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Natural products from plants and applications for pest management

Edited by
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Jesus Simal-Gandara

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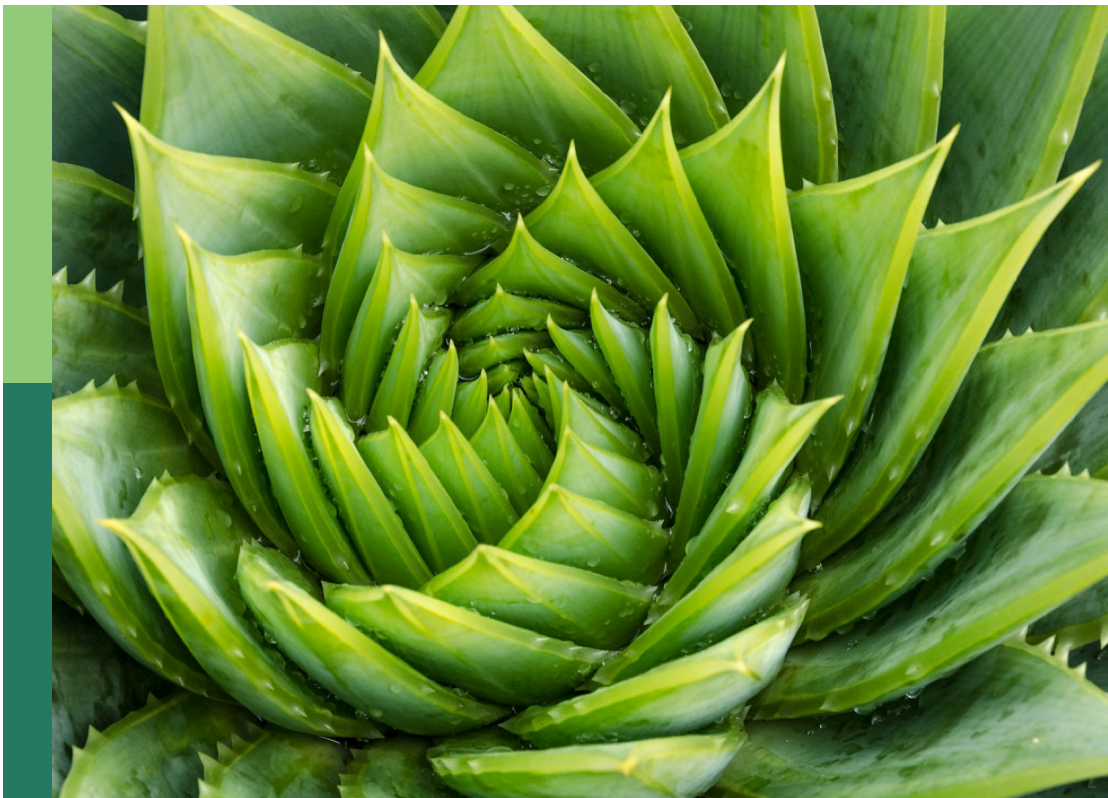

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Natural products from plants and applications for pest management

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Table of contents

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REVIEW
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Prevalent Pest Management Strategies for Grain Aphids: Opportunities and Challenges

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Cereal plants in natural ecological systems are often either sequentially or simultaneously attacked by different species of aphids, which significantly decreases the quality and quantity of harvested grain. The severity of the damage is potentially aggravated by microbes associated with the aphids or the coexistence of other fungal pathogens. Although chemical control and the use of cultivars with single-gene-based antibiosis resistance could effectively suppress grain aphid populations, this method has accelerated the development of insecticide resistance and resulted in pest resurgence. Therefore, it is important that effective and environmentally friendly pest management measures to control the damage done by grain aphids to cereals in agricultural ecosystems be developed and promoted. In recent decades, extensive studies have typically focused on further understanding the relationship between crops and aphids, which has greatly contributed to the establishment of sustainable pest management approaches. This review discusses recent advances and challenges related to the control of grain aphids in agricultural production. Current knowledge and ongoing research show that the integration of the large-scale cultivation of aphid-resistant wheat cultivars with agricultural and/or other management practices will be the most prevalent and economically important management strategy for wheat aphid control.

Keywords: wheat, ecological regulation, resistant cultivar, induced defenses, RNA interference

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INTRODUCTION

Common wheat (*Triticum aestivum* L.) is the third most important staple food crop worldwide, and it is widely cultivated in more than 150 countries throughout the world, occupying approximately 220 million hectares worldwide and feeding approximately 4.5 billion of the world population (FAOSTAT: Food and Agriculture Organization of the United Nations, 2019). Under the scenario of a rapid increase in the human population and a decrease in the area of cropland worldwide, the major challenge for current wheat grain production is reaching a steady annual increase of 2% (Crespo-Herrera et al., 2015). Moreover, wheat plants in agroecosystems are exposed to different pests that cause substantial damage to wheat and severely threaten global food safety. Among them, wheat aphids severely threaten wheat production worldwide; the English grain aphid *Sitobion avenae* Fabricius, the bird cherry-oat aphid *Rhopalosiphum padi* L., the greenbug *Schizaphis graminum* Rondani, and the Russian wheat aphid *Diuraphis noxia* Kurdjumov (Hemiptera: Aphididae), are the most

destructive and most commonly occurring grain aphid species (Elbert et al., 2008; Liu et al., 2012; Crespo-Herrera et al., 2015). These aphids exhibit parthenogenesis and the typical features of R-strategists, which could significantly increase their populations in a short time. Their feeding behaviors involve ingestion of wheat phloem sugar at a high rate and transfer of most phloem sap from their bodily fluids into honeydew (Douglas, 2006), resulting in significant wheat grain yield and quality losses in many wheat production areas around the world (Rabbinge et al., 1981; Liu et al., 2012). In addition, grain aphids are a common vector of barley yellow dwarf virus (BYDV), which causes wheat yellow dwarf disease, one of the most destructive cereal diseases in Europe, Asia and Africa (Fiebig et al., 2004; Tanguy and Dedryver, 2009; Sadeghi et al., 2010). This viral disease further aggravates the problem in cereal crops by increasing the fecundity of grain aphids feeding on virus-infected plants (Fereres et al., 1989; Hu et al., 2013).

Currently, chemical control is still the most important measure to combat grain aphids in agricultural production as it can effectively suppress wheat aphid populations in a short time. Among these chemical insecticides, the neonicotinoid and pyrethroid insecticides are the main option for controlling grain aphids on the global market (Foster et al., 2014; Miao et al., 2014). The widespread and frequent use of neonicotinoid and pyrethroid insecticides in farming significantly stimulates grain aphids to develop insecticide resistance (Foster et al., 2014). The resistance of grain aphids to pesticides has caused a gradual resurgence of these pests. Thus, the damage caused by grain aphids has become a continuous problem in most wheat-producing regions of the world.

To guarantee food safety worldwide, it is imperative to find efficient pest management measures to control the damage from grain aphids. Moreover, over recent decades, genetic and biochemical information used for developing resistance to grain aphids has greatly contributed to a comprehensive way of developing more practical and environmentally friendly control of grain aphids. Therefore, current knowledge and ongoing research about strategies and approaches for sustainable grain aphid management will be synthesized and discussed in this review.

ECOLOGICAL REGULATION OF GRAIN APHIDS

The growing desire for sustainable agriculture has prompted the need to develop more sustainable pest management approaches, such as ecological regulation. Ecological regulation generally refers to the use of agronomic-based management for mediating tripartite plant-pest-biological control agent interactions in agricultural ecosystems, which provides the most economic and environmentally friendly pest management measure (Zhou et al., 2012). Predators or parasitoids therefore play a dominant role in the ecological regulation of pest population growth. There are several biological control agents of grain aphids, including lady beetles (*Adalia bipunctata* L. and *Coccinella septempunctata* L.), green lacewings (*Chrysoperla carnea* Stephens), parasitic wasps (*Aphelinus abdominalis* Dalman and *Aphidius avenae* Haliday), marmalade hoverflies (*Episyrphus balteatus* De Geer), and trombidiid mites [*Allothrombium ovatum* Zhang & Xin (Acari: Trombidiidae)] (Ma et al., 2007; Vandenborre et al., 2011). However, in many cases, the number of predators or parasitoids present in agricultural ecosystems may be insufficient to provide economic management of pests on crops (Ma et al., 2007).

It was demonstrated that intercropping could change the environmental conditions, in a way that increases natural enemy activity, regulates pest population dynamics and minimizes crop damage (Ma et al., 2007; Vandenborre et al., 2011). Intercropping is a traditional agricultural technique of cultivating two or more crop species within the same field. In comparison with monocropping, intercropping could greatly contribute to increased crop production by effectively using environmental resources and suppressing pest outbreaks (Ma et al., 2007). Intercropping of wheat and alfalfa (*Medicago sativa* L.) provides the most practical and economical approach for controlling wheat aphids. For instance, wheat–alfalfa strip cropping significantly increased both the abundance of *A. ovatum* larvae and the parasitization rate of *S. avenae* compared to wheat monoculture (Ma et al., 2007). This could be explained by the fact that strip cropping provided a wetter, shadier soil surface microclimate that caused adult female mites to lay more egg pods and that the non-furrowed areas of the intercropped fields provided a more suitable habitat for mite overwintering (Brust et al., 1986; Zhang and Li, 1996; Ma et al., 2007). The intercropping of wheat and oilseed rape (*Brassica napus* L.) could improve the effective biological control of wheat aphids by increasing the species richness of natural enemies of *S. avenae*, including *E. balteatus* and *A. avenae*, which may control wheat aphid infestation during the early wheat filling stage (Wang et al., 2010). Wheat intercropping with pea (*Pisum sativum* L.) or mung bean (*Vigna radiata* L.) could also support these findings (Xie et al., 2012). This control is likely because the odor of non-hosts could attract a greater number of lady beetles and parasitic wasps to regulate the population dynamics of *S. avenae* colonization on wheat plants than could wheat monoculture. Moreover, intercropping could interfere with the host preference and locating abilities of aphids because the odor released from the non-host overlaps with the odor of the host. Experimental evidence in wheat intercropping with resistant wheat cultivars confirmed that intercropping could be an economic agricultural practice to reduce aphid populations (Zhou et al., 2009). Therefore, intercropping of wheat and other crops or vegetables could be an alternative measure to increase the populations of predators or parasitoids to control the population growth of grain aphids; however, it is challenged by the rapid increase in aphid populations, especially during the filling stage of wheat plants.

In addition, the practice on management of the aphids species of vegetable and crops relied heavily on entomopathogenic fungi, including *Beauveria bassiana*, *Metarhizium anisopliae*, and so on (Kim and Kim, 2008; Bayissa et al., 2017). This could be one of the cost-effective aphid management measures when aphid populations are low, similarly, it is challenged by the rapid increase in aphid populations as well. Moreover, increasing concern regarding the beneficial effects of soil microorganisms

on plant growth and resistance to biotic stresses has led to the widespread use of beneficial microorganisms as biocontrol agents in agricultural practice. The genus *Trichoderma*, such as *Trichoderma harzianum* or *T. atroviride* strain P1, are biocontrol agents for the potato aphid (*Macrosiphum euphorbiae* Thomas). Tomato seeds soaked in a fresh spore suspension of either *T. harzianum* or *T. atroviride* strain P1 resulted in adverse effects on the development period and longevity of aphids by triggering plant resistance responses and/or the release of volatile organic compounds to attract the aphid parasitoid braconid *Aphidius ervi* Haliday (Coppola et al., 2017, 2019). Although few studies have reported that the genus *Trichoderma* of soil microorganisms could mediate the population of grain aphids, the above evidence provides important clues that the soaking cereal seeds in a spore suspension of *Trichoderma* could enhance the resistance of cereal seedlings to grain aphids.

HIGH-VOLTAGE ELECTROSTATIC FIELD (HVEF)-MEDIATED CONTROL MEASURES OF GRAIN APHIDS

Attempts to utilize artificial HVEFs for economical pest control have attracted increasing attention. Initially, direct exposure of seeds to HVEFs was utilized to improve the germination rate, and this practice continues to be used today. In general, crop seeds lose viability during storage, and the longer the storage period prior to cultivation, the greater the amount of reactive oxygen species (ROS) that accumulate (Wang et al., 2009). Directly exposing seeds to an HVEF could activate the antioxidative defense system by increasing antioxidant enzyme activities to increase the viability of seeds (Wang et al., 2009). Moreover, in a recent study, Luo K. et al. (2016) reported that the use of wheat seeds directly exposed to an HVEF could induce biological and physiological changes in the plants, which adversely affected the population growth of the grain aphid *S. avenae*.

In recent decades, large-scale electrical utilization, including long-distance electric power transmission, medical equipment, communication appliances, and so on, has seriously increased the intensity of electrostatic fields that are pervasively present in the environment. Herbivore insects are not only particularly sensitive to environmental alterations but also typically exhibit strong adaptation traits, such as short generation times, high reproductive rates, genetic plasticity, and small body sizes. Therefore, extensive studies have focused on characterizing the adverse effects and adaptive strategies of herbivorous insects to novel electric environments. With direct exposure of herbivorous insects to extreme static electric fields, multitudinous adverse effects can be induced, including chromosome aberrations, paralysis, increased mortality, abnormal propolization, reduced longevity, and possible impairment of colony growth (He et al., 2016). For instance, in our previous studies, when *S. avenae* was directly exposed to an HVEF with an intensity of 4 kV/cm for 20 min, the aphids experienced a significant increase in development time and a reduction in total longevity (He et al., 2016). Those studies have suggested that direct exposure of herbivorous insects to an HVEF is a possible alternative measure to prevent damage caused by these insects. However, the intensity of the current electrostatic environment could not pose serious adverse effects on insects, and establishing extreme static electric fields in agroecosystems would greatly increase production costs. In comparison, direct exposure of seeds to HVEFs is a more reasonable method in agricultural production. To better evaluate the possibility of HVEF exposure as a pest control measure, the direct exposure of seeds and newborn nymphs of *S. avenae* to a 4 kV/cm HVEF for 20 min significantly increased the superoxide dismutase activity but reduced the peroxidase and catalase activities, which indicates that the production of H_2O_2 exceeds the amount that antioxidant enzymes can gradually digest (Luo et al., 2019a). The extensive accumulation of H_2O_2 increases the oxidative stress and even cellular cytotoxicity and reduces the performance of the aphids. Therefore, direct exposure of seeds to HVEFs has the potential to play an important role in the development of alternative economic and environmentally friendly integrated pest management strategies for grain aphids.

PLANT LECTINS AS DEFENSE PROTEINS AGAINST GRAIN APHIDS

Building aphid resistance into wheat plants is considered to be an ideal measure for combating aphids in agricultural production because it is less detrimental to the environment. Compared with cumbersome and time-consuming traditional breeding, adopting recombinant DNA technology to insert resistance into crops is a reliable and effective method to accelerate the breeding of cultivars with substantial insect resistance (Smith and Chuang, 2014). It was demonstrated that plant lectins have the potential to play an important role in the development of integrated pest management strategies (Michiels et al., 2010). Plant lectins are a specific group of proteins with at least one non-catalytic domain that can competitively bind specific carbohydrates, either simple monosaccharides or more complex glycans, resulting in inhibition of the assimilation of sugars in the gut of herbivores (Peumans and Damme, 1995). In addition, plant lectins are highly resistant to proteolysis and can bind to insect proteins, mainly in the gut, and as a consequence, they can be retained within the insect body (Michiels et al., 2010). The above findings suggest that plant lectins can cause adverse effects on the development or fecundity of insects. Thus, genetically modified wheat plants expressing plant lectins have become an important focus in wheat molecular breeding programs.

Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) is the first plant lectin gene successfully engineered into elite wheat cultivars to combat grain aphids in agricultural production (reviews by Michiels et al., 2010). Over the past few decades, considerable progress has been achieved in the genetic expression of GNA in different wheat cultivars through callus bombardment, which gives the plants a higher level of resistance against cereal aphids (Stoger et al., 1999; Hogervorst et al., 2009; reviews by Michiels et al., 2010; Vandenborre et al., 2011). For instance, introducing the GNA gene into the wheat cultivar Bobwhite was shown to exert severe entomotoxic effects on the development and survival of the grain aphid *S. avenae* (Stoger et al., 1999).

In addition, the genetic modification of rice, maize, sugarcane, potato or tobacco plants to express GNA has successfully conferred resistance against different species of aphids (Wang et al., 2005; reviews by Vandenborre et al., 2011).

Although feeding lectins to insects via transgenic plants seems to be a relatively natural system, the potential risk of exposing larvae of different aphid predators, such as lady beetles (*A. bipunctata* and *C. septempunctata*) and green lacewings (*C. carnea*), to GNA has been explored for a long time (Hogervorst et al., 2006). The transfer of entomotoxic effects of GNA along the food chain has potentially increased the intensity of exposure of predators or parasitoids to GNA. The novel environment induced by GNA exposure significantly reduced the fecundity, egg viability and longevity of those aphid predators/herbivores when either feeding on an artificial diet containing GNA or preying on aphids reared on GNA-producing transgenic plants (reviews by Vandenborre et al., 2011). Moreover, feeding on grains or vegetables carrying entomotoxic lectins could trigger local and systemic allergic reactions in many species of mammals (reviews by Vandenborre et al., 2011). Taken together, these results suggest that prior to the development of genetically modified crop varieties expressing plant lectins, it will be necessary to fully understand the mechanism of toxicity of GNA and assess the potential risks of adverse GNA effects on predators and dietary uptake by animals or humans. Unfortunately, relatively few studies have investigated this issue, and the agricultural use of wheat germplasms genetically modified with plant lectins remains relatively rare.

BREEDING PEST-RESISTANT WHEAT CULTIVARS FOR GRAIN APHID CONTROL

In natural agroecosystems, some wheat germplasms have coevolved a range of constitutive defenses to control the damage caused by aphid attackers. The identification of suitable genotypes with constitutive resistance to pests and the introduction of these genotypes into cultivars has resulted in reduced pesticide usage and lower production costs worldwide by controlling damage from pests (Christou et al., 2006). In the last few decades, vast efforts have focused on identifying aphid-resistant genotypes by adopting the terminology of Painter as well as subsequent revisions, and many accessions of common wheat and wheat relatives have been identified as resistant to grain aphids, providing abundant germplasm resources with durable and active resistance to breed wheat cultivars with substantial aphid resistance (Wang et al., 2015). Prior to developing new cultivars, screening suitable aphid-resistant traits from aphid-resistant germplasms would facilitate plant breeders in selecting cultivars with qualified aphid-resistant traits or preferred categories of aphid resistance. These resistance traits include morphological and structural features as well as the synthesis of chemical compounds.

Three major commonly accepted categories exist for the insect resistance traits of plants: tolerance, antibiosis, and antixenosis (War et al., 2012). Among the types of resistance, tolerance is often a complex and polygenic trait that enables plants to compensate or withstand infestation from aphid damage and yield significantly more biomass than a susceptible plant under similar conditions (**Figure 1**). The evaluation of aphid tolerance always adopts the artificial aphid infestation method under field conditions (Hu et al., 2016; Luo et al., 2019b). During the pregenomics era, tolerance has been the preferred type of trait for conventional wheat breeding to obtain high-quality, high-yield, highly resistant cultivars without detrimental effects on human health (Inayatullah et al., 1990). In past decades, the molecular mechanisms of tolerance to many aphid species have been exploited in cultivars of alfalfa, barley, maize, rice, rye, sorghum and wheat (Smith and Chuang, 2014). For instance, wheat plants tolerant to the Russian wheat aphid, *D. noxia*, often exhibit increased photosynthetic rates, growth rates, stored root carbon and/or abilities to shunt stored carbon from roots to shoots (Kerchev et al., 2012; Smith and Chuang, 2014). The gene expression data in *D. noxia*-tolerant plants suggest that photosystem and chlorophyll genes involved in photosynthesis are highly expressed in the foliage of these plants. In a recent study, the results showed that winter wheat plants with higher tolerance to grain aphid infestation upregulated the relative expression of genes associated with photosystem I assembly protein and carbohydrate transfer and conversion several-fold (Luo et al., 2014, 2019b). During the grain-filling stage, large amounts of photoassimilates are transported into the endosperm, contributing to the grain yield, which compensates for the yield loss from the infestation of grain aphids.

Antibiosis is a type of resistance in which the plant produces allelochemicals or toxins, including plant phenolics, flavonoids, tannins, DIMBOA, and proteinase inhibitors, which significantly reduce herbivore growth and development (**Figure 1**). Antixenosis is a type of resistance in which certain characteristics of a plant, such as leaf surface wax, trichomes and cell walls, make it less attractive to herbivores (**Figure 1**; War et al., 2012). In many cases, the resistance of wheat germplasm to aphid feeding is classified into antibiosis resistance and/or antixenosis resistance; however, these effects are always difficult to separate in a single wheat germplasm because the traits associated with antibiosis and antixenosis resistance exhibit cooccurrence or coinheritance in the germplasm (Smith and Chuang, 2014).

To accelerate the process of breeding wheat cultivars with antibiosis resistance and/or antixenosis resistance to grain aphids, molecular marker technologies, such as simple sequence repeats (SSR), have been used in marker-assisted selection (MAS) to screen for aphid-resistant genes in wheat aphid-resistant lines (Bertin et al., 2004; Liu et al., 2012). When considering the closely linked loci of resistance genes, near-isogenic populations developed from crosses between aphid-resistant and aphid-susceptible parents have been successfully used to map and link the loci of aphid resistance genes to various types of molecular markers and develop chromosome maps of resistance genes (Smith and Chuang, 2014). In recent decades, over 10 *D. noxia*-resistant genes and 17 *S. graminum*-resistant genes were identified on wheat chromosomes by different molecular markers (Liu et al., 2001, 2005; Smith et al., 2004; Ricciardi et al., 2010).

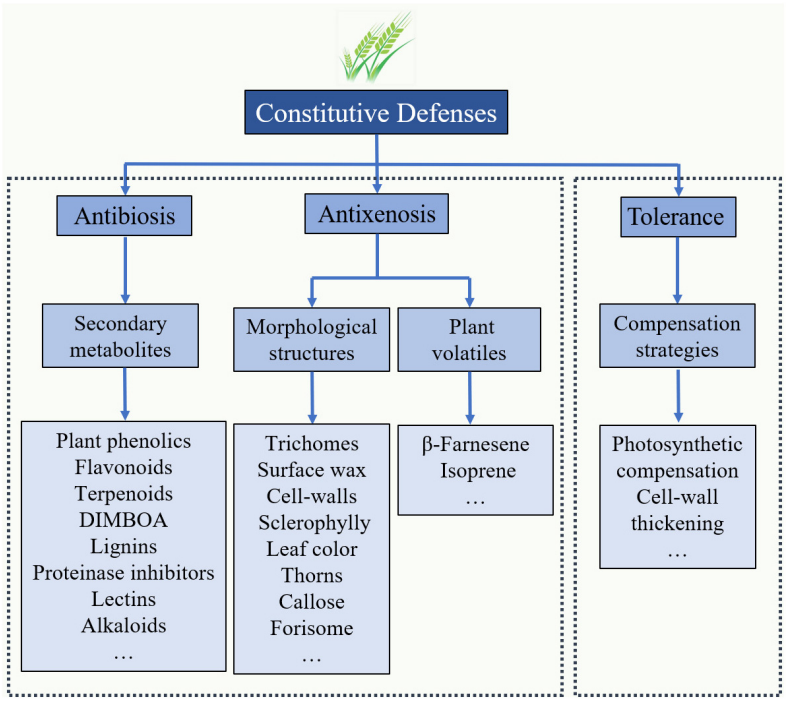

FIGURE 1 | Proposed features and compounds associated with constitutive defense in response to grain aphids in resistant wheat lines. Most of the antibiosis and antixenosis traits exhibited in resistant lines are classified as qualitative traits (controlled by one or a few genes), while the tolerance traits are considered quantitative traits (controlled by numerous genes).

Most aphid resistance characterized in wheat is monogenic and inherited as a dominant trait. For example, the single dominant genes *Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn6*, *Dn7*, *Dn8*, *Dn9*, *Dn2412,* and *Dnx* were reported to confer resistance to Russian wheat aphids (Liu et al., 2001, 2005; Smith et al., 2004; Ricciardi et al., 2010); the candidate genes *Gb2, Gb3, Gb4, Gb5, Gb6, Gb7/Gbx1, Gb8, Gba, Gbb, Gbc, Gbd, Gbx, Gbx1, Gby, Gbz*, and *GbSkl* confer resistance to *S. graminum* (Boyko et al., 2004; Zhu et al., 2005; Aradottir and Crespo-Herrera, 2021). The recessive gene *dn3* from *Aegilops tauschii* (Coss.) has been linked to resistance to *D. noxia*, and the recessive gene *gb1* was the first identified resistance gene to greenbug and originated from *T. durum* (Miller et al., 2001; Dogimont et al., 2010). Recently, one of the *S. avenae* resistance genes *RA-1* was closely linked to the SSR molecular markers *Xwmc179*, *Xwmc553* and *Xwmc201* in the *T. durum* wheat line C273 (Liu et al., 2012). Our recent study revealed that the SSR molecular markers *Xgwm350* and *Xbarc70* are closely linked to an *S. avenae* resistance gene (*Sa2*) in the winter wheat genotype XN98-10-35 (Wang et al., 2015). Both SSR markers are monogenic and inherited as a dominant trait. Previous studies revealed that most of the characterized *D. noxia*, *S. graminum* or *S. avenae* resistance genes present in resistant cultivars have been located on wheat chromosome 7D based on evidence from molecular markers (Wang et al., 2015; Aradottir and Crespo-Herrera, 2021). It was reported that *Ae. tauschii* is the diploid progenitor of the D genome of common wheat and has carried a multitude of resistance genes, including those against wheat stripe rust, powdery mildew, wheat aphids, and so on (Zhu et al., 2005). In addition, these SSR markers will be valuable in MAS for accelerating the process of breeding wheat cultivars with resistance to grain aphids. Moreover, the candidate genes *Rdy2*, *Rdy3*, *Rdy4*, *Bdv1*, *Bdv2*, *Bdv3*, and *Bdv4* for resistance to BYDV have been identified by different molecular markers in barley and wheat cultivars or genotypes (Jarošová et al., 2016; Aradottir and Crespo-Herrera, 2021). However, few studies reported the identification or cloning of the dominant genes associated with *R. padi* resistance in wheat by adopting molecular markers, probably because of the polyphagy and wide host adaptation of *R. padi* (Crespo-Herrera et al., 2014). In addition, most of characterized *R. padi* resistance genes are controlled by quantitative trait loci (QTLs). For instance, Crespo-Herrera et al. (2014) reported three QTLs in the first report on the genetic mapping of *R. padi* resistance in wheat; *QRp.slu.4BL* exhibited antibiosis resistance to *R. padi*, while *QRp.slu.5AL* and *QRp.slu.5BL* exhibited tolerance to *R. padi*. In the same study, QTL *QGb.slu-2DL* located on chromosome 2DL was shown to be associated with *S. graminum* resistance (Crespo-Herrera et al., 2014). More recently, continuing advances in genome-wide association (GWAS) studies have accelerated the pace of the identification of significant markers or QTLs in aphid resistance genes (Joukhadar et al., 2013).

Taken together, the above findings suggest that true resistance genes to grain aphids were naturally found in wheat gene pools, either by introduction, closely related species or coevolution. Notable examples of aphid resistance genes bred into wheat cultivars resistant to *D. noxia*, for instance *Dn4* derived from

wheat line PI 372129, was transferred into several cultivars by adopting cross and backcross techniques, resulting in the release of new wheat cultivars, including "Halt," "Prowers 99," "Prairie Red," and "Yumar" (Smith and Chuang, 2014). Unexpectedly, the transfer of other candidate genes associated with resistance to grain aphids into elite bread wheat lines to construct high-quality wheat germplasm has been relatively unsuccessful.

Similar to chemical control, the practice of breeding for high levels of antibiosis resistance often promotes the development of aphid virulence (reviews by Dogimont et al., 2010; Smith and Chuang, 2014). Additionally, many of the characterized aphid-resistant cultivars are resistant to one species of wheat aphid but are susceptible to other species of aphids (Zhu et al., 2005; reviews by Aradottir and Crespo-Herrera, 2021). For instance, the *T. monococcum* line REB81044 (TM44) is highly resistant to *S. avenae* but susceptible to *R. padi* and *Metopolophium dirhodum* Walker (Tanguy and Dedryver, 2009). These results strongly suggest the need to identify new and diverse aphid resistance genes and genes that confer tolerance or more moderate levels of antibiosis resistance in aphid management, which could be an important hallmark of building plant resistance to aphids, especially in combination with ecological control.

HERBIVORE-MEDIATED INDUCED DEFENSES IN PLANTS IN RESPONSE TO APHID FEEDING

Cereal plants in agroecosystems are often either sequentially or simultaneously attacked by different species of grain aphids (Ni and Quisenberry, 2006). During feeding and probing, their digestive saliva and honeydew always present a multitude of unknown functions of elicitors derived from the aphid itself or their primary endosymbionts, including EF-Tu, chaperone proteins GroEL, and flagellin, which trigger chemical and morphological responses in attacked plants (War et al., 2012; Sabri et al., 2013; Chaudhary et al., 2014; Jaouannet et al., 2014). Among those plant defense responses, the signaling molecules jasmonic acid (JA) and salicylic acid (SA) play a critical role in mediating the signaling networks involved in the induced defense responses to grain aphids and subsequent conspecific or heterospecific colonizers (Smith and Boyko, 2007). Based on most of the present literature available, JA and its derivatives MeJA are the primary phytohormones in plant defense against chewing insects, while the SA signaling pathway is always involved in defense against piercing-sucking insects (Smith and Boyko, 2007; War et al., 2012). Experimental evidence in sorghum and wheat has suggested that aphid infestation induces rapid and transient emission of SA in host plants (Smith and Boyko, 2007). In seedlings, SA can be perceived and bound by a multitude of SA-binding proteins, including catalase (CAT) and ascorbate peroxidase (APX), resulting in the accumulation of H_2O_2 in the apoplastic and symplastic regions of the host (Durner and Klessig, 1995; Tian et al., 2012; Kumar, 2014). H_2O_2 could trigger systemic acquired resistance, which often coincides with a programmed cell death (PCD)-type response and a hypersensitive response (HR) that isolates subsequent aphid colonizers and deprives them of nutrients required for subsequent infestation (Johnson et al., 2003; Mou et al., 2003; Tian et al., 2012; Wu et al., 2012). For instance, our latest study suggested that infestation with *R. padi* significantly increased the expression level of the PR-1 gene associated with SA-dependent responses in the resistant winter wheat line 35-E4 (Luo et al., 2020). Meanwhile, increasing experimental evidence has revealed that aphid infestation triggers the expression of genes related to JA and SA synthesis (**Figure 2**; Zhao et al., 2009; Cao et al., 2014; Luo et al., 2020). For instance, the relative expression of JA synthesis genes, including the *LOX* and *AOS* genes, significantly increased after *R. padi* preinfestation in wheat seedlings of lines 35-E4 and susceptible lines 35-A20 (Luo et al., 2020). The accumulation of JA in wheat seedlings may then be conjugated with the amino acid isoleucine (Ile) to form JA-Ile conjugation with jasmonate-resistant1 (JAR1) (Staswick and Tiryaki, 2004). JA-Ile can be bound by coronatine insensitive 1 (COI1), which promotes the degradation of jasmonate-ZIM domain (JAZ) repressors through the 26S proteasome-mediated pathway (Luo J. et al., 2016). After that, the transcription factor MYC2 in JA signaling was released and positively regulated the transcription of its downstream MYC2-targeted transcription factors to activate JA-induced defense responses, including the expression of the *PDF1.2* (plant defensin 1.2) or *VSP2* (vegetative storage protein 2) genes (Luo J. et al., 2016; Du et al., 2017). However, that study did not determine the expression profiles of marker genes associated with JA-induced defense responses.

Additionally, in many herbivore-plant systems, the interactions between the signaling pathways for SA and JA have been shown to be antagonistic (Shigenaga and Argueso, 2016; Xu et al., 2019; Tan et al., 2021). Over the past decades, a multitude of regulators associated with the antagonistic interaction between SA and JA signaling pathways in plant immune responses have been identified (Pandey et al., 2016; Shigenaga and Argueso, 2016). For instance, MPK4 (mitogen-activated protein kinase 4) positively regulates JA-induced genes such as *PDF1.2* and promotes JA responses while simultaneously suppressing SA biosynthesis and the SA signaling pathway (Petersen et al., 2000; Gao et al., 2008). The central positive regulator of SA signaling, NPR1, can suppress the expression of the genes *PDF1.2* and *VSP2*, markers of the JA signaling pathway (Spoel et al., 2003; Pandey et al., 2016). Additionally, the transcription factor TGA2 acts as an activator of the SA-signaling pathway and as a repressor of JA-responsive genes, probably because TGA2 can bind to the promoter region of ORA59 (octadecanoid-responsive *Arabidopsis* apetala 2/ethylene response factor domain protein 59), which is the master regulator of the JA/ET-induced defense response (Ndamukong et al., 2010; Zander et al., 2014; Pandey et al., 2016). Moreover, herbivore-induced responses in host plants can potentially have a species-specific effect because cultivars generally confer constitutive defense to different species of herbivores at varying levels. For instance, *R. padi* and/or *S. avenae* induced different expression profiles of host JA- and SA-dependent responses in resistant and susceptible winter wheat lines (Luo et al., 2020). Therefore, advances in our understanding of hormone-mediated signaling cascades have

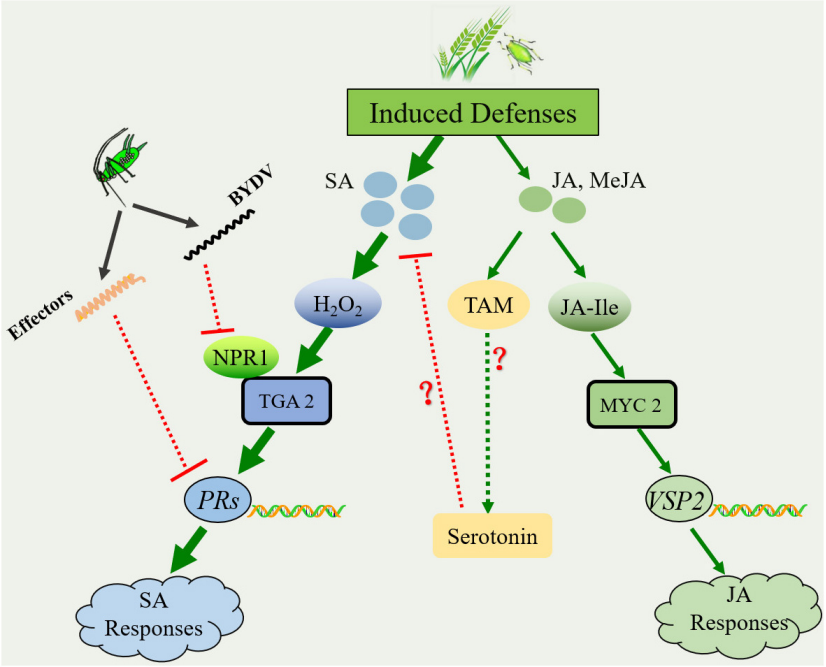

FIGURE 2 | Schematic of the *Sitobion avenae*-wheat interaction during infestation. The colonization of *S. avenae* induces the accumulation of phytohormone molecules, including salicylic acid (SA) and jasmonic acid (JA). SA-mediated defense signaling plays a dominant role in plant defense against subsequent attackers. To diminish SA-dependent responses, JA may promote the synthesis of serotonin. In addition, *S. avenae* could release effectors, and the plant virus carried by the aphids could diminish the host immune response as well. The solid arrow lines represent the pathways supported by experimental evidence from the literature, while the dotted arrow lines represent the pathways predicted from the literature. The red blunt-ends indicate a negative interaction (inhibition) on the SA-mediated plant defense. Red question marks represent the pathways predicted from the literature. H_2O_2, hydrogen peroxide; NPR1, non-expressor of pathogenicity-related genes 1; TGA2, transcription factor TGACG binding II; PRs, pathogenicity-related genes; TAM, tryptamine; and VSP2, vegetative storage protein 2.

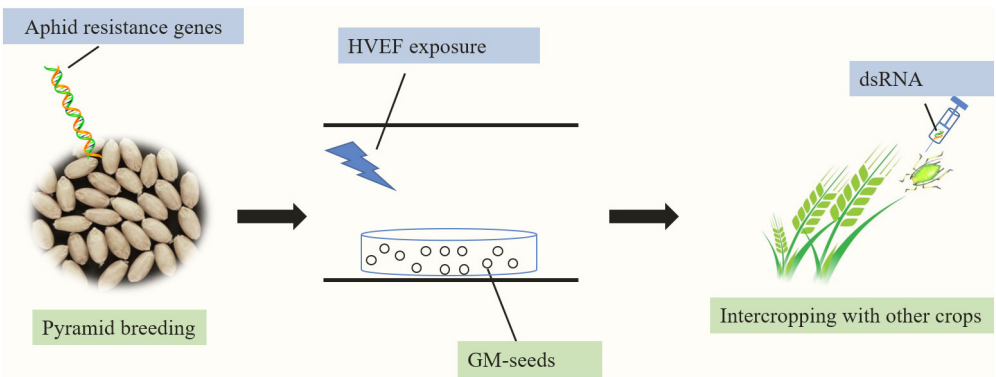

FIGURE 3 | Model summarizing sustainable pest management approaches for cereal aphids in agricultural production. Pyramiding different aphid resistance genes into elite wheat lines to develop aphid-resistant wheat plants and integrating breeding with HVEF exposure of the seeds and intercropping with other crops will be the most promising and effective management strategy for wheat aphid control. The direct transfer of the dsRNA of aphid genes into grain aphids could be a promising aphid control approach.

laid the foundation for understanding the role of these hormones in wheat resistance to aphids.

Moreover, herbivorous insects can elicit low-molecular-weight salivary proteins, known as effector proteins, and release them into the tissue of the attacked plants during feeding (**Figure 2**). Although dozens of salivary proteins have been identified in different species of grain aphids, only a small number of candidate effectors have been characterized (Elzinga and Jander, 2013; Jaouannet et al., 2014). The experimental evidence of the identified effectors in other piercing-sucking pests has shown their function in suppressing plant defenses (Elzinga et al., 2014; Xu et al., 2019). For instance, knocking down the salivary effector *Bt56* in *Bemisia tabaci* significantly reduced the transcript level of marker genes involved in SA signaling in *Nicotiana*

tabacum while upregulating the transcription of the JA response gene PDF1.2 (Xu et al., 2019). Moreover, the predicted functions of effectors including Mp55 and Mp10 in *Myzus persicae* (Sulzer) were found to suppress plant defenses (Elzinga and Jander, 2013; reviews by Jaouannet et al., 2014). Thus, additional effort is required to study the significance and molecular mechanism of salivary proteins in plant-wheat interactions.

In addition, advances in understanding the interactions between wheat and *Fusarium graminearum* Schwabe (anamorph, Hypocreales: Nectriaceae), an economically important cereal pathogen, provide important clues for understanding the role of JA in the suppression of SA-mediated plant defense during wheat-aphid interactions (Drakulic et al., 2015; De Zutter et al., 2017; reviews by Luo et al., 2021). For instance, *F. graminearum* inoculation leads to an upregulation of candidate genes associated with auxin and serotonin biosynthesis in wheat tissue (Qi et al., 2016; Brauer et al., 2019; Su et al., 2021). Based on the available literature, the accumulation of these two compounds probably occurs because of changes in the JA levels in the environment (Qi et al., 2016; Lu et al., 2018; Yang et al., 2018; Su et al., 2021). The potential role of auxin in wheat–*F. graminearum* interactions revealed that auxin and JA acted synergistically to attenuate the SA-dependent responses. Moreover, the experimental evidence attained from a rice-planthopper system revealed that serotonin could enhance the fitness of planthoppers by establishing a competition between the same precursor chorismite and SA (Lu et al., 2018). However, the underlying molecular mechanism of auxin and serotonin in the suppression of SA signaling remains unknown (Luo et al., 2021). Therefore, the significance of JA in the biosynthesis of serotonin and/or auxin after wheat aphid infestation and its role in enhancing the performance of wheat aphids remain to be investigated.

In response to plant immune cascades, aphids and their transmitted viruses attempt to suppress host plant defenses. For instance, wheat plants infected either by *S. graminum* or *S. avenae* carrying BYDV-GAV significantly reduced the expression level of genes associated with JA- and SA-dependent responses in their hosts, including *LOX*, *AOS*, *NPR1*, and *PAL* genes (Kang et al., 2021). In addition, the viral suppressor of RNAi (VSR) 2b protein of cucumber mosaic virus (CMV), carried by the green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), contributes to ROS production and directly interacts with the JAZ protein, thereby suppressing JA-responsive genes such as transcription factors MYC2, MYC3, and MYC4 in *Arabidopsis* (Wu et al., 2017; Guo et al., 2019). However, more experimental evidence will be required to confirm the possibility and mechanism by which wheat aphids and their transmitted viruses suppress SA-mediated defense responses in host plants.

Altogether, those regulators and growth-promoting phytohormones triggered by different attackers could fine-tune the plant immune responses, which further aggravates the problem caused by grain aphids in agroecosystems (**Figure 2**). Therefore, wheat cultivars that incorporate qualified constitutive and induced defenses are preferable for plant breeders to develop novel cultivars with more stable and durable resistance.

RNA INTERFERENCE-BASED APHID CONTROL

Since the discovery that double-stranded RNA (dsRNA) can suppress the transcript abundance of target genes, plant- and insect-mediated RNA interference (RNAi) has been developed as a novel potential approach for pest control (Pitino et al., 2011; Xu et al., 2014; Chung et al., 2018; Yang et al., 2019). Over the past decades, plant-mediated RNAi has knocked down the transcript abundance of critical pest genes in numerous herbivore-plant systems, including cotton bollworm-cotton, corn rootworm-maize, planthopper-rice, aphid-tobacco, and aphid-wheat systems, resulting in the disruption of herbivore performance on plants (Pitino et al., 2011; Xu et al., 2014; Yang et al., 2019). For instance, transgenic wheat plants expressing dsRNA of the *carboxylesterase E4* (*CbE E4*) gene fragment of *S. avenae* showed decreased transcript levels of the *CbE E4* gene and impaired herbivore tolerance to phoxim (O,O-diethyl-O-α-oximinophenyl cyanophosphorothioate) insecticides (Xu et al., 2014). Furthermore, rapid advances in wheat genome sequencing and analysis will facilitate the expression of the dsRNA of many target genes involved in the growth, survival or development of grain aphids in transgenic wheat plants.

In addition, dsRNA could be directly delivered via artificial diets or injected into the hemolymph of insects (Pitino et al., 2011). The preference of those two methods depends largely on the size of the herbivores and the skill of the operator. Previous work confirmed that injection is the most widely adopted method to deliver dsRNA molecules into herbivores such as mosquitoes, beetles, honeybees and grasshoppers, and this method achieves more efficient target gene suppression than the dietary method (Xu et al., 2014). In comparison, delivery via an artificial diet is a non-disruptive technique, preserving the integrity of the treated herbivores, but the precise amount of dsRNA taken up cannot be monitored, resulting in low-efficiency suppression (Sapountzis et al., 2014). Although experimental evidence demonstrated successful direct injection of dsRNA of *Ap-crt* and *Ap-cath-L* genes into the salivary glands of the pea aphid *A. pisum* for silencing the salivary gland proteins, in most cases, the delivery of dsRNA into aphids can be achieved following the oral delivery of RNAi in a filter-sterilized liquid diet, similar to plant phloem sap, supplemented with dsRNA. Attempts to transfer dsRNA into pea aphids have successfully knocked down the expression of aphid genes and suppressed their performance, probably because the pea aphid genome sequence is available. Although the sequences of most grain aphid genomes are not available, the accessibility of the wheat and pea aphid genome sequences would provide valuable evidence for constructing dsRNA of crucial genes of grain aphids.

Endosymbionts are harbored by almost all aphids. *Buchnera aphidicola* is the obligate species, that can synthesize missing essential amino acids and B vitamins and improve the nutritional composition of the restricted diet acquired from plant phloem sap (Douglas, 2014). When bacterial symbionts are eliminated from their insect host by antibiotic treatment, the insects grow poorly and produce few or no offspring (Douglas, 2014). It is,

therefore, very probable that targeting symbiosis-related insect genes by RNAi in the symbiotic aphid-*Buchnera* system may reduce aphid damage. The *amiD* and *ldcA1* genes present in *A. pisum*, associated with protecting *Buchnera* from host attack, were used as templates, and dsRNA fragments were synthesized for use in liquid artificial diets (Chung et al., 2018). The dsRNA fragments, once distributed within aphids, led to a reduction in the amount of the bacterial symbiont *Buchnera* in the pea aphid, with poor aphid performance (Chung et al., 2018). Taken together, feeding of dsRNA molecules targeting critical aphid genes, either by artificial spraying or specifically expressing them in transgenic plants, may be a promising aphid control approach in the future.

CONCLUSION AND FUTURE PERSPECTIVES

In the present review, we summarize the present literature on diverse measures known to suppress grain aphid populations. Based on the available data, we propose that the use of aphid-resistant crop plants integrated with agricultural and/or other management practices will be the most promising and effective management strategy for wheat aphid control (**Figure 3**). In addition, for developing aphid-resistant wheat cultivars, identifying the diverse genes that confer tolerance or more moderate levels of antibiosis resistance is essential for future efforts to improve aphid plant resistance. Moreover, RNAi-mediated aphid control may be an alternative approach for restricting the performance of aphids.

The newly released sequences of common wheat genomes have begun to provide the first real insights into the function and location of grain aphid resistance genes, which will be integrated into elite bread wheat lines to construct high-quality wheat cultivars (Appels et al., 2018). Moreover, the expense and time associated with high-throughput sequencing have been significantly reduced. This will accelerate the process of identifying and utilizing candidate genes with clear molecular mechanisms related to aphid resistance in wheat germplasms. Unfortunately, most of the characterized aphid-resistant cultivars are resistant to one species of wheat aphid but not others. However, wheat aphids are more likely to coinfest different parts of the same plant to obtain nutrients. For example, *S. avenae* prefers to colonize the upper, mature leaves and heads of wheat plants, whereas *R. padi* prefers to colonize the leaf sheaths and the lower leaves (Ni and Quisenberry, 2006). More recently, CRISPR–Cas9 technology has been successfully applied to inactivate crucial genes in cereal crops (Zhang et al., 2016; Kim et al., 2018). Therefore, the combined use of MAS and other molecular breeding measures (pyramiding breeding) is essential for accelerating the breeding of superior cultivars that can withstand attack from different species of grain aphids.

In addition, genetic plasticity not only stimulates grain aphids to evolve insecticide resistance but also serves as the genetic basis for aphids to express virulence to plant genes used in monogenic-based antibiosis resistance. During the plant immune response, the novel feeding effectors secreted by avirulent aphids are sometimes not recognized by the defense system of the resistant plant, and then the virulent aphid overcomes the plant resistance gene or genes in resistant wheat varieties, resulting in outbreaks of grain aphids (Smith and Chuang, 2014). The biotype variation among different RWA isolates and greenbug biotypes supports this conclusion. Historically, more than 11 RWA biotypes and eight greenbug biotypes have been described worldwide (Smith and Chuang, 2014). Although breeding resistant cultivars with multiple, quantitative loci or recessive loci offers a promising approach to delay or avoid aphid virulence, this is a long-term process that can be extremely challenging for plant breeders and entomologists. Altogether, based on the present literature, wheat aphids rapidly evolve virulence to resistant wheat hosts during wheat-aphid interactions, resulting in a need to develop novel strategies for aphid control. Although this method improves the efficiency of downregulation of the expression of grain aphid genes, alternative methods of transferring dsRNA into grain aphids should be explored.

In addition, in agroecosystems, wheat plants are often challenged by different species of pests, including aphid-transmitted viral or other pathogenic diseases, either sequentially or simultaneously. In most cases, there is a "synergistic" relationship between the different species of colonizers. For instance, *S. avenae* pre-exposure significantly facilitates the disease progression of fusarium head blight, a destructive cereal disease. Although the feeding behavior of wheat aphids could trigger hormone-dependent responses in host plants, the role and mechanism of phytopathogen-elicited phytohormones coordinated with other JA or SA signaling pathways to fine tune the plant defense response of wheat remain rudimentary, and further research is required on the crosstalk of complex phytohormonal pathways involved in plant immune responses (Luo et al., 2021). If confirmed, the hormonal crosstalk induced by multiple colonizers would further aggravate the challenge of the ecological regulation of wheat aphid pests in agroecosystems. Therefore, the above working hypothesis triggers important questions for future research and the elucidation of the interaction between aphids and different species of colonizers in ecological regulation of grain aphids and the maintenance of wheat production and grain quality.

AUTHOR CONTRIBUTIONS

KL and ZK conceived the study. KL and XW collected the data and led the writing of the manuscript. KL, ZK, XW, and HZ participated in data interpretation and revised the manuscript. KL prepared the figures. All authors have read and approved the manuscript for publication.

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Effect of Pathogenic Fungal Infestation on the Berry Quality and Volatile Organic Compounds of Cabernet Sauvignon and Petit Manseng Grapes

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The effect of pathogenic fungal infestation on berry quality and volatile organic compounds (VOCs) of Cabernet Sauvignon (CS) and Petit Manseng (PM) were investigated by using biochemical assays and gas chromatography-ion mobility spectrometry. No significant difference in diseases-affected grapes for 100-berry weight. The content of tannins and vitamin C decreased significantly in disease-affected grapes, mostly in white rot-affected PM, which decreased by 71.67% and 66.29%. The reduced total flavonoid content in diseases-affected grape, among which the least and most were anthracnose-affected PM (1.61%) and white rot-affected CS (44.74%). All diseases-affected CS had much higher titratable acid, a maximum (18.86 g/100 ml) was observed in the gray mold-affected grapes, while only anthracnose-affected grapes with a higher titratable acid level (21.8 g/100 mL) were observed in PM. A total of 61 VOCs were identified, including 14 alcohols, 13 esters, 12 aldehydes, 4 acids, 4 ketones, 1 ether, and 13 unknown compounds, which were discussed from different functional groups, such as C6-VOCs, alcohols, ester acetates, aldehydes, and acids. The VOCs of CS changed more than that of Petit Manseng's after infection, while gray mold-affected Cabernet Sauvignon had the most change. C6-VOCs, including hexanal and (*E*)-2-hexenal were decreased in all affected grapes. Some unique VOCs may serve as hypothetical biomarkers to help us identify specific varieties of pathogenic fungal infestation.

Keywords: Cabernet Sauvignon, Petit Manseng, GC-IMS, volatile organic compounds, pathogenic fungal infestation

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INTRODUCTION

Grape (*Vitis vinifera L.*) is one of the oldest horticultural plants in the world that existed since prehistoric times and have survived to the present day (Aşçi et al., 2021; Taskesenlioglu et al., 2022). This species, apart from being one of the most extensively cultivated fruit trees in the world due to its rich biochemical content, is also a fascinating subject for history and evolutionary studies (Kupe et al., 2021). The grape has also been the source of not only nutrition but also of beliefs and symbols in people's daily lives throughout history. Pathogenic fungal infestation is one of the main reasons affecting the development of the grape and wine industry, causing serious economic losses (Alkan et al., 2021). Common pathogenic fungal infestations include gray mold (*Botrytis cinerea*), white rot [*Coniothyrium diplodilla (Speg.) Sacc*], anthracnose (*Colletotrichum sp.*), and others. Gray mold, which is caused by *Botrytis cinerea*, usually kills and destroys berries, resulting in serious losses to many crops (Agudelo-Romero et al., 2015; Rastgou et al., 2022). Grape anthracnose, commonly known as grape bitter rot, causes the fruit to rot and the leaves to develop leaf spots because of infection. White rot often induces serious harm to fruit, which occurs in high temperature and high humidity environments. These pathogenic fungal infestations seriously affect the yield and quality of grapes, resulting in potential harm to the human body and commercial losses in the grape industry. However, precise interactions between various pathogenic fungal infestations and grapes have not been fully explored. Investigating various pathogenic fungal infestations of grapes during growth is critical for exploring the effects of the pathogenic fungal infestations and offering further information on interactions between pathogenic fungi and grapes.

As two popular wine grape varieties in the world, Cabernet Sauvignon (CS) and Petit Manseng (PM) have the exquisite aroma and noble quality as raw materials for red wine and white wine. At present, the serious problem is that pathogenic fungal infestations have negative impacts on the berry's quality and composition. Pathogenic fungal infestations usually lead to rapid physiological changes in berries, such as weight loss, skin color fading, tissue softening, and shortening of shelf-life, which badly reduces the market value of grapes (Vazquez-Hernandez et al., 2018). In addition to such visible quality characteristics, the nutritional value and chemical constituents of grapes will be changed, including more microbial metabolites, sugar degradation, and acid production (Solairaj et al., 2021). Santos et al. (2022) found that Trincadeira wine grapes showed serious symptoms after *Botrytis cinerea* infection, the content of total phenol and total anthocyanin was greatly lower than in healthy samples. An infection experiment conducted by Pons et al. (2018) confirmed that, for Merlot wines, pH and total acidity were the parameters that were systematically influenced by *P. viticola* infection. However, changes in fruit quality are sometimes associated with defensive behavior. Braga et al. (2019) found that phenolic compounds accumulate in infected areas compared with healthy areas, thus, indicating the accumulation of total phenols in resistance response.

Wine aroma is a key criterion for assessing the quality of wine, and the sources of aroma substances in wine are diverse. Among them, variety aroma is the aroma that comes directly from the grapes and characterizes the wine's typicality and origin style. Wine aroma is primarily influenced by the aroma of the varieties, such as volatile organic compounds (VOCs), which are produced by grape metabolism (Dudareva et al., 2013). VOCs include organic categories, such as alcohols, aldehydes, esters, fatty acids, and benzenes, which contribute to producing subtle aroma differences. Simultaneously, pathogenic fungal infestations change the aroma components of grapes by altering VOCs. Pinar et al. (2017) reported that bunch rot mainly caused an increase in the intensities of peach-like/fruity, floral, and liquor-like/toasty aroma notes, which were shown to be related to variations in aroma composition, mainly a modest increase of esters and alcohols. A previous study (Guerche et al., 2006) showed that *Botrytis cinerea* could promote to metabolize some trace volatile substances to produce odor in wine, the volatile metabolites detected in infected grape were mainly 2-methylisoborneol, 1-octene-3-ol, 1-octene-3-one, 2-octene-1-ol, and 2-heptanol. Also, Gadoury et al. (2007) found that powdery mildew accelerated the formation of other pathogenic fungal infestations and increased the contents of ethyl acetate, acetic acid, and ethanol in wine. On the other hand, some experimental trials have demonstrated the capacity of various VOCs produced by plants to inhibit germination and growth of plant pathogens. It has been reported that *Botrytis cinerea* was highly sensitive to the *in vitro* application of monoterpenes, such as (+)-limonene (Simas et al., 2017). However, exposure to (+)-limonene stimulated *in vitro* growth of *Penicillium digitatum*, whereas this fungus was highly inhibited by the application of citral (Simas et al., 2017). Lazazzara et al. (2018) demonstrated that *P. viticola* infection was inhibited in leaf tissues by some VOCs, such as 2-ethylfuran, 2-phenyl ethanol, β-cyclocitral, or trans-2-pentena. In addition, the growth of *Colletotrichum acutatum*, causing citrus post-bloom fruit drop, was moderately inhibited *in vitro* when exposed to linalool (Marques et al., 2015). Consequently, the elucidation of the changes in VOCs of grapes suffering pathogenic fungal infestations during growth is a task of highly practical significance to further explore the impact of pathogenic fungal infestations on grapes.

Gas chromatography-ion mobility spectrometry (GC-IMS) is an advanced technology for the analysis of VOCs, which combines the high separation ability of GC with the rapid response characteristics of IMS (Tuzimski et al., 2016). GC-IMS has the advantages of fast detection speed, simple operation, and easy sample preparation steps (Rousserie et al., 2020). GC-IMS shows rapid response and high sensitivity in the detection of trace volatile and semi-volatile organic compounds in different matrices. Zhao et al. (2022) used GC–IMS with gas chromatography-mass spectrometry technology to identify the flavor components qualitatively and quantitatively, under the various treatment of chiral tebuconazole on the flavor of Merlot and Cabernet Sauvignon wines. GC-IMS shows good application value in flavor analysis, and it is a new technology to detect the changes in volatile components during food processing and storage.

This study aims to investigate the impacts of the various pathogenic fungal infestations on two wine grapes during their growth. To be more specific, biochemical assays and

GC-IMS were used to further explore the effects of pathogenic fungal infestations on berry quality and VOCs in Cabernet Sauvignon and Petit Manseng, then evaluate the influences of gray mold, white rot, and anthracnose. In addition, the VOCs were analyzed from three aspects: (1) pathogenic fungal infestations, (2) volatile compound types, and (3) wine grape varieties. The obtained results provide comprehensive and reliable information for assessing the impacts of pathogenic fungal infestations in wine grapes.

MATERIALS AND REAGENTS

Chemicals and Reagents

The gallic acid, hesperidin, and L (+)-ascorbic acid analytical standards with purities of > 99% were provided by Dr. Ehrenstorfer GmbH (LGC Standards, Augsburg, Germany). Acetone, sodium carbonate, Folin-phenol, diethylene glycol, citric acid, glacial acetic acid, boric acid, metaphosphoric acid, sodium acetate trihydrate, o-Phenylenediamine, thymol blue, activated carbon, sodium tungstate dihydrate, sodium molybdate dihydrate, lithium sulfate, NaOH, HCl, H_3PO_4, and H_2SO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China); all chemicals were analytical grade (> 99%) unless otherwise stated. All standard solutions were stored in brown glass bottles wrapped in aluminum foil to avoid light exposure. Before analysis, the bottles were stored at 4°C. Under these conditions, no degradation was observed for 3 months.

Plant Materials and Treatments

The Cabernet Sauvignon and Petit Manseng grapes used in this study were cultivated in Yantai, Shandong Province, China (E121.39, N37.52). The grapevine trees of Cabernet Sauvignon and Petit Manseng were both 6 years old, with row spacing of 1.8 m × 0.5 m and 1 m × 2 m, respectively. Naturally infected berries with similar severity collected from the vineyard were used to identify consistent berry responses to pathogenic fungal infestations across natural conditions. Grapes were taken after veraison when the berry started to show fungal infection symptoms, and each selected bunch was submitted to a pathological examination for identifying the fungal infection before sample collection. After this, the infection degree of the grapes we chose was as follows: corresponding to healthy tissues and clusters of small lesions (diameter < 2 mm), there were hyphae in the early stage of development and hyphae structure in the middle stage (rarely visible and carefully observed). For CS and PM, the treatment group consisted of grapes with three pathogenic fungal infestations: gray mold (CS-GM, PM-GM), anthracnose (CS-AN, PM-AN), and white rot (CS-WR, PM-WR). The healthy samples were selected as the control groups (CS-CK, PM-CK). Grapes were wrapped in wet gauze after harvest and brought back to the laboratory immediately after refrigeration in a preservative box. The samples were placed in a 4°C-refrigerator before measurement and crushing. All measurements include three replicates, each containing three random clusters. For each grape cluster, the grapes were randomly selected from the shoulder, middle, and bottom of the cluster. One hundred-berry weight, particle size, soluble solids content, titratable acid, and the content of total phenolics, total flavonoids, vitamin C, and tannins were determined.

Determination of Physicochemical Parameters

One Hundred-Berry Weight and Particle Size

One hundred grape berries in each group were chosen randomly to measure 100-berry weight (g), then washed with distilled water and dried by the filter. It was measured by an electronic balance; measurements were repeated three times. Vernier calipers were used to measure the particle size of ten grape berries in each group.

Soluble Solid Content and Titratable Acid

The clear juice (supernatant) extracted was used to determine SSC by using a manual refractometer (ATAGO Company, Fukuoka, Japan) and the results were recorded as the degree of°Brix. TA was determined by titration using 10 ml of diluted juice with the addition of NaOH (0.1 N) and two drops of phenolphthalein until a light pink color was formed (30 s without fading). Finally, the numerical value was expressed by the predominant acid.

Determination of the Phenolic Compounds and Vitamin C in Grapes

Total Phenolics

Total phenolics (TP) content was determined by the modified Folin method (Thimmaiah, 1999). The gallic acid standard sample was dissolved in distilled water and diluted to obtain a standard solution of 0.05 mg/ml. Then, 1 mL (1.2, 1.4, 1.6, 1.8, and 2) of the standard solution was accurately measured, and 0.5 mL of Folin-phenol reagent and 0.5 mL of 10% sodium carbonate solution were added. The distilled water was diluted to 10 ml, and the solution was reacted in a water bath at 25°C for 1 h. The absorbance was measured at 765 nm with the blank reagent as the control to establish the standard curve.

Four-gram sample was weighed, 16 ml of 70% acetone was added, and then, the supernatant was extracted for 3 h and centrifuged for 10 min at 10,000 rpm. A total of 0.2 ml of the sample solution was taken, added with 0.5 ml of Folin-phenol reagent. Then,0.5 ml of 10% sodium carbonate solution was added and was reacted in a water bath at 25°C for 2 h. The absorbance was measured at 765 nm to obtain the TP content.

Total Flavonoids

The experiment was designed to determine Total flavonoids (TF) content following the guideline (NY/T, 2010-2011). The standard solutions of 0 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml of hesperidin (200 mg/L) were absorbed into the test tubes, and then, 5 ml of 90% diethylene glycol solution and 0.1 ml of NaOH (4 M) solution were added. The standard solution (0 mg/L, 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, and 100 mg/L) was incubated in a water bath at 40°C for 10 min, and then cooled for 5 min. The absorbance was measured at 420 nm by ultraviolet spectrophotometer (Shimadzu UV-2450, Kyoto, Japan), and the

standard curve was drawn. Five grams of sample was mixed with NaOH solution, and the PH was adjusted to 13.0. The PH was adjusted by the citric acid solution (20% w/v) to 6 after 30 min, and 5 ml of the solution was mixed with 5 ml of diethylene glycol solution and 0.1 ml of NaOH solution. The absorbance value was determined using the standard curve to calculate the mass concentration of TF.

Total flavonoids (TF) was calculated by hesperidin mass fraction ω, and the value was expressed as mg/100 g using the following formula:

$$\omega = \frac{\rho \times 10 \times 100 \times 1000}{m \times V \times 100} \quad (1)$$

where ρ is hesperidin mass concentration (mg/L); V is determination of absorbed test liquid volume (mL); m is sample weighing mass (g); 10 is color constant volume (ml); 100 is sample extraction volume (ml).

Vitamin C

The VC content of grapes was measured by using the fluorescence method based on (GB 5009.86, 2016). One hundred grams of the grape extract was homogenized after adding 100 g of metaphosphate-acetic acid solution, diluted with the metaphosphate-acetic acid solution, or metaphosphate-acetic acid-sulfuric acid solution. The pH was adjusted to 1.2, 50 ml of supernatant was mixed with 2 g of activated carbon after filtering, and two groups of 10-ml filtrates were taken and added with 5 ml of sodium acetate solution (50% w/v) and 5 ml of boric acid-sodium acetate solution as "sample solution and "sample blank solution," respectively. The ascorbic acid's standard working solution was treated in the same way as "standard solution" and "standard blank solution."

First, 0.5 ml, 1 ml, 1.5 m, and 2 ml of standard solution were absorbed and supplemented with water to 2 ml. In addition, 2 ml of "standard blank solution" was mixed with 5 ml of phthalediamine solution in the darkroom, and the reaction was carried out at room temperature for 35 min. Finally, the fluorescence intensity was measured at 338 nm and 420 nm, and the standard curve was drawn. Two milliliters of "sample solution" and "sample blank solution" with 5 ml of phthalediamine solution were reacted in the darkroom for 35 min reaction at room temperature. The result was measured and the total amount of L (+)-ascorbic acid was determined according to the standard curve. The results were expressed as mg/100 g using the following formula:

$$X = \frac{c \times V}{m} \times F \times \frac{100}{1000} \quad (2)$$

where X is total L (+)-ascorbic acid in sample (mg/100 g); c is L (+)-ascorbic acid mass concentration (μg/mL); V is sample volume (mL); m is actual sample quality (g); F is the sample solution dilution ratio; 100 is conversion coefficient; 1,000 is conversion coefficient.

Tannins

The tannins content of grapes was measured by using a spectrophotometric method based on (NY/T, 1600-2008). Sodium tungstate-sodium molybdate mixed solution was configured, then 1 ml of 0.00 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, and 50 mg/L gallic acid standard solution was absorbed, 5 ml water was added, 1 ml of sodium tungstate-sodium molybdate mixed solution, and 3 ml of sodium carbonate solution (7.5% w/v) were mixed with 0 mg/L, 1 mg/L, 2 mg/L, 3 mg/L, 4 mg/L, and 5 mg/L standard solution. After coloration, the absorbance at 765 nm was measured and the standard curve was plotted. Five grams of sample centrifuged at 8,000 rpm for 4 min after hot water bath extraction, 1 ml of extract was taken and added with 5 ml of water, 1 ml of sodium tungstate-sodium molybdate mixed solution, and 3 ml of sodium carbonate solution. The absorbance at 765 nm was measured, and the concentration of tannin was calculated according to the standard curve. The tannin content in the sample (calculated by gallic acid) was calculated as follows:

$$\omega = \frac{\rho \times 10 \times 10 \times A}{m} \quad (3)$$

where ω is tannin content in samples (mg/100 g); ρ is gallic acid concentration in the determination solution (mg/L); 10 is the constant volume (ml); 10 is the conversion coefficient; A is the sample dilution multiples; m is the sample quality (g).

Volatile Analysis by Gas Chromatography-Ion Mobility Spectrometry

For volatile analysis, the grape sample was homogenized, and 5 g was accurately weighed, then placed in a 20-ml headspace vial and incubated at 40°C for 20 min before sampling. The analysis was performed by using headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) (FlavourSpec®, G.A.S., Dortmund, Germany). The grape samples were incubated at 500 r/min for 20 min at 40°C. Thereafter, 500 μL of gas from the headspace was automatically infused into the heated injector by a syringe in a splitless mode at 85°C. At that point, the samples were directed into an MXT-WAX capillary column (30 m × 0.53 mm × 1 μm, Restek Corporation, Bellefonte, PA, United States). Purified nitrogen (99.999% purity) with a flow rate of 150 ml/min was used as the drift gas for IMS. The temperature of the column and the IMS was 60°C and 45°C, respectively. The carrier gas followed a programmed flow: 2 ml/min for 2 min, increased to 100 ml/min within 20 min, then kept at 100 ml/min for 10 min.

The eluted analytes ionization source was 3H ionization, driven to a drift tube which was run at a constant temperature of 45°C and voltage of 5 kV. C4-C9 *n*-ketones (Sinopharm Chemical Reagent Beijing Co., Ltd., China) was used as references when the retention index (RI) was calculated. Volatile compounds were analyzed according to the differences in their RI and drift time (DT) in the GC × IMS Library from different perspectives.

Date Analysis

A significant difference was evaluated by one-way analysis of variance (ANOVA) followed using Duncan's multiple range test

with a significant level ($P < 0.05$). IBM SPSS statistics (version 20.0, SPSS Inc., Chicago, IL, United States) was employed for significance analysis. The scattered boxplot and principal component analysis (PCA) were implemented by Origin 2021 from Origin Laboratories (available at www.originlab.com). Heatmap with clustering analysis was made by TBtools software (Chen et al., 2020).

The instrument analysis software was composed of Laboratory Analytical Viewer (version 2.2.1, G.A.S. Dortmund, Germany) with its plug-ins: Reporter and Gallery Plot, and GC × IMS Library Search, which were applied to qualitative and comparative detection.

RESULTS AND DISCUSSION

Effects of Pathogenic Fungal Infestations on Physicochemical Parameters of Cabernet Sauvignon and Petit Manseng

One Hundred-Berry Weight and Particle Size

Berry size is widely considered to be a factor in determining the quality of wine grapes. As shown in **Figures 1A–D**. In our study, there were no significant differences between the disease-infected grapes and healthy grapes for the 100-berry weight of CS and PM, while most infected groups decreased slightly. The results showed that the grape infected with anthracnose decreased slightly compared with other disease groups, CS-AN and PM-AN were decreased by 14.03% and 20.02%, respectively. On the other hand, a slight increase was observed in 100-berry weight and particle size for PM, which were 4.49% and 0.34%, respectively, after infection with *Botrytis cinerea*. The effect of pathogenic fungal infestation on berry weight depends on the degree of ontogenic resistance expressed by berries when infected by pathogenic fungi. Gadoury et al. (2003) showed that once the berries reached the diameter of 3 mm (28 days after flowering), powdery mildew did not significantly reduce the weight of the berries. However, the weight of berries was significantly reduced and colonized by the pathogen heavily when berries were infected before resistance. It is speculated that the unobvious change of berry weight may be related to the resistance of grapes when pathogenic fungal infections occurred. The comparison of 100-berry weight and particle size showed that CS-WR > CS-GM > CS-AN and PM-GM > PM-WR > PM-AN.

Soluble Solid Content and Titratable Acid

Soluble solid content (SSC) and TA are related to the taste of wine, which served as important indexes to reflect the quality of the berry and the disease resistance. **Figures 1E–H** show the SSC and TA of the two wine grapes infected with various pathogenic fungi. In CS, the SSC of the infected samples was lower than CS-CK, and CS-AN and CS-GM significantly decreased ($P < 0.05$) by 11.29% and 5.25%, respectively, compared with the healthy samples. Meanwhile, different pathogenic fungal infestations cause a rise of TA content in CS to variable degrees: gray mold (18.86 g/100 ml)>anthracnose (16.01 g/100 ml) > white rot (14.25 g/100 ml). However, the SSC increased after infection in PM, and the maximum (21.80 g/100 ml) of the TA was observed in the PM-AN, and other disease groups were lower than in the healthy group. In a previous study conducted by Stummer et al. (2003), for Cabernet Sauvignon infected with powdery mildew in 2001, the SSC with infection degree greater than 30% has decreased significantly, which was speculated to be related to the high level of powdery mildew infection hindering sugar accumulation, and the TA increased or decreased under different infection degrees. On the other hand, the explanation for the increase in sugar concentration after infection may be associated with the decrease in the volume of diseased berries and the increase in transpiration water loss (Calonnec et al., 2004). Moreover, Girardello et al. (2020) showed that the acidity of Chardonnay grapes increased after infection with erythema, which was supposed to be related to the high concentration of potassium (K) in juice. In summary, it was inferred that our results may be caused by many reasons such as the fruit year, grape variety, pathogenic fungal infestation, the degree of the infection, and so on.

Effects of Pathogenic Fungal Infestation on the Content of the Phenolic Compounds and Vitamin C in Cabernet Sauvignon and Petit Manseng

Total Phenolics

Grape is rich in phenols, and plant polyphenols have been proven to have potential antibiosis activity, which is mainly distributed in the skin, stems, leaves, and seeds of the grape, rather than the juicy middle part (Bruno and Sparapano, 2007). The phenolic compounds mainly include proanthocyanidins, anthocyanins, flavonols, resveratrol, and phenolic acids. As shown in **Figures 2A,B**, for healthy grapes, the TP of CS (13.62 mg GAE/g) was much higher than PM (5.80 mg GAE/g). In CS and PM, a significant difference ($P < 0.05$) was observed in all disease groups compared to the healthy grapes. In comparison to CK, the TP content of the grapes infected with anthracnose increased by 8.25% and 21.61% significantly, while it decreased after infection with white rot. It is worth noting that the TP content in CS and PM presented the opposite effects after infection with gray mold, which showed that CS decreased by 5.02 mg GAE/g but PM increased by 2.02 mg GAE/g compared with CK. A study reported that total phenolic extracted in methanol had a visible downward tendency after infection, and total phenolic extracted in water, mainly hydrophilic compounds such as hydroxybenzoic acid, anthocyanin, flavonoids, or tannin, decreased after infection (Santos et al., 2022). However, wines made from powdery mildew-affected grapes generally have higher phenolic levels than wines made from unaffected grapes (Steel et al., 2013). Bruno and Sparapano (2007) reported that the content of total phenol in grape skins varies with species, soil composition, climate, geographical origin, cultivation methods, and infection exposure. Therefore, it is speculated that different changes were impacted by a range of factors.

Total Flavonoids

Flavonoids have strong antioxidant properties and could be measured to reflect the antioxidant capacity of plants

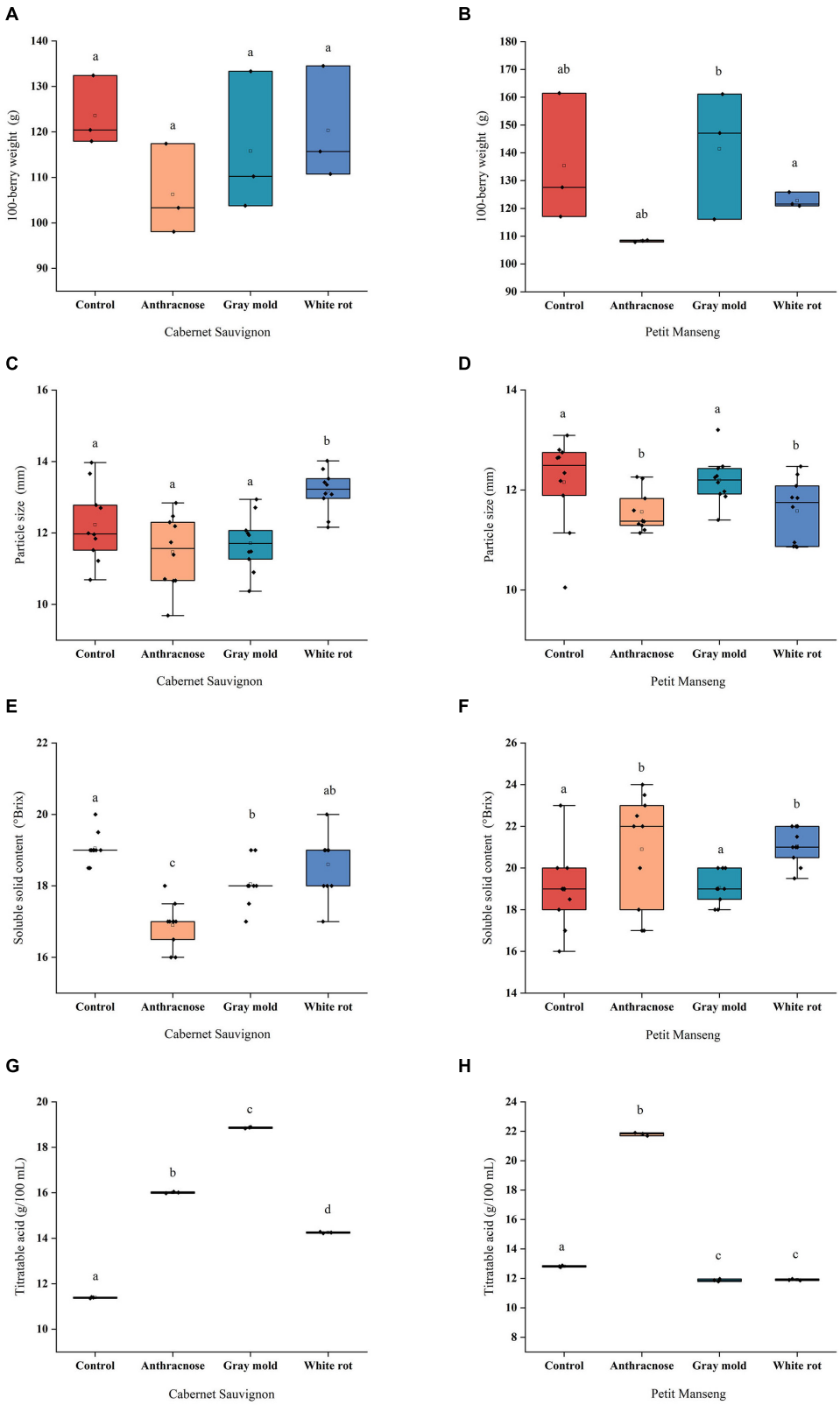

FIGURE 1 | The scattered boxplots show physicochemical parameters of Cabernet Sauvignon and Petit Manseng grapes: **(A, B)** 100-berry weight; **(C, D)** Particle size; **(E, F)** Soluble Solid Content (SSC); **(G, H)** Titratable acid (TA). Different letters (lower case) on the top of the bars represent significant differences among the investigated grape samples. Scattered boxplots show individual data points, median, average value, 25th, and 75th percentile.

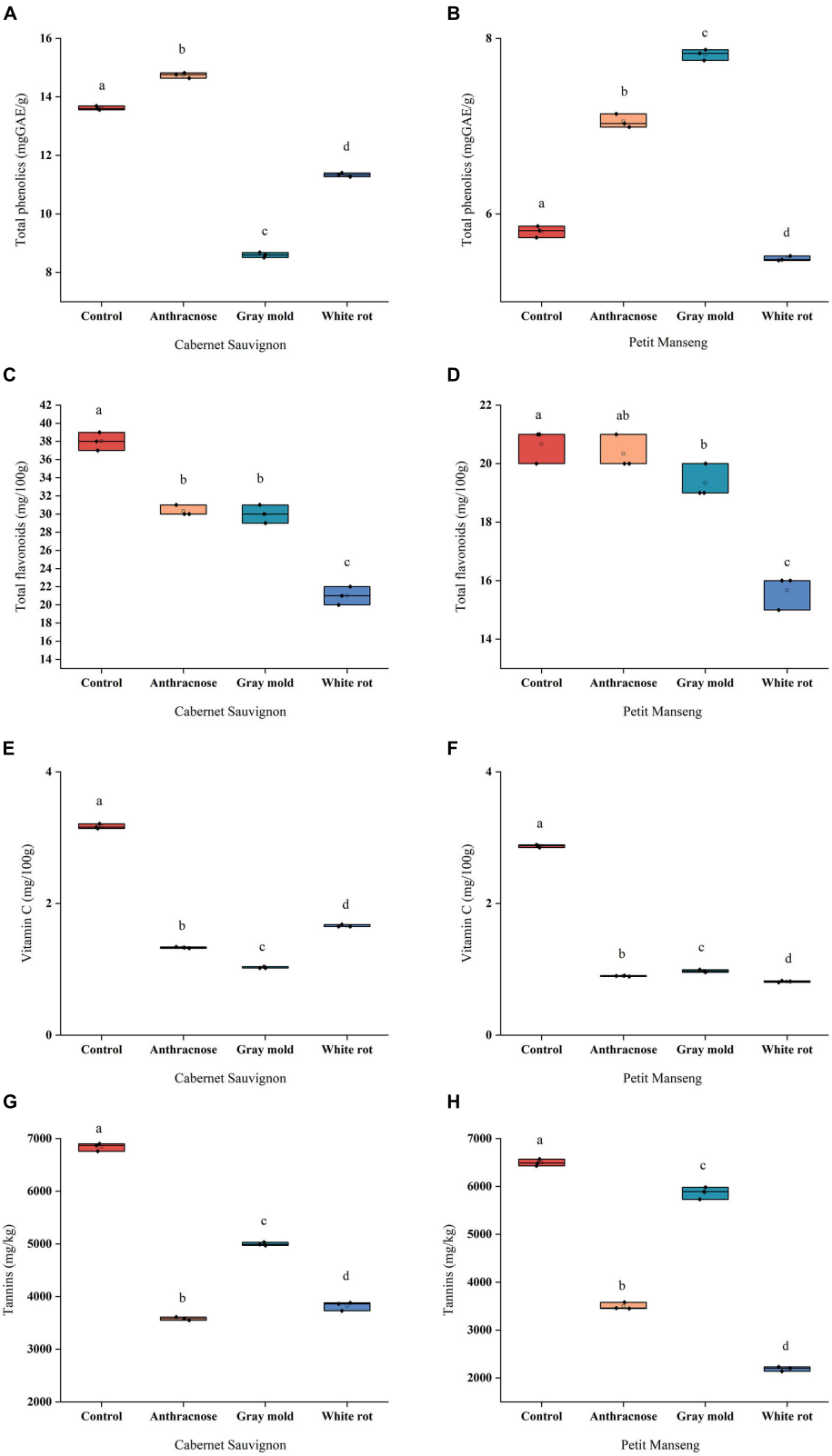

FIGURE 2 | The scattered boxplots show the content of the phenolic compounds and vitamin C in all samples of Cabernet Sauvignon and Petit Manseng grapes: **(A, B)** Total phenolics (TP); **(C, D)** Total flavonoids (TF); **(E, F)** Vitamin C; **(G, H)** Tannins. Different letters (lower case) on the top of the bars represent significant differences among the investigated grape samples. Scattered boxplots show individual data points, median, average value, 25th, and 75th percentile.

(**Figures 2C,D**). The CS-CK (38 mg/100 g) and PM-CK (20.67 mg/100 g) both had the highest content of TF, and almost infected grapes were significantly decreased ($P < 0.05$), except for PM-AN. The TF content decreased to a similar extent after infection with anthracnose and gray mold, the results showed that the content of CS-AN and CS-GM was 30.33 mg/100 g, 30 mg/100 g, and PM-AN and PM-GM were 20.33 mg/100 g, 19.33 mg/100 g, respectively, which suggested that anthracnose and gray mold might have similar effects on the TF. Moreover, flavanols are the most ubiquitous flavonoids in foods, previous study focused on the determination of flavonols of Zinfandel grapes, the findings proved that infected grapes had a lower flavonol content compared to the control group (Blanco-Ulate et al., 2017).

Vitamin C

Vitamin C (VC) is the primary aqueous antioxidant that effectively reduces the damage that reactive oxygen species (ROS) produced. The maximum was found in the control group both in the PM and CS (**Figures 2E,F**). After being invaded by pathogenic fungi, the content of VC in CS and PM decreased significantly ($P < 0.05$), which indicated that the invasion of pathogenic fungi caused a great deal of degradation of VC in grapes, which damaged the nutritional quality. Murria et al. (2018) found that the contents of chlorophyll, carotenoids, and ascorbic acid in infected leaves are lower than those in healthy leaves.

Tannins

Tannins are one of the key factors determining the quality of grapes and wines, which can decide the sensory attributes of wines, such as color, taste, astringency, and bitterness by the content and proportion (Rousserie et al., 2020). As shown in **Figures 2G,H**, There was a significant difference ($P < 0.05$) in tannins of infected grapes compared to healthy grapes in the CS and PM, pathogenic fungal infestation reduced the tannins to a great extent. The grape berry analyses performed in 2010 showed that it greatly decreases skin procyanidins concentrations after infection; it should be noted that the total tannin content in pericarp tissue is considered to contribute to the pre-resistance of berries to pathogenic fungal infestation (Deytieux-Belleau et al., 2009). Previous reports speculated that the tannins were closely related to climatic factors, resulting in stronger responses of plants and more tannins in a warmer environment (Cauduro Girardello et al., 2020). Moreover, tannin has also a certain correlation with grape maturity (Rousserie et al., 2020).

Volatile Organic Compounds Analysis by Gas Chromatography-Ion Mobility Spectrometry

Volatile organic compounds (VOCs) play an indispensable role in the key metabolic pathways involved in plant growth, development, reproduction, and defense (Bouwmeester et al.,

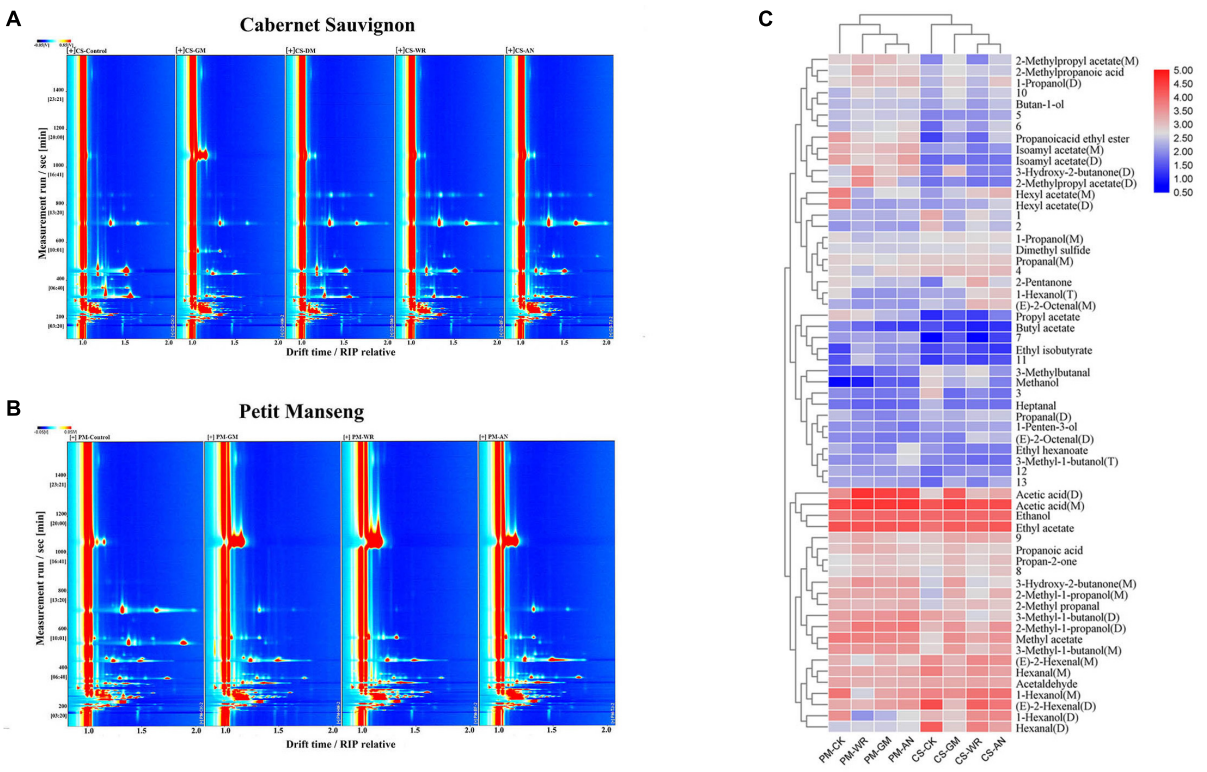

FIGURE 3 | (A, B) Comparison of GC-IMS spectra and **(C)** Heatmap with clustering analysis of VOCs. The numbers in panel **(A, B)** represent VOCs with large differences (the compound names are shown in **Supplementary Table 1**). **(C)** shows the changes in VOCs induced by pathogenic fungal infestation with the control group in Cabernet Sauvignon and Petit Manseng wine grapes.

2019). Once the fruit was infected by pathogenic fungal, VOCs can be used as toxins, defense compounds, energy sources, and infection enhancers (Santos et al., 2022). Understanding the alterations of fruit to pathogenic fungal infestation is essential for the improvement of grapes and for the sustainability of wine production. In this study, VOCs were analyzed from three different aspects: pathogenic fungal infestation, VOC types, and wine grape varieties.

Effects of Pathogenic Fungal Infestations on Volatile Organic Compounds

The GC-IMS spectra of the VOCs in the samples are shown in **Figures 3A,B**, each spectrum represents the sample for each treatment, the Y-axis represents the retention time (s) of gas chromatography, and the X-axis represents the ion migration time. The red vertical line at X-axis 1 is the reactive ion peak (RIP), on both sides of this peak, each point represents a volatile organic compound. The concentration of VOCs was determined by color, blue was the background color, white represents low concentration, and red represents high concentration, that is, the deeper the color, the greater the concentration. It can be intuitively found from the spectrum that after pathogenic fungal infestations, the VOCs of CS and PM both had significant changes. A total of 61 volatile compounds, composed of 14 alcohols, 1 ether, 12 aldehydes, 4 acids, 4 ketones, 13 esters, and 13 unknown compounds, were simultaneously identified (including monomers and polymers) in the samples of CS and PM (**Table 1** and **Supplementary Table 1**), and most of the volatile substances were alcohols, esters, and aldehydes. Among all infected grapes, the variation of VOCs in CS-GM has the greatest difference compared with others. In this group, the concentrations of 9 VOCs decreased, including 6 aldehydes, 2 alcohols, and 1 ether. The concentrations of 19 VOCs increased, including 8 esters, 3 ketones, 3 acids, 4 alcohols, and 1 aldehyde. Among them, 2-methylpropanoic acid, ethyl isobutyrate, 3-hydroxy-2-butanone, acetic acid, and propionic acid increased significantly. As for PM-GM, the concentration of 14 VOCs reduced, including 8 esters, 2 alcohols, 3 aldehydes, and 1 ether, and 11 VOCs were raised, including 4 alcohols, 3 acids, 2 esters, and 2 ketones. Notably, previous studies had explanations for the reduction in 1-hexanol, it is reported that this may be due to the production of noble root wines, which is the result of a unique physiological process (Tosi and Azzolini, 2013). Noble root wine is a sweet wine formed by *Botrytis cinerea* infecting grapes under specific growth conditions. Therefore, we speculated that the grapes infected with *Botrytis cinerea* would generate noble root wine due to certain development circumstances.

In CS-WR, 17 VOCs had a higher level, and 5 VOCs had a lower level compared with healthy grapes, the changes in substances were similar to CS-AN. At the same time, the changes of VOCs in PM-WR and PM-AN were also very close, which speculated that there are some common points in understanding the effects of white rot and anthracnose on grapes. Meanwhile, the contents of acetic acid, 3-hydroxy-2-butanone, propan-2-one, 2-methyl-1-propanol, 1-penten-3-ol, and 1-propanol increased in the CS-WR and PM-WR, which inferred that the changes in aroma components were closely related to the pathogenic fungal infestation. Most esters, dimethyl sulfide, propanal, and n-hexanol increased in CS but decreased in PM, and propionic acid increased only in infected PM. On the other hand, in the grapes with anthracnose, heptanal and methanol decreased greatly in CS, and some substances, such as 1-penten-3-ol, n-hexanol, and propanal were raised in CS while decreased in PM. According to the results, it is speculated that the specific marker VOCs can be further found for specific varieties infected with specific pathogenic fungi. Finally, there was the same change rule in the two grapes, in which the content of hexanal and (*E*)-2-hexenal decreased and acetone increased for all infected grapes.

Effects of Pathogenic Fungal Infestations on Various Types of Volatile Organic Compounds

Heatmap with clustering analysis was shown to clarify the changes of all VOC among the diseases-affected grapes and the control group. In **Figure 3C**, each row showed volatile substances, and M, D, and T in parentheses behind the name of the substance represent the monomer, dimer, and trimer of the substance, respectively. Each column indicated different samples, and the number indicated temporarily unknown compounds. We compared the color difference to determine the variation of VOCs. Blue represents low concentration and red represents high concentration; the more obvious the color was, the more VOC was in the corresponding sample. The samples clustered into the same category indicated a high degree of correlation.

The Changes in C6-Volatile Organic Compounds

In VOCs, green leaf aroma C6-VOCs as one of the main families are derived from the lipoxygenase (LOX) pathway, which is usually induced by biological stress, the precursors are linoleic acid and α-linolenic acid (Gong et al., 2019). In this study, hexanal, (*E*)-2-hexenal, and 1-hexanol are typical C6-VOCs. Among them, hexanal and (*E*)–2-hexenal are C6 unsaturated aldehydes and 1-hexanol is C6 alcohol, which have the characteristics of green grass flavor. The concentrations of hexanal and (*E*)-2-hexenal in all infected grapes decreased, and C6-VOCs were generally associated with plant defense behavior. Shiojiri et al. (2006) showed that overexpression of hydroperoxide lyase (HPL) in Arabidopsis led to higher resistance of transgenic plants to *Botrytis cinerea*, which may be due to the increasing contents of C6 VOCs emitted by plants after infection, reflecting the assumed role of VOCs metabolism in grape defense mechanism. However, our results are in agreement with those reported by Santos et al. (2022), who found that hexanal and (*E*)-2-hexenal decreased greatly both in free or glycosylated after infection with *Botrytis cinerea*, suggesting that C6-VOCs can be used as a stress signal for plant biological stress. Schueuermann et al. (2018) also confirmed the concentration of (*E*)-2-hexenal in *V. vinifera* cv. Chardonnay grapes decreased after infection with *Botrytis cinerea* in two out of three vintages. We speculated that pathogenic fungal infestation could manipulate the level of C6-VOCs to reduce the defense effect of fruit, because low concentrations of (*E*)-2-hexenal may promote mycelium growth (Santos et al., 2022). Moreover, previous studies also showed that green aroma gradually decreased with fruit maturation, which was also a possibility for the decrease

TABLE 1 | Compositions of the volatile substances determined by gas chromatography-ion mobility spectrometry (GC-IMS) analysis.

Count	Compound	CAS#	Formula	MW	RI[a]	Rt[b]	Dt[c]
1	2-Methylpropanoic acid	C79312	$C_4H_8O_2$	88.1	1566.6	1717.688	1.15764
2	Propanoic acid	C79094	$C_3H_6O_2$	74.1	1538.4	1499.028	1.10708
3	acetic acid	C64197	$C_2H_4O_2$	60.1	1462.8	1049.26	1.05129
4	acetic acid*	C64197	$C_2H_4O_2$	60.1	1464.7	1058.641	1.15766
5	1-Hexanol	C111273	$C_6H_{14}O$	102.2	1368.4	701.683	1.32825
6	1-Hexanol*	C111273	$C_6H_{14}O$	102.2	1369.6	704.455	1.65395
7	1-Hexanol**	C111273	$C_6H_{14}O$	102.2	1368.9	702.792	1.99559
8	3-hydroxy-2-butanone	C513860	$C_4H_8O_2$	88.1	1298.6	557.923	1.07285
9	3-hydroxy-2-butanone*	C513860	$C_4H_8O_2$	88.1	1297.0	555.075	1.33145
10	hexyl acetate	C142927	$C_8H_{16}O_2$	144.2	1280.5	528.631	1.3847
11	hexyl acetate*	C142927	$C_8H_{16}O_2$	144.2	1281.0	529.388	1.90012
12	Ethyl hexanoate	C123660	$C_8H_{16}O_2$	144.2	1238.9	468.485	1.3433
13	(*E*)-2-hexenal	C6728263	$C_6H_{10}O$	98.1	1226.5	451.975	1.1769
14	(*E*)-2-hexenal*	C6728263	$C_6H_{10}O$	98.1	1227.1	452.709	1.52571
15	1-Penten-3-ol	C616251	$C_5H_{10}O$	86.1	1168.0	382.14	0.94552
16	isoamyl acetate	C123922	$C_7H_{14}O_2$	130.2	1131.6	344.798	1.30098
17	isoamyl acetate*	C123922	$C_7H_{14}O_2$	130.2	1131.3	344.509	1.74028
18	2-methyl-1-propanol	C78831	$C_4H_{10}O$	74.1	1104.4	319.339	1.17187
19	2-methyl-1-propanol*	C78831	$C_4H_{10}O$	74.1	1103.4	318.471	1.36777
20	Hexanal	C66251	$C_6H_{12}O$	100.2	1101.5	316.735	1.2624
21	Hexanal*	C66251	$C_6H_{12}O$	100.2	1094.7	311.238	1.56219
22	2-methylpropyl acetate	C110190	$C_6H_{12}O_2$	116.2	1023.4	268.504	1.28717
23	2-methylpropyl acetate*	C110190	$C_6H_{12}O_2$	116.2	1021.6	267.449	1.61112
24	2-pentanone	C107879	$C_5H_{10}O$	86.1	992.8	252.367	1.36789
25	ethanol	C64175	C_2H_6O	46.1	935.2	230.801	1.13103
26	propyl acetate	C109604	$C_5H_{10}O_2$	102.1	985.8	249.652	1.47729
27	Propanoic acid ethyl ester	C105373	$C_5H_{10}O_2$	102.1	964.8	241.659	1.45393
28	Ethyl isobutyrate	C97621	$C_6H_{12}O_2$	116.2	973.6	244.977	1.55908
29	ethyl acetate	C141786	$C_4H_8O_2$	88.1	888.0	214.512	1.32965
30	3-Methylbutanal	C590863	$C_5H_{10}O$	86.1	922.4	226.276	1.40082
31	Methyl acetate	C79209	$C_3H_6O_2$	74.1	850.8	202.498	1.19096
32	Propan-2-one	C67641	C_3H_6O	58.1	835.8	197.824	1.11461
33	2-Methyl propanal	C78842	C_4H_8O	72.1	831.2	196.433	1.28088
34	Propanal	C123386	C_3H_6O	58.1	819.6	192.921	1.06504
35	Propanal*	C123386	C_3H_6O	58.1	822.0	193.65	1.14125
36	methanol	C67561	CH_4O	32.0	912.2	222.721	0.98563
37	Acetaldehyde	C75070	C_2H_4O	44.1	778.2	180.93	0.98633
38	butyl acetate	C123864	$C_6H_{12}O_2$	116.2	1081.9	303.09	1.23772
39	3-Methyl-1-butanol	C123513	$C_5H_{12}O$	88.1	1213.8	435.646	1.24493
40	3-Methyl-1-butanol*	C123513	$C_5H_{12}O$	88.1	1214.1	435.999	1.49095
41	3-Methyl-1-butanol**	C123513	$C_5H_{12}O$	88.1	1215.8	438.118	1.78823
42	1-propanol	C71238	C_3H_8O	60.1	1047.9	282.482	1.11188
43	1-propanol*	C71238	C_3H_8O	60.1	1048.5	282.816	1.25422
44	(*E*)-2-octenal	C2548870	$C_8H_{14}O$	126.2	1418.6	852.241	1.34901
45	(*E*)-2-octenal*	C2548870	$C_8H_{14}O$	126.2	1418.5	851.636	1.81337
46	heptanal	C111717	$C_7H_{14}O$	114.2	1191.7	408.576	1.34677
47	butan-1-ol	C71363	$C_4H_{10}O$	74.1	1152.9	366.206	1.18015
48	dimethyl sulfide	C75183	C_2H_6S	62.1	799.5	187.004	0.95792

Dt, drift time; MW, Molecular mass; RI, retention index; Rt, Retention time.
[a]*Retention index.*
[b]*Retention time.*
[c]*Drift time.*
"" Represents dimer.*
*"**" Represents trimer.*

of hexanal and (*E*)-2-hexenal (Agudelo-Romero et al., 2013). Finally, these changes in C6-VOCs are likely to affect the green aroma of grapes.

The Changes in Alcohol

After pathogenic fungal infestations, alcohol-VOCs also changed greatly. Alcohols have physical-chemical properties, which can lead to membrane rupture and interfere with cell metabolism (Yalage Don et al., 2020). In this study, the results showed that 3-Methyl-1-butanol only increased in infected CS, 3-Methyl-1-butanol has the aroma of fusel alcohol and antibacterial properties. Pinar et al. (2017) showed that its presence is usually related to the activity of laccases in *B. cinerea*. Moreover, the existence of fusel alcohol is usually one of the reasons for the rotting smell of grapes. On the other hand, we also observed that increasing ethanol only in PM after pathogenic fungal infestation, and it was at a high level. we speculated that spontaneous fermentation could explain it because there were more yeasts in the microbial group of infected grapes compared with healthy grapes (Magyar, 2011).

The Changes in Ester Acetates

Esters play an important role in wine aromas, as most esters present pleasant aromas. The results showed that acetate accounted for a large proportion of VOCs in this study. Ethyl acetate showed higher levels only in CS after pathogenic fungal infestation, which always contains ethyl acetate. Ethyl acetate presents a strong fruit flavor, which is beneficial to the production of acetic acid, it usually acts as a common adverse metabolite that exists in fruit infected by fungi (Barata et al., 2011). We speculated that infected grapes produce a certain degree of fermentation, and the increase of acetate compounds may promote the colonization of fungi (Boss et al., 2015). In the PM infected with gray mold, white rot, and anthracnose, esters were decreased by 8, 7, and 6, respectively. Only 2 esters increased, which contains ethyl isobutyrate. Ethyl isobutyrate is formed by esterification of ethanol and isobutyrate under acidic conditions, which has an apple aroma. Ethyl isobutyrate may be a specific biomarker for PM after pathogenic fungal infestation, which needs further confirmation in the following experiment.

The Changes in Aldehydes and Acids

In CS, heptanal decreased remarkably and acetic acid increased after pathogenic fungal infestation. Usually, acetic acid is the symbolic volatile of fruit decomposition and decay, which is the pathway to produce ethyl acetate and may be caused by the co-infection of acetic acid bacteria and acid rot (Hall et al., 2018).

The Changes of Unknown Volatile Compounds

In this study, 13 volatile compounds were unidentified from the fingerprints due to information limitations in the built-in NIST database of GC-IMS. The unknown volatile compounds were analyzed deeply for the integrity and reliability of the experiment. Terpenes and norisoprenoids are two of the most

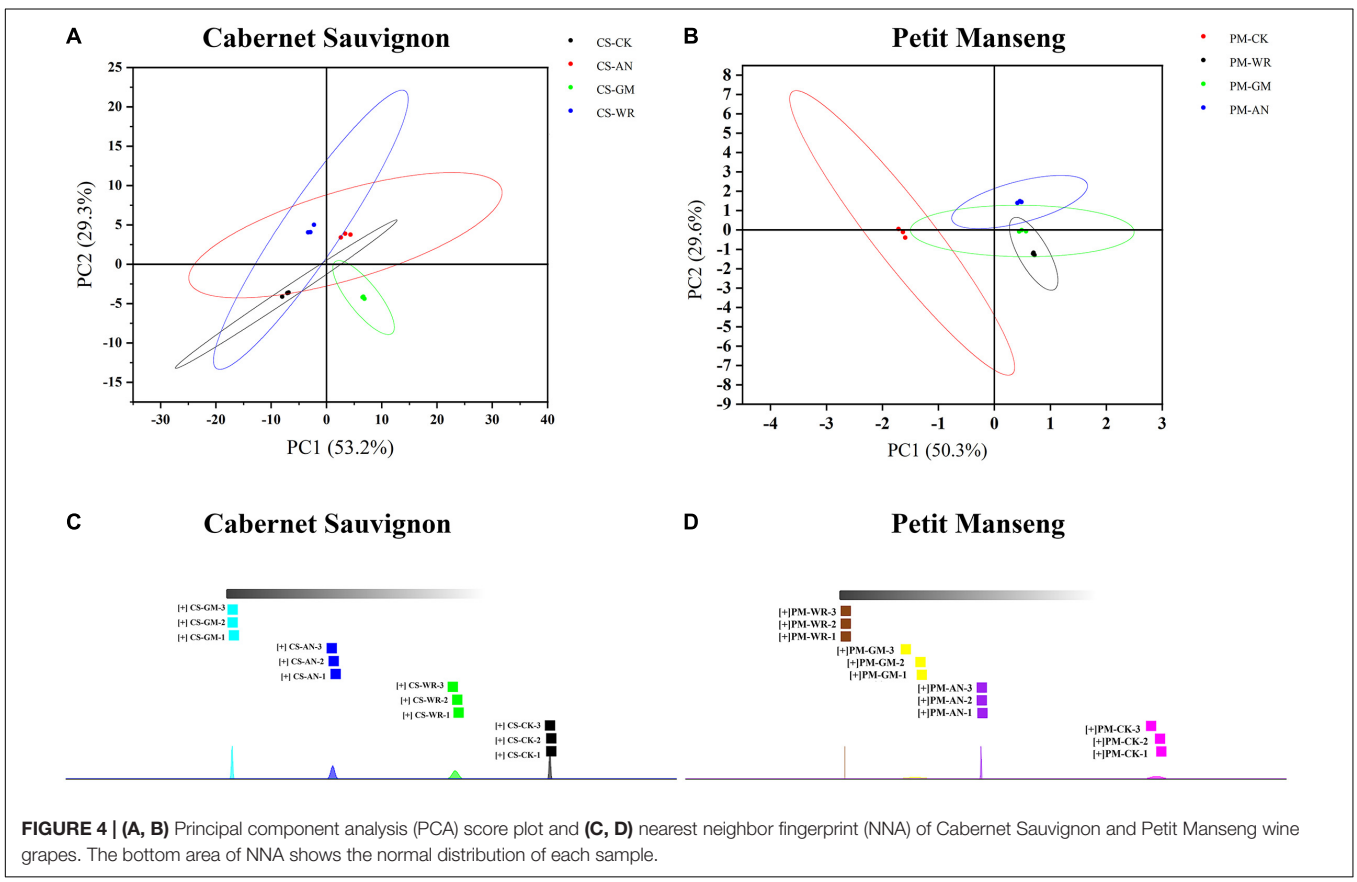

FIGURE 4 | (A, B) Principal component analysis (PCA) score plot and **(C, D)** nearest neighbor fingerprint (NNA) of Cabernet Sauvignon and Petit Manseng wine grapes. The bottom area of NNA shows the normal distribution of each sample.

important aromatic chemicals found in grapes, both in volatile and non-volatile forms, and are known for contributing fruity or flowery notes (González-Barreiro et al., 2015). However, these compounds are found in low concentrations as most of them have very low perception threshold levels (Diéguez et al., 2003). The results showed that the detected unknown compounds had low concentrations, so we speculated that these compounds might be terpenes and norisoprenoids. In CS and PM after infection, compound 10 increased, compound 6 decreased, and compounds 4, 5, 7, and 12 did not change greatly; other numbered compounds may not change consistently and may be due to varietal differences. Among them, in grapes suffering from a pathogenic fungal infestation, compound 11 increased in PM and slightly decreased in CS and compounds 1, 2, and 3 decreased in CS but did not change more in PM.

Differences in Volatile Organic Compounds Present in Different Varieties of Grapes Resulting From Pathogenic Fungal Infestations

The principal component analysis (PCA) and nearest neighbor fingerprint (NNA) (**Figures 4A–D**) intuitively showed the differences between different samples. Different color points represent different samples, the greater the distance between sample points is, the greater the difference is. The PCA of volatile compounds in both healthy and different infected samples was demonstrated in **Figures 4A,B**. The accumulative contribution of the first and second principal components in CS and PM was 82.5% (PC1 was 53.2% and PC2 was 29.3%) and 79.9% (PC1 was 50.3% and PC2 was 29.6%), respectively. The score map clearly illustrated the PCA, comparing the healthy and infected grapes of CS and PM; where the PC1 score variation could be considered as the positive and negative ranges. Meanwhile, the difference in infected samples with different pathogenic fungi could be separated by the different scores of PC1 and PC2. Moreover, the NNA was conducted for further analysis. The fingerprint of the aromatic components of CS and PM was shown in **Figures 4C,D**, according to the similarity of aroma profiles, the samples were divided into various groups. The results showed that the VOCs of CS-GM had the most significant difference compared with others. The difference between VOCs in CS was as follows: gray mold > anthracnose > white rot, while the difference between VOCs in PM was as follows: white rot > gray mold > anthracnose. Also, the VOCs of CS had more alterations after infection than PM. It is not difficult to explain because of the difference in varieties. The strong antibiosis resistance and freshness of PM may indicate that the VOCs have fewer changes after infection, and the total phenolics, total flavonoids, and tannins of healthy grapes in CS are higher than in PM. Therefore, it is normal to explain the different results when two grape varieties were infected with the same pathogenic fungal.

CONCLUSION

In this work, the effect of different pathogenic fungal infestations was investigated on berry quality and VOCs of CS and PM. The quality changed after pathogenic fungal infestation, including the content of VC, TF, and tannins, showed a downward tendency in most of the infected grapes, which is likely because the infestations interfered with the normal physiological metabolism of grapes and changed their composition. Meanwhile, higher levels of TA only appeared in disease-affected CS, and SSC decreased in disease-affected CS but increased in a disease-affected PM; these inconsistent results were speculated to be due to various factors such as varieties and resistance. The results of 100-berry weight showed that there was no significant difference, indicating that pathogenic fungal infestations had little effect on it. The VOCs were investigated by GC-IMS to determine the types and comparative content, a total of 61 VOCs were identified and then investigated from different functional groups, including C6-VOCs, alcohols, ester acetates, aldehydes, and acids. Hexanal and (*E*)-2-hexenal decreased in all infected grapes, which may be due to the C6-VOCs being manipulated to reduce the defense effect of the berry after pathogenic fungal infestation. In disease-affected CS, a higher level of 3-Methyl-1-butanol was related to the activity of laccases in *B. cinerea.*, and only increasing ethanol in disease-affected PM, which was speculated that spontaneous fermentation could explain it because there were more yeasts after infection. These unique VOCs may serve as hypothetical biomarkers to help us identify specific varieties of pathogenic fungal infestations. Furthermore, the results of PCA and NAA showed differences in VOCs present in different varieties of grapes resulting from pathogenic fungal infestation, which indicated that the VOCs of CS changed more than PM after infection, and the VOCs produced by different pathogenic fungal infestations were also different. The difference between VOCs in CS was as follows: gray mold > anthracnose > white rot, while the difference between VOCs in PM was as follows: white rot > gray mold > anthracnose. Finally, this study is beneficial for us to strengthen the understanding of pathogenic fungal infestations during the growth and development of grapes and explore the interaction between pathogenic fungal infestations and grapes. However, the mechanism of VOCs between grapes and pathogenic fungal infestations still needs further research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

XYL: methodology, data curation, and writing the original draft preparation. TL: data curation, resources, and formal analysis. ML: conceptualization, software, and writing the original draft preparation. DC: visualization and investigation. XWL: software and resources. SZ: formal analysis and data curation. XD: conceptualization and validation. JC: supervision, formal analysis, and resources. ZK: conceptualization, resources, writing, reviewing, and editing, project administration, and funding acquisition. JT: validation, writing, reviewing, and editing, supervision, and project administration. 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.2022.942487/full#supplementary-material

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Biological control and plant growth promotion properties of *Streptomyces albidoflavus* St-220 isolated from *Salvia miltiorrhiza* rhizosphere

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Root rot disease caused by *Fusarium oxysporum* is a devastating disease of *Salvia miltiorrhiza* and dramatically affected the production and quality of *Sa. miltiorrhiza*. Besides the agricultural and chemical control, biocontrol agents can be utilized as an additional solution. In the present study, an actinomycete that highly inhibited *F. oxysporum* was isolated from rhizosphere soil and identified as based on morphological and molecular characteristics. Greenhouse assay proved that the strain had significant biological control effect against *Sa. miltiorrhiza* root rot disease and growth-promoting properties on *Sa. miltiorrhiza* seedlings. To elucidate the biocontrol and plant growth-promoting properties of St-220, we employed an analysis combining genome mining and metabolites detection. Our analyses based on genome sequence and bioassays revealed that the inhibitory activity of St-220 against *F. oxysporum* was associated with the production of enzymes targeting fungal cell wall and metabolites with antifungal activities. Strain St-220 possesses phosphate solubilization activity, nitrogen fixation activity, siderophore and indole-3-acetic acid production activity *in vitro*, which may promote the growth of *Sa. miltiorrhiza* seedlings. These results suggest that *St. albidoflavus* St-220 is a promising biocontrol agent and also a biofertilizer that could be used in the production of *Sa. miltiorrhiza*.

KEYWORDS

biocontrol agents, plant growth-promotion, *Streptomyces albidoflavus*, *Salvia miltiorrhiza*, root rot disease

Introduction

Salvia miltiorrhiza is a well-important traditional Chinese medicinal plant with terrific economic, social, and medicinal benefits (Su et al., 2015). Its dried root, called Danshen for its medicinal use, has been used for hundreds of years (Jiang et al., 2019), primarily for the treatment of various cardiovascular and cerebrovascular diseases in China and other Asia countries. In addition, *Sa. miltiorrhiza* is also used as a health-promotion food (Shi et al., 2019). To fit the large demand of Danshen, the planting areas of *Sa. miltiorrhiza* has reached to 100 thousand hectares in China by the year of 2020. However, the production of *Sa. miltiorrhiza* was severely limited by root rot disease caused by *Fusarium oxysporum*. The average incidence of *Sa. miltiorrhiza* root rot disease in China is 10% ~ 30%. Moreover, in some plots where the disease severely happened, the incidence could reach to 80%, causing irreversible losses to farmers (Wang et al., 2018a).

Currently, the root rot disease on *Sa. miltiorrhiza* cannot be effectively controlled by using physical and chemical methods (Ye et al., 2003). Additionally, the long-term overuse of fungicides has caused many adverse effects on environment, animal and human health, soil quality, and pathogen controlling (Wang et al., 2014, 2018a; Raza et al., 2017). Consequently, it is important and urgent to develop alternative methods and agents that are less toxic and more effective in controlling root rot. Utilization of functional microbes that not only antagonistic to phytopathogens but also friendly to environment is considered an economical and effective method to control root rot disease and improve plant health. The use of functional microorganisms and their biological products can provide growers an option to not only avoid the problem of chemical residues on plants and soil, but also to reduce pathogen resistance (Handelsman and Stabb, 1996; Abbas et al., 2020; Sun et al., 2020). Strains of *Streptomyces* are considered as biocontrol agents due to their production of various active compounds with agricultural applications. In addition, they are able to survive in harsh environments and colonize the root of plants belonging to multiple species including *Sa. miltiorrhiza* (Suárez-Moreno et al., 2019; Jose et al., 2021; Wu et al., 2021). Moreover, *Streptomyces* strains have multiple strategies to suppress fungal pathogens such like nutrients competition, cell wall degradation, virulence factors degradation and plant immunity induction (Chen et al., 2018). Certain *Streptomyces* can also improve nutrient absorption and in turn boost plant development by producing auxins, solubilizing inorganic phosphate, fixing nitrogen and other methods (Goudjal et al., 2013; Vijayabharathi et al., 2015; Liu et al., 2016; Raaijmakers and Mazzola, 2016; Jones and Elliot, 2017). *Streptomyces* SCA2-4T, isolated from the rhizosphere soil of prickly pear (*Opuntia stricta*), exhibited a strong antagonistic activity against *F. oxysporum* f. sp. *cubense* tropical race 4 causing banana *Fusarium* wilt (Qi et al., 2021). *Streptomyces* NEAU-S7GS2 isolated from the root of soybean does not only prevent Sclerotinia stem rot of soybean, but also promotes the soybean growth (Liu et al., 2019). Therefore, *Streptomyces* species offers abundant resources of biofungicides or biofertilizers for agricultural usage (Liu et al., 2019).

In the present study, *St. albidoflavus* strain St-220 was isolated from the rhizosphere soil of *Sa. miltiorrhiza*, and was identified based on its morphological and molecular characteristics. Additionally, the plant growth-promoting activity and antifungal activity of St-220 was also evaluated *in vitro* and in greenhouse conditions. To demonstrate the antifungal and growth-promoting mechanisms, we carried out an analysis combining genome mining and metabolites detection based on the genome sequence of St-220.The pathways for synthesis of secondary metabolites including antibiotics and plant growth-promoting compounds were investigated, and genes encoding the antifungal enzymes were also predicted. These results provided essential and deep insights into the biocontrol properties of *St. albidoflavus* St-220.

Materials and methods

Actinomyces and *Fusarium* strains

Salvia miltiorrhiza along with the rhizosphere soil were collected from *Sa. miltiorrhiza* plantation in Laiwu City, Shandong Province, China (36°18′N 117°50′E). The rhizosphere soil of *Sa. miltiorrhiza* were obtained from the root surface. The isolation of actinomycetes was performed according to the methods described previously with modifications (Wang et al., 2021). Briefly, 10 ml of soil suspension containing 1 g rhizosphere soil and 10 ml sterile water was incubated in a shaker at 100 rpm for 30 min, then diluted into 10^{-3} g/ml, 10^{-4} g/ml, and 10^{-5} g/ml. Two hundred microliters of the diluted suspension were added to Gause's agar medium (containing 2% soluble starch, 0.051% K_2HPO_4, 0.025% $MgSO_4$, 0.001% $FeSO_4$, and 2% Agar B, pH 7.2–7.4) amended with 20 μg mL^{-1} nalidixic acid, respectively, and cultured at 28°C. For purification, single colonies grown on the plates were separately transferred to another plates and then stored at −80°C in 20% glycerol. The phytopathogenic fungi *F. oxysporum* was isolated from plant tissues of *Sa. miltiorrhiza* with root rot disease collected from a field in Yuzhou, Henan, in August 2019.

Antagonistic effects of *Streptomyces* strains on *Fusarium oxysporum*

The inhibition ability of *Streptomyces* against *F. oxysporum* was determined using the conventional improved scribe inoculation method (Chen et al., 2018). A mycelium plug of *F. oxysporum* in the center of potato dextrose agar (PDA) plates. *Streptomyces* strains were inoculated by streaking symmetrically at the two sides of the plug, 25 mm to the plate center. Petri dishes not inoculated with *Streptomyces* were used as controls, and three times each experiment was performed. After incubation for 5 ~ 7 days at 28°C, the colony diameters were measured, and the growth

inhibition (GI) was calculated according to the following formula (Qi et al., 2019):

$$\text{Growth inhibition}\ (\text{GI}) = \left[(\text{D} - \text{d}) / \text{D}\right] \times 100\%$$

where D and d represented the diameters of fungal colonies on the control and treated plates, respectively.

Control effect of St-220 on *Salvia miltiorrhiza* root rot disease in greenhouse condition

Before planting, *Sa. miltiorrhiza* seeds were soaked in 75% ethanol for 5 min, and then soaked in 5% bleach for 10 min for surface disinfection. After rinsed with sterile water for three times, the seeds were placed in a culture bottle with a sterile mixture of soil and vermiculite (2:1). To make inoculum, a mycelium plug of *F. oxysporum* was inoculated in PDA liquid culture and incubated in a dark shaker at 28°C 180 rpm for 10 days, then the culture was cloth-filtered and the flow-through was saved as spore suspension, which was then adjusted to 1×10^7 cfu/ml for use. To make cell suspensions of strain St-220, 500 μl of glycerol suspension was inoculated in 500 ml Gause's liquid medium and incubated at 28°C 160 rpm for 10 days. The two-leaf *Sa. miltiorrhiza* seedlings were inoculated by drenching with 10 ml inoculum of *F. oxysporum* (Fo), 10 ml St-220 cell suspension mixed with 10 ml inoculum of *F. oxysporum* (Fo + St), and 10 ml of sterile water (CK), respectively. The inoculated seedlings were grown in a growth chamber with temperature of 30°C/26°C, photoperiod of 12/12 h and 50% humidity. At 30 days after inoculation (DAI), disease symptoms were observed and evaluated using a severity scale: 0 for no symptoms; 1 was suffered disease symptoms less than 20% (only 1 leaf yellowing or wilting); 2 and 3 were plants suffering from disease symptoms in the range of 20%–40% (more than 2 but less than half of the leaves turn yellow or wither) and 40%–80%, respectively; 4 was *Sa. miltiorrhiza* showing severe disease symptoms with only the top 1 to 2 leaves being healthy; level 5 was plants that have died (Li et al., 2022). The disease index (DI) was calculated based on the formula $DI(\%) = \sum\left[(A \times B) \times 100\right] / (C \times 4) \times 100$, where A is the disease scale (0, 1, 2, 3, 4, and 5), B is the number of seedlings at each level of the scale, and C is the total number of seedlings for each treatment. Disease incidence and control efficiency were calculated according to the following formulas:

$$\text{Disease incidence}\,(\%) = \frac{(\text{number of yellow leaves})}{(\text{total plant leaves})} \times 100$$

$$\text{Control efficiency}\,(\%) = \frac{\left(\begin{array}{c}\text{DI of control group} - \\ \text{DI of treatment group}\end{array}\right)}{(\text{DI of control group})} \times 100$$ (Li et al., 2022).

Plant traits including fresh and dry weight of the root and shoot of the seedlings, and the diameter and length of the roots were measured at 30 DAI. Ten seedlings in five culture bottles were inoculated for each treatment, and the experiment was repeated for three times.

In vitro assessment of plant growth promotion traits

To evaluate the growth-promoting properties, the Phosphate solubilization, biological nitrogen fixation, siderophore and indoleacetic acid (IAA) production of the St-220 strain was determined. For this purpose, St-220 was cultured in 100 ml Gause's liquid medium for 5 days at 28°C in an orbital shaker (150 rpm.), and each assay was performed with three biological replicates for each strain.

Phosphate solubilization

An improved Pikovskaya (PVK) solid medium was used to evaluate the ability of strain St-220 on insoluble organic phosphate solubilization. The plate was inoculated with strain St-220 and kept at 28°C for 7 days. Positive phosphate solubilization was evident by a clear halo around strain St-220 (Gupta et al., 1994). Plates inoculated with sterile water were as control. The experiment was repeated three times.

Biological nitrogen fixation

Assay for nitrogen-fixing activity of the strains was performed according to a modified procedure described previously (Roy, 1958): strain St-220 colony was inoculated on nitrogen-free agar medium (Ashby's Nitrogen-free medium) and then incubated at 28°C 7 days for 3 times. That the strain grew after three consecutive transfers indicated nitrogen fixation activity.

Siderophore production

Chrome azurol blue agar was used to assess siderophore production, and the pH was adjusted to 7.2 with KOH as suggested previously (Schwyn and Neilands, 1987). The presence of a yellow halo indicates the production of siderophores.

Indoleacetic acid production

The IAA production activity of St-220 was determined by the method of Salkowski colorimetry (Tang and Bonner, 1948). The activated St-220 was inoculated to 0.5 g/L Gause's agar liquid medium containing tryptophan, and then cultured at 28°C, 150 r/min in a shaker for 7 days to obtain the fermentation broth. One milliliter of the broth was centrifuged at 12,000 rpm for 5 min, then the supernatant was mixed with 2 ml Salkowski reagent containing 15 ml concentrated H_2SO_4, 25 ml distilled water and 0.75 ml of 0.5 M $FeCl_3.6H_2O$ (de Oliveira-Longatti et al., 2014).

After incubation in darkness at room temperature for 30 min, the mixture turned pink when IAA was generated. Serial dilutions

of a standard IAA solution (0, 5, 10, 15, 20, 25, 30, 35, and 40 μg/ml) were used to construct the calibration plot (Abbasi et al., 2019).

Plant growth-promotion experiments

Seeds of *Sa. miltiorrhiza* were disinfected as described previously, and then planted in pots (7 cm × 7 cm × 10 cm) containing 100 g of sterilized soil substrate (nutrient soil: vermiculite = 1:1) for germination. When grew two leaves, seedlings that are similar in height were selected for use. To make inoculum, strain St-220 was grown on Gause's liquid medium and incubated at 28°C 160 rpm for 10 days, then the harvested cell suspension was adjusted to 1×10^7 cfu/ml. For inoculation, 20 ml of St-220 inoculum were applied to each pot, and 20 ml of sterile water was separately applied as negative control. The seedlings were inoculated every 10 days until the plant traits were investigated. Five replicates for each treatment, and the experiment was repeated three times. All the pots were placed in growth chamber at 30/26°C and 12/12 h, 50% humidity. Plant traits including root and shoot fresh weight, total dry weight, length and diameter of the root was measured at 40 DAI.

Sequencing, assembly, annotation, and bioinformatics analysis of the genome of St-220

DNA extraction

To obtain the genomic DNA, a single colony of St-220 was transferred to Gause's liquid medium and then incubated at 28°C at 160 rpm for 5 days. The obtained cell suspension was then centrifuged and the supernatant was yield for DNA extraction by the SDS method (Lim et al., 2016). The DNA purity and quantity were examined by using Qubit® 2.0 Fluorometer (Thermo Scientific). The 16 s rDNA of St-220 was sequenced and compared to existing databases for identification.

Sequencing, assembly, and annotation

The obtained genomic DNA of St-220 was used for the whole genome sequencing by using the Illumina NovaSeq PE150 sequencing platform at Novogene Technology Co., Ltd. (Beijing, China). A series of *de novo* assemblies were carried out with different software (SOAP, SPAdes; Li et al., 2008; Simpson et al., 2009; Bankevich et al., 2012). The protein coding genes (CDSs), rRNA and tRNA were predicted by Glimmer version 3.02, RNAmmer 1.2 and tRNA-scan-SE version 2.0, respectively, (Lowe and Eddy, 1997; Delcher et al., 2007; Lagesen et al., 2007). For gene annotation, BLAST searches was carried out in several databases including NCBI Non-redundant (NR), Clusters of Orthologous Groups (COG; Jensen et al., 2007), Pfam (Finn et al., 2014), Swiss-Prot (Zhou et al., 2021), Carbohydrate-Active enZYmes (CAZy; Zhang et al., 2018), Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2006) and Gene Ontology (GO; Ashburner et al., 2000). The online software antiSMASH version 6.0 was employed to definite antibiotic and secondary metabolite gene clusters (Blin et al., 2021).

Identification and characterizations of strain St-220

Phylogenetic analyses

For identification of St-220, a phylogenetic tree was constructed based on the 16S rDNA and five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) concatenated sequences. Multiple alignment of the sequences and construction of phylogenetic tree using maximum likelihood were generated by using Clustal X (Larkin et al., 2007) and PhyloSuitev1.2.2 (Zhang et al., 2020), respectively. Calculation of orthoANI values (orthologous average nucleotide identity) was performed by JSpeciesWS (Richter et al., 2016) and an online tool ANI-Blast (ANIb) Calculator. The ANIb values were used for assessing two strains are same species. A Genome-to-Genome Distance Calculator (GGDC) web server version 3.0 (Rigden and Fernández, 2022) was used to determine DNA–DNA hybridization (DDH) values *in silico*.

Cultural and morphological characterizations

The morphological characteristics of the St-220 strain were observed under scanning electron microscopy (SEM; model S-3400 N, Hitachi, Ltd., Tokyo, Japan) when grown on PDA medium for 14 days. The mycelium and substrate mycelium characteristics of St-220 were investigated after incubation at 28°C for 14 days on PDA and Gause's agar medium, respectively.

Statistical analysis

Statistical analyses including Student's *t*-tests and ANOVA with Dunnett's test were performed with R scripts. Difference was considered significant when the p value was <0.05.

Results

In vitro antagonistic effects of *streptomyces* strains against *Fusarium oxysporum*

A total of 163 strains of actinomycetes were isolated from the rhizosphere soil of *Sa. miltiorrhiza* in the plantation, and 11 strains showed an inhibitory effect on *F. oxysporum*, of which strain St-220 showed the most obvious inhibitory effect on mycelia growth of *F. oxysporum* (Supplementary Table S1). After 7 days incubation, the *F. oxysporum* incubated with St-220 showed narrow and oval colonies compared to the negative control (Figure 1A). To calculate the inhibition rate, the mean diameters of mycelia colonies were estimated by measuring the perpendicular length of each colony. The mean diameter of *F. oxysporum* mycelia colonies reached to

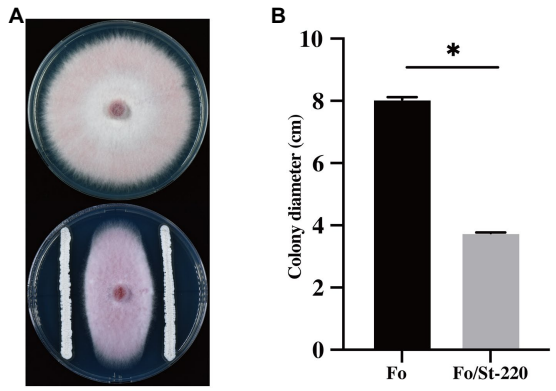

FIGURE 1
The antifungal activity of strain St-220 against *Fusarium oxysporum*. **(A)** *F. oxysporum* colony grew on PDA plate alone (upper) and with St-220 (lower) at 7days after inoculation. **(B)** Colony diameter of *F. oxysporum* in each treatment. Bars with ∗ above are statistically different (p<0.05).

8.50 cm, while that of *F. oxysporum* grown with St-220 reached to 3.46 cm, with an inhibitory rate of 53.40% (Figure 1B).

Control effect of St-220 on root rot disease of *Salvia miltiorrhiza* in greenhouse condition

After treated with cell suspension of strain St-220 for 30 days, *Sa. miltiorrhiza* seedlings in the pathogen treatment group displayed morphological indications of disease, with leaves turning yellow and wilting and roots rotting (Figure 2A). The disease incidence and disease index of the treatment group inoculated with *F. oxysporum* (Fo) were 86.67% and 68.00%, respectively, while the disease incidence and disease index of the treatment group inoculated with *F. oxysporum* and strain St-220 (Fo + St) were 20% and 22.66%, respectively. Strain St-220 significantly ($p < 0.05$) reduced disease incidence by 76.92% and disease index by 66.67% (Supplementary Table S2). Compared with the treatment Fo, the total fresh weight (Figure 2C), dry weight (Figure 2D), shoot height and root length (Figures 2B,E) of the Fo + St treatment significantly increased by 138.45%, 39.73%, 137.43%, and 72.12%, respectively. Meanwhile, root fresh weight, root dry weight, and root diameter were also increased (Figures 2C–F). Therefore, St-220 has the biological control impact on *Sa. miltiorrhiza* root rot in greenhouse condition.

Biological characteristics involved in plant growth-promoting activity of St-220

To explore the potential mechanism of St-220 on plant growth-promoting activity, four biological characteristics of strain St-220 were tested. In phosphate solubilizing activity assay, a distinct circle around the colony was generated after 7 days of strain St-220 growing on PVK medium (Figure 3A), demonstrating that strain St-220 possessed phosphate solubilizing activity. Strain St-220 was able to grow on Ashby's nitrogen-free medium after 3 successive transfers suggesting nitrogen-fixing activity (Figure 3B). The siderophore generating carrier activity of strain St-220 was indicated by the creation of a prominent yellow halo surrounding the colony after 7 days of growth in Chrome Azurol Blue agar (Figure 3C). The IAA production activity of strain St-220 was also determined (Figure 3D). Strain St-220 produced maximum 30.40 μg/ml of IAA at 7 DAI, according to a standard curve based on series dilution [$y = 0.0094x + 0.0430$ ($R^2 = 0.9735$, where y is the absorbance value at wavelength of 530 nm, x is the concentration of IAA)] (Supplementary Figure S1).

Plant growth-promotion activity of St-220 on *Salvia miltiorrhiza*

To investigate the growth-promoting impact of strain St-220 on *Sa. miltiorrhiza*, a greenhouse experiment was performed and the plant traits was assessed at 40 DAI. The results suggested that strain St-220 was able to stimulate *Sa. miltiorrhiza* growth in contrast to non-inoculated plants, since it exhibited increases in shoot height and fresh weight in roots and plants. St-220 significantly increased the root fresh weight, total fresh weight, total dry weight and root dry weight of *Sa. miltiorrhiza* seedlings by 85.22%, 105.50%, 60.88%, and 36.72%, respectively (Figure 4A). Shoot length and root length also showed an increase (Figures 4B-D).

Identification of St-220 strain

After 2-week incubation on PDA, the colony morphology of St-220 revealed a firm surface with white aerial mycelia and faintly whitish-yellow spores (Figure 5A), which is consist with typical morphological characteristics of the *Streptomyces* genus. Both substrate and aerial mycelia were grown well without fragmentation. The flexuous spore chains formed by cylindrical spores were observed under our scanning electron microscope observation (Figure 5B).

The 16S rDNA sequence of St-220 was amplified by PCR and sequenced, and in turn searched in the EzTaxon database, and the strains with high similarity were screened. The sequences of the 16S rDNA and 5 housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) were concatenated and used to construct a phylogenetic tree using the Maximum-Likelihood method with 1,000 bootstraps. The results suggested that strain St-220 and *St. albidoflavus* clustered into a same clade (Figure 5C). To further confirm our result, the Average Nucleotide Identity (ANI) and DNA–DNA hybridization (DDH) values between St-220 and other 13 *Streptomyces* strains were calculated. The genome of

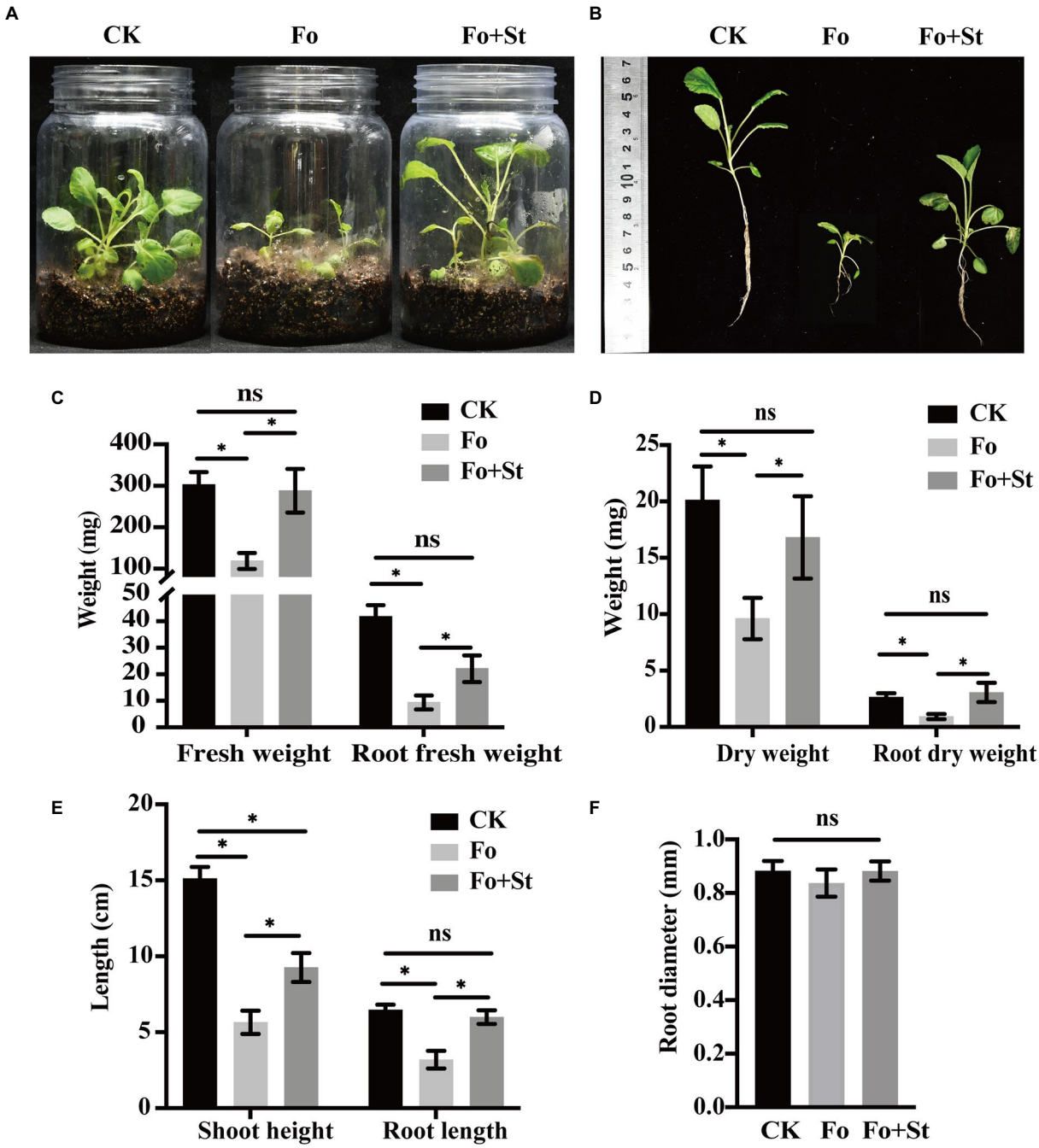

FIGURE 2
Control effect of St-220 on root rot disease of *Salvia miltiorrhiza* seedlings. **(A)** Symptoms of root rot developed on seedlings inoculated with *Fusarium oxysporum* (Fo) and mixture of *F. oxysporum* and St-220 (Fo+St) at 30 DAI, while no symptoms were observed on seedlings inoculated with sterile water (CK). **(B)** The entire plant of the seedlings in CK, Fo, and Fo+St treatment. Measurement of the fresh weight **(C)**, dry weight **(D)**, shoot height, root length **(E)**, and root diameter **(F)** of seedlings inoculated. Data are mean±SE (n=10). Means were compared with ANOVA analysis in combination with Tukey post-test. Means were considered statistically different when $p<0.05$, Bars with * above are statistically different, ns above are not statistically different.

St. albidoflavus showed the highest ANI and DDH value of 98.87% and 93.90, among the test strains, respectively, (Supplementary Table S3), which was greater than the threshold value of 95%~96% and 70 for species delineation (Richter and Rosselló-Móra, 2009). Altogether, strain St-220 is recognized as a new member of the *St. albidoflavus* species.

Genome features of St-220

To have a deep insight in the molecular mechanisms of inhibitory effect and plant growth-promoting, the whole genome of St-220 was sequenced and analyzed. After adapter trimming, the reads were *de novo* assembled into 175 contigs. The genome

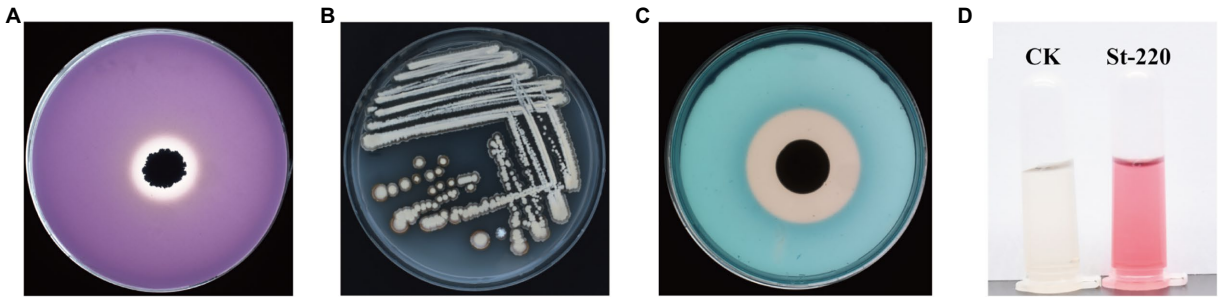

FIGURE 3
Evaluation of strain St-220 for key traits related to direct plant growth-promotion. **(A)** Qualitative phosphate solubilization assay. **(B)** Biological nitrogen fixation activity assay. **(C)** Siderophores qualitative production assay. **(D)** Production of indole acetic acid activity assay.

size of St-220 is 7,310,412 bp with G + C content of 73.41%. The whole genome sequence for St-220 have been deposited in the GenBank database with accession number of JAMFMD000000000. Genomic analysis revealed that the genome of St-220 contained 6,327 CDSs accounting for ~85.43% of the genome (Table 1).

Functional analysis revealed that 5,148, 4,152, 4,798 out of the 6,244 identified CDSs were assigned to COG, GO, and KEGG categories, respectively. In COG categories, the highest ratio the metabolism process was assigned gene numbers with ratio of 36.77%, followed by the category of information storage and processing (17.89%), and the category of cellular processes and signaling (28.61%; Figure 6A). Gene ontology analysis revealed that the category of biological process contained the most GO terms and genes (8,166), followed by molecular function (5,437) and cellular component (2,872; Figure 6B). KEGG pathway analysis showed that the metabolism pathway had the most genes involved, followed by the pathway of environmental information processing (Figure 6C). Additionally, 274 genes were identified in CAZy database and classified into six families. A total of 111 proteins were predicted as belonging to the Glycoside Hydrolase family, of which 88 to Carbohydrate-Binding Modules, 47 to Glycosyl Transferases, 20 to Carbohydrate Esterases, 7 to Auxiliary Activities, and 1 to the Polysaccharide Lyases family (Figure 6D).

Genome analysis of secondary metabolite clusters

In our genome mining analysis, the strain St-220 was predicted on produce a plenty of secondary metabolites. By using the antiSMASH, 21 gene clusters for secondary metabolites biosynthesis were predicted and found located in the chromosome of St-220 (Supplementary Table S4), of which 10 gene clusters were involved in the biosynthesis of metabolites antimicrobial activities including ectoine, desferrioxamine B, surugamide A, antimycin, geosmin, indigoidine, isorenieratene, and candicidin (Figure 7). Furthermore, the Region 12.1 was predicted as involved in the desferrioxamine B and E biosynthesis (Supplementary Table S4), which participated the removal of excess iron in the environment.

Genes associated with fungal cell wall degrading enzymes

The genome of strain St-220 harbors 15 genes encoding enzymes involved in chitin degrading, including six β-N-acetyl hexosaminidase, eight chitinases, and one chitosanase. In addition, St-220 has four chitin-binding proteins belonging to the AA10 family, which enhance the binding abilities of enzymes to insoluble substrates. Four genes in the genome of St-220 were further found to encode endo-1, 3-β-glucanase for degradation of glucan (Supplementary Table S5). Moreover, St-220 contains various genes encoding enzymes that play roles in the degradation of cellulose, protein, and lipids (Supplementary Table S5).

Genes associated with plant growth-promotion

Our genomic analysis identified several genes related to the plant growth-promoting activities of St-220. These genes participated in 3 trp-dependent biosynthesis pathways of indole-3-acetic acid, including the indole acetamide (IAM), the tryptamine (TAM) and the indole-acetonitrile (IAN) pathways. In the IAM pathway, tryptophan is converted to IAM by tryptophan monooxygenase enzyme, and then amidase enzyme converts IAM to IAA. Nine encoding genes associated with the IAM pathway were found in the St-220 chromosome, of which six encoding tryptophan 2-monooxygenase and three encoding amidas (Supplementary Table S6). In the TAM pathway, tryptophan is firstly converted to TAM, then amine oxidase converts TAM to indole-3-acetaldehyde (IAAld), and finally IAAd is converted to IAA by aldehyde dehydrogenase. Two genes encoding monoamine oxidase and four genes encoding aldehyde dehydrogenase were found to be present in the genome of St-220. The fact that St-220 harbors two separate pathways for IAA biosynthesis suggested that the IAA production plays a role in life maintenance and plant growth-promoting activity. Moreover, St-220 also contains a gene encoding putative1-aminocyclopropane-1-carboxylic acid (ACC) deaminase involved in the decomposition of ACC

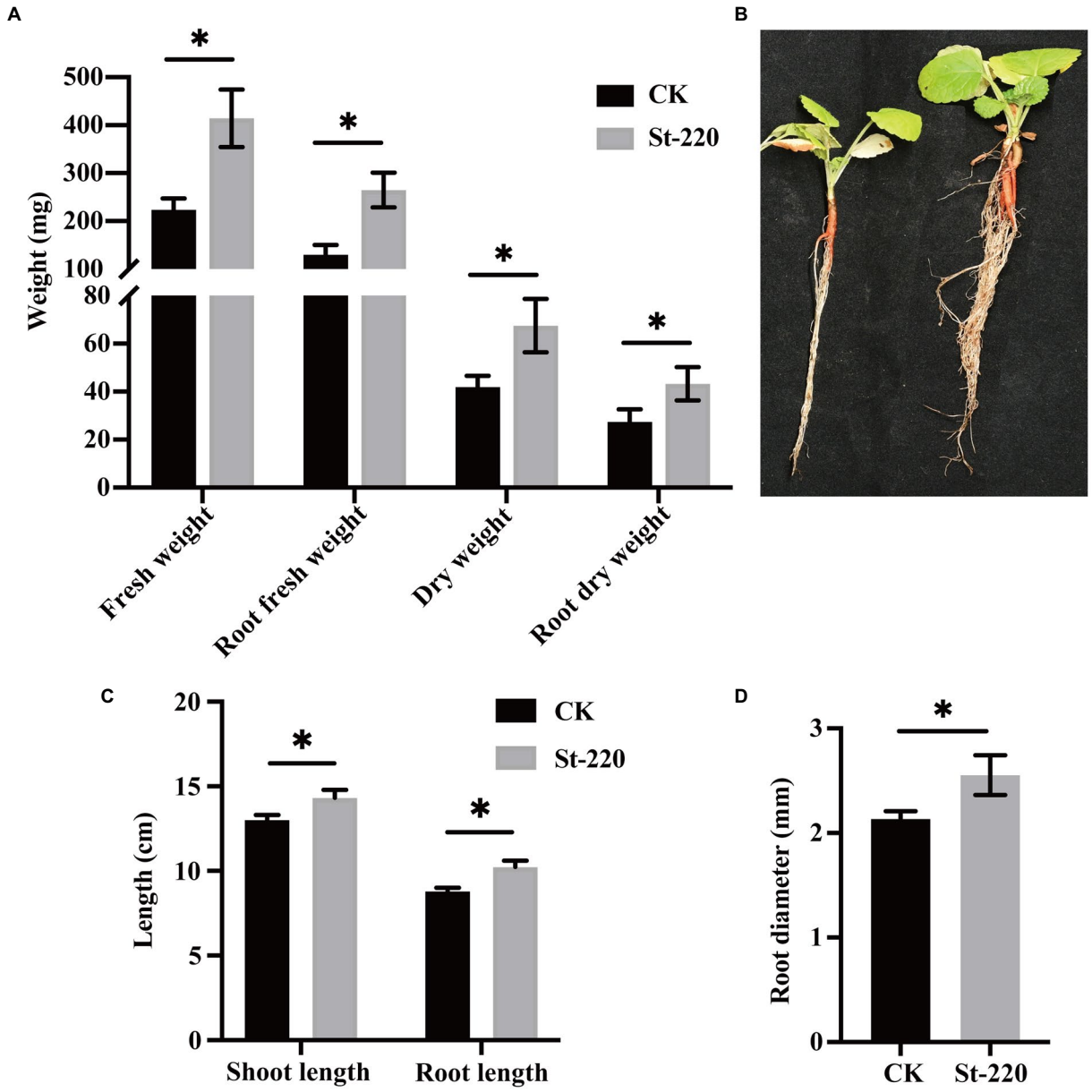

FIGURE 4
The growth-promoting effect of St-220 on *Salvia miltiorrhiza* seedlings. The growth-promoting activity of strains St-220 was measured under greenhouse conditions, and the data were recorded at 40days after inoculation. **(A)** the biomass of *Sa. miltiorrhiza* seedlings. **(B)** overall development of *Sa. miltiorrhiza* seedlings inoculated with sterile water (left) and St-220 (right). The shoot height, root length **(C)** and root diameter **(D)** of *Sa. miltiorrhiza* seedlings inoculated with sterile water and cell suspension of St-220. Data are mean±SE (n=10). Means were considered statistically different when $p<0.05$, Bars with ∗ above are statistically different, ns above are not statistically different.

(Supplementary Table S6) and we made a case that the St-220 could improve the ability of plants to survive under stress conditions by inhibiting ethylene synthesis.

The genome of strain St-220 contains multiple genes involved in the degradation of inorganic polyphosphates and the dissolution of organic phosphates, including a *ppx* gene encoding exopoly phosphatase, a *ppa* gene encoding inorganic pyrophosphatase, and three *phoD* genes encoding alkaline phosphatase. Furthermore, a *pstABCS* cluster involved in the transport and degradation of phosphonates is found in the chromosome of St-220 (Supplementary Table S7).

The genome of Strain St-220 contains one nitrogen fixation protein NifU, and an ammonium transporter protein that was involved in the ability of nitrogen fixation. The strain St-220 genome also contains nine nitrate reductase genes (Supplementary Table S8).

The St-220 genome harbors plenty of genetic elements involved in siderophore biosynthesis and iron complex transport

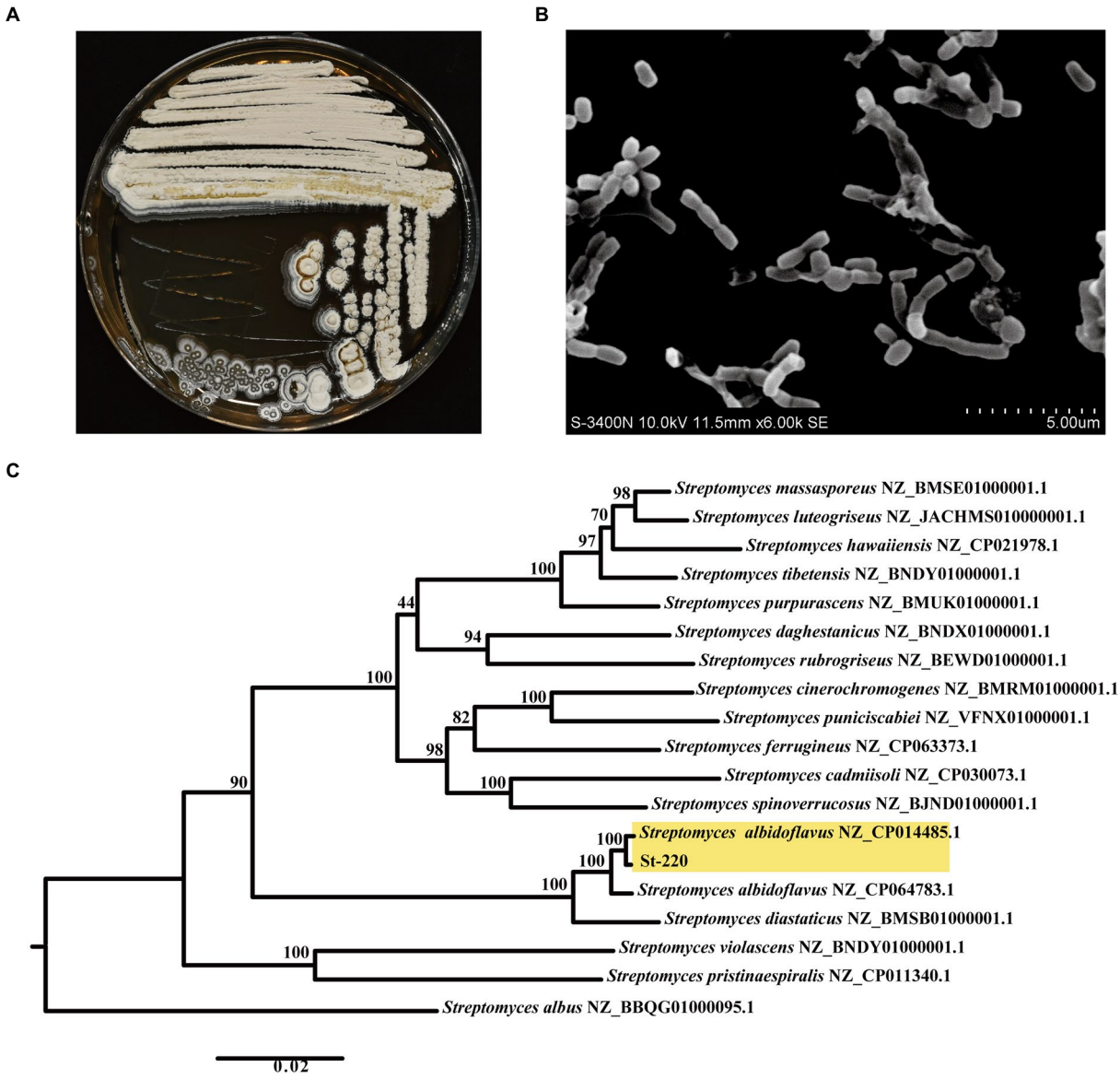

FIGURE 5
Morphological and molecular identification of strain St-220. **(A)** Colony morphology of the strain St-220 on PDA medium after 14days of incubation at 28°C. **(B)** Spores of St-220 observed under scanning electron microscope after incubated on PDA medium for 14days at 28°C. **(C)** A phylogenetic tree using the maximum likelihood method based on the sequences of 16S rDNA and 5 housekeeping genes with 1,000 bootstraps.

(Supplementary Table S9). Moreover, one cluster involved in siderophore biosynthesis is also present in the chromosome sequence of St-220.

Discussion

Root rot disease caused by *F. oxysporum* is one of the most severe soil-borne disease worldwide, and also the main constraint of *Sa. miltiorrhiza* production in China. In the present study, an actinomycete strain St-220 with biocontrol activity was isolated from roots of *Sa. miltiorrhiza* and identified as *Streptomyces albidoflavus*. The strain showed inhibition rate of 53.40% against *F. oxysporum* in the dual culture assay and control effect of 77.33% on root rot disease incidence in greenhouse condition. In addition, *St. albidoflavus* St-220 strain also promoted the growth of *Sa. miltiorrhiza* by increasing biomass including total fresh weight, root fresh weight, total dry weight and root dry weight, as well as shoot and root length. These results indicate that *St. albidoflavus* St-220 is a promising biocontrol agent for the control of root rot disease and biofertilizer for *Sa. miltiorrhiza*.

TABLE 1 Genome features of *Streptomyces albidoflavus* St-220.

Features	Genome
Genome size (bp)	7,310,412
Gene Number	6,327
Gene total length	6,245,418
G + C content (%)	73.58
Genome coverage	85.43
Contings	175
Contings N50 (bp)	71,800
Number of ORFs	6,327
tRNA genes	65
rRNA genes	6
CRISPRs	48
Genomic island	10
Genome accession number	JAMFMD000000000

Streptomyces albidoflavus St-220 have both biological control activity and plant growth-promoting activity

Some *Streptomyces* strains could significantly improve the biocontrol of *Fusarium* root rot disease and promote the growth of plant seedlings (El-Tarabily et al., 2009; Goudjal et al., 2016; Tamreihao et al., 2016; Chen et al., 2021). They were generally identified by three properties: IAA production, the abilities to solubilize phosphate and fix nitrogen, and siderophores production (Vurukonda et al., 2018). IAA is a phytohormone that regulates the growth of plant roots by stimulating the development of root (Lwin et al., 2012), and is also an important trait of plant growth-promoting microorganism. Tomato seedlings significantly increased in fresh and dry weight after treated with IAA producing strain *S. fradiae* (Myo et al., 2019). Phosphorus as a macronutrient is dispensable for plants (Ågren and Weih, 2020). Most of the phosphorus, however, present in the form of insoluble in the soil, and cannot be directly utilized by plants (Rawat et al., 2021). The *Streptomyces* strains with growth-promoting activity can dissolve the insoluble phosphate for plant growth. Inoculation of *Streptomyces* sp. strain 7.1 with inorganic phosphate solubilizing activity significantly increased the fresh weight of roots and stems of rice (Suárez-Moreno et al., 2019). Nitrogen is critical to whole life cycle of plants. The atmospheric nitrogen was transformed into ammonia that could be utilized by plants through nitrogen fixation (Dobbelaere et al., 2003). The siderophores secreted by biocontrol agents could suppress the pathogen and protect plants from pathogen infection by iron-competition and restructuring rhizosphere microbiome (Amano et al., 2011; Yu et al., 2013; Gu et al., 2020). For example, the endophytic *Streptomyces* strains SNL1 and SNL2 producing siderophores have antagonistic activities against *F. oxysporum* f. sp. *cubenese* causing *Fusarium* wilt of banana (Cao et al., 2005). *Streptomyces* can further promote plant mineral nutrient supply by synthesizing siderophores. *Streptomyces* sp. GMKU 3100 producing siderophore was able to promote the growth of rice and mung bean, whereas its siderophore-deficient mutant did not differ from the uninoculated control (Rungin et al., 2012).

Previous studies have revealed that *Streptomyces* strains with above properties showed plant growth-promoting activity. *S. violaceusniger* AC12AB was found to have properties of IAA production, siderophores production, nitrogen fixation and phosphates solubilization. It significantly promoted the potato crop up to 26.8% in field trial (Sarwar et al., 2019). Barley plants inoculated with *S. roseocinereus* MS1B15, a strain with IAA-producing, phosphate solubilizing, and nitrogen-fixing activity, significantly increased shoot and spike length (Chouyia et al., 2020). In this study, application of the St-220 resulted in a significant increase in the biomass of *Sa. miltiorrhiza* seedlings. To elucidate the way that the St-220 promotes the growth, the activities of IAA production, phosphorus solubilization, nitrogen fixation and siderophores production was determined and the synthesis pathway was found in further genomic analysis.

Genomic analysis revealed the potential antifungal and root growth-promoting mechanism of St-220

The strains of *Streptomyces* genus employ their secondary metabolites as weapons to inhibit phytopathogenic fungi (Amin et al., 2021; Hotta, 2021; Mahasneh et al., 2021; Terra et al., 2021). In this study, genome sequencing revealed that the chromosome of the *St. albidoflavus* St-220 contained 21 conserved biosynthesis gene clusters (BGCs), of which 10 showed high similarities in structure with known BGCs encoding terpenes, non-ribosomal peptides, polyketides, siderophores, and ectoines, which had been proven to participate in the regulation of antimicrobial activities of *Streptomyces* strains (van Bergeijk et al., 2020). Among these compounds, the surugamide A, indigoidine Antimycin and Candicidin SF2768 were found to have antifungal activities (Xu et al., 2017; Santos-Beneit et al., 2022), indicating the potential mechanism of the inhibitory effect of St-220 against *F. oxysporum*.

Chitin, the most important component of fungal cell wall, is the preliminary target that biocontrol agents aim at. *Streptomyces* strains produce chitinases to break through the fungal cell wall. For instance, *S. griseus* secret ChiIS, which belongs to glycosyl hydrolase family 19, to inhibit the growth of *Aspergillus nidulans*, *F. culmorum*, and *S. sclerotiorum* (Hoster et al., 2005). Chitinase produced by *Streptomyces* sp. TK-VL_333 showed antifungal activity against *F. oxysporum* (Kavitha and Vijayalakshmi, 2011). The purified and crude chitinase from *S. luridiscabiei* U05 inhibited the growth of *F. oxysporum* and *Alternaria alternata* (Swiontek Brzezinska et al., 2019). In this study, multiple genes (chitinases, β-N-acetyl hexosaminidase, chitosanase) encoding

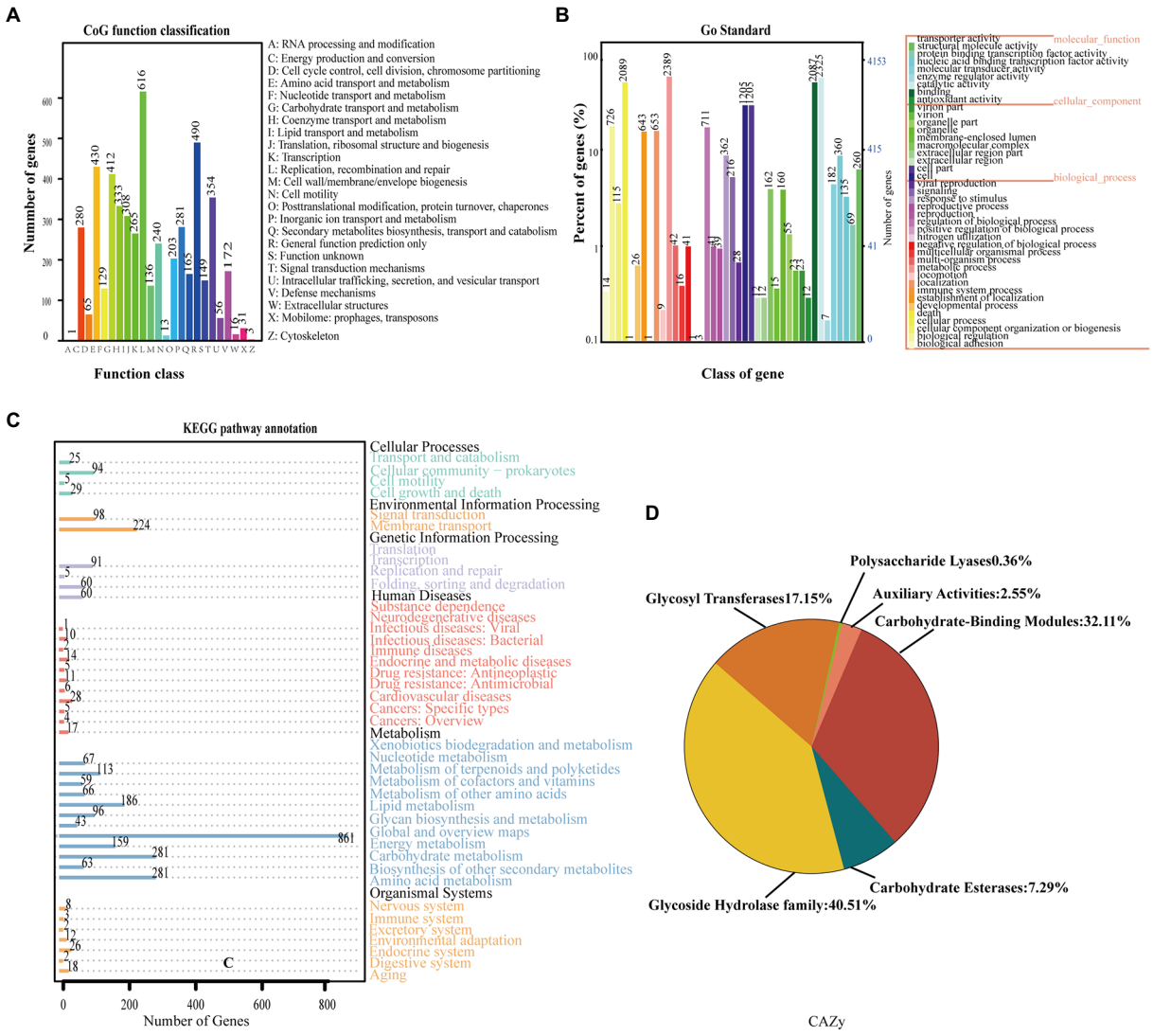

FIGURE 6
Analysis of genome structure and metabolic pathway of strain *Streptomyces albidoflavus* St-220. **(A)** COG annotation of strain *St. albidoflavus* St-220. genome. **(B)** GO functional categories of *St. albidoflavus* St-220. **(C)** Pathway annotation of strain *St. albidoflavus* St-220 genome according to the KEGG database. The vertical axis represented the level two classification of KEGG pathway. The horizontal axis represented the gene number annotated in this classification. Different colors of the columns represented different classifications of KEGG pathway. **(D)** Gene count distributions of carbohydrate-active enzyme (CAZy) families.

enzymes involved in chitin degradation were found in the genome of *St. albidoflavus* St-220, indicating that the St-220 deployed several weapons targeting the fungal cell wall for its biocontrol effect.

The genome mining has also confirmed the potential mechanism of *St. albidoflavus* St-220 on promoting root growth of *Sa. miltiorrhiza*. In our greenhouse assay, *St. albidoflavus* St-220 promoted the growth of *Sa. miltiorrhiza* seedlings by increasing the plant biomass, especially the length, diameter, fresh and dry weight of the plant roots (Figure 4). To have a deep perspective on the root promoting mechanism, we tested and found that *St. albidoflavus* St-220 has the biological characteristics involved in plant promoting activity including phosphate solubilization, nitrogen fixation, IAA production and siderophore production. The actinobacterial strains, such as *St. alfalfae* strain XN-04, *Streptomyces* sp. NEAU-S7GS2, and *St. chartreusis* strain WZS021, have root growth-promoting activities on cotton, soyabean and sugarcane, respectively, and genes related to IAA, siderophores, phosphate solubilization were identified in their genomes (Wang et al., 2018b, Liu et al., 2019, Chen et al., 2021). In various studies, IAA has been shown to increase plant root size and distribution, as well as root hairs, resulting in higher nutrient uptake from the soil (Datta and Basu, 2000; Gumiere et al., 2014; Liao et al., 2017; Ulrich et al., 2021). A number of encoding genes directly involved in the synthesis of indoleacetic acid were found in the genome of

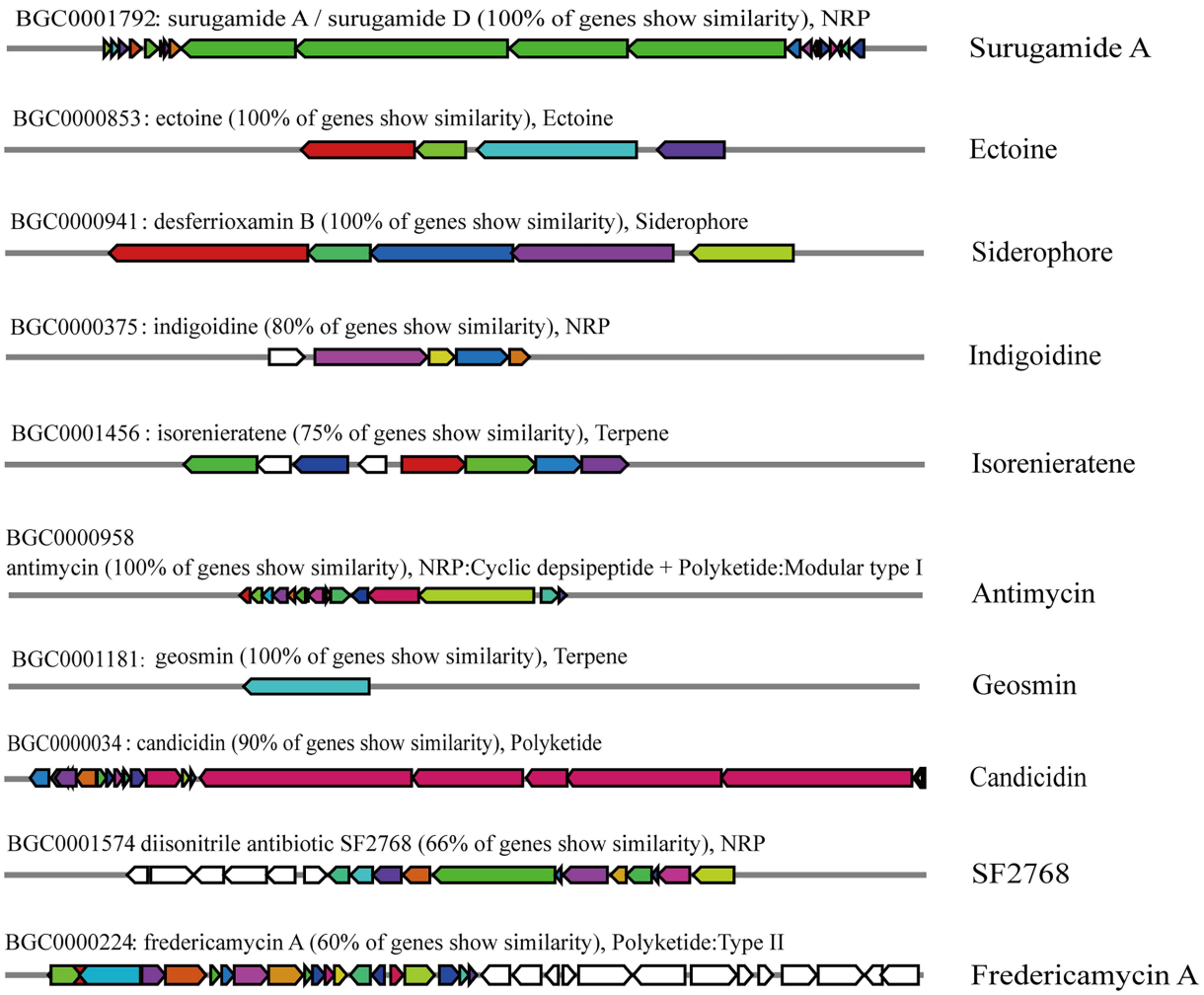

FIGURE 7
Genome-wide analysis of gene clusters related to the biosynthesis of secondary metabolites using the online antiSMASH v6.0 software.

St-220, including two genes encoding monoamine oxidase and four genes encoding aldehyde dehydrogenase. Many plant-associated actinomycetes are able to solubilize phosphorus into a form that can be used by plants by secreting phosphatases and phytases (Suárez-Moreno et al., 2019). In our present study, the genomic sequences of strain St-220 were found to encode acid and alkaline phosphatases, as well as phytases, suggesting a potential root stimulation of St-220. Additionally, 12 genes related to nitrogen fixation were also found in the genome of strain St-220. The nitrogen fixation plays a key role in the promoting activity of biocontrol agents on plant root growth and development (Dobbelaere et al., 2003). Our genome mining confirmed that the *St. albidoflavus* St-220 harbors predicted genes involved in pathways regarding IAA and siderophores production, phosphate solubilization and nitrogen fixation, which may play roles in simulating growth and development of plant roots. Therefore, we speculated that *St. albidoflavus* St-220 promotes plant growth in greenhouse condition through employing genes involved in a variety of metabolites synthesis pathways that may related to growth-promoting effects. Our results revealed the antifungal and growth-promoting activities of the *St. albidoflavus* St-220, and suggested the St-220 could be developed as a promising biological fertilizer.

Conclusion

Strain St-220 has inhibitory activity against *F. oxysporum* causing root rot disease of *Sa. miltiorrhiza*, and also promotes the growth of *Sa. miltiorrhiza* seedlings. The strain was identified as *St. albidoflavus* by its morphological and molecular characteristics. Our genome sequencing identified many pathways involved in synthesis of secondary metabolites with antifungal and growth-promoting activities, indicating the versatility of St-220 for being developed as a BCA against *Fusarium* wilt of *Sa. miltiorrhiza*.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/, JAMFMD010000000.

Author contributions

TW, LG, and LH conceived and designed the experiments. YD and TW performed the experiments and analyzed the data. YD, TW, JJ, YW, CL, KS, JS, BY and CK contributed reagents, materials, and analysis tools. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.976813/full#supplementary-material

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Acylsugar protection of *Nicotiana benthamiana* confers mortality and transgenerational fitness costs in *Spodoptera litura*

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Acylsugars are secondary metabolites that are produced in the trichomes of some solanaceous species and can help control several herbivorous insect pests. Previously, knockout mutations (*asat2* mutants) were shown to significantly reduce the acylsugar content of *Nicotiana benthamiana*, and significantly improve the fitness of six generalist insect herbivores. The current study compared the significant mortality and fitness costs in *Spodoptera litura* conferred by acylsugar protection of *N. benthamiana* (wild-type plants) compared to *S. litura* strains reared in acylsugar-deficient plants with depleted acylsugar biosynthesis. Acylsugar protection prolonged the developmental duration and decreased viability in the larval stages. Further, the fecundity of females and the hatching rate of eggs significantly decreased under acylsugar protection. For F_1 offspring, acylsugar protection still exerted significant negative effects on larval survival rate and fecundity per female. The net reproductive rate and relative fitness of the *S. litura* strain were strongly affected by acylsugar. Altogether, these results indicate that acylsugar could contribute to plant protection due to toxicity to pests, diffused availability, and low environmental persistence. This could represent a complementary and alternative strategy to control populations of insect pests.

KEYWORDS

acylsugar, *Nicotiana benthamiana*, chemical defenses, *Spodoptera litura*, toxicity, fitness cost, transgenerational effects

Introduction

Plants generate molecules with low-molecular mass that are considered secondary metabolites, and they show various mechanisms of defense against different herbivores (Schuman and Baldwin, 2016). In addition to physical characteristics such as low digestibility, spines, and leaf toughness, it has been reported that, in plants, many published metabolites could be used to control insect pests (Bérdy, 2005). However, many insect pests display the ability to resist the defensive traits from metabolites in their preferred species of plants which could be against by more sporadically

distributed chemical defenses. For example, the extensively investigated Brassicaceae provides outstanding instances of plants that generate extra chemical defenses beyond the canonical glucosinolates characteristic of this plant family (Fahey et al., 2001). Two lineages of *Barbarea vulgaris*, glabrous (G-type) and pubescent (P-type), display different content of triterpenoid saponins, and show distinct levels of resistance against *Plutella xylostella* (Agerbirk et al., 2003). *Erysimum* contains cardiac glycosides which negatively affect feeding behavior and oviposition of *Pieris rapae* (Sachdev-Gupta et al., 1990, 1993). Other cases of chemical defenses, such as cucurbitacins in *Iberis umbellate*, alliarinoside in *Alliaria petiolate*, and tropane alkaloids in *Cochlearia officinalis* have been demonstrated previously (Nielsen et al., 1977; Haribal et al., 2001; Brock et al., 2006). These kinds of deterrent or toxic metabolites from various plants can be utilized to enhance resistance to insect pests in crops if reasonable and rational strategies are established with current biotechnologies (Zhou and Jander, 2021).

Acylsugars are insect-deterrent metabolites generated by the family Solanaceae, and are produced and exuded from glandular trichomes of the plants (Goffreda et al., 1988, 1989; Wagner, 1991), resulting in significant negative effects like antibiosis or insect-repellent on various tomato herbivores (Hawthorne et al., 1992; Rodriguez et al., 1993; Juvik et al., 1994; Leckie et al., 2012; Ben-Mahmoud et al., 2018). Similarly, although *Nicotiana benthamiana* has been extensively utilized in the study of plant–microbe interactions (Goodin et al., 2008; Bally et al., 2018), it may not be the most appropriate host plant for studying herbivore–plant interactions (Hagimori et al., 1993; Simón et al., 2003) and the undesirable performance of herbivores on *N. benthamiana* could be partially ascribed to acylsugars (Feng et al., 2021). Specifically, the *Nicotiana* species showing resistance to aphids contained acylsugars, yet acylsugars cannot be measured in the more susceptible species of the genus (Hagimori et al., 1993). Similarly, compared with *Solanum lycopersicum*, the cultivated tomatoes, acylsugars could be detected in the wild tomato species *S. pennellii,* which displayed higher resistance to the pest species *Bemisia tabaci* and *Myzus persicae* (Rodriguez et al., 1993; Marchant et al., 2020). Recently, Feng et al. (2021) reported that changed profiles of acylsugar could reduce levels of resistance to six insect pests such as *B. tabaci*, *M. persicae*, *Macrosiphum euphorbiae*, *Trichoplusia ni*, *Heliothis virescens*, and *Helicoverpa zea*. This type of plant resistance to herbivore pests could be strengthened *via* bioengineering to enhance amounts of defensive metabolites, alter available biochemical pathways, or transfer the biosynthesis of novel types of defensive metabolites into target plants. Nevertheless, present strategies of bioengineering are limited owing to several factors, such as inadequate references for the biosynthetic pathways of plant metabolites, unexpected byproducts originating from plant metabolites, and demands for the spatial specificity of metabolite production to increase resistance to insect pests.

Spodoptera litura (Fabricius), the tobacco cutworm, is one notorious polyphagous and destructive herbivore pest that feeds on various economic and horticultural crops, including cotton, soybeans, tobacco, tomatoes, and peanuts. The extensive range of host plants suggests that *S. litura* could neutralize the traits of resistance of different plants (Shi et al., 2022), and some specific secondary metabolites of the plants significantly inhibit the growth of *S. litura* in the larval stages (Kundu et al., 2018). Because the application of chemical agents has been the primary step against *S. litura* for the most recent few decades, an increasing number of studies has indicated that several field-collected *S. litura* populations have evolved significant levels of resistance to a variety of chemical agents such as carbamate, organophosphate, chlorantraniliprole, pyrethroids, abamectin, indoxacarb, and emamectin benzoate, and the wide application of these chemical agents is no longer a suitable strategy for environment-friendly plant protection (Tong et al., 2013; Saleem et al., 2016; Wang et al., 2018; Xu et al., 2020). Considering that *N. benthamiana* acylsugars showed defensive effects of metabolites against lepidopteran pests, it may be possible to enhance resistance of plant by transgenic methods of transferring biosynthetic pathways (Feng et al., 2021). Typically, establishment of the life-table has been shown as one important method for evaluating and understanding the effects of exogenous elements on the individual and the entire population of insect pests. The analysis of the life-table could be used for precisely estimating the growth rate of the population and the fitness costs, and on this basis, strategies of pest management could be formulated more reasonably (Kliot and Ghanim, 2012). In the present work, mortality and fitness costs in a lab-reared population of *S. litura* with acylsugar protection of *N. benthamiana* were systematically examined, and the results indicated the plant chemical defenses conferred by acylsugar, and these results can supply important data for using acylsugar for controlling pests *via* chemical plant defenses in the field.

Materials and methods

Insects and plants

The reference strain of *S. litura*, Lab-S strain, was used in this study and was reared on an artificial diet in one insect-rearing room without exposure to chemical agents for over 5 years (Zhang et al., 2022). The wild-type (WT) and the acylsugar-deficient *asat2-1* line (ASAT2) plants of *N. benthamiana* were obtained from the Boyce Thompson Institute, Ithaca, New York, USA, and the ASAT2 plants showed an almost complete absence of acylsugar compared to the WT plants (Feng et al., 2021). All plants of WT and the ASAT2 mutant of *N. benthamiana* were reared at 23°C and a 16:8 h light:dark photoperiod in a well-controlled chamber. All bioassays and fitness cost evaluation

work were performed at 26°C under a 16:8 h light:dark photoperiod in a well-controlled growth chamber.

Bioassays

The lethal activity of acylsugar toward various stages of larvae was examined by bioassays. *S. litura* eggs were maintained on an artificial diet, and five larval stages (the 2nd, 3rd, 4th, 5th, and 6th stages), were measured. For each tested instar of larvae, one hundred 12-h-old larvae were selected and fed with the leaves of WT or ASAT2 plants. Ten larvae were placed on one WT or ASAT2 plant as one tested group, and 10 of the tested groups were set as replicas for each bioassay. The immobile larvae in each stage were considered as dead, and the number of larvae that survived was recorded after 48 h. Comparisons were made between the WT and ASAT2 using the Student's *t*-test.

Defensive effects of acylsugar on *S. litura* of F_0

This study evaluated the defensive effects of acylsugar on second-instar larvae of *S. litura*. Six hundred one-day-old second-instar larvae were randomly collected, and 300 of them were fed with *N. benthamiana* leaves of WT plants, while the other 300 were fed with *N. benthamiana* leaves of ASAT2 plants. The total number of deformed pupae was counted, and, within 24 h, all healthy pupae were weighed, and the rate of pupation was recorded. After the adults emerged, 15 pairs of female and male adults were coupled in the first 12-h after emergence, and each couple was placed into one plastic cup (3-cm diameter and 5-cm height). Each of the tested couples was introduced into new plastic cups daily, and the fecundity of each female, oviposition, and egg hatching rate was recorded every day. Comparisons were made between the plants of WT and ASAT2 using the Student's *t*-test.

Transgenerational defensive effects of acylsugar on F_1 offspring

To determine whether acylsugar exerts transgenerational defensive effects on the F_1 population, the egg hatching rate was assessed by sampling 20 egg masses (more than 250 eggs per mass) on the fourth day of the oviposition duration for F_0 females, which were fed on acylsugar (the WT plants) or acylsugar-depleted (the ASAT2 plants) from the second larval instar. Further, 100 collections from four masses of eggs (20–30 eggs from each mass) were utilized to establish the life table for each tested population of *S. litura*. Neonates of the F_1 generation were transferred individually into one plastic tube and fed with artificial diet in the tube. The developmental time of larval-instar stages and survival rates were checked daily, and pupation rate, duration of pupae, the longevity of adults, and emergence rate were recorded every day. Newly emerged males and females of the F_1 generation were coupled and put into one plastic cup for oviposition. The fecundity of females, oviposition duration of females, and hatchability of the eggs were checked daily. Comparisons were made between the WT and ASAT2 using the Student's *t*-test. Net reproductive rate (R_0) and the relative fitness were evaluated according to a previously published method (Wang and Wu, 2014).

Results

Toxicity of acylsugar on different instar larvae in *S. litura*

To confirm if the depletion of acylsugar in the ASAT2 mutants enhances the adaptability of *S. litura* on *Nicotiana benthamiana*, we performed bioassays with the 2nd, 3rd, 4th, 5th, and 6th instars of *S. litura*. When each of the specific instar larvae was put onto the leaves of the ASAT2 mutant or wildtype (WT), survival rates of *S. litura* on WT plants were significantly lower compared to their counterparts reared on the ASAT2 plants (Figure 1). The 2nd instar larvae of *S. litura* on WT plants had the lowest survival rate, ∼53%, while the survival rate of 2nd instar larvae on ASAT2 plants was ∼96% (Figure 1). For other stages of larvae in the bioassays, survival rates of *S. litura* on WT plants decreased more significantly than on ASAT2 plants (Figure 1).

Effect of acylsugar on larvae and adults of *S. litura* F_0 generation

Biological components including survival rate and developmental time, larval, and pupal weight, the fecundity of females, duration of oviposition, and egg hatching rate for the F_0 generation grown from 2nd instar larvae fed with or without acylsugar were studied. Compared to those fed on ASAT2 plants, the survival rate of the second- to sixth instar larvae from the F_0 group fed with WT significantly decreased in each stage (Figure 2A), and their weight significantly decreased in each stage from second instar larvae to pupae (Figure 2B). In comparison with the ASAT2 group, the development time of second- to sixth instar larvae of F_0 fed with WT was significantly prolonged by 2.2 days (Figure 3A). However, pupal duration and female and male longevity were not significantly different between those reared on WT and ASAT2 plants (Figures 3A,B). Further, compared to the mean fecundity of ASAT2-fed females (3,815.53 eggs per female), WT-fed females displayed significantly reduced fecundity, with 2,565.93 eggs per female (Figure 4A). Similarly, a significant decrease in the egg hatch

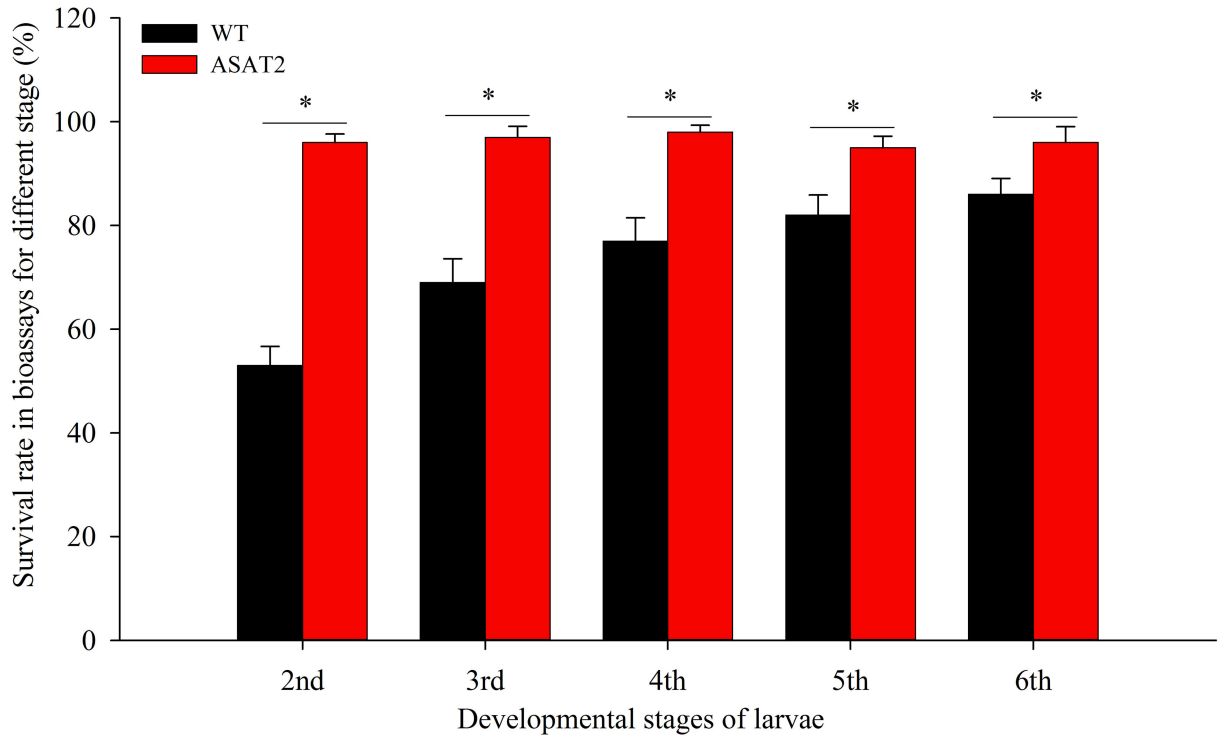

FIGURE 1
Survival rate of bioassays using specific stages of larval instars on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$).

rate of WT-fed females (79.58%) was observed compared with ASAT2-fed females (94.20%; Figure 4C). However, there was no detectable difference in the duration of oviposition between the two populations (Figure 4B).

Transgenerational defensive effects of acylsugar on the F_1 generation

No significant defensive effects of acylsugar on the period of various stages of life were detected between ASAT2-fed and the WT-fed group (Figure 5A). In addition, the pupation and emergence rate did not significantly differ between the two groups (Figure 5B). However, in comparison with the ASAT2-fed group, the larval survival of the WT-fed plant group significantly decreased (Figure 5B). Further, a significant difference in eggs laid per female of F_1 was observed between the ASAT2-fed (4,188.87 ± 267.29) and WT-fed groups (3,356.87 ± 207.54; Figure 6A). On the contrary, no significant difference was observed in other reproduction parameters, such as oviposition duration (Figure 6B) and hatchability of the eggs (Figure 6C). All fitness parameters of F_1 offspring are displayed in Table 1. Relative to the net replacement rate (R_0) of the ASAT2-fed group, the fitness of the WT-fed group was 0.51 (Table 1).

Discussion

Acylsugars exuded by glandular trichomes are considered powerful natural pesticides (Puterka et al., 2003), and can directly kill some species of insect pests (Feng et al., 2021). In this study, we found that although larvae of *S. litura* grow well on the ASAT2 mutant line of *N. benthamiana*, significant insecticidal effects of acylsugar against larvae of *S. litura* were observed in the WT line of *N. benthamiana*. In particular, a 50% lethality effect was detected for the 2nd instar larvae. Similarly, it has been reported that knockout of acylsugar biosynthesis conferred a significantly higher survival rate for *M. persicae* and *B. tabaci* on the ASAT2 mutant line compared with their high mortality in wildtype *N. benthamiana* (Feng et al., 2021). Considering that acylsugars are defensive metabolites generated by various Solanaceae species, in which they provide deterrence against a large range of herbivores, acylsugar-associated herbivore resistance has huge promise against insect pests of tomato such as whiteflies, thrips, and aphids (Goffreda et al., 1988, 1989; Hawthorne et al., 1992; Rodriguez et al., 1993; Juvik et al., 1994; Liedl et al., 1995; Leckie et al., 2012).

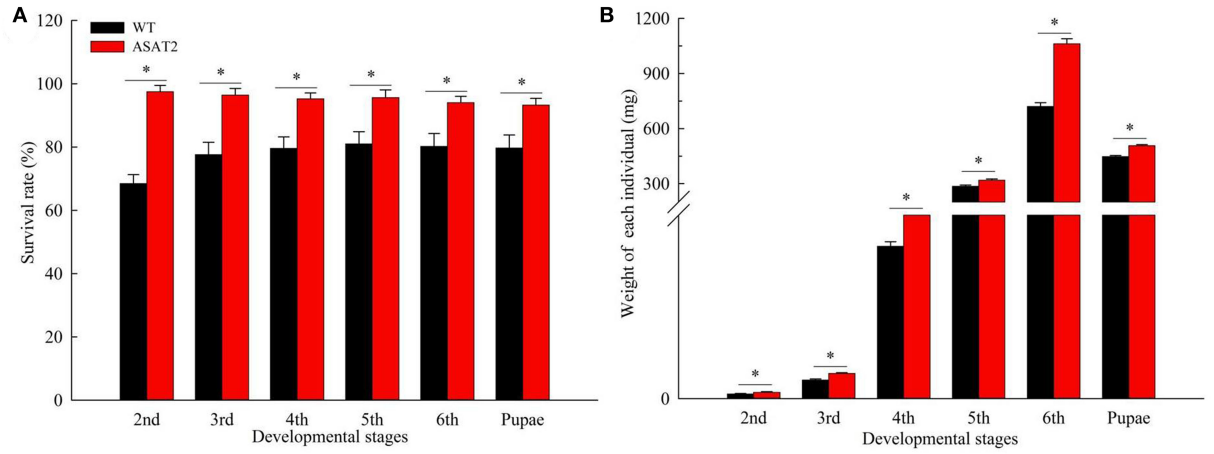

FIGURE 2
Survival rate **(A)** and weight of individual **(B)** in each larval stage of the F_0 generation on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$).

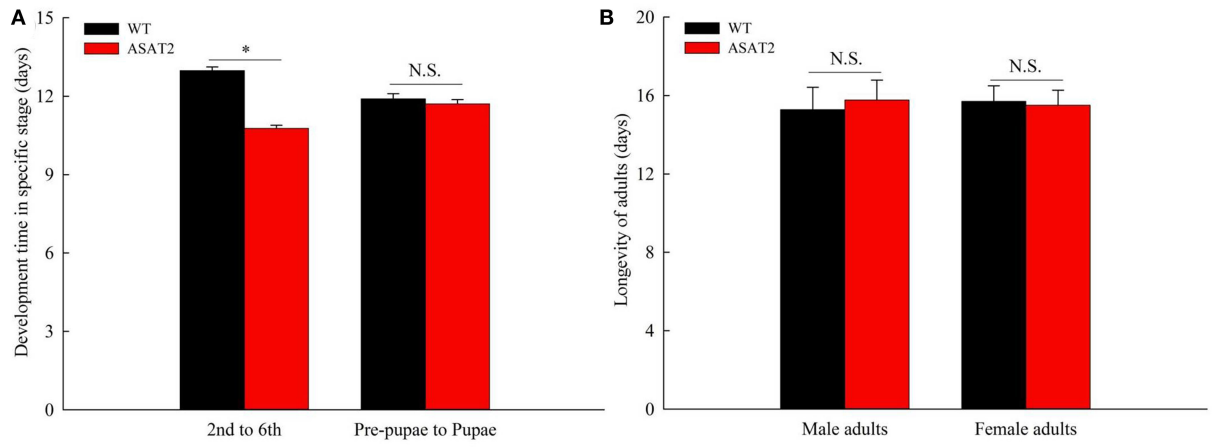

FIGURE 3
Development time **(A)** and longevity of adults **(B)** of the F_0 generation on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$), and n.s. indicates not significant ($P > 0.05$).

Further, acylsugars can negatively affect the fitness of various insect pests by interfering with behaviors such as feeding and oviposition and have detrimental effects on their growth (Simmons et al., 2003; Resende et al., 2006). To investigate the underlying ecological effects of acylsugar on insect pests, we conducted systematic work on the defensive effects on *S. litura*. We observed that acylsugar shows insecticidal effects against *S. litura* larvae from the 2nd to the 6th stage, and it was previously observed that there was a high death rate of sucking insect pests such as *B. tabaci* and *M. persicae* on wild-type plants of *N. benthamiana* (Feng et al., 2021). In the acylsugar-fed group, *S. litura* larvae showed decreased body weight in each larval and pupal stage. They also displayed significant prolongation of the larval period, suggesting that acylsugar not only acts against larvae directly but also suppresses their development. More importantly, fecundity of females and egg hatching rate of the *S. litura* F_1 generation were significantly affected by acylsugar. Similarly, these effects were also observed in *Tetranychus urticae* and *Frankliniella occidentalis* (Lucini et al., 2015; Ben-Mahmoud et al., 2019). It has also been reported that acylsugar could interfere with the oviposition and feeding of *M. persicae* and *Tuta absoluta*, and have detrimental effects on their growth (Simmons et al., 2003; Resende et al., 2006).

In addition to reducing the fitness of *S. litura* during the F_0 generation, the transgenerational effects of acylsugar were

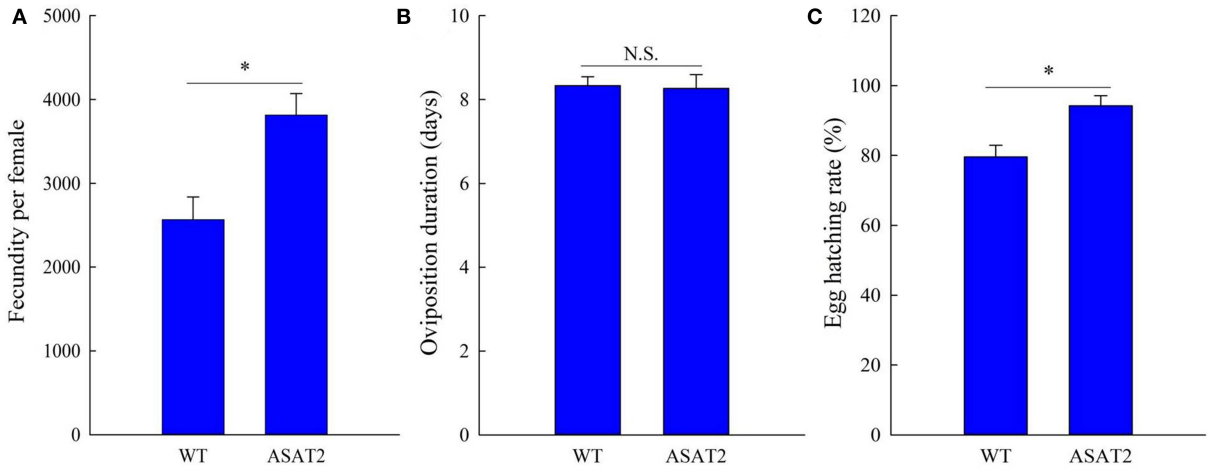

FIGURE 4
Fecundity **(A)**, oviposition duration **(B)**, and egg hatching rate **(C)** of the F_0 generation of *S. litura* on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$), and n.s. indicates not significant ($P > 0.05$).

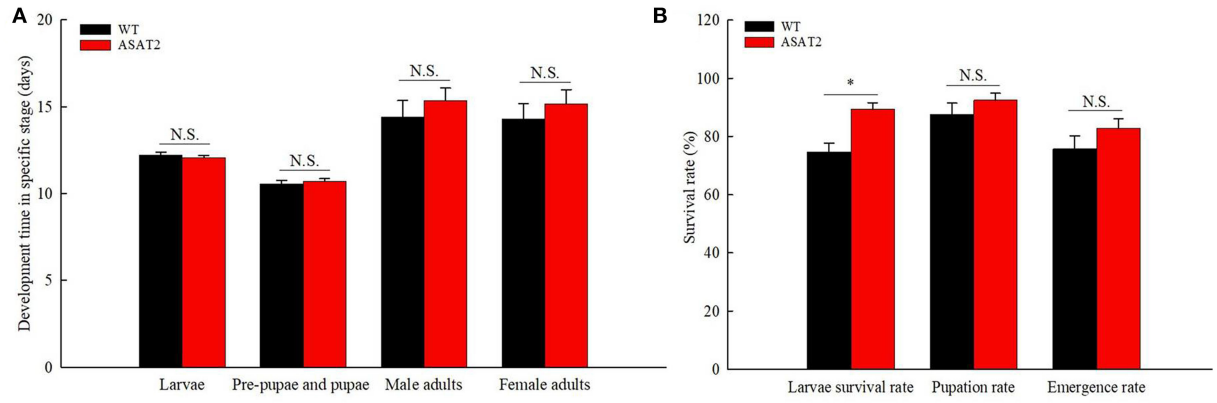

FIGURE 5
Development time **(A)** and survival rate **(B)** of the F_1 generation on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$), and n.s. indicates not significant ($P > 0.05$).

detected. Here, we found that in the F_1 generation of the WT-fed group, the larval survival rate and female fecundity were still significantly suppressed, even though the F_1 generation of *S. litura* was reared on an artificial diet from hatching. A variety of studies have suggested that various chemical agents can affect insect pests by damaging their behavioral or physiological characteristics including longevity, duration of growth, host locating, feeding ability, and fecundity (Desneux et al., 2006; Biondi et al., 2013; Wang et al., 2016, 2017; Qu et al., 2017; Fang et al., 2018; Jam and Saber, 2018; Zhou et al., 2021). Most of these effects could also be transgenerational, indirectly affecting their offspring (Cui et al., 2018), and they could cause alterations in communities and ecosystems (Lu et al., 2012; Mohammed et al., 2019). Thus, the transgenerational effects induced by acylsugar might be contributed to delaying the outbreak of acylsugar in a short term. Recently, biopesticides (natural products) have emerged as a better alternative for pest control (Mostafiz et al., 2020), and acylsugars, one of the products of glandular trichomes that secrete secondary metabolites, could be repellent, toxic, and disturb oviposition and feeding of insect pests. They are involved in tritrophic interactions in plant defenses by tagging herbivores for predation through breaking down volatile acylsugar products (Weinhold and Baldwin, 2011) and efficiently protecting plants from attacks from microbes (Luu et al., 2017). In tomato plants, breeding measures have attempted to control the composition and content of acylsugar for increasing resistance to herbivores, and more enhanced breeding lines have been generated (Leckie et al.,

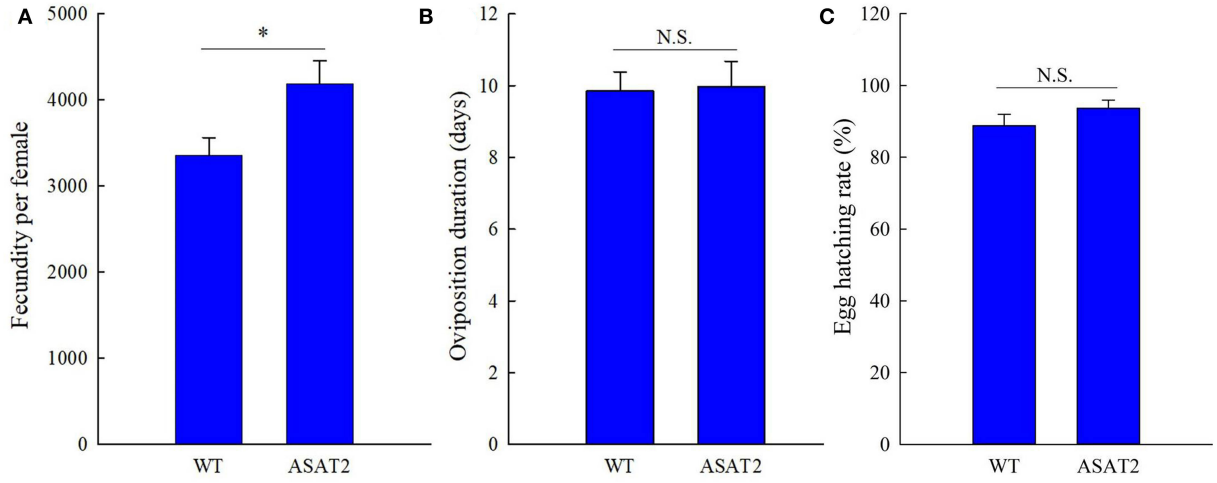

FIGURE 6
Fecundity **(A)**, oviposition duration **(B)**, and egg hatching rate **(C)** of the F_1 generation of *S. litura* on WT and ASAT2 plants of *N. benthamiana*. Values are presented as means ± SE. Asterisks above error bars indicate significant differences ($P < 0.05$), and n.s. indicates not significant ($P > 0.05$).

TABLE 1 Life tables and relative fitness of two tested populations of *Spodoptera litura*.

Life-history parameter	ASAT2	WT
Number of neonates	150	150
Number of pupae	124	98
Number of adults	104	74
Number of female moths	59	40
Mean eggs laid female^{-1}	4,188.87	3,356.87
Egg viability (%)	93.66	88.79
Predicted neonate number of next generation	231,474	119,222
Net replacement rate (R_0)	1,543.16	794.81
Relative fitness	1	0.52

Relative fitness = R_0 (WT-fed)/R_0 (ASAT2-fed).

2012, 2013, 2014; Smeda et al., 2016). Accordingly, acylsugars can provide an alternative to synthetic insecticides for the future environmentally-friendly control of insect pests.

In recent years, novel advances in ecotoxicology have been impacting the assessment of xenobiotic effects (Godfray, 1993; Sedaratian et al., 2013). Demography has been considered as one approach for evaluating the overall effects of xenobiotics because it can illustrate all the impacts of a xenobiotic on a population of insect pests (Hamedi et al., 2010). In addition, combining demography with biological parameters could better predict the impacts of xenobiotics at the population level. Fitness cost is considered as one essential biological component that must be assessed when formulating xenobiotics pest management strategies. The fitness cost can be observed when organisms face niche alteration and must adapt to novel surroundings (Kliot and Ghanim, 2012). In the present study, compared with the ASAT2-fed group, significant the fitness costs resulting from acylsugar displayed a fitness value of 0.52 in the WT-fed group. It has been shown that the more significant fitness cost, the longer it takes for insect pests to develop their populations, which is one vital element of the Integrated Pest Management (IPM) program (Kliot and Ghanim, 2012). Therefore, an overall understanding of fitness costs associated with defensive metabolites of plants could contribute to the design of more effective strategies for pest management against herbivore pests.

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 authors.

Author contributions

RW, BG, and CL conceived and designed the study. RW, BG, QZ, and ZZ performed the experiments and analyzed the data with the help of YL, QY, MZ, and WL. RW wrote the first draft of the manuscript. RW, BG, QZ, and CL participated in manuscript drafting and modification. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Essential oils and plant extracts for tropical fruits protection: From farm to table

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The tropical fruit industry in Malaysia makes up a large proportion of the agriculture sector, contributing to the local economy. Due to their high sugar and water content, tropical fruits are prone to pathogenic infections, providing optimal microorganism growth conditions. As one of the largest exporters of these fruits globally, following other Southeast Asian countries such as Thailand, Indonesia and the Philippines, the quality control of exported goods is of great interest to farmers and entrepreneurs. Traditional methods of managing diseases in fruits depend on chemical pesticides, which have attracted much negative perception due to their questionable safety. Therefore, the use of natural products as organic pesticides has been considered a generally safer alternative. The extracts of aromatic plants, known as essential oils or plant extracts, have garnered much interest, especially in Asian regions, due to their historical use in traditional medicine. In addition, the presence of antimicrobial compounds further advocates the assessment of these extracts for use in crop disease prevention and control. Herein, we reviewed the current developments and understanding of the use of essential oils and plant extracts in crop disease management, mainly focusing on tropical fruits. Studies reviewed suggest that essential oils and plant extracts can be effective at preventing fungal and bacterial infections, as well as controlling crop disease progression at the pre and postharvest stages of the tropical fruit supply chain. Positive results from edible coatings and as juice

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preservatives formulated with essential oils and plant extracts also point towards the potential for commercial use in the industry as more chemically safe and environmentally friendly biopesticides.

KEYWORDS

essential oils, plant extracts, tropical fruits, plant diseases, protection, biopesticides

Introduction

In 2020, the agriculture industry recorded a contribution of 7.4% of Malaysia's Gross Domestic Product (GDP) (Mahidin, 2021). Following rubber, oil palm and paddy, tropical fruits make up a large proportion of the agriculture landscape in Malaysia (Abu Dardak, 2019). It is estimated that roughly 192,000 hectares of agricultural land in Malaysia are used to cultivate tropical fruits. Fruit export in Malaysia is valued at RM1.46 billion (USD 347 million) in 2020, making it one of the most important exported products in the agriculture sector (Abu Dardak, 2022). The fruits exported from Malaysia include seasonal fruits such as durian, rambutan, mango and mangosteen, and those grown all year round like papayas, watermelon and bananas (Arope, 1992). Among the fruits exported, bananas, pineapples, and watermelon make up the majority, with production exceeding 200 metric tons per year (Rozana et al., 2017).

Tropical fruits are prone to diseases such as anthracnose, rotting and mould. Currently, Malaysia's conventional way of managing and treating these diseases is concentrated on the use of chemical pesticides, with more than 50% of farmers preferring this approach instead of other alternatives (Chang, 2021). The inclination for farmers to opt for chemical means may be attributed to their effectiveness and accessibility (Sharifzadeh et al., 2018). However, pesticide residue is increasingly becoming a major safety concern after recorded chemical poisoning and environmental pollution cases. For example, the commonly used pesticides such as propiconazole and various organophosphorus pesticides were found to wind up in Malaysian rivers (Wee et al., 2016; Elfikrie et al., 2020). The loosely regulated use of chemical pesticides thus can pose as threats to the local communities. As a result, research has been actively looking into using nature-derived compounds as alternative organic pesticides.

Essential oils are obtained from distilling aromatic plants and have been gaining interest for their use in aromatherapy. Plant extracts have traditionally been used as flavoring agents and fragrances throughout history. Essential oils often contain bioactive constituents such as esters, terpenes, phenols, aldehydes and ketones, which possess antimicrobial activity (Pauli, 2001). They are relatively safer than commonly used chemical pesticides (Moharramipour and Negahban, 2014; Bhavaniramya et al., 2019; Singh et al., 2019); hence, research has been focused on understanding how the antimicrobial properties of essential oils and plant extracts can be utilized in agriculture as organic biopesticides (Lahlali et al., 2022). Therefore, this review aims to cover the literature on using essential oils and plant extracts as potential pesticides, focusing on plant diseases caused by fungi and bacteria. Bioactive compounds believed to contribute to the antimicrobial activity of essential oils and plant extracts in the management of crops are also reviewed (Figure 1). The review will also summarize how they can be used at different points in the supply chain of getting tropical fruits from the farm to consumers pre and postharvest stages, including processed products such as fresh-cut fruit and fruit juices.

Tropical fruit production in Asian countries and Malaysia

Asian countries have been the main producers and exporters of tropical fruits globally, especially in the European market. Tropical fruits such as banana, mango and pineapple are among the largest cultivated and can be widely found in international markets. Meanwhile, more seasonal tropical fruits such as durian, guava, jackfruit, and mangosteen have lower cultivation and trading activities. According to the Food and Agriculture Organisation of the United Nations, FAO (2021), 2.2 million tons of mangoes were globally exported in 2020, increasing by 2.9% compared to 2019. Similarly, global papaya exports reached 353,000 tons, increasing by 2.7% from 2019. However, global exports of pineapple decreased to 3.1 million tons, representing an 8.2% fall from 2019. The decrease may be due to COVID-19 constraints that negatively impacted the global market in early 2020. Despite the fall in exportation rates, pineapple still recorded the highest global export among the three most cultivated tropical fruits.

The FAO (2021) also reported the gross exportation of tropical fruits in Asian countries from 2018 to 2019. As the largest exporter of mangoes, mangosteen and guava, Thailand recorded 260,100 tonnes of exports in 2018, which increased to 479,600 tonnes in 2019. Meanwhile, India exported 153,300 tonnes in 2018, decreasing to 147,200 tonnes in 2019. Since the

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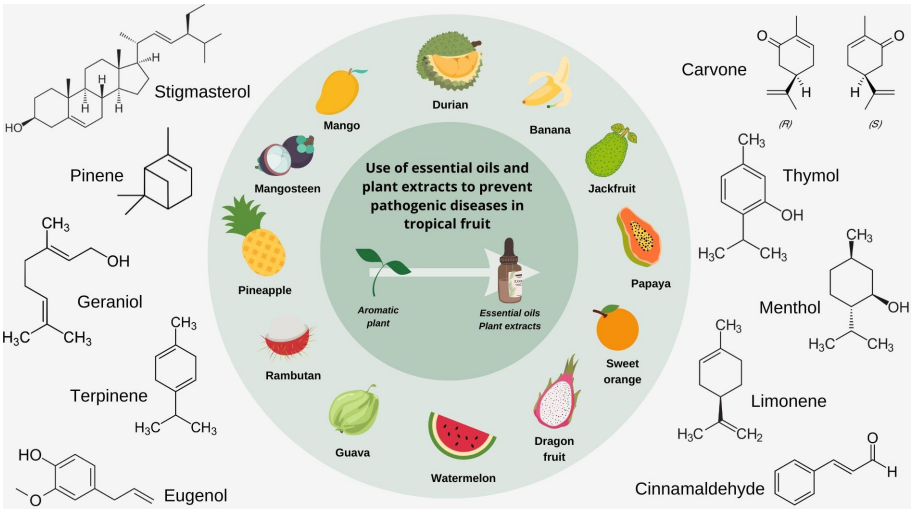

FIGURE 1
Selected tropical fruits and bioactive compounds present in essential oils and plant extracts responsible for observed antimicrobial properties.

fall was recorded before the COVID-19 pandemic took off, the decrease in exportation might be due to postharvest diseases, resulting in the loss of quality of fruits to be exported (Jat et al., 2020). The Philippines is the largest pineapple exporter, with exports reaching 442,100 tonnes in 2018. This number increased to 625,500 tonnes in 2019, while Malaysia exported 19,600 tonnes in 2018, reducing to 17,900 tonnes in 2019. India was the biggest exporter of papaya in 2018 at 18,000 tonnes which further increased in 2019 to 19,000 tonnes, followed by China and Malaysia.

Tropical fruits are important for Malaysia as they are the major source of local income. Over the years, the efforts to produce tropical fruits in Malaysia have been elevated to accommodate an increase in demand in the global market, contributing to the higher revenue recorded from tropical fruit trading activities. As shown in Table 1, Malaysia produced more than 1.5 million tonnes of fruits valued at close to RM 10 million (USD 2.28 million) in 2019. Based on the production and product value, durian has the highest product value than the other 20 local fruits listed. Banana production (325,447 mt) was higher than pineapple production (314,627 mt) but had a lower product value. Since banana can be easily damaged during its harvesting to its transportation process (Cao et al., 2018), the decrease in production value might be due to the quality losses of postharvest fruits during storage and transportation that eventually lead to reduced prices in the market.

From Figure 2, the production trend of tropical fruits in Malaysia can be seen to generally increase over the years from year 2019 to year 2021. This indicates an increased demand for tropical fruits where it will be globally exported to different countries. According to Cao et al. (2018), tropical and subtropical fruits are very vulnerable to the surrounding temperature which will make it further susceptible to fungal

TABLE 1 Production value of major tropical fruits in Malaysia, 2019.

Fruits	Production (mt)	Production Value (RM'000)
Durian	377,251	7,493,882
Banana	325,447	579,295
Pineapple	314,627	621,389
Watermelon	144,147	212,617
Rambutan	55,891	124,489
Papaya	53,681	118,630
Guava	35,962	117,740
Jackfruit	31,281	107,863
Mangosteen	28,764	99,843
Cempedak	27,893	86,096
Duku	24,446	61,058
Dokong	22,913	51,118
Langsat	18,993	40,335
Mango	16,509	39,561
Pomelo	15,133	38,588
Sweet orange	11,006	31,300
Starfruit	8,054	26,415
Dragon fruit	6,879	20,535
Salak	3,443	6,541
Sapodilla	1,828	5,209
Pulasan	966	2,897
Total	1,525,051	9,885,315

Source: Department of Agriculture, Malaysia (2019).

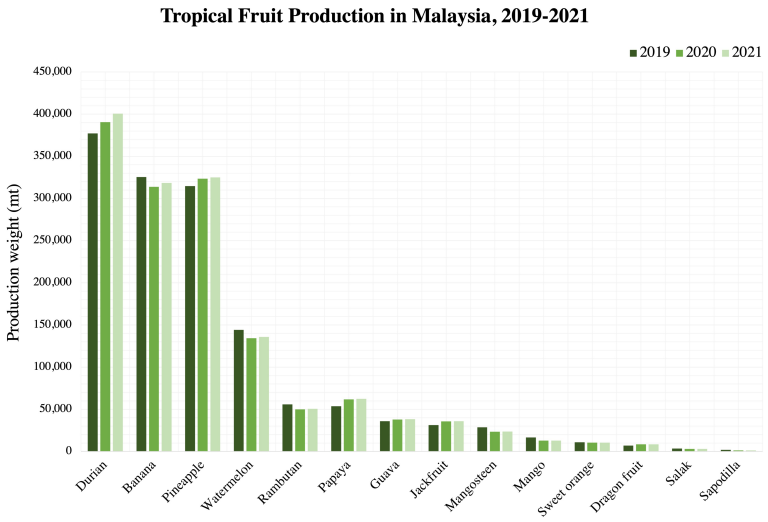

FIGURE 2
Tropical fruit production in Malaysia, 2019-2021. Source: Department of Agriculture, Malaysia (2021).

infections, leading to reduced quality and decay. FAO (2022) reported the decrease of papaya exportation in Malaysia by approximately 4% in year 2022 was partly due to a phytopathogenic bacteria causing the bacterial dieback disease in papaya. Since fluctuations in temperature are inevitable during storage and transportation, there is a need to protect the postharvest tropical fruits to enhance their longevity and consequently, preserve the quality.

Fruits and vegetables are generally considered more perishable than other mass-produced commercial crops like oil palm and rubber. A study of 284 participants found that 93% of consumers consider freshness the top criterion when purchasing and consuming fresh fruits and vegetables (Chamhuri and Batt, 2015). Physical attributes of fruits and vegetables, such as the size, shape, color, and texture, all affected by infections, also contribute to how consumers perceive product quality. This presents a significant challenge for local farmers and exporters to ensure the freshness of crops between getting them from the farm to the consumers. Infections and diseases are also one of the main factors contributing to poor crop yield in Malaysia (Chang, 2021). Due to their higher water content (70-95%), a higher concentration of polysaccharides and significantly increased respiration rate; fruit crops provide a more favorable environment in which microorganisms can thrive. With an average shelf life of 3-5 days, there is a pressing need for the development of highly effective, yet sustainable and safe methods of preserving the freshness of tropical fruits (Wongs-Aree and Noichinda, 2014).

Data and information above indicate the increasing trend in the production of tropical fruits in Malaysia and reveal tropical fruits as important trade products in Southeast Asian countries. In the case of more exotic fruits such as rambutan and durian, the low availability of high-quality fruits as a result of prolonged export times may ultimately drive market prices up, especially in regions where they are not locally cultivated. This then, may affect customer demand, which is inversely proportional to the market price of a product, according to the law of demand. Therefore, the decline in fruit quality during transport and storage necessitate action as the tropical fruit industry play a significant economic role for developing countries in the Southeast Asian region.

Application of essential oils or plant extracts to protect selected tropical fruits

Essential oils and extracts from plants have been studied in various ways for tropical fruit disease management from cultivated to postharvest processing and storage (Supplementary Table 2). The majority of the research done on preharvest disease prevention and treatment is centred around *in vitro* studies. Herein, we reviewed *in vitro* studies utilising a variety of tests. The disc volatilisation method is a popular method of studying the antimicrobial activity of essential oils and extracts (Kloucek et al., 2012). It involves the use of the vapour phase of essential oil of interest through drying on to a piece of material, typically a filter paper. However, to study the effects of essential oils in liquid form, with or without dilution, other techniques such as agar diffusion assays, the poisoned food technique and the broth dilution method can be employed (Balouiri et al., 2016). A small number of studies on the use of these extracts

during cultivation *in vivo* were also reviewed, mainly in the form of a protective vapour treatment. Next, plant extracts and essential oils have been assessed to help prevent spoilage and improve the longevity of postharvest fruit products (El Khetabi et al., 2022). The preferred method of utilising essential oils to increase the shelf life of freshly harvested and cut fruits is through the incorporation into edible coating. Various formulations coatings with essential oils and extracts have previously been studied such as nano-emulsions and biopolymer-based coatings. Finally, they have also been studied as potential preservatives in fruit juices, with an additional focus on the potential effects on the sensory attributes like taste and appearance.

Durian

Durian (*Durio zibethinus*) is an edible fruit belonging to the genus *Durio*. It is grown in the tropics and is characterized by its hard, spiky outer shell. Durian is often exposed to fungal infections, causing various diseases to the crop. *Phytophthora palmivora* is a type of fungus that causes stem canker; *Rhizoctonia solani* is a soil-borne pathogenic fungus that causes leaf blight in durian trees; meanwhile, stem rot in durian trees is caused by *Fusarium solani*. Several studies have been conducted to evaluate the potential antifungal activity of essential oils and plant extracts against these types of fungi *in vitro*. However, to the best of our knowledge, the utilization of essential oils or plant extracts to protect durian *in vivo* has not been studied.

Some essential oils and plant extracts have the potential to be formulated into organic fungicides to prevent stem canker in durian trees by inhibiting the growth of *P. palmivora* as tested *in vitro*. A recent study shows that the vapour of clove and citronella oils can slow down *P. palmivora* growth *in vitro* (Istianto and Emilda, 2021). This is due to the presence of eugenol, a major constituent of volatile clove oil that is believed to possess antifungal properties. Phenolic compounds in clove oil can inhibit the mycelial growth of *P. palmivora* by penetrating the fungal cell membrane and lipids. Hence, the compounds can access the cell's internal contents and disrupt the protein syntheses in the fungal cell (Ansari et al., 2013). At 10 mg/mL concentration, the n-hexane extract of clove buds and clove oil exhibited 90.0% and 72.7% growth inhibition, respectively, against *P. palmivora*. This study also concluded that despite having the same major antifungal compound, the n-hexane extract of clove buds is more suitable for using organic fungicide than clove oil due to its stronger antifungal activity. The synergistic effect of eugenol and other antifungal compounds in the n-hexane extract might contribute to higher efficacy in fungal growth inhibition (Aulifa et al., 2015). Also, the extract of *Cosmos caudatus*, commonly known as king's salad, in ethyl acetate recorded only 15.6% germination of *P. palmivora* compared to the control. This might be due to the ability of ethyl acetate to isolate secondary metabolites such as sesquiterpenes, lactones, stigmasterol and lutein from the crude extract that may contribute to the antifungal activity (Mohd Salehan et al., 2013). According to Garcia-Rellán et al. (2016), *P. palmivora* is sensitive to the essential oil extracted from *Satureja cuneifolia*, an aromatic plant used in Turkey to make herbal tea, as it inhibited 77.1% of the fungal growth at 1 mg/mL concentration. The extract from *Hydnocarpus anthelminthicus*, a tree found in the rainforests of Southeast Asia, also showed complete growth inhibition against *P. palmivora* and *R. solani* at a concentration of 10 mg/mL (Jantasorn et al., 2016). The extract's antifungal properties were mainly attributed to flavonoids, isoflavonoids, phenolics, phenol acids, coumarins and alkaloids.

Apart from preventing stem canker, essential oils and plant extracts can also potentially protect durian trees from leaf blight by inhibiting *R. solani* growth. According to a study carried out by Osman Mohamed Ali et al. (2017), the development of nano emulsions based on a combination of neem and citronella oils is proven to be potential organic fungicides that can control diseases caused by *R. solani*. It was reported that neem and citronella nano emulsions inhibited 40-80% growth of *R. solani* after four days of incubation. Another study reported that more than 80% of *R. solani* growth was inhibited when treated with *Asarum heterotropoides* var. *mandshuricum* essential oil *in vitro* (Dan et al., 2010). The antifungal activity of *Hypericum linarioides* Bosse essential oil against *R. solani* was also evaluated. The acetone and methanol extracts of *H. linarioides* inhibited 43-70% growth of *R. solani*, while pure *H. linarioides* essential oil showed 87.5% growth inhibition at 5 mg/mL (Cakir et al., 2005). In contrast to the previous study by Cakir et al. (2004) that showed α-pinene might contribute to the antifungal properties possessed by *H. linarioides* essential oil, this present study recorded the absence of α-pinene, suggesting the antifungal properties of this essential oil may be contributed by other compounds.

Stem rot and leaf blight caused by *F. solani* and *R. solani* are considered vital diseases in durian cultivation. A study reported that *Piper chaba* Hunter extract containing α-humulene, caryophyllene oxide, viridiflorol, globulol, β-selinene, spathulenol, (E)-nerolidol, linalool and 3-pentanol as antifungal components inhibited 70.3% growth of *R. solani* and 56.6% growth of *F. solani* when tested *in vitro* (Rahman et al., 2011). In another study, the essential oil from *Cuminum cyminum* oil possessed significant antifungal activity against *F. solani* due to pinene, cineole and linalool (Naeini et al., 2010). *Myrcia ovata* Cambessedes essential oil may also be an alternative fungicide to control *F. solani* to prevent stem rot in durian trees. An *in vitro* study showed that *M. ovata* essential oil completely inhibited mycelial growth at a concentration of 30 µL/mL. The authors suggested that this observation may be due to the oil components such as linalool, nerolic acid, geraniol,

neral, geranial, (E)-nerolidol, 1,8-cineole and isopulegol (Sampaio et al., 2016). These studies illustrate that essential oils and plant extracts have the potential to be commercialized as botanical fungicides to protect durians from fungal infections. The efficiency of essential oils and plant extracts to inhibit fungal growth *in vitro* suggests that they may be used as an alternative to synthetic fungicides to control the durian trees' diseases caused by these phytopathogenic fungi.

Banana

Banana (*Musa paradisiaca*, *Musa acuminaia* or *Musa balbisiana*) is an edible fruit produced by plants of the genus *Musa* and grown in the tropics. The banana fruit is commonly eaten raw and is one of the most economically important tropical fruit crops. However, anthracnose and crown rot are postharvest diseases in banana fruits that reduce their quality. These postharvest diseases are caused by *Colletotrichum* spp. and *Fusarium* spp., respectively. Postharvest decay reduces the quality of banana fruits and is one of the biggest factors of economic losses. Several studies have been carried out *in vivo* and *in vitro* to investigate the efficacy of using essential oils and plant extracts in protecting banana fruits from postharvest diseases.

An *in vitro* study reported that the ethanolic extract of *Eucalyptus camaldulensis* at the concentrations of 0.5 mg/mL and 5 mg/mL inhibited the growth of *C. gloeosporioides* by 50% and 98%, respectively (España et al., 2017). Another *in vitro* study reported that the vapours of essential oil extracted from *Cinnamomum cassia*, commonly known as Chinese cinnamon, at volumes of 5 µL and 6 µL per 90x15 mm plate could completely inhibit the growth of *Lasiodiplodia theobromae* and *Colletotrichum musae* respectively. Holy basil oil also recorded complete inhibition against *L. theobromae* growth at 6 µL per 90x15 mm plate. The study used a modified disc volatilization method where a fixed concentration of tested essential oils (200 mg/mL) were added onto filter paper discs and were let to vaporize in sealed Petri plates containing agar inoculated with *L. theobromae* and *C. musae*. It was proposed that eugenol, cinnamyl acetate, humulene, trans-calamenene and caryophyllene present in both *Cinnamomum cassia* essential oil and holy basil oil, can damage the fungal cell wall and cell membrane, thus altering the membrane potential, which eventually leads to growth inhibition (Kulkarni et al., 2021).

In vivo studies need to be done to demonstrate further the potential of using essential oils and plant extracts as organic fungicides against anthracnose. A study showed that the methanolic extract of ginger containing α-curcumene and zingerone as active compounds inhibited more than 80% of *C. musae* growth at a concentration of 5 mg/mL when tested *in vitro*. Banana fruits treated with the extract also recorded a low score of anthracnose severity of 2.2 after five days of storage, compared to the untreated control, which scored 4.8 (Bhutia et al., 2016). Monoterpenes such as citral, L-carvone and citronellal may be used as the active compounds for synthetic fungicides, which completely inhibit *C. musae* conidia germination at concentrations as low as 2 mg/mL, 4 mg/mL, and 2mg/mL, respectively. They are significantly more potent compared to benomyl; a synthetic fungicide commonly used in agriculture that was found to be only able to inhibit 79% of the *C. musae* conidia germination *in vitro*. This shows that these monoterpenes can be formulated into synthetic or chemical fungicides such as benomyl, to enhance their efficacy in killing fungi. Bananas treated with citral showed a 60% reduction of anthracnose lesion diameter compared to the untreated control. It was previously found that citral can negatively affect the tricarboxylic acid cycle, alter mitochondrial morphology, and cause metabolic disorders in pathogenic cells, inhibiting fungal growth and sporulation (Garcia et al., 2008). According to Vilaplana et al. (2018a), bananas treated with 500 µL/L thyme oil showed a 46.4% decay reduction compared to the commercially available fungicide Imazalil, which showed only a 29.4% decay reduction. The authors proposed that the synergistic effect of thymol and carvacrol in thyme oil induced the leakage of the fungal cell membrane, leading to fungal cell tissue deterioration. The fruits also showed better firmness, sensory qualities, and higher weight loss reduction during cold storage than bananas treated with Imazalil. A study reported that incorporating 4 mg/mL cinnamon oil into 100 mg/mL gum arabic can control 80% of anthracnose incidence in postharvest bananas and significantly reduced the weight loss by 89% compared to untreated bananas after 28 days of cold storage. It was also reported that this mixture inhibited 88% of *C. musae* growth when tested *in vitro* (Maqbool et al., 2011). Aloe vera incorporated with garlic oil inhibited 87.7% mycelial growth and 91.2% spore germination when tested *in vitro* against *C. musae*. The mixture was also tested as an antimicrobial coating, which was then found to reduce the incidence and severity of anthracnose by 92.5% and 81%, respectively (Khaliq et al., 2019a).

Crown rot disease is a type of fungal infection that initially occurs at the crown part of bananas and may spread to other parts of the fruit. It is often caused by *Colletotrichum musae* or *Lasiodiplodia theobromae*. Some essential oils and plant extracts have been found to have a similar antifungal activity to commercial fungicides used against crown rot disease. Jahan et al. (2019) reported that the methanol extract of garlic, *A. sativum*, has similar fungicidal activity as chemical fungicides like carbendazim and kanamycin B against crown rot. Spraying emulsions of basil oil on bananas was observed to control anthracnose and crown rot in bananas stored for 21 days. Interestingly, no significant differences were reported compared to benomyl treatment. It also did not affect the physicochemical and sensory properties of treated bananas (Anthony et al., 2003). In another study, eugenol in basil oil controlled crown rot by inhibiting appressorium formation

of *C. musae*, which is crucial to initiating an infection (Siriwardana et al., 2017). Moreover, the synergistic effects of *Cymbopogum nardus* oil and basil oil in a liquid medium are more effective in controlling crown rot in bananas than in benomyl treatment. This may be attributed to various antifungal components such as α-pinene, citronellol, citronellal, eugenol and geraniol (Anthony et al., 2004).

Spraying 40 mg/mL cinnamon and 40 mg/mL thyme oil completely controlled crown rot incidence in bananas. The study also found an 87.1% and 78.7% reduction in crown rot incidence when bananas were treated with sweet almond and bitter almond oil, respectively, without altering the organoleptic properties (Abd-Alla et al., 2014). Complete inhibition of crown rot disease in bananas was recorded when treated with a 250 mg/mL concentration of Zimmu leaf extract without altering organoleptic properties. The extract treatment also was found to have better fungicidal activity than the benomyl in reducing crown rot severity (Sangeetha et al., 2013). The cinnamon extract inhibited 25% of crown rot disease in bananas without affecting postharvest quality (Win et al., 2007). An *in vitro* study carried out by Kamsu et al. (2019) found that cinnamon oil inhibited 100% conidial germination of *C. musae*, *Fusarium incarnatum* and *Fusarium verticillioides* at concentrations of 1025, 950 and 9088 µL/L respectively. *C. musae*, *F. incarnatum* and *F. verticillioides* conidial germination were also completely inhibited by lemongrass oil at 200, 185 and 275 µL/L, respectively. The germination inhibition may be due to the terpenes that act as antifungal compounds, disrupting fungal germination in essential oils. The previous results were validated when Ranasinghe et al. (2002) also found that cinnamon and clove oils possess fungicidal properties against *C. musae*, *L. theobromae* and *F. proliferatum* when tested *in vitro*.

These studies suggested that essential oils and plant extracts have the potential to be organic fungicides in controlling anthracnose and crown rot disease in postharvest bananas. They also can extend the shelf life of postharvest bananas by improving the physicochemical properties without interfering with the organoleptic properties or sensory qualities.

Pineapple

Pineapple (*Ananas comosus*) is a tropical plant from the family *Bromeliaceae*. It has spiky leaves on top and tough leathery skin. Fusariosis is a type of fungal infection that commonly affects pineapple plants. *Fusarium* spp. is the common fungus responsible for fusariosis in pineapples. Fresh-cut pineapples and pineapple juices are susceptible to mould and yeast contamination that causes spoilage. Several studies have been conducted to evaluate the uses of essential oils and plant extracts as natural fungicides to protect pineapples from fusariosis and as alternative preservative methods to control postharvest spoilage.

Essential oils and plant extracts can be used to control fusariosis in pineapples. A recent study reported that monoterpenes such as citral, L-carvone, and citronellal could be a potential alternative for synthetic fungicide as they completely inhibited *Fusarium subglutinans* f.sp *ananas* germination at a concentration as low as 4 mg/mL, 8 mg/mL and 6 mg/mL respectively compared to commercially available chemical fungicide, benomyl that has lower conidia germination inhibition of 42% when tested *in vitro* (Garcia et al., 2008). It shows that citral has a very high antifungal characteristic. It was proposed that citral enter the cell by inducing malondialdehyde, reducing cell membrane elasticity. Then it alters the citric acid cycle and mitochondria morphology which subsequently inhibits fungal growth and sporulation (Luo et al., 2004). A study reported that thyme oil inhibited 100% of *Fusarium verticillioides* mycelial growth at a concentration as low as 250 µL/L when tested *in vitro*. As for *in vivo* study, postharvest pineapples treated with 1000 µL/L thyme oil showed 50.1% disease reduction in 7 days of storage without affecting the sensory quality, higher than fruit treated with chemical fungicide, prochloraz, which showed 32.7% disease reduction (Vilaplana et al., 2018b).

Cutting fruits increases their metabolic activity, thus reducing their shelf life. It also increases the susceptibility to microbial contamination and lowers the quality of the fruit. The development of edible coatings incorporated with essential oils or plant extracts can act as a barrier that protects fresh-cut pineapples from microbial contamination, prolongs shelf life and maintains their quality. Some major essential oil compounds are difficult to incorporate into food due to their lipophilic nature. To overcome this challenge, lipophilic compounds must be emulsified into nano emulsions to be easily incorporated into edible coatings for fruit protection. A study conducted by Prakash et al. (2020) reported that edible alginate coatings incorporated with 0.5 mL/100mL and 1 mL/100mL of citral nano emulsions inhibited *Salmonella typhimurium* and *Listeria monocytogenes* total plate count growth by 4.68 log CFU/g and 2.77 log CFU/g respectively compared to control which recorded higher than 7 log CFU/g. It was also observed that the colour and appearance of the coated cut pineapples were enhanced, which may be contributed by citral inhibiting polyphenol oxidase activity in the coated fruits. This shows that the major compound citral found in various plant essential oils, such as lemongrass essential oil has antimicrobial activity. Another study showed that incorporating 3 mg/mL lemongrass essential oil into alginate coating reduced the weight loss in coated fresh-cut pineapples during storage. It was proposed that the lipophilic nature of essential oils can reduce respiration rate, reducing the weight loss in coated fruits (Azarakhsh et al., 2014). de Araujo et al. (2021) reported that the shelf life of fresh-cut pineapples coated with chitosan incorporated with black pepper (*Piper nigrum*) and Brazilian pepper (*Schinus terebenthifolia*) essential oil was improved by 45 days and recorded 98.4%

efficiency in reducing microbial counts such as *E. coli* and *S. aureus*.

Pineapple juice is susceptible to spoilage caused by mould or yeast contamination. A study reported that sodium benzoate and citrus extract could be used during fruit juice homogenization as antimicrobial preservatives to reduce spores of *Fusarium oxysporum*. Curiously, *F. oxysporum* is a type of fungus that is typically resistant to homogenization. The citrus extract reduced spore counts to 1.14 CFU/mL at a concentration of 1.5 mg/mL and completely removed the spores at a concentration of 3 mg/mL compared to the control, which recorded 6 CFU/mL spore counts. It was proposed that terpenes in the citrus extract can increase peroxide concentration, causing the breakdown of the cell wall and destroying the fungus's vegetative reproduction (Bevilacqua et al., 2012). An investigation by da Cruz Almeida et al. (2018) reported that essential oils from spearmint (*Mentha spicata* L.) and Bowles mint (*Mentha* × *villosa* Huds) can be used in the preservation of pineapple juices against spoilage yeasts. A reduction of *Pichia anomala* and *Saccharomyces cerevisiae* was observed when pineapple juices were treated with 3.75 µL/mL of *M. spicata* essential oil (MSEO) after 48 h of exposure. A reduction in *S. cerevisiae* was observed when treated with 15 µL/mL of *M. x villosa* essential oil (MVEO). This might be due to the antifungal components found in MSEO and MVEO, such as carvone and piperitone oxide. It was proposed that carvone inhibits the proton pump, and the biosynthesis pathway of ergosterol in fungal cells eventually disturbs the cell integrity (Samber et al., 2015). At the same time, piperitone oxide could disrupt the cell membrane hence altering the metabolic activity of the fungus (Ait-Ouazzou et al., 2012; Guerra et al., 2015). These studies present that the antifungal activity of essential oils and plant extracts can be utilized to protect pineapple plants from fusariosis, improve the quality of processed pineapples and control the spoilage in pineapple juices.

Watermelon

Watermelon (*Citrullus lanatus*) is a flowering fruit from the family *Cucurbitaceae*. The crop is cultivated globally but thrives in tropical climates such as that near the equator. It is commonly characterized by a large round fruit protected by a hard outer skin painted with green stripes. Watermelons are exposed to pests and viral infections, while processed watermelons tend to get spoiled due to higher enzymatic reactions. A few studies that have been carried out over the past few years found that essential oils and plant extracts can be utilized to protect fresh and processed watermelons from harmful pests and pathogenic microbes.

Bactrocera cucurbitae, commonly known as the melon fly, is a watermelon pest that causes significant losses to farmers. Melon flies lay their eggs on the watermelon fruit and once hatched, maggots will feed on the fruit, damaging it internally and causing it to quickly rot. Instead of using chemical insecticides to control the infection of *B. cucurbitae*, some plant extracts can be utilized as potential biopesticides that could target the pests at their earlier stage of development. A study was carried out to investigate the larvicidal and pupicidal activities of neem (*Azadirachta indica*), Chinese chaste tree (*Vitex negundo*) and water pepper (*Persicaria hydropiper*) methanolic extracts against *B. cucurbitae in vitro*. It was shown that exposure to *A. indica*, *V. negundo* and *P. hydropiper* recorded high mortality degrees of *B. cucurbitae* (LD_{50} 1.161 mg/cm^2, 2.213 mg/cm^2 and 0.853 mg/cm^2 respectively) in the larvicidal test. *A. indica* and *P. hydropiper* extracts also recorded significantly high pupicidal activities against *B. cucurbitae* (LD_{50} 0.26 mg/cm^2 and LD_{50} 8.70 mg/cm^2 respectively) (Hossain and Khalequzzaman, 2018).

Apart from the damage caused by *B. cucurbitae*, watermelon crops are also susceptible to viral infections such as the watermelon mosaic virus which affects their overall physical features such as yellow spots on leaves, stunted growth, severe discolouration and in extreme cases, necrosis (Xu et al., 2004; Desbiez and Lecoq, 2021). Some plant extracts have been discovered to effectively prevent viral diseases in watermelons by activating defence mechanisms in the treated fruits. A study demonstrated that seed treatment of watermelons followed by six foliar sprays using *Boerhaavia diffusa* root, *Clerodendrum aculeatum* leaf, *Azadirachta indica* leaf, and *Terminalia arjuna* bark extracts recorded 54.2%, 45.6%, 52.0% and 34.8% viral disease reduction, respectively. Increments in vine length, fruit diameter and weight in watermelons treated with these extracts were also observed. The authors proposed that phytoproteins present in the extracts induced a viral resistant mechanism in treated plants by stimulating the production of a viral inhibiting agent (VIA) in the host cells. However, the details of which remain to be fully elucidated. It was also reported that *B. diffusa* may be able to alter the morphology of plant cells to inhibit viral multiplication in host cells (Sharma et al., 2017).

Since fresh-cut and watermelon juices are susceptible to yeasts and mould growth, cinnamaldehyde, mostly found in the essential oil of cinnamon bark, can be used as an antimicrobial agent and employed as an edible coating and preservative. Trans-cinnamaldehyde incorporated into alginate-based coating can act as an antimicrobial compound in multi-layered edible coating to protect fresh-cut watermelons. It was shown that coated fresh-cut watermelons have lower yeasts and mould growth than uncoated fresh-cut watermelons. The coatings also act as a barrier to prevent the respiration rate of the fruit, hence delaying the softening of fresh-cut watermelons and reducing weight loss (Sipahi et al., 2013). Another study also proved the antimicrobial effect of trans-cinnamaldehyde when used as a preservative in watermelon juices, where the solubility was enhanced by nano-emulsification. The study showed that 8 mg/mL trans-cinnamaldehyde inhibited *Salmonella typhimurium* and *Staphylococcus aureus* growth in watermelon

juices and extended the shelf life (Jo et al., 2015). These studies show that essential oils and plant extracts can be used as alternative synthetic pesticides and further developed in food preservation techniques.

Papaya

Papaya (*Carica papaya*) is a tropical fruit from the family *Caricaceae*. It has a sweet taste and juicy flesh, turning orange when ripe. Postharvest papaya is commonly infected by the *Colletotrichum* spp., the fungus which causes anthracnose; meanwhile fresh-cut papayas are prone to mould and yeast contamination. The use of essential oils incorporated into edible coatings has been studied to avoid quality losses of postharvest and fresh-cut papayas.

Coating papayas with *Aloe vera* (AV) can lower the respiration rate of the fruit which subsequently slows down the metabolic process. This helps to delay ripening and increase the shelf life during storage. A study reported that postharvest papayas coated with 50 mL/100 mL of AV gel diluted in distilled water recorded a 2.05% weight loss and 52.29 N of firmness compared to uncoated papayas with a significantly higher 13.2% weight loss and lower firmness of 12.7 N during 15 days of storage. No disease incidence is reported for papayas coated with 50 mL/100 mL AV gel diluted in distilled water after 15 days of storage at a temperature of 28 ± 2°C and 68–70% relative humidity (Mendy et al., 2019). However, a slight increase of relative humidity to a range between 82 to 84%, and decrease of room temperature to 25°C led to 27% disease incidence despite being coated with 100% AV (Brishti et al., 2013). This suggests that although effective, the antimicrobial property of AV gel may be sensitive to fluctuations in room humidity and temperature. Water is generally lost from the papaya fruit through its peel. AV gel coating was found to act as a barrier for water loss, which also contributes to the reduction in fruit weight loss. It was proposed that AV can extend the shelf life of stored fruits by altering their internal environment (Serrano et al., 2005; Valverde et al., 2005). AV gel coating reduces the oxygen availability for oxygen degradation, allowing carotenoid retention.

Another study showed that 20 mg/mL ginger oil incorporated into 100 mg/mL gum arabic used as an edible antimicrobial coating for postharvest papayas recorded lower anthracnose incidence (21%) compared to control (100%) during 28 days of storage. This observation was accompanied by the amelioration of the quality of postharvest papayas without any significant effect on the sensory properties. Using a disease severity scoring scale of 0 to 5, the fungicidal activity of ginger oil was demonstrated when it scored 2.2, less than half compared to the control which reached a maximum score of 5 *in vivo*. This is believed to be due to the antifungal compounds such as α-pinene, 1,8-cineole and borneol present in ginger oil (Ali et al., 2016). These antifungal compounds can lower pathogenic infection by blocking lenticels and cuticles, reducing the respiration and ripening rate of fruits (Martínez-Romero et al., 2006). Limited oxygen availability also reduced the enzyme activity responsible for fruit softening and maintaining fruit firmness (Salunkhe et al., 1991). Postharvest papayas with mesquite-gum based edible coating incorporated with 0.1% (w/w) thyme oil and 0.05% (w/w) Mexican lime essential oil recorded 100% reduction of *C. gloeosporioides* and *Rhizopus stolonifer* infection (Bosquez-Molina et al., 2010). Comparing these two essential oils, Mexican lime oil exhibits higher fungicidal activity than thyme oil, especially when utilized at high concentrations. However, a concentration too high may be poisonous to the fruit which can consequently alter the fruits' tissue ability to inhibit microbial growth. It is then vital to identify the optimal concentration with maximum antifungal properties yet tolerable to the fruits.

Treating postharvest papayas using carboxymethyl cellulose associated with *Lippia sidoides* essential oil delayed rotting and recorded the lowest minimum inhibitory concentration (0.0753 mg/mL) against *C. gloeosporioides* (Zillo et al., 2018). This is due to the presence of thymol and carvacrol in the essential oil that can modify the fungal cell wall and cell membrane to the point of disrupting the essential growth process of the fungus. Maqbool et al. (2011), incorporated of 4 mg/mL cinnamon oil into 100 mg/mL gum Arabic, which controlled anthracnose incidence by up to 71% in postharvest papayas and significantly reduced 81% of the fruit weight loss compared to untreated papayas. Apart from that, it also inhibited 85% of *C. gloeosporioides* growth when tested *in vitro*. These effects are ascribed to the presence of cinnamaldehyde, which limits microbial growth by disrupting the electron transport chain and reacting with nitrogen-containing compounds (Gupta et al., 2008).

Apart from protecting postharvest papayas, essential oils are also useful in reducing mould and yeast contamination in fresh-cut papayas. Fresh-cut papayas coated with cassava starch-based edible coating and 10 mg/mL lemongrass essential oil effectively suppressed yeasts and mould growth by up to 1.48 log CFU/g. They recorded greater weight preservation than uncoated fresh-cut papayas (Praseptiangga et al., 2017). Another study showed that encapsulated trans-cinnamaldehyde could act as an antimicrobial compound when incorporated into multi-layered edible coating without altering the flavour of fresh-cut papayas (Brasil et al., 2012). Moreover, when fresh-cut papayas were coated with 10 mg/mL psyllium gum and sunflower oil, 5 log CFU/g of mould and yeast count was recorded. Contrarily, more than 10 log CFU/g was recorded for the uncoated counterpart. In addition, the hydrophobic property of sunflower oil is can act as a barrier to water vapour loss, leading to reduced weight loss in the coated fruits (Yousuf and Srivastava, 2015). However, it is important to note that the effectiveness of essential oils in protecting fresh-cut papayas only applies to specific time points. Based on these studies, it is shown that essential oils can be utilized to improve the quality in postharvest and fresh-

cut papayas upon storage, mainly through incorporating them into edible external coatings.

Guava

Guava (*Psidium guajava*) is a tropical fruit from the family *Myrtaceae*. It is a small-sized fruit with a crunchy texture with high contents of vitamin C. Guavas are exposed to fruit pests such as *Bactrocera cucurbitae*, leading to fruit rot and spoilage. Yeasts such as *Pichia anomala* and *Saccharomyces cerevisiae* affect the quality of guava juices. Therefore, essential oils and plant extracts' effectiveness in protecting guava from pests, extending the shelf life of postharvest guavas, and reducing spoilage in guava juices were studied.

Incorporating essential oils and plant extracts into edible coating can improve the quality and extend the shelf life of postharvest guavas. Formulation of 10 mg/mL pomegranate peel extract in chitosan coating reduced the transpiration rate in coated guavas due to its lipophilic properties. The low transpiration rate maintains the concentration of internal compounds, resulting in only 29% of ascorbic acid, 8% of total phenol and 12% total flavonoid being lost, thereby delaying of the ripening process during storage (Nair et al., 2018). It was also recorded that postharvest guavas coated with aloe vera gel maintained total flavonoid contents, total antioxidant capacity and sensory properties after 12 days of storage at 27 – 29°C (Kumar et al., 2017). Treating guavas with 2.5 mL/100 mL Tulsi extract incorporated into Arabic gum and sodium caseinate inhibited mould growth during seven days of storage at 28°C (Murmu and Mishra, 2017). Another study reported that 2% cinnamon oil and 20 mg/mL lemongrass oil incorporated into 50 mg/mL Arabic gum and 10 mg/mL sodium caseinate extended guava shelf life up to 40 days. It was proposed that geraniol in lemongrass can slow down polyphenol oxidase (PPO) activity by forming hydrogen bonds with active enzymes which reduce browning in treated guavas (Murmu and Mishra, 2018).

Aloe vera can be used as an edible antimicrobial coating to protect fresh-cut guavas. Lower weight loss and microbial count were reported on fresh-cut guavas coated with aloe vera than on uncoated fresh-cut guavas. This is due to the antimicrobial compounds such as pyrocatechol, cinnamic acid and p-coumaric acid in aloe vera. The coating can also attract and hold water, preventing water loss and weight loss of guavas (Nasution et al., 2015). In addition to the improvement in the quality of pineapple juice post-storage described above, da Cruz Almeida et al. (2018) reported that the essential oils from spearmint (*Mentha spicata L.*) and *Mentha* × *villosa Huds* are also able to preserve guava juice against spoilage yeasts in the same manner. This is due to the fact that these different fruit juices are susceptible to infection by a common pathogen, namely *Saccharomyces cerevisiae*.

Based on these studies, it is believed that essential oils and plant extracts are able to prevent the degradation of phytochemicals leading to extended shelf life of postharvest guavas and prevent contamination in the processed fruit juice. The observations also further illustrate the flexibility of essential oil in preventing a range of unrelated crops from postharvest spoilage. However, pre-cautions need to be taken into account during formulation process, especially when incorporating essential oils into secondary products as high concentrations of essential oil or plant extract can be unfavourable to the sensory attributes of the fruit juice.

Mangosteen

Mangosteen (*Garcinia mangostana*) is a small tropical fruit from the family *Clusiaceae*. It has a purple rind that is both thick and hard to protect a slightly sweet and sour flesh. Postharvest losses of mangosteen can be caused by fungal infections such as *Glomerella cingulata* or gradual fruit ripening and decaying. Several studies have been carried out to investigate the effect of essential oils on mangosteen *in vitro* and *in vivo*.

In vitro studies carried out by Permana et al. (2021) showed that emulsions incorporating virgin coconut oil and cinnamaldehyde can impede the growth rate of *Glomerella cingulata* and can potentially be used as an edible coating for mangosteen. In addition, extracts from various plants such as clove buds, pepper, cinnamon, turmeric, ginger, oregano and thyme may also be potentially used in organic fungicides due to their content of eugenol. Due to its poor stability, Velho et al. (2019) studied the nanoencapsulation of eugenol and the synthetic fungicide, mancozeb, and the consequent antifungal activity against *G. cingulata*. It was found that this mixed formulation had increased antifungal efficacy compared to the free forms of eugenol and mancozeb. The toxicity of the resulting formula was tested, and the authors found that it was safe for plant cells and relatively non-toxic in the soil (da Silva Gündel et al., 2019). Mangosteen treated with 2 mL/L of citronella oil also recorded 20% lower scarring symptoms and a lower ant attack percentage compared to untreated mangosteen. Citronella oil can cause death to ants by damaging its integument. Not only that, the presence of odorous compounds such as citronellal, citronellol and geraniol naturally carry the ability to repel insects (Istianto and Emilda, 2021).

According to Owolabi et al. (2021b), postharvest mangosteen treated with peppermint oil and lime oil formulated with a ratio of 1:3 led to fewer fungal infections. They are also observed to ripen slower which can contribute to prolonged shelf life. Another study also noted that tapioca starch incorporated with peppermint oil and lime oil could be applied on rubberwood boxes to preserve postharvest mangosteen

during transportation (Owolabi et al., 2021a). Limonene, γ-terpinene, terpinolene, eucalyptol, menthone, and menthol are predicted to be the major components of the mixture that contributed to the remarkable antifungal activity. The process of postharvest ripening can also be influenced by the concentration of ethylene. Previously, it was found that essential oils can suppress the 1-aminocyclopropane-1-carboxylic acid synthase oxidase (ACO) transcription gene that is responsible for ethylene production (Owolabi et al., 2021b). This genetic alteration is one of the many notable modes of action in which essential oils can be utilised to improve the longevity of perishable crops. Hence, it is proven that essential oils can be used as natural pesticides and fungicides to maintain the quality of mangosteen.

Mango

Mango (*Mangifera indica*) is a sweet tropical fruit belonging to the family *Anacardiaceae*. It varies greatly in shape, colour and taste. The flesh is typically sweet when ripe. In some Asian regions, the fruit is enjoyed when not fully ripened, where it carries a sharp sour taste. *Colletotrichum* sp. is a common anthracnose-causing agent in many fruit crops, including mangoes. Several studies have elaborated on applying essential oils as edible coating to protect and improve the quality of postharvest mangoes. Antifungal properties and the ability of essential oils and plant extracts incorporated into edible coatings to control respiration rate and act as a barrier to water vapour have also been studied in the management of diseases in postharvest mangoes.

Coatings formulated with essential oils and plant extracts were found to reduce diseases in coated mangoes. A test carried out by de Oliveira et al. (2017) evaluated the antifungal effect of *Mentha piperita* essential oil (MPEO) on *Colletotrichum asianum*, *Colletotrichum dianesei*, *Colletotrichum fructicola*, *Colletotrichum tropicale* and *Colletotrichum karstii*. The synergistic effect of the chitosan coating (5 or 7.5 mg/mL) and *M. piperita* essential oil (MPEO) (0.3, 0.6 or 1.25 µL/mL) inhibited 100% of all *Colletotrichum* sp. growth tested on mango fruits. A large portion of MPEO is made up of monoterpenes, such as menthol and isomenthone, which can disrupt the cellular metabolism of fungal cells (dos Santos et al., 2012). The authors proposed that chitosan may be able to alter the fungal cell membrane permeability, allowing antifungal compounds present in MPEO to act on the fungal cell. Mycelial growth percentages of *Colletotrichum* sp. in the range of from 13.5-85.2% were inhibited by 0.3-2.5 µL/mL of MPEO respectively, *in vitro*. This indicates that the antifungal activity of MPEO is concentration dependent, at least up to a concentration of 2.5 µL/mL. Interestingly, mangoes coated with 5 mg/mL and 0.6 µL/mL of chitosan/MPEO recorded lower anthracnose lesion severity than the synthetic fungicide, difenoconazole. Next, ginger oil was investigated as an antimicrobial additive when it was incorporated into a hydroxypropyl methylcellulose coating. When tested for *C. gloeosporioides* growth, the coating showed 42.6% inhibition and consequently, a 38% anthracnose reduction compared to untreated controls after being stored at 25°C for five days. Weight and firmness were also better preserved compared to uncoated mangoes (Klangmuang and Sothornvit, 2018). According to Zhou et al. (2021), 80 mg/mL galangal essential oil incorporated into carboxymethyl chitosan and pullulan coating also prolonged the shelf life of coated mango up to 9 days. After 15 days of storage, mangoes treated with the carboxymethyl chitosan/pullulan coating incorporated with 80 mg/mL galangal essential oil recorded lower weight loss (8.7%) and greater firmness (3.82N) than uncoated mango.

These results were able to indicate the uses of essential oils in the disease control in mangoes at the postharvest stage. Further studies can be done to evaluate the potential of utilising these natural extracts at different points of the mango supply chain. It may be of interest to fully understand how essential oils can affect the sensory properties of mangoes preharvest due to the fact that mangoes are highly variable in taste.

Sweet orange

Sweet orange (*Citrus X sinensis*) is a hybrid fruit resulting from the cross cultivation of mandarin orange and pomelo. The overall appearance is similar to the typical orange, but sweet oranges are comparably smaller. *Penicillium digitatum* is a type of fungus that causes green mould in postharvest oranges while *Penicillium italicum* causes blue mould. Some fungi such as *Issatchenkia orientalis*, *Meyerozyma caribbica* and *Meyerozyma guilliermondii* are responsible for spoilage in processed orange juice. Several studies evaluated antifungal activity against these postharvest pathogenic fungi by incorporating essential oils or plant extracts into edible coating and preservation methods.

Essential oils incorporated into edible coatings can reduce the disease severity of coated oranges. 0.361 g/mL of pomegranate peel extract incorporated into chitosan and locust bean gum also recorded a green mould incidence reduction (95% and 75%, respectively) when used as coatings for sweet orange compared to uncoated control. The high phenol content in the pomegranate peel extract is believed to be driving the reduction in green mould incidence (Kharchoufi et al., 2018). Another study reported the incorporation of tea tree oil into chitosan coating reduced 50% of *P. italicum* growth on artificially inoculated oranges compared to uncoated fruits. The authors also incorporated bergamot oil into the coating,

which added the effects of preserving the weight and firmness of the fruit (Cháfer et al., 2012).

Adeogun et al. (2016) reported that ethanolic extract of a herbaceous plant in Africa, commonly known as the miracle berries or *Thaumatococcus daniellii*, could potentially be used to protect sweet orange juice against spoilage yeasts *in vitro*. It was reported that the minimum inhibitory concentration for the ethanolic extract of *T. danielli* against *I. orientalis*, *M. caribbica* and M. *guilliermondii* are as low as 0.1, 0.5 and 0.1, respectively. Ethanol can be a useful solvent in extracting plants' antimicrobial compounds of plants to be used as antimicrobial preservatives in fruit juices. As ethanol is acidic, it can make a medium more acidic by donating a hydrogen ion in the aqueous state. Microorganisms present in the medium tend to take up the hydrogen ion, leading to increased concentration of hydrogen ions inside the microbial cells and subsequently, death. Hence, the potency of essential oils or plant extracts to replace chemical fungicides in agriculture and food preservation is proven.

Other tropical fruits: Rambutan, jackfruit, dragon fruit, salak and sapodilla

Rambutan, jackfruit, dragon fruit, salak and sapodilla are fruits that are mostly cultivated in tropical climates, typically within the Southeast Asian region. They, like many other fruit crops, are highly prone to fungal infections. Several studies have been carried out to investigate the efficiency of essential oils and plant extracts against phytopathogenic fungi in the form of edible coating and organic fungicides.

Rambutan (*Nepphelium lappaceum*) has a juicy white flesh protected by typically a red or yellow hairy outer skin. *Oidium nephelii*, is a pathogen of the rambutan fruit, causing powdery mildew at the preharvest stage of cultivation. In addition, rambutan is also susceptible to other more common fungal infections that cause postharvest diseases, such as *Colletotrichum gloeosporioides*, *Gliocephalotrichum microchlamydosporum, and Botryodiplodia theobromae* leading to anthracnose, brown spot and stem end rot, respectively.

The extracts of wood vinegar and *Curcuma longa* (turmeric) were previously studied as alternatives to chemical fungicides in controlling powdery mildew. An *in vitro* study showed that *O. nephelii* germination was completely inhibited when treated with 0.5 µL/mL of wood vinegar extract and 0.5 g/mL extract of *Curcuma longa*. The fungicidal effects of both extracts were further investigated through *in vivo* studies when treatment with *Curcuma longa* and wood vinegar extract recorded 13.8% and 9.3% of infection severity, respectively. This pales in comparison to the untreated control which recorded an infection severity of 61.1% (Preecha et al., 2017). Rambutan fruits treated with clove oil for 13 days exhibited complete inhibition of powdery mildew infection compared to the untreated control *in vitro*, where the latter grew by 40-fold in colony size (Istianto and Emilda, 2021). The reason for this inhibition is believed to result from the alteration of fungal cell surface and structure by clove oil which inhibits the development of the fungus. However, the study also found that at high concentrations (4 mg/mL), rambutan fruit damage was observed, suggesting a potential phytotoxic effect of the clove oil. In postharvest rambutan, cinnamaldehyde was reported to be effective against common pathogens such as *C. gloeosporioides*, *G. microchlamydosporum* and *B. theobromae*. Complete inhibition of mycelial growth and spore germination of all three fungi were recorded when treated with cinnamaldehyde at 0.03 mg/mL and 0.05 mg/mL *in vitro*; meanwhile, an *in vivo* study recorded reduced disease severity in rambutan when treated with the same concentration (1.5 cm lesion diameter) compared to untreated control (4 to 4.5 lesion diameter) (Sivakumar et al., 2002).

Jackfruit (*Artocarpus heterophyllus*) is a tropical fruit characterized by bumpy outer skin, stringy core and multiple seeds with yellow coloured flesh. The fruit has been gaining much attention as a meat replacement in vegetarian and vegan communities, making it an economically important export. They are, however, extremely prone to rotting and spoilage, hence the immediate need for effective control measures during transport and storage. Postharvest rot in jackfruits caused by *Penicillium notatum* may be prevented by using basil (*Ocimum basilicum*), and *Vetiveria zizanioides* essential oils. A study carried out by Atif et al. (2020) reported that vapour treatment using the mixture of *O. basilicum* and *V. zizanioides* essential oils at 25 µL concentration reduced *P. notatum* colony area to 4.2 ± 1 cm^2. It completely suppressed the spore germination after seven days when tested *in vitro*. *P. notatum* growth was also reduced when postharvest jackfruit was treated using the essential oil vapour. The presence of L-carvone and phenolic compounds in the essential oils may play significant roles in the mycelial growth inhibition of *P. notatum*.

Dragon fruit (*Hyelocereus megalanthus*) is a sweet-tasting fruit with small edible seeds. The fruit possesses a soft, scaly outer skin of various colours ranging from red, and purple to yellow. *Alternaria alternata* is a common fungus that causes postharvest disease in dragon fruit; meanwhile, anthracnose in dragon fruit is caused by *C. gloeosporioides* and *Colletotrichum fructicola*. Cinnamon (*Cinnamomum zeylanicum*) and clove (*Eugenia caryophyllus*) essential oils visually inhibited *A. alternata* growth *in vitro* with concentrations as low as 0.25 mg/mL and 0.5 mg/mL, respectively. Meanwhile, dragon fruit treated with 500 µg/mL *E. caryophyllus* essential oil recorded a 31% reduction of mycelial growth compared to untreated fruit

in vivo. A separate study against *C. gloeosporioides* showed that 10.0 mg/mL of ginger extract in ethanol inhibited 88.5% of mycelial growth and 87.5% of conidial germination. Gingerol, the compound responsible for the distinctive ginger taste, may be responsible for the antifungal activity of ginger oil. While effective at controlling the growth of *C. gloeosporioides*, a higher concentration of ginger oil is needed when compared to the commercial fungicide, mancozeb, which recorded an inhibition of 80.7% at a much lower dosage of 2 mg/mL. Dragon fruit treated with 10 mg/mL of turmeric extract and "dukung anak" extract controlled anthracnose incidence postharvest during 28 days of storage compared to control due to the action of curcumin and alkaloids present in the extracts. Gingerol, curcumin and alkaloids can disrupt the fungal cell wall, causing the leakage of electrolytes which leads to the death of the fungus. However, at higher concentrations, "dukung anak" extract and turmeric extract can exhibit phytotoxicity by damaging the fruit's cell tissue and allowing phytopathogenic organisms to attack the damaged fruit (Bordoh et al., 2020). Next, another study reported that 400 µL/L of carvacrol essential oil completely inhibited *C. fructicola in vitro* when treated fruits recorded a lower lesion (7.6 mm) than untreated fruits (27.33 mm). It was also reported that treatment with carvacrol increased the concentration of malondialdehyde, a marker for oxidative stress. The authors proposed that carvacrol can increase the production of reactive oxygen species, leading to lipid peroxidation and increased membrane permeability in *C. fructicola*. It also can modify cell permeability, leading to the unintentional exchange of intracellular components and eventually causing death in fungal cells (Pei et al., 2020).

Salak (*Salacca zalacca*) is a fruit with tough, scaly, and prickly reddish-brown outer skin. The white flesh tastes sweet when fully ripened but tastes sour if consumed unripe. A recent study showed that application of 0.08% (w/w) orange oil vapour in a closed air system completely inhibited *Marasmius palmivorus* and *Thieviolopsis* sp. growth on salacca fruit due to the presence of limonene and subsequently extended the shelf life up to 28 days. Due to its ability to pass through the fungal cell membrane, limonene can disrupt protein synthesis in the fungus and subsequently inhibit fungal sporulation and germination. (Phothisuwan et al., 2021).

Sapodilla (*Manilkara zapota*) is a brown and round or oblong-shaped fruit with a sweet taste with gritty textured flesh, almost like that of a kiwi. Plant extracts can be incorporated into coatings to preserve postharvest sapodilla. Khaliq et al. (2019b) reported that the formulation of 100% aloe vera and 10 mg/mL *Fagonia indica* extract could be applied as a coating to extend the shelf life and preserve sapodilla during storage. Treated sapodilla recorded 9.3% weight loss, higher firmness level (6.67N) and lower decay incidence (4.3%) compared to untreated sapodilla (22.1%, 3.65N and 34.7% respectively). Rambutan, jackfruit, dragon fruit, salak and sapodilla are considered rather exotic fruits, even in regions where they are cultivated. Therefore, studies looking into the use of natural preservatives for the disease management of sapodilla crops remain scarce. With that being said, the limited studies are convincing to demonstrate the versatility of using essential oils and plant extracts as natural disease-controlling mechanisms in a range of tropical fruit crops.

Conclusion and future directions

Essential oils and plant extracts have shown the potential to protect and enhance the quality of pre and postharvest fruits owing to their antimicrobial properties. Some of the essential oils and plant extracts can be used to be formulated as organic fungicides to control diseases in preharvest fruits. As for postharvest fruits, essential oils and plant extracts can be developed into edible coatings incorporated with antimicrobial agents for protection during storage and transportation. Coatings can also be designed for shorter-term storage to prevent rapid spoilage of processed products such as fresh-cut fruits. As essential oils are generally lipophilic while plant extracts are typically extracted in organic solvents, the respiration rate of coated postharvest fruits may be significantly reduced due to the limited exchange of gases. This, in turn, results in the extension of their shelf life, allowing exporters to limit the use of synthetic preservatives. Essential oils and plant extracts may be utilized as organic preservatives for fruit juices, apart from protecting preharvest, postharvest and processed fruits. Hydrophilic compounds with antifungal activities present in essential oils and plant extracts can distribute evenly in fruit juices due to the high water content, increasing their chances of interaction with microorganisms.

The effectiveness of essential oil or plant extract-based edible coating is still, however, dependent on the humidity and temperature of the storage environment; thus, more studies are needed to design more reliable and robust protective coatings effectively. More studies are also warranted to understand the formulation of essential oils and plant extracts and organic pesticides without diminishing their antimicrobial properties. Experiments investigating the antimicrobial properties of these oils and extracts are also concentrated on the direct implications to the pathogens; thus, the effects on the actual fruit need to be thoroughly understood through *in vivo* studies. Finally, although generally considered less toxic than chemical pesticides, the safety of essential oils and plant extracts in formulated coatings must be confirmed before they can be applied in the fruit industry.

Author contributions

Data curation, original draft preparation, writing revisions, editing, visualisation, NI, MM. Resources and review, SM, XS, AA, SC'N. Supervision and project administration, BG, ST, NI, KC. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Author SC'N was employed by company CAIQTEST Malaysia Sdn. Bhd.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.999270/full#supplementary-material

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The insecticidal effect of the botanical insecticide chlorogenic acid on *Mythimna separata* (Walker) is related to changes in MsCYP450 gene expression

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The oriental armyworm *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) can feed on the leaves of many crops, resulting in vast areas of damage and severe losses. Therefore, this insect has become a significant agricultural pest in north Asia. In this study, we fed 3rd instar larvae with artificial diets containing different concentrations of chlorogenic acid and found a significant lethal effect and the mortality increased with increasing chlorogenic acid concentration. Next, we measured the sublethal effect of chlorogenic acid at LC_{20} on the growth and development of *M. separata* larvae. The durations of the 4th and 5th instar were longer than those of the control group (prolonged by 0.8 and 0.6 days, respectively), and the 6th instar was shorter (by 1.1 days). The total survival rate, pupation rate, eclosion rate, sex ratio, and oviposition amount in the LC_{20} chlorogenic acid-treated group were significantly lower than those in the control group. Furthermore, transcriptome analysis of 3rd instar larvae fed various concentrations of chlorogenic acid revealed that several *MsCYP450* genes were significantly up-regulated, and this finding was further validated by qRT-PCR. In addition, various concentrations of chlorogenic acid and different treatment times significantly affected the enzyme activity of CYP450 in 3rd instar larvae. Importantly, dietary ingestion of dsMsCYP450 significantly reduced the mRNA level of *MsCYP450* genes and increased mortality in the presence of chlorogenic acid. Our results revealed that *MsCYP6B6*, *MsCYP321A7*, and *MsCYP6B7-like* play an essential role in the detoxification of chlorogenic acid by *M. separata*. This study provides evidence of control effect by botanical insecticide chlorogenic acid on *M. separata*, and potential detoxification mechanism mediated by P450 of botanical insecticide in arthropods.

KEYWORDS

chlorogenic acid, sublethal effect, P450, RNAi, *mythinma seperata*

1. Introduction

Through long-term co-evolution, insects and plants have formed a relatively stable ecological relationship. Plants not only provide nutrients for phytophagous insects, but also initiate a series of physical and chemical defenses to resist insect feeding. Generally, these defense mechanisms can be divided into two categories: constitutive defense and induced defense (Fornoff and Gross, 2014; Chen, 2015; Fyllas et al., 2022; Sobhy et al., 2022). Both defense mechanisms mainly include physical and chemical defenses. Physical defenses specifically include plant morphology, leaf thickness, and fluff, which negatively impact insect feeding behavior (Gary et al., 2006; Whitehill et al., 2016). Chemical defense refers to physiological and biochemical changes, such as induction of plant phytohormone signaling, a decrease in nutritional components, and production of defense proteins and plant secondary metabolites (Ahn et al., 2007; Body et al., 2019; Shen et al., 2021; Divekar et al., 2022). However, insects have also evolved several behaviors to overcome plant defenses, such as changing feeding strategies and regulating growth rhythm and development. In addition to these behavioral changes, biochemical and molecular characteristics also contribute greatly to adaptation to plant defense systems. For example, phytophagous insects regulate the composition, quantity, and quality of digestive enzymes to overcome the protease inhibitors in plants (Cloutier et al., 2000). Other strategies, such as the inhibition of plant defense injury signals and the detoxification to plant secondary substances, also enable insects to escape from the plant defense system.

Insect cytochrome P450 (CYP450) is a terminal oxidase in the multifunctional oxidase system, and it has a catalytic activity on various substrates. Metabolic resistance is an important mechanism underlying insect resistance to traditional insecticides, and CYP450 plays a crucial role in the detoxification of endogenous and exogenous toxic compounds because of its broad-spectrum substrate specificity (Schuler, 2012; Bao et al., 2016; Xu et al., 2020). It has been reported that plant secondary metabolites and insecticides can induce insect CYP450 gene expression; for example, coumarin can induce overexpression of CYP6B2, CYP6B6, and CYP6B7 in *Helicoverpa armigera* and reduce the sensitivity of the insect to methomyl (Chen et al., 2018). Gossypol can induce high expression of CYP6AB14 and CYP9A98 in *Spodoptera exigua*, and after RNA interference (RNAi)-mediated silencing of these genes, larvae are more sensitive to deltamethrin (Hafeez et al., 2019).

The oriental armyworm, *Mythimna separata* (Walker), is a serious polyphagous and migratory insect pest with strong adaptability, and it shows a preference for high-temperature and -humidity environments (Jiang et al., 2011; Kong et al., 2019). Local outbreaks have caused significant damage to crops, such as corn, rice, and sugarcane (Mishra et al., 2021; Yang, 2021). In addition, some economically important crops such as cotton, beans, and vegetables have also suffered damage (Krempl et al., 2021). The long-term continuous cropping of gramineous crops will increase the probability of crop pests and diseases of *M. separata* (Pang et al., 2021). Chemical treatments are the most common and effective method to control *M. separata* (Wang et al., 2018). However, frequent use of pesticides often leads to serious environmental problems and insecticide resistance (Song et al., 2017). Therefore, there is an urgent need to develop new methods and materials with low toxicity to beneficial organisms and high specificity for target insects.

Plant secondary metabolites, including phenolics and flavonoids, play essential roles in insect resistance (Chen et al., 2015; Chen et al., 2018; Xu et al., 2019). The results from various studies have demonstrated that plant phenolic metabolites such as chlorogenic acid (CGA), methyl jasmonate, and tannic acid negatively affect insect feeding behavior, growth, development, and reproduction, and they may have lethal effects on specific insects (Kundu and Vadassery, 2019; Li et al., 2019; Lin et al., 2021). CGA (C16H18O9) is a dihydroxy phenolic compound that is a common secondary metabolite in plants, including higher dicotyledons and ferns (Xi et al., 2014). CGA has been shown to be involved in plant chemical defenses against insect herbivores (Kundu and Vadassery, 2019); for example, it can be used as a resistance factor for thrips in chrysanthemum (Leiss et al., 2009), and induced biosynthesis of CGA in sweet potato confers resistance against sweet potato weevil (Liao et al., 2020). In addition, CGA is the main component in the anti-insect defenses of *Vernonia anthelmintica* Willd (Liu et al., 2020). Besides protecting plants from herbivores, CGA is also involved in plant growth and development processes, such as shoot organogenesis and fruit ripening (A. Neşe Çokuğraş, and Ebru, 2003; Liu, 2016). In our previous research, we found that the attack of M. separata on sugarcane induced significant accumulation of CGA and that CGA has lethal effect on larvae (Wang et al., 2021). Therefore, CGA is a promising environmentally friendly insecticide that is safer for biological use compared with traditional synthetic pesticides.

Although several studies have focused on CGA-mediated plant chemical defenses against insects and the lethal effect of CGA on target insects (Kundu and Vadassery, 2019; Pan et al., 2020), there is no solid evidence of the role of CGA in inhibiting herbivore attack, and the sublethal effects of CGA on insect development and reproduction remain to be determined. In addition, the effect of the CGA regulatory mechanism on an insect is poorly understood. This study aimed to elucidate the lethal and sublethal effects of CGA on *M. separata* larval growth and development. The effect of CGA on detoxification enzyme activity in *M. separata* and the potential key detoxifying genes were investigated by RNA sequencing (RNA-seq). The findings of this study provide the basis for further understanding the detoxification mechanism of CGA in arthropods and a new method in the management of pests with P450-mediated resistance.

2. Materials and methods

2.1 Insects

M. separata larvae were raised in the lab of National Engineering Research Center for Sugarcane, Fujian A&F University, in a controlled temperature (26 ± 1°C) and fixed photoperiod (L16:D8). Preparation of artificial feed and feeding were performed using the feeding method of Lepidoptera insects described by Cao et al. (Cao et al., 2014).

2.2 Bioassays

In this study, the concentrations of CGA (purchased from Beijing Solarbio Science & Technology Co., Ltd., purity ≥98%) in the artificial diet were 5mg/mL, 10mg/mL, 20mg/mL, 40mg/mL, and 80mg/mL. During the preparation of the artificial diet, all the main materials were mixed under liquid conditions. To prepare the 80mg/mL CGA artificial diet, 0.4 g of CGA was dissolved in 5 mL of 25% absolute ethanol at room temperature, and 15 g of artificial diet was added. The other artificial diets with different concentrations of CGA were prepared in the same way with the appropriate amounts of CGA, and the artificial diet supplemented with 5 mL of 25% absolute ethanol was used as the control. A piece of the artificial diet was placed in each well of a 24-well plate, and one pre-starvation (12 h) 2nd instar larva was placed on the surface of the diet. Three replicates of 24 larvae were tested for every concentration. The same treatment was also for 3rd and 4th instar larvae. Feeding conditions were the same as those in section 2.1. Each day the artificial diet was checked for freshness, and stale food was replaced, and the death of larvae was recorded. The experiment was terminated after five days of treatment, and the statistical data were collected. Larvae were considered dead when they did not respond when stimulated with an ink brush. The LC_{50} for each treatment was determined by Probit analysis in SPSS 18.0.

2.3 Sublethal effects of CGA on the larval development, eclosion rate and fecundity of *M. separata*

The 3rd instar larvae were collected to determine the sublethal effects of CGA on larval growth and development parameters as described by Wang et al. (Wang et al., 2014). LC_{20} was chosen as the concentration for CGA treatment because it resulted in a specific amount of mortality. Healthy 3rd instar larvae were starved for 12 h, then a single 3rd instar larva was placed on the surface of the artificial diet containing the LC_{20} dose of CGA in an individual 25 ml plastic cup and sealed with a lid. Each treatment was performed with 30 larvae, and the treatment was replicated three times. Similarly, twenty 3rd instar larvae were placed on the artificial diet without CGA as a control group, and the treatment was repeated three times. Larvae were examined every day till pupation, and the developmental stage, including molting, pupation, and death, of the larvae was recorded every day. Then we determined the sex of each *M. separata* by pupa and calculated the sex ratio (Chen et al., 2019). After eclosion, the male and female adults were paired and transferred to a cage for mating. After eggs were laid, the egg masses were counted. The date was analysis by t-test in SPSS 18.0.

2.4 Transcriptome analysis

The artificial diets with CGA at LC_{20}, LC_{50}, and LC_{80} were fed to healthy 3rd instar larvae for 5 days, then four to six surviving larvae were randomly selected for further transcriptome analysis with three biological replicates. RNA isolation, cDNA synthesis, library construction, and Illumina sequencing were all performed at Berry Genomics Co., Ltd. (Beijing, China) (Hansen et al., 2010). The RNeasy Micro Kit (Qiagen, Hilden, Germany) was used to isolate total RNA from each sample. RNA purity and concentration were then examined using the NanoDrop 2000, and RNA integrity and quantity were measured using the Agilent 2100 system. Next, an NEB library was established for each sample using mRNA as a template. All libraries were pooled together and subjected to Illumina sequencing with paired-end sequencing. Trinity was used to assemble clean reads, Benchmarking Universal Single-Copy Orthologs (BUSCO) was used to evaluate the integrity of transcript assembly, and Corset program transcripts were used for hierarchical clustering. All unigenes were obtained after assembly, and unigene functional annotation was based on the non-redundant protein sequence (Nr), nucleotide sequence (Nt), protein families (PFAM), KOG and Swiss-Prot databases. Open reading frames (ORFs) were predicted by TransDecoder software with the default setting. Then, paired-end reads were aligned to the unigene sequence using bowtie, and RSEM was used to count the number of reads mapped to each gene and estimate gene expression levels. Differential expression was analyzed using EdgeR. P-values of the results were adjusted to control for the false discovery rate. Genes with $|\log2 (\text{Fold Change})| > 1$ and q value < 0.05 were designated as differentially expressed. Finally, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of differentially expressed gene (DEG) sets were performed using GOseq R and KOBAS 3.0, respectively. GO terms with an adjusted p-value below 0.05 were considered significantly enriched in DEGs.

2.5 Identification and bioinformatics analysis of CYP450 genes

The assembled unigenes were used as queries in searches against the Nr database with a cut-off E-value < 1.0 E^{-5}. The unigenes found in the same BLAST search or that shared high homology with other unigenes were regarded as allelic variants or as different parts of the same gene. The gene with hit result of CYP450 was screened from the Nr results after BLAST. All CYP450 genes of *M. separata* were identified by sequence alignment, and the amino acid sequences were aligned using the default settings in ClustalW 2.0. Then, the CYP450s genes of *M. separata* were compared with those of *H. armigera* and *Spodoptera litura* (obtained from InsectBase 2.0) by performing phylogenetic analysis in MEGA-X.

2.6 Enzyme assays of P450 monoxygenases in *M. separata* larvae treated with CGA

Healthy 3rd instar larvae were starved for 12 h, then transferred to an artificial diet with different concentrations (LC_{20}, LC_{50}, and LC_{80}) of CGA. Feeding conditions were the same as those in section 2.1. Healthy 3rd instar larvae were fed an artificial diet with 25% absolute ethanol as a control. After feeding for 1, 3, 5, and 7 days, ten surviving larvae were randomly selected with three replicates per group to analyze the effects of different concentrations of CGA in the artificial diet and different treatment times on P450 enzyme activities using the CYP450 enzyme assay kit (CK-E93532, Shanghai Enzymatic Biotechnology Co., Ltd.) according to the kit instructions.

2.7 Validation of expression profiles using qRT-PCR

RNA-seq analysis and assays of detoxification-related CYP450 protein activities in *M. separata* treated for different times with different concentrations of CGA revealed that *MsCYP450* genes and CYP450 detoxification proteins were significantly up-regulated. Therefore, we selected seven *MsCYP450* genes that were up-regulated considerably in response to different concentrations of CGA for validation by qRT-PCR using the same RNA that was used in RNA-seq. Primer 5 was used to design specific primer pairs (Table S1), and primers were synthesized by Tsingke Biotechnology Co., Ltd. in China. The cDNA synthesis reaction was performed using the HiScript® II Q RT SuperMix kit with gDNA wiper (Vazyme, China) according to the manufacturer's protocol using 1 μg of total RNA as a template per reaction. QRT-PCR was performed with Hieff® qPCR Green Master Mix (Yeasen, China). Finally, data were analyzed using the $2^{-\Delta\Delta CT}$ method (Sun et al., 2017), and EF-1α was used as a control to correct for sample-to-sample variation. Three technical replicates were performed for each replicate, and the data were expressed as mean ± standard error (SE).

2.8 RNAi in *M. separata*

Fragments of *MsCYP321A7*, *MsCYP6k1-like*, *MsCYP6B6*, *MsCYP324A1*, *MsCYP4V2-like*, *MsCYP6B7-like*, *MsCYP6AE88*, and green fluorescent protein gene (*GFP*) were amplified by PCR using specific primers (Table S2) conjugated with the T7 RNA polymerase promoter (TAATACGACTCACTATAGGG). The T7 Ribomax™ Express RNAi System (Promega, Madison, WI, USA) was used to synthesize double-stranded RNAs (dsRNAs) as described in the manual. Thirty *M. separata* larvae at the late 2nd instar stage were fed an artificial diet containing 100 μg dsRNA in a 24-well plate for 3 days, and fresh dsRNA was added every day. The control group was treated with the same amount of ddH_2O. After 3 days, five living larvae in each group (control, *MsCYP321A7-dsRNA*, *MsCYP6k1-like-dsRNA*, *MsCYP6B6-dsRNA*, *MsCYP324A1-dsRNA*, *MsCYP4V2-like-dsRNA*, *MsCYP6B7-like-dsRNA*, *MsCYP6AE88-dsRNA*, and *GFP-dsRNA*) were collected for total RNA extraction for determination of gene expression. The approximately 25 larvae remaining in each treatment were used for bioassays with CGA at LC_{50} as described above (section 2.3). The number of dead larvae was recorded after CGA application for 6 days. The experiment was replicated three times. After dsRNA treatment with the same method, the growth and development period were determined by 2.3 method that using LC_{20} CGA mixed artificial diet.

3. Results

3.1 The effects of CGA on *M. separata*

The toxicity of CGA against *M. separata* larvae was determined using the feeding method. The mortality was calculated after feeding the 2st, 3rd and 4th instar larvae with an artificial diet containing various concentrations of CGA for 5 days. The results (Table S3) showed that the mortalities of 3rd larvae fed diets with different concentrations of CGA were significantly different after 5 days ($P<0.05$). The higher the concentration of CGA, the higher the mortality. The mortality rate of the 5mg/mL CGA treatment group was 13.33% ± 6.67%, while the mortality rate of the 80mg/mL CGA treatment group reached 80.00% ± 13.33%. Based on the bioassay results, 74.48 mg/mL CGA (LC_{80} dose), 26.29 mg/mL CGA (LC_{50} dose) and 7.15 mg/mL CGA (LC_{20} dose) for 3rd larvae were used for further treatment of larvae.

3.2 Effects of CGA on the growth and development of larvae

The LC_{20} concentration 7.15 mg/mL was used to assess the sublethal effects of CGA on *M. separata* development and reproduction. The duration of development for each instar is shown in Table 1. The results showed that there was no significant difference in the duration of the 3rd instar stage of larvae treated with LC_{20}, but there were significant differences in the durations of the 4th and 5th instars, which were prolonged by 36.87% (0.8 days) and 38.22% (0.6 days), respectively ($P < 0.0001$). However, the duration of the 6th instar larval stage was significantly shortened by 18.15% (1.1 days), and the overall developmental duration of the 3rd to 6th instars was extended by about 0.5 days. Measurement of other growth and development indices of larvae showed that the total survival rate of larvae (90%), eclosion rate (34.09%), sex ratio (0.86), and the number of eggs laid per female (427.8 ± 48.88) of the CGA treatment group were significantly lower than those of the control group. It can be seen that CGA harms the growth, development, and reproduction of larvae.

3.3 Transcriptome analysis of *M. separata*

To assess the potential mechanism underlying the lethal effects of CGA on *M. separata* and potential detoxification pathways, RNA-seq was carried out to identify genes encoding target proteins and potential insecticide detoxification enzymes.

Twelve *M. separata* libraries were sequenced on the Illumina platform and pooled together for assembly. All reads were cleaned, and Trinity was used to conduct quality checks. A total of 310,336,443 reads were assembled into 257,014 transcripts with an N50 length of 1,892. The contigs were assembled into 134,240 unigenes with an average length of 1,176 bp (Table 2) using paired-end joining and gap-filling methods. The length distribution was mainly between 300 and 500 bp (35.66% of sequences); there were no sequences < 300 bp, and 14.91% of sequences were longer than 2 kb (Figure S1).

To annotate unigenes, a BLASTX search of the Nr protein database of the National Center for Biotechnology Information (NCBI), was performed with a cut-off E-value of 10^{-5}. A BLAST hit was obtained for 27,039 distinct sequences (20.1% of the total). Sequences were also used as queries in searches against several other databases, including the Nt, Swiss-Prot, PFAM protein, GO, and KOG databases (Table 2). Based on the best hit in the Nr database, 7300 (27.0%) annotated unigenes had the highest homology to sequences in *H. armigera*. In comparison, fewer matched sequences in *S. litura* (19.7%) and *Heliothis virescens* (16.5%). The fewest sequences matched hits in the more distantly related species *Trichoplusia ni* (6.3%) and *Chilo suppressalis* (5.5%) (Figure S2).

Expression levels of genes in *M. separata* treated with artificial diets containing one of three different concentrations of CGA (LC_{20}, LC_{50}, and LC_{80}) were compared with those of genes in the control group (CK). The comparison LC_{20}vsCK had the most significant DEGs, 1764 (1015 up-regulated and 749 down-regulated). LC_{80}vsCK had the least number of DEGs, of which 967 were up-regulated and 319 were down-regulated. There were 671 up-regulated DEGs and 696 down-regulated DEGs identified in the LC_{50}vsCK comparison (Figure 1A). Analysis of the intersection of DEGs revealed that 229 genes were up-regulated and 173 genes were down-regulated in response to all three CGA treatments (Figures 1B, C).

GO and KEGG enrichment analyses were performed for all DEGs to understand the possible mechanisms underlying gene expression differences between control and CGA-treated *M. separata* larvae. DEGs from the LC_{20}vsCK, LC_{50}vsCK, and LC_{80}vsCK comparisons were all mainly enriched in the GO biological process (BP) term transmembrane transport, the cellular component (CC) term extracellular region, and the molecular function (MF) term oxidoreductase activity (Figure S3).

TABLE 1 Sublethal effects of CGA at LC_{20} on growth and developmental indices of *M. separata*.

Index		Treatments	
		CK	Chlorogenic acid
Developmental duration (days)	3rd instar larva	3.52 ± 0.01^{ns}	3.56 ± 0.03
	4th instar larva	2.17 ± 0.03	2.97 ± 0.07****
	5th instar larva	1.57 ± 0.02	2.17 ± 0.06****
	6th instar larva	7.03 ± 0.09****	5.95 ± 0.09
Larval duration from the 3rd instar (days)		14.27 ± 0.10	14.75 ± 0.11**
Total survival rate of larvae (%)		98%**	90%
Pupation rate (%)		100%^{ns}	97.78%
Eclosion rate (%)		44.9%**	34.09%
Sex ratio (♀/♂)		2.14****	0.86
Number of eggs laid per female		524.8 ± 80.02****	427.8 ± 48.88

Data in the table are mean ± SE. Significance level(t-test):**p<0.01, ****p<0.0001, ns: not significant.

In KEGG enrichment analysis, DEGs from LC_{20}vsCK, LC_{50}vsCK, and LC_{80}vsCK were all enriched primarily in the metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, and chemical carcinogenesis (Figure 2).

3.4 Analysis of the P450 genes responding to CGA in *M. separata*

P450 enzyme activity assays showed that P450 activity was induced by different concentrations of CGA (Figure 3A). P450 activity significantly increased with increasing CGA concentration and reached the maximum at LC_{50}. In addition, we also assessed the effect of duration of LC_{20} CGA treatment on *M. separata* P450 enzyme activity (Figure 3B). The P450 enzyme activity increased significantly post-treatment and reached the maximum at 7 days.

From our transcriptome data, 139 sequences encoding CYP450s were identified, and these sequences corresponded to 61 non-redundant unigenes. Of these, 44 CYP450 genes encoding proteins with more than 200 amino acids identical to annotated CYP450 proteins were used for further analysis (Table S4). The lengths of these CYP450 genes ranged from 662 to 4858 bp. Then, from phylogenetic tree analysis, the CYP450 genes were categorized into four CYP450 (CYP) clans: CYP2, CYP3, CYP4, and the mitochondrial clan (Figure 4). Twenty-eight genes were assigned to the CYP3 clan, which was the most prominent clan; the CYP4 clan was the second largest with 8 genes; 2 genes were assigned to the CYP2 clan; and 6 genes were assigned to the mitochondrial clan, which is only found in animals (Figure 4).

TABLE 2 An overview of the Illumina sequencing of the *M. separata* transcriptome.

Parameter	Value
Total number of raw reads	322,402,739
Total number of clean reads	310,336,443
Total number of clean bases	85.47G
GC percentage	47.78%
Number of transcripts	257,014
Total transcript nucleotides	302,148,057
Mean length of transcripts (bp)	1,176
Number of unigenes	134,240
Total unigene nucleotides	128,131,440
Mean length of unigenes	954
Annotated in NR	27,039
Annotated in NT	30,108
Annotated in KEGG	14,852
Annotated in SwissProt	13,607
Annotated in PFAM	21,624
Annotated in GO	21,623
Annotated in KOG	7,329

To verify the accuracy of the expression profiles obtained by RNA-seq, we used the same RNA sample as template in qRT-PCR analysis to determine the expression levels of seven *MsCYP450* DEGs. As shown in Figure 5, the RNA-seq expression patterns of the DEGs were similar to those determined by qRT-PCR. After treatment with CGA at LC_{20}, LC_{50} and LC_{80}, gene expression was up-regulated. Of the *MsCYP450* genes, *MsCYP321A7* was the most significantly up-regulated. *MsCYP6k1-like*, *MsCYP324A1*, and *MsCYP6AE88* were all expressed at high levels after treatment with different concentrations of CGA. *MsCYP4V2-like* was the most highly expressed after treatment with CGA at LC_{20}, and its expression then decreased with increasing CGA concentration. We also found that the seven *MsCYP450* genes had high expression levels after being treated with CGA at LC_{50}. This is consistent with the observation of the highest activity of CYP450 detoxification enzymes in *M. separata* treated with CGA at LC_{50}.

After continuous ingestion of dsGFP and dsMsCYP450 genes for three days, the late 2nd instar molted to the 3rd instar. Few larvae died after being fed dsRNA, but the level of *MsCYP450* gene expression decreased significantly (43.89%–69.39%) compared with the control group (Figure 6). The lowest expression level was found in larvae fed dsMsCYP6B6, with a 56.11% reduction in expression. Next, approximately 25 surviving larvae exposed to CK (ddH_2O), dsGFP, or dsMsCYP450 genes were used for further bioassay experiments. After 2 days, larval mortality among the dsMsCYP6B6, dsMsCYP321A7, and dsMsCYP6B7-like treatment groups (60.71%, 57.94%, and 47.22%, respectively) was much higher than that in the CK and dsGFP treatment groups (36.45% and 36.90%, respectively, Figure 7). The larval mortality in the dsMsCYP6k1-like, dsMsCYP324A1, dsMsCYP4V2-like, and dsMsCYP6AE88 groups was not significantly different compared with that in the CK and dsGFP treatment groups. After 5 days, the largest larval mortality was dsMsCYP6B6 treatment groups reached 78.97% (Figure S4).

In addition, we also tested larvae growth and development parameter of *M. separata* larvae after different dsRNA treatment showed that (Table S5). Compared with the control group, the 3rd instar duration of the treatment groups was not significantly different, while the 4th and 5th instars began to be significantly prolonged. The longest of 4th instar duration was dsMsCYP321A-7 treatment (3.19 days) and the 5th instar duration was dsMsCYP6B7-like treatment (2.53 days). Compared with the control group, the 6th instar duration of the treatment groups were all shorten and the shortest was dsMsCYP6B6 treatment (5.32 days).

4. Discussion

4.1 Analysis of the insecticidal activity of CGA against *M. separata* larvae

In this study, the effects of different concentrations of CGA on *M. separata* larvae were investigated by adding different

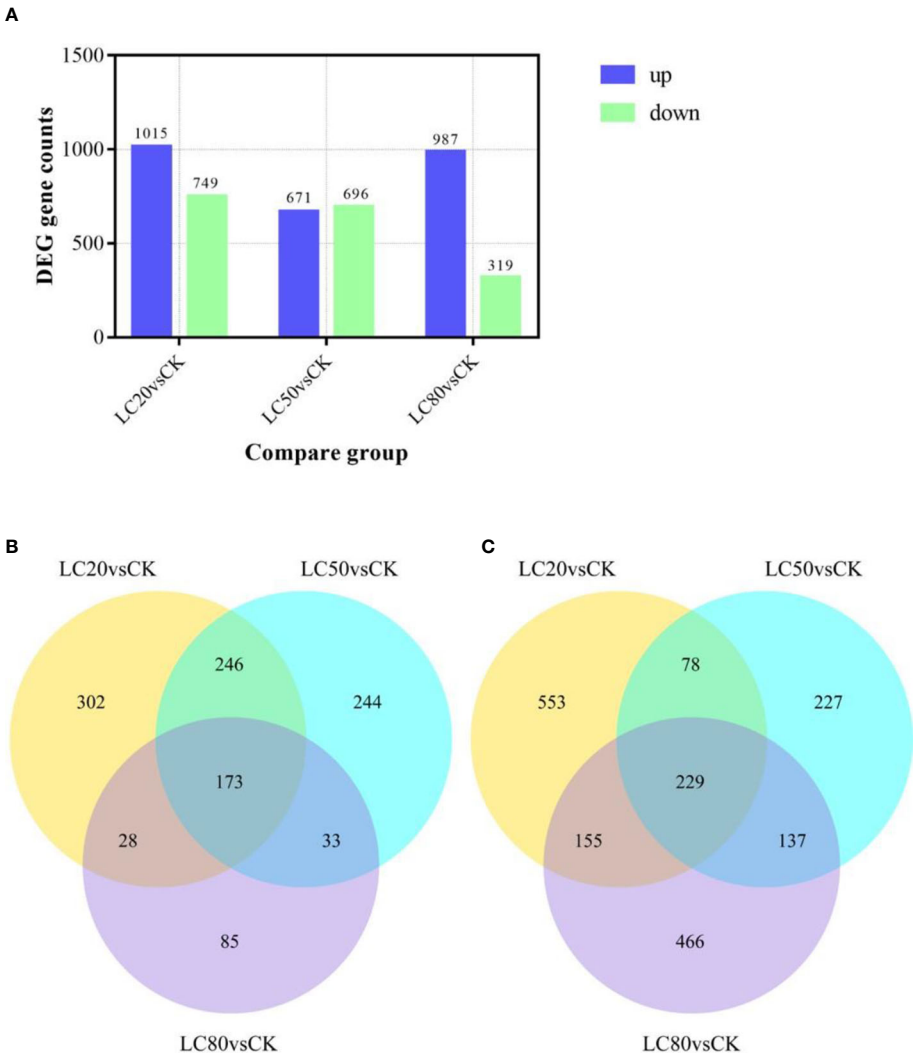

FIGURE 1
The number of DEGs in *M. separata* larvae treated with different concentrations of CGA. **(A)** The number of DEGs in different treatment. The number in the column indicates the number of DEGs. **(B)** The number of DEGs up-regulated by all treatments. **(C)** The number of DEGs down-regulated by all treatments.).

concentrations of CGA to an artificial diet. The larvae in the CGA treatment group started to die after 3 days, and peak death was observed between days 3 and 5. This indicates that CGA has no acute insecticidal effect on *M. separata* larvae within the experimental concentration range of this study, but with prolonged feeding time, CGA may accumulate in larvae and have an insecticidal effect. Similar results have been obtained when treating 3rd instar larvae of *Plutella americana* with 0.500% CGA. The mortality of 5th instar larvae of *P. americana* in the treatment group increased with increasing CGA concentration in the artificial diet and was significantly higher than that in the control group (Pan et al., 2020). Similar results were also obtained when 2nd instar larvae of *Plutella viridis* were treated with 0.3% CGA. The mortality rate increased rapidly from 20% to 50% after 20 days of treatment and reached 100% after 34 days of treatment (Wang et al., 2014).

Generally speaking, botanical insecticides are thought to be eco-friendly and relatively safe. These insecticides have the following main properties: pest selectivity, low risk to non-target organisms, biodegradability, and low risk of inducing insect resistance (Benelli et al., 2019; Zainab and Manfred, 2020). In recent years, many studies have concentrated on the use of plant extracts, particularly biologically active compounds of plant-derived and essential oils, as potential alternatives to commercial insecticides (Isman and Grieneisen, 2014). Therefore, it is necessary to pay attention to the excavation and use of botanical pesticides. Other phenolic substances have shown insectidal activity; for example, 50 μg/mL kaempferol

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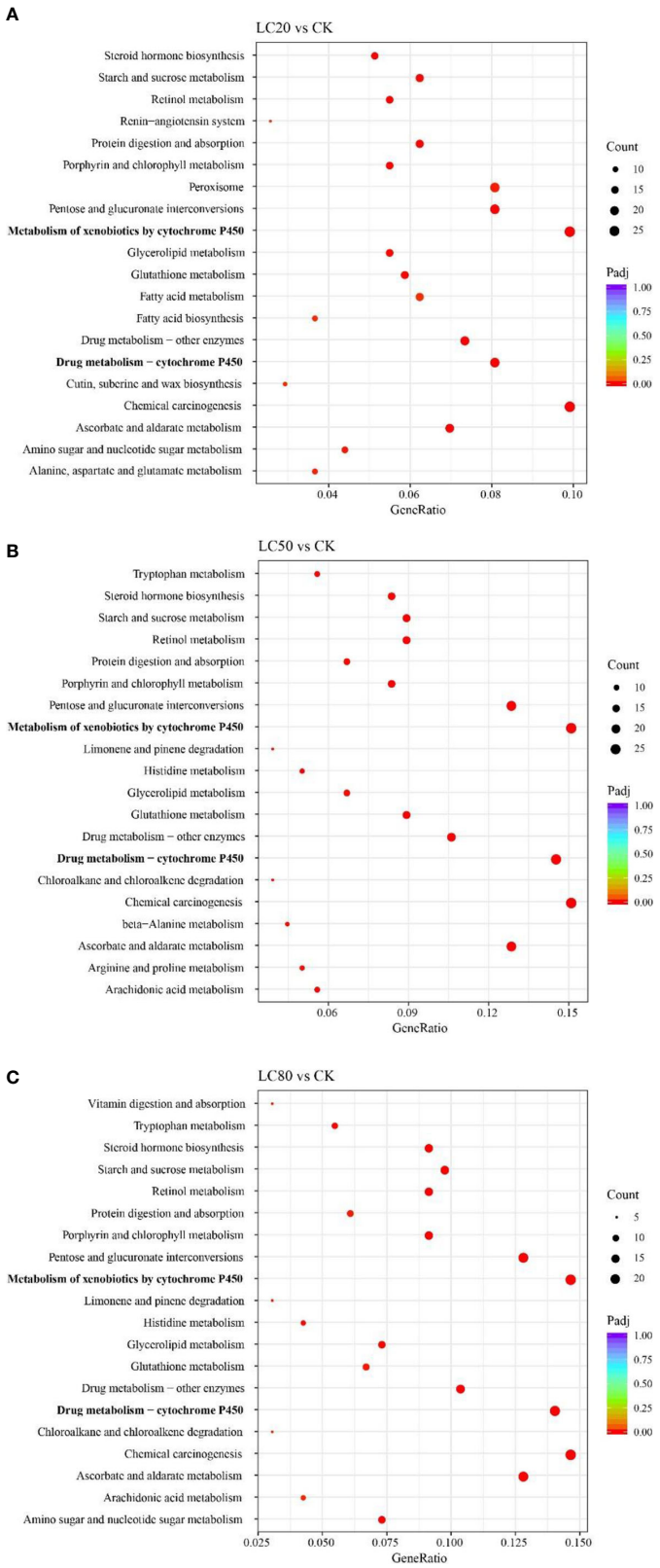

FIGURE 2
KEGG enrichment analyses of DEGs from different comparisons. **(A)** LC_{20}vsCK comparison. **(B)** LC_{50}vsCK comparison. **(C)** LC_{80}vsCK comparison.

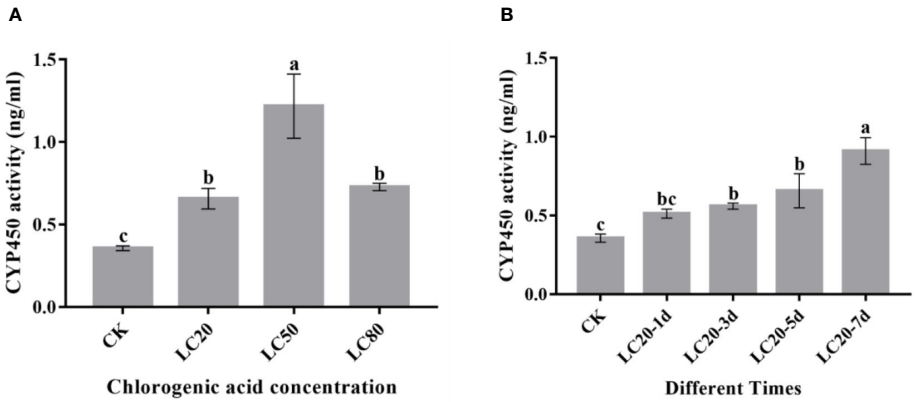

FIGURE 3
The activity of CYP450 proteins in the 3rd instar larvae of *M. separata* fed artificial diets containing CGA. **(A)** Artificial diet containing different concentrations of CGA; **(B)** Artificial diet containing CGA at LC_{20} with different treatment times. Different letters above the bars show significant differences between groups according to Tukey's multiple comparisons tests at $P < 0.05$. Error bars represent the standard deviation (SD) of the means.).

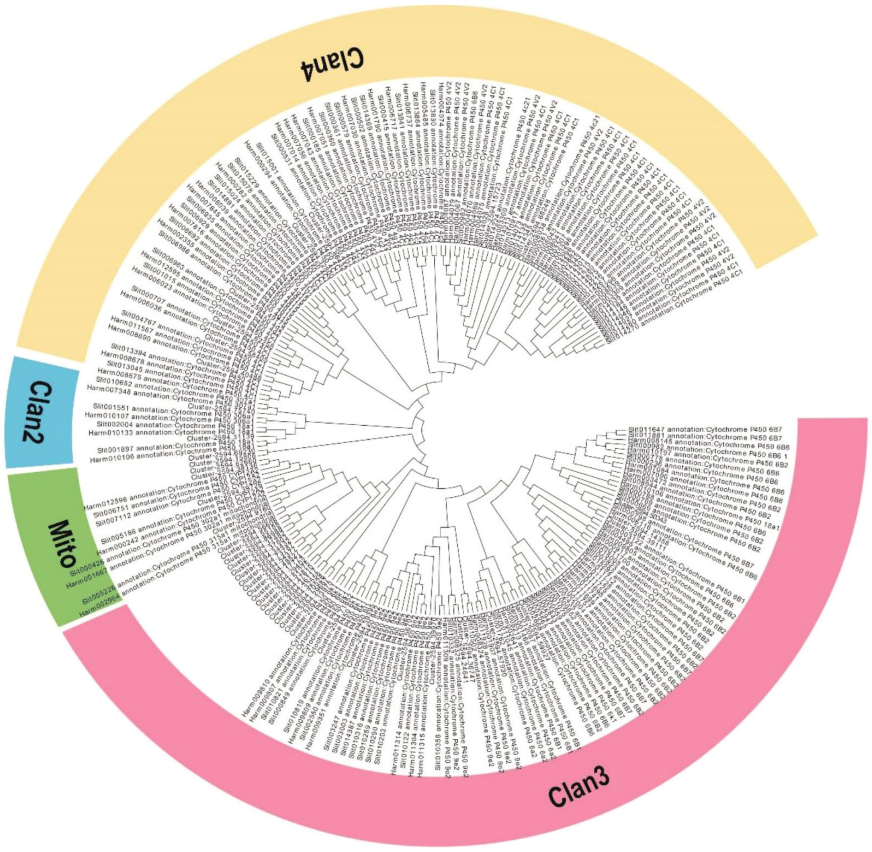

FIGURE 4
Phylogenetic analysis of the CYP450 genes from *M. separata*, *H. armigera*, and *S. litura*. The tree was constructed from multiple sequence alignments using MEGA-X software.

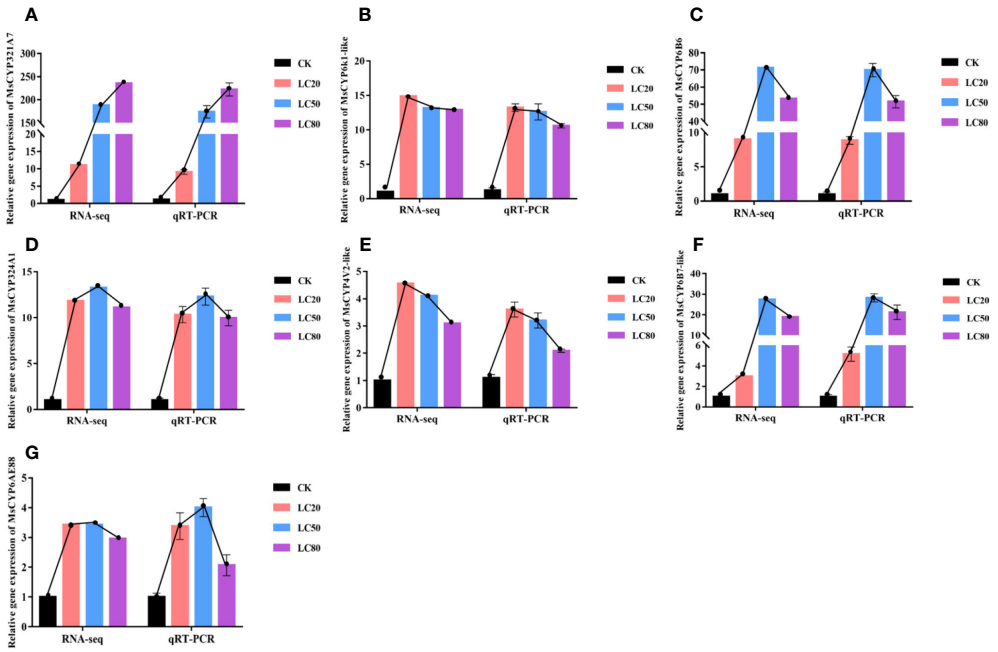

FIGURE 5
Comparison of relative fold change determined from RNA-seq and qRT-PCR analysis of the same samples. (From **A**–**G** the genes are *MsCYP321A7*, *MsCYP6k1-like*, *MsCYP6B6*, *MsCYP324A1*, *MsCYP4V2-like*, *MsCYP6B7-like*, and *MsCYP6AE88*, respectively).

treatment for 72 h, caused 82% mortality of 4th instar larvae of *Culex quinquefasciatus* (Huang et al., 2014); 10 mg/mL periplocosides treatment for 24 h resulted in 76.2% mortality of *Schizaphis graminum* (Rondani) and 37.5% mortality of *M. separata* (Li et al., 2019). The essential oils of *Schinus areira* and *Thymus hyemalis* (in the family Lamiaceae) had insecticidal activity against *Rhipibruchus picturatus* (F.) (Coleoptera: Bruchinae) and *Eceratoniae ceratoniae* Zeller (Lepidoptera: Pyralidae), respectively (Mattar et al., 2022; Adouane et al., 2022).

4.2 Analysis of the sublethal effect of CGA on *M. separata* larvae

Studies to date have shown that CGA has adverse effects on the growth and development of insects and can even be oxidized to more toxic quinones in insects, which have a direct poisoning effect. (Hu et al., 2009; Kundu and Vadassery, 2019) In this study, feeding the 3rd instar larvae with a LC_{20} CGA diet prolonged the duration of *M. separata* larval development (instars 4–5), probably due to a fitness penalty from resisting CGA. Part of the energy intake is used for growth and development, while the other part is used for detoxification metabolism of CGA. But the developmental duration of the mature larvae (6th instar) was shortened, which may be related to the tendency of Lepidoptera to survive poor environments as pupae and the premature pupation of the mature larvae caused by CGA treatment (Deng, 2018). This result was similar to that of previous studies in Lepidoptera, which showed that the duration of *Helicoverpa zea* development was significantly prolonged after ingesting artificial diets containing CGA and caffeic acid (Summers and Felton, 1994), and that CGA reduces the growth, development, and fecundity of *Hyphantria cunea* larvae (Pan et al., 2020). All the results mentioned above show that CGA can prolong the growth period of insects, thereby reducing rate of insect reproduction and the occurrence of disease. In addition, CGA reduced the pupation rate, eclosion rate, sex ratio, and the number of eggs laid per female, indicating that dietary CGA can negatively affect larval development and insect reproduction. These results were consistent with the conclusions reached for other lepidopteran insects. For example, egg production by the gypsy moth is inversely proportional to the phenolic acid content of its food (Tod et al., 2000). *Lymantria dispar* larvae could not complete normal growth and development after feeding on an artificial diet containing tannic acid or CGA. The body weights of the larvae were about 67.2%–75.0% lower than that of the control, and the duration of the larval stage was prolonged by 2–4 times. (Wang et al., 2014). This provides new evidence supporting the hypothesis proposed by Caroline and Simon (Caroline and Simon, 2002) that insects can adapt to different foods by adjusting their reproductive capacity during long-term evolution. After being treated with sulfoxaflor at sublethal

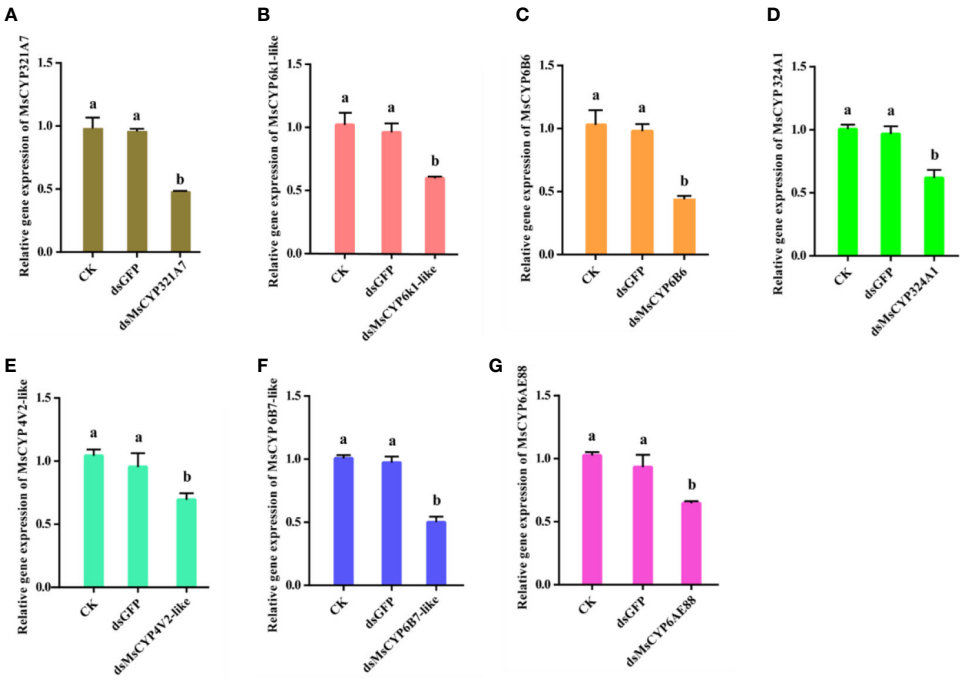

FIGURE 6
The relative gene expression levels of *MsCYP450* genes after dsMsCYP450 gene treatment for 3 days. (From **A**–**G**, the genes are *MsCYP321A7*, *MsCYP6k1-like*, *MsCYP6B6*, *MsCYP324A1*, *MsCYP4V2-like*, *MsCYP6B7-like*, and *MsCYP6AE88*, respectively. Different lowercase letters above the bars indicate a significant difference ($p < 0.05$) based on one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Means ± SE from three replicates are shown).

concentrations, fatty acids, amino acids, and the composition and content of carbohydrates all changed to different degrees, indicating these energy substances mentioned above participate in detoxification metabolism of *M. persicae* to some extent (Zhang et al., 2021). We speculate that the effect of CGA on the growth and development of *M. separata* might be related to some energy substances involved in detoxification metabolism, which needs further research.

4.3 The role of P450 genes in CGA detoxification

Plant secondary metabolites can induce changes in detoxification-related protein activities in phytophagous insects, which may improve the adaptability of these insects (Chen et al., 2015; Wang et al., 2022). CYP450 enzymes are the primary detoxifying enzymes in many organisms. Multiple signaling pathways and critical effector molecules are involved in regulating insect P450s. CYP450 genes play a significant role in detoxification in insects, and insecticide resistance largely depends on the metabolism of exogenous toxic substances by CYP450s (Nauen et al., 2021; Lu et al., 2020). Increased P450 activity is a key mechanism inducing insect resistance (Ye et al., 2022). Wang et al. demonstrated that *AmCYP9q1* plays an important role in the metabolic detoxification of imidacloprid by *Apis mellifera* larvae (Wang et al., 2022). The expression levels of *CYP4L13* and *CYP4M14* genes in the midgut and fat bodies of *Spodoptera frugiperda* increased significantly after larvae were fed exogenous insecticides such as nicotine and flavonoids (Wang et al., 2022). Here, we performed assays of P450 enzyme activity in *M. separata* larvae treated with CGA to investigate the role of *MsCYP450* genes. We found that CGA could induce P450 enzyme activity in *M. separata* larvae fed with different concentrations of CGA for different amounts of time (Figure 3). Studies have shown that P450 can add various chemical groups, including hydroxyl, carboxyl, and amino groups, to toxic secondary metabolites in the insect digestive tract. They can improve the water solubility and reactivity of toxic secondary metabolites and degrade them into less harmful forms (Chung et al., 2007; Cui et al., 2016). In this study, we found evidence that CYP450 enzymes are essential for the detoxification metabolism of CGA in *M. separata*.

In transcriptome analysis, we found 139 *McCYP450* genes, which is a much higher number than those in the lepidopteran insects *H. armigera* (112) and *S. litura* (67) (Zhang, 2018 and Zhang, 2019). Fewer *MsCYP450* genes were identified in this study than in our previous transcriptome sequencing study

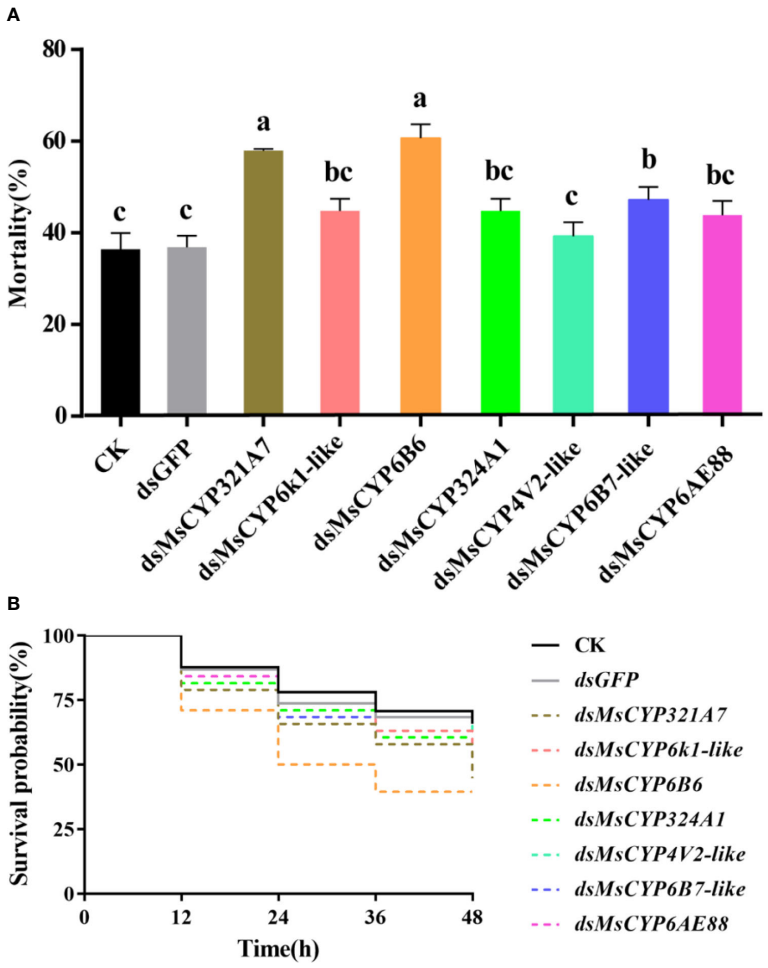

FIGURE 7
The larval mortality and survival rates for *M. separata* larvae after dsRNA treatment. **(A)** Larvae were continuously fed dsRNA for 3 days, and mortality was evaluated for 2 days; **(B)** The survival rates of larvae scored for 2 days after dsMsCYP450 gene treatment for 3 days. Different lowercase letters (a, b and c) above the bars indicate significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Means ± SE from three replicates are shown).

(Wang et al., 2018), possibly because of insufficient sequencing depth or redundant sequencing. According to phylogenetic tree analysis, the 139 *MsCYP450* genes were divided into four clans, of which the CYP3 clan was most closely related to drug resistance metabolism (Wan et al., 2013). By comparing the transcriptomes of *M. separata* treated with different concentrations of CGA with that of the control group, 179 commonly up-regulated genes were identified. Among these up-regulated genes, seven were *MsCYP450* genes and five were from the CYP3 clan. Therefore, we speculated that the seven *MsCYP450* genes might be involved in metabolism. To further confirm the function of these genes, we used the RNAi to knock down their expression. Insect RNAi has been widely used to identify or validate insecticide target genes (Young et al., 2015; Dulbecco et al., 2021) and the use of this technology in *M. separeata* has been reported (Wang et al., 2019). Also this method is widely used in identification of insecticide target. In our study, *M. separata* larvae were continuously fed dsRNA for 3 days, and the mRNA level of *MsCYP450* genes were significantly lower after treatment. The surviving larvae were then exposed to the CGA at LC_{50} for 6 days, and treatment with dsMsCYP6B6, dsMsCYP321A7, and dsMsCYP6B7-like caused a significant reduction in survival compared with the CK and the dsGFP treatment groups. These three *MsCYP450* genes all belonged to the CYP3 clan, which indicates they might play an important role in the detoxification of CGA. The same results have also been observed for similar genes in other insects. Bagchi et al. also demonstrated that CGA significantly induces the CYP450 genes of *Amyelois transitella* and increases the tolerance to CGA (Bagchi et al., 2016), and silencing of the cytochrome P450

gene *CYP321A1* was found to affect tannin detoxification in *S. litura* (Zhao et al., 2021). *CYP6B6* was shown to be involved in esfenvalerate detoxification in the polyphagous insect *H. armigera* (Tian et al., 2017), and *CYP6B7* was shown to play an important role in the resistance of *H. armigera* to fenvalerate (Tang et al., 2007). In this study, we confirmed that *MsCYP6B6, MsCYP321A7*, and *MsCYP6B7-like* play an essential role in the detoxification of CGA in *M. separata*.

In this study, we found that CGA had a lethal effect on *M. separata* and that a sublethal concentration harmed larval growth and development. Seven *MsCYP450* genes that may be involved in the detoxification process were identified by performing P450 enzyme assays and transcriptome analysis. By treating larvae with dsMsCYP450 genes, we determined that MsCYP6B6, MsCYP321A7, and MsCYP6B7-like play a vital role in the detoxification of CGA by *M. separata*. The findings of this functional study of the CGA detoxification genes of this major phytophagous insect provides new insight into this biological process and new targets for agricultural pest control. This study also provides a new method for managing P450-mediated resistance in insect pests.

Data availability statement

The data presented in the study are deposited in the Genebank repository, accession number MsCYP321A7 (OP254196), MsCYP6k1-like(OP254197), MsCYP6B6(OP254198), MsCYP324A1 (OP254199),MsCYP4V2-like(OP254200), MsCYP6B7-like (OP254201),MsCYP6AE88 (OP254202).

Author contributions

D-jL, L-yL, and L-zZ conducted the experiment, D-jL manuscript writing, YF and S-jG data analysis, RW and J-dWdesigned the experiment and funding support. All authors haveread and agreed to the published version of the manuscript.

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Conflict of interest

The 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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Supplementary material

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Protocorm-like-body extract of *Phalaenopsis aphrodite* combats watermelon fruit blotch disease

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Bacterial fruit blotch, caused by the seedborne gram-negative bacterium *Acidovorax citrulli*, is one of the most destructive bacterial diseases of cucurbits (gourds) worldwide. Despite its prevalence, effective and reliable means to control bacterial fruit blotch remain limited. Transcriptomic analyses of tissue culture-based regeneration processes have revealed that organogenesis-associated cellular reprogramming is often associated with upregulation of stress- and defense-responsive genes. Yet, there is limited evidence supporting the notion that the reprogrammed cellular metabolism of the regenerated tissued confers *bona fide* antimicrobial activity. Here, we explored the anti-bacterial activity of protocorm-like-bodies (PLBs) of *Phalaenopsis aphrodite*. Encouragingly, we found that the PLB extract was potent in slowing growth of *A. citrulli*, reducing the number of bacteria attached to watermelon seeds, and alleviating disease symptoms of watermelon seedlings caused by *A. citrulli*. Because the anti-bacterial activity can be fractionated chemically, we predict that reprogrammed cellular activity during the PLB regeneration process produces metabolites with antibacterial activity. In conclusion, our data demonstrated the antibacterial activity in developing PLBs and revealed the potential of using orchid PLBs to discover chemicals to control bacterial fruit blotch disease.

KEYWORDS

***Acidovorax citrulli*, bacterial fruit blotch (BFB), protocorm-like-body, Phalaenopsis orchids, antibacterial activity**

Introduction

Bacterial fruit blotch (BFB) is a serious seedborne pathogen for watermelon and melon worldwide and its outbreaks have caused severe fruit loss in the Americas, Asia, Europe, the Middle and Far East, and Australia (Burdman et al., 2005; Bahar and Burdman, 2010; Burdman and Walcott, 2012). In Taiwan, BFB disease was first reported in 1992-1993 (Tzeng et al., 2010). The recurrence of BFB in 1994 caused more than 60% loss of the watermelon crop nationwide (Tang, 1997; Cheng et al., 2000; Cheng, 2009; Tzeng et al., 2010). The primary source of inoculum is often contaminated seeds (Assouline et al., 1997). Because of the destructive nature of BFB disease, evaluation of pathogen contamination in seed lots prior to their sale and distribution has become a critical practice for seed companies. Based on the National Seed Health System USDA standard (http://www.seedhealth.org/cb1-1), one infected seedling in 30,000 tested seeds can be sufficient to lead to rejection of the entire seed lot. However, seed disinfestations and chemical control only have limited efficacy in controlling BFB (Burdman and Walcott, 2012). Even though seedling grow-out assay is widely used to evaluate seed health, infected seedlings may or may not show disease symptoms. It has been reported that the amount of bacteria present on a seed, environmental conditions, virulence levels of the strains, and the susceptibility of host plants as a whole affect BFB outbreaks (Schaad et al., 2003; Bahar and Burdman, 2010). If infected seedlings are not detected, they might be transplanted into the field and become the primary source of inoculum for field outbreaks. To date, no BFB disease resistant plants have been developed and BFB management remains a major challenge to global watermelon and melon agriculture (Bahar and Burdman, 2010; Islam et al., 2020). Hence, an innovative plan for a BFB management program is a pressing need.

The Orchidaceae represents one of the largest angiosperm families comprising more than 25,000 species that grow in wide range of habitats ranging from rainforest and mountain, to swamp and arctic tundra (Stokstad, 2015). Considering the rich diversity of the orchid species, it is likely that orchids provide a substantial resource of novel compounds for potential application. In fact, orchids have been utilized by humans for thousand years. For example, vanilla orchid *Vanilla planifolia*, probably endemic to tropical forests in Eastern Mexico, is a major source of vanilla (Bory et al., 2008). Additionally, orchids such as *Gastrodia elata*, *Bletilla striata*, and *Dendrobium* species have been used for medicinal purposes in China and other Asian countries for thousands of years (Bulpitt et al., 2007). Despite this background, only a few phytochemicals have been characterized from orchids and the potential of orchid derived-phytochemicals has not been fully explored.

Protocorm-like bodies (PLBs) morphologically resemble the orchid germinated structures, protocorms, but are derived from somatic explants *via* a *de novo* regeneration pathway (Jones and Tisserat, 1990; Chugh et al., 2009; Fang et al., 2022). Because each PLB has the ability to regenerate into an individual plant, PLB-based micropropagation is often used to produce clonal plantlets in the orchid industry (Yam and Arditti, 2009). Our previous comparative transcriptomic studies to dissect the developmental origin of PLB supported that PLB and protocorm share similar molecular signatures and unexpectedly revealed that many genes involved in plant defense responses are specifically enriched in the developing PLB (Fang et al., 2016; Fang, 2021; Fang et al., 2022). Considering the potential functions of PLB-enriched defense related genes, we explored the antimicrobial activity of PLB extract and tested its effects on BFB of watermelon. Our results demonstrate the antibacterial activity of PLB extract and suggest the potential of using the orchid PLBs for developing a reagent to control BFB.

Materials and methods

Pathogen

Acidovorax citrulli Aac153 (*A. citrulli* Aac153), isolated by the Laboratory of Phytopathogenic Bacteria, Department of Plant Pathology, National Chung Hsing University, Taiwan was a kind gift from Dr. Yi-Hsien Lin from National Pingtung University of Science and Technology. *A. citrulli* Aac153 has been shown to cause watermelon fruit blotch disease (Chang et al., 2019). *A. citrulli* Aac153 was stored at -75°C in tryptic soy broth (TSB) supplemented with 15% glycerol (v/v) and allowed to grow on selective medium AacG containing 0.5g/l KH_2PO_4, 2g/l $Na_2HPO_4{\cdot}12H_2O$, 2 g/l $(NH_4)_2SO_4$, 5 g/l L-glutamic acid, 12.5 mg/l bromothymol blue, 15g/l agar, 20 mg/l ampicillin, 25 ppm/l cycloheximde as described previously (Chen, 2014). The single colony was used as inoculum for the primary culture. For subculture, single colony of primary culture was used to inoculate TSB and allowed to grow overnight at 28°C. The overnight culture was then grown on a selective AacG agar plate and the single colony was used as inoculum for the secondary culture. Only the primary and secondary cultures were used for inoculation in all the experiments. This strain produces reproducible, severe symptoms on the commercialized watermelon cultivar China Baby (Chang et al., 2019).

PLB extraction

PLB tissues were homogenized by pestle and mortar in the presence of liquid nitrogen. One gram of pulverized PLB tissues was sonicated in the presence of 5 ml of ethyl acetate (EtOAc) for 30 mins (Branson 8510 DTH). The EtOAc-based PLB extract

was incubated at 55°C for 10 min. Large tissue debris was removed by centrifugation at 3220 x g for 10 min. Supernatant was transferred to a new tube and concentrated by a rotary evaporator (EYELA, USA). The pellet was resuspended in 1 ml 100% MeOH. The PLB extract was then filtered by a 0.2 µm filter (13 mm Acrodisc Syringe filter, Pall) followed by concentration using a CentriVap Vacuum Concentrator (Labconco). The concentrated PLB-extract was flash frozen and stored at -80°C.

Frozen PLB extract was resuspended in 2 ml 30% MeOH. The 1 cc 50 mg Sep-PaK C18 cartridge (Waters) was first equilibrated with 1 ml of 100% MeOH followed by 1 ml of H_2O once and then 1 ml of 30% MeOH once. For fractionation, solid phase extraction (SPE) was carried out by applying 1 ml PLB-based extract onto the equilibrated Sep-PaK C18 cartridge using a step gradient of MeOH-water mixture at a concentration of 30%, 45%, 60%, 80%, and 100% MeOH PLB and the eluents were collected individually. Methanol was allowed to evaporate by CentriVap Vacuum Concentrator (Labconco) and the fractionated PLB extract from 1 g tissues was pooled and resuspended in 100 µl 100% MeOH for seed infestation and pathogenicity assays as described below.

Bacterial growth inhibition assay

Frozen PLB extract from 1 g of PLB tissues (see above) was resuspended directly in 1 ml 100% MeOH. The overnight *A. citrulli* Aac153 culture was pelleted by centrifugation and washed once with 5 ml TSB medium, and diluted to OD_{600} = ~0.05 with TSB medium. The diluted culture was mixed with crude PLB extract (933 µl bacterial culture + 67 µl PLB extract) and aliquoted into 6 technical replicates (100 µl each) to a 96-well microtiter plate and allowed to incubate at 28°C. The OD_{600} was recorded at 0, 15, 19 hours after incubation. For each biological replicate, the absorbance measurements of OD_{600} were recorded in three technical replicates. This experiment was repeated three times.

Disease index scale

Disease symptoms of seedlings at 12 days after transplantation (DAT) were recorded. Normally, 12 DAT watermelon seedlings have two expanded true leaves. Disease index was rated as follows (Figure 1): 0, no symptoms; 1, slight (< 20%) water-soaking or necrotic spots on cotyledons or hypocotyls; 2, increased water-soaking or necrotic spots (>20%) on cotyledons or hypocotyls; 3, expanded water-soaking and necrosis (>50%) on cotyledons or hypocotyls, true leaves often failed to emerge from infected seedlings, for seedlings with emerging true leaves, leaves failed to expand and were often distorted; 4, bent seedlings with necrotic cotyledons and hypocotyls, no true leaves were observed; 5, falling-over seedlings with complete necrotic cotyledons and hypocotyls. Disease severity was calculated as DS (%) = [sum (class frequency × score of rating class)]/[(total number of plants) × (maximal disease index)] × 100.

Seed infestation and pathogenicity assays

Watermelon seeds (China Baby) were purchased from Known-You Seed Company (Taiwan). For seed sterilization, seeds were imbibed in distilled water supplemented with 0.1%

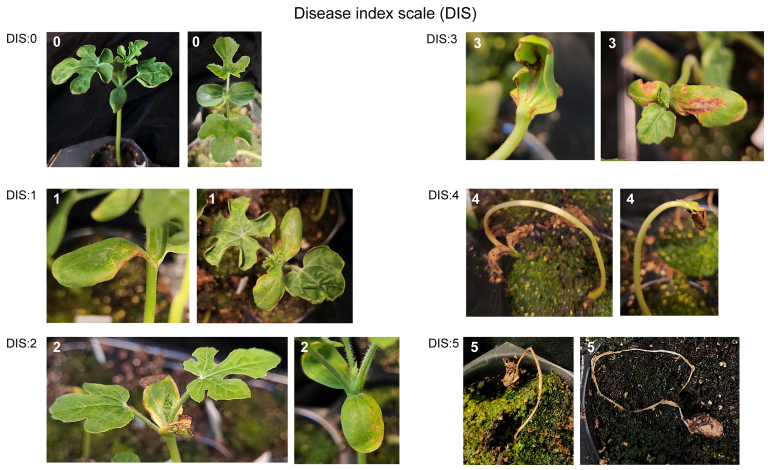

FIGURE 1
The represented disease severity index used to categorize disease symptoms of watermelon seedlings infected with *A. citrulli* Aac153. The numbers indicate the disease index described in Materials and Methods.

Triton X100 and gently shaken for 30 min. Imbibed seeds were sterilized by incubating with 75% ethanol for 5 min followed by washing with sterilized water 5 times. Seeds were allowed to dry in a laminar hood overnight.

For bacterial culture preparation, *A. citrulli* Aac153 culture that grew in 4 ml TSB overnight was pelleted by centrifugation at 3220 x g at 25°C for 10 min, washed once with 5 ml 0.5% carboxymethyl cellulose (CMC), resuspended in 0.5% CMC, and adjusted to OD_{600} to ~0.3. This preparation was used as the bacterial stock for seed infestation and seedling pathogenicity assays.

For seed infestation assay, five sterilized seeds were incubated with *A. citrulli* Aac153 culture in the presence (933 µl diluted bacterial culture + 67 µl fractionated PLB extract) or absence (933 µl diluted bacterial culture + 67 µl methanol) of PLB extract with gentle shaking (200 rpm) at 28°C for 2 h. The infested seeds were allowed to dry in a laminar flow hood overnight. The infected seeds were then resuspended in 1 ml AacG selective medium, incubated at 4°C for 30 min followed by incubation at 37°C for 1 h as described previously (Chen, 2014). Bacteria were concentrated by centrifugation at 15871 x g at 4°C for 10 min. The supernatant was carefully removed and the bacterial pellet was resuspended in 1 ml distilled water. The bacterial suspension was diluted 10 or 100 times by distilled water, plated on AacG selective plates, and allowed to grow at 28°C for three days. For each biological repeat, measurement of colony forming units was performed in three technical replicates. This experiment was repeated three times. A two-tailed Student's t-test was applied. *, $0.05 > p > 0.01$; **, $p < 0.01$. Only the treatments showing statistically significant reduction of bacteria were marked.

For seedling pathogenicity assay, the diluted *A. citrulli* Aac153 culture (OD_{600} to ~0.3) was further diluted 500 times with 0.5% CMC immediately before infection experiment. Eight sterilized seeds were incubated with 1 ml of diluted *A. citrulli* Aac153 culture in the presence (933µl diluted *A. citrulli* Aac153 + 67µl fractionated PLB extract, treatment) or absence (933 µl diluted *A. citrulli* Aac153 + 67µl MeOH, control) of PLB extract at 28°C with gentle shaking (200 rpm) for 24 h. The infested seeds were allowed to germinate in a humidity chamber at 32°C in the dark for 72 h. The germinated seedlings were then transferred to soil and allowed to grow in a growth chamber with a 16-h:8-h light:dark cycle under illumination of ~300 µmol photons $m^{-2}s^{-1}$ at 32°C. Plastic wrap was used to cover soil pots to maintain humidity and removed 5 days after transplantation. Disease symptoms were rated and recorded based on disease index scale (Figure 1) as described previously. The experiment was conducted three times. A two-tailed Student's t-test was applied. **, $0.01 > p > 0.001$; ***, $p < 0.001$.

RNA extraction and RT-qPCR

RNA was extracted as described previously (Fang et al., 2016). Three micrograms of DNA-free RNA were reverse transcribed in the presence of a mixture of oligo(dT) and random primers in a 9:1 ratio using the GoScript Reverse Transcription System (Promega) based on the manufacturer's instructions. Ten microliters of reverse transcription-PCR reaction contained 2.5 µL of 1:20 diluted cDNA, 0.2 µM of primers, and 5 µL of 2x KAPA SYBR FAST master mix (KAPA Biosystems). The amplification program was as follows: 95°C for 1 min, and 40 cycles at 95°C for 5 s and 58°C to 60°C for 20 s. PCR was performed in triplicate. Data are from technical triplicates and the error bars are presented as standard error of the mean. The RNA samples used for RT-qPCR analysis were independent from those for RNA-seq analyses. Primer pairs and the specified annealing temperature used for quantitative PCR are listed in Supplementary Table 1. *UBIQUITIN* was used as an internal control (Lin et al., 2014). The nomenclature of chalcone synthases was based on the previous study (Kuo et al., 2019). List of gene IDs used in this study and their corresponding IDs in the *P. aphrodite* databases are listed in Supplementary Table 2.

Statistical analysis

All experiments were performed three times or as otherwise mentioned in the figure legends. The data are presented as means and standard deviations obtained from at least three replicates of a single experiment. The significant difference between the treatments was analyzed by running a Student's t-test in IBM SPSS v.20.

Results

Plant defense-related genes are specifically upregulated in PLBs

Our previous RNA-seq study investigating the developmental origin of PLBs revealed that Gene Ontology (GO) terms such as oxidation-reduction process, terpene synthase activity, and stress responses are overrepresented in developing PLBs (Fang et al., 2016). The biochemical and biological properties of these GO terms are generally associated with plant defense responses (Field et al., 2006; Almagro et al., 2009; Ton et al., 2009; Gonzalez et al., 2010; Denance et al., 2013; Yang et al., 2013; Savatin et al., 2014). Among them, Phalaenopsis chalcone synthase (*CHS*) and flavonoid 3' hydroxylase (*F3' H*) genes, *PaCHS4*, *PaCHS5*, and *PaF3' H1*, were preferentially upregulated in developing PLBs (Table 1). Chalcone synthase and flavonoid 3' hydroxylase act at the initial steps to produce flavonoids- and isoflavonoid-type phytoalexins (Bak et al., 2011; Dao et al., 2011; Ahuja et al., 2012) that are part of plant defense responses (Ahuja et al., 2012). A

TABLE 1 Plant defense-related genes are enriched in developing PLBs as shown by RNA-seq analysis.

Transcript ID	Annotation	FPKM values										
		30/40 DAP	50/60 DAP	70/80 DAP	90/100/ 120 DAP	140/ 160 DAP	180/ 200 DAP	PLB	Protocorm	Young leaves	Stalk buds	Floral stalks
orchid.id124284.tr400924	PaCHS4	1.7	0.6	0.2	0.4	2.0	2.3	185.9	10.7	1.4	0.8	0.9
orchid.id121282.tr400924	PaCHS5	0.6	0.4	0.1	0.3	2.3	2.2	186.2	15.1	1.3	0.5	0.6
orchid.id17741.tr406385	PaF3' H1	7.56	9.43	25.19	11.91	5.96	10.07	194.62	5.43	7.90	6.39	4.89
orchid.id115099.tr56794	PaCYP71A1	0.2	0.1	0.1	0.7	1.9	1.4	395.4	23.6	0.1	0.2	0.1
orchid.id36575.tr215222	PaWRKY3	0.0	0.0	0.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0
orchid.id184974.tr136611	PaWRKY4	0.0	0.0	0.0	0.0	0.0	0.0	9.1	1.1	0.0	0.0	0.0
orchid.id154271.tr406853	PaECR1	1.1	0.1	0.6	1.2	2.9	1.2	313.0	3.4	0.8	0.8	3.1
orchid.id163617.tr122100	PaPNP1	3.7	1.8	1.5	9.6	0.6	0.3	106.6	3.6	0.9	3.6	0.5
orchid.id156327.tr422593	PaRALF1	1.4	0.6	0.0	0.3	1.2	0.0	159.4	0.2	26.2	20.7	1.4
orchid.id133178.tr112803	PaMLP1	3.1	1.3	0.5	2.4	0.9	2.2	1046.2	7.6	0.2	7.1	6.9
orchid.id148348.tr112803	PaMLP2	4.3	1.4	0.6	3.1	0.9	2.6	1318.7	8.4	0.2	10.6	10.9
orchid.id136038.tr32844	PaPRX1	0.3	0.5	0.1	0.4	0.5	0.3	253.7	27.6	0.1	0.1	0.5
orchid.id123338.tr499847	PaCOMT1	1.4	1.1	1.4	3.0	4.0	3.7	645.1	28.4	1.8	15.0	8.7
orchid.id21743.tr69582	PaCOMT2	0.0	0.0	0.0	0.2	0.0	0.2	28.8	0.0	0.0	0.0	0.0

PLB-enriched *PaCYC71A1*, which is related to Arabidopsis *CYP71A12* (Bak et al., 2011), encodes a putative cytochrome P450 monooxygenase (Table 1). Arabidopsis CYP71A12 takes part in biosynthesis of camalexin, a major phytoalexin important for disease resistance (Millet et al., 2010; Klein et al., 2013; Pastorczyk et al., 2020). Additionally, two PLB-enriched WRKY transcription factors, *PaWRKY3* and *PaWRKY4*, were identified. PaWRKY3 belongs to the group III WRKY transcription factors (Supplementary Figure 1) and is related to Arabidopsis WRKY70 (Wu et al., 2005). Arabidopsis *WRKY70* modulates salicylic acid (SA)- and jasmonic acid (JA)-mediated defense pathways to regulate plant immunity against bacterial pathogens (Li et al., 2004; Li et al., 2006; Zhou et al., 2018). *PaWRKY4*, on the other hand, encodes a group I WRKY transcription factor that is related to Arabidopsis *WRKY33* (Supplementary Figure 1), which is an important regulator for biosynthesis of camalexin and pathogen-associated molecular patterns (PAMP)/pathogen-triggered reactive oxygen species (ROS) and ethylene production (Qiu et al., 2008; Mao et al., 2011; Li et al., 2012; Zhao et al., 2020; Zhou et al., 2020).

These PLB-enriched genes (Table 1) are also associated with other aspects of plant defense responses. For example, *PaECR1* encodes a potential enoyl-coA reductase that has been shown to play roles in plant defense responses in cotton and *P. amabilis* orchid (Fu et al., 2012; Mustafa et al., 2017). A potential PLANT NATRIURETIC PEPTIDE (PEP) encoded by *PaPEP1*A belongs to a family of peptides involved in regulation of defense responses and ion and water homeostasis (Gehring and Irving, 2013; Ficarra et al., 2018). *MAJOR LATEX PROTEIN1* (*PaMLP1*) and *NORCOCLAURINE SYNTHASE1* (*PaNCS1*) belong to pathogen-related 10 (PR10) and Bet v1 proteins (Radauer et al., 2008). MLP proteins are known to be involved in various abiotic and biotic responses (Yang et al., 2015; Wang et al., 2016; Holmquist et al., 2021). NCS proteins catalyze the first committed step of biosynthesis of benzylisoquinoline alkaloids (BIAs) that possess antimicrobial activity (Lee and Facchini, 2010). *PaRALF1*, on the other hand, encodes a protein that is related to Arabidopsis RAPID ALKALINIZATION FACTOR (RALF) peptides, which are found to interact with receptor-like kinase FERONIA to modulate ROS production and plant immune responses (Stegmann et al., 2017; Li et al., 2018; Abarca et al., 2021). The PLB-enriched genes, *PaCOMT1* and *PaCOMT2*, encode putative caffeic acid O-methyltransferases. Rice COMT is reported to have N-acetylserotonin O-methyltransferase (ASMT) activity that converts N-acetylserotonin to melatonin (Byeon et al., 2015), which modulates ROS and SA levels to improve plant responses to various abiotic and biotic stresses (Lee et al., 2015; Khan et al., 2020). Additionally, *PaPRX1* encodes a PLB-enriched peroxidase, which may take part in plant defense responses (O'Brien et al., 2012). The expression patterns of the described PLB-enriched genes have been documented in an independent RNA-seq dataset (Fang et al., 2022) and validated by RT-qPCR analysis in a separate set of samples (Figure 2).

Crude PLB extract slows growth of *A. citrulli* Aac153

Because many plant defense-relates genes were specifically induced in developing PLBs, we hypothesized that the dynamic metabolomic reprogramming of developing PLBs leads to

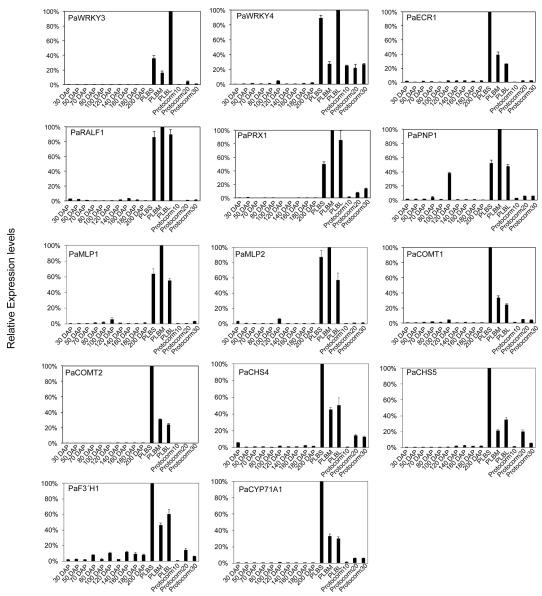

FIGURE 2
Expression profiles of the selected PLB-enriched genes in developing ovaries of *P. aphrodite* collected at 30 to 200 days after pollination (DAP), and developing PLBs and protocorms. Small PLBs (PLBS), medium PLBs (PLBM), large PLBs (PLBL), 10-d-old protocorms (protocorm10), 20-d-old protocorms (protocorm20), and 30-d-old protocorms (protocorm30) are defined as previously described (Fang et al., 2016) by RT-qPCR analysis. Expression was normalized to the Ubiquitin (*PaUBI*) signal. Data are from technical triplicates and the error bars are presented as standard error of the mean. Similar expression pattern was observed in RNA-seq data from two independently collected samples (Fang et al., 2016; Fang et al., 2022).

synthesis of antimicrobial metabolites. Ethyl acetate (EtOAc), which is commonly used for plant metabolite extraction (Tamokou et al., 2012; Oliveira et al., 2013; Lien et al., 2014; Yang et al., 2022), was used to prepare PLB crude extract. The crude extract was then tested for its effect on growth of three plant pathogens including *Acidovorax citrulli* Aac153 (watermelon bacterial fruit blotch disease), *Pectobacterium carotovorum* subsp. *carotovorum* (bacterial soft rot disease), and *Xanthomonas citri* pv. mangiferaeindicae (mango bacterial black spot disease). Because PLB crude extract showed consistent inhibitory effect on growth of *A. citrulli* Aac153 in the preliminary test (data not shown), we decided to focus on *A. citrulli* Aac153. To confirm this inhibitory effect, bacterial growth was monitored over 19 hours. In three separate experiments, PLB crude extract slowed the growth of *A. citrulli* Aac153 15 hours after treatment (Figure 3), indicating the presence of anti-bacterial activity in the developing PLBs. However, growth of *A. citrulli* Aac153 eventually caught up 19 hours after incubation, suggesting other substances may interfere with the inhibitory activity.

PLB extract reduces number of *A. citrulli* Aac153 bacteria associated with watermelon seeds

To enrich and separate the active metabolites from interfering substances, PLB crude extract was fractionated by solid phase extraction (SPE) based on the chemical polarity. Chemicals eluted with different concentrations of MeOH were collected for seed infestation test. Watermelon seeds were inoculated with *A. citrulli* Aac153 in the presence of different PLB eluents (see Materials and Methods). Interestingly, 30% MeOH PLB eluent was shown to be most effective in reducing the number of bacteria associated with watermelon seeds (Figure 4). Only 2.1% to 6.5% of bacterial cells remained after co-incubation with 30% MeOH-water PLB eluent. Co-incubating seeds with the 100% MeOH PLB eluents was also effective in reducing bacterial number but to a lesser extent (between 7.9% to 53.2%) with relatively large variations as compared to the 30% MeOH-water PLB eluents. This indicates

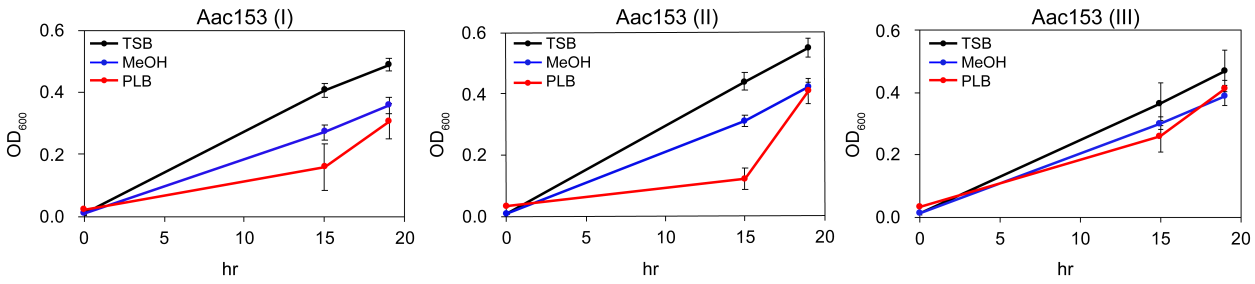

FIGURE 3
Crude PLB extract affected growth of *A. citrulli* Aac153. Aac153, *A. citrulli* Aac153 culture. TSB, cells were allowed to grow in TSB medium. MeOH, cells were allowed to grow in TSB medium containing 6.7% MeOH. PLB, cells were allowed to grow in TSB medium supplemented with 6.7% PLB extract. hr, hour after incubation. I, II, and III represent three independent experiments.

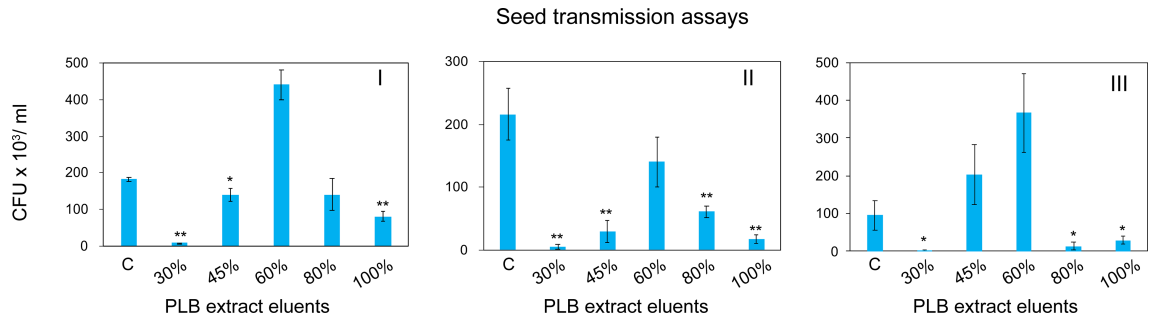

FIGURE 4
Watermelon seed infestation assay. CFU, colony forming unit. CFU Data were collected from 5 pooled watermelon seeds. C, no PLB extract control. 30%, PLB extract eluted by 30% MeOH-H2O. 45%, PLB extract eluted by 45% MeOH-H2O. 60%, PLB extract eluted by 60% MeOH-H2O. 80%, PLB extract eluted by 80% MeOH-H2O. 100%, PLB extract eluted by 100% MeOH-H2O. The experiment was repeated three times with similar results. I, II, and III represent three independent experiment. A two-tailed Student's t-test was applied. *0.05 > p > 0.01; **p < 0.01. Only the treatments showing statistically significant reduction of bacteria were marked.

that compounds with the potent antibacterial activity were enriched in 30% MeOH-water PLB eluent.

PLB extract alleviates disease symptoms caused by *A. citrulli* Aac153

Because 30% MeOH PLB eluent was effective in reducing the number of bacteria attached to the watermelon seeds, we then investigated whether it can protect watermelon seedlings from *A. citrulli* Aac153 infection. To this end, watermelon seeds were incubated with *A. citrulli* Aac153 culture in the presence of 1x or 1/5x of 30% MeOH PLB eluent.

Watermelon seedlings from seeds incubated with CMC or CMC + 6.7% MeOH were used as controls, and no disease symptoms were observed on these seedlings (Figure 5A; Supplementary Figure 2). On the other hand, watermelon seedlings inoculated with *A. citrulli* Aac153 inoculation, showed water-soaking spots and necrotic lesions on the hypocotyl or cotyledons, typical BFB symptoms (Walcott, 2008; Bahar and Burdman, 2010), at 12 days after transplantation (DAP). Co-treatment of 1x 30% MeOH PLB eluent alleviated disease symptoms on the *A. citrulli* Aac153-infected seedlings. Moreover, 1/5 x 30% MeOH PLB eluent was also potent in protecting the *A. citrulli* Aac153-infected seedlings. This experiment was conducted three times and similar results were obtained each time. Disease assessment was quantified by the disease index scale as detailed in Materials and Methods. Disease severity of the *A. citrulli* Aac153-infected seedlings ranged from 65.5% to 81% (Figure 5B). The disease severity of the *A. citrulli* Aac153-infected seedlings treated with 1x 30% MeOH PLB eluent was reduced to 38.0% to 46%. Even 1/5 x 30% MeOH PLB eluent was able to protect the *A. citrulli* Aac153-infected seedlings and disease severity was reduced to 46% to 48.2%. Importantly, PLB treatment at the higher concentration only slightly affected seed germination (p value = 0.03, Table 2). Together, we conclude that PLB-derived metabolites possess the antibacterial activity that protects watermelon seedlings from *A. citrulli* Aac153 infection.

Discussion

Phalaenopsis orchid PLBs possess the antibacterial activity

Accumulated studies have provided molecular evidence linking pluripotency acquisition of plant regeneration processes to activation of defense responses (Chupeau et al., 2013; Ikeuchi et al., 2017; Iwase et al., 2021; Li et al., 2021). Furthermore, defense- or stress-associated pathways are proposed to be part of a gene regulatory network for cell proliferation and organ regeneration (Heyman et al., 2018; Wu et al., 2020a; Zeng et al., 2021). However, there is little evidence to support the notion that the rewired gene regulatory network of regenerated tissues is capable of synthesizing antimicrobial metabolites. Here, we showed that orchid PLB contains antibacterial substances that are potent in slowing the growth of *A. citrulli* Aac153, reducing the number of *A. citrulli* Aac153 associated with watermelon seeds, and protecting watermelon seedlings from severe infection by *A. citrulli* Aac153. Why would the developing PLBs possess the antibacterial activity? PLB is a regenerated structure induced by cutting during tissue culture (Yam and Arditti, 2009). Tissue injury caused by cutting during tissue culture triggers wounding-induced responses that mimic mechanical wounding triggered by herbivores and insects. It is therefore possible that wounding activates responses that contribute to production of antibacterial activity in PLBs. Wounding induced by herbivores and insects is

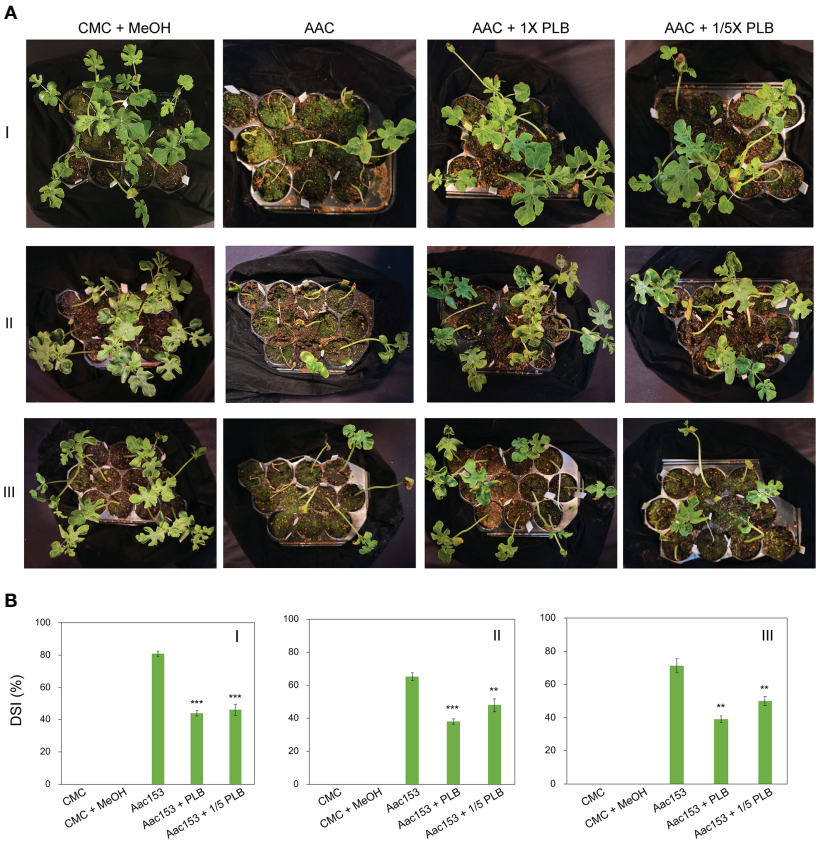

FIGURE 5
Thirty percent MeOH PLB eluent alleviates disease symptoms of watermelon bacterial blotch disease. **(A)** Disease symptoms of *A citrulli* Aac153-infested seedlings at 12 days after transplantion (DAT). **(B)** Disease severity index (DSI) of 12 DAT watermelon seedlings after treatments. CMC + MeOH, seeds were incubated with 6.7% MeOH in CMC medium. Aac153, seeds were incubated with *A citrulli* Aac153 in the presence of 6.7% MeOH. Aac153 + PLB, seeds were incubated with *A citrulli* Aac153 in the presence of 30% MeOH PLB extract. Aac153 + 1/5 PLB, seeds were incubated with *A citrulli* Aac153 in the presence of one-fifth 30% MeOH PLB extract. The experiment was repeated three times. I, II, and III represent three independent experiments. Twenty-two seeds were used in each experiment except CMC and CMC + MeOH treatments. A two-tailed Student's t-test was applied. $^{**}0.01 > p > 0.001$; $^{***}p < 0.001$.

known to trigger *de novo* synthesis of ethylene, jasmonic acid, and abscisic acid (Hyodo et al., 1983; Peña-Cortés et al., 1995; Bergey et al., 1996; Bouquin et al., 1997) that subsequently induce plant immunity responses to protect plants from infection by microbial pathogens (Savatin et al., 2014). Wounding also induces an array of immunity-related transcription factors such as WRKY transcription factors whose functions are to activate plant defense responses and prevent bacterial and fungal infection (Li et al., 2004; Li et al., 2006; Zheng et al., 2006; Pandey et al., 2010; Sarris et al., 2015; Zhou et al., 2018). Coincidently, some of the WRKY transcription factors also play a role in tissues regeneration (Che et al., 2006; Xu et al., 2012; Iwase et al., 2021). In this study, we showed that *PaWRKY3* and *PaWRKY4* (Table 1, Figure 2) are PLB-enriched transcription factors. *PaWRKY3* and *PaWRKY4* are

TABLE 2 Germination rate of watermelon seeds.

	CMC	CMC + MeOH	Aac153	Aac153 + PLB	Aac153 + 1/5 PLB
Germination rate	100.0 ± 0.0%	90.0 ± 9.1%	87.9 ± 8.0%	89.4 ± 6.1%	93.9 ± 3.0%
p value	N/A	0.42	0.15	0.03	0.18

CMC, seeds incubated with CMC medium before germination. CMC + MeOH, seeds incubated with 6.7% MeOH in CMC medium before germination. Aac153, seeds incubated with A. citrulli Aac153 in the presence of 6.7% MeOH. Aac153 + PLB, seeds incubated with A. citrulli Aac153 in the presence of 30% MeOH PLB extract. Aac153 + 1/5 PLB, seeds incubated with A. citrulli Aac153 in the presence of one-fifth 30% MeOH PLB extract. Twenty-two seeds were used in each experiment except CMC and CMC + MeOH control experiments. For CMC and CMC + MeOH treatments, eleven seeds were used in each experiment. The experiment was repeated three times. N/A, not applicable. p values were derived from SPSS Student's t-test analysis.

the homologs of Arabidopsis defense response regulators, *WRKY70* and *WRKY33*, respectively (Li et al., 2017; Zhou et al., 2018). Arabidopsis *WRKY70* is directly regulated by the key immunity signaling regulator NONEXPRESSOR OF PR GENES1 (NPR1) and *wrky70* mutant displayed reduced resistance to the oomycte *Hyaloperonospora parasitica* (Wang et al., 2006; Knoth et al., 2007). Arabidopsis *WRKY33*, on the other hand, is required for pathogen-induced biosynthesis of camalexin and ethylene response (Mao et al., 2011; Li et al., 2012). Considering the antimicrobial activity of PLB extract, we hypothesize that *PaWRKY3* and *PaWRKY4* may be part of cell reprogramming networks in developing PLBs that contribute to the defense activation and accumulation of antimicrobial metabolites. The functions of these *PaWRKY3* and *PaWRKY4* transcription factors remain to be determined.

Phytoalexins are reported to play important roles in combating a broad range of bacterial and fungal pathogens (Glazebrook and Ausubel, 1994; Graham et al., 2007; Ahuja et al., 2012; Schmelz et al., 2014). Because wounding has been reported to induce phytoalexin biosynthesis (Guillet and De Luca, 2005; Naoumkina et al., 2007; Farag et al., 2008), we speculated that phytoalexins make up part of the PLB-based antibacterial metabolites. Since chalcone synthases (*PaCHS4* and *PaCHS5*) and flavonoid 3' hydroxylase (*PaF3' H1*) were specifically upregulated in PLBs, it is possible that flavonoids- and isoflavonoid-type phytoalexins were accumulated to provide the antimicrobial activity. However, we cannot exclude the possibility that other types of phytoalexins or cellular processes provide the inhibitory effect against *A. citrulli* Aac153. The active substance(s) remain to be purified and identified.

Orchid PLBs may enable discovery of novel antimicrobial metabolites

Plants possess a rich repertoire of phytochemicals that are important for plants to combat pathogens and predators, and adapt to biotic and abiotic stresses in the natural environment (Pichersky and Lewinsohn, 2011; Moghe and Last, 2015). The fact that the stress-activated plant regeneration program is often associated with defense-associated cellular activity suggests that the molecular wiring of development and plant immunity processes is overlapping. This notion is supported by a recent study showing that plant development and immunity share a signaling network (Wu et al., 2020b). In addition to a signaling network, we hypothesize that the cellular metabolism is also reprogrammed to accommodate stress-associated organogenesis and plant immunity functions. The available genome and transcriptome databases of *P. aphrodite* (Chao et al., 2017; Chao et al., 2018) provide the molecular basis to discover pathways and to decode the molecular wiring of PLB-associated antimicrobial metabolites (Owen et al., 2017). We suggest that PLBs may be used as a metabolite tap for identifying novel antibacterial compounds. Identification and characterization of the PLB-associated antimicrobial metabolites may provide a new route to harness the chemical diversity of orchid species.

Bacterial fruit blotch disease is a serious threat to the cucurbit (gourd) industry. Even though seed sanitation with hydrochloric acid or peroxyacetic acid have been proven to be effective at eradicating pathogens from infested seeds, seed quality is affected substantially (Hopkins et al., 1996; Hopkins et al., 2003). Moreover, seed disinfestation treatments and chemical control in the field are limited in their ability to reduce the yield losses associated with BFB (Burdman and Walcott, 2012), most likely because the applied chemicals cannot reach bacteria that are associated with developing embryos and seed coats (Rane and Latin, 1992; Hopkins and Thompson, 2002; Dutta et al., 2016). Since PLB-based extract did not affect the viability or germination rate of the watermelon seeds (Table 2), it may serve as an alternative to control watermelon fruit blotch disease.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Author contributions

S-CF conceived the idea and coordinated the study. S-CF, B-LH, and T-PH designed the experiments. S-CF, B-LH, and T-PH analyzed the data. S-CF and T-PH wrote the manuscript. B-LH and J-CC conducted the experiments. All the authors have read and approved the final manuscript.

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Conflict of interest

S-CF and B-LH are named inventors on a patent application pertaining to the technology that was filed by Academia Sinica.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1054586/full#supplementary-material

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Acetylcholinesterase inhibitory activity of sesquiterpenoids isolated from *Laggera pterodonta*

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Plant-derived natural products are important resources for pesticide discovery. Acetylcholinesterase (AChE) is a well-validated pesticide target, and inhibiting AChE proves fatal for insects. Recent studies have shown that the potential of various sesquiterpenoids as AChE inhibitors. However, few studies have been conducted with eudesmane-type sesquiterpenes with AChE inhibitory effects. Therefore, in this research, we isolated two new sesquiterpenes, laggeranines A (1) and B (2), along with six known eudesmane-type sesquiterpenes (3–8) from *Laggera pterodonta*, and characterized their structures and the inhibitory effect they exerted on AChE. The results showed that these compounds had certain inhibitory effects on AChE in a dose-dependent manner, of which compound 5 had the best inhibitory effect with IC50 of 437.33 ± 8.33 mM. As revealed by the Lineweaver-Burk and Dixon plots, compound 5 was observed to suppress AChE activity reversibly and competitively. Furthermore, all compounds exhibited certain toxicity levels on *C. elegans*. Meanwhile, these compounds had good ADMET properties. These results are significant for the discovery of new AChE targeting compounds, and also enrich the bioactivity activity repertoire of *L. pterodonta*.

KEYWORDS

***Laggera pterodonta*, sesquiterpenes, acetylcholinesterase, enzyme kinetics, toxic effects**

1 Introduction

Acetylcholinesterase (AChE) is a critical enzyme performing important functions associated with nerve conduction, involving the catalysis of the degradation process of neurotransmitter acetylcholine and the subsequent termination of its stimulating effect on post-synaptic membrane excitation, through which the enzyme maintains normal nerve impulse transmission in organisms (Fournier and Mutero, 1994). In recent years, AChE has been studied in medicine, chemistry, pesticide and plant protection.

In pest control, AChE is a well-validated pesticide target, and inhibiting AChE proves fatal for insects (Rajashekar et al., 2014). Organophosphorus and carbamate insecticides are the most common AChE inhibitors (Fukuto, 1990). Although they play a great role in pest

control, they can also cause harm to non-target organisms such as humans (Vale, 2015). In addition, due to the long-term use of the same insecticide, pests tend to develop resistance and make the inhibitors ineffective (Vaughan et al., 1997). So, we need to find new inhibitors to solve these problems. Many studies have shown that plant secondary metabolites are the main source of AChE inhibitors. In our previous study, 15 flavonoids isolated from *Eupatorium adenophorum* were discovered to suppress AChE in *Spodoptera litura* and *C. elegans* (Li et al., 2020), and 13 flavonoids isolated from *Ginkgo biloba* were found to inhibit AChE (Ding et al., 2013). Furthermore, we screened more than 200 compounds and the two sesquiterpenoids, parthenolide and tirotundin, extracted from *Chrysanthemum parthenium* and *Tithonia diversifolia*, respectively, elicited a strong inhibitory effect on nematode AChE (Lan et al., 2022). We think this work can provide some ideas for finding new inhibitors from natural products.

Laggera pterodonta (DC.) Benth. which grows in India, the Indochina Peninsula and tropical Africa (Gu et al., 2014), also extensively exist in southwestern China, particularly the Sichuan and Yunnan Provinces. This plant has long been used as folk medicine in China, and has been widely used clinically, with antioxidant, anti-tumor, antibacterial and analgesic effects. Previous studies on its constituents revealed that eudesmane-type sesquiterpene are one of the main secondary metabolites of this plant (Zhao et al., 1997; Yang et al., 2007; Lu et al., 2014; Xie et al., 2021). Sesquiterpenes are an important class of terpenoids with extensive biological activities, which have attracted our attention. A survey conducted by researchers found that 58 sesquiterpenes from various plants showed varying degrees of inhibitory activity against AChE in multiple studies over the past decade, and these findings shed light on the potential of sesquiterpenes to inhibit AChE (Arya et al., 2021). Therefore, we studied the chemical constituents of *L. pterodonta* and took the eudesmane-type sesquiterpene as the research object, trying to find the active compounds that inhibit AChE.

In this research, we isolated two new sesquiterpenes, laggeranines A (1) and B (2), along with six known sesquiterpenes (3–8), and characterized their structures and the inhibitory effect they exerted on AChE. At the same time, in order to understand the mechanism by which these compounds inhibit AChE, only compounds with strong activity levels were selected for kinetic studies. In addition, ADMET prediction is very important for early drug research and development. We also used the admeatSAR platform to make ADMET prediction for these compounds. This study provides experimental and theoretical foundations for novel acetylcholinesterase inhibitors in *L. pterodonta.*

2 Materials and methods

2.1 General experimental procedures

This study obtained chlorpyrifos (≥98% purity) from Sigma Chemical Co. (St. Louis, MO, USA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, ≥98% purity) from Biological Engineering Co. (Huzhou, Zhejiang, China) and acetylcholine iodide (ATChI) (≥98% purity) from Fluka Chemical Co. (Milwaukee, WI, USA). Ultra-pure water (Milli-Q purification system, Millipore, MA) and acetonitrile (HPLC-grade, J.T. Baker, Phillipsburg, NJ) were utilized in semi-preparative HPLC. Additionally, petroleum ether, ethanol, acetone, ethyl acetate, methanol (MeOH), and chloroform of reagent grade were provided by Qingdao Marine Chemical Inc., China.

Bruker 500 and 600 MHz spectrometers were used to measure NMR spectra, using TMS as the endogenous reference. The BioRad FTS-135 spectrometer was utilized to survey IR spectra using KBr pellets, JASCO P-1020 digital polarimeter was employed for analyzing optical rotations, and the Shimadzu UV-2401A for recording UV spectra. The HR-ESI-MS were recorded on a triple quadrupole mass spectromete (Agilent, America). Furthermore, this study utilized silica gel (60–80, 200–300 and 300–400 mesh, Qingdao Marine Chemical Inc, China), SBC MCI gel (75–150 μm, Sci-Bio Chem Co. Ltd., Chengdu, China), Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB) and silica gel H (10–40 μm, Qingdao Marine Chemical Inc, China) for Column Chromatography (CC). The YMC Luna C_{18} reversed-phase column (5 μm; 10 × 250 mm) was utilized for semi-preparative HPLC.

2.2 Plant material

For this study, aerial parts of *L. pterodonta* were collected in July 2017 from Baoshan, Yunnan, China (25°5′N, 99°6′E). Prof. Hua Peng from the Kunming Institute of Botany, Chinese Academy of Sciences (CAS), identified each of our collected samples. Meanwhile, we deposited one voucher specimen (No. 1707016) at the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, CAS.

2.3 Extraction and isolation

Ethanol was added to the extract, containing dried aerial *L. pterodonta* (5 kg), thrice under room temperature (RT), followed by solvent evaporation in a vacuum. The obtained products were then filtered and evaporated to obtain 8 L extracts, which were divided, using equivalent amounts of ethyl acetate and petroleum ether (thrice with each), to obtain ethyl acetate extracts (55 g) and petroleum ether extracts (68 g). Later, a silica gel column (10 × 100 cm) was used for the chromatography of EtOAc extracts and eluted using petroleum ether-acetone (100:1-1:1) to obtain 8 fractions (1–8).

MCI chromatography was conducted to purify fraction 3 (278 mg) and eluted using the MeOH–H2O mixed solution, which generated 4 fractions (3A–3D). We utilized silica gel to purify fraction 3B (102 mg) using petroleum ether-ethyl acetate (50:1–10:1), generating Fr.3B2 (14 mg), which was subsequently purified by semi-preparative HPLC (54% CH3CN within the water) to yield compound 1 (3.4 mg, tR = 15 min).

MCI chromatography was also utilized to purify fraction 4 (45.6 g), which was eluted using the MeOH-H2O mixed solution to yield eight corresponding fractions (4A–4H). Of these, we purified fraction 4D (10.5 g) using the silica gel column and performed the elution using petroleum ether-ethyl acetate (40:1–4:1, stepwise) to obtain another 5 fractions (4D1–4D5). Sephadex LH-20 CC was performed for the chromatography of Fr.4D2 (3.9 g) under MeOH elution, followed by

semi-preparative HPLC, using the 58% acetonitrile solvent system (3 mL/min), to obtain compound 3 (226 mg, tR = 55 min), compound 4 (1.9 g, tR = 59 min) and compound 5 (213 mg, tR = 65 min). Similarly, we used the silica gel column to purify fraction 4E (221 mg), and elution was performed using petroleum ether-ethyl acetate (70:1–4:1, stepwise) to obtain three further fractions (4E1–4E3). Later, Sephadex LH-20 CC was employed to purify Fr.4E2 (46 mg), fraction 4F (2.1 g), and fraction 4G (104 mg) under MeOH elution, followed by semi-preparative HPLC, using the 55%, 47% and 45% acetonitrile solvent systems (3 mL/min), respectively, to yield compound 7 (10.5 mg, tR = 60 min), compound 6 (1.3 g) and compound 8 (76 mg, tR = 19 min), respectively.

The silica gel column was then utilized to purify fraction 7 (708 mg) and eluted using petroleum ether-ethyl acetate (40:1–5:1, stepwise) to generate fractions 7A-7G. Sephadex LH-20 CC was then performed to purify fraction 7C (56 mg) using a mobile phase of [dichloromethane-methanol (1:1)], which yielded two fractions (7C1–7C2). Semipreparative HPLC was adopted to purify Fr.7C2 (15 mg) with the 57% acetonitrile solvent system (3 mL/min) to yield compound 2 (2.8 mg, tR 45 min).

2.3.1 laggeranine A (1)

$[\alpha]_D^{20}$ − 159.13 (*c* 0.16, MeOH); UV (MeOH) Λ_{max} (log ε): 195 (4.17) nm; IR (KBr): 3429, 2930, 2874, 1695, 1623, 1461, 1434, 1384, 1264, 1189, 1151, 1081 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1. HR-ESI-MS: *m/z* 249.1495 $[M-H]^-$ (calcd for $C_{15}H_{22}O_3$, 249.1496).

2.3.2 laggeranine B (2)

$[\alpha]_D^{20}$ − 4.76 (*c* 0.14, MeOH); UV (MeOH) Λ_{max} (log ε): 195 (3.64) nm; IR (KBr): 3434, 2927, 2855, 1714, 1627, 1448, 1383, 1263, 1171, 1123, 1047 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1. HR-ESI-MS: *m/z* 301.1773 $[M + Na]^+$ (calcd for $C_{17}H_{26}O_3$, 301.1774).

TABLE 1 ^1H and ^{13}C NMR Data of Compounds 1 and 2 (δ in ppm, J in Hz, 600 MHz for 1H and 150 MHz for 13C, in Methanol-d4).

Position	1		2	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1a	5.50, d (4.8)	123.9, CH	1.89, td (4.2, 13.8)	37.8, CH_2
1b			1.18, d (13.8)	
2a	4.00, m	65.1, CH	1.51, m	23.2, CH_2
2b			1.61, m	
3a	1.56, dt (1.5, 14.4)	37.5, CH_2	2.14, d (13.8)	34.2, CH_2
3b	1.69, m		2.52, td (4.8, 13.8)	
4	1.90, m	32.4, CH	–	150.8, C
5	–	39.9, C	–	76.2, C
6a	1.62, dd (9.7, 13.4)	41.8, CH_2	1.57, m	39.0, CH_2
6b	1.68, m		2.09, d (12.8)	
7	2.64, m	34.3, CH	2.87, m	38.1, CH
8a	1.74, m	31.1, CH_2	1.52, m	27.4, CH_2
8b	1.83, m		1.67, ddd (3.3, 13.2, 25.8)	
9a	2.05, ddd (2.2, 8.1, 13.7)	29.2, CH_2	1.82, td (3.3, 13.2)	34.5, CH_2
9b	2.49, m		1.10, d (13.2)	
10	–	149.8, C	–	39.7, C
11	–	148.0, C	–	147.0, C
12	–	171.6, C	–	168.8, C
13a	5.56, br. s	122.5, CH_2	5.61, br. s	123.1, CH_2
13b	6.09, br. s		6.14, br. s	
14a	0.87, d (6.8)	16.1, CH_3	4.97, br. s	111.7 CH_2
14b			5.26, br. s	
15	0.88, s	19.6, CH_3	1.04, s	23.1, CH_3
16	–	–	4.21, m	61.8, CH_2
17	–	–	1.30, t (6.0)	14.5, CH_3

The NMR signal abbreviations:br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dt, doublet of triplets; td, triplet of doublet; dd, doublet of doublets; ddd, doublet of dd.
"–" represents quaternary carbon.

2.4 Nematode

This investigation acquired *C. elegans* from the Insect Toxicology Laboratory of Yunnan Agricultural University, Kunming, Yunnan, China, and cultivated them using an oat medium under RT.

2.5 Determination of IC_{50} of compounds to AChE

Third-instar juvenile stage samples of *C. elegans* were thoroughly ground using a glass homogenizer. The homogenate was then dissolved in PBS (pH 7.0, which contained 0.1% Triton X-100) using a suitable volume, followed by a 30-min centrifugation at 6000 r/min under 4°C to harvest supernatants to analyze the enzymes (Ellman et al., 1961; Zhao et al., 2018) [Nematode (3rd instar): 4000 individuals/mL].

After dissolving the eight compounds and chlorpyrifos in acetone, multiple dilutions were made to obtain test concentrations at 5 levels. Thereafter, the test solution of every level (4 μL) was blended with the enzyme solution (96 μL), followed by 2-h incubation at 37°C in a 96-well plate. The final concentrations of compounds were 29.25, 58.50, 117.00, 234.00, and 468.00 μg/mL, respectively. Thereafter, 1.5 mM acetylthiocholine iodide (ATCHI) (50 μL) was added for a further 0.5-h incubation at 37°C. The reaction was finally terminated by adding 0.3 mM 5,5′- dithiobis (2 - nitrobenzoic acid) (DTNB) (50 μL). Afterward, the residual activity of acetylcholinesterase was measured with a microplate reader at 405 nm. Corresponding treatments were then applied to different groups as given below:

1. Treatment group:

Enzyme solution + compound + ATCHI(1.5mM, 50μL) + DTNB(0.3mM, 50μL) .

2. Compound control group:

Enzyme solution + compound + PBS(50μL) + PBS(50μL) .

3. Substrate control group:

Enzyme solution + ATCHI(1.5mM, 50μL) + DTNB(0.3mM, 50μL) .

4. PBS control group:

Enzyme solution + PBS(50μL) + PBS(50μL) .

The inhibition rate (%) was calculated follows:

$$I\,\% = [1 - (\text{Treatment group} - \text{Compound control group})/(\text{Substrate control group} - \text{PBS control group})] \times 100\,\%$$

2.6 Kinetic study on the inhibitory effect of AChE by compound

This study prepared the enzyme solution at multiple gradients (0.125, 0.25, 0.5, and 1.0 U/mL). After adding compound 5 (29.25, 58.50, 117.00, 234.00, and 468.00 μg/mL), the mixed sample was then subjected to a 1-h incubation on a 96-well plate at 37°C (the buffer was used as a substitute for the control group). Later, 1.5 mM ATCHI (50 μL) was added and incubated for a 30-min period, followed by an addition of the 0.3 mM DTNB (50 μL). After 30 s, a microplate reader was utilized to record the OD value at 405 nm (five times at 1-min intervals). The change in the OD value every minute was used to calculate the reaction rate (ΔA/min). Later, the reaction rate curve, as a function of enzyme concentration, was plotted to compare reaction rates (v) among diverse test compound levels. Associations between diverse enzyme levels were utilized to evaluate the inhibitory effect of the compound on nematode AChE (Xiong et al., 2016). In addition, the Lineweaver-Burk double reciprocal graph, demonstrating the reaction rate as a function of enzyme level, was plotted to infer the inhibition type (Guo et al., 2018).

2.7 Toxic effects of compounds on *C. elegans*

In the toxicity analysis, 95 μL of the nematode solution (containing approximately 80 C. *elegans*) along with 5 μL of each compound was added to a 96-well plate. The final concentration of each compound was 0.5 mg/mL, and chlorpyrifos (20 μg/mL) was used as the positive control. The samples were mixed sufficiently, followed by 48-h incubation of plates under RT, and subsequently, the dead nematodes were counted to determine the lethality rate (Roh and Choi, 2008).

2.8 ADMET prediction of compounds

Will compound SMILES format into admetSAR web site (http://lmmd.ecust.edu.cn/admetsar1) prediction module, the small molecule ADMET forecast information can be obtained by clicking on Predict.

2.9 Data analysis

GraphPad Prism 8 was employed for kinetics analysis and IC_{50} value determination of AChE, while SPSS18 was employed for calculating the statistical significance. Data in the bar graph are represented as the mean ± SD. Duncan's new multiple range analysis was conducted to compare and analyze the significance of the difference, where $p<0.05$ indicated for a significant difference. Each experiment was conducted in three independent replicates.

3 Results and discussion

3.1 Structural elucidation of compounds 1-8

This study identified compound 1 as a colorless oily substance, whose molecular formula was determined to be $C_{15}H_{22}O_3$ based on HR-ESI-MS at *m/z* 249.1495 ([M-H] $^-$, calcd, 249.1496). Besides, its IR absorption bands were detected at 3429 cm^{-1} and 1695 cm^{-1}, implying the presence of one conjugated carboxylic acid group. As observed from the ^{1}H NMR spectrum (Table 1), there were signals for three olefinic protons (δ_H 5.50, 5.56, and 6.09) and two methyls groups (δ_H 0.87, 0.88). Both DEPT and ^{13}C NMR spectroscopy analyses (Table 1) identified 15 carbon signals, which included two methyl (δ_C 16.1 and 19.6), five methylenes (δ_C 29.2, 31.1, 37.5, 41.8, and 122.5), four methines (δ_C 32.4, 34.3, 65.1, and 123.9) and four quaternary carbons (δ_C 39.9, 148.0, 149.8, and 171.6). Correlations in the ^{1}H-^{1}H HSQC and COSY diagrams suggested that there were 2 proton-bearing fragments, $CHCHCH_2CHMe$ (a) and $CH_2CHCH_2CH_2$ (b) (Figure 1). In the HMBC spectrum, 2 methyl groups [δ_H 0.87 (3H, d, J = 6.8 Hz) and δ_H 0.88 (3H, s)] exhibited HMBC associations with C-4 and C-5, which indicated their positions within nearby carbons. Meanwhile, the HMBC associations between H_3_14 and C-3 as well as H_3_15 with C-6 revealed the C-14/C-4/C-5 (C-15)/C-6 association. Besides, HMBC associations between H_3_15 and C-10; H-1 and C-9, C-10; H-9 and C-7, C-8, revealed the C-1/C-10(C-5)/C-9 connection. Therefore, as per the associations of H-6 with C-7 and C-11, and H-13 with C-7, C-11, as well as C-12, we determined the compound skeleton. (Figure 1).

As indicated by further HMBC analyses, 1 had a close structural resemblance to tessaric acid, a known eremophilane sesquiterpene (Marcela et al., 1997). The only major discrepancy was the replacement of C=0 group of tessaric acid, with a hydroxyl group of 1, which was inferred from H-1, H-3, H-4 with C-2 cross-peaks in HMBC. The ROESY spectrum (Figure 2) was used to assign the relative configuration for 1, where H-2/H-4 and H-4/H-7 associations suggested that they were co-facial with an α-orientation. Since 1 had a specific rotation ($[\alpha]_D^{20}$ −159.1), similar to that of tessaric acid ($[\alpha]_D^{20}$ −156.2). The specific rotation of tessaric acid was determined by X-ray single-crystal diffraction (Zheng et al., 2003), and the absolute configuration of compound 1 was be the same as that of tessaric acid. Therefore, the structure of compound 1 could be inferred through the above rationale (Figure 3).

Compound 2 was also identified as a colorless oily substance. Based on HR-ESI-MS at *m/z* 301.1773 ([M + Na] $^+$, calcd, 301.1774), we determined the molecular formula to be $C_{17}H_{26}O_3$. As revealed by the IR spectrum, the typical absorption bands were observed for hydroxy (3434 cm^{-1}) and ester carbonyl (1714 cm^{-1}) functional groups. Signals for four olefinic protons (δ_H 4.97, 5.26, 5.61, and 6.14) and two methyls (δ_H 1.04 and 1.30) were observed from the ^{1}H NMR spectrum (Table 1). Besides, 17 carbon signals, which included 2 methyl (δ_C 14.5, 23.1), nine methylenes (δ_C 23.2, 27.4, 34.2, 34.5, 37.8, 39.0, 61.8, 111.7, and 123.1), one methine (δ_C 38.0) and five quaternary carbons (δ_C 39.7, 76.2, 147.0, 150.8, and 168.8), were observed from DEPT and ^{13}C NMR spectroscopy (Table 1). Besides, ^{1}H along with ^{13}C NMR spectroscopy for 2 (Table 1) displayed close resemblance to 5α-hydroxycostic acid (Sanz et al., 1990), but with an additional C_2H_5O group (δ_C 61.8 and δ_C 14.5). 2D NMR data was utilized for subsequent analysis. According to ^{1}H-^{1}H COSY associations, 3 fragments: a (C-1–C-3), b (C-8/C-9), and c (C-16-C17), were present (Figure 1). Moreover, the C_2H_5O group existed in carboxyl (C-12), according to the HMBC association of H-13 with C-7, C-11, C-12; H_3_17 with C-16, as well as H-16 with C-12. The structure of 2 was thereby characterized this way (Figure 3).

For known compounds, ^{1}H NMR and ^{13}C NMR spectra were determined and compared with the data in the literature, the structures were determined to be eudesma-5,12-dien-13-oic acid (3) (Xu et al., 2006), isocostic acid (4) (Cruz and Martinez, 1982), costic acid (5) (Bawdekar and Kelkar, 1965), 5α-hydroxy-4α,15-dihydrocostic acid (6) (Xie et al., 2011), 5α-hydroxycostic acid (7) (Xie et al., 2011), and 3-oxo-di-nor-eudesma-4-en-11-oic acid (8) (Wang et al., 2013). ^{1}H NMR and ^{13}C NMR spectra can be found in supporting materials.

3.2 Determination of IC_{50} of compounds to AChE

To evaluate the inhibitory activity of these compounds on AChE activity, we measured their IC_{50} values with respect to *C. elegans* AChE (chlorpyrifos being utilized as the positive control). IC_{50} represents the compound dose required for 50% inhibition of AChE activity, where a lower value would indicate a more potent inhibition on AChE (Wang et al., 2021). The results showed that those eight compounds inhibited *C. elegans* AChE in a dose-dependent manner, such as compound 5

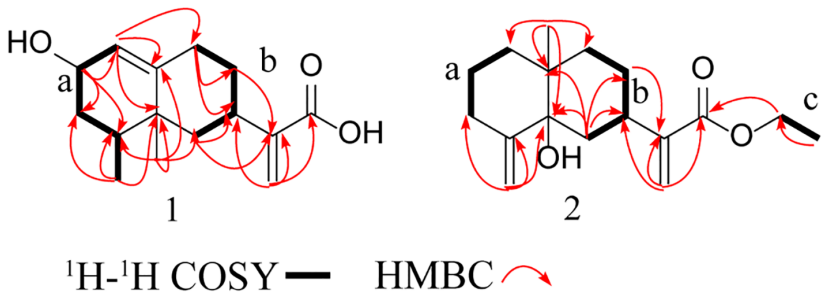

FIGURE 1
Key HMBC, 1 H-1 H COSY, and HMBC correlations of compounds 1 and 2.

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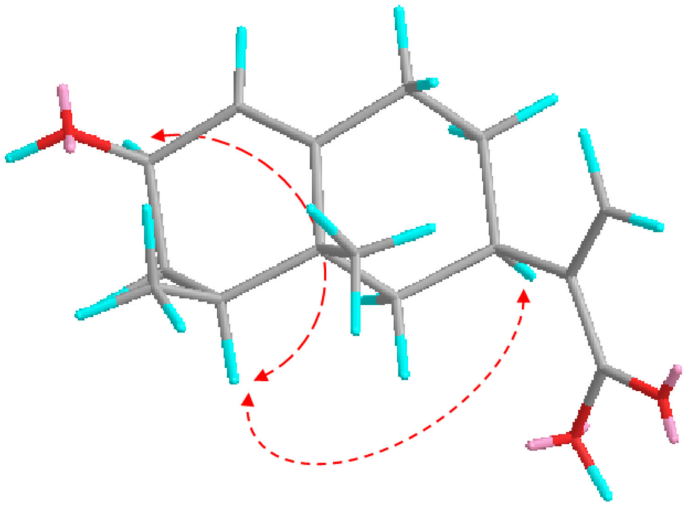

FIGURE 2
Key ROESY correlations of compound 1.

(Figure 4), and the most effective inhibitory effects were compound 5 (IC_{50} = 437.33 ± 8.33 μM), followed by compound 3 (IC_{50} = 464.0 ± 14.74 μM), compound 6 (IC_{50} = 470.33 ± 15.57 μM), compound 7 (IC_{50} = 485.0 ± 11.53 μM), compound 1, compound 4 (IC_{50} < 530 μM), and compounds 2 and 8 (IC_{50} < 710 μM) (Table 2).

Among the 8 compounds, compound 2 showed the worst inhibitory activity, and except compound 2, all the other compounds were eudesmane-type sesquiterpene acids. Therefore, we hypothesized that the carboxyl group in the compound should increase the activity. Compounds 3, 4, and 5 are isomers, while compound 5 shows better activity. Before this, the cytotoxic activity of compounds 4 and 5 against SF9 was also reported, and the same compound 5 showed better activity (Azucena et al., 2005). The difference in their structure lies in the different positions of a double bond. Therefore, we believe that the change of the position of the double bond has certain influence on the activity, and the terminal double bond of the compound should have better activity. The only structural difference between compound 4 and compound 8 is that the C-3 of compound 8 is a carbonyl group, but the activity of compound 4 is significantly higher than that of compound 8. In addition, we found that the hydroxyl group is connected to the skeletons of compounds 1, 6 and 7, but their activities are also not ideal. Can the presence of hydroxyl and carbonyl groups on the eudesmane-type sesquiterpene acid skeleton reduce the inhibitory activity of the compound against AChE? For this purpose, we reviewed studies in the last decade on the inhibition of AChE activity by sesquiterpenoids. However, prior to this study, there were no studies on the inhibition of AChE by the eudesmane-type sesquiterpene acid. Therefore, we hypothesized that the addition of hydroxyl or carbonyl groups to the backbone of eudesmane-type sesquiterpene acid may weaken the inhibitory effect of the compounds on AChE, but this needs to be verified by more experiments.

Compared with various sesquiterpenoids with acetylcholinesterase inhibitory activities reported in the literature, 8 compounds in this study showed unsatisfactory inhibitory activities. Meanwhile, we found that other eudesmane-type sesquiterpenes and their derivatives reported in the literature also showed poor inhibitory activity against AChE, while among the various constituents with AChE inhibitory

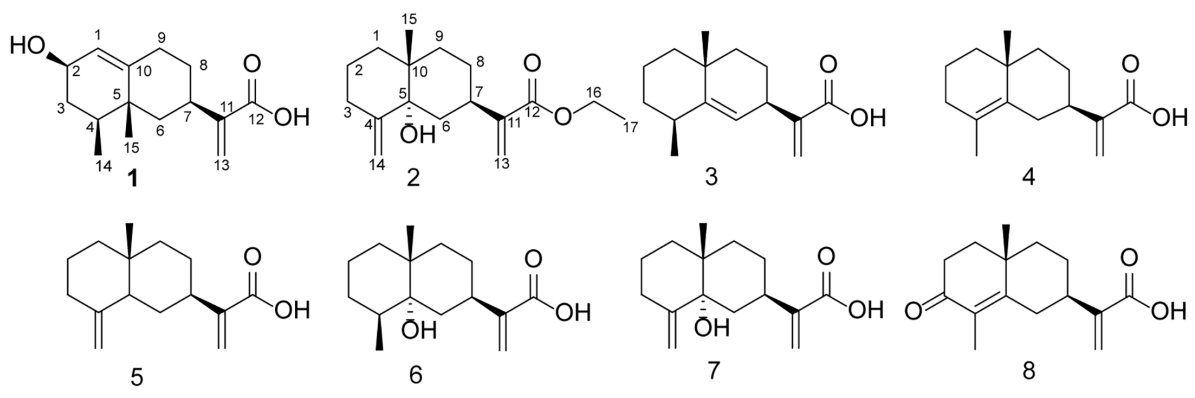

FIGURE 3
Chemical structures of the eight compounds.

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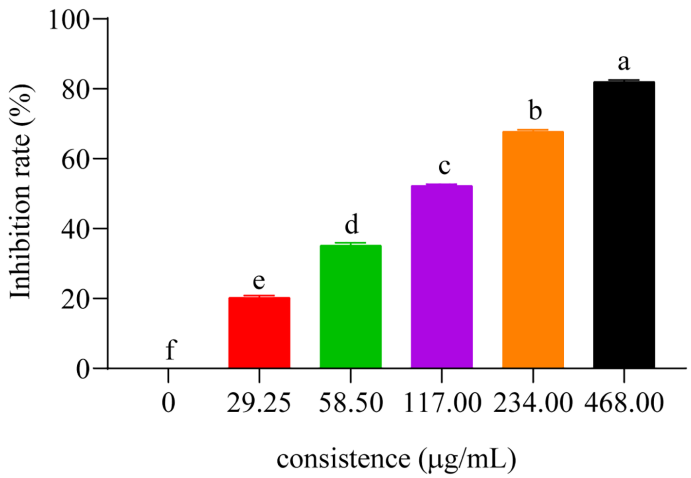

FIGURE 4
The inhibitory effect of compound 5 on the AChE. Concentrations of compound 5 (29.25, 58.50, 117.00, 234.00, and 468.00 μg/mL) for curves are indicated in the column.

activity, various sesquiterpene lactones seemed to be the most promising AChE inhibitors.

3.3 Kinetic study on the inhibitory effect of AChE by compound

To further explore the inhibition mechanism against AChE, we conducted a kinetics analysis on compound 5. The relationship between the maximal reaction initial speed (v) and diverse enzyme levels was analyzed by evaluating the reversible inhibitory effect exerted by compound 5 (Figure 5A). According to Figure 5A, the fitting curve indicated the original rate under diverse levels of compound 5 and a straight line was observed for the enzyme level. Moreover, every straight line intersected at the origin, with the slope dropping as the compound level increased, implying that compound 5 was a reversible inhibitor of AChE.

Under the scenario of compound 5 being identified as a reversible inhibitor, we adopted the double-reciprocal graph (Lineweaver-Burk) to model the maximal original velocity and substrate level to explore the inhibition patterns. According to Figure 5B, each straight line had a unique intercept and slope but intersected within the second quadrant in the Lineweaver-Burk diagram. Meanwhile, as the compound level increased, fitted curves had elevated X-intercepts (-1/Km), Y-intercept (1/Vmax) and slopes, which indicated an increase in the Michaelis constant Km, but a decrease in Vmax. Consequently, compound 5 inhibited AChE in a mixed-type competitive manner, and could be an AChE inhibitor with dual binding sites (Shaik et al., 2019; Tang et al., 2019).

The Dixon plot was made to ascertain the AChE inhibition pattern as well as the dissociation constant (Ki) of our tested compounds. Typically, the Ki value represents the dissociation constant of the enzyme-inhibitor complex, where a lower value indicates a higher affinity of the compound to AChE. Based on the Dixon plot (Figure 6) and the slope in the double reciprocal graph (regarding compound 5 level with maximal original velocity), compound 5 had a Ki value of 24.668 μg/mL (Figure 6).

TABLE 2 IC_{50} of AChE inhibition by compounds.

Compounds	IC_{50} (μM)
1	518.33 ± 5.90^{b}
2	701.33 ± 14.74^{a}
3	464.00 ± 10.00^{d}
4	527.67 ± 8.96^{b}
5	437.33 ± 8.33^{e}
6	470.33 ± 15.57^{cd}
7	485.00 ± 11.53^{c}
8	696.67 ± 12.42^{a}
Chlorpyrifos	7.33 ± 0.58^{f}

The effects of the tested compounds on AChE were repeated in triplicates; IC_{50} values represent the means ± SD; different letters indicate significant differences ($p < 0.05$).

3.4 Toxic effects of compounds on *C. elegans*

To evaluate the level of toxicity on *C. elegans*, we chose eight compounds for analysis. According to the results, the compounds had a potential toxicity level with respect to *C. elegans* at the experimental concentration, where compounds 3 and 5 were highly toxic, with median lethality rates of 60.83 ± 2.6% and 72.92 ± 3.15%, respectively (Table 3). Similarly, compound 2 still showed the lowest toxicity level, which further proved that the carboxyl group in the compound could increase the activity. Compounds 3, 4, and 5 as isomers also showed different toxicity levels, which further confirmed that the change of double bond position had a certain effect on the activity, and the terminal double bond of the compound should have a better activity. At the same time, the toxicity of compounds 1, 6, 7, and 8 with hydroxyl or carbonyl groups in the skeleton is still not ideal, which increases the possibility that the addition of hydroxyl or carbonyl groups to the main chain of the eudesmane-type sesquiterpene acid may reduce the toxicity of the compounds.

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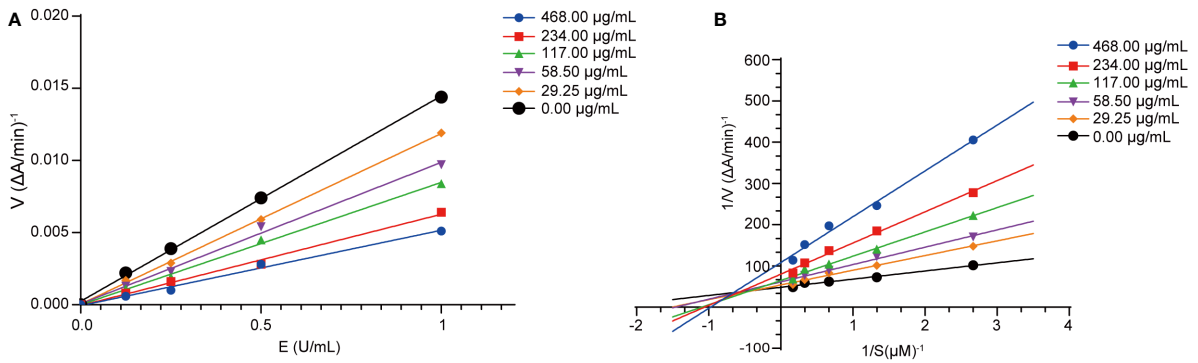

FIGURE 5
Acetylcholinesterase inhibition kinetics analysis of compound 5. **(A)** Hydrolytic activity of acetylcholinesterase concentration under the action of different concentrations of compound 5 (29.25, 58.50, 117.00, 234.00, and 468.00 μg/mL). **(B)** Lineweaver-Burk plots for the inhibition of compound 5 (29.25, 58.50, 117.00, 234.00, and 468.00 μg/mL).

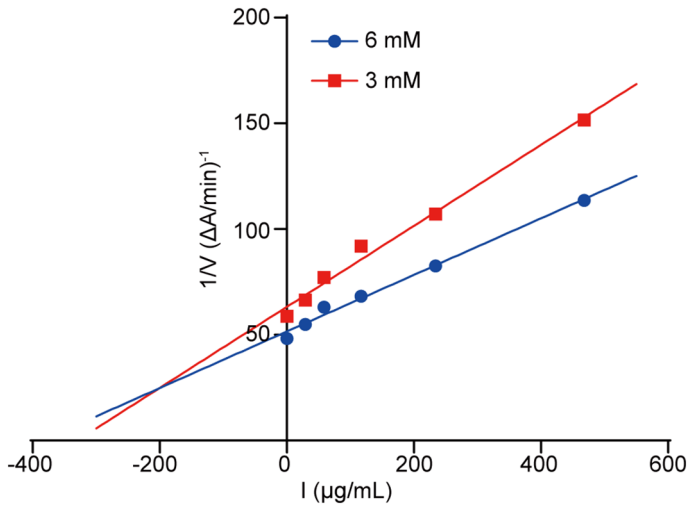

FIGURE 6
Dixon diagram. Dixon plot showing the inhibitory activities of compound 5 against AChE in the presence of different concentrations of substrate. Red and blue lines indicate concentrations of 3 and 6 mM, respectively.

TABLE 3 The lethality of eight compounds to *C. elegans*.

Compounds	Lethality rate (%)
1	38.75 ± 2.50^{d}
2	31.67 ± 0.72^{e}
3	60.83 ± 2.60^{b}
4	30.83 ± 1.91^{e}
5	72.92 ± 3.15^{a}
6	48.75 ± 2.50^{c}
7	50.83 ± 1.91^{c}
8	34.58 ± 1.44^{e}
Chlorpyrifos	81.67 ± 2.60^{f}

The effects of the tested compounds on C. elegans were repeated in triplicates; the values represent the means ± SD; different letters indicate significant differences ($p < 0.05$).

The *in vivo* toxicological results of these compounds were similar to those of AChE inhibition experiments, suggesting that the toxic effects of these compounds on *C. elegans* may be related to their inhibitory activity on AChE to some extent. However, this is not enough to conclude that there was a causal relationship between them. Previous studies have shown that drug toxicity to nematodes occurs in multiple biological tissues (Roh and Choi, 2008). To understand the relationship between AChE activity and toxicity, further experiments are needed for verification.

3.5 ADMET prediction of compounds

The main indexes of ADMET prediction were Blood Brain Barrier (BBB), Human Intestinal Absorption (HIA), Caco-2 permeability (CCP) and Ames mutagenesis (ATT), Carcinogenicity and

TABLE 4 ADMET prediction results for compounds.

Compound	BBB	HIA	CCP	ATT	Carcinogenicity	CYP2D6 inhibitor
1	0.5000−	0.9947+	0.8022+	None	None	non-inhibitor
2	0.7250+	1.000+	0.8954+	None	None	non-inhibitor
3	0.7000+	0.9946+	0.8665+	None	None	non-inhibitor
4	0.7000+	0.9946+	0.8382+	None	None	non-inhibitor
5	0.7250+	0.9950+	0.7249+	None	None	non-inhibitor
6	0.5000−	0.9956+	0.7609+	None	None	non-inhibitor
7	0.7250−	0.9959+	0.6820+	None	None	non-inhibitor
8	0.6500+	0.9929+	0.7555+	None	None	non-inhibitor

BBB "+" represents that drug molecules can easily cross the blood-brain barrier; BBB "–" represents that drug molecules cannot easily cross the blood-brain barrier; a value closer to 1 indicates better permeability to BBB; HIA "+" represents that drug molecules can be absorbed or assimilated through the human intestine, and a value closer to 1 indicates better absorption through the intestine.;CCP "+" means that it can easily penetrate human intestinal cell lines, and the closer the value is to 1, the better CCP permeability is. "None" means that the compound has no mutagenic toxicity or carcinogenicity.

cytochrome CYP2D6 are shown in Table 4. The 8 compounds isolated from *L. pterodonta* were easy to be absorbed or assimilated by the human intestine and could penetrate human intestinal cell lines without mutagenic toxicity or carcinogenicity. This indicates shown that these compounds have good ADMET properties. In addition, except for compounds 1, 6, and 7, which could not easily cross the blood-brain barrier, all other compounds could easily cross the blood-brain barrier. Comparing the structural differences between compounds 1, 6, and 7 and other compounds, it was found that this might be related to the hydroxyl group connected to the skeleton of these three compounds.

4 Conclusions

This study tested novel sesquiterpenes and six known eudesmane-type sesquiterpene acid isolated from *L. pterodonta*, to analyze their inhibitory activity on *C. elegans* AChE. The results showed that all these compounds had certain inhibitory effects on AChE in a dose-dependent manner, of which compound 5 had the best inhibitory effect with IC_{50} of 437.33 ± 8.33 μM. Meanwhile, as revealed by the Lineweaver-Burk and Dixon plots, compound 5 was observed to suppress AChE activity reversibly and competitively. Furthermore, all 8 compounds exhibited certain toxicity levels on *C. elegans*. Finally, the ADMET prediction was carried out for these 8 compounds, and it was found that all compounds had good ADMET properties. Collectively, we believe that these results are significant for the discovery of new AChE targeting compounds, and also enrich the bioactivity activity repertoire of *L. pterodonta*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JL, XQ, and XD conceived and designed the experiments. JL and FL performed the experiments and analyzed the data. XQ, XD, GW, FG, HL, LX, YZ and XH contributed reagents, materials, and analysis tools. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1074184/full#supplementary-material

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Insecticidal and biochemical effects of *Dillenia indica* L. leaves against three major stored grain insect pests

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The Last four decades have witnessed the banning of several synthetic insecticides mainly due to the development of resistance to the target pests and due to hazardous effects on humans and the environment. Hence, the development of a potent insecticide with biodegradable and eco-friendly nature is the need of the hour. In the present study, the fumigant property, and biochemical effects of *Dillenia indica* L. (Dilleniaceae) were studied against three coleopterans stored-products insects. The bioactive enriched fraction (sub-fraction-III) was isolated from ethyl acetate extracts of *D. indica* leaves and found toxic to rice weevil, *Sitophilus oryzae* (L.) (Coleoptera); lesser grain borer *Rhyzopertha dominica* (L.) (Coleoptera) and red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera) with the LC_{50} values of 101.887, 189.908 and 115.1 µg/L respectively after 24 h exposure. The enriched fraction was found to inhibit the function of acetylcholinesterase (AChE) enzyme when tested against *S. oryzae, T. castaneum*, and *R. dominica* with LC_{50} value of 88.57 µg/ml, 97.07 µg/ml, and 66.31 µg/ml respectively, in *in-vitro* condition. It was also found that the enriched fraction caused a significant oxidative imbalance in the antioxidative enzyme system such as superoxide dismutase, catalase, DPPH (2,2-diphenyl-1-picrylhydrazyl), and glutathione-S-transferase (GST). GCMS analysis of the enriched fraction indicates three major compounds namely, 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one, 1,2-Benzisothiazol-3(2H)-one, and Benzothiazole, 2-(2-hydroxyethylthio)-. Finally, we concluded that the enriched fraction of *D. indica* has insecticidal properties and the toxicity may be due to the inhibition of the AChE enzyme in association with oxidative imbalance created on the insect's antioxidant enzyme systems.

KEYWORDS

***Dillenia indica* L., biofumigant, stored grain pests, acetylcholinesterase, antioxidant enzyme system**

Abbreviations: GSH, Reduced glutathione; DPPH, 2, 2, diphenyl, 1, picrylhydrazyl; CDNB, 1, chloro, 2, 4, dinitrobenzene; DTNB, 5, 5, dithio, bis, 2, nitrobenzoic acid; HPLC, High performance liquid chromatography; GCMS, Gas Chromatography Mass Spectrometry; AChE, acetylcholinesterase enzyme; SOD, Superoxide dismutase activity; GST, Glutathione S, transferase

1 Introduction

Every year there is a loss of 5-30 percent of the world's total agricultural food production due to insect infestation on food grains (Rajashekar et al., 2010). The stored grain insects are known to inflict huge damage to stored grains and pulses through the consumption of kernels or accretion of exuviae, webbing, and cadavers (Rajashekar et al., 2012). *Sitophilus oryzae* (L.) (Coleoptera), commonly called rice weevil, and *Rhyzopertha dominica* (L.) (Coleoptera) (common name lesser grain borer), are two of the many primary pests which cause severe global economic losses while red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera), is one of the secondary pests that inflicts damage to stored grain pests in many parts of the world. Generally, chemical-based fumigants are widely used to control the damage caused by insects. But it also brings along several shortcomings such as toxicity to humans and livestock, as well as other non-target organisms, secondary pest outbreaks, pest resurgence, adulteration of food products due to indiscriminate use, erratic supplies, and unavailability at critical periods, high price while also causing several environmental hazards such as ozone depletion. Persistent use of such chemicals also leads to the emergence of resistant strains of the targeted pests (Abubakar et al., 2020). Excessive exposure to chemical pesticides could cause oxidative stress to the human being that ultimately leads to many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, etc. (Huang et al., 2016; Nandipati and Litvan, 2016; Sabarwal et al., 2018). For instance, methyl bromide (now banned) was an effective fumigant used for neutralizing insects on soil and storage structures. Many studies have indicated that prolonged exposure to it has a high effect on the human central nervous system (de Souza et al, 2013; Park et al., 2020). Considering the problems, scientists all over the world are constantly exploring for a safer source for developing eco-friendly bioinsecticides. Plants being one of the richest sources of bioactive molecules may provide potential alternatives to currently used chemical-based approaches (Rajashekar et al., 2012; Singh et al., 2021). Available literature highlights the use of plant-derived botanicals as a source for new insecticides (Miresmailli and Isman, 2014; Rajashekar et al., 2016; Devi et al., 2020; Singh et al., 2021). Therefore, there is a great scope for botanical insecticidal compounds. Providing the best quality seeds for cultivation will enhance productivity thereby providing the best economic and social return. Insect infestation is one of the major factors that affect the viability of seeds meant for prolonged storage. Sometimes the insecticides used to control the stored insects also hamper seed germination. Such things need to be taken care of before deciding the class and dose of the insecticide to be used.

Dillenia indica L. (elephant apple), is a perennial middle-size tree found in tropical, subtropical, and temperate zones. The genus '*Dillenia*' spreads from Madagascar to Fiji Island, and from there it is distributed to Northern and Southern Himalayan slopes, and Southwestern China (Hoogland, 1952). In India, this tree is distributed in the sub-Himalayan tracts, West Bengal, Madhya Pradesh, Assam, North-Eastern India, and South Indian States. This plant has several important biological activities including insecticidal properties (Reddy et al., 2010). They are known to have antidiabetic, antioxidant activity, anti-inflammatory, as well as anticancer properties (Barua et al., 2018). Some literature reported that the spreading of *D. indica* leaves over the stored rice repelled rice weevil (*S. oryzae*) (Bhattacharjee and Ray, 2010). No scientific validation has been provided till date in this aspect. The present study tried to explore the potential insecticidal property of the plant-derived product along with its effect on the antioxidant enzyme system. The study also intended to analyze the phytochemical composition of the bioactive fraction responsible for the fumigant activity. Further, the possible mode of action mechanism was studied with respect to the inhibitory effect on acetylcholinesterase enzyme.

2 Materials and methods

2.1 Collection and preparation of sample

The fresh and matured leaves of *D. indica* were collected from Imphal West, Manipur (N24°49.258', E093°56.411') and authenticated by Dr. Biseshwori Thongam, Scientist-E (Taxonomist), Institute of Bioresources and Sustainable Development, Imphal, Manipur, with voucher number IBSD/M-284. They were properly washed and semi-dried in shade for 4-5 days. The samples were finely powdered using an electric grinder and packed in air-tight poly bags for further use.

2.2 Chemicals

Pyrogallol, Catalase, reduced glutathione (GSH), 2,2- diphenyl-1-picrylhydrazyl (DPPH), 1-chloro-2,4-dinitrobenzene (CDNB), Acetylthiocholine chloride, and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co. (St. Louis, MO, USA); hydrogen peroxide, sodium hydroxide, sodium di-hydrogen phosphate, L-ascorbic acid, and sodium carbonate were obtained from Sisco Research Laboratory, Mumbai, India. Sodium Chloride, Magnesium Chloride, and Tris-base were purchased from Himedia Laboratories, Mumbai.

2.3 Extraction and isolation of bioactive enriched fraction

One kilogram of powdered leaves samples was used for the sequential extraction of phytochemicals in the Soxhlet apparatus. The extraction was done for 8-9 h using different solvents of increasing polarity viz., hexane, petroleum ether, ethyl acetate, chloroform, acetone, and methanol. The solvent extracts were filtered with Whatman paper No. 1 and solvents were evaporated using a rotary vacuum evaporator {Rotavapor R100 (Buchi) Switzerland} under low pressure, at a temperature of 45°C. Each extract was tested for fumigant properties against three stored product insects, viz., *S. oryzae, T. castaneum*, and *R. dominica*. Extract with the highest mortality was further subjected to bioassay-guided isolation of the bioactive enriched fraction using several

chromatographic techniques. Silica gel column chromatography with mesh size 60-120 mesh and glass column of 50 cm length and 3 cm diameter was used for the separation of phytochemicals. The active extract was first eluted with 100% hexane, followed by hexane and ethyl acetate mixture, ethyl acetate, and acetone mixture, and then acetone and methanol mixture at different ratios (75:25; 50:50; 25:75; 0:100). Solvents from all 13 fractions were evaporated under reduced pressure and the residue was dissolved in a known volume of acetone. These solutions were tested for fumigation activity against the three stored product insects. Fractions showing the highest fumigant activity were pooled and subjected to Flash chromatography (CombiFlash Rf^{+} Lumen, Teledyne ISCO, USA) with solvent system hexane and ethyl acetate and 0.5% methanol as a modifier, for further separation of bioactive compounds. The eluted sub-fractions were again tested for fumigation activity against the test insects. The most active enriched fraction based on corrected mortality (Sub-Fraction-III) (Figure 1) was collected and used for further experimental purposes. The enriched fraction was further subjected to semi-preparative high performance liquid chromatography (HPLC) for purification and characterization of the bioactive marker compound(s).

2.4 GCMS analysis of the bioactive marker compounds

The marker compounds isolated from the enriched fraction were identified by Gas Chromatography Mass Spectrometry (Thermo Scientific Trace 1300 Gas Chromatograph & TSQ 8000 DUO Mass Spectrometry) having Quadrapole detector analysis. GC detection was performed at the ionization energy 70eV. The injector and mass transfer line were set at 250°C and 280°C. the carrier gas used was helium at the flow rate of 1ml/min and the injection volume was set at 0.5 µl. The initial column temperature was programmed from 40°C for 1 min to 250°C at a rate of 5°C/min heating ramp and then held at 250°C for 20 min. The compounds were identified using the National Institute of Standards and Technology (NIST) library 2017 based on the comparison of their mass spectra with that of the library.

2.5 Fumigant toxicity

Fumigant toxicity assays were performed following the methodology from Rajashekar et al. (2016). Twenty adults of both sexes of *S. oryzae, T. castaneum*, and *R. dominica* were separately released inside the fumigation chamber of one liter volume capacity. Each chamber was infused with different leaf extract solutions at a fixed concentration of 50 mg/L air to a filter paper already placed inside the chamber. The extract solutions were injected using a Hamilton syringe through a rubber septum fitted to the chamber's lid and the infused filter papers were placed on the under surface of the glass chambers which were checked from direct contact with the insects. An equal volume of pure acetone was used as solvent control. The number of dead insects was determined after 24 h. Dose-response relationship was done for the most active enriched fraction with concentration ranges from 50 to 400 µg/L air. The percentage of corrected mortality was calculated using the Abbott formula equation (Abbott, 1925).

2.6 *In vitro* acetylcholinesterase activity assay

The effect of the enriched fraction on the insect's acetylcholinesterase enzyme (AChE) was studied following Ellman's method with slight modification (Ellman, 1959). The AChE enzyme hydrolyses the substrate acetylthiocholine to produce acetate and thiocholine. Thiocholine reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm. The enzyme activity was tested against crude enzyme extract of *S. oryzae, R. dominica*, and *T. castaneum in -vitro* conditions. The insects (20 adults each) were homogenized using 0.5M Tris-HCl buffer and stored at -20^{0}C. For the study, crude enzyme extract was pre-incubated with the enriched fraction and with standard inhibitor (Pyridostigmine bromide) at different doses of 25, 50, 75, and 100 µg/ml of insect's enzyme extract at 37°C for 30 mins. A microplate reader was used to measure the difference in the absorbance. In a microplate

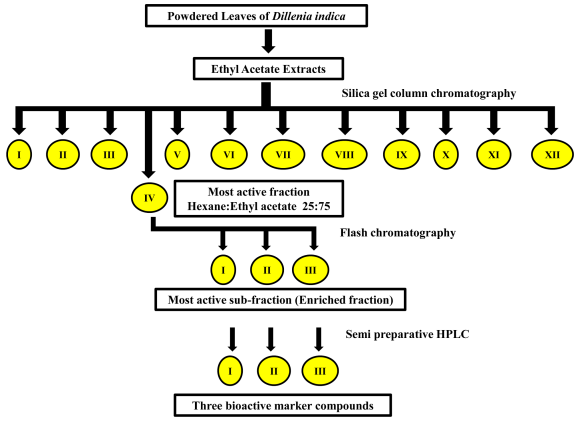

FIGURE 1
Schematic diagram of the isolation of bioactive enriched fraction from ethyl acetate extract of *D. indica* leaves.

well 200 µl of the reaction mixture, 3 µl of 0.1M acetylthiocholine chloride, 10 µl of insect homogenate, and 87 µl of water were added to make the total volume of 300 µl. The reaction mixture is prepared by adding 10.5 ml of cocktail (13 ml of 1M NaCl, 2 ml 1M $MgCl_2$, 10 ml of 0.5M Tris-HCl, and 10 ml of 0.2M EDTA), 3 ml of 1mM DTNB and 6.5 ml of water in a reagent bottle. The reaction is initiated either by adding the treated enzyme or substrate and expressed as percentage inhibition.

Inhibition (%) = 100 - Change of sample absorbance/Change of blank absorbance X 100

2.7 Antioxidant enzymes

2.7.1 Superoxide dismutase activity

The pyrogallol (2mM) autooxidation method described by Marklund and Marklund (1974) was followed for measurement of SOD activity in the tested insects, *S. oryzae* and *T. castaneum*. The reaction mixture contained 2 mM pyrogallol in 0.1M Tris buffer (pH 8.2) and the enzyme. The addition of substrate in the reaction mixture started the reaction and the absorbance was read at 420 nm for 3 min at an interval of 1 min. The SOD activity was expressed as enzyme units/mg protein. The amount of enzyme that inhibits auto-oxidation by 50% is referred to as one unit of enzyme activity.

2.7.2 Catalase activity

The protocol given by Aebi (1983) was used to assay the catalase activity in the tested insects. The reaction mixture contained 3% H_2O_2 in 0.05M phosphate buffer (pH 7.0). The reaction was started by the addition of enzymes and the change in the absorbance at 240 nm was read for 3 min and the activity was expressed as µmole H_2O_2/min/mg protein.

2.7.3 Glutathione-S-transferase

Glutathione S-transferase (GST) activity was measured following the method of Warholm et al. (1985) with CDNB as the substrate. The reaction mixture contains 20 mM GSH and the enzyme (supernatant) in 0.1M phosphate buffer (pH 7.4). The reaction was started by adding 30 mM CDNB and the change in absorbance at 344 nm was monitored in a UV-visible spectrophotometer. The enzyme activity was expressed as µmole CDNB conjugate/min/mg protein.

2.7.4 DPPH radical scavenging assay

The protocol given by Yamaguchi et al. (1998) was used to measure the DPPH radical scavenging activity in *in-vitro* conditions. Briefly, 1 ml of 0.1 mM DPPH solution in 95% ethanol was treated with different concentrations of the active enriched fraction, shaken, and incubated at room temperature for 20 min, and the absorbance was read at 517 nm against a blank. Ascorbic acid was used as the standard to compare the inhibition ability of the enriched fraction to that of the standard. The radical scavenging activity was calculated using the following equation:

$$\textit{Scavenging effect}\ (\%) = [1 - A\ \textit{Sample}\ (517nm)\ /A\ \textit{Control}\ (517nm)]\ x\ 100$$

Total protein content of the sample was measured by the method of Lowry et al. (1951) using BSA as the standard.

2.7.5 Seed germination test

Wheat, *Triticum aestivum* L., and green gram, *Vigna radiata* (L.) R. Wilczek seeds were surface sterilized using 1% sodium hypochlorite for 10 minutes and washed properly with autoclaved distilled water. The 50 sterilized seeds of wheat and green gram were separately kept on Whatman filter paper no. 1 already treated with 100 mg/L and 500 mg/L of enriched fraction and placed in glass petri plates (90X15 mm, borosil, India). The filter paper was kept moist throughout the experimental period by spraying it with distilled water. The germination test was performed for 48 h and 5 days. In the control petri plates, the Whatman filter paper was only soaked with sterilized distilled water. The observation was taken after 48 h and 5 days and the germination percentage was calculated (Rajashekar et al., 2016).

2.8 Data analysis

LC_{50} values were determined using Probit analysis (Finney, 1971) and Statplus 2007 software and computer program SAS (version 6.12, SAS Institute Inc. Cory, NC, USA) were used to analyze the data using One-Way ANOVA ($p<0.05$) by Newman-Keuls test.

3 Result

3.1 Fumigant toxicity test of extracts and enriched fraction

Experimental results reveal that among all the extracts ethyl acetate extract of *D. indica* leaves showed maximum fumigant activities against *S. oryzae, T. castaneum*, and *R. dominica* (Figure 2). Table 1 shows that the bioactive enriched fraction isolated from ethyl acetate extract exhibited toxicity to *S. oryzae, T. castaneum*, and *R. dominica* with the LC_{50} values of 101.88, 198.89, and 115.1 µg/L air respectively after 24 h exposure.

3.2 Compound identification by GCMS

Three major bioactive compounds were separated and eluted from the enriched fraction using semi-preparative HPLC. Gas chromatography mass-spectrometry analysis identified the isolated bioactive as 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (IUPAC name: 6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one), 1,2-Benzisothiazol-3(2H)-one (IUPAC name: 1,2-benzothiazol-3-one), and Benzothiazole,2-(2-hydroxyethylthio)- (IUPAC name:

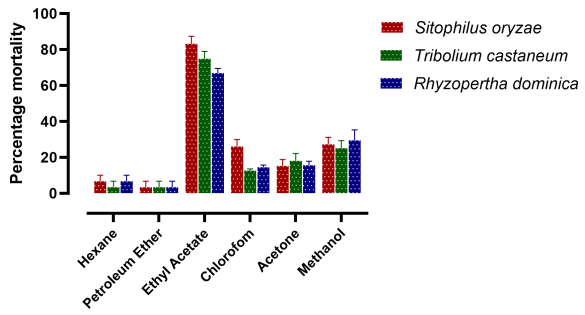

FIGURE 2
Fumigant toxicity of different extracts from *D. indica* leaves, at 50 mg/L, against *S. oryzae, T. castaneum* and *R. dominica*. (n= 4).

2-(1,3-benzothiazol-2-ylsulfanyl)ethanol) with reverse search index (RSI) value of 905, 861, and 941 respectively (Figure 3).

3.3 *In - vitro* inhibition of AChE enzyme

In an *in - vitro* study, we investigated the influence of the bioactive enriched fraction on the insect's acetylcholinesterase enzyme (AChE). The enriched fraction was found to significantly inhibit the AChE activity of *S. oryzae, T. castaneum*, and *R. dominica*. Table 2 reveals various percentage inhibitions of the AChE enzyme in different doses of the enriched fraction. The percentage inhibition of different doses of the enriched fraction was compared with Pyridostigmine (standard AChE inhibitor) at equal concentration. The percentage inhibition value ranges from 20.69% to 55.17%, 12.86% to 51.42%, and 26.28% to 66.67% for different doses with IC_{50} values of 88.57 µg/ml, 97.07 µg/ml, and 66.31 µg/ml on *S. oryzae, T. castaneum*, and *R. dominica*, respectively.

3.4 Effect of the enriched fraction on insect's antioxidant enzyme systems

In the present study, the effect of the enriched fraction on the activities of antioxidant enzymes in the tested insects, viz., *S. oryzae* and *T. castaneum*, were estimated. Figure 4 indicates that the active enriched fraction caused significant impairment in the enzymatic (SOD, Catalase, GST) as well as non-enzymatic (DPPH) antioxidant systems of both *S. oryzae*, and *T. castaneum*. The results showed a significant increase in the activity of SOD, Catalase, and GST. The percentage inhibition of the scavenging activity of DPPH was also found to increase as we increase the concentration of the active enriched fraction (Figure 4).

3.5 Seed germination test

The effect of the bioactive enriched fraction on percentage germination of wheat and green gram seeds were determined. It was found that both treatments i.e., 100 and 500 mg/l have no significant effect on the seed germination at different exposure periods. The percentage of seed germination ranged from 96% to 98.67% and 94.33% to 98.67% at different concentration and exposure time on both wheat and green gram, respectively (Table 3).

4 Discussion

Medicinal and aromatic plants are good sources for many biological activities. Several plant species are known to contain phytochemicals that can be used as insecticides (Rajendran and Sriranjini, 2008; Green et al., 2015; Devi et al., 2021). Natural insecticides are preferable to synthetic chemical-based insecticides because of their eco-friendly nature (Rajashekar et al., 2016; Samada and Tambunan, 2020). Though many plants have been studied for their insecticidal property, there are several more with huge potential yet unexplored. *D. indica* has several important biological properties as compiled and presented by Barua et al. (2018). But no significant scientific research has been done regarding its insecticidal property despite the claims that the plant is traditionally used by farmers to control stored grain pests in the Northeastern parts of India (Bhattacharjee and Ray, 2010). In the present study, we tried to provide scientific validation to those traditional approaches.

The present study revealed that ethyl acetate extract of *D. indica* leaves has maximum fumigant toxicity against *S. oryzae, T. castaneum*, and *R. dominica*. The LC_{50} values of most active enriched fraction were relatively lower than methyl bromide (MeBr), which is a commercially available grain fumigant, with LC_{50} values of 0.67 mg/L and 1.75 mg/L against *Sitophilus zeamais* Motschulsky and *T. castaneum* adults respectively (Liu and Ho,

TABLE 1 Insecticidal activity of the enriched fraction isolated from ethyl acetate extract of *D. indica* leaves against *S. oryzae, T. castaneum* and *R. dominica* by using fumigant assay method. (n= 4).

Insects	LC_{50} value [a,b]	Slope ± SE	Chi square	Degree of freedom
S. oryzae	101.88 ± 10.46	0.1446 ± 0.029 (0.077 – 0.211)	1.22	3
T. castaneum	198.89 ± 15.9	0.1674 ± 0.020 (0.12 – 0.21)	1.67	3
R. dominica	115.1 ± 8.63	0.148± 0.023 (0.096 – 0.2)	1.25	3

[a]LC_{50}= µg/L air.
[b]Values in parenthesis represent confidence limits by probit analysis (Finney, 1971), n=4.

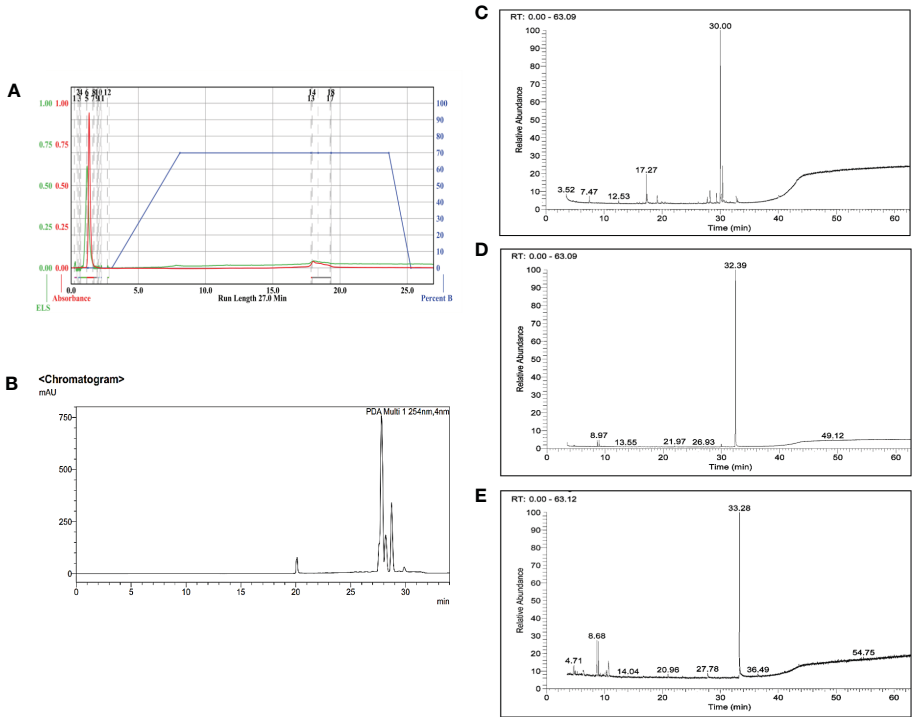

FIGURE 3
Isolation and characterization of the bioactive markers from the enriched fraction **(A)**. Chromatogram from Flash chromatography, **(B)**. Chromatogram from Semi- preparative HPLC, and **(C–E)** Representative GC-MS ion chromatogram of the three isolated bioactive compounds, 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (commonly known as loliolide), 1,2-Benzisothiazol-3(2H)-one, and Benzothiazole,2-(2-hydroxyethylthio)- respectively.

1999). Several similar experiments have been performed on different plants. In a study, Rajashekar et al., 2012 reveal that Coumaran, a natural fumigant isolated from the methanolic extract of leaves of *Lantana camara* (Verbenaceae) has fumigant toxicity against *S. oryzae*, *Callosobruchus chinensis* (Fab.) and *T. castaneum* with LC_{50} values of 0.45 µg/L, 0.38 µg/L, and 0.54 µg/L respectively. The fumigant property of different solvent extracts of *Illicium verum* Hook. f. against *S. zeamais* adults was also reported with the LD_{50} values of the methanol, ethyl acetate, and petroleum ether extracts treatment 7.10, 3.93, and 4.55 mg/l, respectively after 72 h exposure (Li et al., 2013). Essential oils from several aromatic plants also showed fumigant activity. In a study, Devi et al. (2020), reported that essential oils from *Cymbopogon flexuosus* Nees ex Steud. Wats (Poales: Poaceae), *Cymbopogon winterianus* Jowitt ex Bor (Poales: Poaceae), *Cymbopogon martini* Roxb. Wats (Poales: Poaceae), and *Pogostemon cablin* Blanco Benth. (Lamiales: Lamiaceae) exhibited fumigant properties against *S. oryzae*. Essential oils of the Chinese medicinal herb, *Blumea balsamifera* (L.) (Asteraceae) leaves contain 8-cineole, 4-terpineol, and α-terpineol as their main components and were reported to show distinct fumigant toxicity against *S. zeamais* adults (Chu et al., 2013).

The three identified bioactive marker compounds have low molecular weight of 196, 151, and 211 for 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (commonly called as loliolide), 1,2-Benzisothiazol-3(2H)-one, and Benzothiazole,

TABLE 2 Percentage inhibition of enriched fraction on AChE enzyme activity.

Concentration (µg/ml)	Percentage inhibition of AChE enzyme					
	S. oryzae		*T. castaneum*		*R. dominica*	
	Pyridostigmine	Enriched fraction	Pyridostigmine	Enriched fraction	Pyridostigmine	Enriched fraction
25 µg/ml	58.04 ± 1.1^{a}	20.69 ± 1.7^{a}	48.57 ± 2.4^{a}	12.86 ± 1.4^{a}	56.41 ± 2.5^{a}	26.28 ± 2.8^{a}
50 µg/ml	72.99 ± 2.3^{b}	29.89 ± 2.3^{b}	65.71 ± 1.9^{b}	30 ± 3.8^{b}	66.67 ± 1.2^{b}	37.18 ± 1.3^{b}
75 µg/ml	83.33 ± 2.0^{c}	45.4 ± 1.2^{c}	72.85 ± 1.4^{c}	41.43 ± 1.4^{c}	79.48 ± 1.2^{c}	62.82 ± 2.7^{c}
100 µg/ml	94.02 ± 1.0^{d}	55.17 ± 1.5^{d}	88.57 ± 2.8^{d}	51.42 ± 1.4^{d}	92.30 ± 1.0^{d}	66.67 ± 2.6^{d}

Data are given as Mean ± SEM (n=3). Values followed by different letters within the vertical columns are significantly different (P< 0.05) by Duncan's multiple range test.

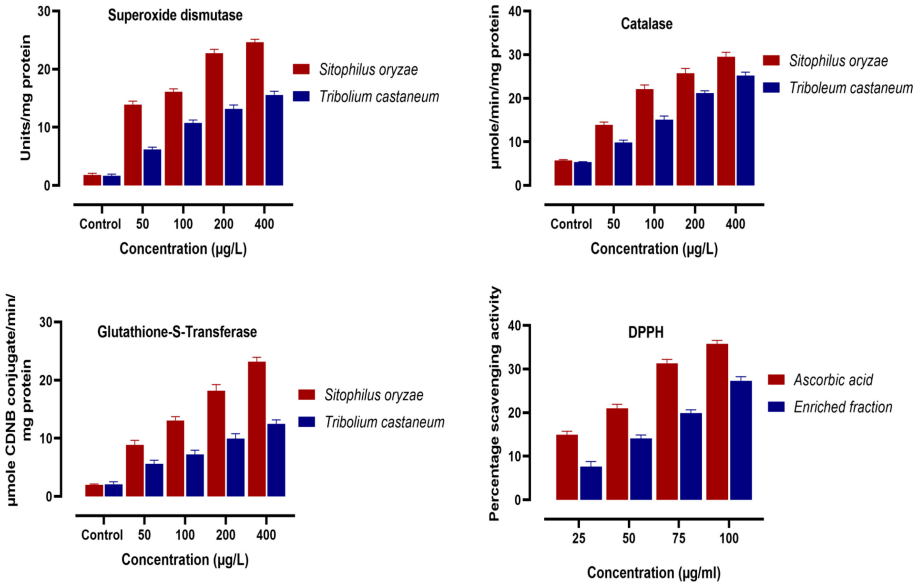

FIGURE 4
Effect of different concentrations of the enrich fraction eluted from ethyl acetate extract of *D. indica* leaves on the activity of enzymatic antioxidants in *S. oryzae*, and *T. castaneum*. (n=4).

2-(2-hydroxyethylthio)-, respectively, which supports its high volatile property. Dias et al. (2020) reported the antioxidant property of Loliolide isolated from *Sargassum horneri*. The mode of action study was mainly based on the changes in the insect's behavior when exposed to the fumigant in the fumigation chamber. In the fumigation toxicity experiment, it was observed that immediately after the treatment, the insects start moving rapidly. This may indicate that the extract is acting on the insect's nervous system like those of organophosphates. Acetylcholine (Ach) is the neurotransmitter that is involved in cholinergic transmission in the brain. AChE enzyme is responsible for the hydrolysis of the neurotransmitter ACh and it is required to rapidly terminate the signaling at neuron junction. Inhibition of the AChE enzyme will cause ACh to bind to the postsynaptic receptor for a longer period causing excessive neuroexcitation. This leads to restlessness, hyperexcitability, tremors, convulsions and paralysis leading to death (Lionetto et al., 2013; Shivanandappa and Rajashekar, 2014). Synthetic insecticides such as organophosphates and carbamates are known to inhibit the acetylcholinesterase enzyme. Therefore, the present study investigated the possible role of the AChE enzyme in the toxicity of the active enriched fraction. The experimental data indicate that the AChE inhibition potential of the enriched fraction was relatively lower than that of the standard. Similar studies have been done by many researchers. In one of the studies, Rajkumar et al., 2019 revealed that the essential oils of *Mentha piperita* L. inhibited the AChE enzyme activity in *S. oryzae*, and *T. castaneum* with LC_{50} values of 29.68%. In a similar study the essential oils of *Ocimum tenuiflorum* (L.) (Lamiales: Lamiaceae) exhibited insecticidal activity *via* inhibiting acetylcholinesterase activity against rice weevil (Bhavya et al., 2018). Coumaran is an active ingredient extracted from *L. camara* which has an inhibitory effect on the insect's AChE enzyme (Rajashekar et al., 2014).

Antioxidant enzyme system provides the primary defense mechanism of a biological system. Any oxidative imbalance could be detrimental to the normal functioning of many metabolic pathways (FontagneÂ-Dicharry et al., 2014). Free radicals or other reactive oxygen species (ROS) are products of normal metabolism or a result of exposure to any external sources such as rays, ozone, cigarette smoking, certain drugs, pesticides, air pollutants, and industrial chemicals (Sule et al., 2022). Oxidative stress is caused due to the imbalance between the

TABLE 3 Effect of the enriched fraction on seed germination of wheat and green gram.

Dosage (mg/l)	Percentage seed germination			
	48 h		5 d	
	Wheat	Green gram	Wheat	Green gram
100	97.3 ± 0.6^{a}	95.33 ± 1.3^{a}	98.67 ± 1.2^{a}	98.67 ± 1.3^{a}
500	96 ± 1.2^{a}	94.33 ± 1.2^{a}	96.67 ± 0.6a	97.33 ± 1.7^{a}
Control	99.33 ± 0.6^{a}	98.67 ± 0.6^{a}	99.33 ± 0.6^{a}	98.67 ± 1.3^{a}

Data are given as Mean ± SEM (n=4). Values followed by same letter within the vertical columns are not significantly different (P< 0.05) by Duncan's multiple range test.

production of free radicals and antioxidant defense in the body. This may lead to chronic and permanent damage to the cell. The bioactive enriched fraction may have caused the production of more free radicals that in turn triggered the system to produce more antioxidant enzymes. This causes a significant increase in the enzymatic activity (SOD, Catalase, GST) as well as non-enzymatic (DPPH) antioxidant systems of both *S. oryzae*, and *T. castaneum* (Figure 4). The superoxide dismutase enzyme is responsible for the removal of toxic ROS with the formation of less toxic hydrogen peroxide and oxygen molecules. Catalase enzymes further detoxify the hydrogen peroxide forming non-toxic water and oxygen molecule (Felton and Summers, 1995; El-Amier et al., 2019). Reduced glutathione (GSH) is a non-enzymatic antioxidant that detoxifies the xenobiotics either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS) and electrophiles or by operating as a cofactor for various enzymes (Lushchak 2012). Glutathione-S-transferase is the enzyme that catalyzes the oxidation of GSH to form oxidized glutathione (GSSH). An increase in GST activity indicated that more of the free radicals have been detoxified. The increased activities of these antioxidant enzymes were dose-dependent (Figure 4).

The important characteristic of natural antioxidants is their ability to scavenge free radicals. Proton-radical scavenging action is an important attribute of antioxidants, which is measured by the DPPH radical scavenging assay. DPPH, a protonated radical, has a characteristic absorbance maximum at 517 nm which decreases in the presence of antioxidants due to the scavenging of the proton radical (Yamaguchi 1998). In our study, the enriched fraction was screened for DPPH radical scavenging activity. *In-vitro* experiment results revealed that the enriched fraction isolated from the ethyl acetate extract of *D. indica* leaves had relatively lower free radical scavenging activity when compared with standard ascorbic acid. The percentage scavenging activity was found to increase as we increase the concentration of the fraction (Figure 4).

In addition, the present study also showed that the enriched fraction has no significant effect on the seed germination of wheat and green gram. This result is desirable as grain protectants should not have any adverse effect on seed germination. In our previous study, we evaluated the mammalian toxicity of the enriched fraction on both male and female BALB/c mice through acute and sub-acute toxicity and revealed no-observed-adverse-effect level (NOAEL) in the experimental BALB/c mice (Singh et al., 2022). This suggested that the enriched fraction of *D. indica* leaves is significantly safer when compared to other commercially available synthetic fumigants.

5 Conclusion

In the present study, for the first time, we have reported the fumigant activity of *D. indica* against *S. oryzae, T. castaneum* and *R. dominica*. The bioactive enriched fraction isolated from ethyl acetate extract of *D. indica* leaves affected the AChE enzyme thereby causing hyperexcitation of the nerve impulse causing paralysis which eventually leads to the death of the insects. The bioactive enriched fraction also causes oxidative imbalance which greatly affects the normal functioning of many metabolic pathways. Three bioactive marker compounds were identified from the enriched fraction, i.e. 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (commonly known as loliolide), 1,2-Benzisothiazol-3(2H)-one, and Benzothiazole,2-(2-hydroxyethylthio)-. Our research finding showed that *D. indica* potentially offers a solution to problems associated with health risks, availability, costs, and resistance as in the case of synthetic pesticides. However, further research is needed to identify the bioactive marker compounds, along with its mammalian toxicity to ensure the safety of human and other non-target mammals. Finally, we concluded that *D. indica* could be used as a source of insecticides from plant origin and could be a viable alternative to synthetic insecticides.

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

KS: conceptualization, validation, formal analysis, investigation, and writing - original draft. AK: manuscript preparation and review. RB: conceptualization, methodology, formal analysis, and review - original draft. YR: project administration, conceptualization, funding acquisition, resources, methodology, supervision, formal analysis, writing - original draft, writing - review and editing, and visualization. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1135946/full#supplementary-material

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Toxicity, baseline of susceptibility, detoxifying mechanism and sublethal effects of chlorogenic acid, a potential botanical insecticide, on *Bemisia tabaci*

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Bemisia tabaci is a threat to agriculture worldwide because of its potential to cause devastating damage to crops. Chlorogenic acid is a bioactive pesticidal phytochemical agent against various insect pests. We here determined the susceptibility of a laboratory strain of *B. tabaci* to chlorogenic acid and other popular insecticides, and the susceptibility of several field-collected populations to chlorogenic acid. Also, cross-resistance to four common insecticides was measured. Chlorogenic acid had the highest toxicity of all tested insecticides, and all the field-collected populations were susceptible to chlorogenic acid, and little cross-resistance was detected between chlorogenic acid and the other tested insecticides. Furthermore, analysis of enzyme activities and expression of P450 genes in *B. tabaci* after treatment with LC_{50} of chlorogenic acid suggested that enhanced P450 activity could be involved in chlorogenic acid detoxification. We subsequently evaluated sublethal effects of chlorogenic acid, and found that treatment with LC_{25} of chlorogenic acid prolonged duration of two developmental stages, reduced fecundity, and decreased survival rates of treated *B. tabaci* compared to untreated insects. Overall, these findings demonstrate strong toxicity and significant sublethal effects of chlorogenic acid on *B. tabaci*, and suggest that overexpression of P450 genes may be associated with chlorogenic acid detoxification.

KEYWORDS

***Bemisia tabaci*, chlorogenic acid, botanical insecticide, metabolic enzymes, cytochrome P450 monooxygenases, sublethal effects**

1 Introduction

Pest management is a necessary aspect of agricultural production. Chemical insecticides are a major pest control measure, and have thus been extensively applied against insect pests for decades, generally with high efficacy. However, this long-term application of chemical insecticides has had detrimental side effects, such as sublethal effects on non-target insects, high levels of chemical residues in the environment and the food web, and ecosystem destruction (Sharma et al., 2020). Bioinsecticides have been suggested as appropriate alternatives to chemical agents owing to their decreased toxicity, high biodegradability, excellent target specificity, and minimal adverse effects on non-target organisms (Wang S. et al., 2022). Plants have developed many environmental adaptations, including physiological alterations, to cope with herbivore attacks. Specialized metabolites are natural plant products that play important roles in safeguarding plants against insect pests; some such compounds have been screened for their potential as commercial pest management products (Divekar et al., 2022). For example, the alkaloid compound caffeine has insecticidal properties; it causes paralysis and intoxication by inhibiting herbivore phosphodiesterase activity, and is therefore regarded as a potential biopesticide (Hollingsworth et al., 2002). Development of botanically-derived pesticides may be a feasible and environmentally sustainable strategy of preventing insect damage to crops.

Several key types of phytochemicals, such as flavonoids and phenolics, have important functions in herbivore resistance (Yao et al., 2019; Xia et al., 2021). Plant phenolic metabolites including chlorogenic acid, tannic acid, and methyl jasmonate show toxicity against insect pests, adversely affecting key physiological processes (Kundu and Vadassery, 2019; Lin et al., 2021; Lin et al., 2022). Chlorogenic acid is reportedly associated with the phytochemical defenses of plants such as *Dendranthema grandiflora* and *Ipomoea batatas* against insect pests such as *Frankliniella occidentalis* and *Cylas formicarius*, respectively (Leiss et al., 2009; Liao et al., 2020). Recently, Wang et al. (2021) reported that chlorogenic acid content was greatly increased as a result of *Mythimna separate* feeding, and that chlorogenic acid displayed significant toxicity against *M. separate* larvae. In recent years, plant-derived pesticidal compounds have become a focus of research attention due to their safety and lack of general environmental toxicity. Chlorogenic acid is one potential botanical insecticide that is highly environmentally friendly compared to common synthetic insecticides.

Insect oxidase systems include cytochrome P450 monooxygenases (P450s), which are multifunctional biocatalysts with broad enzymatic activity on a variety of substrates. Metabolic detoxification is one of the common mechanisms of resistance to various xenobiotics, and P450s are critical in the detoxification of natural and synthetic toxins (Lu et al., 2020; Nauen et al., 2021). Insect exposure to xenobiotics such as pesticides and plant specialized metabolites can induce high expression of P450 genes; for example, in cotton bollworm, coumarin treatment up-regulates the P450 genes *CYP6B7*, *CYP6B6*, and *CYP6B2*, and decreases bollworm susceptibility to methomyl (Chen et al., 2018). Similarly, in *Spodoptera exigua*, gossypol treatment induces high expression of *CYP9A98* and *CYP6AB14*. It was recently reported that several concentrations of chlorogenic acid can induce expression of P450 genes in *M. separate*, and that three P450 genes in particular (*CYP321A7*, *CYP6B6*, and *CYP6B7-like*) may be responsible for detoxifying chlorogenic acid (Lin et al., 2022).

The whitefly *Bemisia tabaci* (Gennadius) is an agriculturally devastating insect pest with high genetic diversity that is distributed worldwide. It has been known to infest more than 600 host plant species, primarily feeding on the phloem (Wang et al., 2017). *B. tabaci* damages plants not only directly but also indirectly; it is capable of transmitting more than 100 different plant viruses through feeding (Wei et al., 2017). Extensive and long-term employment of various synthetic pesticides to control *B. tabaci* worldwide has led to increasing reports of insect resistance to these pesticides (Horowitz et al., 2020); it is thus urgent to identify an alternative that can be used to delay the development of insecticide resistance in an environmentally-friendly manner. In the present study, we confirmed the toxicity of chlorogenic acid in *B. tabaci*, determined the baseline susceptibility of field-sampled *B. tabaci* populations to chlorogenic acid and other pesticides, and assessed pesticide cross-resistance. We found that all field-sampled populations were highly susceptible to chlorogenic acid, and no cross-resistance to the other tested pesticides was observed. We then illustrated the biochemical mechanism of chlorogenic acid action by measuring the activities of glutathione S-transferase (GST), esterase (EST), and P450, and assayed the expression of related genes. Finally, we assessed the sublethal effects of chlorogenic acid on *B. tabaci*. In summary, this study describes the optimal use of chlorogenic acid against *B. tabaci* and lays the foundation for future research and development of chlorogenic acid as a novel botanical pesticide.

2 Materials and methods

2.1 Insects

B. tabaci strain MED-S was originally collected from damaged poinsettia plants (*Euphorbia pulcherrima* Wild. ex Klotz.) in Beijing, China in 2009 (Pan et al., 2011). Four populations of *B. tabaci* that were previously reported to be insecticide-resistant were tested for cross-resistance; these were an abamectin-resistant strain (XZ), an afidopyropen-resistant strain (HD-Afi), a cyantraniliprole-resistant strain (CYAN-R), and a flupyradifurone-resistant strain (FLU-SEL) (Wang et al., 2020a; Wang et al., 2020b; Wang et al., 2022b; Wang et al., 2022c). Populations of *B. tabaci* were collected from six Chinese provinces and tested for baseline susceptibility as previously described (Wang et al., 2022b). All field-collected populations were identified as Mediterranean (MED) cryptic species using a previously published method (Luo et al., 2002). Insects were initially fed on cotton plants *Gossypium hirsutum* (without pesticide exposure) under a 14/10 h light/dark photoperiod at 26 ± 2°C and 55 ± 5% relative humidity (RH). For all assays, adults that were 3 d old or younger were sampled at random; approximately equal numbers of male and female individuals were used.

2.2 Insecticides

All chemical agents tested were of analytical standard grade. Chlorogenic acid (Chemical Abstracts Service [CAS] #327-97-9), abamectin (CAS #71751-41-2), flupyradifurone (CAS #951659-40-8), cyantraniliprole (CAS #736994-63-1), imidacloprid (CAS #138261-41-3), thiamethoxam (CAS #153719-23-4), flonicamid (CAS #158062-67-0), acetamiprid (CAS #160430-64-8), clothianidin (CAS #210880-92-5), nitenpyram (CAS #150824-47-8), and dinotefuran (CAS #165252-70-0) were purchased from Sigma Aldrich (Shanghai, China). Afidopyropen (CAS #915972-17-7) and sulfoxaflor (CAS #946578-00-3) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

2.3 Toxicity of chlorogenic acid to *B. tabaci*

All bioassays were carried out on adult *B. tabaci* individuals using an artificial diet solution as described by Wang et al. (2023). Five separate working concentrations were made for each chemical agent with four replicates per concentration. Thirty to forty *B. tabaci* adults were sampled at random and introduced into a bioassay tube containing insecticide or artificial diet solution without any insecticides (the control), which constituted one replicate. After 96 h in the tube, *B. tabaci* were considered to be dead if they did not move even when touched with a fine-hair brush. Survival and death rates were then calculated and recorded.

2.4 Detoxifying enzyme gene expression and activity assays

Activities of three detoxifying enzymes (GST, EST, and P450) were measured as previously described (Wang et al., 2020a) with slight alterations. The median lethal concentration (LC_{50}) treatment comprised adults that survived treatment with the LC_{50} concentration for 96 h, and the control group was made of insects treated with the control for the same period of time. For each group, 200 mixed-sex *B. tabaci* individuals were sampled as one replicate. Three replicates were sampled for each of the three detoxifying enzymes. Protein content was measured using bovine serum albumin (BSA) as the standard with the method described by Bradford (1976). Based on previous publications regarding P450-mediated pesticide resistance in *B. tabaci* (Wang Q. et al., 2020; Zhou et al., 2020), expression levels of 12 detoxifying genes were measured *via* quantitative reverse transcription (qRT)-PCR as previously described (Wang et al., 2020a): CYP6CX1v1, CYP6CX3, CYP6CX4, CYP6CX5, CYP6CM1, CYP6DW2, CYP6DW3, CYP6DZ4, CYP6DZ7, CYP303A1, CYP4C64, and CYP4G68. Expression data were normalized using *TUB1α* and *EF-1α* as the internal control genes, and the results were conducted in terms of the $2^{-\triangle\triangle Ct}$ method (Pfaffl, 2001). Primer sequences are shown in Supplementary Table S1.

2.5 Sublethal effects of chlorogenic acid on *B. tabaci*

The 25% lethal concentration (LC_{25}) of chlorogenic acid was calculated based on the results of the assay described in Section 2.3. Several fitness parameters were then measured in *B. tabaci* in control and LC_{25}-treated groups. The experiments were carried out as previously described (Wang et al., 2020a) with slight alterations. Briefly, 12 clean cotton plants were evenly divided between two separate insect-proof cages (one control [CK] cage and one LC_{25}-treatment cage). After 96h feeding with LC_{25} or the control by the method of the bioassay, 120 adults of *B. tabaci* that were treated (LC_{25}) were then moved into the LC_{25} cage for egg laying measurements, and 120 untreated *B. tabaci* adults were moved into the CK cage as the control group. After 12 h of oviposition, all the plants were moved out of the two cages, and 10 leaves were recorded from each of the cages. In each of the 20 leaves, 10 eggs were left on each leaf and kept with one leaf clip-cage. All the spots of the eggs on the working leaves were marked, and the cages were placed in the chamber with the room temperature. Newly emerged adults of *B. tabaci* were put onto new leaves with clip cages for fecundity measurements that continued until all tested ones died, and after that the hatch rate of eggs was recorded.

2.6 Statistical analysis

Probit analysis was conducted in PoloPlus (2002) to confirm the significance of the death rate statistics for insects exposed to the series of working concentrations of chlorogenic acid. Resistance ratio (RR) was calculated as LC_{50} (field-collected population)/LC_{50} (MED-S), and levels of pesticide resistance is reported by the published method (Zhang et al., 2022). Specifically, susceptibility with the RR less than 5-fold, low level of resistance with RR from 5- to 10-fold, moderate level of resistance with RR from 10- to 40-fold, high level of resistance with RR from 40- to 160-fold, and very high level of resistance with RR over 160-fold. Significant differences in *B. tabaci* growth duration, viability, fecundity, duration of oviposition, and egg hatchability between the CK and treatment groups were assessed using Student's *t*-test. Differences in detoxifying enzyme activity and gene expression were also assessed with Student's *t*-test. All statistical analyses were conducted using SPSS (2011).

3 Results

3.1 Lethal effects of chlorogenic acid and popular insecticides on *B. tabaci*

The toxicity of chlorogenic acid and 11 other popular chemical agents were confirmed in the susceptible MED-S strain of *B. tabaci* using a feeding method as previously published (Wang et al., 2023) (Table 1). The death rate of the control group was < 5%. The

chemical agent with the highest lethal effect against *B. tabaci* adults was chlorogenic acid (LC_{50} = 0.930 mg/L), followed by cyantraniliprole (LC_{50} = 1.347 mg/L) and flonicamid (LC_{50} = 1.398 mg/L), which also showed excellent toxicity against *B. tabaci*. The other chemical agents had significantly lower toxicity than chlorogenic acid: 3.5 times lower for dinotefuran (LC_{50} = 3.259 mg/L), 3.9 times lower for clothianidin (LC_{50} = 3.656 mg/L), 4.6 times lower for acetamiprid (LC_{50} = 4.299 mg/L), 6.0 times lower for nitenpyram (LC_{50} = 5.574 mg/L), 8.2 times lower for afidopyropen (LC_{50} = 7.617 mg/L), 10.7 times lower for sulfoxaflor (LC_{50} = 9.950 mg/L), 11.3 times lower for flupyradifurone (LC_{50} = 10.495 mg/L), 12.3 times lower for thiamethoxam (LC_{50} = 11.449 mg/L), and 23.1 times lower for imidacloprid (LC_{50} = 21.489 mg/L).

3.2 Baseline susceptibility of field-collected *B. tabaci* to chlorogenic acid and other pesticides

We next tested the baseline chlorogenic acid susceptibility of 12 *B. tabaci* MED populations collected in the field and one laboratory-maintained susceptible strain (MED-S) (Figure 1 and Table S2). Of the field-collected strains, ZZ showed the highest susceptibility to chlorogenic acid (LC_{50} = 0.723 mg/L), whereas the MED-S strain displayed the highest susceptibility overall (LC_{50} = 0.962 mg/L). WQ had the lowest susceptibility to chlorogenic acid (LC_{50} = 3.306 mg/L), followed by TA (LC_{50} = 3.241 mg/L). The resistance ratios of all field-collected strains were less than five-fold different than that of the MED-S strain, indicating a lack of chlorogenic acid resistance in field populations. XZ was confirmed as an abamectin-resistant strain (41.6-fold resistance), HD-Afi was afidopyropen-resistant (174.9-fold resistance), CYAN-R was cyantraniliprole-resistant (99.3-fold resistance), and Flu-R was flupyradifurone-resistant (160.4-fold resistance). However, none of these strains showed chlorogenic acid resistance, suggesting that chlorogenic acid displayed little cross-resistance with these four other pesticides (Table 2).

3.3 Biochemical mechanism of *B. tabaci* response to chlorogenic acid treatment

Chlorogenic acid-treated and control *B. tabaci* were assayed to measure the activity of three detoxifying enzymes: P450, GST, and EST. Compared with the control group, P450 activity was significantly elevated (by 1.9-fold) in the group treated with the LC_{50} concentration of chlorogenic acid; GST and EST activities were increased compared to the control group by 1.3-fold and 1.1-fold, respectively, but the differences were not significant (Table 3). In the control and LC_{50} groups, expression patterns were also analyzed *via* qRT-PCR for 12 P450 genes that have previously been reported as involved in detoxification: CYP6CX1v1, CYP6CX3, CYP6CX4, CYP6CX5, CYP6CM1, CYP6DW2, CYP6DW3, CYP6DZ4, CYP6DZ7, CYP303A1, CYP4C64, and CYP4G68. In comparison to the control group, *CYP6CX3*, *CYP6CX4*, *CYP6DW3*, *CYP4C64*, and *CYP4G68* were significantly up-regulated in the treated insects by 1.9-fold, 2.1-fold, 1.9-fold, 2.4-fold, and 2.0-fold, respectively. In contrast, *CYP6DZ4* was significantly down-regulated (by 10.0-fold) in the LC_{50}-treated group (Figure 2).

3.4 Sublethal effects of chlorogenic acid on *B. tabaci*

In the work of cross-resistance, we assessed the lethality of various concentrations of chlorogenic acid in the MED-S strain, and as shown in the Table 2, LC_{50} value was 0.888 with the Slope ± SE was 1.192 ± 0.133, and X^2 (*df*) was 0.827 (3). Based on the calculation, the value of LC_{25} was 0.241 mg/L, and it was used for

TABLE 1 Median lethal concentration (LC_{50}) of chlorogenic acid and 11 popular insecticides on *Bemisia tabaci*.

Population	N [a]	LC_{50} (95% CL) (mg L^{-1}) [b]	Slope ± SE	X^2 (df)
Chlorogenic acid	614	0.930 (0.656 - 1.200)	1.134 ± 0.135	2.542 (3)
Cyantraniliprole	615	1.347 (1.083 - 1.631)	1.281 ± 0.132	1.036 (3)
Flupyradifurone	617	10.495 (8.248 - 12.794)	1.297 ± 0.135	2.758 (3)
Imidacloprid	621	21.489 (16.285 - 26.696)	1.261 ± 0.134	1.492 (3)
Thiamethoxam	619	11.449 (9.160 - 13.822)	1.347 ± 0.134	1.942 (3)
Sulfoxaflor	626	9.950 (8.373 - 11.815)	1.425 ± 0.131	2.534 (3)
Afidopyropen	612	7.617 (6.095 - 9.164)	1.430 ± 0.140	2.296 (3)
Flonicamid	610	1.398 (1.080 - 1.728)	1.188 ± 0.131	1.605 (3)
Acetamiprid	611	4.299 (3.425 - 5.322)	1.122 ± 0.128	1.188 (3)
Clothianidin	624	3.656 (2.730 - 4.728)	1.596 ± 0.137	3.130 (3)
Nitenpyram	611	5.574 (4.317 - 6.876)	1.203 ± 0.132	1.410 (3)
Dinotefuran	607	3.259 (2.590 - 4.310)	1.067 ± 0.129	1.319 (3)

[a]Number of insects used. [b] CL, confidence limit.

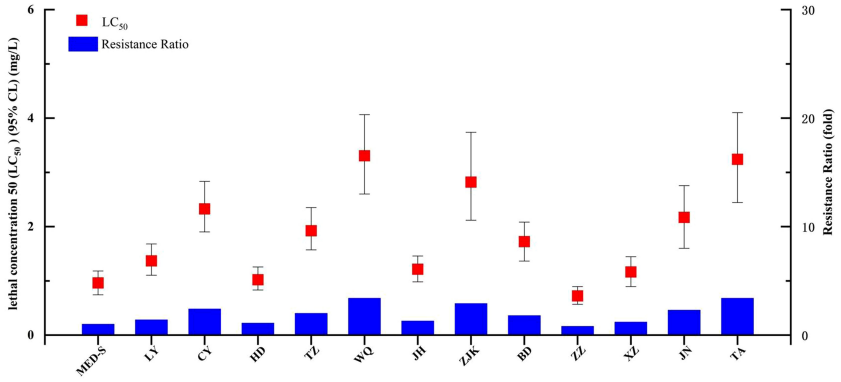

FIGURE 1
Susceptibility of *Bemisia tabaci* populations collected from fields in China to chlorogenic acid.

further evaluation of the sublethal effects of chlorogenic acid on *B. tabaci* development and reproduction. The results showed that treatment of *B. tabaci* adults with the LC_{25} dose significantly decreased the survival rates of F_1 progeny in the neonate to pseudopupae stage and in the pseudopupae to adult stage (Figure 3A). The F_1 progeny of treated insects also showed greatly extended durations of these two developmental stages (Figure 3B). Moreover, treatment with the LC_{25} dose greatly decreased fecundity in female whiteflies; treated females produced 110.93 ± 11.40 eggs each, compared to the 136.87 ± 9.89 eggs produced by females in the control group (Figure 4A). However, there were no significant differences in the duration of oviposition (Figure 4B), 11.57 ± 1.23 days in the treatment group vs. 12.92 ± 1.31 days in the control group, and also in egg hatchability (Figure 4C), 90.11 ± 2.18% in the treatment group vs. 91.93 ± 1.46% in the control group.

4 Discussion

Plant specialized metabolites are considered important candidate compounds in development of botanical insecticides as alternatives to conventional chemical pesticides. However, there is still a dearth of information regarding the bioactivity of botanical toxins against whiteflies. In the present study, we found that the specialized metabolite chlorogenic acid showed higher toxicity than 11 popular commercial insecticides against *B. tabaci* adults (laboratory strain MED-S). Using *B. tabaci* samples collected from the field, we then established the baseline susceptibility of 12 separate populations to chlorogenic acid and assessed cross-resistance to the pesticides abamectin, afidopyropen, cyantraniliprole, and flupyradifurone. All of the tested field-sampled populations were highly susceptible to chlorogenic acid, and chlorogenic acid showed little cross-resistance with abamectin,

TABLE 2 Cross-resistance of *Bemisia tabaci* against chlorogenic acid and four popular insecticides.

Insecticide	Strain	N [a]	LC_{50} (95% CL) (mg/L) [b]	Slope ± SE	$\chi 2$ (*df*)	RR [c]
Chlorogenic acid	MED-S	615	0.888 (0.663 - 1.114)	1.192 ± 0.133	0.827 (3)	
	XZ	615	1.573 (1.156 - 1.986)	1.218 ± 0.136	1.204 (3)	1.8
	HD-Afi	630	1.723 (1.219 - 2.218)	1.172 ± 0.135	1.823 (3)	1.9
	CYAN-R	619	1.011 (0.753 - 1.266)	1.257 ± 0.136	1.211 (3)	1.1
	FLU-SEL	611	2.381 (1.838 - 2.951)	1.155 ± 0.130	0.775 (3)	2.7
Abamectin	MED-S	622	0.10 (0.070 - 0.120)	1.427 ± 0.169	2.701 (3)	
	XZ	615	4.159 (3.130 - 5.180)	1.274 ± 0.137	0.950 (3)	41.6
Afidopyropen	MED-S	617	5.581 (4.140 - 7.075)	1.043 ± 0.128	1.007 (3)	
	HD-Afi	632	976.163 (779.027 - 1221.122)	1.067 ± 0.125	1.289 (3)	174.9
Cyantraniliprole	MED-S	610	1.071 (0.837 - 1.312)	1.266 ± 0.134	1.581 (3)	
	CYAN-R	609	106.402 (83.674 - 129.673)	1.311 ± 0.135	2.828 (3)	99.3
Flupyradifurone	MED-S	616	9.969 (6.770 - 13.134)	1.001 ± 0.130	2.946 (3)	
	FLU-SEL	623	1599.386 (1250.543 - 1957.451)	1.265 ± 0.132	1.140 (3)	160.4

[a]Number of insects used. [b] CL, confidence limit. [c] Resistance ratio (RR) = LC_{50}(strain XZ, HD-Afi, CYAN-R, or FLU-SEL)/LC_{50}(strain MED-S).

TABLE 3 Metabolic enzyme activity in control (CK) *Bemisis tabaci* individuals and those treated with the median lethal concentration (LC_{50}) of chlorogenic acid.[a].

Treatment	P450s activity		ESTs activity		GSTs activity	
	pmol min^{-1} mg^{-1}	Ratio [b]	nmol min^{-1} mg^{-1}	Ratio [b]	nmol min^{-1} mg^{-1}	Ratio [b]
CK	0.68 ± 0.16		311.06 ± 18.93		40.26 ± 10.31	
LC_{50}	1. 32 ± 0.24 *	1.9	329.75 ± 25.32	1.1	51.07 ± 11.12	1.3

[a]Mean activity values in a single column followed by an asterisk are significantly different ($p < 0.05$). [b] Ratio = activity in individuals treated with the LC_{50} dose/activity in CK individuals.

afidopyropen, cyantraniliprole, and flupyradifurone. Although there have been few previous investigations into the toxicity of chlorogenic acid against *B. tabaci*, chlorogenic acid reportedly exerts excellent lethal effects against various insect pests such as *M. separata*, *Hyphantria cunea*, and *Lymantria dispar* (Wang et al., 2014; Pan et al., 2020; Lin et al., 2022). These characteristics make chlorogenic acid a promising candidate botanical pesticide for use as a more environment-friendly option in field applications compared to synthetic insecticides.

Previous studies of *B. tabaci* have indicated that metabolic resistance to popular chemical agents involves increased activity of P450 enzymes and up-regulation of P450 genes (Zhou et al., 2020; Wang et al., 2020a; Wang et al., 2020c). Here, we selected 12 candidates of detoxifying P450 genes and measured expression levels after chlorogenic acid treatment. After treatment with the LC_{50} dose for 96 h, P450 enzyme activity was greatly induced; furthermore, five P450 genes were significantly up-regulated and one was down-regulated in comparison with the untreated control group. We thus concluded that those six genes (*CYP6CX3*, *CYP6CX4*, *CYP6DW3*, *CYP4C64*, *CYP4G68*, and *CYP6DZ4*) were involved in detoxifying chlorogenic acid. P450 genes have crucial detoxification functions in many insects; pesticide resistance relies primarily on xenobiotic metabolism *via* cytochrome (CY) P450s

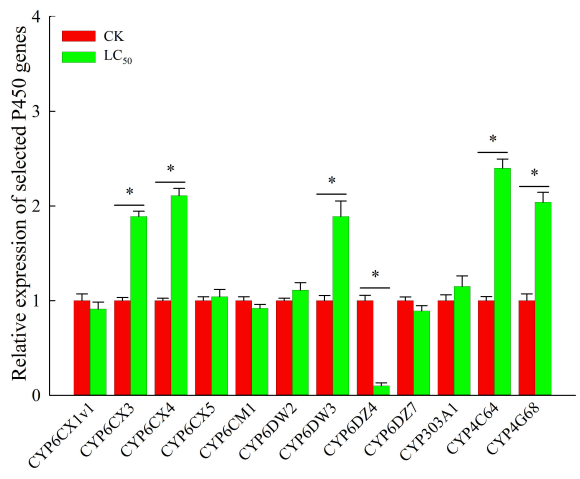

FIGURE 2
Expression profiles of 12 cytochrome P450 genes that may be involved in chlorogenic acid detoxification in *Bemisia tabaci* adults. Red, control (CK) individuals. Green, individuals treated with the median lethal concentration (LC_{50}) of chlorogenic acid. Values are presented as the mean ± standard error. *$p < 0.05$ (Student's *t*-test).

(Lu et al., 2020; Nauen et al., 2021). Similarly, phytochemicals can induce changes in the expression levels of detoxification-related P450 genes. For example, two P450 genes, *CYP4M14* and *CYP4L13*, are significantly up-regulated in *Spodoptera frugiperda* larvae after exposure to flavonoids and nicotine (Wang et al., 2022c). It was recently reported that chlorogenic acid can induce P450 enzyme activity and that the genes *CYP6B7-like*, *CYP321A7*, and *CYP6B6* are responsible for chlorogenic acid detoxification in *M. separata* (Lin et al., 2022). We therefore speculate that these genes, some of which were significantly up-regulated in *B. tabaci* after chlorogenic acid treatment, may be detoxification genes; furthermore, the insecticidal effects of chlorogenic acid against *B. tabaci* may be due to P450 gene suppression, preventing detoxification and thus resulting in insect death.

Plant-derived pesticides not only have lethal capacity, but also affect insect physiological functions such as behavior, viability, reproduction, and development at sub-lethal concentrations (Toledo et al., 2019; Piri et al., 2020). For example, treatment of *B. tabaci* with the LC_{25} dose of the phytochemical compound β-asarone can prolong the developmental duration, decrease viability, and significantly reduce the rate of reproduction (Wang et al., 2022a).We here found that treatment with the LC_{25} dose of chlorogenic acid had several effects on *B. tabaci*: it prolonged the duration of two developmental stages; decreased survival rates of nymphs, pseudopupae, and adults; and significantly decreased female fecundity. These results were consistent with those of previous publications, which have indicated that the duration of *M. separate* larval growth is significantly prolonged after treatment with the LC_{20} dose of chlorogenic acid (Lin et al., 2022). Moreover, in *Helicoverpa zea*, the developmental duration can be prolonged by exposure to caffeic acid and chlorogenic acid (Summers and Felton, 1994). These previous findings combined with the results of the present study indicate that chlorogenic acid can extend the duration of insect developmental stages, decrease rates of pupation and eclosion, and alter the sex ratio of populations and the fecundity of females; chlorogenic acid thus negatively affects development and reproduction of multiple insect pests.

In conclusion, we found that chlorogenic acid displays excellent lethal effect on *B. tabaci* in the both lab-rear strain and field-collected populations. No cross-resistance to four popular insecticides, and five P450 genes that may be involved in the detoxification process was identified in the work. Moreover, it is important to clarify the sublethal effects of a pesticidal agent as part of an overall assessment of its suitability for field applications. The present study reveals novel insights into the sublethal effects of

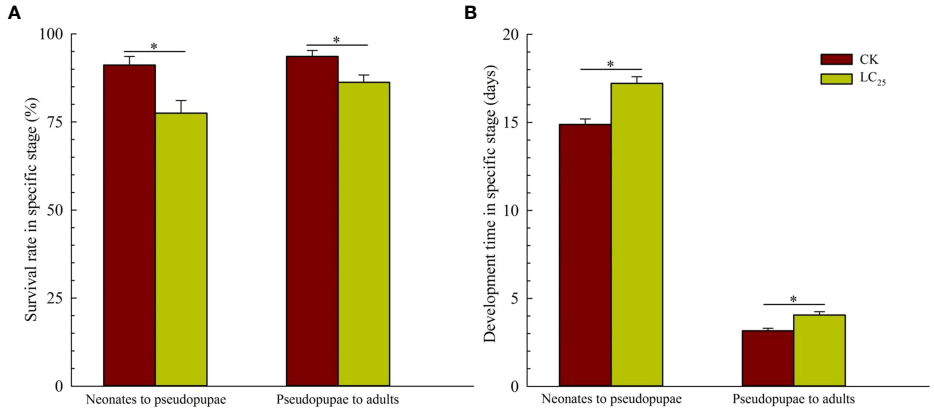

FIGURE 3
Survival rates **(A)** and developmental stage durations **(B)** in the F_1 generation of *Bemisia tabaci*. Dark red, control (CK) individuals. Gold, individuals treated with the 25% lethal concentration (LC_{25}) of chlorogenic acid. Values are presented as the mean ± standard error. *$p < 0.05$ (Student's *t*-test).

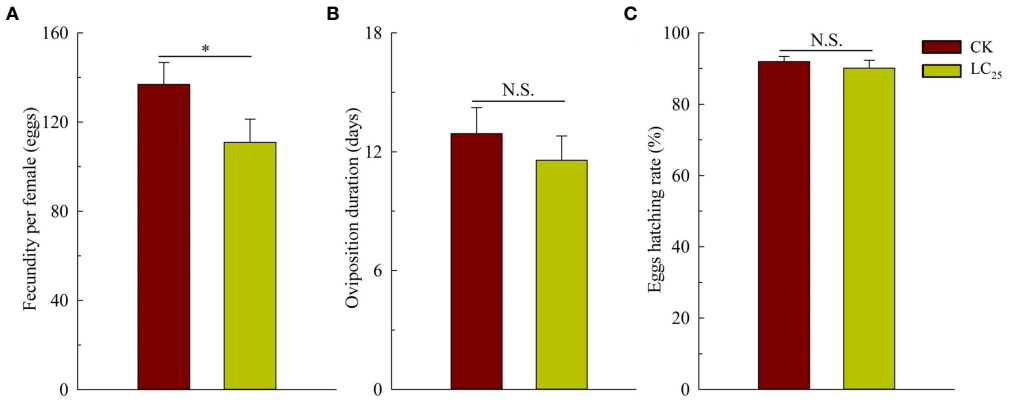

FIGURE 4
Fecundity **(A)**, oviposition duration **(B)**, and egg hatching rate **(C)** of the F_1 generation of *Bemisia tabaci*. Dark red, control (CK) individuals. Gold, individuals treated with the 25% lethal concentration (LC_{25}) of chlorogenic acid. Values are presented as the mean ± standard error. *$p < 0.05$ and n.s. indicates not significant ($p > 0.05$) (Student's t-test).

chlorogenic acid on whiteflies, promoting efficacious use of this compound and contributing to decreased whitefly management costs and crop yield losses due to herbivory.

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 authors.

Author contributions

Conceptualization, RW, JW and CL. Methodology, RW and QZ. Software, QZ. Validation, CQ. Formal analysis, QW. Investigation, RW, QZ and JW. Resources, CQ. Data curation, QW. Writing—Original draft preparation, RW. Writing—review and editing, RW, JW and CL. Visualization, RW. Supervision, RW, JW and CL. Project administration, RW and CL. Funding acquisition, RW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1150853/full#supplementary-material

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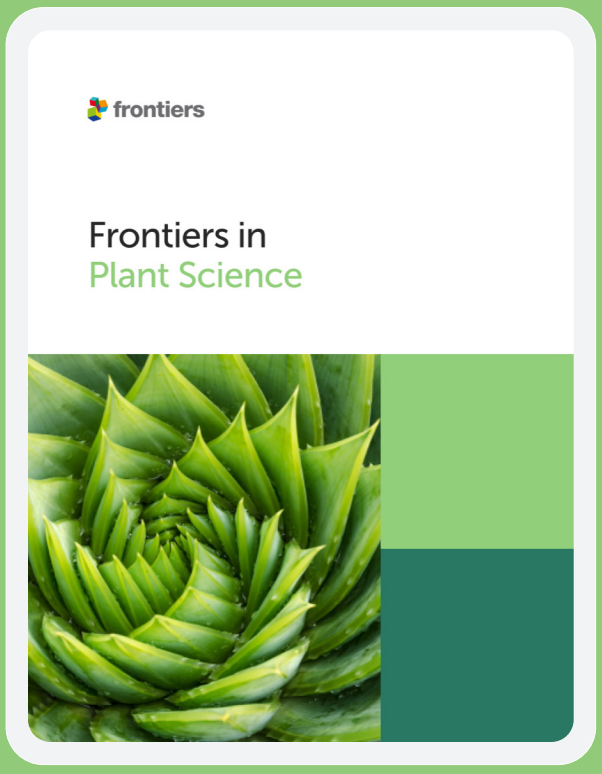

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