et al. (2020). An mRNA Vaccine against SARS-CoV-2 — Preliminary Report. *N. Engl. J. Med.* 383, 1920–1931. doi: 10.1056/NEJMoa2022483

Jiao, J., Gai, Q.-Y., Wang, W., Luo, M., Zu, Y.-G., Fu, Y.-J., et al. (2016). Enhanced astragaloside production and transcriptional responses of biosynthetic genes in *Astragalus membranaceus* hairy root cultures by elicitation with methyl jasmonate. *Biochem. Eng. J.* 105, 339–346. doi: 10.1016/j.bej.2015.10.010

Jiao, J., Gai, Q.-Y., Wang, W., Zang, Y.-P., Niu, L.-L., Fu, Y.-J., et al. (2018). Remarkable enhancement of flavonoid production in a co-cultivation system of *Isatis tinctoria* L. hairy root cultures and immobilized *Aspergillus niger*. *Ind. Crops Prod.* 112, 252–261. doi: 10.1016/j.indcrop.2017.12.017

Kad, A., Pundir, A., and Sood, H. (2018). Elicitation of salidroside under tissue culture conditions in the *trans*-himalayan plant *Rhodiola imbricata*. *WSEAS Transact. Biol. Biomed.* 16, 75–89.

Kannan, N., Manokari, M., and Shekhawat, M. S. (2020). Enhanced production of anthraquinones and phenolic compounds using chitosan from the adventitious roots of *Morinda coreia* Buck. and Ham. *Ind. Crops Prod.* 148:112321. doi: 10.1016/j.indcrop.2020.112321

Karuppaiya, P., and Tsay, H. S. (2020). Enhanced production of podophyllotoxin, kaempferol, and quercetin from callus culture of *Dysosma pleiantha* (Hance) Woodson: An endangered medicinal plant. *Biotechnol. Appl. Biochem.* 67, 95–104. doi: 10.1002/bab.1810

Karwasara, V. S., Jain, R., Tomar, P., and Dixit, V. K. (2010). Elicitation as yield enhancement strategy for glycyrrhizin production by cell cultures of *Abrus precatorius* Linn. *In Vitro Cell. Dev. Biol. Plant* 46, 354–362. doi: 10.1007/s11627-010-9278-7

Keyaerts, E., Vijgen, L., Maes, P., Neyts, J., and Ranst, M. V. (2004). *In vitro* inhibition of severe acute respiratory syndrome coronavirus by chloroquine. *Biochem. Biophys. Res. Commun.* 323, 264–268. doi: 10.1016/j.bbrc.2004.08.085

Keyaerts, E., Vijgen, L., Pannecouque, C., Van Damme, E., Peumans, W., Egberink, H., et al. (2007). Plant lectins are potent inhibitors of coronaviruses by interfering with two targets in the viral replication cycle. *Antiviral Res.* 75, 179–187. doi: 10.1016/j.antiviral.2007.03.003

Khan, I., Khan, M. A., Shehzad, M. A., Ali, A., Mohammad, S., Ali, H., et al. (2020). Micropropagation and production of health promoting Lignans in *Linum usitatissimum*. *Plants* 9:728. doi: 10.3390/plants9060728

Khan, M. A., Ali, A., Mohammad, S., Ali, H., Khan, T., Jan, A., et al. (2020). Iron nano modulated growth and biosynthesis of steviol glycosides in *Stevia rebaudiana*. *Plant Cell Tissue Organ Cult.* 143, 121–130. doi: 10.1007/s11240-020-01902-6

Khan, M. A., Khan, T., and Ali, H. (2019a). Plant cell culture strategies for the production of terpenes as green solvents. *Ind. Appl. Green Solvents* 50, 1–20. doi: 10.21741/9781644900239-1

Khan, M. A., Khan, T., Riaz, M. S., Ullah, N., Ali, H., and Nadhman, A. (2019b). "Plant cell nanomaterials interaction: growth, physiology and secondary metabolism," in *Comprehensive Analytical Chemistry*, eds S. K. Verma and A. K. Das (Amsterdam: Elsevier), 23–54. doi: 10.1016/bs.coac.2019.04.005

Khan, T., Abbasi, B. H., Khan, M. A., and Azeem, M. (2017). Production of biomass and useful compounds through elicitation in adventitious root cultures of *Fagonia indica*. *Ind. Crops Prod.* 108, 451–457. doi: 10.1016/j.indcrop.2017.07.019

Khan, T., Ullah, M. A., Garros, L., Hano, C., and Abbasi, B. H. (2019c). Synergistic effects of melatonin and distinct spectral lights for enhanced production of anti-cancerous compounds in callus cultures of *Fagonia indica*. *J. Photochem. Photobiol. B Biol.* 190, 163–171. doi: 10.1016/j.jphotobiol.2018.10.010

Kim, D., Kim, S., Jeong, I., Kim, J.-B., Lee, G.-J., Kang, S.-Y., et al. (2009). Improvement of ginsenoside production by *Panax ginseng* adventitious roots induced by γ-irradiation. *Biol. Plant.* 53:408. doi: 10.1007/s10535-009-0079-y

Kim, D. E., Min, J. S., Jang, M. S., Lee, J. Y., Shin, Y. S., Park, C. M., et al. (2019). Natural bis-benzylisoquinoline alkaloids-tetrandrine, fangchinoline, and cepharanthine, inhibit human coronavirus OC43 infection of MRC-5 human lung cells. *Biomolecules* 9:696. doi: 10.3390/biom9110696

Kim, D. S., Song, M., Kim, S.-H., Jang, D.-S., Kim, J.-B., Ha, B.-K., et al. (2013). The improvement of ginsenoside accumulation in Panax ginseng as a result of γ-irradiation. *J. Ginseng Res.* 37, 332–340. doi: 10.5142/jgr.2013.37.332

Kim, J. Y., Kim, Y. I., Park, S. J., Kim, I. K., Choi, Y. K., and Kim, S.-H. (2018). Safe, high-throughput screening of natural compounds of MERS-CoV entry inhibitors using a pseudovirus expressing MERS-CoV spike protein. *Int. J. Antimicrob. Agents* 52, 730–732. doi: 10.1016/j.ijantimicag.2018.05.003

Kim, Y.-S., Hahn, E.-J., Murthy, H. N., and Paek, K.-Y. (2004). Effect of polyploidy induction on biomass and ginsenoside accumulations in adventitious roots of ginseng. *J. Plant Biol.* 47, 356–360. doi: 10.1007/BF03030551

Kusakari, K., Yokoyama, M., and Inomata, S. (2000). Enhanced production of saikosaponins by root culture of *Bupleurum falcatum* L. using two-step control of sugar concentration. *Plant Cell Rep.* 19, 1115–1120. doi: 10.1007/s002990000240

Ledford, H. (2020). Coronavirus breakthrough: dexamethasone is first drug shown to save lives. *Nature* 582:469. doi: 10.1038/d41586-020-01824-5

Levenson, E., and Howard, J. (2020). *What We Know About Moderna's Coronvirus Vaccine and How it Differs From Pfizer's. CNN*. Available online at: https://edition.cnn.com/2020/12/17/health/moderna-vaccine-what-we-know/index.html (accessed February 12, 2021).

Li, S.-Y., Chen, C., Zhang, H.-Q., Guo, H.-Y., Wang, H., Wang, L., et al. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Res.* 67, 18–23. doi: 10.1016/j.antiviral.2005.02.007

Li, Y.-J., Guo, Y., Yang, Q., Weng, X.-G., Yang, L., Wang, Y.-J., et al. (2015). *Flavonoids casticin* and chrysosplenol D from *Artemisia annua* L. inhibit inflammation *in vitro* and *in vivo*. *Toxicol. Appl. Pharmacol.* 286, 151–158. doi: 10.1016/j.taap.2015.04.005

Lin, S.-C., Ho, C.-T., Chuo, W.-H., Li, S., Wang, T. T., and Lin, C.-C. (2017). Effective inhibition of MERS-CoV infection by resveratrol. *BMC Infect. Dis.* 17:144. doi: 10.1186/s12879-017-2253-8

Lubbe, A., Seibert, I., Klimkait, T., and Van Der Kooy, F. (2012). Ethnopharmacology in overdrive: the remarkable anti-HIV activity of *Artemisia annua*. *J. Ethnopharmacol.* 141, 854–859. doi: 10.1016/j.jep.2012.03.024

Lung, J., Lin, Y. S., Yang, Y. H., Chou, Y. L., Shu, L. H., Cheng, Y. C., et al. (2020). The potential chemical structure of anti-SARS-CoV-2 RNA-dependent RNA polymerase. *J. Med. Virol.* 92, 693–697. doi: 10.1002/jmv.25761

Marchev, A. S., Yordanova, Z. P., and Georgiev, M. I. (2020). Green (cell) factories for advanced production of plant secondary metabolites. *Crit. Rev. Biotechnol.* 40, 443–458. doi: 10.1080/07388551.2020.1731414

Maxmen, A. (2020). More than 80 clinical trials launch to test coronavirus treatments. *Nature* 578, 347–348. doi: 10.1038/d41586-020-00444-3

Nadeem, M., Ahmad, W., Zahir, A., Hano, C., and Abbasi, B. H. (2019). Salicylic acid-enhanced biosynthesis of pharmacologically important lignans and neo lignans in cell suspension culture of *Linum ussitatsimum* L. *Eng. Life Sci.* 19, 168–174. doi: 10.1002/elsc.201800095

Nature. (2020). Redeploying plant defences. *Nat. Plants* 6:177. doi: 10.1038/s41477-020-0628-0

Nazir, M., Asad Ullah, M., Mumtaz, S., Siddiquah, A., Shah, M., Drouet, S., et al. (2020). Interactive effect of melatonin and UV-C on phenylpropanoid metabolite production and antioxidant potential in callus cultures of purple basil (*Ocimum basilicum* L. var. s *purpurascens*). *Molecules* 25:1072. doi: 10.3390/molecules25051072

Olgunsoy, P., Ulusoy, S., and Akçay, U. Ç. (2017). Metabolite production and antibacterial activities of callus cultures from rosa damascena mill. Petals. *Marmara Pharm. J.* 21, 590–597. doi: 10.12991/marupj.319331

Orihara, Y., and Furuya, T. (1990). Biotransformation of 18β-glycyrrhetinic acid by cultured cells of *Eucalyptus perriniana* and *Coffea arabica*. *Phytochemistry* 29, 3123–3126. doi: 10.1016/0031-9422(90)80169-H

Osman, N. I., Sidik, N. J., and Awal, A. (2018). Efficient enhancement of gallic acid accumulation in cell suspension cultures of *Barringtonia racemosa* L. by elicitation. *Plant Cell Tissue Organ Cult.* 135, 203–212. doi: 10.1007/s11240-018-1456-z

Park, J.-Y., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, S.-J., Kim, D., et al. (2012a). Diarylheptanoids from *Alnus japonica* inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biol. Pharm. Bull.* 35, 2036–2042. doi: 10.1248/bpb.b12-00623

Park, J.-Y., Kim, J. H., Kim, Y. M., Jeong, H. J., Kim, D. W., Park, K. H., et al. (2012b). Tanshinones as selective and slow-binding inhibitors for SARS-CoV cysteine proteases. *Bioorg. Med. Chem.* 20, 5928–5935. doi: 10.1016/j.bmc.2012.07.038

Park, J.-Y., Ko, J.-A., Kim, D. W., Kim, Y. M., Kwon, H.-J., Jeong, H. J., et al. (2016). Chalcones isolated from *Angelica keiskei* inhibit cysteine proteases of SARS-CoV. *J. Enzyme Inhib. Med. Chem.* 31, 23–30. doi: 10.3109/14756366.2014.1003215

Patil, R. A., Lenka, S. K., Normanly, J., Walker, E. L., and Roberts, S. C. (2014). Methyl jasmonate represses growth and affects cell cycle progression in cultured Taxus cells. *Plant Cell Rep.* 33, 1479–1492. doi: 10.1007/s00299-014-1632-5

Pavlov, A., and Bley, T. (2018). *Bioprocessing of Plant in Vitro Systems*. Berlin: Springer. doi: 10.1007/978-3-319-54600-1

PMI (2020). *Medicago Develops a Plant-Based Vaccine for Coronavirus. Philip Morris International*. Available online at: https://www.pmi.com/media-center/news/medicago-develops-a-plant-based-vaccine-for-coronavirus (accessed February 12, 2021).

Pourebad, N., Motafakkerazad, R., Kosari-Nasab, M., Akhtar, N. F., and Movafeghi, A. (2015). The influence of TDZ concentrations on in vitro growth and production of secondary metabolites by the shoot and callus culture of *Lallemantia iberica*. *Plant Cell Tissue Organ Cult.* 122, 331–339. doi: 10.1007/s11240-015-0769-4

Ptak, A., Morańska, E., Saliba, S., Zieliński, A., Simlat, M., and Laurain-Mattar, D. (2017). Elicitation of galanthamine and lycorine biosynthesis by *Leucojum aestivum* L. and *L. aestivum* 'Gravety Giant' plants cultured in bioreactor RITA§. *Plant Cell Tissue Organ Cult.* 128, 335–345. doi: 10.1007/s11240-016-1113-3

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R. M., et al. (2016). Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules* 21, 182–182. doi: 10.3390/molecules21020182

Razavizadeh, R., Adabavazeh, F., and Komatsu, S. (2020). Chitosan effects on the elevation of essential oils and antioxidant activity of *Carum copticum* L. seedlings and callus cultures under in vitro salt stress. *J. Plant Biochem. Biotechnol.* 29, 473–483. doi: 10.1007/s13562-020-00560-1

Roberts, S. C. (2007). Production and engineering of terpenoids in plant cell culture. *Nat. Chem. Biol.* 3, 387–395. doi: 10.1038/nchembio.2007.8

Ryang, J., Yan, Y., Song, Y., Liu, F., and Ng, T. B. (2019). Anti-HIV, antitumor and immunomodulatory activities of paclitaxel from fermentation broth using molecular imprinting technique. *AMB Express* 9:194. doi: 10.1186/s13568-019-0915-1

Ryu, Y. B., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, J.-Y., Kim, D., et al. (2010a). Biflavonoids from *Torreya nucifera* displaying SARS-CoV 3CLpro inhibition. *Bioorg. Med. Chem.* 18, 7940–7947. doi: 10.1016/j.bmc.2010.09.035

Ryu, Y. B., Park, S.-J., Kim, Y. M., Lee, J.-Y., Seo, W. D., Chang, J. S., et al. (2010b). SARS-CoV 3CLpro inhibitory effects of quinone-methide triterpenes from *Tripterygium regelii*. *Bioorg. Med. Chem. Lett.* 20, 1873–1876. doi: 10.1016/j.bmcl.2010.01.152

Saeed, S., Ali, H., Khan, T., Kayani, W., and Khan, M. A. (2017). Impacts of methyl jasmonate and phenyl acetic acid on biomass accumulation and antioxidant potential in adventitious roots of *Ajuga bracteosa* Wall ex Benth., a high valued endangered medicinal plant. *Physiol. Mol. Biol. Plants* 23, 229–237. doi: 10.1007/s12298-016-0406-7

Sajjalaguddam, R. R., and Paladugu, A. (2015). Phenylalanine enhances Quercetin content in In vitro cultures of *Abutilon indicum* L. *J. Appl. Pharm. Sci.* 5, 080–084. doi: 10.7324/JAPS.2015.501014

Santoro, V. M., Nievas, F. L., Zygadlo, J. A., Giordano, W. F., and Banchio, E. (2013). Effects of growth regulators on biomass and the production of secondary metabolites in peppermint (*Mentha pi-perita*) micropropagated *in vitro*. *Am. J. Plant Sci.* 4, 49–55. doi: 10.4236/ajps.2013.45A008

Santos, P. M., Figueiredo, A. C., Oliveira, M. M., Barroso, J., Pedro, L. G., Deans, S. G., et al. (1998). Essential oils from hairy root cultures and from fruits and roots of *Pimpinella anisum*. *Phytochemistry* 48, 455–460. doi: 10.1016/S0031-9422(98)00022-3

Schmitt, J., and Petersen, M. (2002). Influence of methyl jasmonate and coniferyl alcohol on pinoresinol and matairesinol accumulation in a Forsythia× intermedia suspension culture. *Plant Cell Rep.* 20, 885–890.

Schoeman, D., and Fielding, B. C. (2019). Coronavirus envelope protein: current knowledge. *Virol. J.* 16:69.

Shaikh, S., Shriram, V., Khare, T., and Kumar, V. (2020). Biotic elicitors enhance diosgenin production in *Helicteres isora* L. suspension cultures via up-regulation of CAS and HMGR genes. *Physiol. Mol. Biol. Plants* 26, 593–604.

Shakya, P., Marslin, G., Siram, K., Beerhues, L., and Franklin, G. (2019). Elicitation as a tool to improve the profiles of high-value secondary metabolites and pharmacological properties of *Hypericum perforatum*. *J. Pharm. Pharmacol.* 71, 70–82.

Shen, X., Du, Q., Xu, Y., and Lu, Y. J. E. J. O. B. (2012). Stimulation of trans-resveratrol biosynthesis in *Vitis vinifera* cv. Kyoho cell suspension cultures by 2, 3-dihydroxypropyl jasmonate elicitation. *Electron. J. Biotechnol.* 15:3.

Shi, L., Wang, C., Zhou, X., Zhang, Y., Liu, Y., and Ma, C. (2013). Production of salidroside and tyrosol in cell suspension cultures of *Rhodiola crenulata*. *Plant Cell Tissue Organ Cult.* 114, 295–303.

Shintani, D., and Dellapenna, D. (1998). Elevating the vitamin E content of plants through metabolic engineering. *Science* 282, 2098–2100.

Shrestha, A., Pandey, R. P., and Sohng, J. K. (2019). Biosynthesis of resveratrol and piceatannol in engineered microbial strains: achievements and perspectives. *Appl. Microbiol. Biotechnol.* 103, 2959–2972.

Skrzypczak-Pietraszek, E., Piska, K., and Pietraszek, J. (2018). Enhanced production of the pharmaceutically important polyphenolic compounds in *Vitex agnus* castus L. shoot cultures by precursor feeding strategy. *Eng. Life Sci.* 18, 287–297.

Srivastava, M., Singh, G., Sharma, S., Shukla, S., and Misra, P. (2019). Elicitation enhanced the yield of glycyrrhizin and antioxidant activities in hairy root cultures of *Glycyrrhiza glabra* L. *J. Plant Growth Regul.* 38, 373–384.

Stark-Lorenzen, P., Nelke, B., Hänßler, G., Mühlbach, H., and Thomzik, J. (1997). Transfer of a grapevine stilbene synthase gene to rice (*Oryza sativa* L.). *Plant Cell Rep.* 16, 668–673.

Teptat, P., Tansakul, P., and Sakunphueak, A. (2020). *Effects of Sequential and Simultaneous Double Elicitation on Aloe-Emodin Production in Cassia Tora Root Cultures*. Available online at: https://www.researchsquare.com/article/rs-38327/v1 (accessed September 15, 2020).

Thanh Le, T., Andreadakis, Z., Kumar, A., Gómez Román, R., Tollefsen, S., Saville, M., et al. (2020). The COVID-19 vaccine development landscape. *Nat. Rev. Drug Discov.* 19, 305–306.

Tirillini, B., Ricci, A., Pintore, G., Chessa, M., and Sighinolfi, S. (2006). Induction of hypericins in *Hypericum perforatum* in response to chromium. *Fitoterapia* 77, 164–170.

Vafadar, F., Amooaghaie, R., Ehsanzadeh, P., Ghanadian, M., Talebi, M., and Ghanati, F. (2020). Melatonin and calcium modulate the production of rosmarinic acid, luteolin, and apigenin in *Dracocephalum kotschyi* under salinity stress. *Phytochemistry* 177:112422.

Veerashree, V., Anuradha, C., and Kumar, V. (2012). Elicitor-enhanced production of gymnemic acid in cell suspension cultures of *Gymnema sylvestre* R. Br. *Plant Cell Tissue Organ Cult.* 108, 27–35.

Verpoorte, R., Contin, A., and Memelink, J. (2002). Biotechnology for the production of plant secondary metabolites. *Phytochem. Rev.* 1, 13–25.

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 30, 269–271.

Wang, W., Zhao, Z.-J., Xu, Y., Qian, X., and Zhong, J.-J. (2006). Efficient induction of ginsenoside biosynthesis and alteration of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by using chemically synthesized 2-hydroxyethyl jasmonate. *Appl. Microbiol. Biotechnol.* 70, 298–307.

Weathers, P. J., Towler, M. J., and Xu, J. (2010). Bench to batch: advances in plant cell culture for producing useful products. *Appl. Microbiol. Biotechnol.* 85, 1339–1351.

Wen, C.-C., Kuo, Y.-H., Jan, J.-T., Liang, P.-H., Wang, S.-Y., Liu, H.-G., et al. (2007). Specific plant Terpenoids and Lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *J. Med. Chem.* 50, 4087–4095.

Wilczañska-Barska, A., Królicka, A., Głód, D., Majdan, M., Kawiak, A., and Krauze-Baranowska, M. (2012). Enhanced accumulation of secondary metabolites in hairy root cultures of *Scutellaria lateriflora* following elicitation. *Biotechnol. Lett.* 34, 1757–1763.

Wink, M. (2020). Potential of DNA intercalating alkaloids and other plant secondary metabolites against SARS-CoV-2 causing COVID-19. *Diversity* 12:175.

Wongwicha, W., Tanaka, H., Shoyama, Y., and Putalun, W. (2011). Methyl jasmonate elicitation enhances glycyrrhizin production in *Glycyrrhiza inflata* hairy roots cultures. *Z. Naturforschung C J. Biosci.* 66, 423–428.

Worldometer (2020). *Coronavirus*. Available online at: https://www.worldometers.info/coronavirus/ (accessed November 14, 2020).

Wu, C.-Y., Jan, J.-T., Ma, S.-H., Kuo, C.-J., Juan, H.-F., Cheng, Y.-S. E., et al. (2004). Small molecules targeting severe acute respiratory syndrome human coronavirus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10012–10017.

Wurtzel, E. T., and Kutchan, T. M. (2016). Plant metabolism, the diverse chemistry set of the future. *Science* 353, 1232–1236.

Yang, Q.-Y., Tian, X.-Y., and Fang, W.-S. (2007). Bioactive coumarins from *Boenninghausenia sessilicarpa*. *J. Asian Nat. Prod. Res.* 9, 59–65.

Yarmolinsky, L., Huleihel, M., Zaccai, M., and Ben-Shabat, S. (2012). Potent antiviral flavone glycosides from *Ficus benjamina* leaves. *Fitoterapia* 83, 362–367.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., et al. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303–305.

Yi, L., Li, Z., Yuan, K., Qu, X., Chen, J., Wang, G., et al. (2004). Small molecules blocking the entry of severe acute respiratory syndrome coronavirus into host cells. *J. Virol.* 78, 11334–11339.

Yu, K.-W., Gao, W., Hahn, E.-J., and Paek, K.-Y. (2002). Jasmonic acid improves ginsenoside accumulation in adventitious root culture of *Panax ginseng* CA Meyer. *Biochem. Eng. J.* 11, 211–215.

Yu, M.-S., Lee, J., Lee, J. M., Kim, Y., Chin, Y.-W., Jee, J.-G., et al. (2012). Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13. *Bioorg. Med. Chem. Letters* 22, 4049–4054.

Yu, Y., Zhang, W.-B., Li, X.-Y., Piao, X.-C., Jiang, J., and Lian, M.-L. (2016). Pathogenic fungal elicitors enhance ginsenoside biosynthesis of adventitious roots in *Panax quinquefolius* during bioreactor culture. *Ind. Crops Prod.* 94, 729–735.

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SARS-CoV-2 Antigens Expressed in Plants Detect Antibody Responses in COVID-19 Patients

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Background: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has swept the world and poses a significant global threat to lives and livelihoods, with 115 million confirmed cases and at least 2.5 million deaths from Coronavirus disease 2019 (COVID-19) in the first year of the pandemic. Developing tools to measure seroprevalence and understand protective immunity to SARS-CoV-2 is a priority. We aimed to develop a serological assay using plant-derived recombinant viral proteins, which represent important tools in less-resourced settings.

Methods: We established an indirect ELISA using the S1 and receptor-binding domain (RBD) portions of the spike protein from SARS-CoV-2, expressed in *Nicotiana benthamiana*. We measured antibody responses in sera from South African patients (n = 77) who had tested positive by PCR for SARS-CoV-2. Samples were taken a median of 6 weeks after the diagnosis, and the majority of participants had mild and moderate COVID-19 disease. In addition, we tested the reactivity of pre-pandemic plasma (n = 58) and compared the performance of our in-house ELISA with a commercial assay. We also determined whether our assay could detect SARS-CoV-2-specific IgG and IgA in saliva.

Results: We demonstrate that SARS-CoV-2-specific immunoglobulins are readily detectable using recombinant plant-derived viral proteins, in patients who tested positive for SARS-CoV-2 by PCR. Reactivity to S1 and RBD was detected in 51 (66%) and 48 (62%) of participants, respectively. Notably, we detected 100% of samples identified as having S1-specific antibodies by a validated, high sensitivity commercial ELISA, and optical density (OD) values were strongly and significantly correlated between the two assays. For the pre-pandemic plasma, 1/58 (1.7%) of samples were positive, indicating a high specificity for SARS-CoV-2 in our ELISA. SARS-CoV-2-specific IgG correlated significantly

with IgA and IgM responses. Endpoint titers of S1- and RBD-specific immunoglobulins ranged from 1:50 to 1:3,200. S1-specific IgG and IgA were found in saliva samples from convalescent volunteers.

Conclusion: We demonstrate that recombinant SARS-CoV-2 proteins produced in plants enable robust detection of SARS-CoV-2 humoral responses. This assay can be used for seroepidemiological studies and to measure the strength and durability of antibody responses to SARS-CoV-2 in infected patients in our setting.

Keywords: SARS-CoV-2, COVID-19, serology, ELISA, plant expression

INTRODUCTION

The current global pandemic, caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in over 115 million cases and at least 2.5 million deaths, as of 02 March 2021. SARS-CoV-2 was first detected in December 2019 in Wuhan, a city in the Hubei province of China, and is thought to originate from zoonotic transmission of a bat coronavirus (Tan et al., 2020; Zhu et al., 2020). Coronavirus disease 2019 (COVID-19), the resultant disease, is commonly associated with fever, cough, and fatigue, and in severe cases, pneumonia and respiratory failure (Chan et al., 2020).

SARS-CoV-2 is a 30 kB positive-stranded RNA virus that is a member of the *Betacoronavirus* genus and the subgenus *Sarbecovirus* (Letko et al., 2020). The genus harbors human pathogens that cause respiratory infections, namely the highly virulent SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), as well as the circulating "common cold" human coronavirus (hCoV)-OC43 and hCoV-HKU1 (Su et al., 2016). Betacoronaviruses express four essential structural proteins, namely the spike (S) glycoprotein, membrane (M) protein, envelope (E) protein, and nucleocapsid (N) protein, as well as multiple accessory and non-structural proteins (Neuman et al., 2011; Lu et al., 2020). The S glycoprotein is a homotrimer that protrudes from the surface of the viral particles (Tortorici and Veesler, 2019), and interacts with the human cell receptor angiotensin converting enzyme 2 (ACE2) through the receptor-binding domain (RBD), gaining viral entry into the host cell (Li, 2016; Letko et al., 2020; Walls et al., 2020). S is cleaved by host cell proteases into two subunits: the S1 subunit which harbors the RBD and enables binding to host cell receptors, and the S2 subunit that is important for fusion with the host cell membrane (Walls et al., 2020; Wrapp et al., 2020).

The S1 subunit is highly immunogenic, and its RBD portion is the main target of neutralizing antibodies, thus becoming the focus of serological studies (Amanat et al., 2020; Huang et al., 2020; Liu et al., 2020; Okba et al., 2020). Recently, potent neutralizing antibodies isolated from the convalescent sera of SARS-CoV-2 patients were demonstrated to be protective against disease from high-dose SARS-CoV-2 challenge in a small animal model (Rogers et al., 2020), suggesting the potential for therapeutic interventions as well as inferring that recovered SARS-CoV-2 patients may be afforded protection from re-infection by neutralizing antibody responses. Amanat et al. (2020) showed a strong correlation between the neutralizing antibody response and ELISA endpoint titers against S, suggesting the use of serological assays in estimating the percentage of infected people who have neutralizing antibodies that protect them from re-infection or disease.

Serological assays that can detect antibody responses to SARS-CoV-2 are critical for answering pressing questions regarding immunity to the virus. It is not known what proportion of infected individuals elicit antibodies to SARS-CoV-2, if antibodies serve as correlates of protection, and if so, what the threshold of binding or neutralizing titers are that will provide immunity, and the duration of these responses. Serological assays such as ELISA can assist in answering these questions. These assays need to be both sensitive as well as demonstrate high specificity for SARS-CoV-2, and not give false positives due to cross-reactivity with widely circulating hCoVs NL63, 229E, OC43, and HKU1. While the N protein is more conserved among coronaviruses, the S protein sequence has lower sequence conservation. The S1 portion is 21–25% identical at the amino acid level to circulating hCoVs (Okba et al., 2020). Thus, serological assays using the full-length S protein, S1 subunit, or RBD portion as antigens have shown good specificity with little cross-reactivity to NL63 and 229E (Amanat et al., 2020; Rosales-Mendosa et al., 2020) compared to the use of N protein (Rosales-Mendosa et al., 2020).

Purified recombinant proteins are essential for the establishment of serological assays. Numerous protein expression systems exist, each with their own advantages and limitations. These include bacterial, mammalian, yeast, insect, and plant-based systems (Yin et al., 2007; Shanmugaraj et al., 2020). Plant-based systems have several advantages over more widely used conventional protein expression systems. Most notably, they are rapid, cost-effective, and support post-translational modifications similar to mammalian cell systems, making them attractive protein expression systems particularly in low-income settings (Maliga and Graham, 2004; Shanmugaraj et al., 2020). Historically, their major disadvantage was low yield (Shanmugaraj et al., 2020); however, advances in plant technology, including transient expression systems and viral vectors, have led to improvements in protein yield (Kapila et al., 1997; Yamamoto et al., 2018). Additionally, SARS-CoV S1 protein expressed in tomato and tobacco plants demonstrated good immunogenicity in mice (Pogrebnyak et al., 2005). Together, these studies highlight the potential of plant-based expression systems for

the development of serological assay reagents as well as vaccines for the current SARS-CoV-2 pandemic.

In this study, we describe the development of an ELISA that enables detection of antibodies directed at the S1 subunit and the RBD portion of the SARS-CoV-2 S glycoprotein, generated through a plant-based expression system.

MATERIALS AND METHODS

Cloning and Expression of Recombinant Proteins

The S1 portion and receptor binding domain (RBD) of the spike protein of SARS-CoV-2 Wuhan-Hu-1 isolate (GenBank: MN908947.3) were produced by Cape Bio Pharms, Cape Town, South Africa. Briefly, *Nicotiana benthamiana* codon-optimized DNA encoding S1 (aa 14–698) and an extended region containing the RBD (aa 281–698) were synthesized commercially (Genscript). Both genes were fused at their C-terminal region to the fragment crystallizable region (Fc) of rabbit IgG1 (Genbank: L29172.1) and subsequently cloned into Cape Bio Pharms' proprietary vector, pCBP2. *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) was used to carry agroinfiltration. Growth of recombinant *A. tumefaciens* and vacuum infiltration of *N. benthamiana* plants were performed as described previously (Maclean et al., 2007). Three days post-infiltration, leaves were homogenized in the presence of phosphate buffered saline (PBS) at a 2:1 ratio buffer:leaf material. Cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C, and the clarified supernatant was used for expression analyses and purification by Protein A affinity chromatography.

For purification, the extract was filtered through a 0.22 μm cellulose nitrate filter (Sartorius) before loading onto a pre-equilibrated 5 ml column packed with POROS MabCapture A resin (Thermo Fisher). The column was then washed with 10 column volumes of wash buffer (PBS, pH 7.5) and bound proteins eluted using elution buffer (0.1 M glycine, pH 2.5). Eluted fractions were captured in 1/10th volume of neutralization buffer (1 M Tris, pH 8.5) and then pooled and applied to a 10 K molecular weight cutoff Amicon centrifuge tube (Millipore) for buffer exchange against PBS and sample concentration.

SDS-PAGE and Western Blot

Expression and purity of recombinant S1 and RBD fusion proteins were evaluated by western blot and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified samples were added to sample loading dye NuPAGE LDS sample buffer and reducing agent (both Invitrogen) and heated to 70°C for 10 min. Samples were loaded into pre-cast polyacrylamide gels (Bolt 4–12% Bis-Tris Plus; Invitrogen) and run at 200 V for 40 min. Visualization of protein samples on acrylamide gels was performed using Coomassie Brilliant Blue G250 stain (Merck). Gels were stained overnight with agitation, and destaining solution (30% methanol and 10% acetic acid) was added for 1 h at room temperature. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a dry transblotter (Invitrogen). The membrane was blocked for 30 min (PBS containing 5% fat free milk and 0.1% Tween 20) at room temperature, followed by incubation with mouse anti-rabbit horseradish peroxidase (1:5,000; Sigma) for 1 h at 37°C with agitation. The membrane was washed four times using wash buffer (PBS with 0.1% Tween 20) at room temperature for 15 min, and developed using TMB solution (1-step ultra TMB-blotting solution, Thermo Scientific) in the dark for 30 min.

Volunteer Recruitment and Sample Collection

Samples were collected from SARS-CoV-2 infected volunteers (n = 77) recruited from Gauteng and the Western Cape provinces of South Africa from 10 April 2020 to 26 May 2020. Volunteers had previously undergone a reverse transcriptase PCR (RT-PCR) test for SARS-CoV-2 from an upper respiratory tract (nose/throat) swab collected into viral transport media. Swabs were processed through approved assays in accredited public and private clinical laboratories. Inclusion criteria were age >=18 years and a confirmed positive PCR for SARS-CoV-2 on the national database of the National Health Laboratory Services (NHLS). Of the 77 participants, 34 (44%) had a second positive PCR result recorded within a week after the first positive test. With respect to disease severity, five participants were asymptomatic, 23 had mild disease (characterized by mild upper respiratory tract symptoms), 38 had moderate disease (defined by gastrointestinal symptoms or lower respiratory tract symptoms), and two had severe disease (admission to hospital). Serum and saliva samples were collected between 8 and 70 days after the first positive PCR test. An additional 101 volunteers who had a negative RT-PCR test were included in the Euroimmun testing (described below). Ethical approval for these studies was obtained from the Human Research Ethics Committee (HREC) of the University of Witwatersrand (M200468) and the University of Cape Town (210/2020). All participants provided written, informed consent.

Pre-pandemic plasma (n = 58) was obtained from banked human samples that were collected from participants recruited from Cape Town, South Africa in 2011–2012, from a study protocol approved by the HREC of the University of Cape Town (158/2010). Storage consent was provided by all participants, and approval for use of the samples in this study was obtained from the HREC, UCT. Samples came from participants who were HIV-infected (n = 27) or HIV-uninfected (n = 31). All participants had tested positive for exposure to *Mycobacterium tuberculosis* based on a positive IFN-γ-release assay (QuantiFERON-TB Gold In-Tube), i.e., were classified as having latent tuberculosis infection. The median age was 26 years [interquartile range (IQR): 22–34 years] and 44/58 (76%) were female. All HIV-infected individuals were antiretroviral treatment (ART)-naive, with a median CD4 count of 591 cells/mm^3 (IQR: 511–749).

All studies were conducted in a BSL-2+ laboratory environment under approval of the University of Cape Town's Institutional Biosafety Committee (IBC007-2020). All samples

were treated with 1% Triton-X100 (Sigma) for 60 min at room temperature to inactivate any potentially live virus in the samples (Remy et al., 2019).

Enzyme-Linked Immunosorbent Assay

The ELISA protocol was adapted from a published protocol (Stadlbauer et al., 2020). Briefly, 96-well plates (Nunc MaxiSorp, Thermo Fisher) were coated at 4°C overnight with 50 µl of varying concentrations (1–4 µg/ml) of purified recombinant RBD or S1 proteins in PBS or bicarbonate buffer (both Sigma). The following day, plates were washed five times using an automated plate washer and incubated at room temperature in blocking solution [1% casein or 3% non-fat powder milk prepared in PBS with 0.1% Tween 20 (PBS-T)]. After 1 h, the blocking solution was discarded and 100 µl of serum, plasma, or saliva samples (at 1:50 dilution for sera/plasma and 1:10 for saliva) were added for 2 h at room temperature. Next, plates were washed five times and incubated with goat anti-human IgG (Fc-specific) peroxidase conjugate (1:5,000; IgG-HRP, Sigma), or goat anti-human IgA (α-chain specific), F(ab')$_2$ fragment peroxidase conjugate (1:5,000; IgA-HRP, Sigma) or goat anti-human IgM peroxidase conjugate (1:2,000; IgM-HRP, Southern Biotech) for 1 h at room temperature. The plate was then developed using 100 µl O-phenylenediamine dihydrochloride (OPD; Sigma) for 12 min before the reaction was stopped with 50 µl 3 M hydrochloric acid (HCl, Sigma). The plates were read at 490 nm using a Versamax microplate reader (Molecular Devices) using SoftMax Pro software (version 5.3). A cutoff for positivity was set at 2 SD above the mean optical density (OD) of pre-pandemic samples. For determining endpoint titers, 2-fold serial dilutions were performed for 20 PCR+ samples and 40 pre-pandemic controls. Area under the curve (AUC) was determined and the positivity threshold was calculated as before, mean + 2 SD. All patient samples from SARS-CoV-2 RT-PCR+ volunteers were also analyzed using the anti-SARS-CoV-2 ELISA (IgG; Euroimmun), in an independent laboratory. Samples from 101 RT-PCR-volunteers were also tested on the same platform. The Euroimmun assay uses the S1 domain of the spike protein, expressed in mammalian cells. The assay was conducted according to the manufacturer's instructions. Results were determined as a ratio of the OD signal of the samples to the average OD signal of calibrators, and are expressed as OD to calibrator ratio, as per the manufacturer's recommendations. A ratio <0.8 was considered as negative, >0.8 to <1.1 as indeterminate or borderline, and >1.1 as positive.

Statistical Analysis

Statistical analyses were performed in Prism (GraphPad, version 8). Nonparametric tests were used for all comparisons. The Friedman test with Dunn's multiple comparison test was used for matched comparisons; the Mann-Whitney *U* unmatched and Wilcoxon matched pairs *t*-tests were used for unmatched and paired samples, respectively. Spearman Rank tests were used for all correlations. AUC was calculated in Prism. A value of $p < 0.05$ was considered statistically significant.

All data in this manuscript can be found in **Supplementary Table 1**.

RESULTS

SARS-CoV-2 Antigen Expression in Plants

The S1 and RBD portions of the Spike protein of SARS-CoV-2 were expressed in *N. benthamiana* as fusions to the rabbit IgG Fc tag. Western blot and SDS-PAGE analysis revealed expression of purified S1 (**Figures 1A,B**) and RBD (**Figures 1C,D**) at the expected protein sizes of ~140 and ~100 kDa, respectively. Higher molecular weight bands of ~280 and ~200 kDa indicated possible dimer formation of S1 and RBD, respectively. In addition, lower molecular weight bands indicated potentially multiple cleavage products of S1 and RBD in the preparations.

Participant Description

Serum samples were collected from 77 volunteers who had previously tested positive for SARS-CoV-2 by PCR. The demographic and clinical characteristics of the participants are summarized in **Table 1**. Just over half the participants were female, and the median age was 39 years. The date of onset of symptoms was not available, but samples were taken a median of 6 weeks after SARS-CoV-2 PCR positivity. The majority of patients (79%) experienced mild or moderate

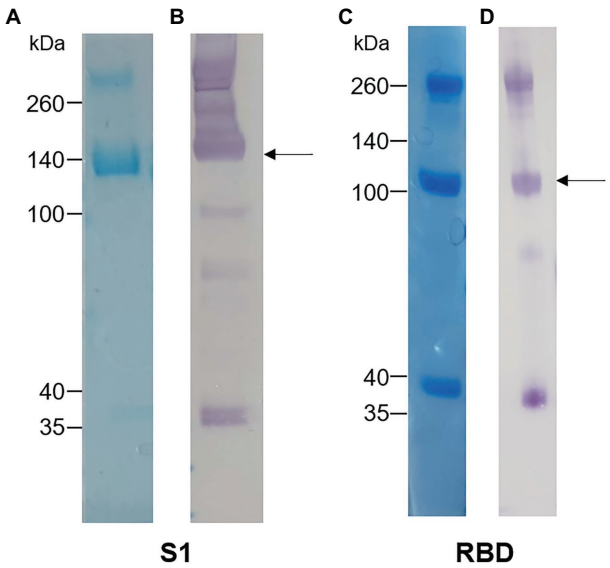

FIGURE 1 | Analysis of plant-expressed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike antigens after Protein A purification. **(A)** Coomassie-stained SDS-PAGE gel and **(B)** Western blot of S1-rabbit Fc fusion protein (2 µg of concentrated elution fraction). Lines on the left indicate molecular weight marker (Spectra Multicolor Broad range protein ladder) in kDa. The arrow indicates the expected size for recombinant S1 protein (~140 kDa). **(C)** Coomassie-stained SDS-PAGE gel and **(D)** Western blot of RBD-rabbit Fc fusion protein (5 µg of concentrated elution fraction). Arrows indicate expected size for RBD-rabbit Fc conjugate (~100 kDa).

COVID-19 disease. We also included 58 archived plasma samples from HIV-infected and uninfected individuals collected prior to the pandemic (2011–2012) as negative controls for our assay. For the commercial Euroimmun test kit, an additional 101 SARS-CoV-2 PCR negative participants were included as controls.

Optimization of the ELISA Assay

The in-house ELISA diagnostic assay in this study was developed from the published protocol (Stadlbauer et al., 2020).

TABLE 1 | Characteristics of Coronavirus disease 2019 (COVID-19) patients (n = 77).

Sex female, *n* (%)	42 (55)
Age (years)[a]	39 (29–50)
Time since positive PCR test (days)[a]	42 (29–52)
Disease severity, *n* (%)[b]	
Asymptomatic	5 (7)
Mild	23 (30)
Moderate	38 (49)
Severe	2 (3)

[a]*Median and interquartile range.*
[b]*Not available for n = 9 participants.*

To establish a robust and sensitive in-house ELISA, we optimized several parameters, including S1 and RBD antigen coating concentration, as well as the coating and blocking buffers. Coating concentrations of 1, 2, and 4 μg/ml S1 and RBD were compared for SARS-CoV-2-specific IgG detection in four SARS-CoV-2 convalescent volunteers and three pre-pandemic samples. Two and 4 μg/ml demonstrated a significantly higher reactivity than 1 μg/ml for both S1 and RBD (**Figures 2A,B**; p = 0.0005 and p = 0.004, respectively, using the Friedman test with Dunn's test for multiple comparisons), with little increase in the background (negative control) signal. Thus, 2 μg/ml was selected for subsequent assays. Coating of ELISA plates with antigen in different coating buffers, namely PBS and bicarbonate buffer, was also assessed (**Figure 2C**). No differences were detected, so PBS was selected for our procedure. A comparison of the blocking buffers PBS with 0.1% Tween-20 (PBS-T), PBS-T with 1% casein and PBS-T with 3% non-fat milk powder was performed (**Figure 2D**). PBS-T with 1% casein was selected based on background signal and positivity trends. We also determined the optimal titer of secondary antibody IgG-HRP (1:5,000), as well as optimal serum dilution (1:50; data no shown).

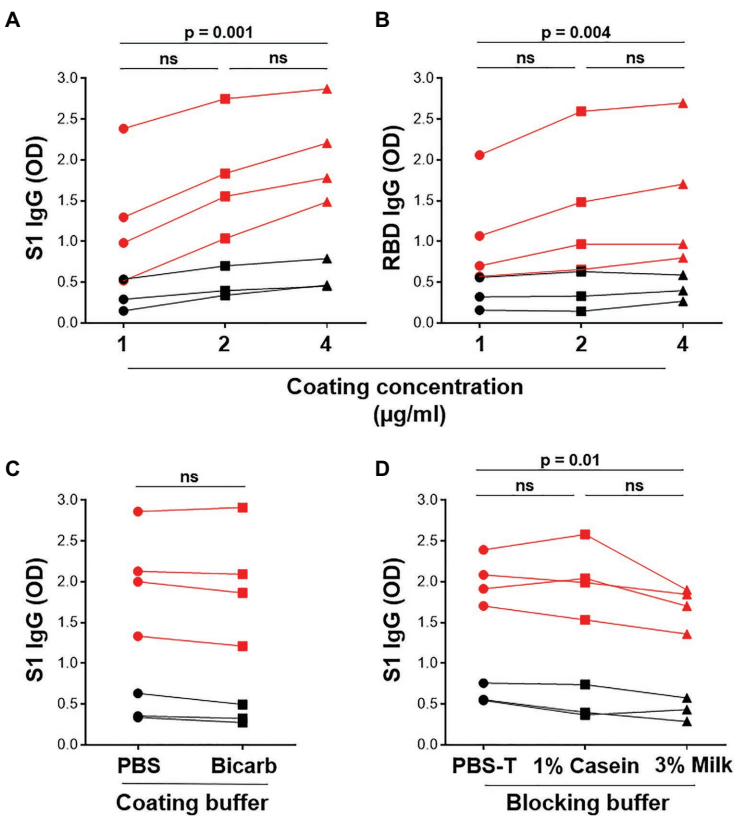

FIGURE 2 | Optimization of ELISA antigen coating concentration, coating buffer, and blocking buffer. The effect of antigen coating concentration (1, 2, and 4 μg/ml) was tested for **(A)** S1 and **(B)** RBD, using serum samples from SARS-CoV-2 positive convalescent participants (n = 7). Statistical analyses were performed using the Friedman test with Dunn's test for multiple comparisons. **(C)** Comparison of phosphate buffered saline (PBS) and bicarbonate buffer for coating viral antigens. Statistical analyses were performed using a Wilcoxon matched pair's test. **(D)** The effect of different blocking solutions. Statistical analysis was performed using the Friedman test with Dunn's test for multiple comparisons.

Plant-Produced S1 and RBD Proteins Are Suitable for ELISA Detection of SARS-CoV-2 Antibodies

In order to test whether plant-produced SARS-CoV-2 antigens were able to detect virus-specific antibodies from infected patients, we screened convalescent sera from 77 volunteers who had recovered from COVID-19. Individuals were tested for reactivity against both S1 and RBD antigens by a standard indirect ELISA based on a published protocol (Stadlbauer et al., 2020). Archived pre-pandemic plasma samples from 58 individuals, including 27 HIV-infected persons, were used to test the background reactivity to SARS-CoV-2 S1 and RBD. The threshold for positivity was set at 2 SD above the mean optical density (OD) of the pre-pandemic samples.

Of the 77 COVID-19 convalescent serum samples, 51 (66%) tested positive for SARS-CoV-2-specific IgG against S1, and 48 (62%) tested positive against RBD (**Figures 3A,B**). In contrast, only 1/58 pre-pandemic plasma samples showed reactivity above the positivity cutoff. As expected, S1 and RBD IgG OD values correlated strongly (r = 0.977; p < 0.0001; data not shown). In order to independently validate our results, the same PCR+ sera were run in a separate laboratory in a blinded manner, using a commercial IgG ELISA (Euroimmun) based on S1 antigen (**Figure 3C**). That assay included 101 PCR-sera, two of which were positive, and may represent false negative PCR tests. All samples that were positive by the commercial ELISA test for SARS-CoV-2 S1 antibodies were positive in our assay (42/77). We detected nine additional samples that were positive in our assay, two of which had high OD values well above our threshold for positivity, and six that were also positive for RBD-specific IgG. We demonstrated a strong and significant direct correlation for sample OD values between the two assays (r = 0.89, p < 0.0001, Spearman Rank test, **Figure 3D**). Of note, we found no association between SARS-CoV-2-specific IgG OD values and disease severity or days post PCR positivity (data not shown). Thus, our ELISA using plant-produced recombinant viral proteins performed similarly to a highly sensitive and specific commercial SARS-CoV-2 ELISA using S1 antigen from a mammalian expression system.

Determination of Immunoglobulin Titers and Isotypes

We next determined the titers of SARS-CoV-2-specific IgG, IgM, and IgA responses in a subset of 20 SARS-CoV-2

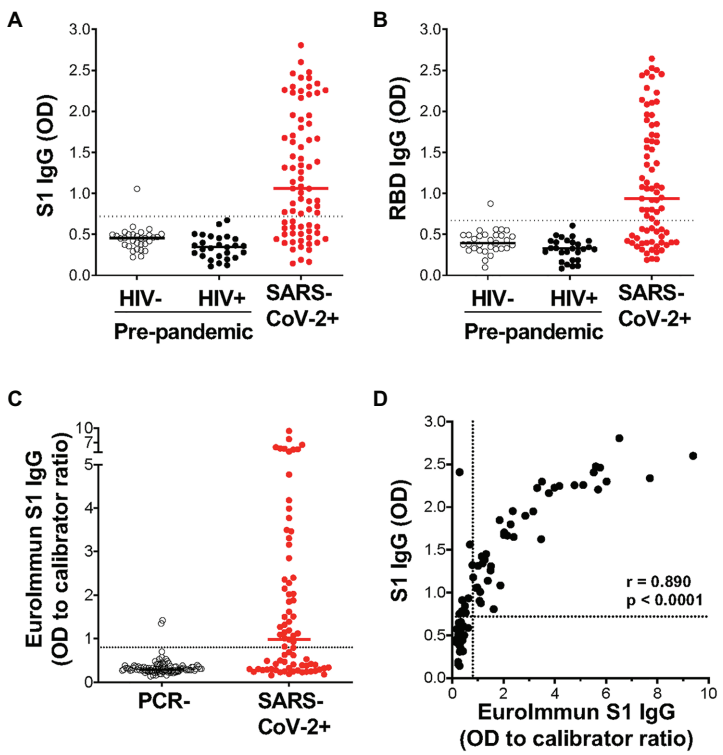

FIGURE 3 | Detection of IgG using plant-expressed SARS-CoV-2 spike antigens in COVID-19 convalescent volunteers and pre-pandemic controls using an in-house ELISA. Reactivity to plant-expressed S1 **(A)** and RBD **(B)** in pre-pandemic samples from HIV-uninfected individuals (n = 31), HIV-infected individuals (n = 27), and SARS-CoV-2 PCR positive volunteers (n = 77). Dotted lines indicate threshold for positivity, calculated as the mean optical density (OD) + 2SD of the pre-pandemic samples. **(C)** Reactivity in Euroimmun IgG S1 of the same SARS-CoV-2 PCR positive volunteers (n = 77) and a set of PCR negative sera (n = 101). Results are expressed as OD to calibrator ratio, as per the manufacturer's recommendations. The dotted line is at 0.8, above which samples are indeterminate or borderline (>0.8 and <1.1) or positive (>1.1). **(D)** Correlation of the OD values for S1-specific IgG in our in-house ELISA and the commercial Euroimmun IgG S1 ELISA assay. Statistical analyses were performed using a non-parametric Spearman rank correlation. Each dot represents one individual.

convalescent serum samples and 40 pre-pandemic samples. Assays were performed on serially diluted samples (**Figures 4A–F**) to determine endpoint titers and AUC values for quantitative interrogation of the data (**Figures 4G–L**). S1-specific IgG was detected in sera of 15/20 individuals (75%), IgM in 13/20 (65%), and IgA in 12/20 (70%) of individuals (**Figures 4G–I**). The median AUCs of IgG, IgM, and IgA were significantly higher in convalescent individuals compared to pre-pandemic ($p < 0.0001$ for all, Mann-Whitney U test). Results for RBD-specific IgG were similar (**Figures 4J–L**). Interestingly, of the five SARS-CoV-2 convalescent sera that tested S1 IgG negative, three had S1-specific IgM and one had S1-specific IgA. Similarly, of the four samples negative for RBD-specific IgG, three were positive for IgM and one

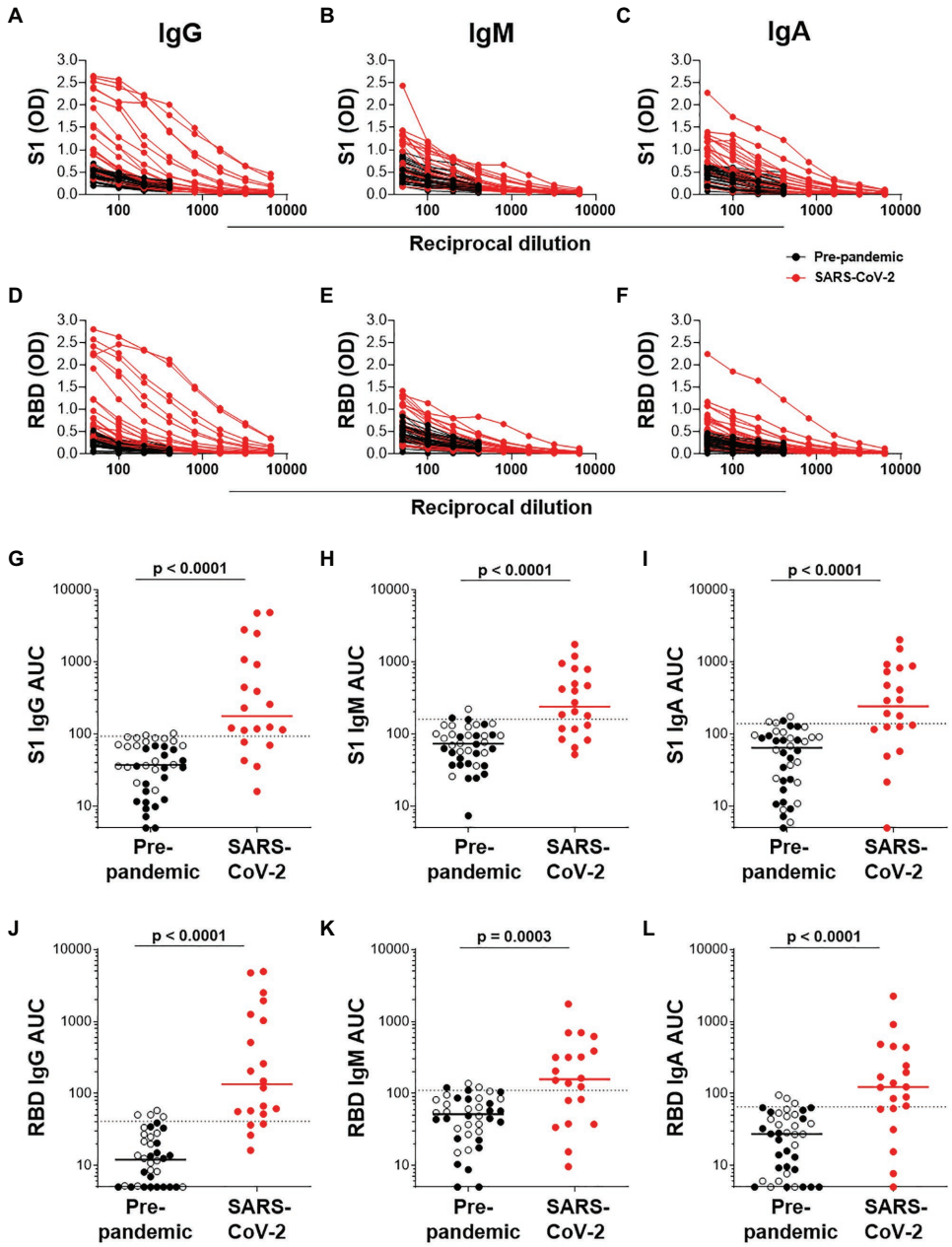

FIGURE 4 | Semi-quantitative detection of S1- and RBD-specific IgG, IgM, and IgA. Two-fold dilution series of sera for detection of S1-specific IgG **(A)**, IgM **(B)**, and IgA **(C)** and RBD-specific IgG **(D)**, IgM **(E)**, and IgA **(F)**. COVID-19 convalescent volunteers ($n = 20$) are indicated in red, and pre-pandemic controls ($n = 40$) are indicated in black. **(G–I)** and **(J–L)**; Data from the same experiment as in **(A–C)** and **(D–F)**, respectively, but plotted as area under the curve (AUC). Horizontal lines represent median values. Dotted lines indicate the threshold for positivity. Statistical analyses were performed using a Mann-Whitney U test. A value of $p < 0.05$ was considered statistically significant.

was double positive for IgM and IgA. Therefore, SARS-CoV-2 S1-specific antibodies were detected in 19/20 convalescent samples and RBD-specific antibodies in 20/20 samples.

Further examination of S1-specific antibody isotypes revealed that approximately one-third of individuals were positive for IgG, IgM, and IgA (n = 7/19), a smaller proportion had both IgG and IgM or IgG and IgA (n = 3 and 4, respectively), while some individuals were positive for only IgG (n = 1), IgM (n = 3), or IgA (n = 1; **Figure 5A**). RBD-specific isotypes gave similar results (**Figure 5B**). There was a significant correlation

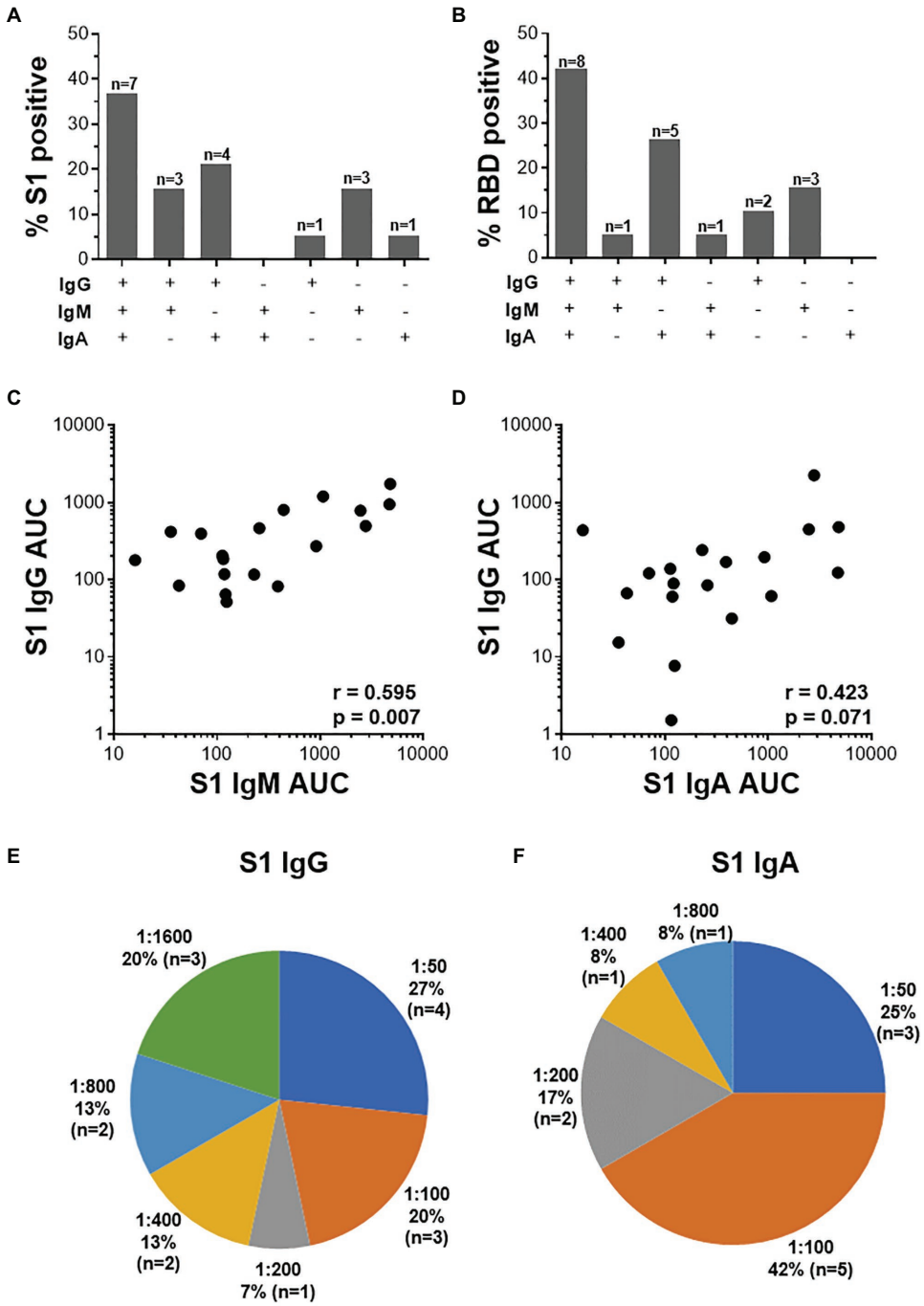

FIGURE 5 | The relationship between IgG, IgM, and IgA responses to S1 and RBD SARS-CoV-2 antigens. **(A)** Proportions of COVID-19 convalescent volunteers mounting different combinations of IgG, IgM, and IgA specific for S1 (**A**; n = 19) and RBD (**B**; n = 20). Relationship between S1-specific IgG and IgM **(C)** and IgG and IgA **(D)**. Statistical analyses were performed using a non-parametric Spearman rank correlation. Proportion of convalescent volunteers with endpoint titers for IgG **(E)** and IgA **(F)** of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1,600.

between S1-specific IgG and IgM ($r = 0.595$, $p < 0.007$, Spearman Rank test, **Figure 5C**) and anti-RBD ($r = 0.045$, $p < 0.045$; data not shown). S1-specific IgG showed a trend toward a correlation with IgA ($r = 0.423$, $p = 0.07$; **Figure 5D**), while RBD-specific IgG correlated significantly with IgA ($r = 0.635$, $p < 0.003$; data not shown). There was no correlation between IgM and IgA responses for either S1 or RBD (data not shown).

Endpoint titers for S1- and RBD-specific IgG, IgM, and IgA were determined. S1-specific IgG endpoint titers in 33% of the samples were high (20% at 1:1,600 and 13% at 1:800), 13% were moderate (1:400), and the majority (54%) of samples had low titers (27% at 1:50, 20% at 1:100, and 7% at 1:200; **Figure 5E**). S1-specific IgA titers were lower than IgG and only two individuals have a titer of 1:800 or 1:400 each, and the remaining 84% had low titers (=<1:200; **Figure 5F**). IgM titers for both S1 and RBD were all low (=<1:100; data not shown). RBD-specific titers for IgG and IgM were similar to those S1, with the exception of two donors who had titers of 1:3,200 (data not shown).

Detection of SARS-CoV-2-Specific Antibodies in Saliva

Given that virus-specific serum antibodies were readily detectable using plant-produced SARS-CoV-2 antigens, we investigated the detection of salivary IgG and IgA using our assay. We compared antibody responses to SARS-CoV-2 antigens in paired saliva and serum from 10 participants. In these preliminary analyses, 1/7 samples that had detectable S1-specific serum IgG also demonstrated S1 IgG positivity in saliva (**Figure 6A**). Additionally, 2/5 IgA+ sera exhibited virus-specific IgA in saliva. An additional IgA+ sample was detected in saliva but absent from the serum (**Figure 6B**). This indicated that IgA was more readily detectable in saliva than IgG. Further analyses to determine robust thresholds for positivity of saliva immunoglobulins will be performed going forward. These preliminary results demonstrate the potential of our ELISA to detect antibodies to SARS-CoV-2 in saliva.

DISCUSSION

There is a critical need for the development of serological tests to detect SARS-CoV-2 antibodies. Population seroprevalence studies to estimate the extent of pandemic spread in communities, and studies defining protective immunity to SARS-CoV-2, all depend on reliable serological tests. In addition, serological assays are required for the development and evaluation of an effective vaccine. Ideally, such tests need to be cost-effective and easy to scale up to be beneficial in low-income settings. In this study, we describe the establishment of an indirect SARS-CoV-2 antibody ELISA using the S1 and RBD antigens of the spike protein of SARS-CoV-2 expressed in *N. benthamiana*. S protein domains were selected because they are highly immunogenic and the primary target for neutralizing antibodies (Berry et al., 2010; Chen et al., 2020). Using sera from convalescent volunteers with a PCR-confirmed past SARS-CoV-2 infection, we detected SARS-CoV-2-specific IgG, IgA, and IgM to viral S1 and RBD. Our results were highly concordant with a widely used, high sensitivity, and specificity commercial S1 IgG ELISA kit (Euroimmun).

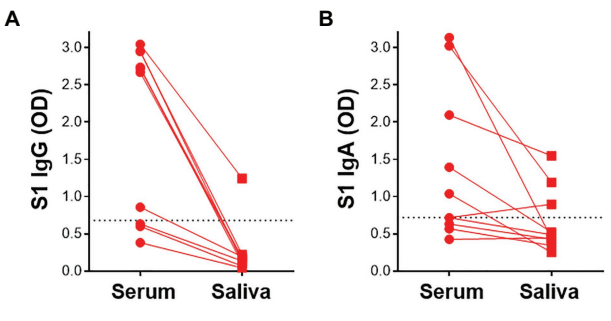

FIGURE 6 | Detection of S1-specific antibodies in saliva. Comparison of paired serum and saliva S1-specific IgG **(A)** and IgA (**B**; $n = 10$). Dotted lines indicate the positivity threshold for serum.

A range of expression systems exist for the generation of the recombinant proteins required for serological assays. Plant protein expression systems have some advantages over more widely-used mammalian or insect cell systems, as they do not require expensive media or growth conditions (Shanmugaraj et al., 2020). They are also advantageous over bacterial or yeast systems in that they may support post-translational modifications similar to that of mammalian cell lines, and lack contaminating pathogens or endotoxins that pose a problem when purifying desired proteins (Maliga and Graham, 2004; Shanmugaraj et al., 2020). Lack of correct protein glycosylation and recombinant protein yield are cited as disadvantages to using plants to express protein. However, *N. benthamiana* is favored for protein expression due to its rapid generation of biomass, a defective post-transcriptional gene silencing system, and the extensive range of engineering strategies, including glycoengineering, that can be applied along its secretory pathway; all of which may overcome the challenge of low yield (Margolin et al., 2020). SARS-CoV-2 Spike is heavily glycosylated (22 sites), and different patterns of glycosylation between plant and mammalian expression systems may impact antigen recognition and thus test sensitivity (Rosales-Mendoza et al., 2020; Watanabe et al., 2020). The glycans in SARS-CoV-2 are made up of complex and high-mannose configurations; however, the structure of high-mannose glycans is conserved across eukaryotes (Capell et al., 2020), potentially mitigating differences between plant and mammalian-produced Spike. Furthermore, we used the S1 and RBD portions of Spike, which have fewer of the glycosylation sites present (7 and 1, respectively; Rosales-Mendosa et al., 2020) compared to full length Spike. Nonetheless, glycan-specific antibodies may be missed using plant-produced antigens and we may underestimate antibody reactivity in clinical samples. On the other hand, plant glycans, unlike those from mammalian cells, do not contain sialic acid (Bohlender et al., 2020). While the implications of this are not fully elucidated, sialic acid may shield epitopes (Galili, 2020), potentially resulting in increased reactivity to plant-produced proteins. Our data show comparable results to a commercial assay using mammalian-expressed Spike, suggesting that the effects of differential glycosylation and sialylation of antigens on assay sensitivity are not substantial;

nevertheless, further studies are warranted. Thus, there is great potential to use plant-based expression systems for the rapid generation of serological assay reagents and even vaccines for pandemics, including the current global SARS-CoV-2 pandemic.

Using our ELISA with plant-derived recombinant viral proteins, we detected S1-specific IgG in 66.2% and RBD-specific IgG in 62.3% of individuals who had tested positive for SARS-CoV-2 by PCR in the past. Responses between the two protein fragments were highly correlated, as predicted, and the small difference in reactivity was not unexpected, given the greater number of epitopes in the larger S1 domain. Our sensitivity appears lower than that reported in the literature, with a seroprevalence of 90.1–100% in individuals confirmed to have been SARS-CoV-2-infected by PCR (Amanat et al., 2020; Beavis et al., 2020; Liu et al., 2020; Long et al., 2020), and a lower seroprevalence (65.8%) in those who were diagnosed <14 days before serological testing (Pollán et al., 2020). However, we obtained highly concordant results between our assay and a validated commercial ELISA. In fact, the reported manufacturer's sensitivity of the Euroimmun S1-specific IgG ELISA is 94.4%. This suggests that the lack of S1-specific IgG detection from some recovered COVID-19 patients in our cohort is more likely due to low or absent S1-specific IgG antibody at the time of sampling, rather than a lack of sensitivity in our assay. Antibodies in SARS-CoV-2 infection predominantly target S and N, and a range of commercial and in-house immunoassays have been developed (Houlihan and Beale, 2020). While the sensitivity to detect infection using these antigens does not appear to differ during acute infection, reports are now emerging that S antibodies persist for longer than N antibodies (Grandjean et al., 2020; Fenwick et al., 2021), consistent with the original SARS infection (Chia et al., 2020). In addition to the waning of N antibodies in the post-acute/convalescent phase, Fenwick et al. (2021) also reported that trimeric S protein detected 9–31% more seropositives than monomeric S1 and N, indicating a considerable underestimation of true seroprevalence. That study included the Euroimmun S1 IgG ELISA used in the present study. We may thus have detected a higher seroprevalence in our sample set had we used a trimeric S protein as antigen, rather than monomeric S.

With regard to specificity, we detected IgG cross-reactivity to SARS-CoV-2 in 1/58 (1.7%) of pre-pandemic plasma samples from a cohort of HIV-infected and uninfected volunteers with latent TB infection, giving a specificity of 98.3%. Cross-reactive antibody responses, while lower in magnitude, have been reported in SARS-CoV-2 unexposed individuals (Khan et al., 2020), and likely result from past infections with common circulating hCoVs. Thus, our assay for SARS-CoV-2-specific IgG performs as well as a widely used commercial kit in terms of sensitivity and specificity, and is suitable for serological studies of humoral responses in the current pandemic.

Several factors may affect antibody detection after SARS-CoV-2 exposure. Timing of sampling is important, with IgM typically arising first, peaking 2–3 weeks after symptom onset (Long et al., 2020). IgG is typically detected after IgM in serum, peaking at roughly the same time (Huang et al., 2020). However, in SARS-CoV-2 infection, antibodies may not follow this typical pattern of seroconversion (Long et al., 2020; Seow et al., 2020) and seroconversion to a single Ig subclass has been described (Seow et al., 2020). SARS-CoV-2-specific antibodies are rapidly and readily induced after infection, but the kinetics may be influenced by multiple factors, such as cross-reactive serum antibodies as well as memory B cells from hCoVs (Hartley et al., 2020; Morgenlander et al., 2021). This appears similar to other hCoVs, including SARS-CoV and MERS-CoV (Huang et al., 2020). Interestingly, when investigating isotype responses in addition to IgG, we showed that a further 4/20 (20%) donors had S1-specific IgA or IgM. Thus, in our initial screen where 34% of individuals who had previously tested positive for SARS-CoV-2 by PCR had no detectable IgG responses, 20% may have had isotype responses other than IgG. A recent study showed that combined detection of IgG, IgM, and IgA increased the overall detection of SARS-CoV-2 antibodies, enabling better identification of infected individuals with low antibody levels (Faustini et al., 2020).

A further factor in detection of antibodies to SARS-CoV-2 is waning of the response over time, which has potentially important consequences for the duration of protective immunity and the risk of reinfection. One study showed a decrease in IgG in half of patients tested, calculating an overall half-life of 36 days for IgG (Ibarrondo et al., 2020). Waning of binding antibody responses to S and RBD has been reported soon after their peak, particularly IgM and IgA antibodies, but IgG responses have shown persistence for greater than 90 days post-illness onset (Seow et al., 2020; Wajnberg et al., 2020). A limitation of our study was that we did not have information on the date of COVID-19 symptom onset in our cohort, limiting our analyses to time post PCR positivity, which did not yield a relationship with antibody positivity or OD value. Additional factors that may also influence antibody generation and kinetics include disease severity, age, and comorbidities. We found no relationship between increasing disease severity and antibody positivity or OD value, likely due to the fact that the majority of our study participants had mild to moderate COVID-19.

We determined endpoint titers of binding antibodies to S1 and RBD in a subset of 20 convalescent participants in our cohort. Several studies have demonstrated that binding antibody titers against S correlate with neutralization capacity (Amanat et al., 2020; Okba et al., 2020; Premkumar et al., 2020). A recent study reporting S-specific IgG titers in almost 20,000 patients screened for eligibility as convalescent plasma donors demonstrated that 70% of IgG+ donors had high titers (>1:960) of antibodies (Wajnberg et al., 2020). Importantly, 100% of those with titers >2,880 exhibited neutralizing activity (ID_{50} of >1:10). Although we performed our study on a much smaller sample size, we detected titers of S1 or RDB-specific IgG of up to 1:3,200. However, the majority of donors (54%) had titers below 1:200, and only a third of samples had high titers >1:800. Unsurprisingly, IgA and IgM titers were lower than IgG titers, and did not exceed 1:800 for IgA and 1:400 for IgM. Further studies characterizing antibody titers in recovered COVID-19 patients in our setting are warranted.

Saliva is a non-invasive specimen that can be self-collected and thus represents an attractive sample type for large-scale sampling such as in seroprevalence studies. We demonstrate

that our ELISA can detect SARS-CoV-2-specific IgG and IgA not only in serum, but also in saliva. Further optimization and validation will be required to establish the conditions for optimal detection of antibodies in saliva, including the use of pre-pandemic saliva samples. Recent studies have reported the detection of S-specific antibodies in saliva (Faustini et al., 2020; Randad et al., 2020). Faustini et al. (2020) suggested that the use of both serum and saliva samples increased the detection of SARS-CoV-2 antibody responses, reporting substantial discordance between the two sample types. Although preliminary, our results provide the basis for investigating the detection of SARS-CoV-2 antibodies in saliva using antigens expressed in plants.

In conclusion, our study demonstrates that recombinant SARS-CoV-2 proteins produced in plants enable the robust detection of SARS-CoV-2-specific antibodies. One of our aims was to develop a cost-effective serological assay for both large-scale seroepidemiology as well as research studies of SARS-CoV-2 humoral immunity. We achieved this by making use of plants for the production of viral antigens, which has the benefit of rapid scale-up, and sourcing reagents that were available locally and thus available at a lower cost. Our ELISA can be used to evaluate SARS-CoV-2 seroprevalence and describe the kinetics of the humoral immune response in infected individuals. Serological studies in a setting like ours, in South Africa, where comorbidities such as HIV and TB are highly prevalent, are underexplored and can benefit from this assay.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Faculty of Health Sciences Human Research Ethics Committee, University of Cape Town. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WB, MM, MT, JW, FP, SB, EM, LS, and JB: conceived and designed the study and experiments. WS and IS: provided support and critical protocol review. MM, MT, JW, AD, SJ, NM, MG, FP, and SB: performed the experiments. MM, AD, SJ, NM, MG, SI, RN, RK, and WB: analyzed the data. FP, SI, RN, RK, MT, JW, MM, and WB: wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.589940/full#supplementary-material

REFERENCES

Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T. H. O., Chromikova, V., McMahon, M., et al. (2020). A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat. Med.* 26, 1033–1036. doi: 10.1038/s41591-020-0913-5

Beavis, K. G., Matushek, S. M., Abeleda, A. P. F., Bethel, C., Hunt, C., Gillen, S., et al. (2020). Evaluation of the EUROIMMUN anti-SARS-CoV-2 ELISA assay for detection of IgA and IgG antibodies. *J. Clin. Virol.* 129:104468. doi: 10.1016/j.jcv.2020.104468

Berry, J. D., Hay, K., Rini, J. M., Yu, M., Wang, L., Plummer, F. A., et al. (2010). Neutralizing epitopes of the SARS-CoV S-protein cluster independent of repertoire, antigen structure or mAb technology. *MAbs* 2, 53–66. doi: 10.4161/mabs.2.1.10788

Bohlender, L. L., Parsons, J., Hoernstein, S. N. W., Rempfer, C., Ruiz-Molina, N., Lorenz, T., et al. (2020). Stable protein sialylation in physcomitrella. *Front. Plant Sci.* 11:610032. doi: 10.3389/fpls.2020.610032

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K., Schillberg, S., and Christou, P. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Chan, J. F. W., Yuan, S., Kok, K. H., To, K. K.-W., Chu, H., Yang, J., et al. (2020). A familial cluster of pneumonia associated with the 2019 novel

coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet* 395, 514–523. doi: 10.1016/S0140-6736(20)30154-9

Chen, W. H., Hotez, P. J., and Bottazzi, M. E. (2020). Potential for developing a SARS-CoV receptor-binding domain (RBD) recombinant protein as a heterologous human vaccine against coronavirus infectious disease (COVID)-19. *Hum. Vaccines Immunother.* 16, 1239–1242. doi: 10.1080/21645515.2020.1740560

Chia, W. N., Tan, C. W., Foo, R., Kang, A. E. Z., Peng, Y., Sivalingam, V., et al. (2020). Serological differentiation between COVID-19 and SARS infections. *Emerg. Microbes Infect.* 9, 1497–1505. doi: 10.1080/22221751.2020.1780951

Faustini, S. E., Jossi, S. E., Perez-Toledo, M., Shields, A., Allen, J. D., Watanabe, Y., et al. (2020). Detection of antibodies to the SARS-CoV-2 spike glycoprotein in both serum and saliva enhances detection of infection. medRxiv [Preprint]. doi: 10.1101/2020.06.16.20133025

Fenwick, C., Croxatto, A., Coste, A. T., Pojer, F., André, C., Pellaton, C., et al. (2021). Changes in SARS-CoV-2 spike versus nucleoprotein antibody responses impact the estimates of infections in population-based seroprevalence studies. *J. Virol.* 95:e01828–20. doi: 10.1128/JVI.01828-20

Galili, U. (2020). Amplifying immunogenicity of prospective Covid-19 vaccines by glycoengineering the coronavirus glycan-shield to present alpha-gal epitopes. *Vaccine* 38, 6487–6499. doi: 10.1016/j.vaccine.2020.08.032

Grandjean, L., Saso, A., Torres Ortiz, A., Lam, T., Hatcher, J., Thistlethwayte, R., et al. (2020). Long-term persistence of spike antibody and predictive modeling of antibody dynamics following infection with SARS-CoV-2. medRxiv [Preprint]. doi: 10.1101/2020.11.20.20235697

Hartley, G. E., Edwards, E. S. J., Aui, P. M., Varese, N., Stojanovic, S., McMahon, J., et al. (2020). Rapid generation of durable B cell memory to SARS-CoV-2 spike and nucleocapsid proteins in COVID-19 and convalescence. *Sci. Immunol.* 5:eabf8891. doi: 10.1126/sciimmunol.abf8891

Houlihan, C. F., and Beale, R. (2020). The complexities of SARS-CoV-2 serology. *Lancet Infect. Dis.* 20, 1350–1351. doi: 10.1016/S1473-3099(20)30699-X

Huang, A. T., Garcia-Carreras, B., Hitchings, M. D. T., Yang, B., Katzelnick, L. C., Rattigan, S. M., et al. (2020). A systematic review of antibody mediated immunity to coronaviruses: kinetics, correlates of protection, and association with severity. *Nat. Commun.* 11:4704. doi: 10.1038/s41467-020-18450-4

Ibarrondo, F. J., Fulcher, J. A., Goodman-Meza, D., Elliott, J., Hofmann, C., Hausner, M. A., et al. (2020). Rapid decay of anti-SARS-CoV-2 antibodies in persons with mild Covid-19. *N. Engl. J. Med.* 383, 1085–1087. doi: 10.1056/NEJMc2025179

Kapila, J., De Rycke, R., Van Montagu, M., and Angenon, G. (1997). An Agrobacterium-mediated transient gene expression system for intact leaves. *Plant Sci.* 122, 101–108. doi: 10.1016/S0168-9452(96)04541-4

Khan, S., Nakajima, R., Jain, A., Assis, R. R., Jasinskas, A., Obiero, J. M., et al. (2020). Analysis of serologic cross-reactivity between common human coronaviruses and SARS-CoV-2 using coronavirus antigen microarray. bioRxiv [Preprint]. doi: 10.1101/2020.03.24.006544

Letko, M., Marzi, A., and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat. Microbiol.* 5, 562–569. doi: 10.1038/s41564-020-0688-y

Li, F. (2016). Structure, function, and evolution of coronavirus spike proteins. *Annu. Rev. Virol.* 3, 237–261. doi: 10.1146/annurev-virology-110615-042301

Liu, W., Liu, L., Kou, G., Zheng, Y., Ding, Y., Ni, W., et al. (2020). Evaluation of nucleocapsid and spike protein-based enzyme-linked immunosorbent assays for detecting antibodies against SARS-CoV-2. *J. Clin. Microbiol.* 58, e00461–20. doi: 10.1128/JCM.00461-20

Long, Q.-X., Liu, B.-Z., Deng, H.-J., Wu, G.-C., Deng, K., Chen, Y., et al. (2020). Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat. Med.* 26, 845–848. doi: 10.1038/s41591-020-0897-1

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395, 565–574. doi: 10.1016/S0140-6736(20)30251-8

Maclean, J., Koekemoer, M., Olivier, A. J., Stewart, D., Hitzeroth, I. I., Rademacher, T., et al. (2007). Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J. Gen. Virol.* 88, 1460–1469. doi: 10.1099/vir.0.82718-0

Maliga, P., and Graham, I. (2004). Plant biotechnology: molecular farming and metabolic engineering promise a new generation of high-tech crops. *Curr. Opin. Plant Biol.* 7, 149–151. doi: 10.1016/j.pbi.2004.01.016

Margolin, E. A., Strasser, R., Chapman, R., Williamson, A.-L., Rybicki, E. P., and Meyers, A. E. (2020). Engineering the plant secretory pathway for the production of next-generation pharmaceuticals. *Trends Biotechnol.* 38, 1034–1044. doi: 10.1016/j.tibtech.2020.03.004

Morgenlander, W. R., Henson, S. N., Monaco, D. R., Chen, A., Littlefield, K., Bloch, E. M., et al. (2021). Antibody responses to endemic coronaviruses modulate COVID-19 convalescent plasma functionality. *J. Clin. Invest.* 11:146927. doi: 10.1172/JCI146927

Neuman, B. W., Kiss, G., Kunding, A. H., Bhella, D., Baksh, M. F., Connelly, S., et al. (2011). A structural analysis of M protein in coronavirus assembly and morphology. *J. Struct. Biol.* 174, 11–22. doi: 10.1016/j.jsb.2010.11.021

Okba, N., Muller, M., Li, W., Wang, C., Geurts vanKessel, C., Corman, V., et al. (2020). SARS-CoV-2 specific antibody responses in COVID-19 patients. medRxiv [Preprint]. doi: 10.1101/2020.03.18.20038059

Pogrebnyak, N., Golovkin, M., Andrianov, V., Spitsin, S., Smirnov, Y., Egolf, R., et al. (2005). Severe acute respiratory syndrome (SARS) S protein production in plants: development of recombinant vaccine. *Proc. Natl. Acad. Sci. U. S. A.* 102, 9062–9067. doi: 10.1073/pnas.0503760102

Pollán, M., Pérez-Gómez, B., Pastor-Barriuso, R., Oteo, J., Hernán, M. A., Pérez-Olmeda, M., et al. (2020). Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. *Lancet* 396, 535–544. doi: 10.1016/S0140-6736(20)31483-5

Premkumar, L., Segovia-Chumbez, B., Jadi, R., Martinez, D. R., Raut, R., Markmann, A., et al. (2020). The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci. Immunol.* 5:eabc8413. doi: 10.1126/sciimmunol.abc8413

Randad, P. R., Pisanic, N., Kruczynski, K., Manabe, Y. C., Thomas, D., Pekosz, A., et al. (2020). COVID-19 serology at population scale: SARS-CoV-2-specific antibody responses in saliva. medRxiv [Preprint]. doi: 10.1101/2020.05.24.20112300

Remy, M. M., Alfter, M., Chiem, M. N., Barbani, M. T., Engler, O. B., and Suter-Riniker, F. (2019). Effective chemical virus inactivation of patient serum compatible with accurate serodiagnosis of infections. *Clin. Microbiol. Infect.* 25, 907.e7–907.e12. doi: 10.1016/j.cmi.2018.10.016

Rogers, T. F., Zhao, F., Huang, D., Beutler, N., Burns, A., He, W., et al. (2020). Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. *Science* 369, 956–963. doi: 10.1126/science.abc7520

Rosales-Mendoza, S., Marquez-Escobar, V. A., Gonzalez-Ortega, O., Nieto-Gomez, R., and Arévalo-Villalobos, J. I. (2020). What does plant-based vaccine technology offer to the fight against COVID-19? *Vaccines (Basel)* 8:183. doi: 10.3390/vaccines8020183

Seow, J., Graham, C., Merrick, B., Acors, S., Steel, K. J. A., Hemmings, O., et al. (2020). Longitudinal evaluation and decline of antibody responses in SARS-CoV-2 infection. medRxiv [Preprint]. doi: 10.1101/2020.07.09.20148429

Shanmugaraj, B., Malla, A., and Phoolcharoen, W. (2020). Emergence of novel coronavirus 2019-nCoV: need for rapid vaccine and biologics development. *Pathogens* 9:148. doi: 10.3390/pathogens9020148

Stadlbauer, D., Amanat, F., Chromikova, V., Jiang, K., Strohmeier, S., Arunkumar, G. A., et al. (2020). SARS-CoV-2 seroconversion in humans: a detailed protocol for a serological assay, antigen production, and test setup. *Curr. Protoc. Microbiol.* 57:e100. doi: 10.1002/cpmc.100

Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., et al. (2016). Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends Microbiol.* 24, 490–502. doi: 10.1016/j.tim.2016.03.003

Tan, W., Zhao, X., Ma, X., Wang, W., Niu, P., Xu, W., et al. (2020). A novel coronavirus genome identified in a cluster of pneumonia cases—Wuhan, China 2019−2020. *China CDC Wkly.* 2, 61–62. doi: 10.46234/ccdcw2020.017

Tortorici, M. A., and Veesler, D. (2019). Structural insights into coronavirus entry. *Adv. Virus. Res.* 105, 93–116. doi: 10.1016/bs.aivir.2019.08.002

Wajnberg, A., Amanat, F., Firpo, A., Altman, D. R., Bailey, M. J., Mansour, M., et al. (2020). SARS-CoV-2 infection induces robust, neutralizing antibody responses that are stable for at least three months. medRxiv [Preprint]. doi: 10.1101/2020.07.14.20151126

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281.e6–292.e6. doi: 10.1016/j.cell.2020.02.058

Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S., and Crispin, M. (2020). Site-specific glycan analysis of the SARS-CoV-2 spike. *Science* 369, 330–333. doi: 10.1126/science.abb9983

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C.-L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507

Yamamoto, T., Hoshikawa, K., Ezura, K., Okazawa, R., Fujita, S., Takaoka, M., et al. (2018). Improvement of the transient expression system for production of recombinant proteins in plants. *Sci. Rep.* 8, 1–10. doi: 10.1038/s41598-018-23024-y

Yin, J., Li, G., Ren, X., and Herrler, G. (2007). Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *J. Biotechnol.* 127, 335–347. doi: 10.1016/j.jbiotec.2006.07.012

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

Conflict of Interest: FP and SB are employed by Cape Bio Pharms.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The C-Terminal Half of SARS-CoV-2 Nucleocapsid Protein, Industrially Produced in Plants, Is Valid as Antigen in COVID-19 Serological Tests

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Background: The fight against the current coronavirus disease 2019 (COVID-19) pandemic has created a huge demand of biotechnological, pharmaceutical, research and sanitary materials at unprecedented scales. One of the most urgent demands affects the diagnostic tests. The growing need for rapid and accurate laboratory diagnostic tests requires the development of biotechnological processes aimed at producing reagents able to cope with this demand in a scalable, cost-effective manner, with rapid turnaround times. This is particularly applicable to the antigens employed in serological tests. Recombinant protein expression using plants as biofactories is particularly suitable for mass production of protein antigens useful in serological diagnosis, with a neat advantage in economic terms.

Methods: We expressed a large portion of the nucleoprotein (N) derived from SARS-CoV-2 in *Nicotiana benthamiana* plants. After purification, the recombinant N protein obtained was used to develop an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to SARS-CoV-2 in human sera. To validate the ELISA, a panel of 416 sera from exposed personnel at essential services in Madrid City Council were tested, and the results compared to those obtained by another ELISA, already validated, used as reference. Furthermore, a subset of samples for which RT-PCR results were available were used to confirm sensitivity and specificity of the test.

Results: The performance of the N protein expressed in plants as antigen in serologic test for SARS-CoV-2 antibody detection was shown to be highly satisfactory, with calculated diagnostic sensitivity of 96.41% (95% CI: 93.05–98.44) and diagnostic

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specificity of 96.37 (95% CI: 93.05–98.44) as compared to the reference ELISA, with a kappa (K) value of 0.928 (95% CI:0.892–0.964). Furthermore, the ELISA developed with plant-derived N antigen detected SARS-CoV-2 antibodies in 84 out of 93 sera from individuals showing RT-PCR positive results (86/93 for the reference ELISA).

Conclusion: This study demonstrates that the N protein part derived from SARS-CoV-2 expressed in plants performs as a perfectly valid antigen for use in COVID-19 diagnosis. Furthermore, our results support the use of this plant platform for expression of recombinant proteins as reagents for COVID-19 diagnosis. This platform stands out as a convenient and advantageous production system, fit-for-purpose to cope with the current demand of this type of biologicals in a cost-effective manner, making diagnostic kits more affordable.

Keywords: COVID-19, SARS CoV-2 nucleocapsid protein, biofactories, plant expression, molecular farming, ELISA coronavirus

INTRODUCTION

The present SARS-CoV-2 coronavirus pandemic is the most important global sanitary crisis humankind has faced in the last 100 years. Since its emergence in Asia at the end of 2019, the new virus has already hit all continents, producing harm of remarkable magnitude both in public health, and in the World economy. The pandemic has also underscored the need of rapid and flexible responses in the industrial capacities required to produce large amounts of tools and reagents for diagnosis, therapies, and prevention (vaccines) of new viral diseases in a cost-effective way.

Serological tests are essential tools for the detection of antibody responses to SARS-CoV-2 infection. They play an essential role not only in diagnosis but also in the evaluation of the seroprevalence in exposed populations and in research studies aimed to provide answers to still open key questions regarding the complete characterization of the course of the infection such as, for instance, the duration of antibodies. In this regard, enzyme-linked immunosorbent assays (ELISAs) are widely used in most diagnostic and research applications due to their long-proved efficiency, easiness, and high throughput capacity.

Many serological assays have been made available recently for detection of SARS-CoV-2 specific antibodies (Hoste et al., 2020; Rikhtegaran Tehrani et al., 2020; Whitman et al., 2020). However, their relatively high cost still prevents many laboratories, especially in developing countries, from their broad use. Therefore, approaches enabling significant reductions in production costs for these assays are highly desirable. Specifically, as the most expensive component of these assays, the production of the recombinant viral proteins used as antigens would benefit largely of more cost-efficient processes, leading to more affordable serological tests.

A particularly attractive system for cost-efficient recombinant protein production is based on their expression in plants, often named Plant Molecular Farming, which has gained importance and relevance over the years. Nowadays, several proteins with pharmaceutical and other industrial applications are already being produced in plants, particularly in *Nicotiana benthamiana*. The favorable features of this industrial approach have been widely and frequently reviewed, among which speed of production in very large amounts and comparative low costs of productions stand out (Ma et al., 2003; Williams et al., 2014, 2016; Moon et al., 2019; Capell et al., 2020; McDonald and Holtz, 2020). In the particular case of SARS-CoV-2 proteins, a few examples showing their production in plants have been published very recently (Diego-Martin et al., 2020; Siriwattananon et al., 2021). A previous approach to produce plant-made viral proteins that could be potentially used as antigens has been shown for the related SARS-CoV, where antibody binding to immobilized plant-produced virus antigen was demonstrated using a limited panel of serum samples (Demurtas et al., 2016).

Many serological assays for SARS-CoV-2 antibody detection rely upon the use of the viral multifunctional nucleoprotein (N) (McBride et al., 2014; Hoste et al., 2020; Tré-Hardy et al., 2021), expressed in recombinant forms using different expression systems. We have undertaken the production of a large portion of SARS-CoV-2 N protein in *N. benthamiana*, through its transient expression from a vector derived from *Tobacco mosaic virus* (TMV) and shown its suitability to detect antibodies using a wide panel of human serum samples obtained in early 2020. For that, we developed an ELISA test which was used to analyze the presence of antibodies to SARS-CoV2 N antigen in the panel sera. The results were compared with those obtained using an already validated ELISA test commercially available.

MATERIALS AND METHODS

Cloning of SARS-CoV-2 N Gene Into Plant Expression Vector TMV-PSN

Generation of Infectious RNA

The cDNA encoding for a truncated N form (termed "NucleoProtein Coronavirus-2," abbreviated, NPC-2) of SARSCoV-2 which codes for its amino acids 231–419 (GenBank YP_009724397.2) was designed with a His-tag at its C-terminus. The synthetic sequence was made by GENEART AG optimizing

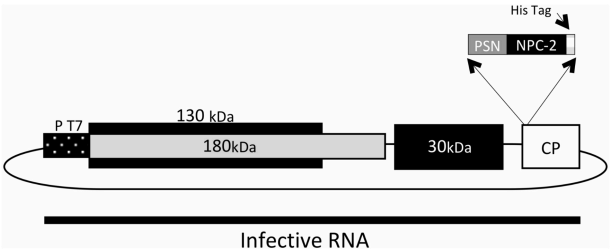

FIGURE 1 | Schematic diagram of the expression vector TMV-PSN-NPC-2 DNA. The coding sequence of SARSCoV-2 Nucleoprotein (NPC-2) was modified by the addition of C-terminal His tag and inserted into the TMV vector. An 82 bp fragment corresponding to the signal peptide (PSN) was included in position N-terminal. Infectious cDNA clone was transcribed from the T7 bacteriophage RNA promoter (PT7), and the RNA used to infect *N. benthamiana* plants. The TMV genome has four open reading frames (ORF). ORF 1 of 130 kDa with Methyltransferase and Helicase function, ORF 2 of 180 kDa with Methyltransferase, Helicase function and RNA-dependent RNA polymerase (RdRp) function. ORF 3 of 30 kDa corresponding to Movement Protein (MP) and ORF 4 of 17 kDa corresponding to Coat Protein (CP).

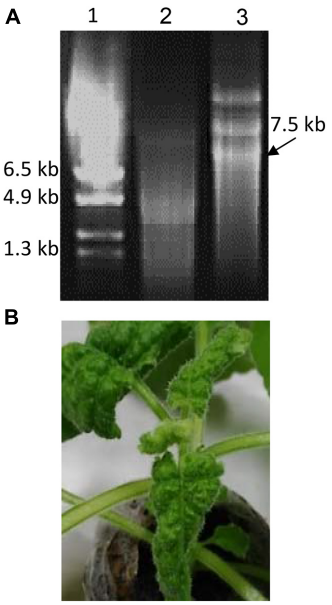

FIGURE 2 | Production of infectious RNA from TMV-PSN-NPC2 clone and inoculation of *N. benthamiana* plants **(A)** *In vitro* transcription (IVT), ethidium bromide-stained 0.5% agarose gel. 1: RNA marker (Promega G319A), 2: RNA control kit, 3: RNA TMVPSN-NPC2. The RNA transcribed (7.5 kb) was used for plant inoculation. **(B)** Infectivity of the recombinant virus. The picture shows a plant at 8 dpi (days post inoculation). Leaves with symptoms of infection reflected by the mild leaf deformation with variable mottling grade.

for codon usage in *N. benthamiana* plants. The sequence was cloned into TMV expression vector (GENEWARE System) under the control of a duplicated promoter of the capsid protein (CP) gene. The extensin signal peptide (PSN) from *Nicotiana tabacum*, which directs the recombinant NPC-2 toward the plant endoplasmic reticulum (**Figure 1**), was also included.

Plasmid DNA of the stable clone TMV-PSN-NPC-2 was purified using QIAGEN's plasmid purification kit, according to the manufacturer's instructions.

Capped infectious RNA was prepared *in vitro* from plasmid TMV-PSN NPC-2 clone with the mMESSAGE mMACHINE^{TM} T7 Transcription Kit (INVITROGEN). Each *in vitro* transcription reaction (IVT) was made with 0.625 μl plasmid DNA (0.2 μg/μl), 0.125 μl water, 0.25 μl 10× Buffer, 1.25 μl 10× nucleotides (CAP) and 0.25 μl 10× T7 RNA polymerase, in a 2.5 μl final volume. The reaction was incubated for 2 h at 37°C. RNA was quantified by electrophoresis in a 0.5% ethidium bromide-stained agarose gel (**Figure 2A**).

Inoculation of *Nicotiana benthamiana* Plants With the Infectious Transcript

Three hundred *N. benthamiana* plants, 18–28 day-old, were inoculated by gently scraping their leaves with 50 μl of diluted infectious RNA [2.5 μl of IVT reaction in 100 μl of 1× FES buffer (43 mM glycine, 139 mM K_2HPO4, 1% sodium pyrophosphate, 1% bentonite and 1% of celite, w/v)]. Plants were kept at 25°C in growth chambers (60% humidity, 16/8 photoperiod).

Infected leaves from 5 dpi symptomatic plants were pooled and extracted by grinding in 150 ml of 50 mM phosphate buffer, pH 7.5, with 0.04% (w/v) of sodium metabisulphite. The crude extract was then used to inoculate 2,000 *N. benthamiana* plants in a greenhouse (70% humidity, 16/8 photoperiod). Infection and NPC-2 expression were assessed by ELISA with polyclonal antibodies against TMV (AGDIA, CAB 57400) and SARSCoV-2 N protein (Invitrogen, PA5-81794) according to the manufacturers' instructions.

Stability of the recombinant virus during infection was evaluated by Immunocapture Reverse Transcription Polymerase Chain Reaction (IC-RT-PCR) according to Nolasco et al. (1993). To amplify the NPC-2 insert from viral vector, two specific oligonucleotides were designed hybridizing outside of the vector cloning site (cTMVF 50 -CGT GTG ATT ACG GAC ACA ATC C-30 and cTMVR 50 -TAC TGT CGC CGA ATC GGA TTC G-30). Thereby, the expected size of the complete construction (TMV–PSN–NPC-2) is 820 bp and for the "empty" vector (TMV–PSN) 226 bp. The infected plants with viral RNA were used to infect plants by mechanical inoculation. The stability of the recombinant viral vector was evaluated in this way in plants at 8- and 12-days post inoculation (dpi).

Purification and Quantification of Recombinant NPC-2

Symptomatic leaves were harvested 11–13 dpi and blender-ground in 100 mM potassium phosphate buffer, pH 7.3–7.6, 100 mM NaCl, 0.04% (v/v) Triton ×100, 5 mM BME, and 0.1 mM PMSF (1:3 w/v). The homogenate was filtered through three cloth layers and centrifuged at 6,000 × *g* to remove cell debris and insoluble material. The soluble extract was subjected to a two-step purification process involving a first stage by Ni^{2+}-sepharose affinity chromatography, and a second stage by Cation Exchange in a HiTrap SP Sepharose FF 5 ml (AKTA Xpress System -GE), according to Williams et al. (2016). The

absorbance (280 nm wavelength) peaks were analyzed on a 15% SDS-PAGE and by western blot (as described below). Purified NPC-2 protein was quantified by SDS-PAGE and UV spectrophotometry by Nanodrop.

Molecular and Antigenic Characterization of NPC-2

The purified recombinant NPC-2 protein was subjected to SDS-PAGE gel analysis (100–500 ng/lane) and stained with Coomassie Blue. The bands with a migration in the gel around 21 kDa, were excised. Their tryptic fragments were analyzed by MALDI-TOF in an external service (SCSIE-Universidad de Valencia).

The purified NPC-2 was subjected to Western blot analysis, probed with a commercial polyclonal antibody specific for SARSCoV-2 N protein (N-full length SARSCoV-2 Nucleocapsid Polyclonal Antibody, Invitrogen PAS81794). For that, recombinant NPC-2 protein (0.3–3.0 μg) was separated by SDS-PAGE and electrotransferred into a nitrocellulose membrane, which was blocked with TBS buffer supplemented with 5% (w/v) skimmed milk, and subsequently incubated with the commercial anti-SARSCoV-2 N polyclonal antibody, diluted 1:2,000, for 1 h. The development was done after incubation with alkaline-phosphatase-conjugated goat anti-rabbit IgG (97048 Abcam), 1:200, 1 h, followed by substrate addition (NBT/BCIP).

Serological Validation of Plant NPC-2 With Samples From SARS-CoV2-Infected and Non-infected Individuals

Serum Samples

In this study we used a total of 416 human serum samples from the "Program of Surveillance and Early Detection of COVID 19 in essential services personnel in the city of Madrid" collected in April 2020, when the city of Madrid was severely hit by the pandemic, which were provided by the Institute of Public Health of the Madrid City Council. Samples were classified as positive (n = 193) or negative (n = 223) to SARS-CoV-2 antibodies on the basis of their result with a commercially available ELISA (INgezim COVID 19 DR, Ingenasa). already validated, with 100% diagnostic sensitivity (95% confidence interval, CI = 97.7–100%) and 98.2% diagnostic specificity (95% CI = 97–99.1%) (Hoste et al., 2020). A subset of the samples corresponded to patients with confirmed SARS-CoV-2 infection by a positive result by Real Time Reverse Transcription Polymerase Chain Reaction RRT-PCR (Corman et al., 2020) in oropharyngeal samples at the time of serum collection, or previously (**Table 1**).

TABLE 1 | Serum classification by commercial serological assay and PCR confirmation.

	Serum samples
Antibody + /confirmed by PCR	86
Antibody + /not confirmed by PCR	107
Antibody -/confirmed by PCR	7
Antibody -/not confirmed by PCR	216
Total	416

Indirect NPC-2 ELISA

Enzyme-linked immunosorbent assay plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with 2 μg/well of purified recombinant NPC-2 protein antigen in 0.1 M carbonate/bicarbonate buffer pH 9.6 and maintained at 37°C for 45 min. Thereafter, wells were blocked with phosphate buffered saline, pH 7.4, 0.05% (v/v) Tween 20 (PBST), containing 3% (p/v) skimmed milk, for 1 h at room temperature (RT). The plates were incubated with serum samples diluted 1:200 in PBST with 1% skimmed milk, for 2 h at RT. Duplicates of positive (sera from a confirmed COVID 19 patient), and negative controls were included in each plate. Plates were washed three times with PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Thermofisher), diluted 1:5,000 in PBST, for 1 h at RT. Finally, after three additional washes with PBST, plates were incubated with peroxidase substrate (TMB-MAX, Neogen Corporation) for 5 min in the dark at RT. Reaction was stopped by adding 0.5 M H_2SO_4. Optical density (OD) was measured in each well at 450 nm in an ELISA microplate reader. Results were valid when OD in negative control wells was below 0.8 OD units and the OD in positive control wells was over 2.50 OD units.

Statistical Analysis

Data were statistically analyzed using a receiver-operator characteristics (ROC) curve analysis using the GraphPad Prism 6 software. The kappa statistic, analyzed by the same software, was used to measure the strength of agreement between the results obtained by the indirect ELISA test with recombinant NPC-2 protein antigen and the reference commercial ELISA.

Sensitivity of the tests (NPC-2 ELISA and reference ELISA) was calculated as the proportion of positive samples by the method used as reference (reference ELISA or samples from patients with previous positive PCR result) that tested also as positive in the evaluated test.

Specificity was calculated as the proportion of negative samples by reference ELISA which tested negative in NPC3 ELISA.

RESULTS

Cloning of SARSCoV-2 N Gene Into Plant Expression Vector TMV-PSN

Production of Infectious RNA

The DNA sequence of SARSCoV-2 N (Gen Bank YP_009724397.2, aa 231–419), designed with His-tag C-terminal and optimized to achieve its correct expression in *N. benthamiana* plants was successfully cloned into TMV-PSN vector and the recombinant TMV-PSN-NCP2 clone was confirmed by sequencing. *In vitro* 6 kb RNA transcripts produced from the recombinant TMV-PSN-NCP2 clone were used to infect plants by mechanical inoculation (**Figure 2A**). Evident symptoms of infection such as leaf deformation with variable mottling grade were visible after 6–8 dpi (**Figure 2B**).

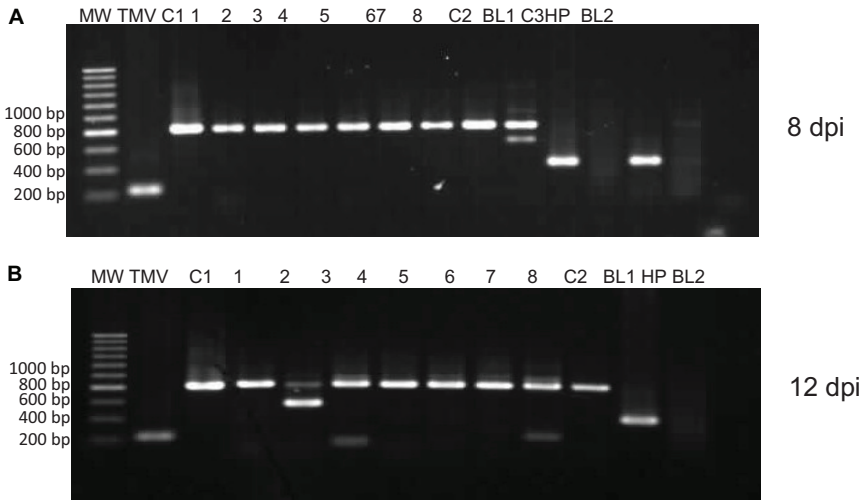

FIGURE 3 | Stability of NPC-2 by IC-RT-PCR. Amplification of an 820-bp fragment, which is the size expected for the recombinant clone, was observed in 100% of the plants infected. MW, hiperladder 200 bp; TMV, empty vector, C1, TMV-PSN-NPC2 clone (820 pb). 1–8, Infected plant, C2, TMV-PSN-EGF clone (397 pb), BL1, negative PCR control; C3, infected plant with TMV-PSN-EGF (IC-RT-PCR positive control). HP: healthy plant, BL2: negative control of IC-RT-PCR. **(A)** Infected plants to 8 dpi and **(B)** infected plants to 12 dpi.

Stability of TMV-PSN NPC-2 in *Nicotiana benthamiana* Plants

The stability of the TMV-PSN-NPC2 construct plasmid was analyzed in the inoculated *N. benthamiana* plants by IC-RT-PCR at 8 and 12 dpi. Amplification of an 820-bp fragment, which is the size expected for the recombinant clone, was obtained in 100% of the plants analyzed with TMV-PSN-NPC-2 while in the different controls included, namely, extracts from plants infected with the empty vector (TMV-PSN) and from plants infected with another unrelated construct (TMV-PSN EGF) the amplicons observed were of 226 and 397 bp, respectively, corresponding with their expected sizes (**Figure 3**).

The IC-RT-PCR assay not only showed that 100% plant leaves analyzed had the expected size to insert NPC-2 (820 bp) but also that 1 in 8 plants, both in 8 and 12 dpi, showed a partial deletion of the insert (600 bp).

Purification From *Nicotiana benthamiana* Leaves and Yield Evaluation

The recombinant protein NPC-2, was purified from leaf extracts, using two successive chromatographic steps, Ni^{2+}-affinity and cationic exchange. After Ni^{2+}-affinity chromatography, more than 90% of the NPC-2 protein was recovered from the soluble plant extract, with over 80% purity (**Figure 4A**). NPC-2 migrated as a double band with an apparent molecular weight 21.8 kDa, in agreement with our predictions based on amino acid sequence.

After the second purification step, cation exchange column chromatography, one major protein peak was observed on a 0–1 M NaCl gradient (**Figure 4B**). The recombinant protein typically eluted at 30.2% of elution buffer (0.3 M NaCl approximately). Fractions were pooled, concentrated and quantified by spectrophotometry. Upon Western blot analysis probed with anti SARS CoV-2 N antibody, the 20–22 kDa bands were antigenically identified as N-derived NPC-2. We estimate a final NPC-2 yield of 200 mg/kg of fresh tissue, with over 95% homogeneity (**Figures 4C,D**).

MALDI-TOF analysis of the two bands recognized by the polyclonal SARS CoV-2 N antibody gave the same peptide mass finger printing pattern as the C-terminal (aa 231–419) of SARS CoV-2 N protein (data not shown).

Serological Validation of Plant-Produced NPC-2 With Serum Samples From SARS-CoV2-Infected and –Non-infected Individuals

An indirect ELISA was developed based on the recombinant NPC-2 protein from *N. benthamiana* plants (hereinafter, NPC-2 ELISA). The ELISA was optimized for IgG antibody detection in serum samples (data not shown). A total of 416 human serum samples were analyzed, that, in the absence of a serological gold standard method, were previously classified as positive or negative on the basis of the results obtained with a commercial ELISA (hereinafter "reference ELISA") that has proven to be highly sensitive and specific (Hoste et al., 2020). Importantly, the method chosen as reference is also based on the use of a recombinant N derived from SARS-CoV2. The results obtained with the NPC-2 ELISA (expressed as corrected OD values, i.e., O.D. ratios relative to negative serum control result) were compared with those from the reference ELISA. To compare the performance of the NPC-2 ELISA to that of the reference ELISA, a receiver-operator characteristics (ROC) analysis was used (**Figure 5A**). The area under the curve (AUC) of the NPC-2 ELISA was 0.9831 [95% confidence interval (CI): 0.971–0.995] indicating that this method has a very high diagnostic accuracy.

Based on the ROC curve, a cut-off of four was set (**Figure 5B**). Using this value, a diagnostic specificity of 96.41% (95% CI:

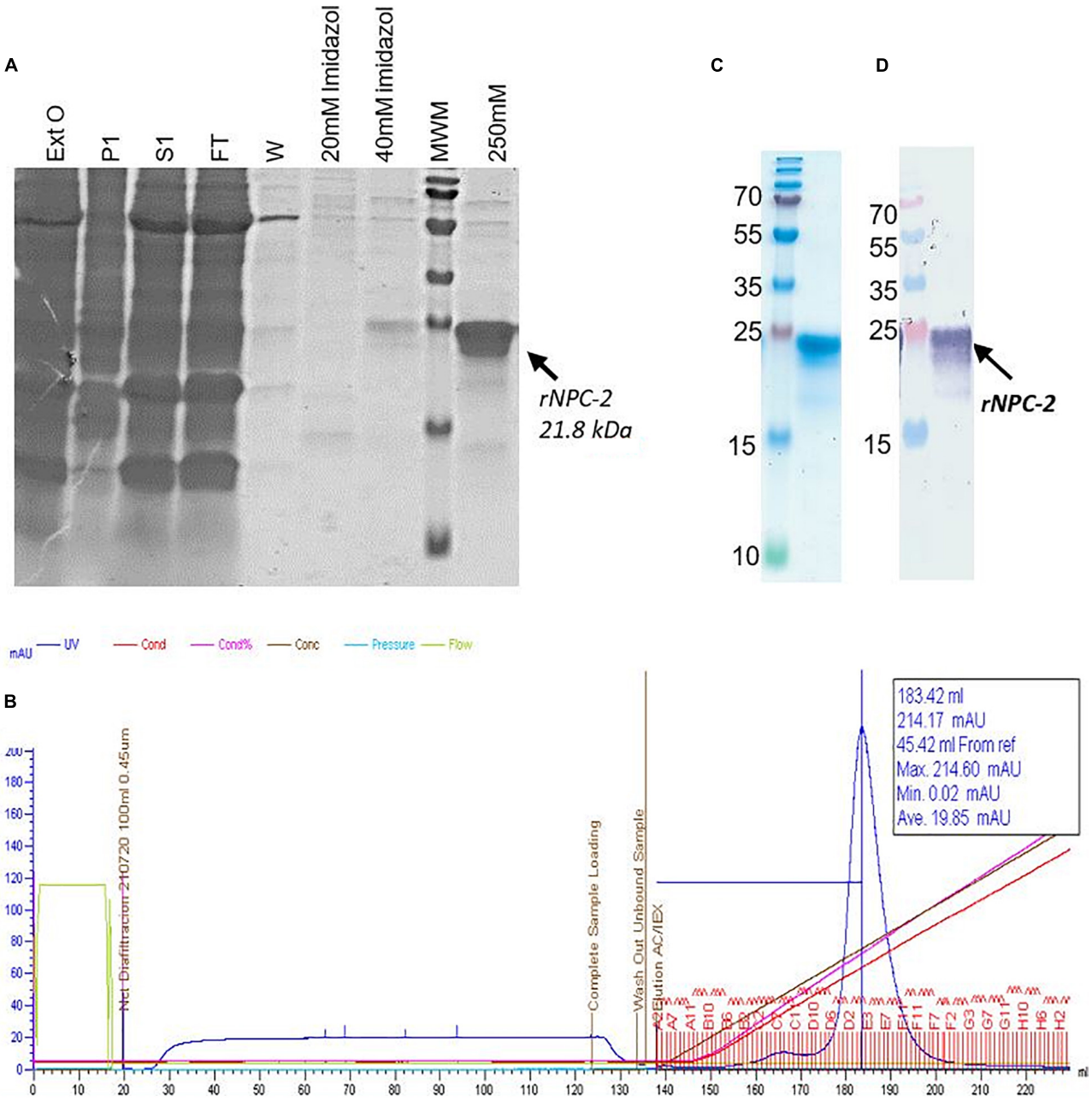

FIGURE 4 | Analysis of purified plant-produced recombinant NPC-2. See main text for details of the purification procedure. **(A)** Coomassie stained SDS-PAGE of Affinity Chromatography (IMAC-resin Ni2 + Sepharose Fast Flow). Fifteen μl of: Ext-0 plant extract (Ext0), pellet (P1), soluble plant extract (S1), FT flowthrough (FT), washed (W) and imidazole gradient (20, 40, and 250 mM) were loaded. The NPC-2 protein was eluted with more than 80% of purity and migrate in SDS-PAGE (reducing conditions) as a protein of 21.8 kDa. **(B)** Fractions recovered from Cation Exchange Chromatography, (Hitrap SP FF SP) corresponding to recombinant NPC2. **(C)** Final Purity of recombinant NCP2 obtained from NaCl gradient analyzed by SDS-PAGE stained with Coomassie Blue. **(D)** Western blot analysis of recombinant protein NCP2 identified by Nucleocapsid Polyclonal Antibody (Invitrogen PAS81794).

93.05–98.44) and sensitivity of 96.37 (95% CI: 93.05–98.44) were achieved (**Figure 5B** and **Table 2**). Using the kappa statistic to measure the concordance of results between techniques, a value of K = 0.928 was obtained, indicating an almost perfect agreement between reference and the NPC-2 ELISA (Landis and Koch, 1977).

As a step further in the validation of the NPC-2 ELISA, the results obtained in a subset of serum samples selected as "true positive" i.e., from individuals with a positive PCR result either in the same day of serum collection or in previous analyses, were analyzed. **Table 3** shows a comparison of the results obtained in these true positive samples by NPC-2 ELISA and reference ELISA

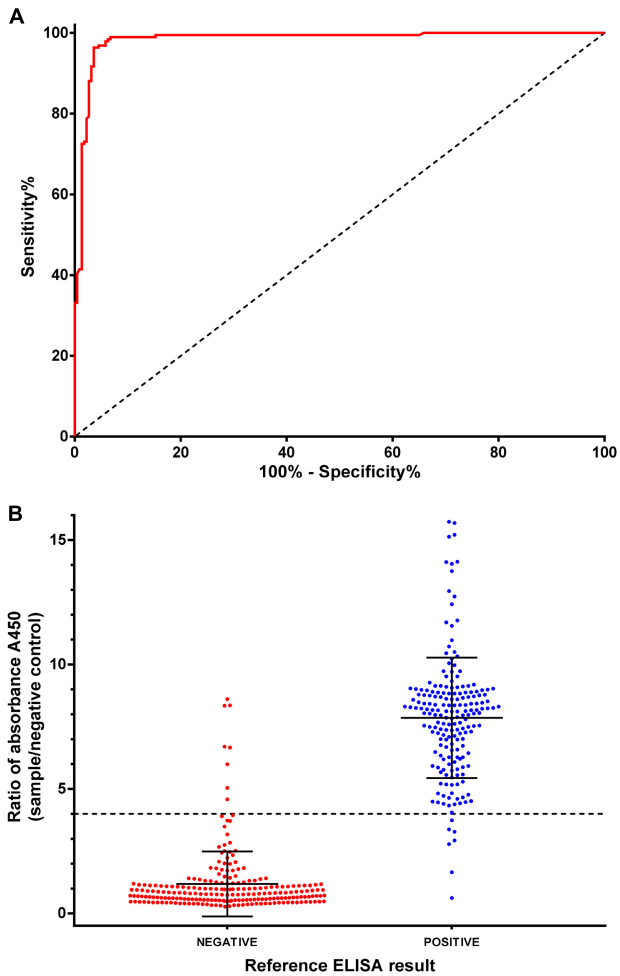

FIGURE 5 | ELISA assay validation and diagnostic performance. **(A)** Receiver operating characteristic (ROC) analyses of the Indirect NPC-2 ELISA in 416 human sera, positive or negative by reference ELISA (INgezim COVID-19 DR, Ingenasa). **(B)** Distribution of NPC-2 ELISA protocol results by sample classification (negative or positive in reference ELISA test).

TABLE 2 | Diagnostic sensitivity (DSe), diagnostic specificity (DSp) and kappa coefficient (K) of the NPC-2 ELISA compared to the reference ELISA.

		NPC-2 ELISA		
		Positive	**Negative**	**Total**
Reference ELISA	Positive	186	7	193
	Negative	8	215	223
	Total	194	222	416
DSe	96.41% (95% CI: 93.05–98.44)			
DSp	96.37 (95% CI: 93.05–98.44)			
K value	0.928 (95% CI:0.892–0.964)			

tests. NPC-2 ELISA detected 90.32% (84/93) of true positive sera, whereas the reference ELISA detected 92.47% (86/93), that is, the NPC-2 ELISA detected only a slightly lower proportion of true positive samples. In fact, only two samples with a positive result in the reference ELISA showed a negative result with NPC-2 ELISA, further confirming that both methods have a similar capacity to detect serologically positive COVID-19 cases.

TABLE 3 | Comparison of ELISA assays in determining SARS-CoV-2 antibodies in serum samples from patients confirmed by RT-PCR.

		NPC-2 ELISA		
		Positive	**Negative**	**Total**
Reference ELISA	Positive	84	2	86
	Negative	0	7	7
	Total	84	9	93

DISCUSSION

The present paper reports a fast, efficient, and easily scalable expression system for the large-scale production of recombinant SARSCoV-2 nucleoprotein (N), useful as antigen for serologic tests, in plants of *N. benthamiana*. The availability of such production systems for viral proteins at a massive scale has become already a critical issue in the fight against COVID-19. Indeed, it is expected to become even more relevant in the years to come when epidemiological studies will need to be undertaken at a global level to conduct serological surveillance studies of the human population, as well as in animal reservoirs. The N protein of the virus is one of the several viral proteins potentially useful as antigenic markers of infection, together with S, E, and M, and probably the most widely used at this respect (Liu et al., 2020; Rikhtegaran Tehrani et al., 2020). Each one of these has pros and cons, so probably a global picture will be obtained through their combined use. Importantly, antibodies to N are not present in individuals vaccinated with S-based vaccines, so unlike S-based serologic tests, which effectively detect antibody responses elicited either by infections or by vaccines (mostly based on the development of immune responses to S protein, involved in seroneutralization and thus protection from SARS-CoV2 infections), N-based serologic test will be expected to react to antibodies raised by actual infections, and not vaccinations (as long as they are carried out using S-protein-based immunogens), which might be an advantage for some applications, e.g., in seroprevalence studies focused on infections, during ongoing or after vaccination programs. Some of the main characteristics of the N protein for these purposes have been recently reviewed (Li and Li, 2021).

The production of SARS-CoV-2 recombinant N protein has been addressed so far mostly in *Escherichia coli* (Algaissi et al., 2020). However, this prokaryote expression system frequently fails to fold correctly proteins that are to be acting in a complex eukaryotic environment. This incorrect folding usually results in the expression of insoluble conformations, forming inclusions, useless in most biotechnological applications. This problem has been found for the N protein. Plants are a good alternative for the production of eukaryotic proteins, particularly using transient expression systems based on viral vectors (Gleba et al., 2007; Pogue et al., 2010). In fact, some SARS-CoV-2 proteins

have already been made in plants (Burnett and Burnett, 2019; Sainsbury, 2020; Makatsa et al., 2021; Siriwattananon et al., 2021), including N (Diego-Martin et al., 2020), although the suitability of plant-made N for detection and diagnosis of antibodies in samples of human sera has not been tested yet. In addition to these technical advantages, plant production of recombinant proteins presents an important economic advantage in terms of production costs, which becomes an important aspect when massive production is required. It has been estimated that these costs represent a mere 0.1% of mammalian cell-based platforms, or 2–20% of systems based on bacteria (Yao et al., 2015).

In this study, the C-terminal domain of the N protein (aa 231–419) has been expressed in *N. benthamiana* using the TMV-PSN vector, already used by our group for the expression of other proteins (Williams et al., 2016, 2014). The protein with a MW of 21.9 kDa was purified from soluble plant extract with a final yield of approximately 200 mg recombinant protein per kg of fresh tissue. This is a better yield in comparison with our previous results obtained by us for human vascular endothelial growth factor VEGF (Williams et al., 2016), and also a better expression level than the one obtained by others in plants for this protein (Diego-Martin et al., 2020). An initial number of 300 plants grown under controlled conditions (growth chambers) allowed us to generate enough inoculum to infect 11,000 plants in the greenhouse. This means processing 16 kg of plant tissue per week. With a yield of 200 mg per kg of tissue, and based on our previous experience in the recovery of proteins from the soluble plant extract, we estimate a final yield of *ca.* 4 g recombinant protein per 16 kg of fresh tissue per week (12 g per month). Just to give an idea of the potential of this production system, one ELISA plate using the format described in this study requires 0.2 mg antigen, so 12 g may yield enough antigen to fill 60,000 plates and analyze approximately 5,400,000 serum samples. According to our own calculations the approach followed in this study might reduce the production costs of serologic tests significantly, making them more affordable, especially in low-income countries. Only two purification steps were required to obtain >95% purity: A first initial affinity chromatography capture, followed by a second stage of polishing through cationic exchange. After the two processes, over 90% of the protein was recovered. The recombinant protein was identified by MALDI-TOF and proven to be recognized by a commercial antibody raised against the full viral protein. No sign of protein degradation was observed in the purification process. One of the main drawbacks of viral vectors for protein production in plants is the stability of the foreign gene in the plant cell during their replication cycle. This particular issue was addressed by IC-RT-PCR carried out on infected plants 8–12 dpi, that is, the period coinciding with tissue harvest. We found a remarkably good stability with 12.5% of the plants showing partial deletions. Under our growth conditions, plants at 12 dpi were reaching a weight of 1.2–1.8 g/leaf.

The ability of plant-made recombinant N protein to be recognized by antibodies was evaluated in 416 serum samples from infected and non-infected individuals collected during the first epidemic wave (April, 2020) affecting Spain in the context of a surveillance study focused on voluntarily participating active staff from the essential services of Madrid City Council (police, firemen, emergencies and other essential services). This panel of samples was reflecting the immunological status of this cohort of staff occupationally exposed to virus infection in a context of high virus transmission. Moreover, RT-PCR from nasal swabs was performed in each individual at the time of blood sample collection and, in some cases, also earlier, in order to verify their infection status. Thus, it was possible for a subset of serum samples used in this study to ensure they came from ongoing infections. It is widely known that there is a lag period between the detection of SARS-CoV-2 RNA in nasopharyngeal tract and the development of antibodies in serum. Thus, it has been observed that some RT-PCR positive individuals yield negative results in serologic assays (Pollán et al., 2020). This may explain that from 97 RT-PCR-positive individuals, seven had undetectable antibodies in serum by the reference ELISA and

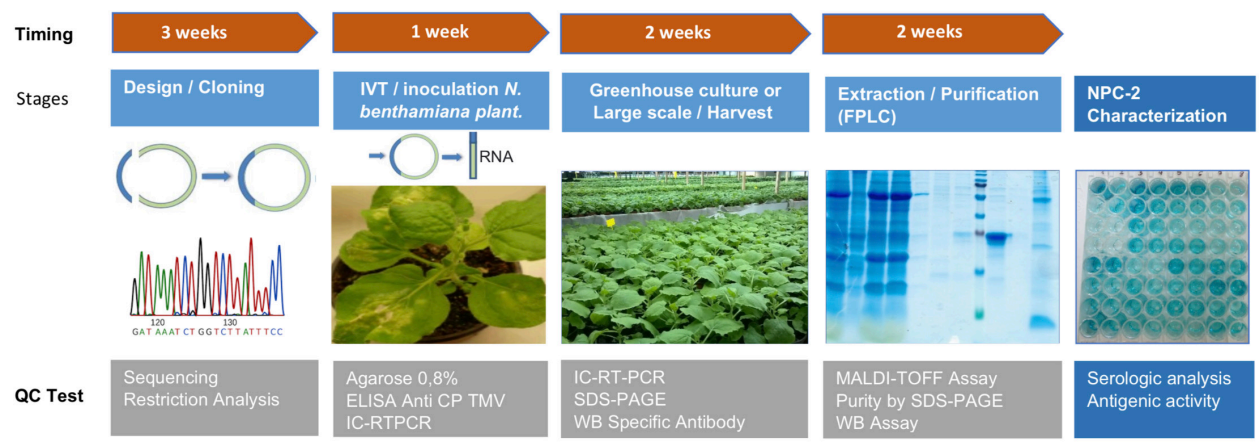

FIGURE 6 | Flowchart of the complete process of production of NPC-2 from *N. benthamiana* plants. The upper timeline describes the extent of different stages: Design/Cloning (3 weeks), *in vitro* transcription and production of inoculum (1 week), plant greenhouse culture of large scale (2 weeks), Extraction/Purification of final product NPC-2 from plants (2 weeks). The lower boxes show the quality control tests associated with each stage.

two more in the NPC-2 ELISA. Indeed, these two sera that yield different results in the two ELISAs may be due to the nature of each test: on the one hand, the reference ELISA is based on a double recognition (DR), which means that the specific antibodies in the sample are first captured by immobilized antigen, and after several washing steps, are detected by the addition of soluble HRP-conjugated antigen which binds to the free remaining antigen-binding sites in the antibodies. This type of ELISA can detect IgG, IgA, and IgM class antibodies, whereas the indirect NPC-2 ELISA used in this study was designed to detect only IgG. Therefore, it is likely that immunoglobulins other than IgG may be present in the two samples detected only in the reference test and went unnoticed in the NPC-2 ELISA. It is worth reminding here that although the indirect ELISA developed for this study was limited in this and probably other aspects, the aim of the study was to show the aptitude of the NPC-2 developed in plants as antigen valid for serological methods, rather than developing a fully functional ELISA method. Once the NPC-2 has been shown useful for detection of human IgG, it is highly likely that it would work in the same way in improved serological methods, including other ELISA formats such as double recognition (DR), competition, IgM (or IgA)-capture ELISAs. Indeed, this plant-produced antigen may also be used to develop some alternative on-site diagnostics not requiring specific equipment (multiwell plate readers), e.g., colloidal gold-based test strips, in order to make the serological diagnosis even more affordable.

Differential IgG levels could be detected by ELISA in sera from COVID-19 patients with a similar or a better sensitivity, as compared to other systems using recombinant N protein, as the reference ELISA used in this work. The assay showed a high sensitivity in patients recently infected so it can represent an interesting approach for diagnostic and epidemiological studies. The high purity, sensitivity in antibody detection, and very low level of endotoxins in the final protein preparation should make it a powerful diagnostic tool. From an industrial standpoint, the yields obtained in the purification process would allow to manufacture recombinant protein for millions of diagnostic reactions kits in 8 weeks (**Figure 6**).

Very recently, another study has been published showing the usefulness of other plant-produced SARS-CoV-2-derived antigens, the S (or "spike") protein and its Receptor-Binding Domain (RBD) (Makatsa et al., 2021). Apart from the antigens used, the main difference with our study is that Makatsa et al. used a lower number of samples in the validation (77 positive, 58 negative), that IgA and IgM were also tested, and that IgA/IgG in saliva were also evaluated. Overall, plant-expressed S and RBD from SARS-CoV-2 performed well at detecting SARS-CoV-2-specific immunoglobulins, with slightly better sensitivity for S. We confirm and extend this result to N, with sensitivities, measured in RT-PCR confirmed cases, higher that those obtained with S or RBD, although the different selection of sample donors in both studies may impair a direct comparison of the results. As discussed above, serologic tests based on S (or RBD) and N are both necessary and complement each other when it comes to differentiating infection- from vaccination induced antibodies. In addition, the S protein accumulates mutations in a higher rate than in the N protein (Kaushal et al., 2020), where variable sites affect its N-terminal but not its C-terminal half, used in this study as antigen. Therefore, NCP-2-based immunoassays would be less prone to sensitivity losses due to infections by SARS-CoV2 variants.

Although not specifically tested in this study, cross-reaction with other coronaviruses currently circulating in humans (i.e., excluding SARS-1, which is not circulating anymore) is not expected due to the low percentages of identity observed in the amino acid sequence of homologous regions of our truncated version of SARS-CoV-2 nucleoprotein (NPC-2) in the human coronaviruses, such as MERS (51%), OC43 (37%), HKU1 (36%), NL63 (29%), and 229E (27%).

Upon validation of the results and technology presented in this work, we should be in a position to tackle the production of other SARS-CoV-2 proteins. This would allow increased specificity in the detection of virus variants.

Finally, it is worth to remark that in this study, technology derived from the first virus ever described (TMV, 1892) is used to produce a protein from the last human described virus (SARS-CoV2, 2019) at an industrial scale, and enables the development of cost-efficient serological tests useful to fight its infection, and INGENASA for providing the ELISA kits used as reference technique.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PL, LW, FP, and MJ-C conceived this article. LW, SJ, and FL designed the experiments. LW and PL drafted the first manuscript with support from FP. SJ, AR, SG, AS, IB, FL, and LW carried out the experiments. MM-C and BP-G contributed to sample management and procurement. LW and FL analyzed data and performed statistical analyses. All authors discussed the results and contributed to the final manuscript.

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REFERENCES

Algaissi, A., Alfaleh, M. A., Hala, S., Abujamel, T. S., Alamri, S. S., Almahboub, S. A., et al. (2020). SARS-CoV-2 S1 and N-based serological assays reveal rapid seroconversion and induction of specific antibody response in COVID-19 patients. *Sci. Rep.* 10:16561. doi: 10.1038/s41598-020-73491-5

Burnett, M. J. B., and Burnett, A. C. (2019). Therapeutic recombinant protein production in plants: challenges and opportunities. *Plants People Planet* 2, 121–132. doi: 10.1002/ppp3.10073

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K. C., and Schillberg, S. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25, 635–643. doi: 10.1016/j.tplants.2020.04.009

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., et al. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro. Surveill.* 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045

Demurtas, O. C., Massa, S., Illiano, E., De Martinis, D. D., Chan, P. K. S., Di Bonito, P. D., et al. (2016). Antigen production in plant to tackle infectious diseases flare up: the case of SARS. *Front. Plant Sci.* 7:54. doi: 10.3389/fpls.2016.00054

Diego-Martin, B., González, B., Vazquez-Vilar, M., Selma, S., Mateos-Fernández, R., Gianoglio, S., et al. (2020). Pilot production of SARS-CoV-2 related proteins in plants: a proof of concept for rapid repurposing of indoor farms into biomanufacturing facilities. *Front. Plant Sci.* 11:612781. doi: 10.3389/fpls.2020.612781

Gleba, Y., Klimyuk, V., and Marillonnet, S. (2007). Viral vectors for the expression of proteins in plants. *Curr. Opin. Biotechnol.* 18, 134–141.

Hoste, A. C. R., Venteo, A., Fresco-Taboada, A., Tapia, I., Monedero, A., López, L., et al. (2020). Two serological approaches for detection of antibodies to SARS-CoV-2 in different scenarios: a screening tool and a point-of-care test. *Diagnostic Microbiol. Infect. Dis.* 98:115167. doi: 10.1016/j.diagmicrobio

Kaushal, N., Gupta, Y., Goyal, M., Khaiboullina, S. F., Baranwal, M., and Verma, S. C. (2020). Mutational frequencies of SARS-CoV-2 genome during the beginning months of the outbreak in USA. *Pathogens* 9:565. doi: 10.3390/pathogens9070565

Landis, J. R., and Koch, G. G. (1977). The Measurement of observer agreement for categorical data. *Biometrics* 33, 159–174. doi: 10.2307/2529310

Li, D., and Li, J. (2021). Immunologic testing for SARS-CoV-2 infection from the antigen perspective. *J. Clin. Microbiol.* 59, e2160–e2120. doi: 10.1128/JCM.02160-20

Liu, W., Liu, L., Kou, G., Zheng, Y., Ding, Y., Ni, W., et al. (2020). Evaluation of nucleocapsid and spike protein-based enzyme-linked immunosorbent assays for detecting antibodies against SARS-CoV-2. *J. Clin. Microbiol.* 58, e461–e420. doi: 10.1128/jcm.00461-20

Ma, J. K. C., Drake, P. M. W., and Christou, P. (2003). The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* 4, 794–805.

Makatsa, M. S., Tincho, M. B., Wendoh, J. M., Ismail, S. D., Nesamari, R., Pera, F., et al. (2021). SARS-CoV-2 antigens expressed in plants detect antibody responses in COVID-19 patients. *Front. Plant Sci.* 12:589940. doi: 10.3389/fpls.2021.589940

McBride, R., van Zyl, M., and Fielding, B. C. (2014). The coronavirus nucleocapsid is a multifunctional protein. *Viruses* 6, 2991–3018. doi: 10.3390/v6082991

McDonald, K. A., and Holtz, R. B. (2020). From farm to finger prick-a perspective on how plants can help in the fight against COVID-19. *Front. Bioeng. Biotechnol.* 8:782. doi: 10.3389/fbioe.2020.00782

Moon, K. B., Park, J. S., Park, Y. I., Song, I. J., Lee, H. J., Cho, H. S., et al. (2019). Development of systems for the production of plant-derived biopharmaceuticals. *Plants* 9:30. doi: 10.3390/plants9010030

Nolasco, G., De Blas, C., Torres, V., and Ponz, F. (1993). A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J. Virol. Methods* 45, 201–218.

Pogue, G. P., Vojdani, F., Palmer, K. E., Hiatt, E., Hume, S., Phelps, J., et al. (2010). Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnol. J.* 8, 638–654. doi: 10.1111/j.1467-7652.2009.00495.x

Pollán, M., Pérez-Gómez, B., Pastor-Barriuso, R., Oteo, J., Hernán, M. A., Pérez-Olmeda, M., et al. (2020). Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. *Lancet* 396, 535–544. doi: 10.1016/S0140-6736(20)31483-5

Rikhtegaran Tehrani, Z., Saadat, S., Saleh, E., Ouyang, X., Constantine, N., DeVico, A. L., et al. (2020). Performance of nucleocapsid and spike-based SARS-CoV-2 serologic assays. *PLoS One.* 15:e0237828. doi: 10.1371/journal.pone.0237828

Sainsbury, F. (2020). Innovation in plant-based transient protein expression for infectious disease prevention and preparedness. *Curr. Opin. Biotechnol.* 61, 110–115. doi: 10.1016/j.copbio.2019.11.002

Siriwattananon, K., Manopwisedjaroen, S., Kanjanasirirat, P., Budi, P. P., Rattanapisit, K., Shanmugaraj, B., et al. (2021). Development of plant-produced recombinant ACE2-Fc fusion protein as a potential therapeutic agent against SARS-CoV-2. *Front. Plant Sci.* 11:604663. doi: 10.3389/fpls.2020.604663

Tré-Hardy, M., Wilmet, A., Beukinga, I., Favresse, J., Dogné, J. M., Douxfils, J., et al. (2021). Analytical and clinical validation of an ELISA for specific SARS-CoV-2 IgG, IgA, and IgM antibodies. *J. Med. Virol.* 93, 803–811. doi: 10.1002/jmv.26303

Whitman, J. D., Hiatt, J., Mowery, C. T., Shy, B. R., Yu, R., Yamamoto, T. N., et al. (2020). Evaluation of SARS-CoV-2 serology assays reveals a range of test performance. *Nat. Biotechnol.* 38, 1174–1183. doi: 10.1038/s41587-020-0659-0

Williams, L., Deana, A., Romero, A., Molina, A., and Lunello, P. (2014). High-level production of active human TFPI-2 Kunitz domain in plant. *Protein Expr. Purif.* 96, 14–19. doi: 10.1016/j.pep.2014.01.011

Williams, L., Lunello, P., Arantxa, A., Bronchalo, I., Romualdo, A., Jurado, S., et al. (2016). Potential application of plant-derived bioengineered human VEGF for tissue regeneration. *Curr. Pharm. Biotechnol.* 17, 458–464.

Yao, J., Weng, Y., Dickey, A., and Wang, K. Y. (2015). Plants as factories for human pharmaceuticals: applications and challenges. *Int. J. Mol. Sci.* 16, 28549–28565. doi: 10.3390/ijms161226122

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