ACCELERATING GENETIC GAINS IN PULSES

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ACCELERATING GENETIC GAINS IN PULSES

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EDITORIAL
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Editorial: Accelerating Genetic Gains in Pulses

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Editorial on the Research Topic

Accelerating Genetic Gains in Pulses

Legumes, members of the Fabaceae/Leguminosae family, are the third largest family of higher plants with almost 20,000 species belonging to 650 genera, and are ubiquitous all over the world. Among all legumes, pulse crops or food legumes fall into the four clades of the subfamily Papilionoideae which include aeschynomenoids/dalbergiods, genistoids, hologalegina, and phaseoloids/millettoids. They are distinctive due to their positive impact on agricultural and environmental sustainability and have a prominent role in promoting human and animal health, soil amelioration, cropping system diversification, and sustenance of rural livelihoods (Pratap et al., 2021a). These also provide protein isolates that are increasingly being used in the food industry as functional ingredients suitable for vegan diets (Robinson et al., 2019). The inclusion of pulses in rotation with cereals helps to improve system yields, enhance net carbon sequestration, and lower the carbon footprint. Nonetheless, in addition to being an excellent source of protein, starch, and micronutrients, pulses also contain anti-nutritional compounds that can interfere with the absorption of minerals (Moore et al., 2018) and also the digestion of protein (Clemente et al., 2015).

Realizing their importance, significant research has been dedicated to their genetic amelioration, thereby turning them into mainstream crops from so-called "orphan legumes". Classical plant breeding methods led to the development of more than 3,800 improved varieties of different pulse crops globally, with improved attributes of grain yield, crop duration, stress resistance, nutrition quality, etc. However, despite this effort, the increase in average pulse yields (from 637 to 1,009 kg/ha) has been modest compared to dramatic increases in cereal productivity (from 1,353 to 4,074 kg/ha) between 1961 and 2017 (Kumar et al., 2020). Among legumes, Koester et al. (2014) studied 80 years of historical data of soybean breeding at the Crop Research and Education Center in Urbana, USA and reported a genetic gain of 26.5 kg ha^{-1} $year^{-1}$, attributing the gain in grain yield to increases in light interception, energy conversion, and partitioning efficiencies. Productivity gains in pulses have been recorded when especially considered along with the markedly reduced duration of the improved varieties, leading to increased cropping intensity, while genetic gains have been recorded for traits imparting resistance to major biotic and abiotic stresses, herbicide tolerance, larger seeds, and improved nutritional quality. This resulted in the growth, in terms of production and productivity, in major pulse-producing countries. For example, India witnessed the highest growth in production in mung bean (178%), followed by chickpea (125%), urdbean (90%), pigeonpea (51%), and lentil (34%) in the last 15 years (Gaur, 2021). Notably, breeding in most pulses has remained confined to the exploitation of genetic variation within the primary gene pool, which has resulted in a narrow genetic base in most of them.

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Therefore, there is a need to focus on exploiting the genetic and genomic resources made available through draft genome sequences, high-throughput genotyping and phenotyping tools, data management services, and bioinformatics resources. The need to make use of available information in different pulses provided the impetus for this Research Topic.

Globally, chickpea (*Cicer arietinum* L.) is the second largest pulse crop, cultivated by smallholder farmers in more than 50 countries. In recent years, remarkable progress was made in developing novel genomic tools in chickpea including the draft genome sequence (Varshney et al., 2018), millions of single nucleotide polymorphism (SNP) markers (Thudi et al., 2016; Varshney et al., 2019), and cost-effective genotyping platforms including low- to high-density SNP arrays (Roorkiwal et al., 2020). Likewise, quantitative trait loci (QTLs) and markers associated with abiotic and biotic stresses were also identified which facilitated development of superior cultivars through marker-assisted breeding. Thudi et al. reported novel genetic loci associated with root morphological traits, as well as phosphorus-acquisition and use efficiency in chickpea through genome-wide association mapping. They reported an SNP locus (Ca1_12310101) on Ca1 associated with physiological P-use efficiency, shoot dry weight, and shoot P content. They also identified genes related to shoot P concentration, physiological P-use efficiency, specific root length, and manganese concentration in mature leaves. Jha et al. identified major QTLs and potential candidate genes for heat stress tolerance and reported a genomic region on CaLG07 harboring QTLs explaining > 30% of the phenotypic variation for days to pod initiation, 100-seed weight, and for nitrogen balance index explaining > 10% PVE.

The MutMap approach targets discovery of mutant genes for assessing gene function and is based on BSA analysis of mutant progenies obtained in the/an F_2 population (Etherington et al., 2014). It relies on the cross between the mutant and its wild-type, and thereby directly targets the causal SNPs generated during mutagenesis which are responsible for phenotypic behavior (Tribhuvan et al., 2018). Manchikatla et al. reported development of markers associated with early flowering and enhanced seed size in chickpea through the MutMap approach. They identified a single unique genomic region on Ca6 (between 9.76 and 12.96 Mb) harboring 31, 22, 17, and 32 SNPs with a peak of SNP index = 1 for low bulk for flowering time, high bulk for flowering time, high bulk for 100-seed weight (HSW), and low bulk for HSW, respectively. They developed two markers, viz., Ca6EF10509893 for early flowering and Ca6HSDW10099486 for HSW, and validated them using the candidate SNPs.

Madurapperumage et al. discussed chickpea as a source of essential fatty acids and focused on plant lipids, their functions, and benefits for human health. They reviewed the chemical analysis of essential fatty acids and possible breeding targets to enrich them which could be possible by phenotyping diverse chickpea germplasm; candidate genes responsible for quantitative trait loci mapping using genome-wide association mapping were identified.

Crop wild relatives, landraces, and exotic germplasm are highly useful for introgression of novel variation to widen the genetic base of the elite gene pool leading to incremental gains over the breeding cycles (Pratap et al., 2021a). They also harbor positive QTLs for improving agronomic traits. Toker et al. reported a new *Cicer* species proposed as *Cicer turcicum* and explained its potential to improve various traits in chickpea including heat tolerance and bruchid resistance.

The breeding cycle usually takes 7–10 years for development of a new cultivar depending upon various factors (Kumar et al., 2020). Speed breeding can improve genetic gains in crop improvement programs by increasing the number of plant generations in a year, subsequently reducing the length of the breeding cycle (Chiurugwi et al., 2019). Fang et al. demonstrated a cost-saving speed-breeding methodology in soybean integrating an off-site nursery, a fresh-seeding method, and marker-assisted selection. Using the above combination they could obtain at least four generations in a year against one achieved through conventional methods. Croser et al. demonstrated the effectiveness of collaborative breeding efforts toward deployment of innovative breeding technologies for developing and rapidly introgressing imidazolinone group 2 herbicide tolerance into an Australian *desi* chickpea cultivar. They elaborated that their inter-institutional collaborative efforts could save a period of 3 years.

Root systems have an important role in water and nutrient acquisition, and plant root system architecture (RSA) directly controls the plant health and survival (Sozzani and Iyer-Pascuzzi, 2014). *In situ* methods are available to facilitate non-destructive spatial and temporal investigations into root systems grown in soil. Mung bean [*Vigna radiata* (L.) Wilczek] is a short duration and widely adaptable pulse crop known for its easily digestible high-quality protein (Pratap et al., 2021b), and high initial growth vigor which warrants study for the underlying mechanisms of faster water and nutrient uptake. Singh and Bell reported genotypic variability for RSA in mung bean and their physiological relationships with shoot growth dynamics. Early maturing varieties exhibited rapid root elongation rates and leaf area development. This resulted in more vigorous root and shoot growth during initial crop stages with the early varieties recording deeper, longer, and lighter roots. Rohilla et al. reported 10 marker-trait associations in mung bean significant for yellow mosaic disease, caused by mung bean yellow mosaic India virus, and four seed yield-related traits, viz., days to flowering, days to maturity, plant height, and number of pods per plant. They grouped different genotypes into three major clusters and three genetically distinct sub-populations with one admixture sub-population.

Sadras et al. quantified the genetic gain in lentil yield in the last three decades in Australia and observed the variation in the expression of genetic gain with the environment. They reported that yield did not increase in farmers' fields above the level of 1.2 t ha^{-1} over three decades; this could be attributed to non-mutually exclusive reasons including lack of genetic gain in yield, lack of progress in agronomic practices, lesser adoption of superior technologies, and expansion of lentil to low-yielding environments. Tripathi et al. reported

development of a core set of lentil germplasm comprising 170 accessions (137 Indian and 33 exotic) from the Indian gene bank which could be efficiently deployed in lentil improvement programs.

Minor pulses hold tremendous nutritional significance although these are produced and consumed locally and there is generally no exchange of materials between countries unless they are already cultivating and consuming these minor pulses (Ahlawat et al., 2016). Among the minor pulses, faba bean (*Vicia faba* L.) is the fourth most important cool season legume consumed locally in Asia, America, and Mediterranean countries. The genome information is scarce in this crop mainly due to the intrinsic difficulties of assembling/annotating its big genome size of 13 Gb (Maalouf et al., 2021). Adhikari et al. reviewed the development and role of conventional and molecular breeding tools for accelerating genetic gain in faba bean and suggested that the availability and use of DNA markers such as vicine-convicine (vc^-) and herbicide tolerance in breeding programs have encouraged breeders and given confidence in marker-assisted selection. Closely linked QTLs for biotic and abiotic stress tolerance are available and could be used to enhance the efficiency of the selection process. Omomowo and Babalola reviewed the constraints and prospects of improving cowpea productivity to ensure food and nutritional security and environmental sustainability, taking a special interest in Africa. They elaborated on recommended methods to achieve extreme growth in productivity. Chu et al. elucidated the role of *VaSDC1* in modulation of flavonoid metabolic pathways in seed coat color in adzuki bean (*Vigna angularis* L.) and genetically mapped it in the interval between simple-sequence repeat (SSR) markers *Sca326-12*, *Sca326-4*, and *BAgs007* on chromosome 3 using an F_4 population.

Of late, there has been a surge in the demand for plant-based proteins globally, even in those countries who have not normally been large consumers of pulses. This warrants a focus on strategies leading to a significant production improvement of pulses and their nutritional quality enhancement. Overall, the 14 articles published in this special issue reported new innovations/contributions toward genetic improvement of pulse crops and the knowledge gained could be further deployed toward development of new superior lines leading to improved genetic gains. The articles also highlight development of new markers, useful marker trait associations, new useful species, etc. Nonetheless, it is also evident that strategically important minor legumes still lag behind other pulses and require a research impetus toward development of new genomic information and deployment of molecular tools for their improvement. Extensive studies are required to quantify precise genetic gains in pulses with respect to yield and nutrition traits which will help in developing strategies for targeted breeding.

AUTHOR CONTRIBUTIONS

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

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Cicer turcicum: A New *Cicer* Species and Its Potential to Improve Chickpea

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Genetic resources of the genus *Cicer* L. are not only limited when compared to other important food legumes and major cereal crops but also, they include several endemic species with endangered status based on the criteria of the International Union for Conservation of Nature. The chief threats to endemic and endangered *Cicer* species are over-grazing and habitat change in their natural environments driven by climate changes. During a collection mission in east and south-east Anatolia (Turkey), a new *Cicer* species was discovered, proposed here as *C. turcicum* Toker, Berger & Gokturk. Here, we describe the morphological characteristics, images, and ecology of the species, and present preliminary evidence of its potential utility for chickpea improvement. *C. turcicum* is an annual species, endemic to southeast Anatolia and to date has only been located in a single population distant from any other known annual *Cicer* species. It belongs to section *Cicer* M. Pop. of the subgenus *Pseudononis* M. Pop. of the genus *Cicer* L. (Fabaceae) and on the basis of internal transcribed spacer (ITS) sequence similarity appears to be a sister species of *C. reticulatum* Ladiz. and *C. echinospermum* P.H. Davis, both of which are inter-fertile with domestic chickpea (*C. arietinum* L.). With the addition of *C. turcicum*, the genus *Cicer* now comprises 10 annual and 36 perennial species. As a preliminary evaluation of its potential for chickpea improvement two accessions of *C. turcicum* were field screened for reproductive heat tolerance and seeds were tested for bruchid resistance alongside a representative group of wild and domestic annual *Cicer* species. *C. turcicum* expressed the highest heat tolerance and similar bruchid resistance as *C. judaicum* Boiss. and *C. pinnatifidum* Juab. & Spach, neither of which are in the primary genepool of domestic chickpea. Given that *C. arietinum* and *C. reticulatum* returned the lowest and the second lowest tolerance and resistance scores, *C. turcicum* may hold much potential for chickpea improvement if its close relatedness supports interspecific hybridization with the cultigen. Crossing experiments are currently underway to explore this question.

Keywords: *Cicer*, new species, genetic resources, heat tolerance, bruchid resistance

HIGHLIGHTS

- We found that a new species endemic to East Anatolia, Turkey, which we have described and illustrated.
- The new species belongs to the same group with *C. arietinum* L., *C. reticulatum* Ladiz., and *C. echinospermum* P.H. Davis in the genus *Cicer* L. (Fabaceae) according to ITS sequencing.
- Based on preliminary studies, *C. turcicum* is tolerant to some abiotic and biotic stresses including heat, and bruchid that could be used in interspecific crosses to improve domesticated chickpea.

INTRODUCTION

The genus *Cicer* L. has a Rand Distribution, with a center of diversity scattered around the fringes of Africa as the continent has dried over the past few million years (Pokorny et al., 2015). *Cicer* species are from the Atlas Mountains and Canary Islands, in the Ethiopian highlands, to the Balkans and Caucasia, and into South and Central Asia. The richest density of *Cicer* species occur in the Anatolia-Turanian phytogeographic region (van der Maesen, 1972). The genus, despite earlier classifying in the tribe Vicieae Alefeld (1859), has been classified in its own tribe, Cicereae Alef. (Kupicha, 1977; Nozzolillo, 1985; van der Maesen, 1987). In a *Cicer* monograph, van der Maesen (1972) recognized 39 *Cicer* species including 31 perennials and eight annuals, including domesticated chickpea (*Cicer arietinum* L.). Since 1972 to 2007, the following *Cicer* species including *C. heterophyllum* Contandr., Pamukc. & Quezel (Contandriopoulos et al., 1972) from Mediterranean region of Turkey, *C. reticulatum* Ladiz. (Ladizinsky, 1975) from south eastern Turkey, *C. canariense* A. Santos & G.P. Lewis from the Canary Islands (Santos-Guerra and Lewis, 1986), *C. rassuloviae* Linczevski (Czrepanov, 1981), *C. laetum* Rassulova & Sharipova (Rassulova and Sharipova, 1992), and *C. tragacanthoides* Jaubert & Spach var. *turcomanicum* Popov from Turanian region were added to the genus (van der Maesen, 1984; van der Maesen et al., 2007). *C. uludereensis* Donmez (2011), *C. floribundum* Fenzl. var. *amanicola* M. Ozturk & A. Duran, *C. heterophyllum* Contandr., Pamukc. & Quezel var. *kassianum* M. Ozturk & A. Duran and *C. incisum* (Willd.) K. Maly subsp. *serpentinica* M. Ozturk & A. Duran were more recently added as new perennial *Cicer* taxa (Ozturk et al., 2011, 2013). Throughout that period only a single new annual wild *Cicer* species was added. *C. reticulatum,* now considered the wild progenitor of domesticated chickpea, was discovered in Dereici, Savur district, Mardin province, Turkey by Ladizinsky (1975). As a result of these discoveries, by 2020 the number of species in the genus *Cicer* was recognized as 45 species with nine annuals and 36 perennials. Importantly, as outlined below, only two of previously known eight annual wild *Cicer* species (*C. reticulatum* and *C. echinospermum* P.H. Davis) are in the primary and secondary gene pools of cultivated chickpea and are readily inter-fertile with chickpea (Ladizinsky and Adler, 1976a; van der Maesen et al., 2007; Smykal et al., 2015).

Among the annual *Cicer* species, *C. arietinum* is the sole species under domestication and worldwide grown in 60 countries with production quantity of 17.2 million tons from an area of 17.8 million ha in 2018 (FAOSTAT, 2020). Domesticated chickpeas with two varietal groups such as *desi* having pigmented plants, flowers and seeds and *kabuli* having non-pigmented plants, flowers and seeds were mainly grown in Indian sub-continued and Mediterranean region, respectively (Penmetsa et al., 2016). They are a significant source of protein, carbohydrates, vitamins, minerals and unsaturated fatty acids. Chickpeas not only possess characteristics for a balanced diet, especially for poor populations throughout the world (Jukanti et al., 2012; Pradhan et al., 2014; Upadhyaya et al., 2016; Jimenez-Lopez et al., 2020; Sab et al., 2020), but are also important for sustainable agriculture since fixing atmospheric nitrogen to soil via special bacteria provides rotational value to subsequent crops (Afonso-Grunz et al., 2014; Marques et al., 2020b). With climate change, the continued importance of chickpeas depends on their capacity to adapt to adverse environments (Roorkiwal et al., 2014; Ahmad et al., 2016; Deokar and Tar'an, 2016; Pang et al., 2017; Marques et al., 2020a). Gross production value of domesticated chickpea in 2016 has been estimated to be about 5.9 billion $ in the world (FAOSTAT, 2020).

Germplasm resources of annual *Cicer* are not only very limited when compared to cereals and other important food legumes (Berger et al., 2003; Smykal et al., 2015; Foyer et al., 2016; Dwivedi et al., 2019) but also some include several endemic species with endangered status based on the criteria of the International Union for Conservation of Nature (Ozturk, 2011; Talip et al., 2018; Tekin et al., 2018). This is very relevant for chickpea, given the limited diversity of the cultigen, and the ongoing need for new sources of diversity to exploit in crop improvement (Abbo et al., 2003). Currently only two annual *Cicer* species (*C. reticulatum* and *C. echinospermum*) are crossable to cultivated chickpea, but domesticated chickpea is not crossable with other species in the tertiary genepool including *C. bijugum* K.H. Rech., *C. chorassanicum* (Bge) Popov, *C. cuneatum* Hochst. ex Rich, *C. echinospermum, C. judaicum* Boiss., *C. pinnatifidum* Jaub. & Spach, *C. reticulatum,* and *C. yamashitae* Kitamura (van der Maesen et al., 2007). *C. bijugum, C. echinospermum, C. pinnatifidum*, and *C. reticulatum* are native species of Anatolia and Middle-Eastern regions, while *C. cuneatum* occurs in Ethiopia, south-east of Egypt, north of Sudan and Saudi Arabia, *C. chorassanicum* and *C. yamashitae* are distributed to north and north-east of Iran and Afghanistan, and *C. judaicum* is grown in Middle-Eastern region (Robertson et al., 1995; Berger et al., 2003). While *C. judaicum* was incorrectly listed in Turkey (Robertson et al., 1995), only *C. bijugum, C. echinospermum, C. pinnatifidum*, and *C. reticulatum* have been found in Anatolia, Turkey (Davis, 1970; van der Maesen, 1972; Berger et al., 2003; Ozturk, 2011; Ozturk et al., 2011).

In an effort to expand on these limited crop wild relative resources for chickpea, a *Cicer* collection mission focusing particularly on *C. echinospermum* and *C. reticulatum* was undertaken largely in south-eastern and eastern Turkey from 2013 to 2015 (Toker et al., 2014; Berger et al., 2017, 2018; von Wettberg et al., 2018), with opportunistic side trips from

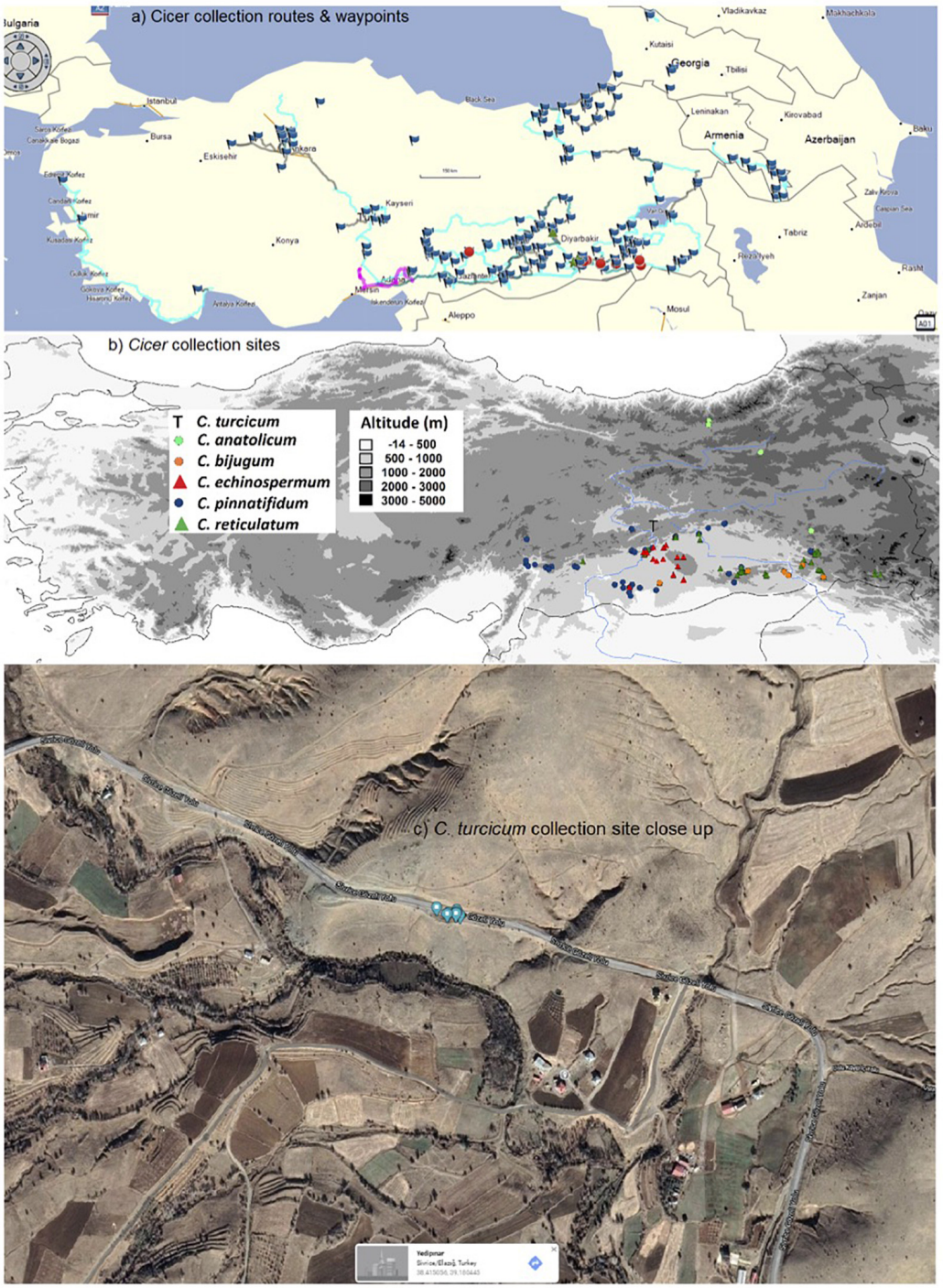

FIGURE 1 | West Asian *Cicer* survey (2013–18) routes and waypoints **(a)**, collection sites classified by species **(b)**, and close-up of the sole *C. turcicum* collection site at Yedipinar collection site, Sivrice district, Elazig province, Turkey. (Image from Google maps, Map data @2021, Australia) **(c)**.

2016 to 2018 (**Figure 1**). During the collection mission, *ca* 590 accessions of *C. bijugum, C. echinospermum, C. pinnatifidum*, and *C. reticulatum* were collected from 91 sites and partially evaluated for their adaptive traits (Kahraman et al., 2017; Talip et al., 2018; von Wettberg et al., 2018; Reen et al., 2019; Berger et al., 2020; Newman et al., 2020). This mission covered a huge range of locations throughout Turkey and beyond and collected a new species thus far unknown to the scientific world at only a single site (**Figure 1**). In the present study we propose this new species as *C. turcicum*, describe its known distribution and

ecology, its morphological characteristics and relatedness to other *Cicer* species using internal transcribed spacer (ITS) sequencing. Finally, we undertake a preliminary evaluation for its utility for chickpea improvement by studying the species tolerance to heat in the reproductive stage and seed resistance to the bruchid, *Callosobruchus chinensis* L.

MATERIALS AND METHODS

Cicer Survey and Collection Missions

Cicer survey and collection missions were conducted from 2013 to 2018 focusing largely on eastern and south-eastern Anatolia with opportunistic side trips through central and western Turkey, southern Armenia, central and western Georgia (**Figure 1a**). Populations were surveyed in early spring so that plants could be identified using floral characteristics. This entailed random survey of potential collection sites (see waypoints in **Figure 1a**) by 1–5 scientists searching for any wild *Cicer* species, with a focus on *C. echinospermum* and *C. reticulatum,* and opportunistically recording the presence of any wild *Lens* and *Pisum* relatives (Smýkal et al., 2018). *Cicer* leaf material was collected on a single plant basis to facilitate genetic studies, all samples being individually geo-referenced using a Garmin Montana 650 (von Wettberg et al., 2018). Geo-referenced soil samples were also taken at this time. Mature seeds were collected on an individual plant basis in late spring/early summer and geo-referenced as before.

Collection Site Climatic Data

Collection site climate data (altitude, monthly mean, minimum and maximum temperature, and precipitation) was extracted at 30 s resolution (ca. 1 km grid) from WorldClim ([1]Hijmans et al., 2005). Additional climate descriptors (monthly mean frost days, rain days, precipitation coefficients of variance, relative humidity, sun hours, wind speed) were extracted at 10 min resolution (ca. 12 km grid) ([2]New et al., 2002). Similar climate data were also extracted directly from the weather station at the Elazig airport (892 m asl), 24.1 km distant from the Yedipinar collection site (1,548 m asl) and at lower elevation (892 vs. 1,548 m), courtesy of the Turkish State Meteorological Service (TSMS, 2020).

Site-specific bioclimatic variables such vegetative and reproductive phase rainfall were calculated from these data by defining when plants typically emerged, flowered and matured at each collection site using observation and local feedback crosschecked against seasonal rules imposed on the monthly climate data, and details were given by Upadhyaya et al. (2011).

Identification

Cicer turcicum specimens were compared with the species with the closest resemblance (*Cicer pinnatifidum* and *C. judaicum*) and with specimens at Akdeniz University herbarium. All parts of the specimens were recorded using a ruler with 0.5 mm precision. Photographs were taken with a Sony Alpha 700 digital camera.

[1]http://www.worldclim.org

[2]http://www.cru.uea.ac.uk/cru/data/hrg/

Taxonomic Treatment

According to results of the assessment of morphological including description and habitat with its ecology and molecular data on ITS sequences, the new species were taxonomically classified and evaluated. Also, it was compared to the related species including *C. pinnatifidum, C. judaicum, C. echinospermum*, and *C. reticulatum*.

Conservation Status

Conservation status was suggested according to plant population and the IUCN threat category (IUCN, 2014).

DNA Extraction, PCR, and Sequencing

Cicer arietinum (ILC 8262 and ICC 8617), *C. reticulatum* (AWC 602), *C. turcicum*, *C. pinnatifidum* (AWC 503 and AWC 505), *C. judaicum* (PI 458559) and *C. cuneatum* were grown under controlled conditions in greenhouse for molecular analysis.

Fresh leaves were stored at –20°C until DNA extraction. Total genomic DNA was extracted using the CTAB method of Doyle and Doyle (1990). DNA concentrations were estimated on 1% agarose gels stained with ethidium bromide. For this study, the nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S rDNA and ITS2) was used to evaluate the relationships between species. The ITS region was amplified using primers ITS 4 and ITS 5 (White et al., 1990). The PCR analysis was carried out with 1 U of *Taq* DNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada) in the supplied reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 40 ng of template DNA, and ddH_2O to a final volume of 15 μL. PCR amplification conditions were as follows: an initial pre-denaturation step at 94°C for 5 min, 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. Amplification was performed on a Bioneer thermocycler (MyGenie^{TM}). PCR products were electrophoresed on a 1.5% agarose gel run at 75 V in 1 × TAE buffer and visualized under UV light after staining with ethidium bromide. Sequencing was carried out at Macrogen Inc., Europe via BM Laboratories Ltd., with direct sequencing in both directions using the amplification primers. All sequences were manually edited using Chromas v. 2.6.5 (McCarthy, 1996–1998) and aligned in Bioedit v. 7.0.5.3 (Hall, 1999). Double peaks were represented by IUPAC ambiguity codes in the species of *C. turcicum* in the alignment. Sequences were submitted to GenBank.

Screening for Heat Tolerance

Cicer turcicum phenology and heat tolerance was compared against a range of wild and domestic *Cicer* accessions (**Table 1**) in a common garden experiment at the Akdeniz University campus Antalya, Turkey (30° 44′ E, 36° 52′ N, 51 m asl). The experiment was conducted in a screenhouse, with plants sown directly into the loam soil for 2 years from 2018–2019 to 2019–2020. Soil properties were given by Kivrak et al. (2020). Water holding capacity, organic matter, soil nitrogen, zinc, and iron were determined to be at low levels, $CaCO_3$ and pH were, 26.5 and 7.69%. The experimental design was RCBD with three replications using plots 2 m in length with row spacing of

100 cm, sown on 27th December 2018 in the first year and 29th December 2019 in the second year in order to expose the plants to heat stress during their reproductive phase. Plant phenology (flowering, podding, maturity) was observed at 2–3 day intervals and accessions screened for heat tolerance using a visual 1–9 scale at podset (**Table 2**). Plants were irrigated with drip irrigation system at 3-day intervals in order to prevent the confounding effects of drought.

Screening for Resistance to the Bruchid

Callosobruchus chinensis L. maintained at the Department of Plant Protection, Akdeniz University, Antalya, Turkey were used in a no-choice test after Erler et al. (2009) and Eker et al. (2018). Insect rearing was carried out with susceptible chickpea seeds at 26 ± 2°C and 65 ± 5% RH in complete darkness. To rear fresh adults of a uniform age, seeds with eggs were put in clean jars filled with a large number of chickpea seeds which were checked every day for insect health.

Ten seeds of an accession each of *C. arietinum, C. pinnatifidum*, *C. judaicum*, and two accessions of *C. turcicum* (**Table 1**) were placed in a separate glass jar of one liter. For each accession, three replications were used. Ten pairs (10♀ and 10♂) of day-old adults of the brıchid were put into each jar. Then glass jars were covered with a gauze cloth in order to anticipate the flight of the insects and to allow air circulation. The bruchids were forced to feed only the seeds of one accession in a jar. After a week oviposition, the adult insects were carefully removed from each jar. Oviposition in each jar was controlled using a stereo-microscope and number of eggs laid by the insect were counted for each accession separately. The jars were controlled daily for adult emergence for 30 days.

Assessment for resistance to the pulse bruchid was evaluated by recording number of eggs per seed, number of holes per seed, percentage of seed damage and seed weight loss in each accession in no-choice test. The number of eggs per seed was recorded with the stereo-microscope. The number of holes was assessed by the round holes with the "flap" on seed coat. Percentage of seed damage was counted as the damaged seeds for each accession, and then data were converted into percentage as damage incidence according to Khattak et al. (1995):

$$Damage\ incidence\ (\%)\ =$$

$$(No\ of\ seeds\ damaged/Total\ no\ of\ seeds)\ \times\ 100$$

The damage incidence was classified according to **Table 2**. A similar scale in *Cicer* and *Pisum* species was successfully used by Eker et al. (2018) and Esen et al. (2019), respectively. Seed weight loss was determined the following formula (Khattak et al., 1995):

$$Total\ loss\ (\%)\ =\ \ (n_2 - n_1)\ /n_2\ \ 100,$$

where n_2 and n_1 are the initial weight of seeds before the test and the weight of the damaged seeds after the test, respectively.

Data Analyses

The phylogenetic tree was constructed with the Maximum Parsimony (MP) method using MEGA v. 7 (Kumar et al., 2018),

TABLE 1 | Germplasm evaluated for bruchid (B) resistance and heat tolerance (H) experiments (Exp) at Akdeniz University.

Species	Accession ID	Origin	Collection site	Latitude	Longitude	Notes	References	Exp
C. arietinum	ACC 100	Tur	Akdeniz Uni			Kabuli, bruchid susceptible check	Eker et al., 2018	B
C. arietinum	ACC 1054	Tur	Akdeniz Uni			Kabuli check, heat-sensitive check, cold tolerant	Ceylan et al., 2019	H
C. arietinum	ILC 8262	Esp				Kabuli check, heat-sensitive check, cold tolerant	Singh et al., 1992; Canci and Toker, 2009a	H
C. arietinum	ILC 8617	ICARDA	Rabat			Kabuli check, heat-sensitive check, cold tolerant	Singh et al., 1995; Canci and Toker, 2009a	H
C. judaicum	PI 458559	Pal	Beit Sira, Ramla	32.00	34.83	Wild check,	Robertson et al., 1995; Canci and Toker, 2009a	H, B
C. pinnatifidum	IG 72984	Tur	Gaziantep	37.05	37.25	Wild check,	Toker, 2005; Canci and Toker, 2009a	H, B
C. pinnatifidum	IG 72986	Tur	Gaziantep	37.05	37.25	Wild check,	Toker, 2005; Canci and Toker, 2009a	H
C. reticulatum	IG 72971	Tur	Savur, Mardin	37.30	40.73	Wild check,	Robertson et al., 1995; Canci and Toker, 2009a	H
C. turcicum	AWC 404	Tur	Yedipinar, Elazig	38.42	39.18	New annual *Cicer* species		H, B
C. turcicum	AWC 551	Tur	Yedipinar, Elazig	38.42	39.18	New annual *Cicer* species		H, B

TABLE 2 | A visual quantitative 1–9 scale for resistance to a/biotic stresses evaluated in Exps 1 and 2.

Scale	Reaction category	Heat tolerance	Resistance to bruchid
1	Very highly resistant	Very good vigor and 100% pod setting and filling	Damage incidence is 0% and no holes observed
2	Highly resistant	Good vigor and 96–99% pod filling	Damage incidence is about 2–5%
3	Resistant	Good vigor and 86–95% pod filling	Damage incidence is 6–10%
4	Moderately resistant	Moderate vigor and 76–85% pod filling	Damage incidence is 11–20%
5	Moderate	Poor vigor and 51–75% pod filling	Damage incidence is 21–30%
6	Moderately susceptible	Lack of vigor and 26–50% pod filling	Damage incidence is 31–40%
7	Susceptible	Lack of vigor and 11–25% pod filling	Damage incidence is 41–50%
8	Highly susceptible	Lack of vigor and 1–10% pod filling	Damage incidence is 51–90%
9	Very highly susceptible	No flowering or podding	Damage incidence is more than 91%

under heuristic searches with 100 random addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping, saving no more than 100 trees with length ≥ 1 per replicate, automatically increasing the maximum number of trees saved. Bootstrapping was performed using the same settings and 1,000 replicates, but without branch swapping. For the phylogenetic analyses, available sequences of *C. echinospermum* (AB198910.1) and *C. bijugum* (AJ237701.1) were retrieved from GenBank for comparison. Also, the sequences data belongs to *P. sativum* L. (L36637.1) and *L. culinaris* Medik. subsp. *orientalis* (Boiss.) Ponert (AJ441321.1) were used as outgroups in the phylogenetic analyses.

Visual scale data were converted to percentage and then used to perform analysis of variance (ANOVA) using Genstat V20 software, nesting accessions within species. Residual plots were generated to detect errors and confirm common and independent variance. For each stressor, significant differences between the accessions were studied using LSD and Duncan multiple range tests.

FIGURE 2 | *C. turcicum in situ* at Yedipinar collection site, Sivrice district, Elazig province, Turkey on 12th June 2015. Specimen is both flowering and podding, with some mature pods.

TABLE 3 | Number of survey sites in which wild *Cicer* species were found, categorized by country and species.

Country	Armenia	Georgia	Turkey	Total
Total survey sites	25	8	209	242
Annual wild *Cicer* species				
C. reticulatum			40	40
C. echinospermum			18	18
C. bijugum			7	7
C. pinnatifidum			38	38
C. turcicum			1	1
Perennial wild *Cicer* species				
C. anatolicum	1		4	5
C. isaricum			1	1

RESULTS

Collection of *C. turcicum*

Plant specimens and mature seeds were collected near Yedipinar collection site in the Sivrice district (Elazig Province, Turkey) on 12th June 2015 (**Figure 1**). Plants were flowering and podding, with some mature pods (**Figure 2**).

Cicer turcicum appears to be a rare species, found only once among the 242 sites surveyed in Turkey, Armenia and Georgia (**Table 3**). The Yedipinar collection site is remote from other known occurrence of annual wild *Cicer* (**Figure 1b**), 38 km from the closest *C. pinnatifidum*, 46 km from *C. reticulatum*, 49 km from *C. echinospermum*, and 124 km from *C. bijugum*.

Species Biology and Habitat Characterization

Cicer turcicum is an East Anatolian endemic in the Irano-Turanian phytogeographic region. Habitat is a hilly area with some trees cover on the slopes ranging from isolated oak woodlands, oak/juniper forest and some pine plantations. The plants were located in a tight cluster in a light brown sandy loam on a S-facing rubble slope adjacent to the Sivrice-Gozeli road (38.4174N, 39.1783E) in moderately dense annual vegetation at 1,544–1,553 m elevation (**Figure 1c**).

Based on *in-situ* field observation made during the *Cicer* survey and collection mission, *C. turcicum* phenology seems most similar to *C. reticulatum* and somewhat later than *C. pinnatifidum.* Most of the latter species observed close to the Yedipinar collection site had mature, shattered pods at this time (see also subsequent phenology data from common garden comparison). On this basis we expect *C. turcicum* to germinate

TABLE 4 | Characteristics of the sole *C. turcicum* collection site at Yedipinar village, Sivrice district, Elazig province, Turkey based on geographic data extracted from Garmin Montana 650 and climate data from WorldClim (Hijmans et al., 2005) and 10 min Climatology (New et al., 2002), calculated over the indicated growing season phases.

Descriptor	Site mean	Pre-season (July-Oct)	Veg phase (Oct-May)	Rep phase (May-June)	Season total
Latitude (°d)	38.4175				
Longitude (°d)	39.1782				
Elevation (m)	1548				
Mean temp (°C)			3.9	17.1	6.8
Min temp (°C)			−6.6		
Max temp (°C)				26.0	
Temp change (°C/day)				0.1	
Frost days (sum)			94	1	94
Precipitation (sum, mm)		15	554	88	642
Precipitation CV (%)			59	81	64
Rain days (sum)			82	17	98
Rel humidity (%)			66	44	61
Sun hrs (%/day)			52	75	58

with the opening autumn rains (October), start flowering in late April/early May and mature from mid-June onward like other annual *Cicer* species. Climate at the collection site of *C. turcicum* is typically Mediterranean, with arid summers and cold winters. The area is relatively cool, with snow cover 5 months of the year, reflecting the relatively high elevation (**Table 4**). Accordingly, *C. turcicum* receives most of its seasonal rainfall during the vegetative phase, characterized by frequent, reliable precipitation, high relative humidity, and low sunshine (**Table 4** and **Figures 3A,B**). Vegetative mean temperatures are very low and there is a high incidence of frost (**Table 4**). The mean reproductive phase climate is mild, with relatively low temperatures, a low rate of temperature increase, and relatively frequent rainfall (**Table 4**). Monthly mean temperatures from climate databases do not capture the climatic extremes that are likely to exert strong selection pressure on endemic plant species. This is demonstrated by data from the nearby (albeit considerably lower altitude) Elazig airport weather station which shows that temperatures can range from <-20°C to >40°C in the vegetative and reproductive phases, respectively (**Figure 3A**). Given the much greater elevation of the Yedipinar collection site, it is likely that minimum temperatures may range even lower than this, while reproductive phase temperatures may not be as extreme.

Taxonomy-Morphology of *C. turcicum*

Description

Annual; stem semi-prostrate up to 45 cm long, procumbent branches at base, completely pubescent, glandular hairs. Leaves imparipinnate with 7 pairs of leaflets; rachis 3–5 × 0.7–1.1 cm in outlines; petiol 5–6 mm; leaflets pubescent, fairly close, opposite or not, shortly petiolulate 0.5 mm, oblong-elliptic, 5–6 (−7) × 2–4 mm, and a single leaflet at base of rachis (arrow in **Figures 4a,e**), basal 1/5 part entire; teeth 9–11 (−14), acute. Stipules pubescent, four unequal teeth, each teeth triangular, 2–3 × 4–6 mm (**Figure 4b**). Inflorescence generally 1-flowered (seldom double-flowered), axillary racemes; peduncle pubescent, 4–10 mm, ending in an arista, 2 mm; bracts linear, 0.5 mm; pedicel pubescent, 4–8 mm. Calyx hardly dorsally gibbous at the base, pubescent, 3–6 mm, teeth triangular-lanceolate, 2–4 mm. Corolla veined, glabrous, purple-magenta, fading into blue-violet and magentaroadly ovate, when old; standard (wexillum), emarginated at apex, attenuate at base, 8–10 × 6–8 mm; wings (alae) obovate, strongly auriculate at base, 6–7 × 2–3 mm; keel (carina) rhomboid, 4–5 × 1.5–2.5 mm. Stamens diadelf (9+1), filaments 5–6 mm long (fused part 4 mm, free part 1.5–2 mm, upturned). Ovary ovoid, 6 mm long, densely glandular pubescent; style ca. 2–4 mm, upturned. Pods rectangular ovate at base, 15–18 × 6–9 mm, stylus and stamens persistent when old, 3–4 seeds, shattered when ripe. Seeds triangular-arietinoid, distinctly bilobular, beaked, 5–6 × 4–5 mm, hilum 0.5–1 mm, seed coat greenish-dark brown, tuberculated (**Figures 4c,d**).

Cicer turcicum is completely different from *C. pinnatifidum, C. judaicum, C. echinospermum*, and *C. reticulatum* because of gross morphology and seed size/shape differences (**Figure 4** and **Table 5**). Flowers, pods and seeds of the new species are larger than those of *C. pinnatifidum* and *C. judaicum*, while they were smaller than those of *C. echinospermum* and *C. reticulatum* (**Table 5**). The new species can easily be distinguished by differences in leaflets (one of leaflets at the base of leaf is single), stipules (Crown-shaped), and seeds (greenish-dark brown and tuberculate) from *C. pinnatifidum, C. judaicum, C. echinospermum*, and *C. reticulatum* (**Figure 4**).

Taxonomic Treatment

Based on morphological and molecular data allowing comparison to the related samples, it was decided that the specimens collected from Elazig belongs to a new species. This species was named *C. turcicum* and taxonomically put in subgenus *Pseudononis* M. Pop. and section *Cicer* M. Pop. (van der Maesen et al., 2007; Ozturk et al., 2013).

Cicer turcicum Toker, Berger & Gokturk, sp. nov. (**Figures 4a–d**).

Type: —TURKEY. B7 Elazig: Sivrice, Yedipinar around (38.4174N, 39.1783E) at 1,544–1,553 m elevation, in June 2015,

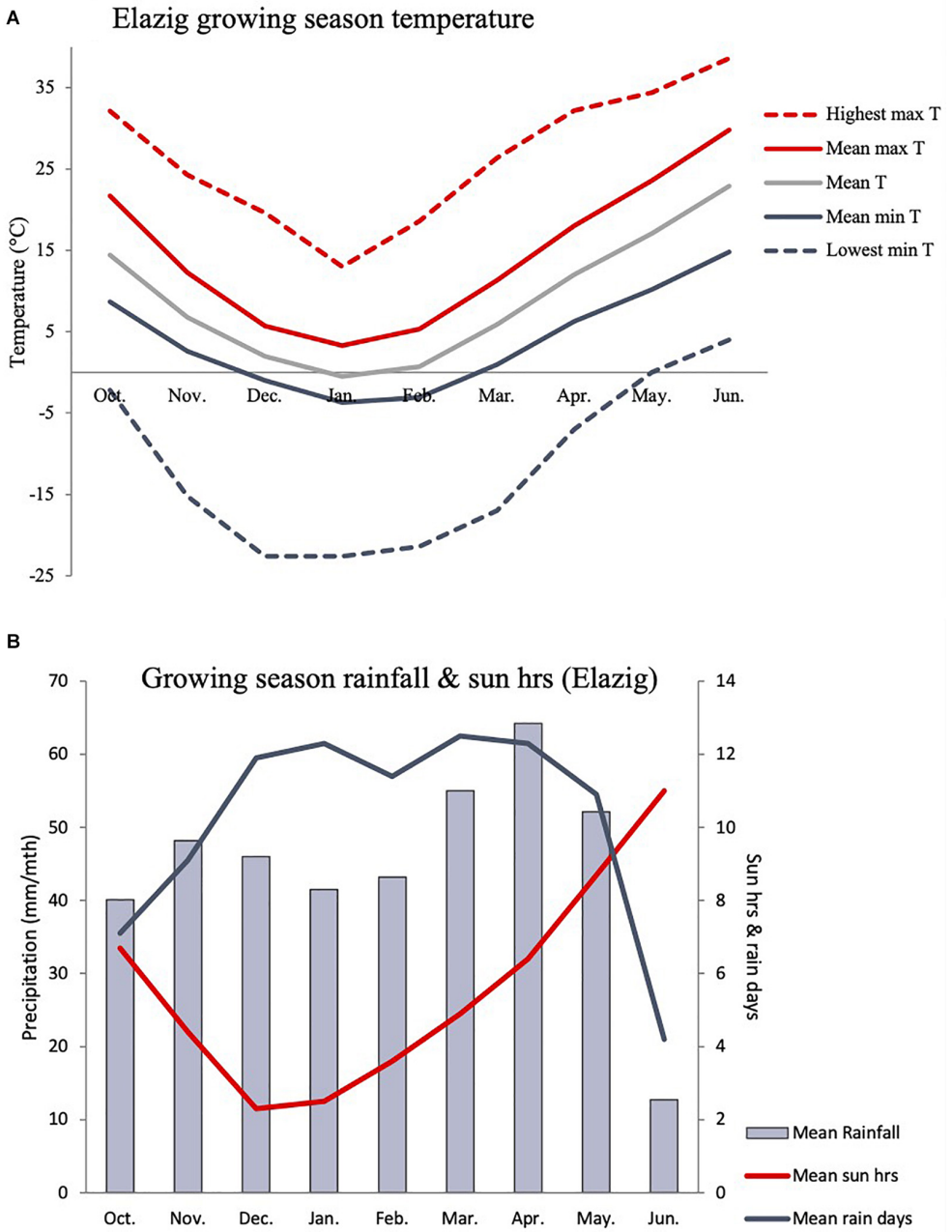

FIGURE 3 | Indicative *C. turcicum* growing season temperature **(A)**, precipitation and sun hours **(B)** based on long term monthly data (1981–2010) from the Elazig airport weather station (892 m asl), located 24.1 km from the Yedipinar collection site (1,548 m asl) at lower elevation (892 vs. 1,548 m). *C. turcicum* germinates in October, flowers in April/May and matures in June/July based on field observations and phenology data from common garden evaluation (see **Figure 6**).

Toker, Berger (1001) & Gokturk (holotype Akdeniz University herbarium!, isotypes PAMUH!, ANK!, HUB!, GAZI!).

Etymology

The specific epithet is derived from the name of Turkey.

Alignment and Sequence Characteristics

Nucleotide sequences were deposited in GenBank (accessions MW424513-MW424518). The ITS region (ITS1-5.8S gene-ITS2) in *Cicer* ranged from 692 to 704 bp. The aligned length for the ITS dataset was 662 positions, with 58 informative sites and 118 variable sites. In total, 43 diagnostic single nucleotide polymorphisms and one tri-nucleotide deletion were observed in the aligned dataset. No intraspecific variation was observed in *C. arietinum*, *C. turcicum*, and *C. pinnatifidum*. *C. turcicum* showed seven single nucleotide identities to the sequences of *C. arietinum, C. reticulatum*, and *C. echinospermum* (**Table 6**), positions: (45, 78, 103, 204, 457, 472, 474). This species had

FIGURE 4 | Leaves of *C. pinnatifidum*, *C. turcicum* and *C. judaicum* (**a**, left to right). Single leaflet at the base of leaves of *C. turcicum* (red arrow). Stipules of *C. judaicum*, *C. pinnatifidum*, and *C. turcicum* (**b**, left to right). Seeds of *C. reticulatum*, *C. echinospermum*, and *C. turcicum* (**c**, left to right). Seeds of *C. judaicum*, *C. pinnatifidum*, and *C. turcicum* (**d**, left to right). Shoots of *C. pinnatifidum*, *C. judaicum*, and *C. turcicum* (**e**, left to right).

an identical nucleotide with *C. pinnatifidum* in the position of 98. Additionally, their three nucleotide deletions (GAC, position: 205–207) were shared. *C. turcicum* had double peaks in direct sequences, so additive characters were represented in the positions of 536 and 588 (**Table 6**). These characters were not observed in any other species.

Phylogenetic Analysis of the ITS Region

The MP analysis resulted in 10 equally parsimonious trees (length: 133) with a consistency index (CI) of 0.898, a retention index (RI) of 0.917 and a rescaled index (RC) of 0.868. In the phylogenetic tree, four major groups were observed (**Figure 5**), one of which included *P. sativum* and *L. culinaris* subsp. *orientalis* as out-group, while the rest were taxa in the genus *Cicer*. Group I, consisting of *C. arietinum, C. reticulatum, C. echinospermum*, and *C. turcicum,* was supported by a 98% bootstrap value in the parsimony tree. This group revealed two subgroups (**Figure 5**). There was a strong support that *C. turcicum* was in the different group from *C. arietinum, C. reticulatum, C. echinospermum* (bootstrap support, 99%). Group II included *C. pinnatifidum*, *C. bijugum*, and *C. judaicum*. This group showed a bootstrap value of 98%. Group III only consisted of *C. cuneatum*.

TABLE 5 | Comparison of *C. turcicum* for diagnostic characteristics with *C. pinnatifidum, C. judaicum C. echinospermum*, and *C. reticulatum*.

Characteristics	*C. judaicum**	*C. pinnatifidum**	*C. turcicum*	*C. echinospermum**	*C. reticulatum****
Leaves (no)	(7–9) 11–13	4–9 (11)	13–14	7–11	8–15
Leaflets (mm)	4–7 (9) × 2–5 (8)	4–11 (12) × 2–5 (7)	5–6 (7) × 2–4	4–9 (11) × 2–5	5–11 (15) × 2–4
Stipules (no of teeth)	3	6–7	4	3–5	4–5
Seeds (mm)	3–4 × 3–4	4–6 × 3–5	5–6 × 4–5	7 × 5	5–9 × 4–6
Pods (mm)	10–13 × 5–6	10–15 × 6–8	15–18 × 6–9	15–20 × 10–12	12–16 × 8–12
Leaflet position	Opposite or not	Opposite or not	Opposite or not but a single leaflet at base	Opposite or not	Opposite or not
Distribution**	Levant (Isr, Pal, Leb, Syr)	Levant, S & SE Anatolia	E Anatolia (1 location)	SE Anatolia	SE Anatolia

*, **, and *** Data were obtained by van der Maesen (1972); Berger et al. (2003), and Ladizinsky (1975), respectively.

TABLE 6 | Species diagnostic differences in ITS region.

Species	Position in alignment										
	45	**78**	**98**	**103**	**204**	**205–207**	**457**	**472**	**474**	**536**	**588**
C. arietinum	A	C	A	C	T	GAC	G	C	T	T	A
C. reticulatum	A	C	A	C	T	GAC	G	C	T	T	A
C. echinospermum	A	C	A	C	T	GAC	G	C	T	T	A
C. turcicum	A	C	G	C	T	GAC	G	C	T	**Y***	**R***
C. judaicum	C	T	A	–	G	–	A	T	C	T	A
C. pinnatifidum	C	T	G	–	G	–	A	T	C	T	A
C. cuneatum	C	T	A	–	G	–	A	T	C	T	A
C. bijugum	C	T	A	–	G	–	A	T	C	T	A

*Additive characters (double peaks in direct sequences) are represented by IUPAC codes in bold.

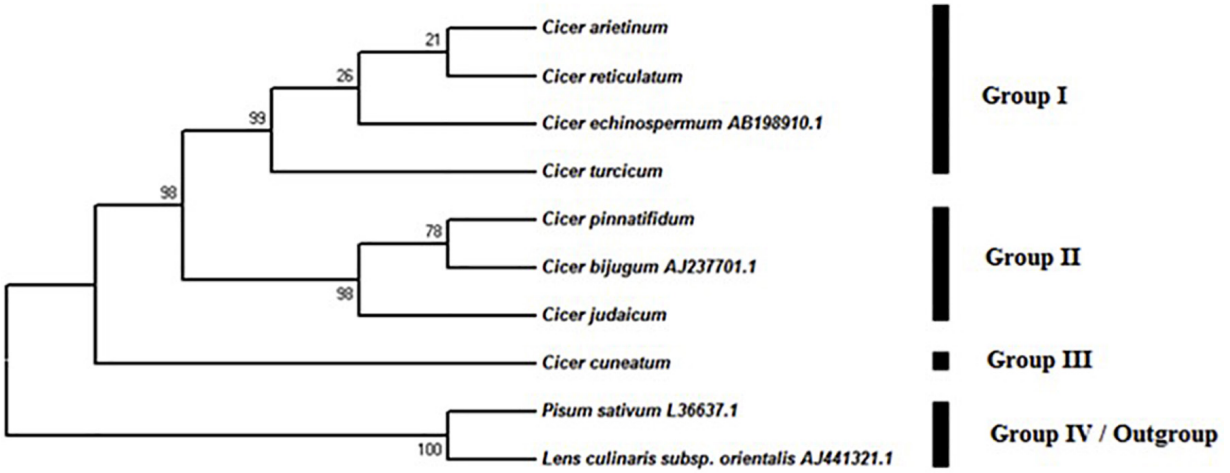

FIGURE 5 | Phylogenetic tree from the maximum parsimony analysis based on the sequence of ITS region in *Cicer* taxa.

Phenology

Common garden evaluations in the Akdeniz University screenhouse in 2018/19 and 2019 confirmed field observations made at the Yedipinar collection site regarding the typical Mediterranean winter annual phenology of *C. turcicum*. In the 2018/19 experiment, *C. turcicum* flowered and podded slightly later than *C. reticulatum*, followed by the remaining annual wild *Cicer* species, while in the following year there were no significant differences among any of the annual wild *Cicer* species (**Figure 6**). Domestic chickpea covered a wider range, the cultivar Ompar and ILC 8262 returning intermediate flowering and podding dates, while ILC 8617 was consistently 7–10 days later in both years (**Figure 6**, $P < 0.001$). *C. turcicum* matured relatively early, particularly in the 2019/20 experiment, where it was earlier than *C. judaicum,* an accession of *C. pinnatifidum* and particularly *C. reticulatum* (**Figure 6**). In 2018/19, wild *Cicer* maturity was more evenly distributed, with only *C. reticulatum* maturing at a later date than the rest of the group. *C. arietinum*

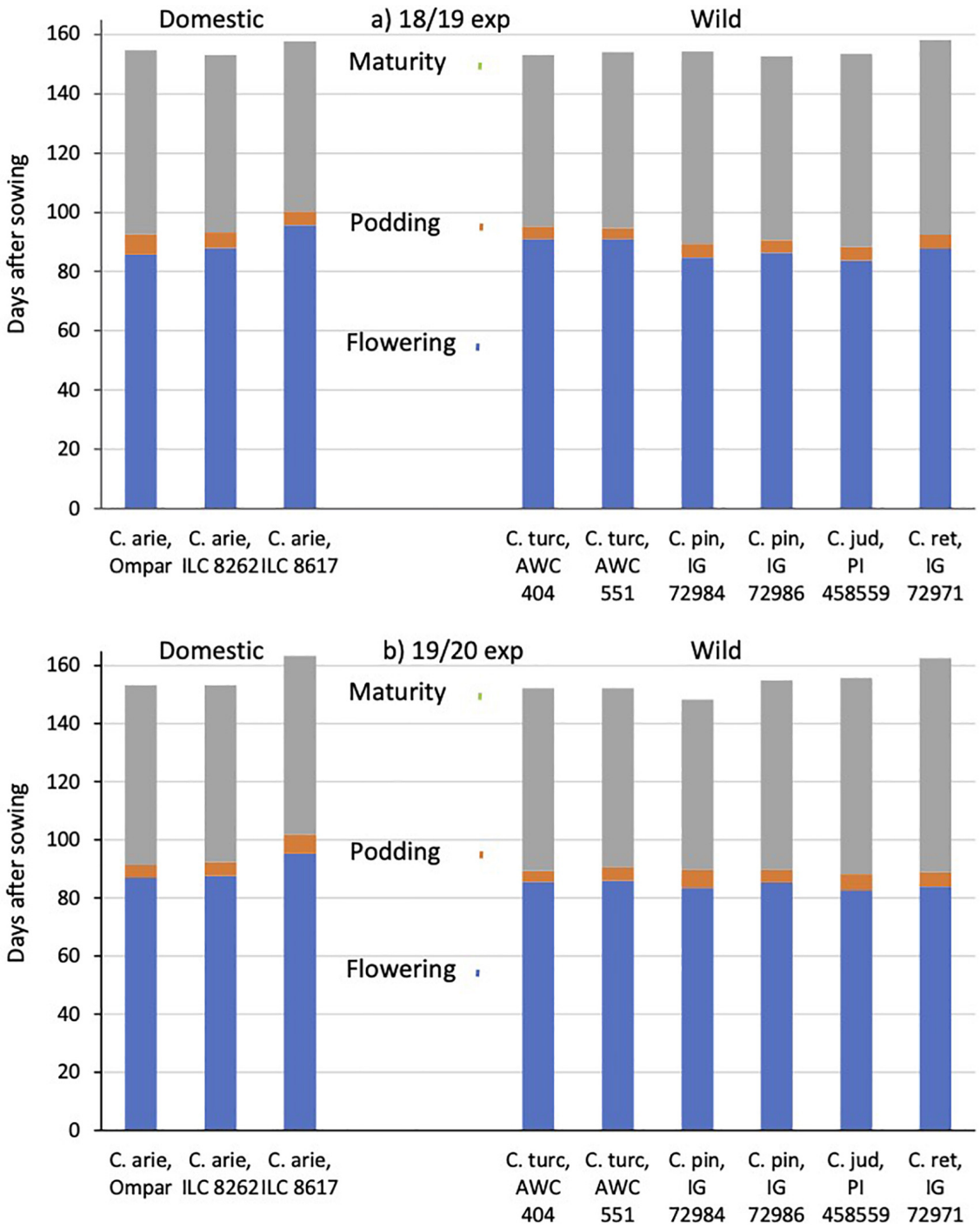

FIGURE 6 | *C. turcicum* phenology (flowering, podding, maturity) compared to related annual wild and domestic *Cicer* species. Data is from Mediterranean cool-season common garden screenhouse comparisons at Akdeniz University, **(a)** 2018/19, **(b)** 2019/20. Color-coded vertical lines represent accession least significant differences (LSD $P < 0.05$) for flowering (2.6–2.7 days), podding (2.4–2.7 days) and maturity (1.9–2.4 days). Abbreviations: C. arie, *C. arietinum*; C. turc, *C. turcicum*; C. pin, *C. pinnatifidum*; C. jud, *C. judaicum*; C. ret, *C. reticulatum*.

maturity dates followed the other phenological data, Ompar and ILC 8262 maturing early (similar to *C. turcicum*), +while ILC 8617 was consistently 3–10 days later (**Figure 6**, similar to *C. reticulatum*).

Heat Tolerance

Despite the broad phenological similarities described above (**Figure 6**), there were dramatic differences in pod setting under elevated reproductive phase temperatures between wild

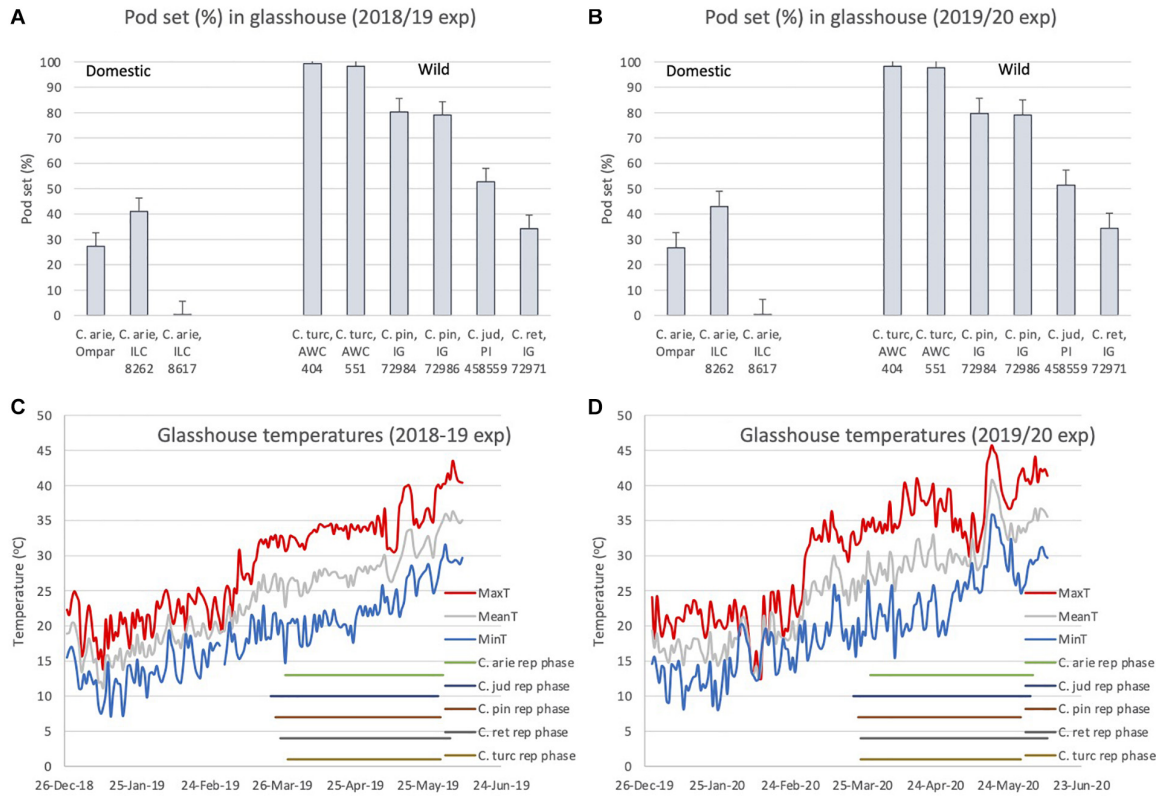

FIGURE 7 | *C. turcicum* pod set percentage **(A,B)** under high reproductive phase temperatures **(C,D)** compared to related annual wild and domestic *Cicer* species. Data is from Mediterranean cool-season common garden screenhouse comparisons at Akdeniz University, **(A,C)** 2018/19, **(B,D)** 2019/20. Error bars represent accession least significant differences (LSD $P < 0.05$). Reproductive phase lengths (flowering to maturity) are shown individually for each species **(B)**. Abbreviations: C. arie, *C. arietinum*; C. turc, *C. turcicum*; C. pin, *C. pinnatifidum*; C. jud, *C. judaicum*; C. ret, *C. reticulatum*.

and domestic *Cicer* species in both years (**Figure 7**). ANOVA indicated large species differences across years ($P < 0.001$), without interaction ($P < 0.574$), and smaller differences between varieties within species ($P < 0.001$), again without interaction over years. Thus, while pod set percentage means of all wild *Cicer* species were greater than in domestic chickpea ($P < 0.001$), *C. turcicum* > *C. pinnatifidum* > *C. judaicum* > *C. reticulatum* (**Figures 7A,B** $P < 0.05$). Pod set in domestic chickpea germplasm varied from 0% in ILC 8617 to 43% in ILC 8262, the latter variety setting a greater proportion of pods than IG 72971 ($P < 0.05$), the sole representative of *C. reticulatum* in this experiment. Analysis of the diurnal temperatures ranges recorded during the experiment demonstrated that heat escape resulting from variable phenology was not a factor in these inter-specific differences (**Figures 7C,D**). Mean temperatures increased linearly throughout the reproductive phase (2018/19, 0.11°C/day, $r^2 = 0.71$; 2019/20, 0.13°C/day, $r^2 = 0.62$) from *ca.* 26°C at flowering to > 35°C at maturity (**Figures 7C,D**). While temperature maxima fluctuated more on a daily basis, with weaker linear trends (2018/19, 0.09°C/day, $r^2 = 0.49$; 2019/20, 0.10°C/day, $r^2 = 0.36$), all species experienced maxima > 40°C during podding in both years, and none escaped sharply rising temperatures toward the end of the growing season in either year (**Figures 7C,D**).

Resistance to Bruchid

Orthogonal contrasts revealed striking wild-domestic differences in bruchid resistance, accounting for all of the significant species differences. Seed damage was far lower in wild compared to domestic *Cicer*, whether measured as the number of holes on the seed coat (**Figure 8**, $P < 0.001$), percentage of seeds damaged ($P < 0.001$) or in terms of seed dry matter consumed by the bruchids ($P < 0.031$). As a result, bruchid egg production was far lower on wild compared to domestic *Cicer* (**Figure 8**, $P < 0.001$). There were no significant differences among wild *Cicer* for any of these traits, nor between the two *C. turcicum* accessions evaluated in the present study ($P_{diff} = 0.452–0.976$).

DISCUSSION

In the present study we introduce *C. turcicum*, a new annual wild *Cicer* species hitherto unknown to science. *C. turcicum* appears to be a rare species, thus far recorded only in a single location in Elazig province, SE Anatolia, at a considerable distance from the nearest known wild *Cicer* population (**Figure 1**). The Yedipinar collection site has a realtively high elevation (*ca* 1,550 m) and exposes *C. turcicum* to an extreme temperature range throughout the growing season, from very cold winters

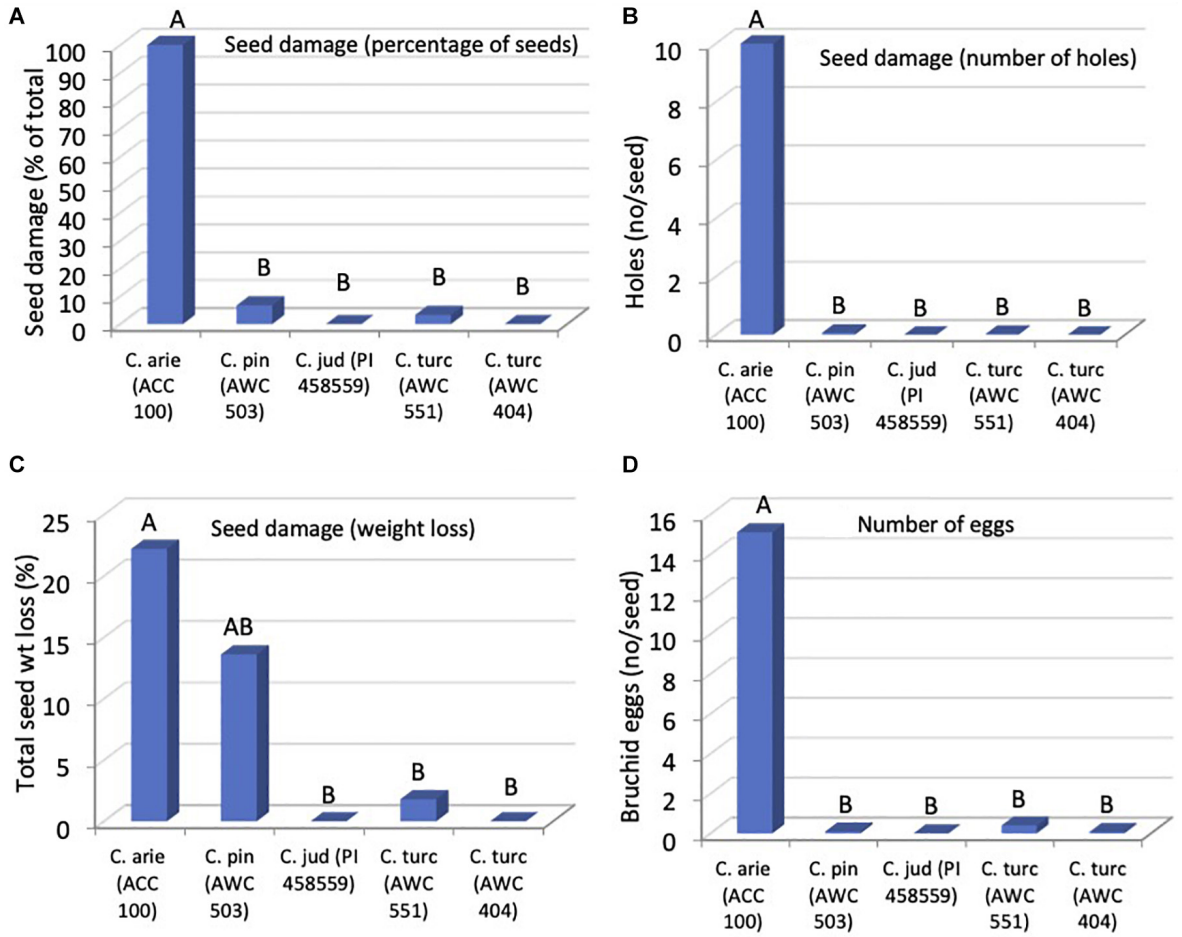

FIGURE 8 | Bruchid resistance in wild compared to domestic *Cicer* species in terms of seed damage **(A)**, number of holes **(B)**, weight loss **(C)**, and number of eggs **(D)**, from a no-choice feeding test at Akdeniz University. Letters represent accession group membership from Duncan multiple range test, different letters indicate significant difference ($P < 0.05$).

to hot, dry summers. *C. turcicum* has a distinct morophology that separates it from wild relatives, paticularly leaflet and seed size, distribution and shape (see **Figure 4** and **Table 5**), while ITS sequencing suggests it to be closely related to *C. arietinum*, *C. reticulatum*, and *C. echinospermum* (**Figure 5**). Common garden evaluation demonstrates that *C. turcicum* has a typical annual wild *Cicer* phenology, but appears to be more tolerant of reproductive heat stress than its wild relatives, and similarly resistant to bruchid feeding.

These findings raise a number of interesting implications and questions that need to be followed up. Arguably the most important of these is species rarity. The 2013–2018 *Cicer* mission surveyed 242 sites in detail, geo-referencing the presence/absence of wild crop legume relatives (*Cicer*, *Pisum*, *Lens*) and noting associated species. The fact that *C. turcicum* was only found at a single location underlines its relative scarcity. However, while the region immediately south of the Yedipinar collection site has been comprehensively surveyed (**Figure 1**), there were very few sites in Elazig province itself, particularly the areas surrounding Yedipinar to the north. A population of *C. pinnatifidum* was found at Tepekoy, 38 km to the west of Yedipinar, while *Lens* was found between Maden and Ergani, 42 km to the east of Yedipinar. Clearly, there is more work to do to establish the *C. turcicum* distribution. However, at this stage, with only a single collection site identified, it may be prudent to place *C. turcicum* under the IUCN threat category "Critically Endangered (CR)" (IUCN, 2014) because its estimated area of occupancy is less than 10 km^2, population size is estimated to be less than 50 mature individuals, and is under threat of heavy grazing pressure [CR B2; C2a(i)] given its proximity to Yedipinar and the Sivrice-Gozeli road (**Figure 1c**). In the meantime, we suggest that further survey missions focusing on Elazig province be undertaken as a matter of urgency.

While the identification of any new species is of in interest in its own right, the fact that *C. turcicum* is both an annual and appears to be closely related to *C. arietinum*, the single domesticated *Cicer* species makes it all the more important because annual *Cicer* species are relatively uncommon and its relatedness to chickpea opens new questions regarding the domestication of this crop. The ITS-sequencing phylogeny

presented in this study reflects the current taxonomic status of the species. Thus, *Pisum* and *Lens* were outgroups, reflecting their status as genera in the tribe Fabeae Rchb. referred to as Vicieae (Schaefer et al., 2012), while all the *Cicer* species were broadly clustered in Cicereae (Javadi and Yamaguchi, 2004; Schaefer et al., 2012). The within *Cicer* species clustering closely followed the known genepool (GP) classification:

(1) GP1: *C. arietinum* (domesticated chickpea) and *C. reticulatum* (Ahmad, 1999). Hybridization in the primary gene pool (GP1) is straightforward, progeny are fully fertile due to good chromosome pairing, alien gene transfer is achievable from wild to domesticated chickpea with traditional methods (Ladizinsky and Adler, 1976a,b; Adak et al., 2017; Koseoglu et al., 2017).
(2) GP2: *C. echinospermum*. Species in GP2 can be crossed with domesticated chickpeas and produced at least some fertile progeny, while hybrids are weak, partly sterile, and recovery of progeny in subsequent generations is difficult due to post fertilization problems (Mallikarjuna et al., 2011). Hybridization success varies between accessions (Kahraman et al., 2017). The proximity of *C. turcicum* to *C. echinospermum* in the ITS dendrogram (**Figure 5**) suggests that it is likely to be a member of GP2. To confirm this a hybridization program crossing *C. turcicum* with *C. arietinum* and *C. echinospermum* should be established.
(3) GP3. Species in GP3 are difficult to cross successfully with domesticated chickpeas (Ahmad et al., 1988; Badami et al., 1997; Clarke et al., 2006; Abbo et al., 2011) and include *C. bijugum, C. judaicum, C. pinnatifidum,* and *C. cuneatum*. Our ITS phylogeny places *C. cuneatum* in a separate cluster from the other GP3 species, and is in agreement with an earlier RAPD-derived phylogeny (Ahmad, 1999).

The discovery of *C. turcicum* at a single location in Yedipinar location, Sivrice district, Elazig province underlines the importance of Turkey as a center of biodiversity, particularly of the wild relatives of domesticated crops including chickpea. Turkey includes over 30% endemic species of approximately 12,000 natural vascular plant taxa in the world including 3,788 endemics (Guner et al., 2012). These are well documented using a grid system (Ture and Bocuk, 2010) and are distributed in different phytogeographical regions that intersect in Anatolia. A total of 17 *Cicer* taxa including domesticated chickpea, *C. anatolicum, C. bijugum, C. echinospermum, C. floribundum var. floribundum, C. floribundum var. amanicola, C. heterophyllum var. heterophyllum, C. heterophyllum var. kassianum, C. insicum subsp. incisum, C. incisum subsp. serpentinica, C. isauricum, C. montbretti, C. pinnatifidum, C. reticulatum, C. oxydon, C. turcicum*, and *C. uludereensis* are known to occur in Anatolia. The distribution of both extant *Cicer* species and their archeological remains suggest that Anatolia is not only the primary gene center of the genus *Cicer*, but also the cradle of the genus in terms of species richness.

Finally, the preliminary discovery of heat tolerance and bruchid resistance in *C. turcicum* add value to it's role as a donor in crop improvement should it be readily crossable with chickpea, or as experimental material to study responses to these stresses if it is not readily crossable. Heat stress causes yield loss in chickpea: day temperatures > 32°C reduces pod set (Basu et al., 2009). The incidence of heat stress in chickpea is predicted to rise in line with the 2–3°C temperature rise expected as a result of climate change in the near future (IPCC, 2007; Hatfield and Prueger, 2015). Although a number of studies have been carried out on heat tolerance in cultivated chickpea (Canci and Toker, 2009b; Krishnamurthy et al., 2011; Upadhyaya et al., 2011; Devasirvatham et al., 2015; Farooq et al., 2017; Paul et al., 2018) and its wild relatives (Canci and Toker, 2009a) yet they have generally found insufficient variation to meet this challenge. The observed heat tolerance of *C. turcicum* aligns well with the climate of the site of origin, characterized by an extreme temperature range. Note that the evidence for heat tolerance in *C. turcicum* is particularly compelling because the temperature data indicates that all species were subject to the same high reproductive phase temperature range, meaning that there were no heat escape opportunities. Nor is it likely that *C. turcicum* was more tolerant than the remaining wild species because of faster pod set, given that it's seed size is larger than both *C. pinnatifidum* and *C. judaicum*. Vegetative frost and reproductive chilling tolerance are also sorely lacking in domestic chickpea (Berger et al., 2012). Given, the cold nature of the of *C. turcicum* collection site, it is possible that this species may also harbor useful cold tolerance.

Bruchid resistance is also rare in domestic chickpea. Although more than 3,000 chickpea accessions were evaluated for resistance to *C. chinensis* at the International Center for Agricultural Research in the Dry Areas (ICARDA), no resistance was found in *kabuli* types. However, while some resistant *desi* chickpea with thick, rough or tuberculate seed coats have been identified (Reed et al., 1987), wild species such as *C. echinospermum* were found to be "immune" or free from damage (Eker et al., 2018). Annual *Cicer* species have already been screened for resistance to seed bruchid prior to the present study, and all accessions of *C. echinospermum* (100%), some accessions of *C. bijugum* (42.9%), *C. judaicum* (12.8%), and *C. reticulatum* (5%) were outlined to be free from the insect damage (Singh et al., 1998).

CONCLUSION

The following conclusions can be drawn from the present study:

- *C. turcicum* is a new, morphologically and genotypically distinct annual *Cicer* species which appears to be rare and found in different, climatically extreme environments than its *Cicer* relatives.
- ITS sequencing places it within the secondary genepool of domestic chickpea; this needs to be confirmed by crossing studies.
- Preliminary evaluation shows *C. turcicum* to harbor heat tolerance and bruchid resistance, but needs to be confirmed with wider evaluation.

The above list suggests that *C. turcicum* will be useful for chickpea improvement if the species can be successfully crossed with the cultigen, but that it also represents an interesting opportunity for domestication and trait discovery studies if that is not the case. Regardless, *C. turcicum* is rare, and needs better understanding/protection. We suggest further survey and collection focusing on Elazig province in SE Anatolia, and registration in a "Critically Endangered (CR)" IUCN threat category.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: Sequences were submitted to GenBank with the accession numbers of AB198910.1.

AUTHOR CONTRIBUTIONS

CT, JB, and RSG designed the studies. JB, BA, and AK found the new species. CT and RSG described the new species. DS and HS performed the molecular study. TE and HS conducted heat tolerance and bruchid studies. CT, JB, RSG, and EW wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Novel Genes and Genetic Loci Associated With Root Morphological Traits, Phosphorus-Acquisition Efficiency and Phosphorus-Use Efficiency in Chickpea

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Chickpea—the second most important grain legume worldwide—is cultivated mainly on marginal soils. Phosphorus (P) deficiency often restricts chickpea yields. Understanding the genetics of traits encoding P-acquisition efficiency and P-use efficiency will help develop strategies to reduce P-fertilizer application. A genome-wide association mapping approach was used to determine loci and genes associated with root architecture, root traits associated with P-acquisition efficiency and P-use efficiency, and any associated proxy traits. Using three statistical models—a generalized linear model (GLM), a mixed linear model (MLM), and a fixed and random model circulating probability unification (FarmCPU) —10, 51, and 40 marker-trait associations (MTAs), respectively were identified. A single nucleotide polymorphism (SNP) locus (Ca1_12310101) on Ca1 associated with three traits, i.e., physiological P-use efficiency, shoot dry weight, and shoot P content was identified. Genes related to shoot P concentration (NAD kinase 2, dynamin-related protein 1C), physiological P-use efficiency (fasciclin-like arabinogalactan protein), specific root length (4-coumarate–CoA ligase 1) and manganese concentration in mature leaves (ABC1 family protein) were identified. The MTAs and novel genes identified in this study can be used to improve P-use efficiency in chickpea.

Keywords: chickpea, genome-wide association study, phosphorus-acquisition efficiency, phosphorus-use efficiency, root traits, genetic mapping

INTRODUCTION

Phosphorus (P) is an essential nutrient for crop production. Using spatially explicit global maps for more than 100 crops, MacDonald et al. (2011) reported that 29% of the global cropland area is P deficient, while 71% has surplus P. For example, 42% of field soil in India is low in P, 38% is medium and 20% is high (Motsara, 2002). Excessive P fertilizer and manure application in industrialized

countries have led to low P-use efficiency (PUE), with surplus P retained in soil as residual P (Syers et al., 2008) or lost to the environment where it is causing significant water quality problems. Globally 51–86% more P input will be needed for sustainable crop production by 2050 (Mogollón et al., 2018), unless we work toward more P-efficient crops and cropping systems (Cong et al., 2020).

Chickpea (*Cicer arietinum* L.), is one of the most important grain legumes cultivated by smallholder farmers in more than 50 countries. Advances in chickpea genomics in the last decade have made large-scale genomic resources available to the chickpea research community including molecular markers (Thudi et al., 2011; Hiremath et al., 2012), genetic maps (Nayak et al., 2010), genome sequences (Varshney et al., 2013b), and resequencing of several germplasm lines (Thudi et al., 2016a,b; Varshney et al., 2019). These resources have improved our understanding of both abiotic (Varshney et al., 2014) and biotic stress tolerance in chickpea (Sabbavarapu et al., 2013) and enabled fine mapping of traits (Jaganathan et al., 2015; Kale et al., 2015). Furthermore, the resources have been successfully used to develop new varieties with enhanced tolerance or resistance (see Varshney et al., 2013a; Mannur et al., 2019; Bharadwaj et al., 2020; Roorkiwal et al., 2020). Twelve chickpea genotypes with well-known responses under drought and irrigation were evaluated for profuse root length density (RLD) in surface soil and root dry weight (RDW) and root:shoot ratio (RSR) in deeper soil layers (Purushothaman et al., 2017). This study revealed that drought stress increased RLD below 300 mm soil depth, deep RDW, and RSR, but decreased root diameter. Moisture-conservation practices and optimum P levels to enhance PUE were reported recently in chickpea grown in vertisols in central India (Chaudhary et al., 2018). In soybean, root length is positively correlated with P accumulation in well-watered and water-stressed conditions (He et al., 2017). Phenotypic plasticity and genetic variability in root architectural traits of chickpea and their role in drought tolerance using a novel semi-hydroponic system have been reported (Chen et al., 2017). A recent study revealed that root system plasticity affects P acquisition efficiency, PUE, and photosynthetic PUE in 266 chickpea genotypes (Pang et al., 2018c). Root exudates like carboxylate enable P acquisition from the soils which are low in available P. Manganese concentration in mature chickpea leaves is positively correlated with the amount of rhizosheath carboxylates, offering an easily measurable proxy for assessing rhizosheath carboxylates in 100 chickpea genotypes grown under low P availability (Pang et al., 2018a). In the context of global climate change, Pang et al. (2018b) summarized the factors affecting PUE and enhancing P-acquisition efficiency in legumes, and key areas for future research. Understanding the genetics of these traits, identification of genomic regions, molecular markers and or marker trait associations for PUE efficiency related traits will help improving these traits through marker-assisted selection or genomics-assisted breeding.

Various type of molecular markers have been used to establish marker-trait associations (MTA) in chickpea. For instance, 1,072 Diversity Arrays Technology (DArTs), 651 single nucleotide polymorphisms (SNPs), 113 gene-based SNPs, and 36 simple sequence repeats (SSRs) were used to establish 312 MTAs for drought- and heat-tolerance related traits (Thudi et al., 2014). SNPs were used extensively for association studies in chickpea due to their abundance and amenability for high-throughput genotyping (Diapari et al., 2014; Jadhav et al., 2015; Upadhyaya et al., 2016; Varshney et al., 2019; Sab et al., 2020).

In legumes, genome-wide association studies (GWAS) are gaining momentum. A recent study reported two SNP markers tightly linked to seed iron (Fe) and one to seed zinc (Zn) concentration in lentils (*Lens culinaris* Medik.) (Khazaei et al., 2017). Another study reported 159 quantitative trait nucleotides (QTNs) and 52 candidate genes associated with the photosynthetic response to low-P stress in soybean [*Glycine max* (L.) Merr.] (Lü et al., 2018). In chickpea, association mapping between SNP markers and seed copper (Cu), P, and potassium (K) concentrations identified eight SNPs associated with variation in three nutrients in more than two environments (Ozkuru et al., 2018). Similarly, seed mineral concentration in pea (*Pisum sativum* L.; Gali et al., 2019), Fe chlorosis in soybean; Mamidi et al., 2014; Assefa et al., 2020), and Fe bioavailability in cooked dry beans (*Phaseolus vulgaris* L.; Katuuramu et al., 2018) have been mapped. Novel genes involved in the accumulation of P in *Lotus japonicas* have been reported using GWAS analysis (Giovannetti et al., 2019).

In view of above, this study was conducted to undertake GWAS analysis to identify MTAs for (i) root architectural traits evaluated in a high-throughput semi-hydroponic root phenotyping platform, and (ii) root morphological and physiological traits related to P-acquisition efficiency and P−use efficiency under low P supply. This is the first study that reports genomic regions associated with above mentioned traits by using three different models, namely fixed and random model circulating probability unification (FarmCPU), mixed linear model (MLM), and generalized linear model (GLM) in the GAPIT-R package. The reference genome has been used to identify the candidate genes in the identified MTAs associated with above traits.

MATERIALS AND METHODS

Germplasm Lines and Phenotyping

The chickpea reference set (Upadhyaya et al., 2008) comprising of 300 diverse accessions (267 landraces, 13 advanced lines and cultivars, 7 wild *Cicer* accessions, and 13 accessions with unknown biological status) was used for phenotyping datasets as following: (i) 270 genotypes of the reference set were evaluated for 30 root architectural traits in a semi-hydroponic phenotyping system (Chen et al., 2017); (ii) Two hundred and sixty-six genotypes (including 255 from the chickpea reference set along with 11 Australian chickpea cultivars (Ambar, Almaz, Neelam, Genesis 079, Genesis 090, Genesis 509, Genesis 836, Genesis Kalkee, PBA Boundary, PBA Slasher and PBA Striker) were evaluated for P-acquisition efficiency and P−use efficiency with P supplied as insoluble $FePO_4$ (Pang et al., 2018c); and (iii) a selected subset of 100 chickpea genotypes of the reference set—showing visual differences in plant size or leaf symptoms of P deficiency—phenotyped for shoot/root morphological and

physiological traits to understand the relative roles of root morphology and physiology in P-acquisition efficiency (Pang et al., 2018a,c).

Genotyping and Determining Population Structure

The SNP dataset, based on whole-genome resequencing data on the reference dataset was filtered for missing values (≥20%) and minor-allele frequency <5% using vcftools and imputed by BEAGLE-5.0 (Browning and Browning, 2016; Varshney et al., 2019). As the number of genotypes were different in data sets, Population structure was separately determined for the chickpea reference set and 91 genotypes (from the 100 selected subset of 199 genotypes) using ADMIXTURE v1.3.0 (Zhou et al., 2011).

Genome-Wide Association Analysis

GWAS for Root Traits and Seven P-Acquisition Efficiency Traits

To identify significant MTAs and avoid spurious associations of the 270 genotypes mentioned above, 233 genotypes (with phenotyping data and genotyping data) were considered for GWAS analysis. GWAS analysis was performed using 698,183 SNPs (SNP calls obtained based on aligning 233 genotypes to reference genome CDC Frontier) and phenotyping data for 37 traits (i.e., 30 root traits and seven P-acquisition efficiency traits). MTAs were determined using three models, namely fixed and random model circulating probability unification (FarmCPU), mixed linear model (MLM), and generalized linear model (GLM) in the GAPIT-R package (Lipka et al., 2012).

GWAS for Biochemical Traits/Proxy Traits for PUE

A total of 706,865 SNPs and phenotyping data generated on 91 chickpea germplasm lines was for analysis in GAPIT-R using FarmCPU and MLM models to determine MTAs for biochemical traits.

The Bonferroni correction threshold of 7.07E-08 was used to avoid spurious associations. The genes involving significant SNP markers were aligned against the NCBI non-redundant (nr) protein database taxon Viridiplantae using BLASTX, to obtain functional annotations. GO and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway identification were conducted on these sequences in the KEGG pathways in-built in BLAST2GO. The SNPEff- 4.3T open source program was used for variant annotation and prediction of significant SNP effects.

RESULTS

Population Structure and Genome-Wide Association Study (GWAS)

Using genome-wide SNP data for 233 chickpea genotypes (with both genotypic and phenotypic data), three subpopulations were identified (**Supplementary Figures 1A,B**) using ADMIXTURE. Similarly, in the smaller subset of 91 genotypes, three subpopulations were identified (**Supplementary Figures 1C,D**). Three statistical models—GLM, MLM, and FarmCPU—enabled identification of 10, 51, and 40 MTAs, respectively (**Table 1** and **Supplementary Table 1**) after applying Bonferroni and FDR corrections. Spurious MTAs were excluded by examining Q/Q plots. Forty-two of the MTAs identified by the GLM and MLM models were robust (>10% of the phenotypic variation explained); the phenotypic variation explained by the MTAs identified in the FarmCPU model was not computed. The GLM model identified MTAs for four traits (root growth rate, root mass density, specific root length, and shoot P concentration); this low number of traits could be due to the many spurious MTAs (**Supplementary Figure 2**). The FarmCPU model identified MTAs for the most traits, followed by the MLM model.

GWAS Signals for Root Architectural Traits

Fifty-seven MTAs were identified for 19 root architectural traits phenotyped in our earlier studies (Chen et al., 2017; Pang et al., 2018c; **Table 1**). The FarmCPU model identified one significant MTA each in root depth zone 1 (TRL_z1; cm) and root depth zone 2 (TRL_z1; cm) on Ca3 and Ca6 for taproot length (i.e., root depth, z1 + z2), one significant MTA (Ca3_26114159) for root mass (RM, mg), two significant MTAs (Ca4_29694614 and Ca6_57970784) for branch density (BD; cm^{-1} taproot), and one significant MTA (Ca2_6340118) for branch intensity (BI; cm^{-1} root). No significant MTAs were detected for the above root traits in the GLM and MLM methods. Nevertheless, the MLM model identified six significant MTAs and FarmCPU identified one MTA for root mass ratio (RMR), and the MLM and FarmCPU models identified two significant MTAs (one each) for root length ratio (RLR_top/sub) and three significant MTAs (two and one, respectively) for average branch length (ABL; cm per branch). The FarmCPU model identified one significant MTA each for root diameter s2 (RD_s2; mm), subsoil root diameter (RD_sub; mm), and topsoil root diameter (RD_top; mm) on Ca6, Ca1, and Ca2, respectively. The FarmCPU model also identified two significant MTAs for water-use efficiency (WUE; A/g_s) on Ca6 and Ca8. Based on the physical position of the SNP loci (MTAs) associated with different traits on Ca4, none of the MTAs was mapped in "*QTL-hotspot_a*" or "*QTL-hotspot_b*" that harbors several drought-tolerance-related root traits (Kale et al., 2015). The MTAs revealed key root traits for efficiently acquiring soil resources and adapting to drought and other abiotic stresses. Of the 13 significant MTAs (12 from MLM and one from GLM) identified for RGR (cm d^{-1}), six (46.2%) were identified on Ca3 and three (23.1%) on Ca6. Six MTAs identified on Ca6 explained 3.6–10.0% of the phenotypic variation associated with five genes (Ca_08259, Ca_01151, Ca_01152, Ca_01156, and Ca_16553). Both statistical models (GLM and MLM) detected one significant MTA (Ca1_4716136), which explained higher phenotypic variation (11.8–13.1%) than the other MTAs (**Supplementary Table 1**). Eight significant MTAs were identified for specific root length (SRL, cm) in all three models; of these, one MTA on Ca7 (Ca7_3606123), explaining about 17.8% of the phenotypic variation, was consistent in all three models used for GWAS analysis. The MTA was associated with the Ca_03107 gene that encodes pectinesterase/pectinesterase inhibitor PPE8B. Six

TABLE 1 | Summary of marker trait associations identified using three statistical models namely GLM, MLM and FarmCPU.

Trait	Code	GLM			MLM			FarmCPU	
		No. of MTAs	*p*-value	R2	No. of MTAs	*p*-value	R^2	No. of MTAs	*p*-value
Average branch length	(ABL; cm per branch)	–	–	–	2	5.92×10^{-8} to 3.69×10^{-8}	5.01–8.24	1	5.02×10^{-8}
Branch density	(BD; cm^{-1} taproot)	–	–	–	–	–	–	2	6.41×10^{-10} to 2.67×10^{-10}
Branch intensity	(BI; cm^{-1} root)	–	–	–	–	–	–	1	7.51×10^{-9}
Root diameter s2	(RD_s2; mm)	–	–	–	–	–	–	1	5.14×10^{-9}
Subsoil root diameter	(RD_sub; mm)	–	–	–	–	–	–	1	7.2×10^{-14}
Topsoil root diameter	(RD_top; mm)	–	–	–	–	–	–	1	7.46×10^{-12}
Root growth rate	(RGR; cm d^{-1})	1	2.2×10^{-8}	13.13	12	5.62×10^{-15} to 1.9×10^{-8}	9.2–11.78	–	–
Root length ratio	(RLR_top/sub)	–	–	–	–	–	–	1	1.06×10^{-8}
Root length s2	(RL_s2; cm)	–	–	–	–	–	–	1	1.59×10^{-8}
Root mass	(RM; mg)	–	–	–	–	–	–	1	3.86×10^{-8}
Root mass ratio	RMR	–	–	–	6	4.4×10^{-25} to 1.73×10^{-8}	20.86–30.12	1	3.8×10^{-8}
Root_2	Root diameter (mm)	–	–	–	1	1.77×10^{-12}	7.52	–	–
Root tissue density	(RTD; mg cm^{-3})	2	2.88×10^{-9} to 6.73×10^{-8}	14.23 to 16.95	–	–	–	2	7.18×10^{-15} to 4.58×10^{-10}
Shoot dry weight	SDW (mg)	–	–	–	–	–	–	1	6.31×10^{-9}
Specific root length	(SRL; m g^{-1} dry mass)	3	2.48×10^{-9} to 5.94×10^{-8}	17.2 to 20.31	2	1.88×10^{-10} to 1.44×10^{-9}	17.63	3	2.99×10^{-11} to 4.06×10^{-9}
Taproot length zone1	(TRL_z1; cm)	–	–	–	1	7.15×10^{-8}	13.59	1	7.15×10^{-8}
Taproot length zone2	(TRL_z2; cm)	–	–	–	1	1.48×10^{-11}	9.64	1	1.48×10^{-11}
WUE	WUE (A/gs)	–	–	–	–	–	–	2	2.80×10^{-11} to 1.85×10^{-8}
Total rhizo dry soil (g plant-1)	Total rhizo (g $plant^{-1}$)	–	–	–	6	5.66×10^{-17} to 1.34×10^{-11}	5–17.63	–	–
Phosphorous-utilization efficiency		–	–	–	13	4.45×10^{-18} to 8.41×10^{-15}	20.23–26.16	1	2.13×10^{-9}
Physiological P-use efficiency	PPUE (μmol g^{-1} P s^{-1})	1	5.46519E-08	11.95	–	–	–	–	–
Shoot phosphorous concentration	mg g^{-1}	3	2.85×10^{-9} to 7.65×10^{-8}	27.67 to 40.33	2	5.23×10^{-9} to 2.85×10^{-9}	27.69–32.97	–	–

(Continued)

TABLE 1 | Continued

Trait	Code	GLM			MLM			FarmCPU	
		No. of MTAs	*p*-value	R2	No. of MTAs	*p*-value	R^2	No. of MTAs	*p*-value
Shoot P content	Shoot P content	–	–	–	5	8.2×10^{-9} to 7.7×10^{-8}	39.41.3	1	1.68×10^{-13}
Carboxylate_2	Carboxylate conc (μmol g^{-1} root DW)	–	–	–	–	–	–	1	5.93×10^{-8}
Ci	Ci	–	–	–	–	–	–	1	8.52×10^{-10}
Citric_2	Citric (μmol $plant^{-1}$)	–	–	–	–	–	–	1	1.42×10^{-9}
Malonic (μmol $plant^{-1}$)	Malonic (μmol $plant^{-1}$)	–	–	–	–	–	–	1	4.82×10^{-10}
Mn concentration in mature leaves	Mn_ML	–	–	–	–	–	–	7	6.08×10^{-21}
P_ML	P_ML (mg g^{-1})	–	–	–	–	–	–	1	6.08×10^{-21}
P_Pn	Pn_area (μmol m^{-2} s^{-1})	–	–	–	–	–	–	1	2.6×10^{-8}
Pn_mass	Pn_mass (μmol g^{-1} s^{-1})	–	–	–	–	–	–	1	3.57×10^{-9}
Rhizo-pH		–	–	–	–	–	–	2	1.72×10^{-15} to 2.24×10^{-8}
Specific rhizosheath weight	(g g^{-1} root DW)	–	–	–	–	–	–	1	2.97×10^{-8}
Total MTAs		10			51			40	

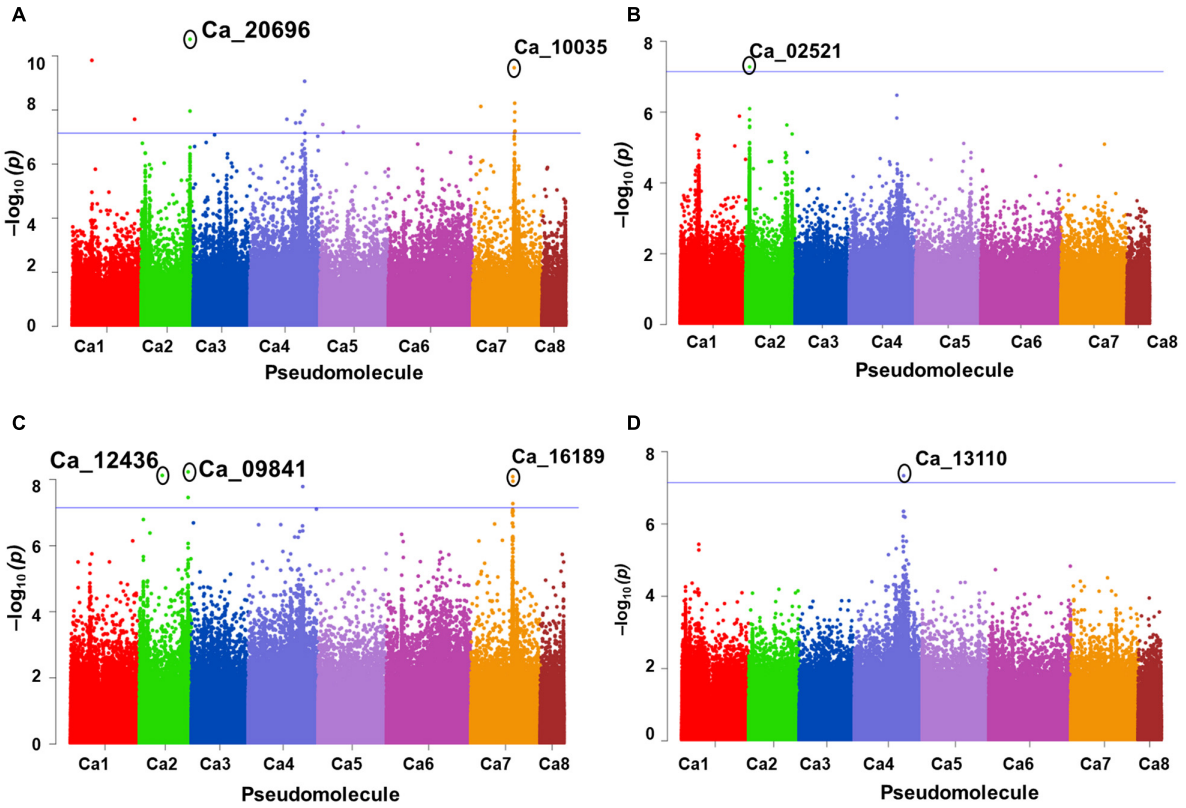

FIGURE 1 | GWAS signals for phosphorus (P) -acquisition and P-use efficiency-related traits. **(A)** Shoot P concentration, **(B)** physiological P-use efficiency, **(C)** P-utilization efficiency, and **(D)** total shoot P content. Three different statistical models GLM, MLM, and FarmCPU were used to identify the MTAS. The significant MTAs were determined using Bonferroni correction (**Table 1**).

MTAs for total rhizosheath dry soil (g plant^{-1}) were identified on Ca8 (3), Ca5 (1), Ca2 (1), and Ca1 (1).

GWAS Signals for P-Acquisition Efficiency and PUE-Related Traits

A total of 10 significant MTAs (three based on GLM and seven based on MLM) for shoot P content—six on Ca4, two on Ca6 and one each on Ca7 and Ca2 were identified (**Figure 1A**). Two significant MTAs on Ca4 (Ca4_38518152 and Ca4_8269508) were identified with the GLM and MLM models (**Supplementary Table 1**). The 15 MTAs identified—14 from MLM and one from FarmCPU—explained 5–21% of the phenotypic variation. Five of the MTAs were identified on Ca6 followed by Ca7 (4), Ca4 (2), Ca2 (2), Ca1 (1), and C3 (1) (**Figure 1B**). A SNP locus, Ca7_33808891, associated with physiological P-Use efficiency present in gene Ca_16189 on Ca7 explained 20.23% PVE (**Figure 1C**; **Supplementary Table 1**). A SNP locus, Ca4_38518152, associated with Shoot phosphorus content present in gene Ca_13110 on Ca4 explained 31.5% PVE (**Figure 1D**; **Supplementary Table 1**). In the case of physiological PUE one significant MTA (Ca1_12310101) on Ca1 explaining 12.0% of the phenotypic variation was identified. A single nucleotide polymorphism locus (Ca1_12310101) on Ca1 associated with three traits, i.e., physiological P-use efficiency, shoot dry weight, and shoot P content was identified (**Figure 2**).

GWAS Signals for Proxy Traits

One significant MTA for P concentration in mature leaves (P_ML; mg g^{-1}) was identified on Ca4. Seven significant MTAs for Mn concentration in mature leaves were identified on Ca2 (3), Ca4 (2), and Ca7 (**Figure 3**). The SNP loci on Ca7 (Ca7_32383349) and Ca4 (Ca4_1791932) were associated with manganese concentration in mature leaves with two different *p*-values (**Figure 3**), while three SNPs associated with the Mn_ML on Ca2 (Ca2_7561143, Ca2_866639, and Ca2_359984). One MTA each for citric (μmol plant^{-1}), Ci, carboxylate conc (μmol g^{-1} root dw), malonic (μmol plant^{-1}), Pn_area (μmol m^{-2}s^{-1}), Pn_mass (μmol g^{-1} s^{-1}), specific rhizosheath weight (g g^{-1}root DW) were identified using FarmCPU. No significant MTAs were identified for these traits using GLM and MLM models.

DISCUSSION

Understanding the genetics of traits associated with enhanced PUE, including P-acquisition efficiency and P-use efficiency, is essential for its manipulation. In our earlier studies on chickpea, we gained insight into root system architecture, shoot/root traits

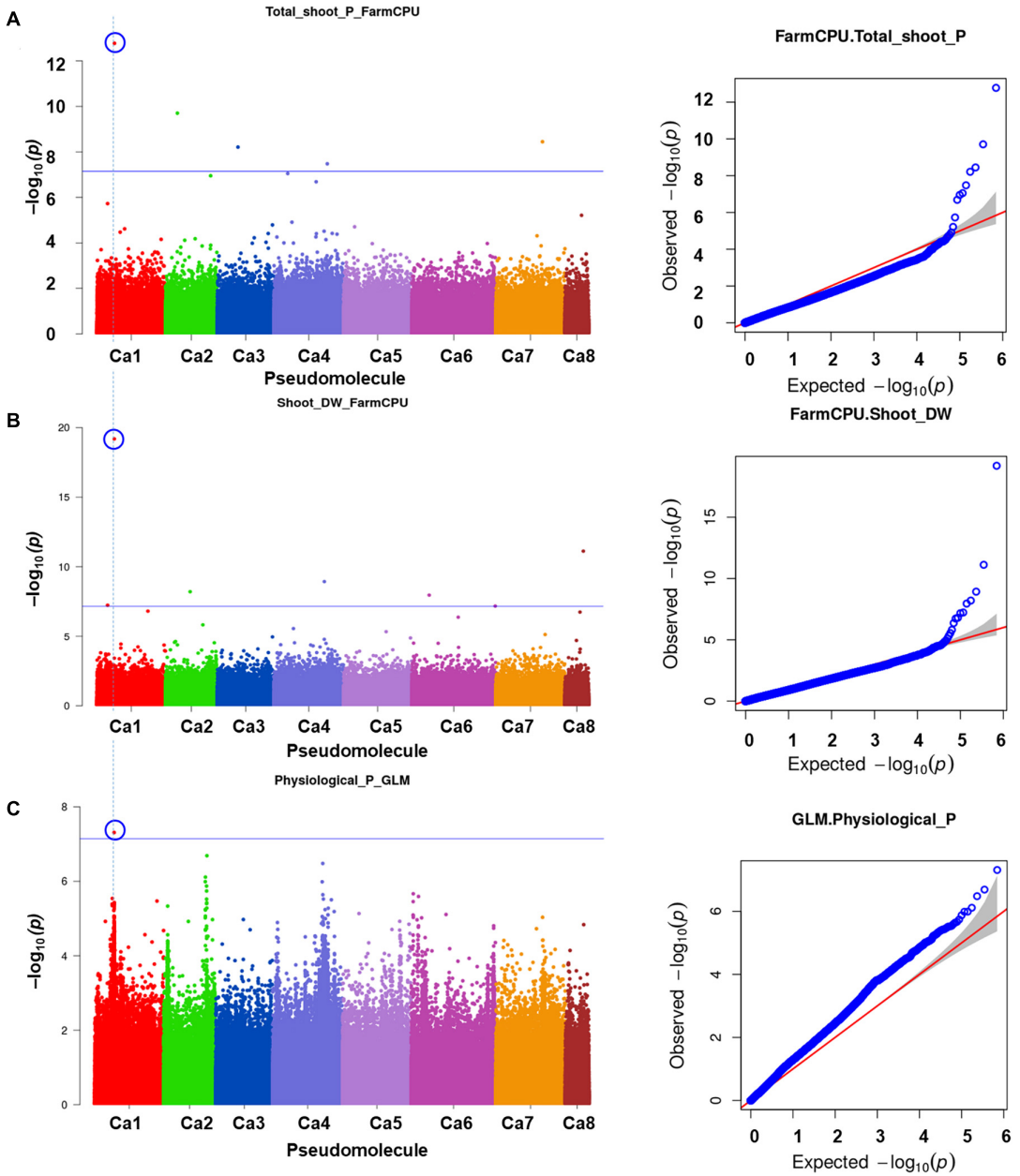

FIGURE 2 | An SNP locus (Ca1_12310101) on Ca1 showing association with three traits **(A)** physiological phosphorus (P)-use efficiency, **(B)** shoot dry weight, and **(C)** shoot P content. Three different statistical models GLM, MLM, and FarmCPU were used to identify the MTAS. The significant MTAs were determined using Bonferroni correction (**Table 1**).

associated with P-acquisition efficiency and P-use efficiency, and associated proxy traits. Many SNPs associated with root-hair length in P-sufficient and P-deficient conditions have been reported recently (Kohli et al., 2020); here we report SNPs associated with PUE and P-acquisition efficiency related root traits as well as proxy traits, which can be deployed for breeding.

As population structure is important for avoiding spurious associations, we identified three subpopulations using ADMIXTURE in the reference set (233 genotypes) and a smaller subset (91 genotypes). Similarly, earlier studies reported three subpopulations using different marker systems (Thudi et al., 2014) and genome-wide SNP markers (Varshney et al., 2019). In the smaller subset of 91 genotypes, we also identified three subpopulations (**Supplementary Figures 1A–D**). Four subpopulations were recently reported in a diverse set of 186 genotypes (including 20 Iranian landraces and 166 Kabuli advanced breeding lines from ICRISAT and ICARDA) using DArTseq markers (Farahani et al., 2019). Two subpopulations were reported in a set of 92 (77 landraces and five elite cultivars) chickpea germplasm lines that represent arid, semi-arid, and

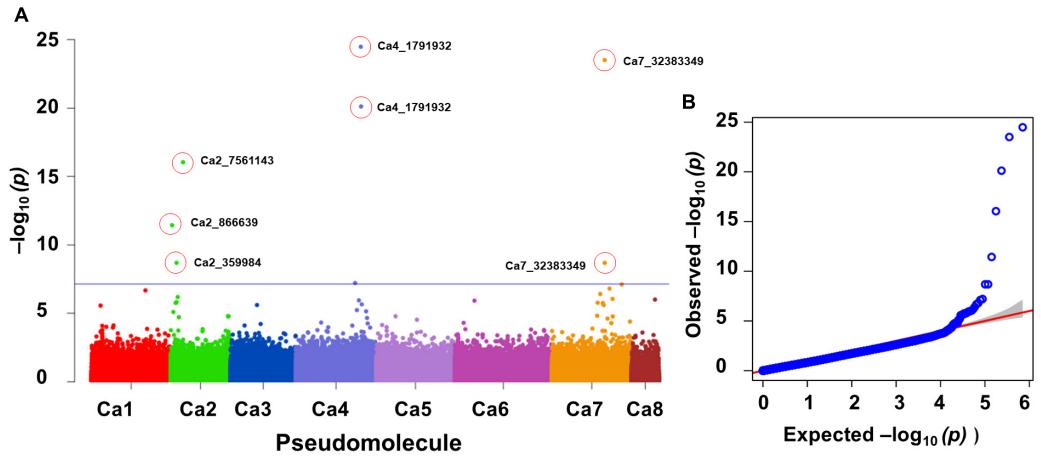

FIGURE 3 | GWAS signal for manganese concentration in mature leaves. Of seven MTAs identified, three on Ca2 were unique and two each on Ca4 and Ca7 were the same SNP loci associated with the trait at different significance levels. **(A)** Manhattan plot showing significant MTAs and **(B)** Q-Q plots for the trait. Three different statistical models GLM, MLM, and FarmCPU were used to identify the MTAS. The significant MTAs were determined using Bonferroni correction (**Table 1**).

tropical climates using 8,113 genotyping-by-sequencing based SNPs (Sani et al., 2017).

Three statistical models (GLM, MLM, and FarmCPU) were used to identify genome-wide association signals for root architectural traits, P-acquisition efficiency and PUE-related traits. Similarly, a recent study used different statistical models to compare selected traits with different heritabilities in soybean and maize—the FarmCPU model provided a closer number of QTL than those in the literature and known genomic regions (Kaler et al., 2020). In this study, identification of significant MTAs and determining their usefulness for chickpea improvement was a major focus, rather than comparative utilities of different statistical models. In the present study, significant MTAs for 17 of the 30 root traits analyzed was reported. Earlier, in chickpea, MTAs for drought-tolerance-related root traits were based on screening a reference set in polyvinyl chloride pipes or semi-automated root screening facility at ICRISAT (Thudi et al., 2014; Varshney et al., 2019). In this study, MTAs for root traits studied under semi-hydroponic conditions and the role of root and shoot traits in P-acquisition efficiency and PUE are reported.

The MTA (Ca1_4716136) identified for RGR, is present in a gene (Ca_00555) that encodes a receptor-like cytosolic serine/threonine-protein kinase RBK2 involved in protein phosphorylation in *Arabidopsis thaliana*[1]. Rac-like GTP-binding protein (ARAC5), with 87.8% homology, is localized on the plasma membrane of *Arabidopsis thaliana* root tips. The Ca_01156 gene encodes Acyl-CoA-binding domain-containing protein 4; in *Arabidopsis thaliana*; overexpression of Acyl-CoA -binding protein 3 (ACBP3) results in leaf senescence (Xiao et al., 2010). In chickpea, the pectinesterase gene was downregulated in the roots of salt-tolerant genotypes (Kaashyap et al., 2018). Pectinestarases also play a key role in root-hair initiation and elongation (Cosgrove, 2016). Similarly, another MTA on Ca1 (Ca1_8712480) explained about 20% of the phenotypic variation detected in the GLM and MLM models. The MTA was present in the Ca_02941 gene that encodes 4-coumarate–CoA ligase 1. In tobacco, root length increased by 64% compared with the wildtype, on overexpression of *Fm4CL-like 1* [4-coumarate:CoA ligase 4 (*4CL-like 1*) from *Fraxinus mandshurica*] under mannitol-simulated drought stress (Chen et al., 2019).

Two MTAs (Ca4_38518152 and Ca4_8269508) for shoot P content were present in two genes, Ca_13110 and Ca_08315, which encode NAD kinase 2, chloroplastic-like isoform X2 and piezo-type mechanosensitive ion channel homolog isoform X2, respectively. NAD kinase 2 is involved in phosphorylation. In general, NADK genes show tissue specificity in expression. In *Arabidopsis*, *NADK2* is expressed in leaves, while *TaNADK2* is highly expressed in wheat pistils, caryopses, and endosperm during the reproductive stage (Li et al., 2018). All MTAs for shoot P content were robust and explained 27.7–41.4% of the phenotypic variation. QTL for shoot P content and PUE were located on chromosomes 3 and 4, respectively (Hammond et al., 2009). Of the five MTAs on Ca6, two were in the same gene (Ca_10411), encoding dynamin-related protein 1C. The MTA identified in the FarmCPU model, Ca1_16163105 on Ca1, was present in the Ca_06938 gene that encodes organic cation/carnitine transporter 4-like (OCT), which is involved in homeostasis in animals and has been well-studied in *Arabidopsis thaliana*—disruption of *AtOCT1* affects root development (Lelandais-Brière et al., 2007). Four major pathways in *Lupinus albus* that contribute to PUE are carbon fixation, cluster-root formation, soil P mobilization, and cellular P reuse (Xu et al., 2020). A recent effort to understand the genetic basis of photosynthesis and PUE affecting yield reported that three major QTL (*q14-2, q15-2*, and *q19-2*) explained 6.6–58.9% of the phenotypic variation (Li et al., 2016). Furthermore, the gene that encodes purple acid phosphatase within the *q19-2* region (*Glyma.19G193900*) is a potential candidate for regulating both soybean PUE and photosynthetic capacity. A total of 159 QTNs within 31 genomic regions and genes associated

[1]https://www.uniprot.org/uniprot/Q8RXC8

with photosynthesis-related traits under P stress conditions were genotyped in 2,019 soybean accessions using 292,035 high-quality SNPs and phenotyped under adequate- and low-P conditions for 2 years (Lü et al., 2018). The MTA (Ca1_12310101) for physiological PUE is present in a gene that encodes fasciclin-like arabinogalactan protein 12 (Ca_02521); in *Arabidopsis*, this gene is involved in cell wall biogenesis (**Figure 2C**). Further, this SNP locus was associated with total shoot P content and shoot DW in different models (**Supplementary Table 1** and **Figure 2**). Such shared associations were also reported using different models in mungbean PUE (Reddy et al., 2020). A recent study reported that low expression of selected fasciclin-like arabinogalactan protein genes led to kernel abortion in maize (*Zea mays*) and *Arabidopsis thaliana* seeds (Cagnola et al., 2018). In cereals such as rice, SNP loci on chromosomes 1, 4, 11, and 12 are associated with PUE (Wissuwa et al., 2015).

Pang et al. (2018a) reported that root foraging and root physiology, such as the exudation of carboxylates into the rhizosphere, are important strategies for plant P acquisition efficiency. A positive correlation was also identified between mature leaf Mn concentration and rhizosheath carboxylate amount relative to root DW, and hence the carboxylate-releasing P-mobilizing strategy was proxied by foliar Mn concentration in a large set of chickpea germplasm under low P supply (Pang et al., 2018a). The MTA for Mn concentration in mature leaves identified on Ca4 was in gene Ca_14893 that encodes zinc finger BED domain-containing protein RICESLEEPER 2-like protein. Carboxylate exudation is an important physiological root trait that enables plants to mine soil P (Lambers et al., 2008, 2015; Richardson and Simpson, 2011). Carboxylate concentrations in the rhizosheath are positively correlated with shoot P content (Pang et al., 2018a). A SNP locus associated with carboxylate amount was identified in the rhizosheath (μmol g^{-1} root DW) associated with a Ca_11019 gene that encodes for ABC1 family protein, and is an integral part of the membrane. The FarmCPU model identified two significant MTAs (Ca4_15651907 and Ca8_230753) in RhizoPH.

CONCLUSION

In summary, the SNP loci associated with more than one trait were identified. For instance, Ca1_12310101 on Ca1 is associated with three traits (i.e., physiological PUE, shoot DW, and shoot P content), Ca2_31290805 on Ca2 is associated with P utilization and total root length, Ca4_37796452 on Ca4 is associated with RMR and shoot P content, Ca3_6798755 on Ca3 is associated with RGR and TRLz1, and Ca7_5414752 on Ca7 is associated with leaf intracellular CO_2 concentration and WUE. The MTA for Mn in mature leaves identified on Ca4 was in gene Ca_14893 that encodes zinc finger BED domain-containing protein RICESLEEPER 2-like protein. The MTAs reported in this study can be used in chickpea breeding programs to enhance PUE.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: CNSA (https://db.cngb.org/cnsa/) of CNGBdb with accession code CNP0000370.

AUTHOR CONTRIBUTIONS

KHMS and RKV conceived the study. MT and DK performed GWAS. YC and JP generated the phenotyping data. PB, MRo, AC, MRy, and HL contributed to the resources and the writing. MT, RKV, and KHMS prepared the manuscripts. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.636973/full#supplementary-material

Supplementary Figure 1 | Example of spurious associations detected for taproot length (TRL, mm) in the GLM model, with no associations detected in the MLM and FarmCPU models.

Supplementary Figure 2 | Population structure. **(A,B)** Three sub-populations among 233 genotypes phenotyped for root and phosphorus-related traits, and **(C,D)** three sub-populations among 91 genotypes phenotyped for shoot/root morphological and physiological traits. Each colored vertical line represents proportions of ancestral populations (K) for each individual. Optimum K value determined using ADMIXTURE's cross-validation procedure.

Supplementary Table 1 | Details of significant MTAs with different traits and functional annotation of SNP loci.

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ORIGINAL RESEARCH
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Australian Lentil Breeding Between 1988 and 2019 Has Delivered Greater Yield Gain Under Stress Than Under High-Yield Conditions

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The contemporary lentil (*Lens culinaris* ssp. *culinaris*) industry in Australia started in the late 1980s. Yield in farmers' fields averages 1.2 t ha^{-1} nationally and has not increased over three decades. Lack of yield progress can be related to a number of non-mutually exclusive reasons: expansion of lentil to low-yielding environments, lack of genetic gain in yield, lack of progress in agronomic practices, and lack of adoption of superior technologies. The aims of this study were to (i) quantify the genetic gain in lentil yield since 1988, (ii) explore the variation in the expression of genetic gain with the environment, and (iii) identify shifts in crop phenotype associated with selection for yield and agronomic adaptation. We grew a historic collection of 19 varieties released between 1988 and 2019 in eight environments resulting from the factorial combination of two sowing dates, two water regimes, and two seasons. Across environments, yield varied 11-fold from 0.2 to 2.2 t ha^{-1}. The rate of genetic gain averaged 20 kg ha^{-1} year^{-1} or 1.23% year^{-1} across environments and was higher in low-yield environments. The yield increase was associated with substantial shifts in phenology. Newer varieties had a shorter time to flowering and pod emergence, and the rate of change in these traits was more pronounced in slow-developing environments (e.g., earlier sowing). Thermal time from sowing to end of flowering and maturity were shorter in newer varieties, and thermal time from pod emergence to maturity was longer in newer varieties; the rate of change in these traits was unrelated to developmental drivers and correlated with environmental mean yield. Genetic gain in yield was associated with increased grain number and increased harvest index. Despite their shorter time to maturity, newer varieties had similar or higher biomass than their older counterparts because crop growth rate during the critical period increased with the year of release. Genotype-dependent yield increased over three decades in low-yield environments, whereas actual farm yield has been stagnant; this suggests an increasing yield gap requiring agronomic solutions. Genetic improvement in high-yield environments requires improved coupling of growth and reproduction.

Keywords: crop growth rate, biomass, genetics, harvest index, phenology, phenotype

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INTRODUCTION

Australia currently produces over 300,000 t of lentils annually and contributes to approximately 10% of global trade, whereas Canada produces over 3 Mt and accounts for 50% of trade. The contemporary lentil industry in Australia started in the late 1980s with the introduction of late flowering, low-yielding forage types, and after a lag phase, acreage increased linearly since the mid-1990s (**Figure 1A**). Production increased in parallel to acreage (**Figure 1B**), whereas national average yield remained stagnant at 1.2 t ha^{-1}, with large variation from failed crops to ∼2 t ha^{-1} (**Figure 1C**). In comparison, the acreage of the Canadian lentil industry grew exponentially since its inception, and increases in both acreage and yield contributed to an increase in production (**Figures 1D–F**).

Lack of progress in lentil average national yield in Australia can be related to several non-mutually exclusive reasons: expansion of the crop to drier, lower-yielding environments; lack of genetic improvement in yield; lack of progress in agronomic practices; and lack of adoption of superior technologies. Most of the Australian lentil is grown in the medium rainfall areas (350–450 mm year^{-1}) of southern Australia, in particular, the sandy loam soils in South Australia and the alkaline gray cracking clays of Victoria. These regions feature winter-dominant rainfall, with a combination of drought, frost, and heat restricting the yield of pulses (Sadras et al., 2012; Lake et al., 2016, 2021). Supported by better agronomy (Llewellyn et al., 2012), pulses in the Mallee have increased from 7% in 2006 to 24% in 2017; this increase was at the expense of fallow, which declined from 18 to 2%, and pasture, which declined from 18 to 12% (Moodie and Brand, 2019). In comparison with the more productive Wimmera (440 mm year^{-1})[1], where lentil yield can reach more than 4.5 t ha^{-1}, yields in the Mallee (300 mm year^{-1})[2] are up to ∼3.5 t ha^{-1}. Hence, expansion of the crop into drier areas has likely contributed to stagnant national average yield. A strong focus on lentil herbicide tolerance to improve weed management may have also had indirect consequences for yield (Mao et al., 2015; McMurray et al., 2019).

Here, we focus on genetic improvement. Despite recognized limitations, retrospective studies comparing historic collections of varieties are routinely used with two objectives—to quantify the rate of genetic gain of a given breeding program and to uncover phenotypic changes associated with selection for yield (Austin et al., 1980; Slafer, 1994; Fischer et al., 2014; Tamagno et al., 2020). The assumption underlying the second objective is that making explicit the realized phenotypic change can guide further improvement. The absolute rate of genetic gain (kilograms per hectare per year) is often higher in environments with higher yield potential (Austin et al., 1980; Sadras et al., 2016), whereas the relative rate of genetic gain (percentage per year) is mostly independent of the environment (Fischer et al., 2014); quantifying the environmental influence on the expression of genetic gain in yield is thus important. The aims of this study were to (i) quantify the genetic gain in lentil yield since 1988, (ii) explore variation in the expression of genetic gain with the environment, and (iii) identify shifts in the crop phenotype associated with selection for yield and agronomic adaptation.

[1]http://www.climatekelpie.com.au/wp-content/uploads/2019/10/040-Wimmera-VIC-Climate-Guide.pdf

[2]http://vro.agriculture.vic.gov.au/dpi/vro/vrosite.nsf/pages/rainfall#:$\sim$:text=Median%20annual%20rainfall%20ranges%20from,parts%20of%20the%20mountainous%20regions

MATERIALS AND METHODS

Experimental Design, Varieties, and Environments

We reanalyze the results of experiments reported by Lake and Sadras (2021), including 19 varieties released and used in the Australian lentil breeding program between 1988 and 2019 (**Table 1**). Crops were grown in eight environments with an 11-fold variation in yield from 0.2 to 2.2 t ha^{-1}. Lake and Sadras (2021) emphasized yield components from a physiological perspective; here, we focus on yield and phenotypic shifts with the year of release.

Trials were established on a calcic luvisol soil at Roseworthy (−34.5, 138.69). Briefly, environments resulted from the combination of two seasons (2018, 2019), two sowing dates, and two water regimes. Early sowings were on April 24, 2018, and April 29, 2019, and the late sowings on June 6, 2018, and June 24, 2019. Early-sown crops were irrigated or rainfed until June 26, 2018, and August 1, 2019, when rainout shelters were deployed to exclude rainfall until harvest, whereas late-sown crops were irrigated or rainfed. Hereafter, we refer to irrigated treatment as "wet" and rainfed and rainout shelter treatments as "dry." Sowing date was assigned to the main plot, water regime to subplot, and varieties randomized within subplots with three replicates per treatment. Each experimental plot comprised six rows, 0.23 m apart, 5 m long, with a target plant density of 120 plants m^{-2}.

Phenology, Yield, Biomass, Crop Growth Rate, and Harvest Index

Crops were phenotyped for phenology, crop growth rate, yield, and its components: biomass, harvest index, grain number, and grain size.

We scored phenology twice weekly to determine the time from sowing (S) to 50% of the plants within the plot at flowering (F), pod emergence (PE), end of flowering (EoF), and maturity (M). Phenological stages are expressed on a thermal time scale with a base temperature of 0°C (Summerfield et al., 1985). The ratio PE-M:S-M was taken as a measure of the grain filling period in relation to the total cycle.

We measured biomass and crop growth rate non-destructively using the Canopeo app (Patrignani and Ochsner, 2015), which provides a two-dimensional measure of canopy coverage, combined with canopy height to return a three-dimensional trait. We used a calibration derived from a separate trial, in which we regressed actual biomass *vs*. Canopeo × height. Canopeo

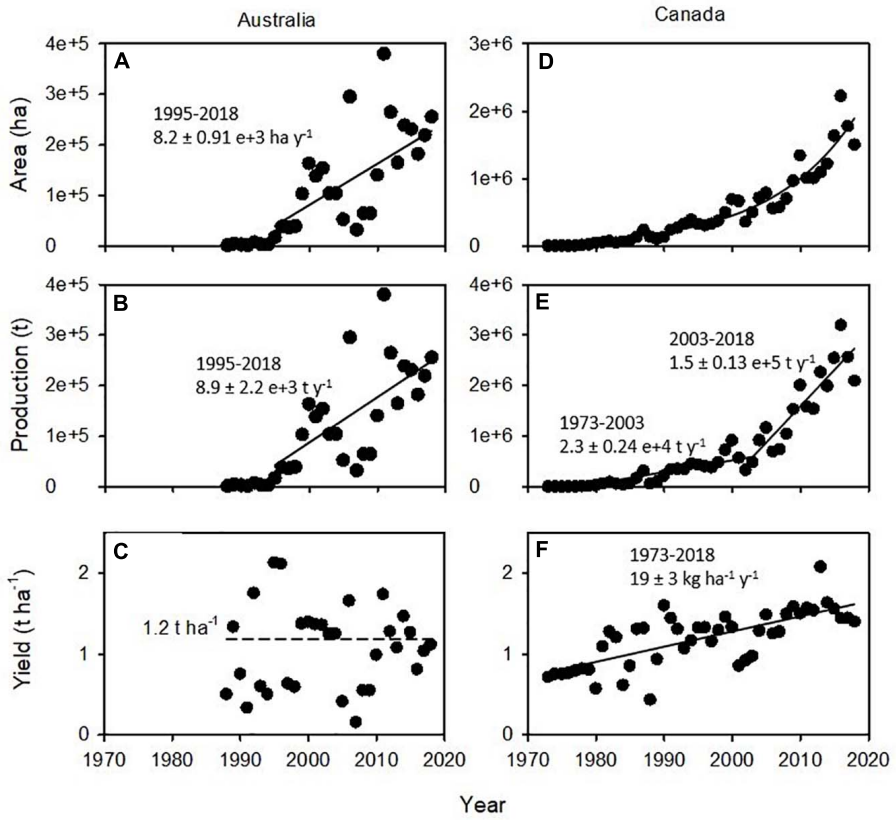

FIGURE 1 | Area, production, and yield of lentils in **(A–C)** Australia and **(D–F)** Canada. In **(A,B,E,F)**, slopes and standard errors are shown for the fitted least-square regressions. In **(A,B,E)**, inflection points were identified fitting piece-wise models. Note difference in scales between Australia and Canada for area and production. Source: FAOSTAT, July 2020.

photographs were taken looking down from 140 cm every 7–10 days.

At maturity, we harvested shoots in 1-m^2 sections from the four central rows of the plot to determine grain yield and its components. Harvest index was derived from shoot biomass and grain yield. Further details of methods are in Lake and Sadras (2021).

Data Analysis

We tested trait response to variety, environment, and the interaction using analysis of variance with Genstat (20th edition). Best linear unbiased predictions were calculated with Multi Environment Trial Analysis with R for Windows version 6.0. We calculated the genetic rate of change as the slope of the least-square regression between trait and year of release. We calculated actual rates, e.g., kilograms per hectare per year for yield, and rates relative to the newest variety (Fischer et al., 2014). Rates were calculated for data pooled across all environments and for each environment separately. Environmental dependence in the expression of genetic shifts in yield and other traits was explored by plotting the rate of genetic change against the environmental mean of yield and the environmental mean of the trait. We report p-value as a continuous quantity and Shannon information transform [$s = -\log_2(p)$] as a measure of the information against the tested hypothesis (Greenland, 2019).

RESULTS

Growing Conditions

Table 2 summarizes growing conditions and yield in the eight environments. Growing-season rainfall + irrigation ranged from 117 mm for the early-sown, dry crop in 2018, to 332 mm for the early-sown, wet crop in 2019. Across varieties, yield ranged from 21 g m^{-2} for early-sown, dry treatment in 2018, to 221 g m^{-2} for early-sown, wet treatment in 2018. Across varieties, average yield was positively associated with growing season rainfall ($y = -18.1 + 0.59\ x$, $R^2 = 0.50$; $p = 0.052$, $s = 4.3$) and with minimum temperature ($y = -90.8 + 38.2\ x$, $R^2 = 0.69$; $p = 0.010$, $s = 6.6$).

Phenology

All phenostages varied with variety, environment, and their interaction (**Supplementary Table 1**). **Table 3** shows absolute and relative rates of change of phenological traits for the pooled data. Across environments, thermal time from sowing to flowering,

pod emergence, end of flowering, and maturity were all shortened with the year of release. In contrast, the thermal time between pod emergence and maturity and the proportion of the season between pod emergence and maturity both increased with the year of release.

Figure 2 shows the rate of change of phenological traits with the year of release as a function of (a) the environmental mean for the trait and (b) the environmental mean for yield. The environmental mean of the trait captures temperature, photoperiod, and water influences on development, empirically defining slow- and fast-developing environments. For example, the environmental mean thermal time to flowering ranged from 1039°Cd in the late-sown wet treatment 2019 to 1451°Cd in the early-sown wet treatment in 2019 (**Table 2**). The rates of change in thermal time to flowering and to pod emergence were stronger, i.e., more negative, in environments favoring slower development (**Figures 2A,C**). For example, the rate of change in flowering changed from −1.20 to −0.46% year^{-1} with environmental means from 1411 to 1167°Cd. The rates of change in thermal time to flowering and maturity were proportional to environmental mean yield (**Figures 2F,H,J**) and unrelated to the environmental mean of the phenostage (**Figures 2E,G,I**). Thermal time from pod emergence to maturity relative to thermal time from sowing to maturity was related to the environmental mean for both duration of phenostage and yield (**Figure 3**).

Yield and Its Components

Yield varied ninefold with variety (**Table 1**) and 10-fold with environment (**Table 2**), with no interaction between environment and variety (**Supplementary Table 1**). Across environments, yield increased with the year of release at 20 kg ha^{-1} year^{-1} or 1.23% year^{-1} (**Table 3**). The rate of genetic gain in yield declined linearly with increasing environmental mean yield (**Figure 4A**).

Grain number varied fourfold with variety and 10-fold with the environment, with a significant interaction between environment and variety (**Supplementary Table 1**). Across environments, grain number increased with the year of release at 34 seeds m^{-2} year^{-1} or 0.92% year^{-1} (**Table 3**). The rate of change in grain number with the year of release was higher in low-yielding environments (**Figure 4B**). Grain size varied with variety (twofold) and with the interaction between environment and variety (**Supplementary Table 1**). Across environments, grain size increased by 0.40 mg seed year^{-1} or 0.96% year^{-1} (**Table 3**). The rate of genetic change in grain size was unrelated to environmental mean yield (**Figure 4C**).

Shoot biomass at maturity varied little between varieties (<1.5-fold) and varied ∼5-fold with environment, with no interaction between environment and variety (**Supplementary Table 1**). Across environments, the absolute rate of change in biomass with the year of release was close to zero, and the relative rate was 0.38% year^{-1} (**Table 3**). The association between the

TABLE 1 | Seed type, phenology, and yield of 19 lentil varieties.

Variety[a]	Type	Year of release	Thermal time from sowing to (°Cd)				Yield (g m^{-2})
			Flowering	Pod emergence	End of flowering	Maturity	
Indianhead[b]	Red	1988	1546 ± 81.0	1679 ± 56.8	1940 ± 70.5	2193 ± 73.8	19 ± 6.9
Matilda	Green	1993	1273 ± 64.2	1374 ± 38.5	1706 ± 72.5	2034 ± 87.0	120 ± 17.1
Aldinga	Red	1995	1315 ± 68.7	1451 ± 42.0	1761 ± 68.0	2094 ± 76.4	129 ± 16.8
Northfield	Red	1995	1368 ± 80.9	1515 ± 51.9	1751 ± 64.9	2080 ± 78.6	129 ± 23.7
Nugget	Red	2000	1296 ± 70.0	1431 ± 45.3	1726 ± 67.0	2033 ± 84.4	99 ± 13.9
Boomer	Green	2008	1251 ± 53.3	1360 ± 34.8	1736 ± 68.3	2046 ± 78.9	101 ± 10.4
Nipper	Red	2008	1346 ± 78.9	1469 ± 46.6	1746 ± 68.7	2045 ± 82.0	128 ± 18.7
PBA Flash	Red	2009	1272 ± 58.3	1371 ± 36.9	1728 ± 66.2	2041 ± 76.3	140 ± 19.5
PBA Blitz	Red	2010	1096 ± 31.9	1236 ± 23.8	1602 ± 44.3	1969 ± 82.7	131 ± 14.5
PBA Jumbo	Red	2010	1275 ± 64.2	1396 ± 39.7	1722 ± 64.2	2022 ± 78.2	146 ± 22.7
PBA Ace	Red	2011	1208 ± 45.7	1321 ± 27.8	1717 ± 68.6	2008 ± 80.9	116 ± 14.2
PBA Bolt	Red	2011	1191 ± 44.3	1320 ± 27.6	1693 ± 61.3	2028 ± 80.3	141 ± 14.7
CIPAL0901[c]	Red	2013	1130 ± 38.5	1258 ± 26.7	1637 ± 55.1	1983 ± 85.6	153 ± 15.2
PBA Hurricane	Red	2013	1225 ± 45.6	1337 ± 32.8	1679 ± 59.7	2028 ± 77.0	124 ± 16.3
PBA Giant	Green	2014	1168 ± 42.5	1289 ± 28.2	1706 ± 66.7	2025 ± 77.7	97 ± 11.5
PBA Greenfield	Green	2014	1249 ± 49.9	1375 ± 33.0	1742 ± 64.7	2046 ± 76.1	110 ± 20.4
PBA Jumbo2	Red	2014	1216 ± 57.0	1344 ± 32.0	1734 ± 67.0	2013 ± 78.5	121 ± 13.6
CIPAL1504[c]	Red	2018	1239 ± 51.8	1369 ± 37.7	1753 ± 68.5	2056 ± 79.0	141 ± 25.8
CIPAL1701[c]	Red	2019	1106 ± 41.0	1238 ± 23.8	1676 ± 72.5	1963 ± 87.5	180 ± 22.5

Values are BLUPs ± standard error across eight environments.

[a] *Original study of Lake and Sadras (2021) comprised 20 varieties, including Commando. Here, we exclude Commando because it was not used in Australian breeding.*

[b] *Indianhead was an imported variety used extensively in the early stages of the breeding program (Inder et al., 2008).*

[c] *CIPAL lines have not been released as varieties but have been tested in National Variety Trials (NVT), the precursor stage to release. Year of release has been estimated for these lines based on the usual time spent in NVT. Idrissi et al. (2019) used a similar criterion to project the year of release of promising lentil lines in the Moroccan breeding program. BLUPs, best linear unbiased predictions.*

TABLE 2 | Mean yield across varieties and growing conditions in eight environments resulting from combinations of season, sowing date, and water regime.

Season	Sowing date	Water regime	Yield (g m^{-2})	Seasonal rainfall + irrigation (mm)	Daily average for the critical period					Thermal time from sowing to (°Cd)			
					Tmax (°C)	Tmin (°C)	Radiation (MJ m^{-2})	VPD (kPa)	PTQ (MJ m^{-2}°C^{-1})	*F*	PE	EoF	*M*
2018	Early	Wet	221	289	16.9	7.1	9.9	0.7	0.8	1411	1575	2318	2835
	Early	Dry	21	117	16.9	4.9	12.7	0.8	1.2	1337	1467	1670	2008
	Late	Wet	110	240	21.6	4.5	18.2	1.3	1.5	1167	1280	1570	1827
	Late	Dry	77	203	21.7	4.1	18.8	1.4	1.6	1160	1282	1537	1769
2019	Early	Wet	145	332	20.8	5.9	14.8	1.2	1.2	1451	1585	2069	2362
	Early	Dry	71	156	18.4	3.8	14.5	1.0	1.4	1408	1519	1740	2057
	Late	Wet	131	324	23.5	6.2	19.7	1.6	1.5	1039	1131	1350	1582
	Late	Dry	183	218	25.6	7.6	19.4	1.8	1.3	1127	1223	1533	1840

Tmax is maximum temperature, Tmin is minimum temperature, VPD is vapor pressure deficit, and PTQ is photothermal quotient. Critical period was between flowering and 200°Cd after flowering, averaged across varieties.

relative rate of change in biomass and environmental mean yield was weak and negative (**Figure 4D**).

The crop growth rate in the critical period varied 2.5-fold with variety and fourfold with the environment, with no interaction between environment and variety (**Supplementary Table 1**). Across environments, the crop growth rate increased with the year of release at 0.07 kg ha^{-1}°Cd^{-1} year^{-1} or 1.46% year^{-1}. The rate of change in crop growth rate with the year of release was higher in more stressful environments (**Figure 4E**).

Harvest index varied sixfold with variety and 3.5-fold with the environment and also varied with the interaction between environment and variety (**Supplementary Table 1**). Across environments, the harvest index increased 0.0042 year^{-1} or 1.25% year^{-1} (**Table 3**). The rate of increase in harvest index with the year of release almost halved between the lowest and highest yielding environments (**Figure 4F**).

DISCUSSION

Genetic Gain in Yield Was Stronger in Stressful Environments

Our measured genetic gain for Australian lentils between 1988 and 2019 averaged 20 kg ha^{-1} year^{-1} or 1.23% year^{-1} across eight environments. It compares with the rate of 18–27 kg ha^{-1} year^{-1} for Ethiopian lentil in two environments (Bogale et al., 2015); 31–35 kg ha^{-1} year^{-1} for Moroccan lentil (Idrissi et al., 2019); 11–17 kg ha^{-1} year^{-1} for kabuli (Tadesse et al., 2018), and 32 kg ha^{-1} year^{-1} for desi chickpea in Ethiopia (Bekele et al., 2016).

Contrary to the observation that relative rates of genetic gain are independent of the environment in cereals (Fischer et al., 2014), here, we found that the expression of genetic gain in lentil yield was stronger under stress and often close to zero in high-yielding environments (**Figure 4A**). The rates of genetic change in the main drivers of yield, including grain number, crop growth rate, and harvest index, were also larger in low-yielding environments (**Figure 4**). Consistent with our finding, well-managed National Variety Trials in southern Australia, which benchmark current and new germplasm, show no improvement in either maximum or environmental mean yield between 2009 and 2018 (**Supplementary Table 1**). For lentils in Ethiopia, the rate of genetic gain in yield relative to the newest variety was 0.80% year^{-1} in an environment of 1.3 t ha^{-1} average yield and 0.92% year^{-1} in an environment of 4.8 t ha^{-1} (Bogale et al., 2015). For lentils in Morocco, the rate of genetic gain relative to the local check was 0.68% year^{-1} in a dry environment (200–350 mm year^{-1}) compared with 1.0% year^{-1} in a wetter environment (300–500 mm year^{-1}). We conclude that the proposition of environment-independent relative rates of genetic gain cannot be generalized.

The higher rate of genetic gain in low-yielding environments partially associates with the late phenology of early introductions. The breeding program has continually decreased time to flowering, podding, and maturity (**Figure 2** and **Table 3**) as earliness is critical for yield in short, dry seasons (Silim et al., 1993; Kumar et al., 2012). Similarly, breeding has focused on

taller and more upright crops to facilitate improved machine harvest in drier environments with actual gains of 0.12 cm year^{-1} (data not shown); lower crop growth rate and shorter plants of earlier varieties would impact yield under dry or short-season conditions (Erskine, 2009; Muehlbauer et al., 2009).

Higher Proportion of Time From Pod Emergence to Maturity, Higher Harvest Index, and Higher Crop Growth Rate in the Critical Period Offset Earlier Flowering and Maturity

Genetic gain in yield in Mediterranean, East Asian, and Sub-Saharan African environments has been associated with earlier flowering in lentils, chickpea, and wheat (Siddique et al., 1989; Erskine et al., 1994; Berger et al., 2004, 2006; Sadras and Lawson, 2011; Bogale et al., 2015). This is an important adaptation, achieving yield before the concurrent water and thermal stress later in the season (Thomson et al., 1997; Erskine et al., 2011).

We found three traits that offset the reduction in yield associated with shorter time to flowering and maturity: a longer period from pod emergence to maturity relative to crop duration (sowing to maturity), an increased harvest index, and an increased growth rate during the critical period. Harvest index was partially related to the extended period from pod emergence to maturity.

In indeterminate lentil, early flowering, combined with a lengthening of the reproductive period, increases the probability of grain set and filling to occur in favorable conditions while maintaining vegetative growth. However, a lengthening of the reproductive period may have negative effects under extreme stress, with Syrian research showing reproductive duration was negatively associated with lentil yield (Silim et al., 1993). For our set of varieties and environments, there was a negative association between time to flowering and time between pod emergence and maturity in the longer duration environments, with no relationship in the stress environments (**Supplementary Table 2**). This is a reflection of the later flowering, earlier Australian releases being adapted from material originating in longer season environments where they can flower later and extend reproduction.

Genetic Gain in Yield Primarily Associated With Growth Rate, Grain Number, and Harvest Index

The average rate of genetic gain in yield, 1.23% year^{-1}, compares with the rate of change of 1.46% year^{-1} for growth rate, 0.92% year^{-1} for grain number, and 1.25% year^{-1} for harvest index. In soybean, early gains in yield were driven by increased biomass and harvest index (Koester et al., 2014; Suhre et al., 2014), and allometric analysis further highlights the improvement in reproductive allocation (Tamagno et al., 2020). Lentil can grow large dense canopies and tend to suffer from a low harvest index, particularly in higher-yielding conditions (Kusmenoglu and Muehlbauer, 1998; Hanlan et al., 2006; Lake and Sadras, 2021). Phenotypes adapted to the main producing regions of Canada are assumed to combine moderate biomass and high harvest index (Hanlan et al., 2006). Averaged across environments, CIPAL 1701 had the highest harvest index at 0.33, and the average across varieties was 0.23 compared with reported maxima 0.44–0.59 (Whitehead et al., 2000; Malhi et al., 2007; Unkovich et al., 2010); the maximum for our dataset (0.54) indicates an opportunity for improvement.

Grain size in Canadian lentil (Muehlbauer, 1974) and kabuli chickpea in India (Gowda et al., 2011) was negatively correlated with yield. Australian breeding between 1988 and 2019 has achieved both increased grain size and yield (**Table 3**). In United States soybean improvement, grain size increased initially (Specht and Williams, 1984), but more recent work shows grain number has driven yield gain (Tamagno et al., 2020); this is also the case for Canadian soybean (Voldeng et al., 1997); and Ethiopian common bean (Bezaweletaw et al., 2006).

Trait Combinations Are Feasible

The indeterminate nature of lentils provides opportunities and challenges with large environmental variation in biomass. As biomass has low heritability, selection for crop growth rate

TABLE 3 | Absolute and relative rate of genetic change (±SE) for lentil traits in varieties released between 1988 and 2019.

Trait	Absolute	Relative (% year^{-1})
Yield	20 ± 6.9 kg ha^{-1} year^{-1}	1.23 ± 0.28
Thermal time sowing to flowering	−9 ± 1.6°Cd year^{-1}	−0.78 ± 0.08
Thermal time sowing to pod emergence	−4.9 ± 1.7°Cd year^{-1}	−0.72 ± 0.08
Thermal time sowing to end of flowering	−4.9 ± 2.9°Cd year^{-1}	−0.27 ± 0.05
Thermal time sowing to maturity	−4.5 ± 3.6°Cd year^{-1}	−0.22 ± 0.04
Thermal time pod emergence to maturity	4.9 ± 2.6°Cd year^{-1}	0.56 ± 0.13
Ratio thermal time pod emergence-maturity/sowing-maturity	0.003 ± 0.0007 year^{-1}	0.73 ± 0.11
Crop growth rate	0.07 ± 0.02 kg ha^{-1}°Cd^{-1} year^{-1}	1.46 ± 0.35
Biomass	16 ± 21 kg ha^{-1} year^{-1}	0.38 ± 0.15
Harvest index	0.004 ± 0.001 year^{-1}	1.25 ± 0.25
Grain number	34 ± 18 seeds m^{-2} year^{-1}	0.92 ± 0.31
Grain size	0.40 ± 0.08 mg seed^{-1} year^{-1}	0.96 ± 0.20

Rates are the slope of least-square regressions between trait and year of release for data pooled across eight environments. Relative rate is percentage of the latest variety.

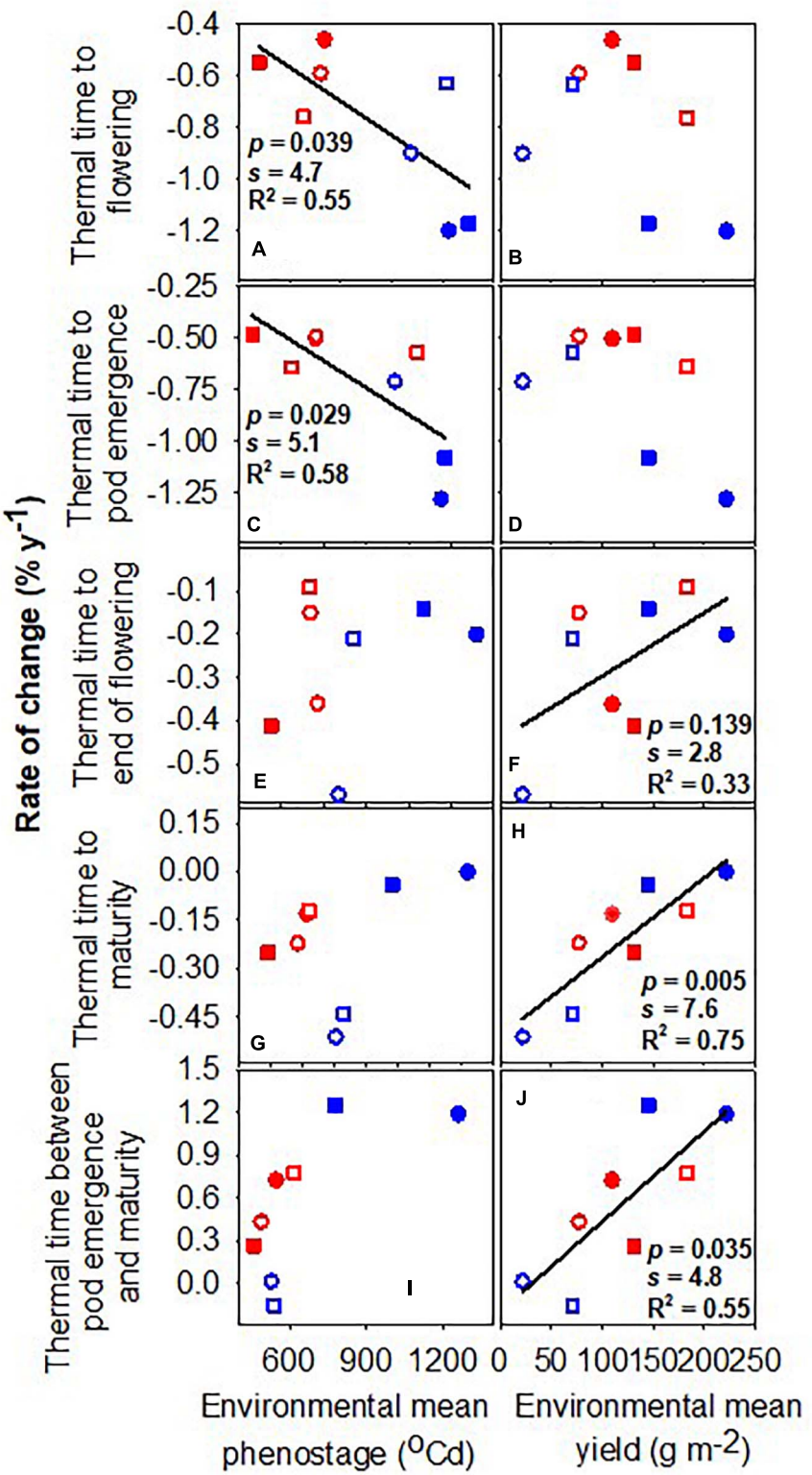

FIGURE 2 | Rate of change of thermal time from sowing to flowering, pod emergence, end of flowering and maturity, and the duration between pod emergence and maturity against the environmental mean phenostage **(A,C,E,G,I)** and the environmental mean yield **(B,D,F,H,J)**. Lines are least-square regressions and are only presented where $p < 0.05$, $s > 4.3$. Rates are relative to the newest variety. Symbols are: blue (2018), red (2019), circles (early sowing), square (late sowing), open (rainfed), closed (irrigated).

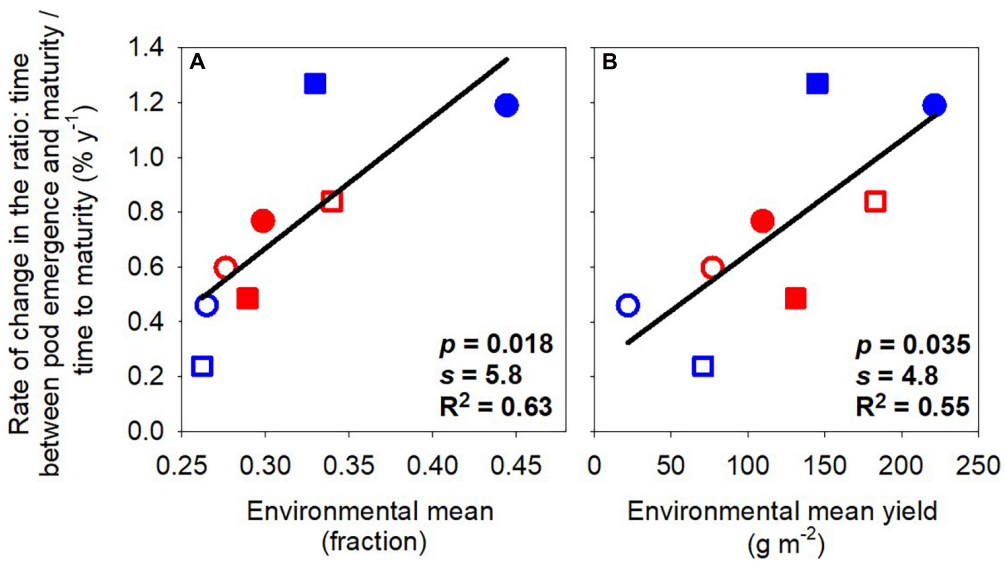

FIGURE 3 | Rate of change of the ratio: time between pod emergence and maturity/time to maturity against environmental mean of the ratio **(A)** and environmental mean yield **(B)**. Lines are least-square regressions. Rates are relative to the newest variety. Symbols are: blue (2018), red (2019), circles (early sowing), square (late sowing), open (rainfed), closed (irrigated).

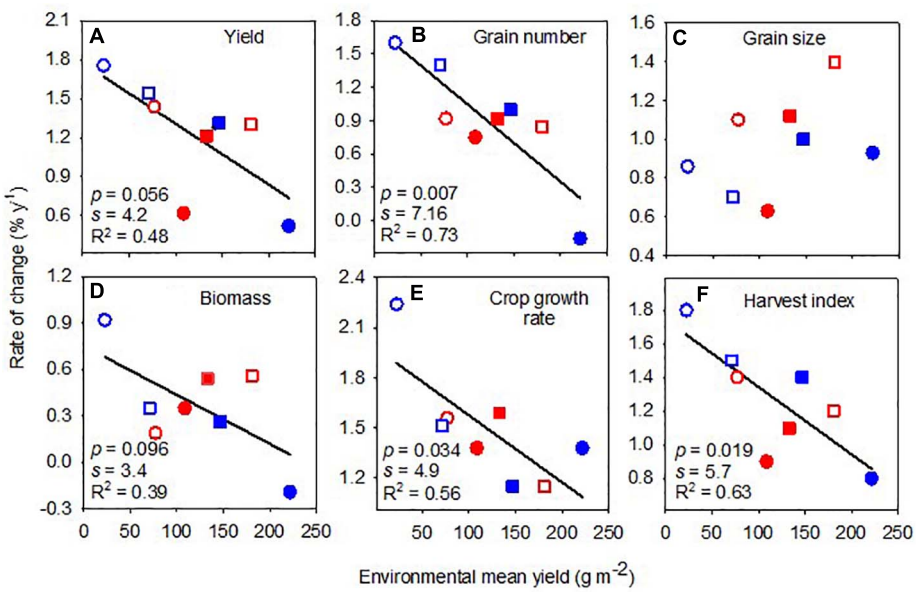

FIGURE 4 | Rate of change of yield **(A)**, grain number **(B)**, grain size **(C)**, biomass **(D)**, crop growth rate **(E)**, and harvest index **(F)** against environmental mean yield. Lines are least-square regressions. Rates are relative to the newest variety. Symbols are: blue (2018), red (2019), circles (early sowing), square (late sowing), open (rainfed), closed (irrigated).

in physiologically meaningful windows and harvest index are likely to be effective in increasing yield (Lake and Sadras, 2021). In short-season Mediterranean environments, combining early flowering and longer reproductive duration may improve harvest index and reduce problems associated with excessive vegetative growth. Successfully combining these traits may provide genetic gains in yield with less risk of a trade-off between yield in high- and low-yielding environments. Selection for early flowering is desirable in shorter Mediterranean environments, but there is a limit to how far flowering can be advanced against frost risk in the target population of environments (Lake et al., 2021). A longer flowering window can offset yield losses from limited frosts, but regular frosts may be more problematic, particularly in shorter seasons.

CONCLUSION

Over the three decades of Australian lentil breeding and for our sample of varieties and environments, genetic gain in yield was 20 kg ha^{-1} year^{-1} or 1.23% year^{-1}. The estimated genetic gain in yield was larger in lower-yielding environments. This genetic gain combined with improved agronomy has allowed the spread of lentils into lower rainfall regions of Australia, increasing rotational options and allowing more diverse cropping systems (Llewellyn et al., 2012; Moodie and Brand, 2019). The lack of improvement in the national average yield over this period is partially related to the expansion of the crop to intrinsically lower-yielding environments. Further improvements in lentil production require the adoption of improved practices to close the gap between water-limited and actual yield and a stronger focus in breeding for superior combinations of crop growth rate, biomass, and harvest index for higher yield potential.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

VS contributed to the planning and analysis of research and the writing of the manuscript. GR contributed to the writing of the manuscript. LL contributed to the planning, performing, analysis of research, and the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.674327/full#supplementary-material

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MutMap Approach Enables Rapid Identification of Candidate Genes and Development of Markers Associated With Early Flowering and Enhanced Seed Size in Chickpea (*Cicer arietinum* L.)

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Globally terminal drought is one of the major constraints to chickpea (*Cicer arietinum* L.) production. Early flowering genotypes escape terminal drought, and the increase in seed size compensates for yield losses arising from terminal drought. A MutMap population for early flowering and large seed size was developed by crossing the mutant line ICC4958-M3-2828 with wild-type ICC 4958. Based on the phenotyping of MutMap population, extreme bulks for days to flowering and 100-seed weight were sequenced using Hi-Seq2500 at 10X coverage. On aligning 47.41 million filtered reads to the CDC Frontier reference genome, 31.41 million reads were mapped and 332,395 single nucleotide polymorphisms (SNPs) were called. A reference genome assembly for ICC 4958 was developed replacing these SNPs in particular positions of the CDC Frontier genome. SNPs specific for each mutant bulk ranged from 3,993 to 5,771. We report a single unique genomic region on Ca6 (between 9.76 and 12.96 Mb) harboring 31, 22, 17, and 32 SNPs with a peak of SNP index = 1 for low bulk for flowering time, high bulk for flowering time, high bulk for 100-seed weight, and low bulk for 100-seed weight, respectively. Among these, 22 SNPs are present in 20 candidate genes and had a moderate allelic impact on the genes. Two markers, Ca6EF10509893 for early flowering and Ca6HSDW10099486 for 100-seed weight, were developed and validated using the candidate SNPs. Thus, the associated genes, candidate SNPs, and markers developed in this study are useful for breeding chickpea varieties that mitigate yield losses under drought stress.

Keywords: MutMap, early flowering, chickpea, 100 seed weight, candidate genes and SNPs

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important annual grain legume crop predominantly cultivated on residual soil moisture in the arid and semi-arid areas of the world. Global annual cultivation of chickpea is over 14.56 million ha with a total production of 14.77 million tons (FAOSTAT, 2017, accessed on January 26, 2020). Chickpea seeds are rich in protein (17–20%), minerals (phosphorus, calcium, magnesium, iron, and zinc) (Jukanti et al., 2012; Sab et al., 2020), and carotenoids; chickpea also improves soil health by adding atmospheric nitrogen (20–40 kg N ha^{-1}) through symbiosis (Joshi et al., 2001). Climate changes during the recent past have been posing serious threats to chickpea production and causing about 19% yield losses (Kadiyala et al., 2016).

In India, chickpea is grown in a wide range of agro-climatic niches. Based on crop duration, these regions can be classified as short-duration (Southern/peninsular India), medium-duration (Central India), and long-duration (Northern India) environments. In general, chickpea matures in a wide time frame of 80–180 days. However, in 66% of chickpea-growing areas, the available crop-growing season is about 80–120 days as they are exposed to abiotic stresses such as drought and heat toward the grain-filling stage. A major shift in the chickpea area (about 3 million ha) from Northern India (cooler, long-season environment) to Southern India (warmer, short-season environment) has been observed during the past four decades. As a result, no major boost in the total production of chickpea has been substantiated.

Terminal drought is considered as one of the most important constraints to chickpea production, and almost 40–50% yield losses were observed globally (see Roorkiwal et al., 2020). The number of days to flowering is an important trait for crop adaptation and productivity, especially in arid and semi-arid regions that experience terminal drought conditions. Early phenology, an adaptation-related trait, helps in the adaptation of chickpea to short-season environments as early flowering genotypes escape terminal (end of season) stresses (drought, high/low temperature) (see Berger et al., 2006). Therefore, the ability to manipulate flowering time is an essential component of chickpea improvement. Seed size/weight is an important yield-contributing trait, and therefore, in past, major breeding emphasis was on improving this trait (Gaur et al., 2014). As a result, early flowering desi and kabuli genotypes were identified through germplasm characterization (Upadhyaya et al., 2007); the low-resolution quantitative trait loci (QTLs) have been reported for flowering time and seed size (Varshney et al., 2014c; Upadhyaya et al., 2015; Verma et al., 2015; Mallikarjuna et al., 2017). Further, efforts were also made to understand the genes and pathways involved in flower development in chickpea (Singh et al., 2013), including through a gene expression atlas (Kudapa et al., 2018). Although the QTLs mapped within large genomic intervals limit the identification of potential candidate genes and their use in marker-assisted selection, in recent years, using a marker-assisted backcrossing approach several high-yielding and drought-tolerant lines in different genetic backgrounds of chickpea have been released for cultivation (Varshney et al., 2013a; Bharadwaj et al., 2021). Molecular breeding lines with enhanced resistance to biotic stresses were also developed (Varshney et al., 2014b; Pratap et al., 2017; Mannur et al., 2019).

The majority of the QTL mapping and gene isolation approaches using traditional approaches are time-consuming and low-throughput methods. Nevertheless, for more than a decade, the next-generation sequencing (NGS) technologies facilitated understanding of the genetics of complex traits at a faster pace in cereals and legumes (Thudi et al., 2020; Jaganathan et al., 2020). In the case of chickpea, apart from sequencing the genome (Varshney et al., 2013b) and several hundred germplasm lines (Thudi et al., 2016a,b; Varshney et al., 2019b), traits were fine-mapped (Kale et al., 2015; Singh et al., 2016). For decades, forward genetic approaches that rely on molecular characterization of altered phenotypes have been one of the driving forces for crop improvement. In the case of crops with a narrow genetic base, such as chickpea, the creation of allelic variation through mutations and the identification of causal variants will be a potential alternative that can overcome the existing production barriers. MutMap is one of the novel gene mapping approaches that allows rapid identification of causal nucleotide changes of mutants by whole-genome resequencing of pooled DNA of mutant F_2 progeny derived from crosses made between candidate mutants and the parental line (Abe et al., 2012; Fekih et al., 2013). This new NGS-based technique has been successfully applied in crop plants for rapid identification of the candidate gene as well as the QTL responsible for agronomically important traits (Abe et al., 2012; Megersa et al., 2015; Takagi et al., 2015; Fang et al., 2016; Klein et al., 2018; Tran et al., 2020).

Here, we report for the first time in chickpea, deployment of the MutMap approach that enabled us to rapidly identify genes and single nucleotide polymorphisms (SNPs) associated with early flowering and seed size. In addition, we also report the development and validation of markers that can be used for selection in chickpea breeding programs for improving these traits.

MATERIALS AND METHODS

Development and Phenotyping of MutMap Population

To identify phenotypically distinct mutant lines for early flowering and larger seed size, a set of 100 mutant lines from a TILLING (target-induced local lesions in the genome) population developed through ethyl methanesulfonate (EMS) mutagenesis of desi chickpea genotype ICC 4958 (unpublished ICRISAT) was phenotyped for these traits at ICRISAT (17.5111° N, 78.2752° E).

ICC 4958 is a drought-tolerant accession available from the ICRISAT germplasm collection. It was collected from Jabalpur, Madhya Pradesh, India, in 1973, and it was among the over 1,500 germplasm accessions screened for drought resistance at ICRISAT Center between 1978 and 1983. It is being used as a donor parent for introgressing drought tolerance-related traits

and that produces high yields in low productivity, short-duration, terminal drought-prone environments, e.g., those in peninsular India (Varshney et al., 2013a; Bharadwaj et al., 2021).

A set of 45 simple sequence repeat (SSR) markers distributed equally across the genomes was used to identify the genetic similarity among the selected lines. SSR genotyping was performed as described earlier (Thudi et al., 2011). PCR products were denatured and size-fractioned using capillary electrophoresis on an ABI 3730 DNA Genetic Analyzer (Applied Biosystems, United States). Based on allelic data, the mutant line with > 95% similarity to ICC 4958 was selected as the female parent. A MutMap population was developed crossing ICC 4958-M3-2828 (with large seed size and early flowering) and ICC 4958. F_{1s} were selfed to produce F_2 seeds. These F_2 seeds were sown in the field during crop season 2017–2018 at ICRISAT. The F_2 population was scored for days to flowering (DF) and 100-seed weight (SDW).

Isolation of DNA and Sequencing of ICC 4958 (Wild Type) and Trait Bulks

Genomic DNA was extracted from the leaves of F_2 individuals using the NucleoSpin Plant II kit (Macherey-Nagel, Dren, Germany). An equimolar concentration of DNA from 15 F_2 plants with high phenotypic values was pooled together as high bulk, and similarly, DNA from low phenotypic values was pooled together as low bulk. Thus, four extreme bulks, two for each trait, were prepared for WGRS along with wild-type parent ICC 4958 separately. About 5 μg of the pooled DNA was used for the preparation of a sequencing library of average insert size 200–500 bp, according to the protocol for the Paired-End DNA Sample PrepKit (Illumina, United States). The library was sequenced to 10X of genome coverage with the Illumina HiSeq 2500 platform (Illumina, United States).

Alignment of Short Reads to Reference Sequences and SNP Calling

Initially, a reference-based sequence of the ICC 4958 wild type was generated by aligning the sequence data generated to the CDC Frontier reference genome (Varshney et al., 2013b) as described in the study by Abe et al. (2012). In brief, 59 million paired-end short reads from ICC 4958 wild type and four mutant pools were used for the analysis. The quality checks for these reads were performed using FastQC v0.11.8 (Andrews, 2010), and Trimmomatic v0.39 (Bolger et al., 2014) was used to filter poor-quality reads and remove potential adapter contamination. For this, Illumina adapters and primers sequences were used by Trimmomatic for trimming, followed by iterative removal throughout the read length with mean base Phred qualities > 30 in 5-bp sliding windows. Remaining sequences with lengths < 35 bp after trimming were discarded as well as orphan single-end reads. These high-quality short reads were pooled and aligned with MAQ to the CDC Frontier reference sequence. Alignment files were converted to SAM or BAM files using SAMtools (Li et al., 2009) and applied to a filter pipeline (Kosugi et al., 2013) for the identification of reliable SNPs. This filter pipeline was developed to maximize true SNP detection and minimize false SNP calling by (i) the removal of paired-end reads of insert size > 325 bp, (ii) calling SNPs only for genomic regions covered by a minimum of three reads for homozygous SNPs and five reads for heterozygous SNPs and a maximum of threefold of average read depth over the genome, and (iii) calling SNPs only on sites with an averaged Illumina Phred-like quality score ≥ 20. Using this pipeline, we identified 332,395 reliable SNPs between ICC 4958 reads and the CDC Frontier reference sequence. On the basis of this result, we generated an ICC 4958 reference sequence by replacing CDC Frontier nucleotides with those of ICC 4958 at 332,395 sites. To remove the effect of SNPs irrelevant to the mutant screen, we generated and used a reference sequence of the same wild-type ICC 4958 that was used for mutagenesis. We further refined this reference sequence by taking a consensus of cumulative genome sequences of the mutants.

Paired-end sequence reads of bulked DNA of mutant F_2 progeny were aligned to the ICC 4958 reference sequence, and SNPs were scored as homozygous SNPs (with SNP index ≥ 0.9) and heterozygous SNPs (with SNP index ≥ 0.3 and < 0.9). We further excluded common SNPs shared by at least two mutant lines as well as G→A or C→T transitions (as they are most frequent in EMS mutagenesis). After identifying the genomic regions harboring a cluster of SNPs with an SNP index of 1, we relaxed the condition of the filter to consider all SNPs (caused by all the transition and transversion) in the region as candidate SNPs for the causal mutation. SNP index plot regression lines were obtained by averaging SNP indices from a moving window of five consecutive SNPs and shifting the window one SNP at a time. The *x*-axis value of each averaged SNP index was set at a midpoint between the first and the fifth SNP.

Primer Designing and Validation

The candidate SNPs with a SNP index = 1 were targeted for designing allele-specific markers. WASP, a web-based tool, was used for designing allele-specific primers (Wangkumhang et al., 2007)[1]. A total of 82 desi chickpea genotypes (47 for early flowering and 48 for seed size) were selected for marker validation. PCR was carried out in a 5 μl volume containing 10 ng of DNA, 1X buffer, 200 μM dNTP, 2.5 mM $MgCl_2$, 1–5 picomole forward and reverse primers, and 0.1 U of *Taq* polymerase. PCR was performed using Perkin Elmer 384-well Thermal cyclers (Applied Biosystems, United States) and involved a touchdown PCR. Touch down PCR cycles involved initial denaturation at 94°C for 5 min followed by 10 cycles of denaturation at 94°C for 20 s, 60°C for 30 s, 72°C for 30 s that decreases 1°C per cycle; then 35 cycles of 94°C for 20 s, at an optimized annealing temperature of each primer pair (51–58°C) for 30 s, 72°C for 30 s; final extension of 72°C for 20 min, and hold at 4–10°C forever. The PCR products were checked on 1.2% agarose gel containing 0.5 μl/10 ml ethidium bromide (10 mg/ml) with a 100-bp DNA ladder by running it at a constant voltage of 90 V for 25 min. The amplification was visualized under UV illumination using the Uvi-Tech gel documentation system (DOL-008.XD, England).

[1]http://bioinfo.biotec.or.th/WASP

RESULTS

MutMap Population for Early Flowering and Large Seed Size

Based on phenotyping of 100 mutant lines from the TILLING population, 25 lines that were phenotypically distinct from the wild-type ICC 4958 for flowering time and seed size were identified. In order to identify a mutant line that is > 95% similar to ICC 4958 wild type at the genome level, 25 lines along with wild type were genotyped using 25 SSR markers that are equally distributed across the genome (**Supplementary Table 1**). Based on SSR marker data, a dendrogram (**Supplementary Figure 1**) was constructed using DARWin5 (Perrier et al., 2003). The mutant ICC4958-M3-2828 with > 95% similarity to the ICC 4958 wild type and phenotypically distinct for flowering time and seed size was selected for developing a MutMap population. A total of 28 F_1 seeds were harvested by crossing ICC4958-M3-2828 and ICC 4958 wild type from July to September 2017 in the greenhouse at ICRISAT. During the crop season 2017–2018, F_1s were advanced to F_2 and a total of 204 F_2 seeds were harvested.

Phenotypic Diversity in MutMap Population and Preparation of Trait Bulks

A total of 204 F_2 plants were phenotyped for early flowering and 100-seed weight during the crop season 2018–2019 in the field. The MutMap population had high phenotypic variability for both flowering time and seed size **(Supplementary Table 2** and **Supplementary Figures 2A,B**). A negative correlation ($R = -0.13$) was observed among these traits (**Supplementary Figure 2C**). DNA from 15 F_2 progeny that displayed early flowering (27–34 days, as EF pool) and late flowering (59–60 days, as LF pool) was combined. Similarly, we also combined DNA from 15 F_2 progeny that displayed high 100-seed weight (43.0–46.2 g, as HSDW pool) and low 100-seed weight (27.0–41.0 gm, as LSDW pool) and subjected them to whole-genome sequencing using the Illumina HiSeq2500 platform (**Supplementary Table 3**).

Whole-Genome Sequencing and Alignment of Short Reads

Both wild-type ICC 4958 and four trait bulks were sequenced to ∼10X of genome coverage. For each trait detail on the number of raw reads, filtered reads, mapped reads, average coverage, and average quality are presented in **Table 1**. In the case of flowering time-related pools, we obtained 44,907,030 and 37,699,572 cleaned bases for EF pool and LF pool, respectively. Similarly, for seed size-related pools, 43,818,340 and 50,125,538 cleaned bases were obtained for the HSDW pool and LSDW pool, respectively (**Table 1**). On aligning 47.41 million filtered reads to the CDC Frontier reference genome, 31.41 million reads were mapped and 332,395 SNPs were called. These SNPs were used to develop a consensus reference genome sequence for ICC 4958 by replacing them in the particular positions of CDC Frontier. The filtered bulk sequenced paired-end reads were aligned and SNPs were called against this reference assembly that yielded alignment results as follows: 27.12 M reads for the LF pool, 31.57 M reads for the EF pool, 35.30 M reads for the HSDW pool, and 30.81 M reads for the LSDW pool (**Table 1**).

Trait-Associated Genes and SNPs

The Illumina short reads obtained for the four bulks were separately aligned to the reference sequence of ICC 4958 and then compared to the SNPs of each mutant bulk against wild-type ICC 4958 to identify the SNPs specific for each mutant bulk as well as their distribution on each pseudomolecule (**Table 2**). The SNP index for each SNP was also calculated. The number of SNPs among the bulks ranged from 3,993 (EF pool) to 5,771 (HSDW pool). In the case of EP pool flowering bulk, MutMap revealed 3,993 and 5,081 SNPs, of which 872 and 25 were candidate sites for the EF and LF pools, respectively, with a SNP index ≥ 0.8 (**Table 2**). While in the case of seed size bulks, MutMap revealed 4,777–5,771 SNPs, of which 771 and 1,078 were candidate sites for the LSDW and HSDW pools, respectively, with a SNP index ≥ 0.8 (**Table 2**). These SNPs were presumably the candidate mutations. However, it was not possible to pinpoint causal mutations from so many candidates. Further, for each bulk, SNP index vs. SNP genomic position graphs for the eight chickpea pseudomolecules were generated as shown in **Supplementary Figures 3–6**. The SNP index plots were very similar between the mutant and wild-type bulks across the entire genome. Nevertheless, a single genomic region on Ca6, between 9.76 and 12.96 Mb with a peak of SNP index 1, was identified in all four mutant bulks overlapping in this region that is missing from the wild-type bulk (**Table 3**). As expected, the SNP index was close to 0 across the genome, but within the unique genomic region identified on Ca6, between 9.76 and 12.96 Mb, its value was greater than zero. This was the only region that exhibited a SNP index difference of > 0 that is significant between the mutant and wild-type bulks. After identifying the region specific to mutant bulk, with a SNP index = 1, the SNPs therein (Ca6, between 9.76 and 12.96 Mb) were scrutinized in detail. Accordingly, we found a total of 38, 22,17,32 SNPs with a SNP index = 1 in the case of EF (**Figure 1**), LF, HSDW (**Figure 2**), and LSDW pools,

TABLE 1 | Summary of data generated and aligned on wild and mutant pools.

Wild/mutant pools	Raw reads	Filtered reads	Number of reads mapped	Average coverage (X)	Average mapping quality (%)
ICC 4958 (wild)	63,541,248	47,414,372	31,418,911	9.28	42.51
Days to flowering (Low; LDF)	58,196,006	44,907,030	31,571,460	9.49	42.38
Days to flowering (High; HDF)	51,747,822	37,699,572	27,123,905	7.97	42.53
100-seed weight (Low; LSDW)	58,041,216	43,818,340	30,815,440	9.19	42.37
100-seed weight (High; HSDW)	65,129,934	50,125,538	35,307,049	10.59	42.55

TABLE 2 | Identification and distribution of associated SNPs in the genome.

Trait (bulk)	Number of SNPs	SNPs with a SNP index > 0.8	Pseudomolecules							
			Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7	Ca8
Days to flowering (Low)	5,081	872	864	503	486	856	626	860	735	151
Days to flowering (High)	3,993	25	667	385	395	648	508	705	556	129
100-seed weight (High)	5,771	1078	812	491	498	775	596	817	643	145
100-seed weight (Low)	4,777	771	1,068	566	552	960	653	999	794	179

respectively in the region for the mutant bulk (**Table 3**). Of the 102 candidate SNPs, 41 were unique candidate SNPs and 33 were found in more than one bulk. On annotation of the 74 SNPs (41 unique and 33 in more than one bulk), 48, 16, 7, and 3 were intergenic, intronic, synonymous, and missense SNPs, respectively (**Supplementary Table 4**). Among 22 SNPs with a SNP index = 1, 44 were CT, and eight were GA transitions in the case of LF pool (**Supplementary Table 5**). We identified 31 SNPs with a SNP index = 1 in the case of the EF pool, of which 17 were CT and 14 GA transitions (**Supplementary Table 6**). Among 31 SNPs with a SNP index 1, a SNP (Ca6_10099486) present in the gene Ca_08581 that encodes putative importin beta-3 (AtKPNB1), in a previous study upregulation of AtKPNB1 led to early flowering in Arabidopsis (Luo et al., 2013). Similarly, 17 and 32 mutations were identified with a SNP index = 1, in HSDW and LSDW pools, respectively (**Supplementary Tables 7, 8**). Further, among 22 SNPs, two SNPs are in the gene Ca_08530, which encodes aspartokinase homoserine dehydrogenase involved in the homoserine biosynthetic process, in the phosphorylation process, and in the oxidation–reduction process (**Table 4**).

Markers for Early Flowering and Large Seed Size

Of 102 candidate SNPs on Ca6, between 9.76 and 12.96 Mb, 74 candidate SNPs were targeted to design primer pairs using WASP (see text footnote 1). A total of 12 allele-specific primer pairs were designed, and the primer sequence information is provided in **Supplementary Table 9**. Twelve primer pairs were initially amplified on a set of eight chickpea genotypes. Of twelve primer pairs, seven primer pairs had amplification on all eight genotypes tested. However, allele-specific amplification was obtained for Ca6HSDW10099486, Ca6HSDW9890335, Ca6HSDW9828083, and Ca6EF10509893. Hence, these 4 markers were validated on 82 select chickpea germplasm lines (47 for early flowering and 48 for seed size). As a result, one marker each for EF (Ca6EF10509893; **Figure 3A**) and HSDW (Ca6HSDW10099486; **Figure 3B**), with allele-specific amplification and high accuracy in the tested germplasm lines, has the potential to be used for improving the early flowering and seed size in chickpea. A clear significant difference ($p < 0.05$) between the amplified and non-amplified genotypes based on their phenotypic values for Ca6EF10509893 and Ca6HSDW10099486 can be visualized in **Figures 3C,D**, respectively. Nevertheless, these markers need to be tested on large germplasm sets for their efficiency before being used in early-generation selection in chickpea breeding programs.

TABLE 3 | Summary of SNPs with SNP index 1 on chromosome Ca6.

Trait	Region on Ca6 (Mb)	Total region (Mb)	SNPs with a SNP index = 1
Days to flowering (Low)	9.77–12.96	3.19	31
Days to flowering (High)	9.77–11.09	1.32	22
100-seed weight (High)	9.76–10.68	0.92	17
100-seed weight (Low)	9.82–10.68	0.86	32

DISCUSSION

Early flowering and seed size are the two important traits in chickpea as short-duration cultivars can escape terminal drought and high/low-temperature stresses, and enhanced seed size increases the yield to compensate for yield loss due to drought stress. Although early flowering accessions of desi and

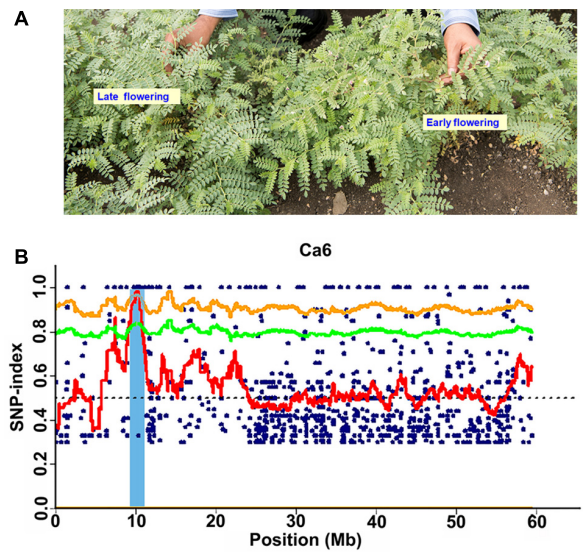

FIGURE 1 | Phenotypic variation for flowering time and identification of candidate genomic region using MutMap approach. **(A)** Representative picture showing the variation in flowering in the MutMap population developed crossing ICC 4958-M3-2828 × ICC 4958 (wild). **(B)** A genomic region on Ca6 x-axis indicates the physical position of the chromosome, and the y-axis indicates the average SNP-index in a 2 Mb interval with a 10 kb sliding window.

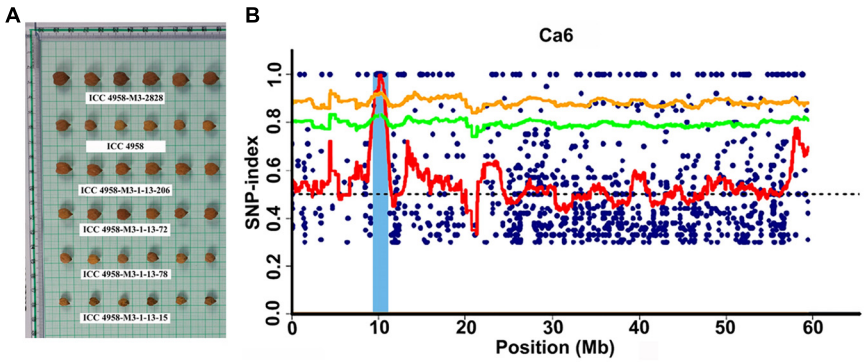

FIGURE 2 | Phenotypic variation for seed size and identification of candidate genomic region using MutMap approach. **(A)** Representative picture showing the variation in seed size in the MutMap population developed crossing ICC 4958-M3-2828 × ICC 4958 (wild). **(B)** A genomic region on Ca6 x-axis indicates the physical position of the chromosome, and the y-axis indicates the average SNP-index in a 2 Mb interval with a 10 kb sliding window.

kabuli types have been identified from germplasm collections (Upadhyaya et al., 2007) and super-early lines (ICCV 96029 and ICCV 96030) have been developed, there is a need for the identification of candidate genes and causal SNPs to accelerate the development of climate-resilient chickpea varieties. In the past, early flowering genes and their allelic relationships were reported based on the understanding of trait genetics (Gaur et al., 2014). Major QTLs for 100-seed weight were reported and were also fine-mapped (Varshney et al., 2014c; Jaganathan et al., 2015; Kale et al., 2015). However, none of the studies focused on the identification of candidate genes and causal SNPs responsible for flowering time and seed size.

The development of climate-resilient cultivars will make small-holder agriculture profitable in the anticipated climate change scenarios. In addition to the integration of multidisciplinary approaches in breeding, the adoption of a 5Gs breeding approach will accelerate genetic gains as well as meet the future demands of nutritious food (Varshney et al., 2018, 2020). In the case of legumes, sequence-based breeding in the post-genome sequence era has improved the efficiency of developing climate-resilient cultivars considerably (Varshney et al., 2019a). In this study, we report the identification of genes and SNPs using a MutMap approach, as well as the development of markers for use in chickpea breeding programs toward the development of cultivars with early flowering and large seed size.

We developed a MutMap population by crossing ICC 4958-M3-2828 to ICC 4958 (wild type) to identify the candidate genes and causal SNPs for early flowering and large seed size. A previous MutMap approach and its variants were successfully deployed to localize the position of genes for agronomically important genes in cereals such as rice (Abe et al., 2012; Hu et al., 2016; Deng et al., 2017; Yuan et al., 2017; Nakata et al., 2018), sorghum (Jiao et al., 2018), and also in cucumber (Xu et al., 2018). This is the first study deploying a MutMap approach in chickpea. In this study, we demonstrated that MutMap is a powerful approach to identify causal homozygous mutation in bulked F_2 plants selected for a phenotype of interest. Although the MutMap method was initially considered for mapping of monogenic recessive (mutations) gene-controlled traits, it is now possible to map dominant mutations through progeny testing and bulking of homozygous dominant F_2 individuals. In total 3,32,395 SNPs were used to develop a consensus reference genome sequence by replacing them at a particular position on the CDC Frontier genome. The sliding window analysis was applied to identify the trait linked SNPs with a SNP index value = 1. The extreme bulks sequenced reads were aligned to a consensus reference genome through a sliding window approach (moving averages). The SNPs in the sequenced F_2 population were in heterozygous state, show a 1:1 segregation pattern, 50% SNPs were mutant type, and the remaining 50% SNPs were wild type represented by a SNP index value of 0.5. If the F_2 population SNPs are in homozygous condition, then these are linked to the mutant phenotype (100% mutant reads, 0% wild-type reads represented as the SNP index value = 1). A SNP index value of 1 or near to 1 indicates the causal mutant SNP linked to the trait of interest, whereas a value of 0.5 indicates SNP not linked to the trait. The SNPs possess a SNP index value of 1 or near to 1 can be successfully targeted for marker development that can potentially be used in breeding. The number of SNPs among the bulks ranged from 3,993 (EF pool) to 5,771 (HSDW pool). Nevertheless, we identified only 102 candidate SNPs with a SNP index = 1. Interestingly, a genomic region harboring the candidate SNPs for all four bulks was on Ca6. In previous reports on chickpea, major QTLs for flowering time were reported on CaLG04 in the genomic region referred to as "*QTL-hotspot.*" Nevertheless, major QTLs corresponding to flowering time genes *efl-1* from ICCV 96029, *efl-3* from BGD 132, and *efl-4* from ICC 16641 were mapped on CaLG04, CaLG08, and CaLG06, respectively (Mallikarjuna et al., 2017). This indicates that flowering is a complex process coordinated by environmental and endogenous factors to ensure plant reproduction in appropriate conditions (Kumar et al., 2016). The "*QTL-hotspot*" was reported on Ca4 from 9.1 to 16.1 Mb (Varshney et al., 2014a). The relative positions of *efl1* and *efl2* genes mapped by Mallikarjuna et al. (2017) to "*QTL-hotspot*" were determined using the primer sequence of flanking markers NCPGR21 and GAA47 using *blastn -task "blastn-short.*" The marker for *efl1* (NCPGR21) was found to be present inside

TABLE 4 | Summary of candidate genes in the genomic region on chromosome Ca6 harboring candidate SNPs with SNP-index = 1.

Reference/ consensus base	SNP position	Gene	Sequence description	Biological process	Cellular component	Molecular function
C/T	10,685,694	Ca_08529	Subtilisin-like protease SDD1-like protein	Proteolysis	–	Serine-type endopeptidase activity
C/T	10,670,959	Ca_08530	Aspartokinase-homoserine dehydrogenase	Homoserine biosynthetic process, phosphorylation, oxidation–reduction process	–	Aspartate kinase activity, homoserine dehydrogenase activity, NADP binding
C/T	10,678,606	Ca_08530	Aspartokinase-homoserine dehydrogenase	Homoserine biosynthetic process, phosphorylation, oxidation–reduction process	–	Aspartate kinase activity, homoserine dehydrogenase activity, NADP binding
G/A	10,509,893	Ca_08544	Probable magnesium transporter NIPA1	Magnesium ion transmembrane transport	Early endosome, plasma membrane, integral component of membrane	Magnesium ion transmembrane transporter activity
G/A	10,498,876	Ca_08547	Beta-galactosidase 9	Carbohydrate metabolic process	Vacuolar membrane, plant-type cell wall, integral component of membrane	Beta-galactosidase activity, carbohydrate binding
C/T	10,432,389	Ca_08552	TBC1 domain family member 13-like	Intracellular protein transport, activation of GTPase activity	Intracellular	GTPase activator activity, Rab GTPase binding
G/A	10,394,606	Ca_08553	UV radiation resistance-associated-like protein	Protein targeting to vacuole, SNARE complex assembly, multivesicular body sorting pathway	Endosome, cytosol, integral component of membrane	SNARE binding
G/A	10,259,516	Ca_08563	Mediator of RNA polymerase II transcription subunit 10b-like	Regulation of transcription by RNA polymerase II	Mediator complex	Transcription coregulator activity
G/A	10,226,669	Ca_08566	Serine/threonine-protein phosphatase PP2A catalytic subunit isoform X2	Protein dephosphorylation	–	Phosphoprotein phosphatase activity
C/T	10,190,456	Ca_08570	Acyl-activating enzyme 17, peroxisomal protein, putative	–	Integral component of membrane	Catalytic activity
G/A	10,099,486	Ca_08581	Importin beta-3, putative	NLS-bearing protein import into nucleus, ribosomal protein import into nucleus	Nuclear membrane, nuclear periphery	Nuclear localization sequence binding, Ran GTPase binding
G/A	10,040,897	Ca_08587	Acyl-activating enzyme 17, peroxisomal protein, putative	–	Integral component of membrane	Catalytic activity
C/T	9,828,083	Ca_08609	Hypothetical protein glysoja_010758	–	–	–
G/A	10,599,353	Ca_08537	S-Adenosyl-L-homocysteine hydrolase	One-carbon metabolic process, S-adenosylhomocysteine catabolic process	Cytosol	Adenosylhomocysteinase activity, NAD binding
G/A	10,498,876	Ca_08547	Beta-galactosidase 9	Carbohydrate metabolic process	Vacuolar membrane, plant-type cell wall, integral component of membrane	Beta-galactosidase activity, carbohydrate binding
G/A	10,259,516	Ca_08563	Mediator of RNA polymerase II transcription subunit 10b-like	Regulation of transcription by RNA polymerase II	Mediator complex	Transcription coregulator activity
C/T	10,190,456	Ca_08570	Acyl-activating enzyme 17, peroxisomal protein, putative	–	Integral component of membrane	Catalytic activity
G/A	10,099,486	Ca_08581	Importin beta-3, putative	NLS-bearing protein import into nucleus, ribosomal protein import into nucleus	Nuclear membrane, nuclear periphery	Nuclear localization sequence binding, Ran GTPase binding
G/A	10,040,897	Ca_08587	Acyl-activating enzyme 17, peroxisomal protein, putative	–	Integral component of membrane	Catalytic activity
C/T	10,025,005	Ca_08590	Receptor-like protein kinase	Protein phosphorylation	Integral component of membrane	Protein serine/threonine kinase activity, ATP binding
G/A	9,890,335	Ca_08601	LRR receptor-like kinase family protein	Protein phosphorylation	Integral component of membrane	Protein kinase activity, ATP binding
C/T	9,828,083	Ca_08609	Hypothetical protein glysoja_010758	–	–	–

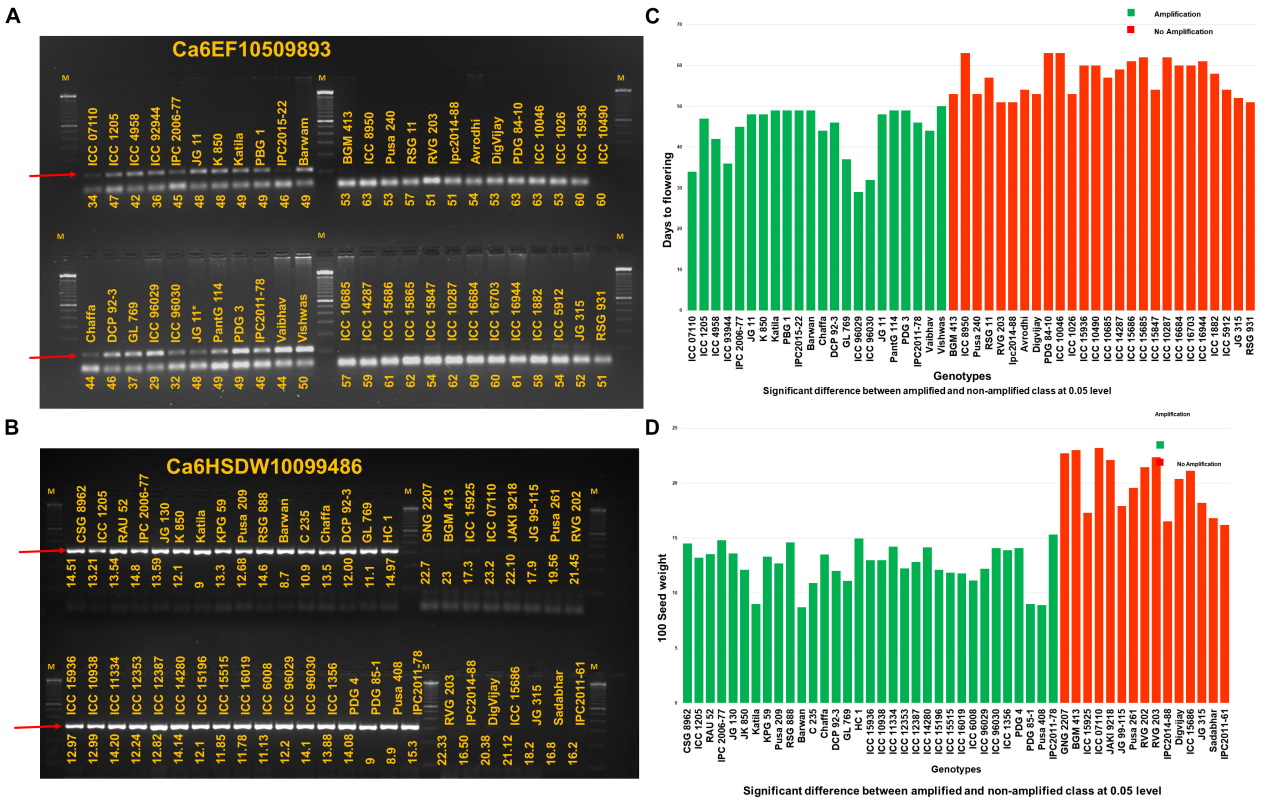

FIGURE 3 | Markers developed for early flowering and seed size **(A)** The marker allele-specific marker Ca6EF10509893 for early flowering shows amplification in early flowering genotypes and no amplification in late flowering genotypes and **(B)** similarly, in the case of allele specific marker Ca6HSDW10099486 for 100-seed weight amplification can be seen and no amplification in case of genotypes with high 100-seed weight. M is the 100-bp marker. Allele-specific amplicons are indicated with the red color arrow. A clear significant difference ($p < 0.05$) between the amplified and non-amplified genotypes based on their phenotypic values for Ca6EF10509893 and Ca6HSDW10099486 can be visualized in **(C,D)**, respectively.

the "*QTL-hotspot*" at ∼10 Mb on Ca4, whereas the marker for *efl2* (GAA47) was found present ∼818.1kb upstream of the "*QTL-hotspot*" on the genome at ∼8.3 Mb on Ca4. Further, the flowering time genes are distributed throughout the genome and are dependent on the genetic background. Genome-wide distribution of flowering time genes is not uncommon and was recently also reported in cucurbits (Yi et al., 2020).

On annotating the candidate SNPs in this genome region on Ca6, we identified that these SNPs are located within candidate genes that are involved in flowering time as well as in seed development. For instance, the candidate SNPs, Ca_10137361 and Ca6_11657245, present in Ca_08579 and Ca_25060 genes, respectively, are associated with calmodulin sensing Ca^{2+} signals and are reported to be involved in flowering time (Kumar et al., 2016). The SNP Ca6_10099486, present in gene Ca_08581 that encodes putative importin beta-3, was reported to play a key role in drought tolerance in *Solanum tuberosum* (Xu et al., 2020). AtKPNB1, which is a member of the Arabidopsis importin family, was reported to be a gene associated with ABA sensitivity at germination, early seedling development, drought tolerance, and stomatal closure regulation; it is expressed in various organs and any specific tissues in listed organs such as leaves, roots, and flowers (Luo et al., 2013). Further, a SNP Ca6_10685694, present in gene Ca_08529, encodes for subtilisin-like protease SDD1 (STOMATAL DENSITY AND DISTRIBUTION-1) and SDD1-like transcripts in *Solanum chilense* and *Solanum lycopersicum*. SDD1 is also known to play an important role in early leaf and flower development in both tomato species (Morales-Navarro et al., 2018). Similarly, a SNP Ca6_10498876, present in gene Ca_08547 that encodes beta-galactosidase9 to be expressed during fruit ripening, plays a major role in abscission, early onset of growth, and development processes in flowers and fruitlets (Wu and Burns, 2004). The genes Ca_08587 and Ca_08570 encode for the acyl-activating enzyme 17 (AAE17) reported to having a functional role in seed development. The Ca_08530 gene encodes aspartokinase-homoserine dehydrogenase (AK/HSD) enzyme involved in aspartate kinase activity, homoserine dehydrogenase activity, and NADP binding activity. AK/HSD-GUS gene has been reported to be expressed in actively growing tissues and seed development. A SNP Ca6_10685694, present in the gene Ca_08529 located on Ca6, encodes subtilisin-like protease (SBT) SDD1-like protein. SBTs have been shown to control diverse developmental processes like stomatal distribution and density (Berger and Altmann, 2000; Von Groll et al., 2002). Two allele-specific markers, Ca6EF10509893 for early flowering and

Ca6HSDW10099486 for 100-seed weight, developed in this study were also validated on a select set of chickpea germplasm lines. These markers can be further tested on a larger germplasm panel with the potential to be converted to high-throughput assays for early-generation selection in chickpea breeding programs.

CONCLUSION

MutMap has the advantage of both bulk segregant analysis and WGRS (whole-genome resequencing) approaches and enables the identification of candidate genes and causal SNPs. In the present study, we report 102 candidate SNPs in 22 candidate genes. The candidate genes identified in this study are involved in early flowering as well as enhanced seed size. Further, we also report the development and validation of markers for use with chickpea. Testing of these markers on a large and diverse panel of genotypes will be required prior to use in breeding programs for improving these traits.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA715624.

AUTHOR CONTRIBUTIONS

MT conceived the project and secured funding. PM performed the experiments and prepared the first draft. BPM developed the MutMap population. DK, PB, and AWK performed the bioinformatics analysis. RP performed the experiments and phenotyped the population. UCJ contributed germplasm for marker validation. RKV, AC, and PS contributed the resources. MT and RKV wrote the review, edited manuscript and finalized the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.688694/full#supplementary-material

Supplementary Figure 1 | Dendrogram constructed based on the 25 SSR markers distributed across chickpea genome indicates more than 95% similarity between ICC 4958 (wild type) and ICC 4958-M3-2828.

Supplementary Figure 2 | Frequency distribution and correlation among days to flowering and 100-seed weight. Histogram showing the frequency distribution of days to flowering **(A)** and 100-seed weight **(B)**. A negative correlation of 0.13 was observed between these traits.

Supplementary Figure 3 | Single nucleotide polymorphism **(**SNP) index plots for all eight pseudomolecules for early flowering pool. The red line indicates the sliding window average of 2-Mb interval with an increment of 10 kb for SNP index.

Supplementary Figure 4 | Single nucleotide polymorphism index plots for all eight pseudomolecules for late flowering pool. The red line indicates the sliding window average of 2 Mb interval with an increment of 10 kb for SNP index.

Supplementary Figure 5 | Single nucleotide polymorphism index plots for all eight pseudomolecules for large seed size pool. The red line indicates the sliding window average of 2 Mb interval with an increment of 10 kb for SNP index.

Supplementary Figure 6 | Single nucleotide polymorphism index plots for all eight pseudomolecules for small seed size pool. The red line indicates the sliding window average of 2 Mb interval with an increment of 10 kb for the SNP index.

Supplementary Table 1 | Allelic data of 25 SSR markers distributed on the genome generated on mutant and wild lines.

Supplementary Table 2 | Phenotyping data generated on MutMap population derived from ICC 4958-M3-2828 × ICC 4958.

Supplementary Table 3 | Summary of the extreme bulks prepared for flowering time and seed size mutant based on phenotyping data generated on MutMap population derived from ICC 4958-M3-2828 × ICC 4958.

Supplementary Table 4 | Summary of non-redundant candidate SNPs in the genomic region of Ca6 and their annotations.

Supplementary Table 5 | Summary of SNPs identified in late flowering pool.

Supplementary Table 6 | Summary of SNPs identified in early flowering pool.

Supplementary Table 7 | Summary of SNPs identified in large seed size pool.

Supplementary Table 8 | Summary of SNPs identified in small seed size pool.

Supplementary Table 9 | Details of primer pairs designed for candidate SNPs.

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VaSDC1 Is Involved in Modulation of Flavonoid Metabolic Pathways in Black and Red Seed Coats in Adzuki Bean (*Vigna angularis* L.)

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Seed coat colour is an important nutritional quality trait. Variations in anthocyanins and flavonoids induce the diversity of seed coat colour in adzuki bean (*Vigna angularis* L.). Red seed coat and black seed coat are important adzuki bean cultivars. Insights into the differences of flavonoid metabolic pathways between black and red adzuki bean are significant. In this study, we explored that the difference in seed coat colour between the red (Jingnong6) and the black (AG118) is caused by the accumulation of anthocyanins. The RNA-sequencing (RNA-Seq) and real-time reverse transcription (qRT)-PCR results showed that the *Vigna angularis* L. seed coat color (*VaSDC1*) gene, an R2R3-MYB transcription factor, should be the key gene to regulate the black and red seed coat colours. In three different colouring staes of seed development, *VaSDC1* was specifically expressed in the black seed coat (AG118) landrace, which activates the structural genes of flavonoid metabolic pathways. As a result, this caused a substantial accumulation of anthocyanins and created a dark blue-black colour. In the red (Jingnong6) seed coat variety, low expression levels of *VaSDC1* resulted in a lower accumulation of anthocyanins than in AG118. In addition, *VaSDC1* was genetically mapped in the interval between simple-sequence repeat (SSR) markers *Sca326-12*, *Sca326-4,* and *BAgs007* on chromosome 3 using an F_4 segregating population derived from the cross between Jingnong6 and AG118. These results will facilitate the improvement of nutritional quality breeding in adzuki beans.

Keywords: adzuki bean, *VaSDC1*, flavonoid metabolism, seed coat colour, transcriptome, qRT-PCR

INTRODUCTION

The adzuki bean (*Vigna angularis* L.), one of the major pulse crops in the genus *Vigna*, is mainly planted and consumed in East Asia and carries an important economic value (Yoshida et al., 2008; Shi et al., 2016). The cultivation area of adzuki bean in China is approximately 22,000 ha, making China the largest producer in the world. China is the original centre of the adzuki bean, with the

largest number of genetic resources (Ning et al., 2009; Liu et al., 2013). The adzuki bean is widely used in a variety of foods (e.g., paste in pastries, desserts, cakes, porridge, adzuki bean rice, jelly, adzuki milk, ice lollies, and ice cream) for at least a billion people (Itoh et al., 2004a,b; Lestari et al., 2014). It is consumed during traditional celebrations such as the Chinese Spring Festival and Japanese weddings (Yousif et al., 2007; Horiuchi et al., 2015).

Adzuki beans are rich in starch (53.14%), protein (22.72%), iron (7.4 mg/100 g), zinc (4.0 mg/100 g), dietary fibre (12–13%), B vitamins and folic acid (Tjahjadi et al., 1988). Phenolic acids and flavonoids extracted from adzuki bean exhibited significant antioxidant, immune-regulatory and radical-scavenging activities (Amarowicz and Pegg, 2008; Yao et al., 2015). Adzuki beans possess strong 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$) free-radical-scavenging capacity and α-glucosidase inhibitory activity. Significant positive correlations ($P < 0.01$) between the antioxidant activity and total phenolic acids, and between total flavonoids and free caffeic acid contents were observed (Shi et al., 2016). The abundant phenolic substances in the adzuki bean were reported to have the strong free-radical-scavenging capacity, which can prevent and control oxidative damage caused by inflammation, atherosclerosis, cardiovascular disease and even cancer (Sato et al., 2005a,b; Hori et al., 2010).

The phenolic compounds mainly include phenolic acids and flavonoids. Flavonoids showed differences and variations during seed ripening and harvesting (Raffo et al., 2004). The phenolic content changed remarkably during the ripening process (Amiot et al., 1986; Romero et al., 2002; Bouaziz et al., 2004).

Legume seeds with dark colour (black, red and brown) possess a significantly higher phenolic acid content than lighter coloured (yellow, green and white) ones. There were numerous antioxidant activities of different legume accessions (Amarowicz and Pegg, 2008). The antioxidant activity was strongly correlated with a total phenolic acid content, and total phenolic acid content has been confirmed to correlate reasonably with seed coat colour (Xu et al., 2010). Seed coat colour influenced the synthesis and accumulation of phenolic compounds in the adzuki bean. The concentration of phenolic compounds correlated with the values of seed colour (Kim et al., 2011). Coloured seeds (e.g., black and red) had higher antioxidant activities than colourless seeds (e.g., white and beige) in common beans (Madhujith et al., 2010). We published a draft of the adzuki bean genome by whole-genome shotgun sequencing on the Jinnong6 variety (Yang et al., 2015). We analysed the genetic relationships of seed coat colour using 12 F_2 or F_3 hybridised combinations derived from eight seed coat colours and predicted the genetic model of adzuki bean seed coat colours, though that the difference between black and red is controlled by B locus and T/Y locus (Chu et al., 2021). The *VaScB* gene controlling the black seed coat trait (SDC1) was mapped onto chromosome 3 (Li et al., 2017). *VaScB* should be the same locus as the B locus but still needs to be verified. Black is the darkest seed coat colour in adzuki bean with the highest flavonoid content. Red is the most common seed coat colour in cultivated adzuki beans. Exploration of the regulatory mechanism between black and red seed coats and the synthesis pathway of bioactive flavonoid of adzuki bean is important to improve the antioxidant properties and quality breeding in adzuki beans.

In this study, the precise differences in gene expressions in the flavonoid metabolic pathways between red seed coat Jingnong6 and black seed coat AG118 were revealed during the different colouring stages of seed development, utilising RNA-sequencing (RNA-Seq) and real-time reverse transcription (qRT)-PCR. *VaSDC1*, an R2R3-MYB transcription factor, was suggested to lead to the difference between red and black seed coats in adzuki bean through activating the structural genes of flavonoid metabolic pathways. VaSDC1 has high homology with AtMYB75/90 in amino acid sequences that influenced the colour of Arabidopsis that was explored by phylogenetic analysis. The morphological marker SDC1 between black and red seed coats was mapped in the interval of simple-sequence repeat (SSR) markers *Sca326-12*, *Sca326-4,* and *BAgs007*, and *VaSDC1* was found in the same interval. Insight on *VaSDC1* was important to understand the regulatory mechanism of the adzuki bean seed coat colour and flavonoid metabolism. These SSR markers may be used as an assistant for the selection of the potential antioxidant properties and quality breeding in adzuki bean.

MATERIALS AND METHODS

Plant Materials

The improved varieties of adzuki bean (Jingnong6) with red seed coat colour and the landrace (AG118) with black seed coat colour were utilised for RNA-Seq and qRT-PCR. Jingnong6, AG118 and their 209 recombinant inbred lines of F_4 that were used for genetic mapping were grown at the Experimental Farm of Beijing University of Agriculture (BUA) in 2018. The colouration of the adzuki bean seed coat is a diffusion process from hilum to whole seed coat. We divided the colouration of adzuki bean seed coat into three stages. The hilum was only coloured in stage 1. The whole seed coat began to be coloured preliminarily in stage 2. The whole seed coat was deeply coloured in stage 3. We collected the seed coat of Jingnong6 and AG118 during the three colouring stages, respectively. All samples were immediately frozen in liquid nitrogen and then stored at −80℃.

The 209 recombinant inbred lines of F_4 (106 dominant black seed coat individuals and 103 recessive red/light brown seed coat individuals) derived from the F_2 population of a cross between Jingnong6 and AG118 by the single seed descent method (Horiuchi et al., 2015) were used to map the *VaSDC1* gene.

Identification of Pigments

As described earlier (Li et al., 2020), seed coat tissues were grounded into powder; then, flavonoids and carotenoids were extracted using methanol. An equal volume of water and dichloromethane were added to the methanol extract and thoroughly mixed. Finally, the samples were centrifuged at 13,000 rpm to separate flavonoids and carotenoids into the supernatant liquid (aqueous) and the denser liquid (non-aqueous).

Relative Quantification of Anthocyanin by Ultraviolet-Visible (UV/Vis) Spectroscopy

Seed coat tissues were grounded into powder in liquid nitrogen and then resuspended in 0.1 mol/L hydrochloric acid. The hydrochloric acid extract was sealed in a beaker, followed by a 4-h incubation at 32℃. Finally, samples were filtered and measured at a wavelength of 530 nm with an ultraviolet spectrophotometer. The absorbance of 0.1 mol/L hydrochloric acid at a wavelength of 530 nm served as the blank control. Absorbance reading of 0.1 mol/L of each sample at a wavelength of 530 nm was served as a measurement unit of anthocyanin relative content.

Identification of Anthocyanin by Liquid Chromatography-Electrospray Ionisation–Tandem Mass Spectrometry (LC-ESI-MS/MS) Analysis

The seed coats of Jingnong6 and AG118, collected at the third colouring stages, were grounded into a powder and extracted with 1 ml of 70% aqueous methanol by overnight incubation at 4°C. The extracted solution was centrifuged for 10 min at 10,000 g. Extracts were absorbed, and the supernatant was filtered. Extracts were determined using LC-ESI-MS/MS analysis. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on the Triple quadrupole-linear ion trap mass spectrometer (Q TRAP) LC/MS/MS system, operated in positive ion mode and controlled by the Analyst 1.6 software (ABI Sciex, United States). Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of multiple reaction monitoring (MRM) transitions was monitored for each period according to the metabolites eluted within that period.

Total RNA and DNA Extraction

Total RNA was extracted from 0.1 g of each powdered tissue with EASYspin plus Total RNA Isolation Kit (Aidlab, China), according to the instructions of the manufacturer, with minor modifications. Genomic DNA was extracted from 0.1 g of a leaf taken from each F_4 line by the improved cetyltrimethylammonium bromide (CTAB) method. The integrities of total RNA and DNA were further assessed by 1% agarose gel electrophoresis. Concentrations and purity of RNA and DNA were assayed by NanoDrop^{TM} 8000 Spectrophotometer (Thermo Fisher Scientific, United States).

Illumina Transcriptome Library Preparation, Sequencing, and Expression Level Estimation

To reveal the transcriptome expression profile during seed coat colouring, total RNA from Jingnong6 and AG118 seed coats was extracted during three seed coat colouring stages and used for RNA-Seq (**Figure 1**). Three biological replicates were evaluated for each variety and stage, totalling 18 samples (each replicate was composed of samples mixed from more than three individuals). Messenger RNA (mRNA) was enriched by magnetic beads with oligo(dT) and fragmented with fragmentation buffer. The first-strand complementary DNA (cDNA) was synthesised by random hexamers, and the second-strand cDNA was synthesised by adding buffer, deoxynucleotide mix (dNTPs), Ribonuclease H (RNaseH) and DNA polymerase I. The purified double-stranded cDNA was then repaired at the end. A tail was added and sequenced with a sequencing connector for PCR amplification. The final products of PCR were sequenced using Illumina HiSeq^{TM} 4000. We used an in-house Perl script to process the raw data to obtain the clean data. After obtaining clean reads, hierarchical indexing for spliced alignment of transcripts (HISAT) (Kim et al., 2015) was used to compare clean reads to the reference genome (Yang et al., 2015). Gene expression levels were estimated *via* the fragments per kilobase of transcript per million mapped reads (FPKM) method using RNA-Seq by expectation-maximisation (RSEM).

FIGURE 1 | Seed coat colouring degree of Jingnong6 and AG118 in different colouring stages.

Gene Mapping

SSR Hunter version 1.3 and Oligo version 7 were used for the primer design of 12 SSR primer pairs (**Supplementary Table 1**). DNA was diluted to the same concentration (50 ng/μl). The reaction mixture contained 5 μl PCR mix, 1 μl DNA, 0.4 μm of each forward and reverse primer and double-distilled water (ddH_2O) to make up the final volume to 10 μl. Amplifications were performed with the following programme: 95℃ for 5 min; 40 cycles of 95℃ for 30 s; 55℃ for 30 s 72℃ for 30 s and 72℃ for 10 min. PCR products were separated on 8% polyacrylamide gel electrophoresis (PAGE), silver stained and viewed. As a morphological marker, SDC1 was used to construct a linkage map with SSR markers.

Quantitative Real-Time PCR Analysis

The 1,000 ng of the total RNA was reverse transcribed using PrimeScript RT Reagent Kit (RR037A, TaKaRa, Japan) following

the instructions of the manufacturer. The cDNA was diluted 10-fold with nuclease-free water for qRT-PCR analysis. qRT-PCR was conducted using LightCycler® 96 Plates and performed on the LightCycler 96 SW 1.1 (Roche Molecular Systems, Germany). The reaction mixture contained 10 μl ChamQTM Universal SYBR® qPCR Master Mix (#Q711, Vazyme, China), 1 μl 10-fold diluted cDNA, 0.4 μm of each forward and reverse primer (**Supplementary Table 2**) and ddH_2O to make up the final volume to 20 μl. Amplifications were performed with the following programme: 95℃ for 30 s; 40 cycles of 95℃ for 10 s and 60℃ for 30 s, and melting curve analysis conditions (95℃ for 10 s, 65℃ increased to 97℃ with temperature increments of 0.11℃ every 1 s). No-template reactions were used as negative controls, and each sample was assessed in three technical replicates. Gene transcription levels of seed coats from the Jingnong6 and AG118 during the three colouring stages were calculated using three biological replicates (each replicate contained the mixed seed coats of six individuals). An actin housekeeping gene of the adzuki bean was used for normalisation.

Data Analysis

Heatmap analysis was used to display the gene expression profile using OmicShare tools.[1] The relative expression levels of these genes were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Neighbour-joining (NJ) phylogenetic trees were constructed with bootstrap values estimated from 1,000 replicate runs using molecular evolutionary genetics analysis version 7.0 (MEGA7) (Kumar et al., 2016) to analyse the phylogenetic relationships among the *VaSDC1* gene of the adzuki bean and the genes from *Arabidopsis*. A linkage map was constructed using JoinMap version 4.0 with a logarithm of the odds (LOD) threshold of 3.0. The Kosambi mapping function was used to convert recombination frequencies for mapping distances (Kosambi, 1944). Seed coat colour trait was calculated in the genetic map as a qualitative trait.

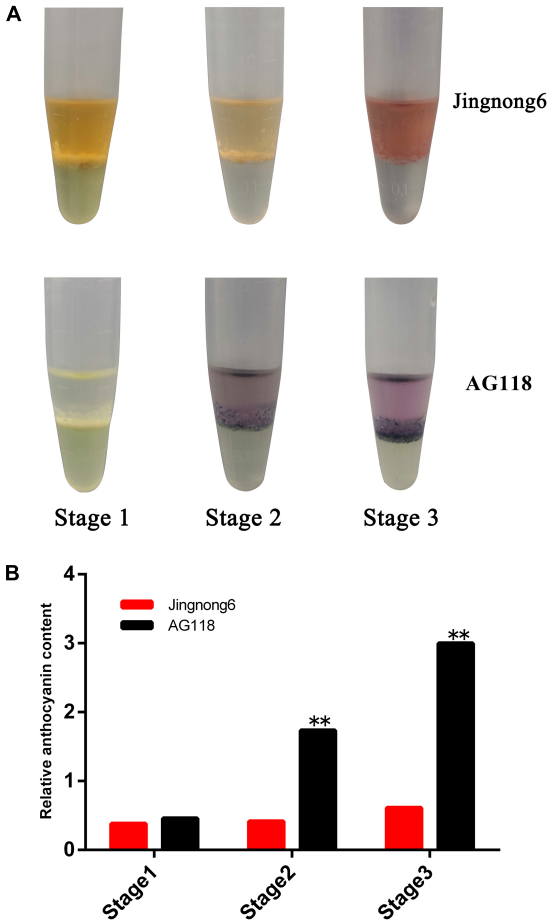

FIGURE 2 | Identification of colouring substance and content in seed coats of Jingnong6 and AG118. **(A)** Identification of colouring substances in the seed coats of different colouring stages; **(B)** relative anthocyanin content of Jingnong6 and AG118 seed coats in different colouring stages. $^{**}P \leq 0.01$.

RESULTS

Differences Between the Adzuki Bean Seed Coat Colour and Pigment Content During the Seed Colouring Stages

The morphological analysis showed an obvious difference in colour depth during the three seed coat colouring stages between the Jingnong6 and AG118, as the seed coat colour is gradually deepened from stage 1 to stage 3 (**Figure 1**). Determination of pigments showed that the difference between the red seed coat of Jingnong6 and the black seed coat of AG118 depended on anthocyanins but not carotenoids (**Figure 2A**).

In addition, the level of accumulation of anthocyanin was increased, corresponding to the colour deepening in successional colouring stages. In the AG118 landrace, the anthocyanin content quadrupled from stage 1 to stage 2 and doubled from stage 2 to stage 3. However, it only exhibited a small increase from stage 1 to stage 3 in Jingnong6 (**Figure 2B**). The seed coats of Jingnong6 and AG118 at stage 3 were collected and assayed for the final compositions of anthocyanins using LC-ESI-MS/MS analysis. A total of nine different anthocyanin metabolites divided into two categories were detected (**Figure 3**). Four kinds of centaurins were detected in stage 3 of both Jingnong6 and AG118. However, the concentration of the four centaurins in AG118 is much higher than that in Jingnong6. While five kinds of delphinidins were almost undetectable in stage 3 of Jingnong6, a much higher concentration of delphinidin was detected in stage 3 of AG118.

Expression Profile of Structural Genes in the Flavonoid Metabolic Pathways

The RNA-Seq produced 735.1563 Mbs of clean reads from 18 libraries, and the expression level (expected number of FPKM) of each isoform was calculated with a mapping ratio of 81.24–92.56%.

The genes involved in the different colouring stages of Jingnong6 and AG118 were screened from the transcriptome dataset. Heatmap analysis was used to illustrate the expression

[1]https://www.omicshare.com/tools/home/index/index.html

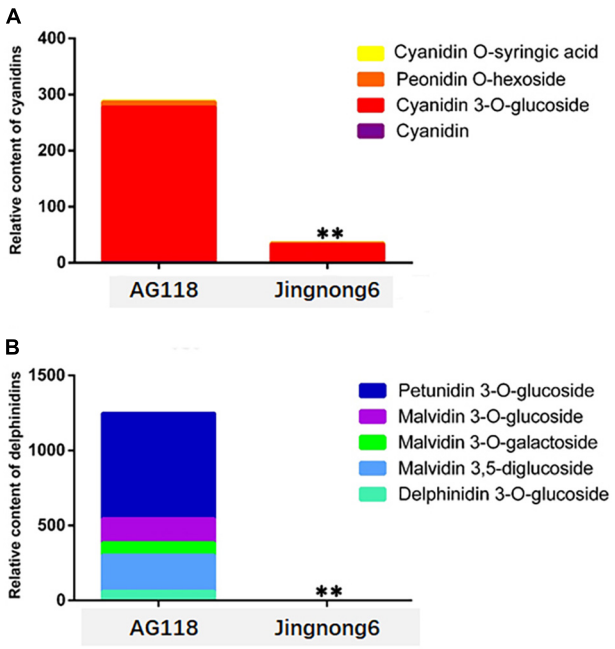

FIGURE 3 | Identification and quantification of the pigments during stage 3 in seed coat colouring of Jingnong6 and AG118. *$^{**}P \leq 0.01$*.

profiles of Jingnong6 and AG118 structural genes in the biosynthesis of flavonoid based on their FPKM values. The results showed that multiple structural genes in the flavonoid metabolic pathways, including chalcone synthase (*VaCHS*), chalcone isomerase (*VaCHI*), flavanone 3-hydroxylase (*VaF3H*), dihydroflavonol-4-reductase (*VaDFR*), anthocyanidin synthase (*VaANS*), anthocyanidin 3-O-glucosyltransferase (*VaBZ1*), flavonoid3′-hydroxylase (*VaF3'H*), flavonoid3′5 ′-hydroxylase (*VaF3′5 ′H*) and R2R3-MYB (*VaSDC1*), had different expression models in seed coat colouring stages between Jingnong6 and AG118. The expression levels of these structural genes in the flavonoid metabolic pathways observably increased from stage 1 to stage 3 in AG118 but had only small changes from stage 1 to stage 3 in Jingnong6 (**Figure 4A**).

To validate the RNA-Seq results, we analysed the expression levels of these structural genes from stage 1 to stage 3 in Jingnong6 and AG118 by qRT-PCR. The expression patterns of these genes were very similar to the RNA-Seq results (**Figure 4B**). These results validated the relevance of the RNA-Seq data. In addition, qRT-PCR results had better gene expression consistency.

Identification of *VaSDC1* by Gene Mapping

We initially mapped the SDC1 trait (i.e., the difference between black and red seed coat colours) on a 1,454 kb physical interval between the initial position of the short arm and *s342-127390* of chromosome 3 (Li et al., 2017). *VaSDC1* was located in this range. To finely map the *VaSDC1* gene, which leads to the difference in the seed coat colour between Jingnong6 and AG118, 12 pairs of SSR primers were developed that span the 1,454 kb interval, encompassing the genomic sequence between the initial positions of chromosome 3 and *s342-127390*. Three (25%) SSR motifs were found to be polymorphic in the two parental lines (**Supplementary Table 1**). The colour difference between black and red seed coats was regarded as a morphological marker and named SDC1 (i.e., the difference between the black seed coat and red seed coat in F_4 segregating population from the cross between Jingnong6 and AG118) and was used for gene mapping. Using three SSR markers, *Sca326-12*, *Sca326-4,* and *BAgs007*, the morphological marker SDC1 was found to be located between the SSR markers *Sca326-12* and *BAgs007* at a distance of 4.3–3.1 cm, respectively (**Figure 5B** and **Supplementary Table 3**).

DISCUSSION

The adzuki bean is also called "The Red Small Bean" in China. The seed coat colour of most cultivars is red including Jingnong6. AG118 is a rare landrace of the black seed coat. In other legume crops, seed coat colour has been shown to correlate with polyphenol content (Amarowicz and Pegg, 2008). During the colouring process of Jingnong6 and AG118, with the deepening of seed coat colour, the anthocyanin content increased gradually. The anthocyanin content of AG118 was much higher than that of Jingnong6, at each colouring stage. In stage 3, the contents of anthocyanins, delphinidins and their derivatives of AG118 seed coat were significantly higher than those of Jingnong6. Seed coat morphological analysis and anthocyanin content determination indicated that colouration of the seed coat in the adzuki bean is a process of gradual accumulation of anthocyanins.

During the three seed coat colouring stages, the expression levels of the structural genes of the flavonoid metabolic pathway in AG118 were significantly higher than that of Jingnong6 as indicated by RNA-Seq and verified by qRT-PCR. The expression levels of the structural genes in the flavonoid biosynthesis pathway can directly influence the accumulation of anthocyanin, which determines seed coat colour (Lepiniec et al., 2006). We found that expression levels of *VaF3'H* and *VaF3'5'H* were significantly higher in AG118 compared to Jingnong6 during the colouring stages. *VaF3'H* and *VaF3'5'H* are able to turn the final product into cyanidin, delphinidin and their derivatives in the flavonoid metabolic pathways but not pelargonidin. This result corresponds to our content analysis of cyanidin, delphinidin and their derivatives. Structural genes *VaCHS*, *VaCHI*, *VaF3H*, *VaDFR*, *VaANS*, *VaBZ1*, *VaF3'H,* and *VaF3'5'H* in the flavonoid biosynthesis pathway had different expression trends during the three colouring stages of Jingnong6, but their expression level increased significantly from stage 1 to stage 3 in AG118 (**Figure 4**). The results indicated that the expression of these genes was activated in AG118.

VaSDC1 has the same expression pattern as these structural genes described above. *VaSDC1* is an R2R3-MYB transcription factor, which displays the highest homology with AtMYB75/90/113/114 (**Figure 5A**) in amino acid sequence. *AtMYB75/90* (i.e., *AtPAP1/AtPAP2*) was reported to be able to regulate the structural genes in the flavonoid metabolic pathways and further influence the colour in tomato (Li et al., 2018). In conclusion, *VaSDC1* might be the key

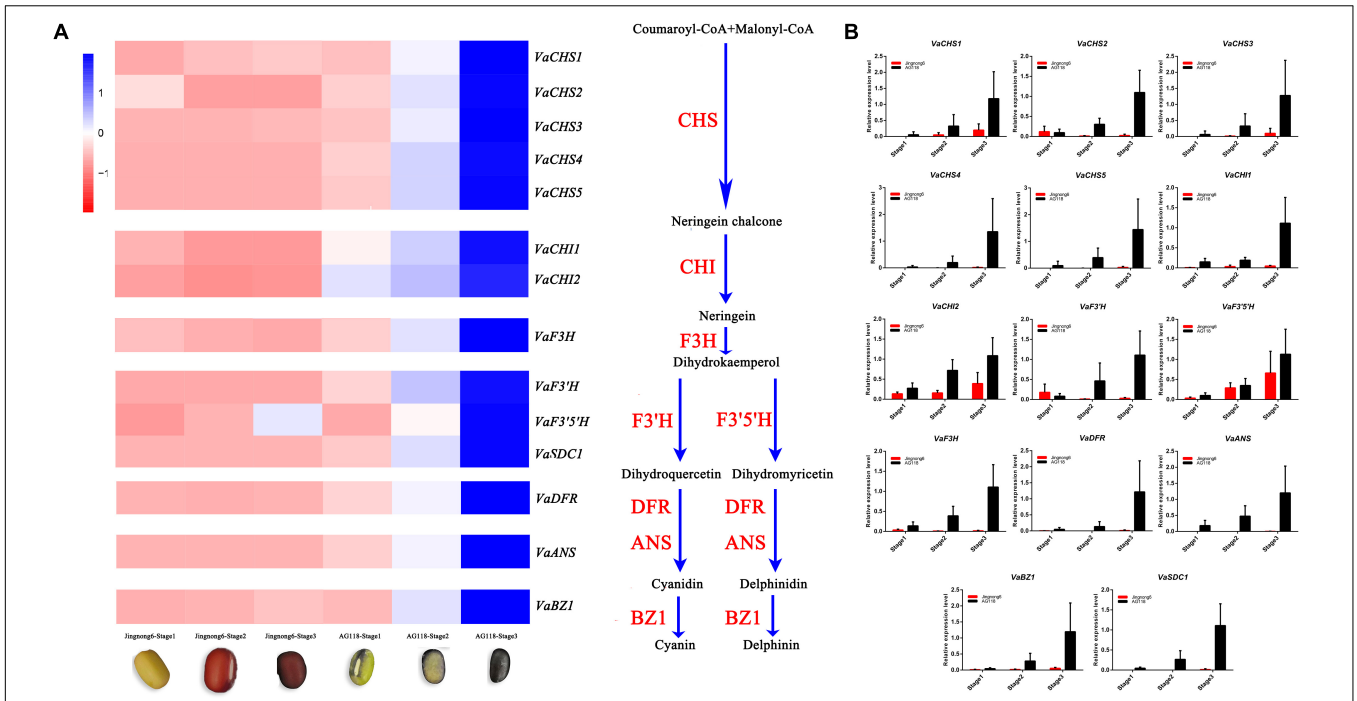

FIGURE 4 | Differences in gene expression levels of Jingnong6 and AG118 seed coats. **(A)** The expression profiles of flavonoid metabolic pathway structural genes in Jingnong6 and AG118 from stage 1 to stage 3 by RNA-Seq; **(B)** the expression profiles of structural genes of flavonoid metabolic pathways verified by qRT-PCR (mean ± SD from three biological replicates).

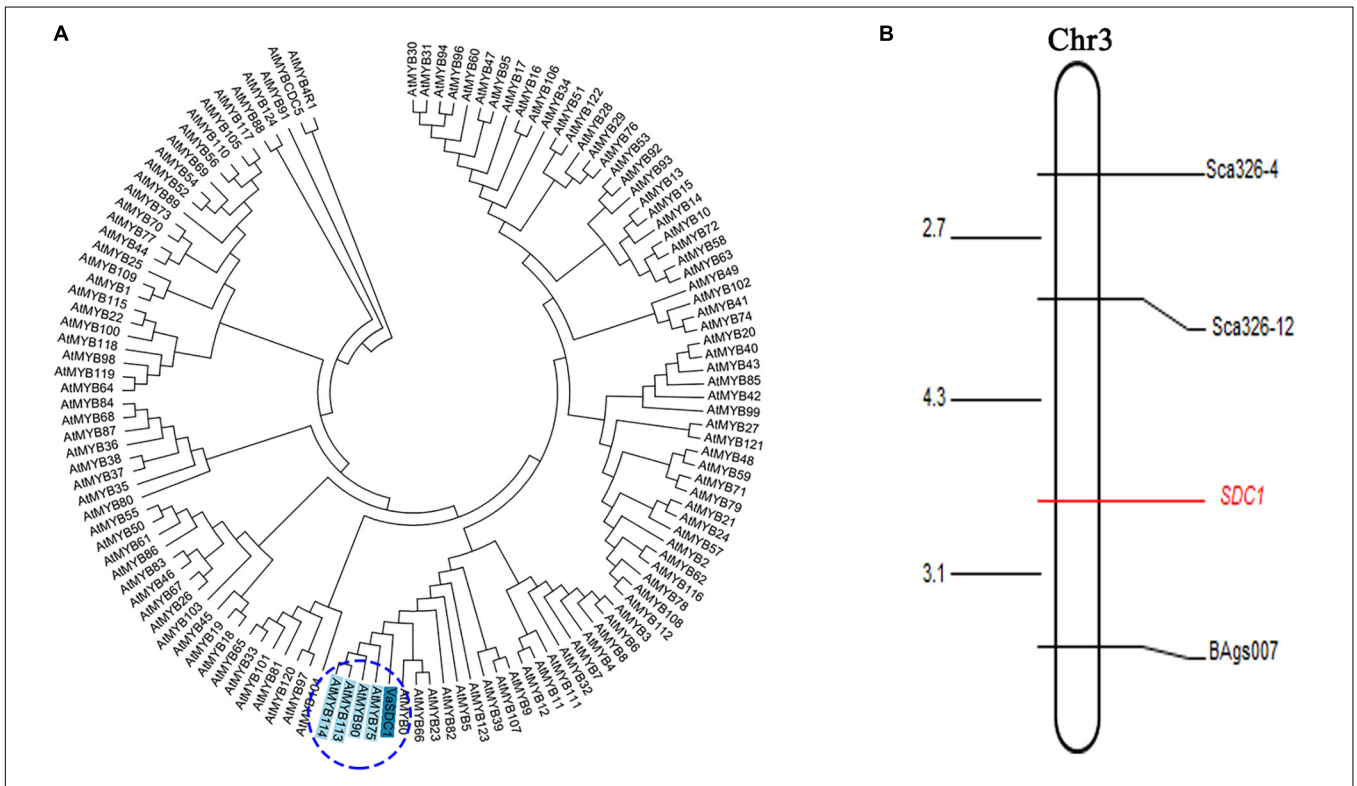

FIGURE 5 | Further proof of the involvement of *VaSDC1* in the colouration difference between Jingnong6 and AG118. **(A)** Neighbour-joining (NJ) phylogenetic tree based on amino acid sequences *VaSDC1* and all R2R3-MYBs of *Arabidopsis*; **(B)** linkage map between SDC1and SSR markers.

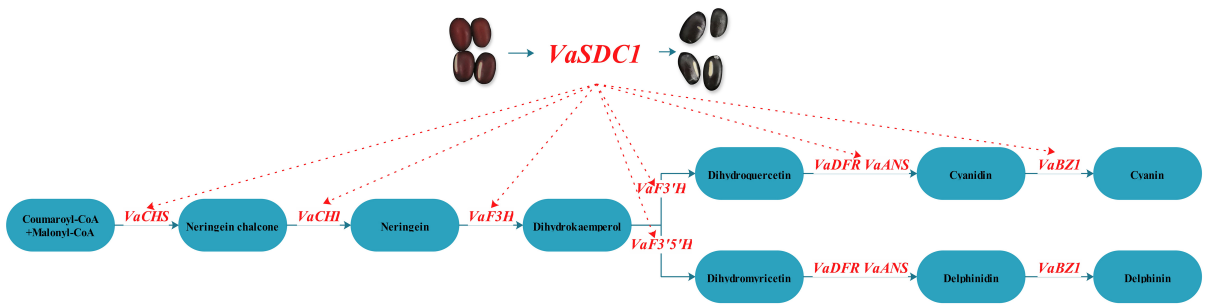

FIGURE 6 | Hypothetical model of the molecular mechanism of the flavonoid metabolic pathway in the red Jingnong6 and the black AG118.

factor that leads to the difference between black and red seed coat colours in adzuki beans. UDP-glucose:flavonoid 3-O-glucosyltransferase (*UGT78K1*) gene caused soybean black seed coat colour (Kovinich et al., 2010). *VaSDC1*, as a transcription factor, can increase the accumulation of flavonoids by activating the expression of structural genes in the flavonoid metabolic pathway and change the adzuki bean seed coat colour. *VaSDC1* is different from the *UGT78K1* gene leading to black seed coat colour in soybean. This result was significant for the breeding of antioxidant quality in adzuki beans.

Based on a previous study, we mapped the black seed coat trait SDC1, which is controlled by a single gene onto chromosome 3 (Li et al., 2017). In order to further identify whether *VaSDC1* was the key factor regulating the difference in the seed coat colour between Jingnong6 and AG118, SSR markers were used to narrow the mapping interval of SDC1 (**Figure 5B**). As a morphological marker, SDC1 was further mapped in the interval between *Sca326-12* (1,211,665 bp from the initial position of chromosome 3) and *BAgs007* (2,482,806 bp from the initial position of chromosome 3), which contains the *VaSDC1* gene. To a certain extent, the mapping result of SSR markers confirms our hypothesis that *VaSDC1* is the key factor regulating the colour difference between Jingnong6 and AG118. However, further verification is required.

A hypothetical model of the molecular mechanism of the flavonoid metabolic pathway between black seed coat AG118 and Jingnong6 was predicted (**Figure 6**). The high expression of *VaSDC1* in AG118 may activate the expression of structural genes in the flavonoid metabolic pathways, further promote the accumulation of anthocyanin and lead to the difference between black AG118 and red Jingnong6. We developed three molecular markers linked to *VaSDC1*. These results enriched our understanding of seed coat colouration mechanisms. Furthermore, molecular markers can lead to the improvement of nutritional quality in adzuki beans.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://bigd.big.ac.cn/gsa, PRJCA004579.

AUTHOR CONTRIBUTIONS

PW designed and managed the project and revised the manuscript. KY and LWC coordinated the project and experiments. LWC analysed all the data and wrote the primary manuscript. PZ collected all seed coat samples, prepared the DNA, mapped the *VSDC1* gene using SSR markers, and analysed the transcriptome. KLW took part in measuring the anthocyanin content and analysed structural gene expression levels. BZ and YSL cultivated and managed the experimental accessions, segregated population and identified the phenotypes. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.679892/full#supplementary-material

Supplementary Table 1 | The description of genes and primers used in this study.

Supplementary Table 2 | The description of primers for SSR markers.

Supplementary Table 3 | Information about markers for gene mapping.

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Major QTLs and Potential Candidate Genes for Heat Stress Tolerance Identified in Chickpea (*Cicer arietinum* L.)

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In the context of climate change, heat stress during the reproductive stages of chickpea (*Cicer arietinum* L.) leads to significant yield losses. In order to identify the genomic regions responsible for heat stress tolerance, a recombinant inbred line population derived from DCP 92-3 (heat sensitive) and ICCV 92944 (heat tolerant) was genotyped using the genotyping-by-sequencing approach and evaluated for two consecutive years (2017 and 2018) under normal and late sown or heat stress environments. A high-density genetic map comprising 788 single-nucleotide polymorphism markers spanning 1,125 cM was constructed. Using composite interval mapping, a total of 77 QTLs (37 major and 40 minor) were identified for 12 of 13 traits. A genomic region on CaLG07 harbors quantitative trait loci (QTLs) explaining >30% phenotypic variation for days to pod initiation, 100 seed weight, and for nitrogen balance index explaining >10% PVE. In addition, we also reported for the first time major QTLs for proxy traits (physiological traits such as chlorophyll content, nitrogen balance index, normalized difference vegetative index, and cell membrane stability). Furthermore, 32 candidate genes in the QTL regions that encode the heat shock protein genes, heat shock transcription factors, are involved in flowering time regulation as well as pollen-specific genes. The major QTLs reported in this study, after validation, may be useful in molecular breeding for developing heat-tolerant superior lines or varieties.

Keywords: chickpea, heat stress, genotyping-by-sequencing, normalized difference vegetation index, days to pod initiation

INTRODUCTION

Given the global climate changes, heat stress is becoming a major challenge to crop production and food safety. As per Intergovernmental Panel on Climate Change, the current rate of global warming is 0.2°C per decade and is predicted to reach 1.5°C between 2,030 and 2,052 (https://www.bbc.com/news/newsbeat-48947573). Such an increase in temperatures leads to heat stress and costs the global economy US$2.4 trillion a year (https://news.un.org/en/story/2019/07/1041652). More than

15% of the global land area becomes exposed to high levels of heat stress with an additional 0.5°C increase to the 2°C (Sun et al., 2019). Heat stress, besides affecting producers directly, also reduces labor productivity (Kjellstrom, 2016), further compounding the effects of increasing temperature on crop yields. In recent years, shifts toward more sustainable and healthy diets, which are typically characterized by high consumption of vegetables and legumes, have been evidenced (Scheelbeek et al., 2018).

Chickpea (*Cicer arietinum* L.) is an important cool season grain legume crop cultivated in the arid and semi-arid regions across the globe. It is an excellent source of proteins, essential amino acids, vitamins, and minerals (Jukanti et al., 2012). Major chickpea producing countries are India, Australia, Pakistan, Turkey, Russia, Myanmar, Iran, Mexico, Canada, and USA. In India, Madhya Pradesh, Maharashtra, Rajasthan, Karnataka, Uttar Pradesh, and Andhra Pradesh are the major chickpea growing states. Although India is the largest producer of chickpea, in order to attain self-sufficiency by 2050, the chickpea production in the country needs to reach 16–17.5 Mt from an area of about 10.5 Mha with an average productivity of 15–17 q/ha (Dixit et al., 2019). Drought and heat, the two most important environmental factors, can cause more than 70% yield loss in chickpea (Varshney et al., 2019). Traditionally, chickpea requires prolonged winter for better growth and cultivation in the northern states of India. However, in the northern states, the pulse area especially chickpea cultivation was reduced due to the green revolution. Southern and Central India, where significant chickpea area increased, are exposed to drought and heat stresses. The rise in ambient air temperature (≥35°C) that coincides with the reproductive processes leads to various anomalies in reproductive events, especially during fertilization, pod formation, and pod filling in chickpea (Devasirvatham et al., 2013; Kaushal et al., 2013; Gaur et al., 2014).

The genetic mechanism of heat stress in different crop plants has been reviewed extensively (see Janni et al., 2020). In general, the impact of heat stress depends on the intensity, duration of exposure, and degree of the elevated temperature. In the case of legumes like chickpea, heat stress has deleterious effects on the morphology, physiology, and reproductive growth (Sita et al., 2017). The effects of heat stress on the development of various male and female tissues in different legume species have been reviewed recently (Liu et al., 2019). In the case of legume crops, heat shock proteins (*HSP*), *HSP* gene families, and various metabolites were reported to control heat stress response (see Janni et al., 2020). Heat stress adversely affects pollen viability, fertilization, and seed development, which leads to a reduced harvest index consequently, and these events greatly impact chickpea yield. In the cool season, legumes such as chickpea, lentil, faba bean, and field peas, the temperature above 30°C lead to yield losses (Jiang et al., 2015; Bishop et al., 2016; Bhandari et al., 2017). As heat stress is a complex trait governed by many genes/QTLs, breeding for heat stress tolerance in chickpea remains challenging (Krishnamurthy et al., 2011; Devasirvatham et al., 2013). Therefore, the effects of heat stress on chickpea growth, development, and yield are important to understand by observing agronomic traits to develop high-temperature-tolerant cultivars.

Genomic revolution, during the last two decades, simplified understanding of the complex responses to biotic and abiotic stress in several crop plants (Roorkiwal et al., 2020; Thudi et al., 2020). Chickpea research community has access to genome sequence (Varshney et al., 2013), genome-wide variations among diverse germplasm lines at the sequence level (Thudi et al., 2016a,b; Varshney et al., 2019) for trait dissection, and the development of climate-resilient chickpea varieties (Mannur et al., 2019; Bharadwaj et al., 2020). The genotyping-by-sequencing (GBS) approach has been extensively used for single-nucleotide polymorphism (SNP) discovery and mapping traits in several crops for genetic research and breeding applications (Chung et al., 2017), including chickpea (Jaganathan et al., 2015; Thudi et al., 2020). Besides proteomic and metabolomic approaches to understanding the molecular mechanism of heat tolerance (Parankusam et al., 2017; Salvi et al., 2018), efforts were made to map QTLs and markers associated with heat tolerance in chickpea (Thudi et al., 2014; Paul et al., 2018a; Varshney et al., 2019; Roorkiwal et al., 2020).

In this study, we reported the construction of a high-density genetic map using SNPs derived from the GBS approach and major QTLs for phenological, physiological, yield, and yield-related traits based on phenotyping of recombinant inbred line (RIL) population (DCP 92-3 × ICCV 92944) under two environments (normal and late sown) for 2 years (2017–2018 and 2018–2019). In addition, we also reported the potential candidate genes implicated for heat tolerance in the QTL regions.

MATERIALS AND METHODS

Plant Material

A biparental mapping population, comprising 184 F_7 RIL lines, derived from the cross DCP 92-3 × ICCV 92944 segregates for heat tolerance was used for identifying genomic regions and candidate genes for heat tolerance. DCP 92-3 is a logging and *Fusarium* wilt-resistant variety released by the Indian Council of Agricultural Research (ICAR)-Indian Institute of Pulses Research (IIPR), Kanpur, Uttar Pradesh, India for cultivation in Punjab, Haryana, Delhi, Northern Rajasthan, and Western Uttar Pradesh. Pollen viability at a critical temperature of 35°C differentiates the heat-sensitive and heat-tolerant genotypes. Based on physiological, biochemical, yield, and yield-related trait studies conducted earlier (Gaur et al., 2012; Kumar et al., 2012; Kaushal et al., 2013; Bhandari et al., 2020), the chickpea genotype ICCV 92944 was reported as a heat-tolerant genotype and was released as BARI Chola-10 in Bangladesh, as Yezin 6 in Myanmar, and as JG 14 in India and is performing well under late sown conditions.

Phenotyping of Recombinant Inbred Line Population

In the case of chickpea, the optimal temperature for its growth ranges between 10 and 30°C. Chickpeas are sensitive to heat stress particularly at the reproductive phase (flowering and seed development). A few days of exposure to high temperatures

(35°C or above) during the reproductive phase can cause heavy yield losses through flower and pod abortion. Late sowing, a simple and effective field screening technique for reproductive-stage heat tolerance in chickpea developed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, was adopted for phenotyping the RILs for heat stress tolerance. The F_7 RILs (184 individuals) and parents DCP 92-3 × ICCV 92944 were evaluated for two consecutive years 2017–2018 and 2018–2019 under normal sown environment (NS; second week of November) and late sown or heat stress environment (HTS; third week of December) at ICAR-IIPR, Kanpur, Uttar Pradesh, India (26° 26′ 59.7228″ N and 80° 19′ 54.7356″ E). The experiments were conducted under field conditions in a plot admeasuring 3 × 0.6 m, and the distance between plants is 10 cm. The RIL population was evaluated in augmented block design along with the parents DCP 92-3 and ICCV 92944 and two elite chickpea genotypes JG 11 and ICC 4958. All the individuals of the population were apportioned into a total of 10 blocks along with the four checks replicated in each block. The maximum, minimum, and mean temperatures were recorded weekly during the entire cropping season for both years (**Supplementary Figure 1**). The mapping population was phenotyped for physiological traits like normalized difference vegetation index (NDVI; using GreenSeeker, Optical Sensor Unit, 2002 114 NTech Industries, Ukiah, USA), nitrogen balance index (NBI, using DUALEX® optical leafclip meter), NBI® combines chlorophyll and flavonols (related to nitrogen/carbon allocation) measured by using DUALEX® optical leafclip meter, chlorophyll content (CHL, using DUALEX® optical leafclip meter ng/mm^2) and cell membrane stability (CMS, %), yield, and yield-related traits [(total filled pods per plant (FP), biological yield per plant (BYPP, g), seed yield per plant (SYPP, g), harvest index (HI, %), and 100 seed weight (100SDW, g)]. To avoid the biasness, the mean of 10 individual plants was sampled for seed yield/plant taken from each planted genotype instead of seed yield/m^2 per plot. Furthermore, the mean of 10 plants randomly chosen from each line was used for recording the abovementioned traits for all the individuals of mapping population under NS and HTS for both years. Two irrigation and same agronomic package of practice were followed for both NS and HTS sown genotypes for both years. NDVI was measured as per the following formula NDVI = NIR–RED\NIR + RED (Myneni et al., 1995), and CMS was measured as per the formula used by Blum and Ebercon (1981). CMS = 100–membrane injury index (MII), where MII is calculated as a ratio of C1 and C2, with C1 and C2 denoting the electrolytes measured at 40 and 80°C, respectively.

Statistical Analyses

Analysis of Variance, Best Linear Unbiased Prediction (BLUP), and Heritability

The ANOVA for the RIL population was performed using GenStat (17th Edition), for individual environments using the mixed model analysis. For each trait and environment, the analysis was performed considering entry and block (nested within replication) as random effects and replication as fixed effects. To pool the data across environments and to make the error variances homogeneous, the individual variances were estimated and modeled for the error distribution using the residual maximum likelihood (ReML) procedure. The *Z*-value and *F*-value were calculated for random effects and fixed effects, respectively. Broad-sense heritability was calculated as $H^2 = Vg/(Vg + Ve/nr)$, as suggested by Falconer et al. (1996), and pooled broad-sense heritability was estimated as $H^2 = Vg/\{(Vg) + (Vge/ne + Ve/(ne \times nr))\}$, as suggested by Hill et al. (2012), where H^2 is the broad-sense heritability, Vg is the genotypic variance, Vge is the G × E interaction variance, Ve is the residual variance, ne is the number of environments, and nr is the number of replications.

DNA Extraction, Genotyping, and Single-Nucleotide Polymorphism Calling

DNA from 184 RILs, along with the parents, was isolated from 2-week-old seedlings following the high-throughput mini-DNA extraction method (Cuc et al., 2008). The quality of DNA was checked by using 0.8% agarose gel electrophoresis, and the quantity was assessed by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc., USA). The GBS approach was used for SNP calling between the parents and genotyping the RILs as described by Elshire et al. (2011). GBS libraries from the parental lines and RILs were prepared using *ApeKI* endonuclease (recognition site: G/CWCG), followed by ligation with uniquely barcoded adapters using T4 DNA ligase enzyme. Such digested ligated products from each sample were mixed in equal proportion to construct the GBS libraries, which were then amplified, purified to remove excess adapters, and used for sequencing on HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). Sequence reads from raw FASTQ files were used for SNP identification and genotyping using the reference-based GBSv2 analysis pipeline implemented in TASSEL v5.0 (Bradbury et al., 2007). In brief, all reads that begin with one of the matched barcodes immediately followed by the expected four base remnants of the enzyme cut site are sorted, de-multiplexed, and trimmed to first 64 bases starting from the enzyme cut site. Reads containing N within the first 64 bases after the barcode are rejected. The remaining good quality reads (called as tags) were aligned against the draft genome sequence of chickpea using Bowtie2 software. The alignment file was then processed by using the GBSv2 analysis pipeline for SNP calling and genotyping.

Linkage Map Construction and Identification of QTLs

In order to construct the genetic map, all markers were grouped into eight linkage groups with the logarithm of odds (LOD) threshold of 5.0. Marker order within a linkage group was assigned using the regression mapping algorithm with a maximum recombination frequency of 0.4 at a LOD of three and a jump threshold of five. The ripple command was fine-tuned by adding each marker locus to confirm the final marker order. The Kosambi mapping function was used to calculate the map distance in centimorgan (cM). The segregation distortion and chi-square (χ^2) values were detected using JoinMap V4.0, and markers with heterozygosity and significant segregation distortion were excluded ($p < 0.001$) from the analysis. The linkage map was constructed using ICIMapping 3.2 software

(Meng et al., 2015). The QTL analysis was conducted for NS 2017, NS 2018, NS pooled data, HTS 2017, HTS 2018, and HTS pooled data together with the genotyping data and genetic map information using software windows QTL Cartographer version 2.5 (Wang et al., 2012). The composite interval mapping (CIM) analysis was conducted by scanning the intervals of 1.0 cM between markers and putative QTLs with a window size of 10.0 cM and by using the parameters of model six and 1,000 times of permutation with the 0.05 significance level along with the function of "Locate QTLs" option to locate QTLs.

Identification of Candidate Genes Within QTL Confidence Intervals

Based on the physical position of the SNPs/markers flanking the QTL regions, the candidate genes present within the determined QTL intervals were retrieved from the draft genome sequence (CaGAv1.0) of chickpea (Varshney et al., 2013). The identified genes in QTL intervals were searched against NCBI-nr protein database using BLAST program. The gene ontology (GO) terms associated with the genes were searched for GO terms, using BLAST2GO software (Conesa et al., 2005).

RESULTS

Phenotypic Performance and Genetic Variability of the Parents and Mapping Population

A considerable amount of genetic variation for various phenological, yield, and yield-related traits was observed in both the parents and the derived RILs under NS and HTS environments for both years. The descriptive statistics are shown in **Table 1**. Transgressive segregates in both directions were observed for days to flower initiation (DFI), FP, and SYPP traits in the RIL population (**Figure 1**). The Combined ANOVA indicated the presence of significant genetic variability in the evaluated RILs under both NS and HTS. High to moderate heritability (98.2–61.3%) under NS for both years and 73.3–98.4% heritability under HTS for both years were recorded. Only low heritability of 38.2 and 47.9% for HI was observed under HTS during 2017–2018 and 2018–2019. However, high heritability (77.6–84.7%) was noted under NS conditions.

Relationships Among Different Traits

To investigate the relationship among different traits, we calculated the pairwise correlations among different traits within each environment (NS and HTS). During 2017–2018, under HTS environment, a positive and high significant correlation was observed between DFI with that of days to pod initiation (DPI) ($p < 0.01$) and days to maturity (DM) ($p < 0.01$) (**Supplementary Table 1**). Similarly, during 2018–2019, under HTS environment, a positive and high significant correlation was also observed between DFI with that of DPI ($p < 0.01$) and DM ($p < 0.05$) (**Supplementary Table 2**). Furthermore, during 2017–2018, under HTS environment, NBI and CHL were found to possess a positive and high significant correlation ($p < 0.01$). However, no significant correlation was observed during 2018–2019 under HTS environment. A number of filled pods (NFP) and SYPP had a significant and positive correlation under heat stress environments during both years. Furthermore, NPF has a significant positive correlation with BYPP in 2017–2018 and with HI in 2018–2019 (**Supplementary Tables 1, 2**). Nevertheless, 100SDW possesses a positive and high significant correlation with HI and SYPP during both years under HTS environment (**Supplementary Tables 1, 2**). Similar positive and high significant correlations were also observed under NS environments in both years as well as pooled data of NS environments for the abovementioned traits (**Supplementary Tables 3–6**).

Single-Nucleotide Polymorphisms-Based Genetic Map

A total of 49.89 Gb (49 million reads) clean GBS reads were generated using HiSeq2500 on the RIL population derived from DCP 92-3 × ICCV 92944. The number of reads generated per individual ranged from 0.86 to 5.3 million. A total of 3,425,458 genome-wide SNPs were identified on aligning the data to CDC Frontier reference genome (Varshney et al., 2013) using TASSEL-GBS pipeline. After excluding ambiguous SNP calls, SNPs that are monomorphic among the parental genotypes, and SNPs with segregation distortion, a total of 7,947 polymorphic SNPs were used for the linkage map analysis using ICIM. As a result, a genetic map comprising 788 SNPs distributed on eight linkage groups (CaLG01–CaLG08) spanning 1,125 cM was constructed (**Figure 2**; **Supplementary Tables 7, 8**; **Supplementary Figure 2**). The CaLG06 had the highest proportion of the mapped SNPs (23.4%; 185 SNPs), whereas CaLG08 had the lowest proportion of the mapped SNPs (7.6%; 60 SNPs) and the largest linkage group CaLG01 spanned 191 cM, whereas the smallest linkage group CaLG08 spanned 68 cM.

QTLs for Heat Stress Tolerance Traits

By using CIM, a total of 77 QTLs (37 major QTLs and 40 minor QTLs) were identified for 12 of 13 traits phenotyped for two seasons (2017–2018 and 2018–2019) and two environments (NS and HTS). Of 77 QTLs, 37 QTLs were major explaining ≥10% phenotypic variation (PVE), and 40 QTLs were minor explaining <10% PVE (**Table 2**). A positive value of the additive variance of a given QTL indicates that the female parent (DCP 92-3) has a positive effect on the trait; while a negative value indicates that the male parent (ICCV 92944) having a positive effect on the trait.

QTLs for Phenological Traits

Under the HTS environment, in the case of DFI, two QTLs each were identified during 2018–2019 and pooled data of 2017–2018 and 2018–2019 on CaLG06 and CaLG08. The PVE ranged from 7.48 to 8.96%. While in the case of DM, all three QTLs (PVE 8.96–18.13%) identified were in 2017–2018 and under HTS environment on CaLG01. In the case of DFI, an additive effect for QTLs on CaLG06 ranged from −2.84 to −2.94 (**Table 2**; **Figure 3**).

TABLE 1 | Phenotypic performance of heat sensitive (DCP 92-3), tolerant (ICCV 92944), RILs, and heritability of traits evaluated under normal and heat stress environments.

Traits	Environment	DFI (d)	DM (d)	DPI (d)	DPF (d)	NDVI	NBI	CHL (ng/mm^2)	CMS (%)	FP	BYPP (g)	100SDW (g)	HI (%)	SYPP (g)
DCP92-3	NS 2017	56.35	136.50	74.39	22.31	0.58	23.77	24.23	46.37	72.18	31.88	15.23	65.67	21.18
	HTS 2017	49.56	103.28	65.32	11.32	0.51	23.30	22.39	38.32	41.20	19.43	13.16	41.37	7.74
	NS 2018	53.42	133.00	74.31	17.40	0.62	24.17	23.64	55.69	54.89	26.63	12.11	57.81	15.84
	HTS 2018	40.20	98.90	51.87	15.19	0.47	17.69	23.52	34.09	25.02	11.97	12.95	34.69	3.20
ICCV92944	NS 2017	45.02	118.80	60.66	18.98	0.63	22.99	25.22	53.81	82.06	45.51	26.55	67.28	31.02
	HTS 2017	51.35	101.40	62.09	11.13	0.52	22.11	25.78	43.83	47.30	17.82	17.77	54.59	9.96
	NS 2018	40.92	116.50	60.71	17.60	0.62	23.35	24.98	58.86	59.51	32.51	21.84	65.07	21.30
	HTS 2018	39.52	91.80	53.61	16.25	0.51	17.88	23.43	33.16	28.60	12.57	16.97	41.71	4.20
Means of RILs	NS 2017	57.40	127.70	78.80	18.20	0.61	17.65	30.23	56.90	88.40	35.80	21.00	66.20	25.70
	HTS 2017	47.00	97.30	60.10	12.50	0.38	18.00	28.70	45.20	44.70	21.30	20.70	49.40	10.40
	NS 2018	56.10	125.70	74.60	17.50	0.61	22.55	28.00	54.40	86.60	38.00	20.40	64.80	24.70
	HTS 2018	43.90	100.40	57.20	14.90	0.36	17.76	23.60	41.40	36.40	18.30	19.60	44.30	7.80
Range of RILs	NS 2017	53.9–59.91	124.5–130.4	74.5–82.42	12.12–24.76	0.38–0.75	11.2–25.6	17.1–45.3	44.9–66.9	79.7–97.69	29.8–41.3	14.6–31.77	57.8–71.66	20.5–31.18
	HTS 2017	39.7–51.3	94.8–100.22	51.7–64.4	8.5–19.68	0.3–0.54	14.8–24.1	15–44	42–42–50.4	38–51.12	15.8–28.22	13.7–31.85	45.7–55.69	8.84–13.04
	NS 2018	52.5–61	122.3–129.9	70.7–79.2	9–24.7	0.41–0.59	18.2–28.7	17.5–37.4	36.0–66.6	69–104.18	32.4–49.35	12.7–31.56	52.14–70.53	19.65–33
	HTS 2018	33.7–50.75	88.5–107.6	47.5–63.96	11–21.4	0.25–0.52	17.3–18	23.3–24	30.5–52.7	24.5–45.47	13.8–24.83	13–29.21	39.8–49.74	4.17–11.757
Heritability%	NS 2017	86.80	86.60	94.50	93.60	93.50	76.40	94.90	82.10	61.30	70.30	97.40	77.60	74.20
	HTS 2017	85.80	73.60	86.50	96.60	90.60	91.20	98.50	78.50	80.00	83.20	98.40	47.90	67.37
	NS 2018	87.80	83.90	92.00	98.00	77.20	80.80	88.90	92.50	80.50	77.10	98.20	84.70	73.24
	HTS 2018	92.30	95.30	91.60	90.80	90.90	86.60	80.40	68.80	84.40	83.90	96.20	38.20	77.90

DFI, days to flower initiation; DM, days to maturity; DPI, days to pod initiation; DPF, days to pod filling; NDVI, normalized difference vegetation index; NBI, nitrogen balance index; CHL, chlorophyll content; CMS, cell membrane stability; FP, filled pods; BYPP, biological plant yield, 100SDW, 100 seed weight; HI, harvest index, SYPP, seed yield per plant.

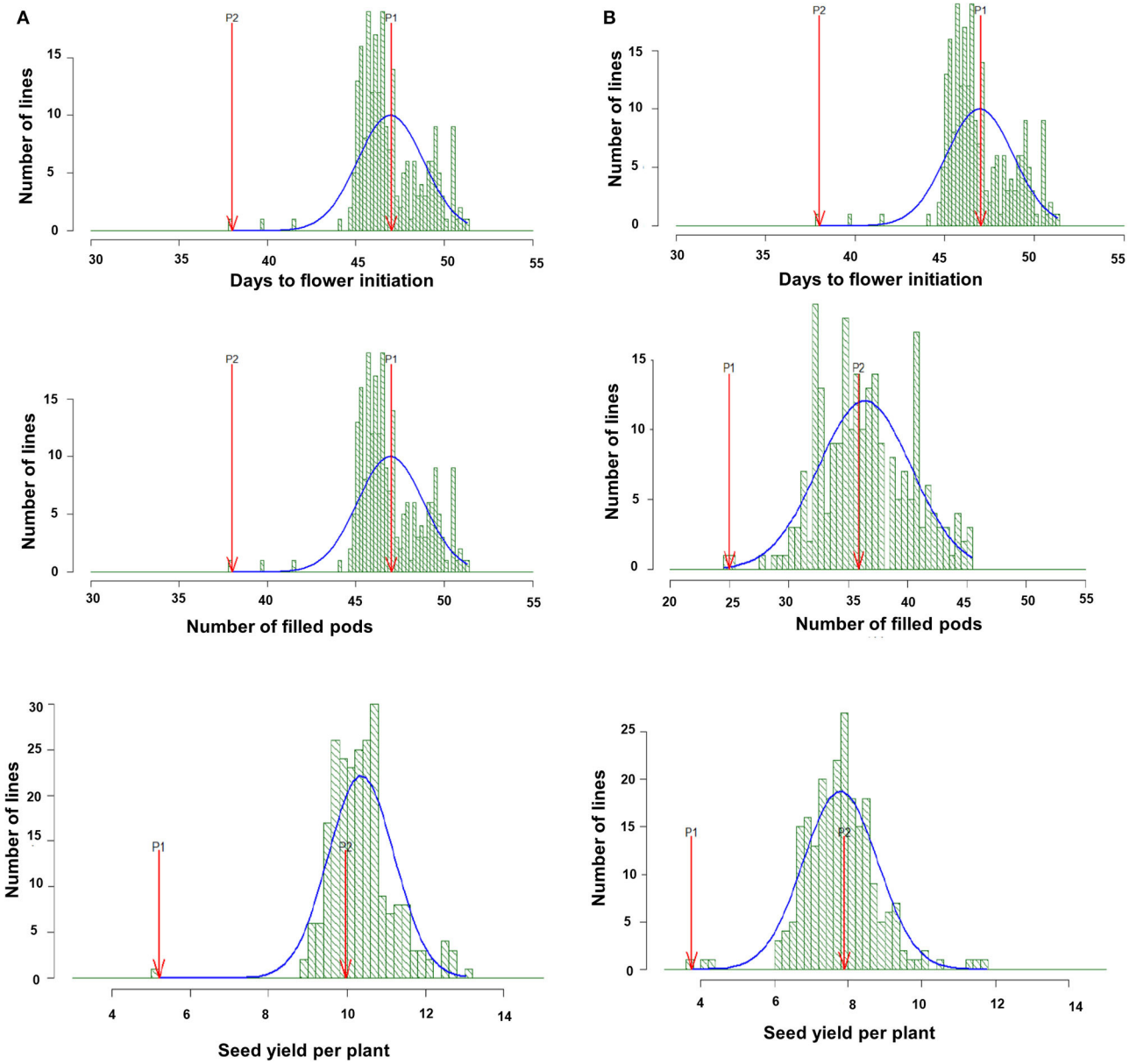

FIGURE 1 | Frequency distribution of days to flower initiation (DFI, d), total filled pods per plant (FP), and seed yield per plant (SYPP, g) in RIL population derived from DCP 92-3 × ICC 92944 and evaluated, under heat stress environment 2017–18 **(A)** and under heat stress environment 2018–19 **(B)**.

QTLs for Physiological Traits

A total of 36 (17 major and 19 minor) QTLs were identified for physiological traits with PVE ranging from 6.69% to 34.02%.

Normalized Difference Vegetation Index (NDVI)

A total of 16 QTLs (seven major with PVE 10.27–34.02% and nine minor with PVE 6.69–9.85%) were identified for NDVI, out of which six were identified based on HTS environment and 10 were identified based on NS environment. Interestingly, for this trait, QTLs were identified on all linkage groups except CaLG07. Furthermore, the majority of QTLs (25%) were present on CaLG02 that explained 8.84–26.31% PVE. However, the QTL on CaLG01 that explained the highest PVE (34.02%) among all QTLs identified for this trait was based on pooled HTS environment (**Figure 3A**).

Nitrogen Balance Index (NBI)

A total of 10 QTLs (five major with PVE 10.26–13.93% and five minor with PVE 7.39–9.95%) were identified for NBI. Among these 10 QTLs, five were on CaLG08, three on CaLG07, and two on CaLG06. Of five QTLs identified on CaLG08, four QTLs were flanked by SCA8_6301805 and SCA8_11012719 markers and one

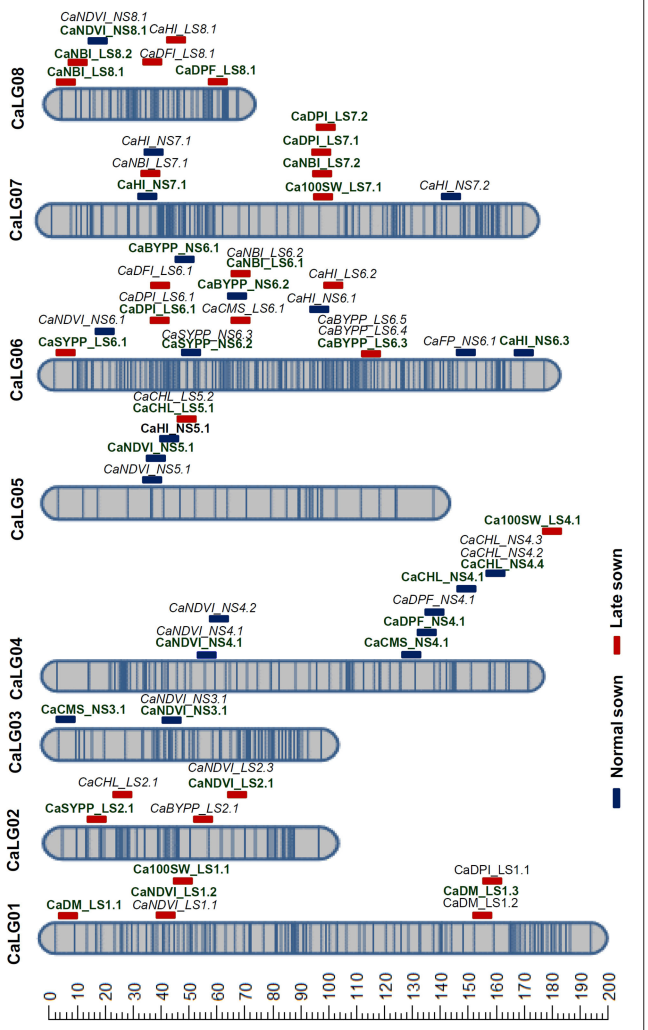

FIGURE 2 | Genetic map and QTLs for heat tolerance-related traits. A genetic map comprising 788 SNPs distributed in eight linkage groups (CaLG01–CaLG08) spanning 1,125 cM was constructed using RIL population derived from DCP 92-3 × ICCV 92944. The blue and red-colored bars indicate the QTLs identified under normal and late sown conditions, respectively. The major QTLs are indicated in bold font and the minor QTLs in italics.

QTL was flanked by SCA8_6301805–SCA8_11012719 markers. Furthermore, among these five QTLs, two each were identified based on the pooled data from HTS environments and HTS of 2018–2019 and one based on HTS 2017–2018 (**Figure 3B**).

Chlorophyll Content (CHL)

Of the seven QTLs identified for CHL, four QTLs were on CaLG04, two were on CaLG05 and one on CaLG02. Under HTS 2018, one minor QTL (PVE 8.14%) was identified on CaLG02. Similarly, under HTS pooled data, one major QTL (PVE 15.04%) and one minor QTL (PVE 6.78%) were identified for CHL on CaLG05 flanked by SCA5_30627756–SCA5_1154130 and SCA5_1154130–SCA5_11665932 markers, respectively (**Table 2**; **Figure 3C**). Furthermore, under NS 2017, one major QTL (PVE 33.52%) and one minor QTL (PVE 8.26%) were identified for CHL on CaLG04 flanked by SCA4_48715028–SCA4_14861717 and SCA4_48720330–SCA4_48714912 markers, respectively. In addition, one major QTL (PVE 19.71%) and one minor QTL (PVE 9.92%) were identified on CaLG04 based on pooled data under NS.

Cell Membrane Stability (CMS)

Of three QTLs identified for CMS, one QTL each was on CaLG03, CaLG04, and CaLG06. Among these QTLs, two were identified based on pooled data from the NS environment and one based on pooled data from the HTS environment.

QTLs for Yield and Yield-Related Traits

Eighteen major and 16 minor QTLs were identified for yield and yield-related traits with PVE ranging from 5.88 to 43.49% (**Table 2**).

Days to Pod Initiation

Under HTS environments (2017–2018, 2018–2019, and pooled data), a total of 6 QTLs (three major with PVE 10.33–43.49% and three minor with PVE 5.88–8.45%) were identified for DPI. Out of these, one QTL was on CaLG01, three on CaLG06, and two on CaLG07. Furthermore, all QTLs identified on CaLG06 were flanked by SCA6_39028647–SCA6_43908965 markers, while QTLs on CaLG07 were flanked by SCA7_9555338–SCA7_47907019 markers. However, the QTL explaining the highest proportion of PVE was present on CaLG07 (**Figure 4**).

Days to Pod Filling and Number of Filled Pods

Of three QTLs (two major with PVE 11.96–11.97% and one minor with PVE 9.38%), two were on CaLG04 and one was on CaLG08. The major QTL on CaLG08 was identified under HTS 2018, while the remaining two were based on the pooled data of the NS environment. However, all three QTLs were flanked by different markers (**Table 2**). One minor QTL (PVE 6.6%) for NFP was identified on CaLG06 based on the pooled data of the NS environment.

Seed Yield per Plant (g)

A total of four QTLs (three major with PVE 11.88–18% and one minor with PVE 8.66%) were identified for SYPP, of which two major QTLs were under the HTS 2018 environments and the remaining two were based on pooled data of the NS environment.

Biological Yield per Plant (g)

A total of eight QTLs (three major with PVE 10.7–11.16% and five minor with PVE 6.92–9.43%) were identified for BYPP. Of eight QTLs, five were identified in the HTS environments of 2017–2018 and 2018–2019 and based on pooled data, and three were identified in the NS environments and based on pooled data. Among these QTLs, seven QTLs were present on CaLG06 and one on CaLG02. Furthermore, all eight QTLs were flanked by different markers (**Table 2**; **Figure 4A**).

TABLE 2 | Summary of QTLs identified for phenological, physiological, yield, and yield-related traits using RIL population derived from DCP 92-3 × ICCV 92944.

Trait	Season	Environment	Linkage group	QTL name	Position (cM)	Left marker	Right marker	PVE%	Additive effect
PHENOLOGICAL TRAITS									
Days to flowering initiation (DFI)	2018–19	Late sown	CaLG06	CaDFI_LS6.1	37.11	SCA6_43908965	SCA6_39028647	8.01	−2.94
	2018–19	Late sown	CaLG08	CaDFI_LS8.1	42.71	SCA8_7197652	SCA8_14126483	7.80	−1.33
	Pooled	Late sown	CaLG06	CaDFI_LS6.1	37.11	SCA6_43908965	SCA6_39028647	8.96	−2.85
	Pooled	Late sown	CaLG08	CaDFI_LS8.1	42.71	SCA8_7197652	SCA8_14126483	7.48	−1.15
Days to maturity (DM)	2017–18	Late sown	CaLG01	CaDM_LS1.1	7.11	SCA1_888	SCA1_30956998	18.13	0.98
	2017–18	Late sown	CaLG01	CaDM_LS1.2	152.61	SCA1_19586410	SCA1_19572921	8.96	−0.57
	2017–18	Late sown	CaLG01	CaDM_LS1.3	154.81	SCA1_11502160	SCA1_19572921	15.78	−0.84
PHYSIOLOGICAL TRAITS									
Chlorophyll content (CHL, ng/mm^2)	2017–18	Normal sown	CaLG04	CaCHL_NS4.3	151.51	SCA4_14861717	SCA4_48715028	33.52	4.12
	2017–18	Normal sown	CaLG04	CaCHL_NS4.3	151.51	SCA4_48714912	SCA4_48720330	8.26	3.31
	2018–19	Late sown	CaLG02	CaCHL_LS2.1	38.31	SCA2_30364073	SCA2_30370411	8.14	−0.03
	Pooled	Late sown	CaLG05	CaCHL_LS5.1	44.01	SCA5_1154130	SCA5_30627756	15.04	3.81
	Pooled	Late sown	CaLG05	CaCHL_LS5.2	44.31	SCA5_11665932	SCA5_1154130	6.78	4.43
	Pooled	Normal sown	CaLG04	CaCHL_NS4.1	142.91	SCA4_14861717	SCA4_48715028	19.71	2.92
	Pooled	Normal sown	CaLG04	CaCHL_NS4.2	150.11	SCA4_48715028	SCA4_48714912	9.92	2.74
Cell membrane stability (CMS, %)	2017–18	Normal sown	CaLG04	CaCMS_NS4.1	133.61	SCA4_48720031	SCA4_11271232	10.33	1.91
	Pooled	Late sown	CaLG06	CaCMS_LS6.1	67.21	SCA6_10020187	SCA6_10626699	7.75	2.86
	Pooled	Normal sown	CaLG03	CaCMS_NS3.1	0.01	SCA3_8852605	SCA3_9063118	11.37	−3.66
Nitrogen balance index (NBI)	2017–18	Late sown	CaLG08	CaNBI_LS8.3	3.81	SCA8_11012719	SCA8_6301805	11.44	1.35
	2017–18	Late sown	CaLG08	CaNBI_LS8.1	0.01	SCA8_6301805	SCA8_15284963	9.95	1.01
	2018–19	Late sown	CaLG08	CaNBI_LS8.2	1.01	SCA8_6301805	SCA8_15284963	13.93	−0.05
	2018–19	Late sown	CaLG07	CaNBI_LS7.2	97.01	SCA7_47907019	SCA7_9555338	11.94	−0.18
	2018–19	Late sown	CaLG07	CaNBI_LS7.1	34.61	SCA7_44149643	SCA7_28235343	9.31	0.05
	Pooled	Late sown	CaLG08	CaNBI_LS8.2	1.01	SCA8_6301805	SCA8_15284963	11.46	2.20
	Pooled	Late sown	CaLG06	CaNBI_LS6.1	69.71	SCA6_10671035	SCA6_10020177	10.26	1.51
	Pooled	Late sown	CaLG08	CaNBI_LS8.1	0.01	SCA8_6301805	SCA8_15284963	9.95	1.92
	Pooled	Late sown	CaLG06	CaNBI_LS6.2	70.71	SCA6_10671035	SCA6_10020177	8.96	1.42
	Pooled	Late sown	CaLG07	CaNBI_LS7.1	34.61	SCA7_44149643	SCA7_28235343	7.39	−2.16
Normalized difference vegetation index (NDVI)	2017–18	Late sown	CaLG02	CaNDVI_LS2.1	65.41	SCA2_31975221	SCA2_8484804	26.31	0.03
	2017–18	Late sown	CaLG02	CaNDVI_LS2.2	66.01	SCA2_16462107	SCA2_31975187	8.84	0.03
	2017–18	Normal sown	CaLG04	CaNDVI_NS4.1	68.31	SCA4_16039554	SCA4_15942274	11.45	0.05
	2017–18	Normal sown	CaLG04	CaNDVI_NS4.2	69.21	SCA4_47389419	SCA4_15935131	7.03	0.05
	2018–19	Late sown	CaLG02	CaNDVI_LS2.1	65.41	SCA2_31975221	SCA2_8484804	22.94	0.03
	2018–19	Late sown	CaLG02	CaNDVI_LS2.2	66.01	SCA2_16462107	SCA2_31975187	9.85	0.04
	2018–19	Normal sown	CaLG03	CaNDVI_NS3.1	48.41	SCA3_4871529	SCA3_18799532	10.73	0.00
	2018–19	Normal sown	CaLG08	CaNDVI_NS8.1	18.61	SCA8_11729896	SCA8_12875512	10.27	0.00
	2018–19	Normal sown	CaLG08	CaNDVI_NS8.2	18.91	SCA8_11269673	SCA8_11729896	9.21	0.00
	2018–19	Normal sown	CaLG03	CaNDVI_NS3.1	48.41	SCA3_4871529	SCA3_18799532	9.11	0.00
	2018–19	Normal sown	CaLG06	CaNDVI_NS6.1	20.01	SCA6_57720446	SCA6_57760410	6.69	0.00
	Pooled	Late sown	CaLG01	CaNDVI_LS1.2	44.21	SCA1_8682204	SCA1_33504088	34.02	−0.06
	Pooled	Late sown	CaLG01	CaNDVI_LS1.1	42.21	SCA1_33504088	SCA1_40495126	9.39	−0.06
	Pooled	Normal sown	CaLG05	CaNDVI_NS5.2	36.11	SCA5_12124749	SCA5_22672234	10.40	0.04
	Pooled	Normal sown	CaLG05	CaNDVI_NS5.1	35.11	SCA5_12124749	SCA5_22672234	9.47	0.04
	Pooled	Normal sown	CaLG04	CaNDVI_NS4.1	68.31	SCA4_16039554	SCA4_15942274	7.41	0.03
YIELD AND YIELD RELATED TRAITS									
Days to pod initiation (DPI, d)	2017–18	Late sown	CaLG07	CaDPI_LS7.2	98.01	SCA7_47907019	SCA7_9555338	43.49	−1.38
	Pooled	Late sown	CaLG07	CaDPI_LS7.1	97.01	SCA7_47907019	SCA7_9555338	10.52	−4.80
	Pooled	Late sown	CaLG06	CaDPI_LS6.1	37.11	SCA6_43908965	SCA6_39028647	10.33	−2.75
	2017–18	Late sown	CaLG06	CaDPI_LS6.1	37.11	SCA6_43908965	SCA6_39028647	5.88	−1.72

(Continued)

TABLE 2 | Continued

Trait	Season	Environment	Linkage group	QTL name	Position (cM)	Left marker	Right marker	PVE%	Additive effect
	2018–19	Late sown	CaLG06	CaDPI_LS6.1	37.11	SCA6_43908965	SCA6_39028647	8.45	−2.71
	Pooled	Late sown	CaLG01	CaDPI_LS1.1	153.61	SCA1_19586410	SCA1_19572921	8.14	−1.03
Days to pod filling (DPF, d)	2018–19	Late sown	CaLG08	CaDPF_LS8.1	67.41	SCA8_1742959	SCA8_3665619	11.97	−1.06
	Pooled	Normal sown	CaLG04	CaDPF_NS4.2	136.61	SCA4_48657505	SCA4_48720031	11.96	1.29
	Pooled	Normal sown	CaLG04	CaDPF_NS4.1	138.11	SCA4_48714724	SCA4_48657505	9.38	1.10
Number of filled pods (FP)	Pooled	Normal sown	CaLG06	CaFP_NS6.1	141.40	SCA6_34028484	SCA6_36622908	6.60	3.41
100 seed weight (100SW, g)	2017–18	Late sown	CaLG07	Ca100SW_LS7.1	97.01	SCA7_47907019	SCA7_9555338	31.30	4.33
	Pooled	Late sown	CaLG01	Ca100SW_LS1.1	46.21	SCA1_8682204	SCA1_33504088	37.23	2.73
	Pooled	Late sown	CaLG04	Ca100SW_LS4.1	159.71	SCA4_40568556	SCA4_14861717	36.34	2.85
	Pooled	Late sown	CaLG07	Ca100SW_LS7.1	97.01	SCA7_47907019	SCA7_9555338	33.48	4.11
Seed yield/plant (SYPP, g)	2018–19	Late sown	CaLG02	CaSYPP_LS2.1	22.51	SCA2_22704770	SCA2_35770691	18.00	−0.50
	2018–19	Late sown	CaLG06	CaSYPP_LS6.1	12.21	SCA6_35796441	SCA6_2512179	13.97	−1.60
	2018–19	Normal sown	CaLG06	CaSYPP_NS6.2	52.31	SCA6_9908036	SCA6_10234443	11.88	1.60
	2018–19	Normal sown	CaLG06	CaSYPP_NS6.3	53.01	SCA6_9993257	SCA6_9908036	8.66	1.38
Biological yield/plant (BYPP, g)	2017–18	Late sown	CaLG06	CaBYPP_LS6.5	115.31	SCA6_7929647	SCA6_7939281	10.79	1.24
	2018–19	Normal sown	CaLG06	CaBYPP_NS6.1	52.31	SCA6_9908036	SCA6_10234443	11.16	1.72
	Pooled	Normal sown	CaLG06	CaBYPP_NS6.1	52.31	SCA6_9908036	SCA6_10234443	10.70	2.16
	2017–18	Late sown	CaLG06	CaBYPP_LS6.3	114.01	SCA6_7939281	SCA6_7929339	9.37	1.14
	2018–19	Late sown	CaLG02	CaBYPP_LS2.1	55.91	SCA2_35860429	SCA2_29590953	7.23	−2.11
	2018–19	Late sown	CaLG06	CaBYPP_LS6.4	115.01	SCA6_7939281	SCA6_7929339	6.92	0.67
	Pooled	Late sown	CaLG06	CaBYPP_LS6.5	115.31	SCA6_7929647	SCA6_7939281	7.46	0.94
	2018–19	Normal sown	CaLG06	CaBYPP_NS6.2	58.71	SCA6_10672468	SCA6_10231199	9.43	1.56
Harvest index (HI, %)	2017–18	Normal sown	CaLG05	CaHI_NS5.1	42.11	SCA5_30627756	SCA5_41304451	18.69	2.30
	2017–18	Normal sown	CaLG07	CaHI_NS7.1	35.81	SCA7_36854123	SCA7_44149692	12.38	−2.53
	2018–19	Normal sown	CaLG06	CaBYPP_NS6.3	170.81	SCA6_52007475	SCA6_44667261	39.31	−2.26
	2018–19	Late sown	CaLG06	CaHI_LS6.2	100.21	SCA6_8170633	SCA6_7835024	7.31	−0.62
	Pooled	Late sown	CaLG08	CaHI_LS8.1	43.11	SCA8_14325980	SCA8_7197652	7.10	2.02
	2017–18	Normal sown	CaLG07	CaHI_NS7.2	142.71	SCA7_42355015	SCA7_30768244	9.24	3.24
	2018–19	Normal sown	CaLG06	CaHI_NS6.1	84.21	SCA6_7722925	SCA6_9536577	8.08	−1.69
	Pooled	Normal sown	CaLG07	CaHI_NS7.1	35.81	SCA7_36854123	SCA7_44149692	8.92	−2.40

PVE (%) = percent phenotypic variation explained; a positive value means the female parent (DCP92-3) having a positive effect on the trait. A negative value means the male parent (ICCV 92944) having a positive effect on the trait.

PVE (%) = percent phenotypic variation explained; a positive value means the female parent (DCP92-3) having a positive effect on the trait. A negative value means the male parent (ICCV92944) having a positive effect on the trait.

Harvest Index (%)

Of eight QTLs, one minor QTL each was identified for HI under the HTS environment of 2018 and pooled data of both years, while the remaining six QTLs were on the NS environments of 2017–18 and 2018–19 and pooled data of both years. Among six QTLs under the NS environments, three QTLs were major with PVE 12.38–39.31% and three were minor with PVE 8.08–9.24%. Furthermore, among eight QTLs, three QTLs were located on CaLG06, three on CaLG07, and one each on CaLG05 and CaLG08.

100 Seed Weight (g)

A total of three major QTLs were identified for 100SDW under HTS 2017 (one QTL) and based on pooled data (three QTLs) under HTS environments for both years. Among four QTLs, two were located on CaLG07 (**Figure 4B**), while one each was located on CaLG01 and CaLG04. Furthermore, the PVE for these four QTLs ranged from 31.3 to 37.23%.

Candidate Genes in QTL Regions

Mining of the candidate genes for heat tolerance revealed 1,498 genes in 24.82 Mb (8.68–33.50 Mb) region on CaLG01, 1,162 genes in 23.49 Mb (8.48–31.98 Mb) region on CaLG02, 1,408 genes in 25.71 Mb (14.86–40.57 Mb) region on CaLG04, 140 genes in 4.88 Mb (39.03–43.91 Mb) region on CaLG06, and 2,074 genes in 38.35 Mb (9.56–47.91 Mb) region on CaLG07 (**Supplementary Table 9**). Based on functional categorization, many genes were found to be associated with biological processes in these genomic regions. Using GO classification, we further identified a total of 32 candidate genes (7 on CaLG01, 3 on CaLG02, 14 on CaLG04, and 8 on CaLG07) known to function, directly or indirectly, as heat–stress response

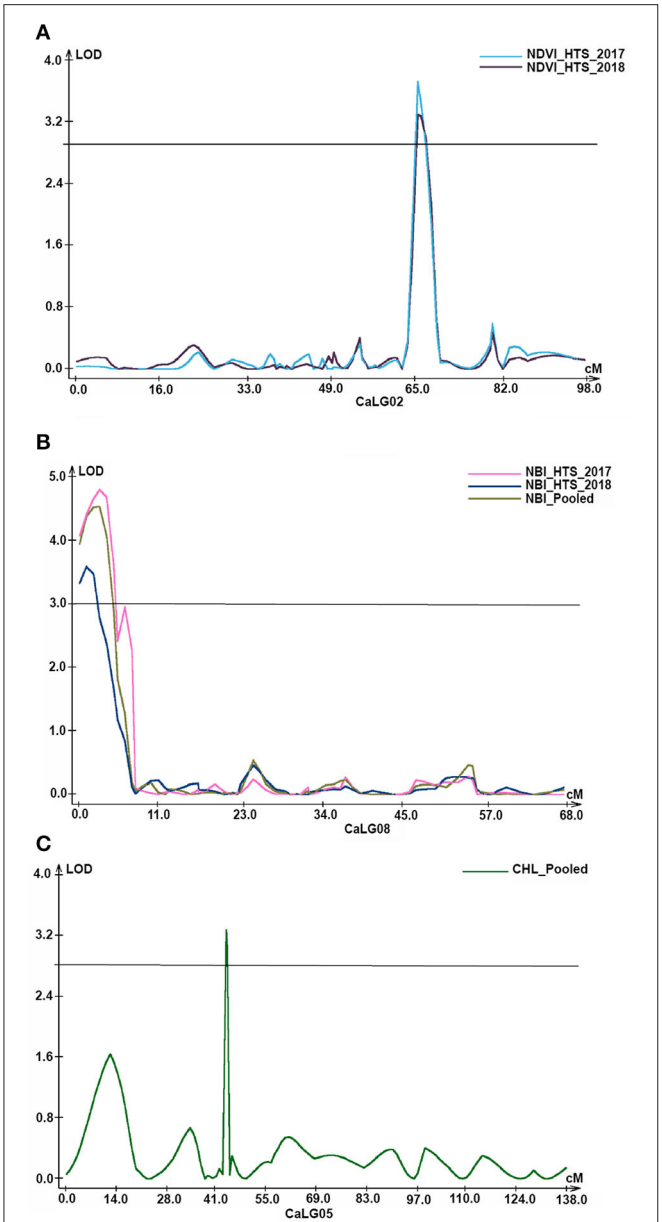

FIGURE 3 | Genomic regions with major QTLs for physiological traits. **(A)** Under heat stress environments of 2017–18 and 2018–19, two QTLs explaining 8.84% and 9.85% PVE, respectively, for normalized difference vegetation index (NDVI) were identified on CaLG02; **(B)** similarly, in addition to QTLs under two heat stress environments, QTLs for nitrogen balance index (NBI) were also identified based on pooled data on CaLG08; **(C)** a major QTL explaining 15.04% PVE for chlorophyll content on CaLG05 based on pooled data of under heat stress environments of 2017–18 and 2018–19.

genes in chickpea (**Table 3**). Among seven genes on CaLG01, six genes were encoding heat shock proteins, while one gene was encoding pollen-specific leucine-rich repeat extensin-like protein 1. While in the case of CaLG02, of three selected candidate genes, Ca_16007 encoded pollen-specific leucine-rich repeat extensin-like protein 1, Ca_24649 encoded a truncated transcription factor CAULIFLOWER A-like, and Ca_22033 encoded heat shock protein-binding protein. Among 14 selected candidate genes on CaLG04, six genes were pollen-specific, four were related to heat shock protein, three were DnaJ heat shock amino-terminal domain protein, and one was related to protein PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 isoform X1 (**Table 3**). The eight genes on CaLG04 encode heat shock protein/heat shock factor protein HSF24-like (Ca_18924, Ca_16239, and Ca_09277), pollen-specific leucine-rich repeat extensin-like protein 1/pollen receptor-like kinase 3 (Ca_16434 and Ca_16155), protein EARLY FLOWERING 3/flowering time control protein FY (Ca_10118 and Ca_17996), and calmodulin-binding heat-shock protein (Ca_13761) (**Table 3**).

DISCUSSION

In the context of climate change, every degree increase in aerial temperature has a severe impact on crop production, especially on chickpea that is predominantly cultivated on residual soil moisture in marginal environments (Gaur et al., 2014). Therefore, understanding the nature, impact, and molecular mechanisms of heat stress tolerance will help in designing strategies to overcome production losses. In chickpea, previously, very few studies were focused on understanding the nature, impact, and existing diversity in germplasm lines as well as identifying the genomic regions responsible to some extent. In this study, we reported major QTLs and novel genes in these genomic regions, which are associated with responsible for heat stress tolerance.

Late sowing, a simple and effective field screening technique for reproductive-stage heat tolerance in chickpea developed at the ICRISAT, Patancheru, India, was adopted for phenotyping the RILs for heat stress tolerance. The late sowing approach was adopted earlier in understanding the genetic variability for heat stress among genotypes as well as in identifying the genomic regions responsible for heat stress tolerance; for instance, in cool season crops, namely, chickpea (Paul et al., 2018b), wheat (Sareen et al., 2020), brassica (Branham et al., 2017), and rice (Prasanth et al., 2016). As the selection was based on yield *per se* results in a slower response because of genotype × environmental interactions, we also phenotyped the mapping population for physiological traits like CMS, NDVI, NBI, and CHL, which could be used as an indirect selection criterion to improve heat tolerance in chickpea as this was used in other crop plants. A sufficient amount of genetic variability for various phenological, physiological, and yield-related traits was noted in both the parents, and the RIL population was studied under NS and HTS environments for both years. The similar results were also reported earlier in chickpea based on evaluating the germplasm lines as well as one RIL population under HTS environment (Krishnamurthy et al., 2011; Devasirvatham et al., 2013; Paul et al., 2018b). High heritability for physiological traits like CMS, NDVI, NBI, and CHL contents under HTS environments indicates that the selection for heat tolerance relying on these traits could be effective. Earlier, heat stress was reported to reduce the total CHL and showed moderate to high heritability for

TABLE 3 | Key heat stress responsive genes in the QTL regions.

Gene				Pseudomolecule		Sequence description
Name	**Start**	**End**	**Length (bp)**	**Start**	**End**	
Ca_18341	24514080	24515481	1,401	Ca1_8682204	Ca1_33504088	Heat shock protein
Ca_02777	10118666	10120375	1,709	Ca1_8682204	Ca1_33504088	Heat shock protein
Ca_06915	16486764	16488806	2,042	Ca1_8682204	Ca1_33504088	Alpha-crystallin domain of heat shock protein
Ca_24217	18375189	18377243	2,054	Ca1_8682204	Ca1_33504088	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_06900	16670592	16673062	2,470	Ca1_8682204	Ca1_33504088	Heat shock protein
Ca_02832	9661308	9664099	2,791	Ca1_8682204	Ca1_33504088	Heat shock 70 kDa protein
Ca_22117	21727236	21731181	3,945	Ca1_8682204	Ca1_33504088	Heat shock-like protein, putative
Ca_16007	17402067	17404092	2,025	Ca2_8484804	Ca2_31975221	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_24649	15993671	15997430	3,759	Ca2_8484804	Ca2_31975221	Truncated transcription factor CAULIFLOWER A-like
Ca_22033	16232678	16236688	4,010	Ca2_8484804	Ca2_31975221	Heat shock protein-binding protein
Ca_20135	22124562	22125716	1,154	Ca4_14861717	Ca4_40568556	Heat shock transcription factor A3
Ca_05385	17413769	17415327	1,558	Ca4_14861717	Ca4_40568556	Heat shock protein
Ca_25302	26380392	26382057	1,665	Ca4_14861717	Ca4_40568556	Heat shock protein
Ca_17137	20149055	20150949	1,894	Ca4_14861717	Ca4_40568556	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_22444	35651353	35653252	1,899	Ca4_14861717	Ca4_40568556	DnaJ heat shock amino-terminal domain protein
Ca_17160	20521283	20523227	1,944	Ca4_14861717	Ca4_40568556	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_20459	27199342	27201308	1,966	Ca4_14861717	Ca4_40568556	Heat shock protein
Ca_21304	27740485	27742509	2,024	Ca4_14861717	Ca4_40568556	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_15124	37590074	37592294	2,220	Ca4_14861717	Ca4_40568556	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_14182	30066661	30069132	2,471	Ca4_14861717	Ca4_40568556	DnaJ heat shock amino-terminal domain protein
Ca_14827	36505593	36508070	2,477	Ca4_14861717	Ca4_40568556	Pollen-specific LRR extensin-like protein
Ca_05401	17218852	17221940	3,088	Ca4_14861717	Ca4_40568556	DnaJ heat shock amino-terminal domain protein
Ca_14004	19020031	19024609	4,578	Ca4_14861717	Ca4_40568556	Pollen protein Ole E I-like protein
Ca_14192	30295389	30312868	17,479	Ca4_14861717	Ca4_40568556	Protein PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 isoform X1
Ca_18924	28388535	28390538	2,003	Ca7_9555338	Ca7_47907019	Heat shock protein
Ca_16434	38877066	38879095	2,029	Ca7_9555338	Ca7_47907019	Pollen-specific leucine-rich repeat extensin-like protein 1
Ca_16239	26957077	26959156	2,079	Ca7_9555338	Ca7_47907019	Heat shock protein
Ca_16155	33278245	33280606	2,361	Ca7_9555338	Ca7_47907019	Pollen receptor-like kinase 3
Ca_10118	31400709	31404217	3,508	Ca7_9555338	Ca7_47907019	Protein EARLY FLOWERING 3
Ca_09277	12362555	12367223	4,668	Ca7_9555338	Ca7_47907019	Heat shock factor protein HSF24-like
Ca_13761	36747534	36754463	6,929	Ca7_9555338	Ca7_47907019	Calmodulin-binding heat-shock protein
Ca_17996	41387378	41400231	12,853	Ca7_9555338	Ca7_47907019	Flowering time control protein FY

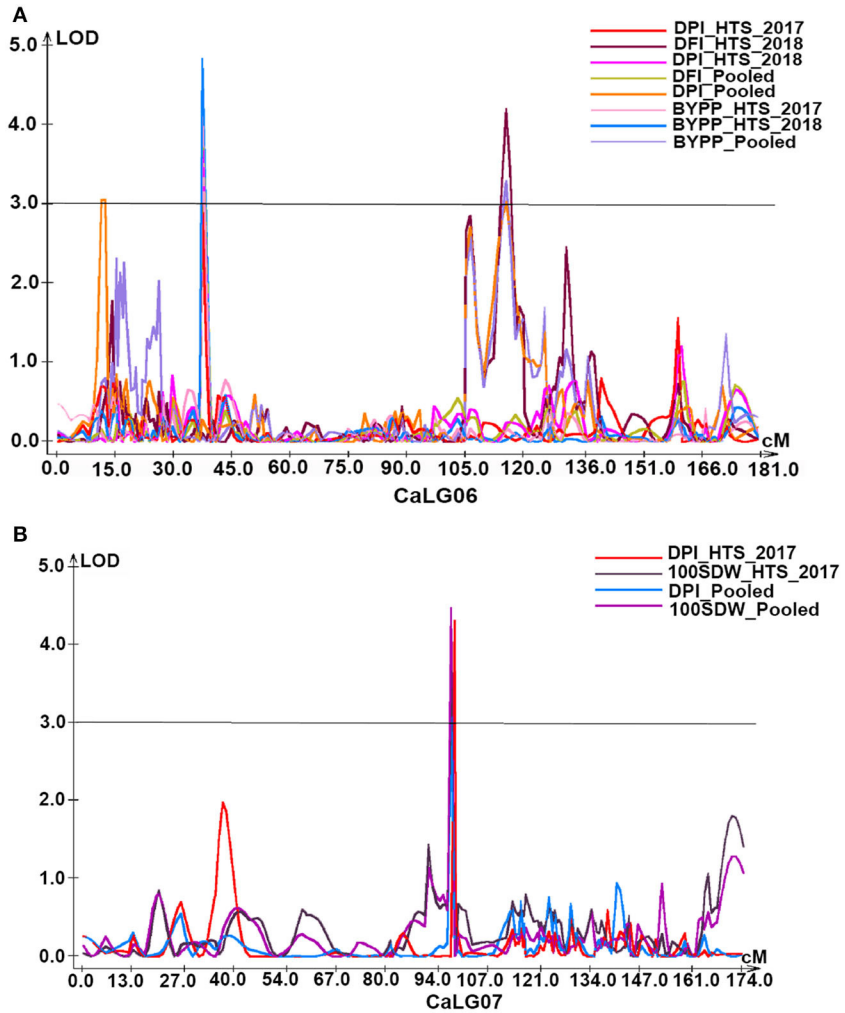

FIGURE 4 | Genomic regions with major QTLs for yield and yield-related traits. **(A)** Under heat stress environments of 2017–18 and 2018–19 as well as based on pooled data of these two environments QTLs for days to pod initiation (DPI, d) and biological yield per plant (BYPP, g) were mapped on CaLG06. In addition, days to flower initiation (DPI, d) of two QTLs for normalized difference vegetation index (NDVI) were identified explaining 8.84% and 9.85% PVE, respectively, on CaLG02; **(B)** QTLs for days to pod initiation (DPI, d) and 100 seed weight (100SDW, g) under stress environment of 2017–18 as well as based on pooled data of both heat stress environments of 2017–18 and 2018–19 were co-localized on CaLG07.

NDVI, CMS, and CHL content under stress condition in wheat (Bhusal et al., 2018; Condorelli et al., 2018; Pradhan et al., 2020), maize (Naveed et al., 2016), carrot (Nijabat et al., 2020), and pea (Tafesse et al., 2020). Low heritability for HI trait under HTS environments observed in this study indicates that the election for this trait will not enhance yield under stress. As yield traits remain the primary objective for improving the heat tolerance in all crop plants including chickpea, a positive and significant correlation among the yield and yield-related traits especially, FP, SYPP, and BYPP could serve as an important parameter for developing heat-tolerant chickpea genotype.

In this study, using a RIL population with a dense genetic map and phenotyping under NS and HTS allowed us to precisely identify major QTLs for heat stress in chickpea. Our genetic map has approximately 3-fold more markers compared to the previous study reporting the QTLs for heat stress tolerance (Paul et al., 2018a). In addition to reporting QTLs for phenological, yield, and yield-related traits under heat stress environments, to the best of our knowledge, this study is the first comprehensive study that reports QTLs for physiological traits like CHL, NBI, NDVI, CMS, and NPP in chickpea. A better understanding of phenology in response to heat stress will enable designing the breeding strategies. Minor QTLs for DFI were identified on CaLG06 and CaLG08, while a major QTL was identified for DM on CaLG01. Physiological traits like CMS, NDVI, and NBI, to date, have been used as proxy for grain yield under stress mostly in cereals (ElBasyoni et al., 2017; Bhusal et al., 2018; Condorelli et al., 2018; Getahun et al., 2020; Khanna-Chopra and Semwal,

2020). In this study, we reported major QTLs for these traits in chickpea, which may be used, after validation, for marker-assisted breeding for heat stress tolerance in chickpea.

In the case of chickpea, four flowering time genes (*efl-1* from ICCV 96029, *efl-3* from BGD 132, and *efl-4* from ICC 16641) and their allelic relationships were reported (Gaur et al., 2015), and major QTLs corresponding to these genes were mapped on CaLG04, CaLG08, and CaLG06, respectively (Mallikarjuna et al., 2017). Furthermore, marker trait associations for flowering time were reported earlier (Thudi et al., 2014; Upadhyaya et al., 2015; Varshney et al., 2019). However, in this study, we reported QTLs for DFI on CaLG06 and CaLG08 under heat stress environments as well as based on the pooled data of 2 years. Furthermore, interestingly, QTLs for DPI and DFI co-localized or mapped in the same genomic region under HTS environments of both years as well as based on pooled data. These observations indicate that introgression of one of the traits simultaneously improves both the traits, which are key for achieving resilience to heat stress.

Earlier two major genomic regions harboring QTLs for heat tolerance-related traits were mapped on CaLG05 and CaLG06; however, none on the QTLs explained >20% PVE (Paul et al., 2018a). Nevertheless, HI in this study explained >30% PVE. Similarly, CaLG04 also harbored QTLs for five traits (CHL, CMS, NDVI, DPF, and 100SDW), among these QTLs for traits CHL and 100SDW explained >30% PVE. Except HI (PVE 39.13%, NS 2018), none of the QTLs mapped on CaLG06 had PVE >15%. Similarly, QTLs for traits like CHL, NDVI, and HI were mapped on CaLG05 which explained 6.78–18.69% PVE. Earlier, a genomic region refereed as "*QTL-hotspot*" was reported to harbor several QTLs for different drought tolerance-related traits including 100SDW on CaLG04 (Varshney et al., 2014). A genomic region on CaLG07 harbors QTLs explaining >30% PVE for DPI and 100SDW as well as QTL for NBI explaining >10% PVE. For 100SWD, a total of four major QTLs were identified on CaLG01, CaLG04, and CaLG07 under HTS, and no QTLs were detected under NS. For SYPP trait, two major QTLs were identified on CaLG02 and CaLG06 under HTS. Only one QTL was identified on CaLG06 under NS. However, yield-related QTLs were not consistently recorded under all the conditions suggesting their environmental specific expression. Likewise, QTLs contributing to pods per plant, seed yield per plant, and seed weight were reported on CaLG01 and CaLG06 (Bajaj et al., 2015; Srivastava et al., 2016). In the case of cowpea, four QTLs were identified for pod set number per peduncle under HS and markers, which were utilized in breeding applications (Lucas et al., 2013; Pottorff et al., 2014). Similarly, in lentils, a major QTL controlling the seedling survival and pod setting traits under heat stress was noticed (Singh et al., 2017). In addition, QTLs for SYPP and BYPP (full names) were mapped in the same genomic region under NS environments of both years as well as based on pooled data. For yield and yield-related traits like DPI, DPF, and SYPP under HTS major alleles were contributed by ICCV 92944. For the 100SDW trait under HTS major alleles were contributed by DCP 92-3. On the other hand, almost all of the traits DPI, BYPP, and SYPP were contributed by DCP 92-3 under NS. For the trait HI under NS major alleles was contributed by both parents. In addition to these major QTLs, several QTLs were identified that were environmentally specific under NS and HTS, which only appeared in this study in the first year (NS or HTS). A total of nine major QTLs were located on CaLG06, which highlight the importance of this region in the heat tolerance mechanism in chickpea. Some QTLs were largely affected by environmental factors and that could be detected in only one season, and for these QTLs, further verification should be required.

HSP genes play a pivotal role in heat stress tolerance. In this study, 32 genes were identified in the QTL regions of CaLG01, CaLG02, CaLG04, and CaLG07. Similarly, in the case of soybean, 38 Hsfs were identified that were located on 15 soybean chromosomes (Li et al., 2014). *HSP* gene families were reported to be involved in drought and heat stress responses in soybean seedlings (Zhang et al., 2015; Das et al., 2016; Liu et al., 2019). *HSP90* gene families in five legumes and expression profiles in chickpea were reported earlier (Agarwal et al., 2016). Furthermore, based on genome-wide association studies, especially eight flowering time-regulating genes (*efl1*, *FLD*, *GI*, *Myb*, *SFH3*, *bZIP*, *bHLH*, and *SBP*) were reported (Upadhyaya et al., 2015). The genes reported in this study can be further explored for haplotypes based on the germplasm sequence information available in the public domain that has the potential for genetic improvement of the trait (Varshney et al., 2020). In addition, pollen-specific leucine-rich repeat extensin-like protein 1 genes identified in the QTL regions were reported to synergistically maintain pollen tube cell wall integrity; thus, they play critical roles in pollen germination and pollen tube growth (Wang et al., 2018). Recently, cloning of SHY in tomato, a pollen-specific gene that encodes a leucine-rich repeat (LRR) protein, demonstrated its role in a signal transduction pathway mediating pollen tube growth (Guyon et al., 2004).

CONCLUSIONS

In this study, we identified a total of 37 major QTLs across the genome for 12 traits. DFI, DPI, and DM are the key traits for escaping the heat stress in chickpea especially reproductive heat stress that hampers chickpea production. In this study, we reported major QTLs explaining >30% PVE for these key traits that contribute to yield under heat stress. In addition, we also reported for the first time major QTLs for proxy traits (physiological traits like CHL, NBI, NDVI, and CMS). Furthermore, 32 candidate genes in the QTL regions that encode the *HSP*, heat shock transcription factors, genes are involved in flowering time regulation as well as pollen-specific genes. The major QTLs reported in this study may be useful in molecular breeding for developing heat-tolerant superior lines or varieties.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI repository, BioProject ID PRJNA695065 (http://www.ncbi.nlm.nih.gov/bioproject/695065).

AUTHOR CONTRIBUTIONS

UJ and MT conceived the idea. UJ, HN, and RJ conducted the experiments. UJ performed the statistical analysis. MT, UJ, RP, and RV prepared the manuscript. VV and PB performed the GBS analysis. NS contributed to the development and phenotyping of mapping population. AC and RV contributed to consumables and the generation of genotyping data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.655103/full#supplementary-material

Supplementary Figure 1 | Mean weekly minimum and maximum day temperature (°C) recorded during the crop season of 2017–2018 and 2018–2019. Temperature >30°C during reproductive stages especially at podding stage and pod filling were recorded, which is more than the critical temperatures that hamper the production of chickpea.

Supplementary Figure 2 | High density genetic map comprising 788 single-nucleotide polymorphism markers mapped on eight chromosomes of chickpea. The map distance is indicated as a common scale for all eight linkage groups (CaLG01–CaLG08) on the left side figure and marker names on the right side of each linkage group.

Supplementary Table 1 | Correlations among different traits under normal sown environments of 2017–2018.

Supplementary Table 2 | Correlations among different traits under late sown or heat stress environments of 2017–2018.

Supplementary Table 3 | Correlations among different traits under normal sown environments of 2018–2019.

Supplementary Table 4 | Correlations among different traits under late sown or heat stress environments of 2018–2019.

Supplementary Table 5 | Correlations between different traits based on pooled data of 2 years under normal sown environments.

Supplementary Table 6 | Correlations between different traits based on pooled data of 2 years under late sown or heat stress environments.

Supplementary Table 7 | Genotyping-by-sequencing–single-nucleotide polymorphism (GBS–SNP) marker data used for construction of genetic map for recombinant inbred line population derived from DCP 92-3 (heat sensitive) × ICCV 92944 (heat tolerant).

Supplementary Table 8 | Distribution of SNPs on the genetic map derived from DCP 92-3 × ICCV 92944.

Supplementary Table 9 | Genes identified in the genomic regions harboring major QTLs on CaLG01, CaLG02, CaLG04, CaLG06, and CaLG07.

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Speed-Breeding System in Soybean: Integrating Off-Site Generation Advancement, Fresh Seeding, and Marker-Assisted Selection

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Speed breeding by artificial control of photothermal conditions facilitates generation advancement but was limited in scale and cost. In this study, we demonstrated a cost-saving off-site summer nursery pattern, taking full advantage of shorter daylength and higher temperature with lower latitude compared to the origin of the soybean cultivars used in the study. This substantially reduced the generation cycles under totally natural conditions. Using this approach, two generations of soybean cultivars from Northeastern Spring Planting Region (NE) and Yellow-Huai-Hai Valleys Summer Planting Region (YHH) were successfully obtained in Beijing and Hainan, respectively, compared to one generation in origin. Fresh-seeding method was also used to further shorten the generation duration by 7–10 days, thereby allowing at least four generations per year. Using DNA markers to define haplotypes of maturity genes *E1–E4*, we proposed a model to predict the optimum adaptation region of the advanced generation lines. Taken together, we present a speed-breeding methodology combining off-site nursery, fresh-seeding method, and marker-assisted selection, aimed at accelerating soybean improvement.

Keywords: generation advancement, off-site nursery, fresh seeding method, marker-assisted selection, speed breeding system

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the important oil crops in China due to its industrial use and domestic consumption. There is an urgent demand for high-yielding soybean cultivars to reduce the importation from other countries. In developing superior cultivars, cross-breeding is an effective breeding method in a great majority of crops, including soybean, and usually more productive and/or with other desirable characteristics from rich genetic variation by hybridization (Gai et al., 2015). Nonetheless, cross-breeding in soybean is time-consuming, generally requires at least eight generations from the crossing of selected parents to genetically stable lines for selection and evaluation. More than four generations are required to develop pure lines in this process. This slow development rate is attributed partially to the lengthy generation cycles, which

seriously retards the improvement in soybean yield, quality, and resistance to pest and diseases (Forster et al., 2014; Shuai et al., 2017; Fang and Han, 2019). Hence, shortening the breeding cycle through accelerating generation turnover is an issue of major importance in soybean breeding.

Greenhouse strategy allows specific adjustments regarding daylength, temperature, CO_2 concentration, and other climatic factors to adapt or accelerate plant development and finally shortens the generation time (Wu, 1961; Kothari et al., 2011; De La Fuente et al., 2013; Alahmad et al., 2018; Ghosh et al., 2018; Li et al., 2018; Watson et al., 2018; Jahne et al., 2020). However, with the supplements of equipment and electricity, greenhouse strategy is costly and size limited. Besides, since 1960s, winter nursery in Hainan Island has been widely used, which provides soybean breeders an opportunity to get one more generation during the off-season in north China (from November to April). Even so, two generations per year in Hainan is not enough, hence there still is an urgent need for a more economic and efficient strategy to speed up soybean breeding.

Another attempt to shorten the generation time is the fresh-pod–picking method (Fang and Han, 2019). As the plants reach R6 or full-seed stage, the most-mature pods are picked, and the seeds dried before sowing. In combination with single-seed descent (Chahal and Gosal, 2002), the generation time could be extremely reduced through harvesting small number of immature seeds to sidestep the ripening stage (Chang and Han, 2000).

Ineffective selection could be made directly based on phenotypes during off-season nursery, because phenotypic variation for important agronomic traits (such as growth period, plant height, and yield) among the populations would decrease (Fei et al., 2009). Instead, marker-assisted selection (MAS) could be used as a supplement for the phenotypic selection, while desirable individuals would be selected by genotypes without phenotypes of homozygous lines (Xu and Crouch, 2008). Previous studies showed that haplotype combinations at the *E1–E4* loci can explain more than 60% of variation in flowering time (Liu et al., 2008; Xia, 2013) and that there is a high correlation between *E1* and *E4* genotypes and growth period, and latitudinal adaptability (Jia et al., 2014; Jiang et al., 2014) in soybean. Therefore, *E1–E4* genotype identification of hybrid progenies during speed breeding, instead of phenotype identification, could facilitate the breeding process.

In this study, we developed a flexible protocol called off-site summer nursery to shorten the generation length under natural conditions, using the progenies of cross combinations from different ecotype cultivars as breeding materials. Besides, we found that the fresh-seeding method can further shorten the generation interval in soybean by sidestepping the drying duration of fresh seeds. We also proposed a prediction model of growth period phenotypes in advance with *E1–E4* genotypes after generation advancement and production of genetically stable lines. This significantly improved the selection efficiency. All together, we presented a speed-breeding system combining off-site generation advancement, fresh seeding, and MAS, which greatly reduces generation length and facilitates soybean breeding and research programs at low cost.

MATERIALS AND METHODS

Summer Nursery

Soybean hybrid progenies of diverse ecotypes from Northeastern Spring Planting Region (NE) and Yellow–Huai–Hai Valleys Summer Planting Region (YHH) were used in this study (**Supplementary Table 1**). Soybean hybrid progenies from NE were generated by crossing of cultivars from Mohe (Heilongjiang province), Ganhe (Inner Mongolia), Suihua (Heilongjiang province), Changchun (Jilin province), and Shenyang (Liaoning province), while soybean hybrid progenies from YHH were generated by crossing of cultivars from Beijing and Xinxiang (Henan province).

Summer nursery trials were carried out in two parts. Part one: the hybrid progenies from NE were sown on May 9, 2018, in Beipuchang farm of Chinese Academy of Agricultural Sciences (CAAS) in Beijing (39°58′N, 116°20′E). Part two: the hybrid progenies from YHH were sown on May 9, 2018, in Nanbin Farm of CAAS in Sanya, Hainan province (18°27′N, 109°11′E) (**Figure 1A**).

Fresh pods with the fastest ripening and plumpest seeds on each plant, were harvested after more than 80% of the plants reached the full-seed stage (R6). The pods were dried for 7–10 days in the shade at a temperature of 25–35°C and humidity $\leq$ 30%. Finally, seeds were detached from dried pods and sown immediately following the same procedure. During the experiment, growth stages were recorded as described by Fehr and Caviness (1977).

Fresh Seeding Tests

Five soybean varieties, including an early maturing variety Heihe 27, three intermediate maturing varieties Zhonghuang 70, Zhonghuang 325, and Zhonghuang 314, and a late maturing variety Zigongdongdou were used as materials. The varieties were supplied by Ministry of Agriculture and Rural Affairs (MARA) Key Laboratory of Soybean Biology, Institute of Crop Sciences, CAAS.

Seeding was done in Nanbin Farm on November 11, 2018. Harvesting was done by handpicking at six pod developmental stages: (I) Early-seed pod (ES): Pods containing approximately 20% of their maximum weight in fresh seeds at R5.5 stage; (II) Medium-seed pod (MS): Pods containing approximately 40% of their maximum weight in fresh seeds at R5.7 stage; (III) Full-seed pod (FS): Pods fully filled with green seeds at R6 stage; (IV) Green-yellow pod (GYP): Pods with a maximum weight of fresh seeds at R6.5 stage; (V) Yellow pod (YP): Pods with a brownish skin that was not yet round at R7 stage; and (VI) Brown pod (BP): Pods with a brown skin and matured seeds at R8 stage. At each harvest stage, three replicates of 100 seeds were randomly selected to measure the weight and moisture content of seeds and pods before and after oven-drying at 44°C for 96 h. The remaining seeds or harvested pods were dried in two schemes: (1) seeds were carefully extracted from the pods before drying (depodded); (2) seeds were dried inside intact pods (podded). The seeds or pods were left air-dried at an ambient temperature (27 ± 2°C).

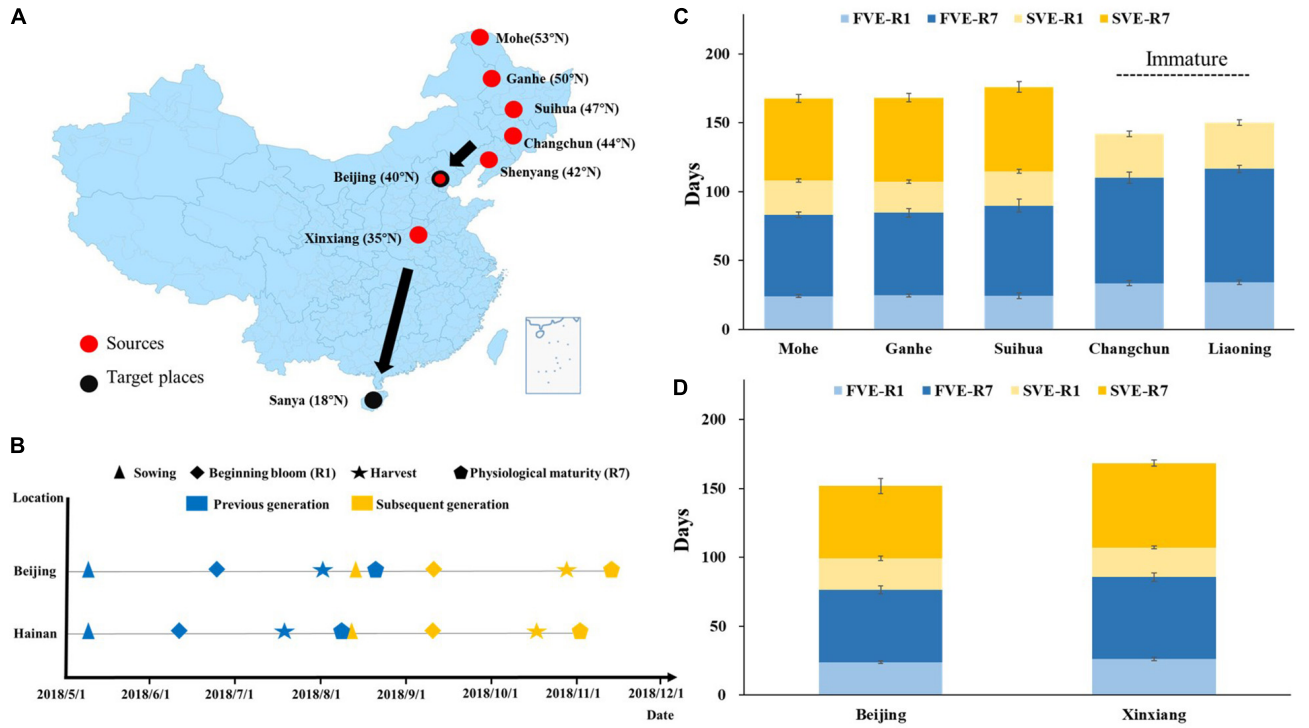


FIGURE 1 | Development of summer nursery in soybean. **(A)** The material sources and planting locations in summer nursery. The progenies of 34 cross combinations from Mohe, Ganhe, Suihua, Changchun, and Shenyang were planted in Beijing. The progenies of eight cross combinations from Beijing and Xinxiang were planted in Hainan. **(B)** Timeline of soybean development during summer nursery in Beijing and Hainan. Blue color symbols represent the developmental stages of the first generation, and the yellow color symbols represent that of the subsequent generation. Developmental stages of summer-sown soybean progenies **(C)** from Northeastern Spring Planting Region or **(D)** from Yellow–Huai–Hai Valleys Summer Planting Region planted in Hainan. F, first generation; S, subsequent generation; VE, emergence; R1, beginning bloom; R7, beginning maturity.

Seed germination tests were performed as described by the International Seed Testing Association (1999) protocols with some modifications. Three replicates of 50 seeds were used. The percentage of normal seedlings was determined as the radicle emerged from the seed coat more than 1 mm.

Analysis of variance (ANOVA) was performed by Microsoft Excel 2016. Means were separated by Duncan's multiple range test at $P < 0.05$, where the F-test was significant.

Genotyping and Phenotyping

A total of 262 hybrid progenies (F_6) from Mohe were genotyped for *E1–E4* maturity genes. They were also evaluated on the field for growth period traits in two locations.

InDels were detected by fragment-specific PCR and gel electrophoresis, and SNP sites were detected by Kompetitive Allele-Specific PCR (KASP), according to a previously reported protocol (Liu et al., 2020). All primers for sequencing and genotyping are provided in **Supplementary Table 2**. The PCR amplification products were scanned by FAM/VIC/ROM of BiOTek (SYNERGY/H1 microplate reader), and scanned data were detected by Kluster Caller typing software.

The hybrid progenies (F_6) were sown on May 9, 2018, in Ganhe Farm, Molidawa Banner, Inner Mongolia (49°27′N, 124°40′E) and Beijicun Village, Mohe City, Heilongjiang province (53°27′N, 122°24′E), respectively, for phenotypic evaluation (growth period). According to descriptions of growth stage in Fehr and Caviness (1977), VE, R1, and R7 were investigated.

RESULTS

Off-Site Summer Nursery Shortens the Soybean Breeding Cycle

To explore whether the hybrid progenies from different ecological regions can achieve two generations in summer in lower latitude with shorter day length, hybrid progenies from NE and YHH were planted in Beijing and Sanya under natural conditions, respectively. The daylength and ambient temperature in Beijing and Sanya during soybean summer nursery in 2018 are shown in **Supplementary Figure 1**.

The hybrid progenies from Mohe, Ganhe, and Suihua were sown on May 9, 2018. The plants reached the beginning bloom stage (R1) at 24.2 ± 1.2, 24.6 ± 1.0, and 24.4 ± 2.2 days after emergence (DAE), respectively (**Figure 1C**). They began maturity at 83.2 ± 1.9, 84.6 ± 3.1, and 89.8 ± 4.6 DAE, and were harvested by fresh-pod picking on August 1, 2018 (79 DAE). The subsequent generations of these materials were sown on August

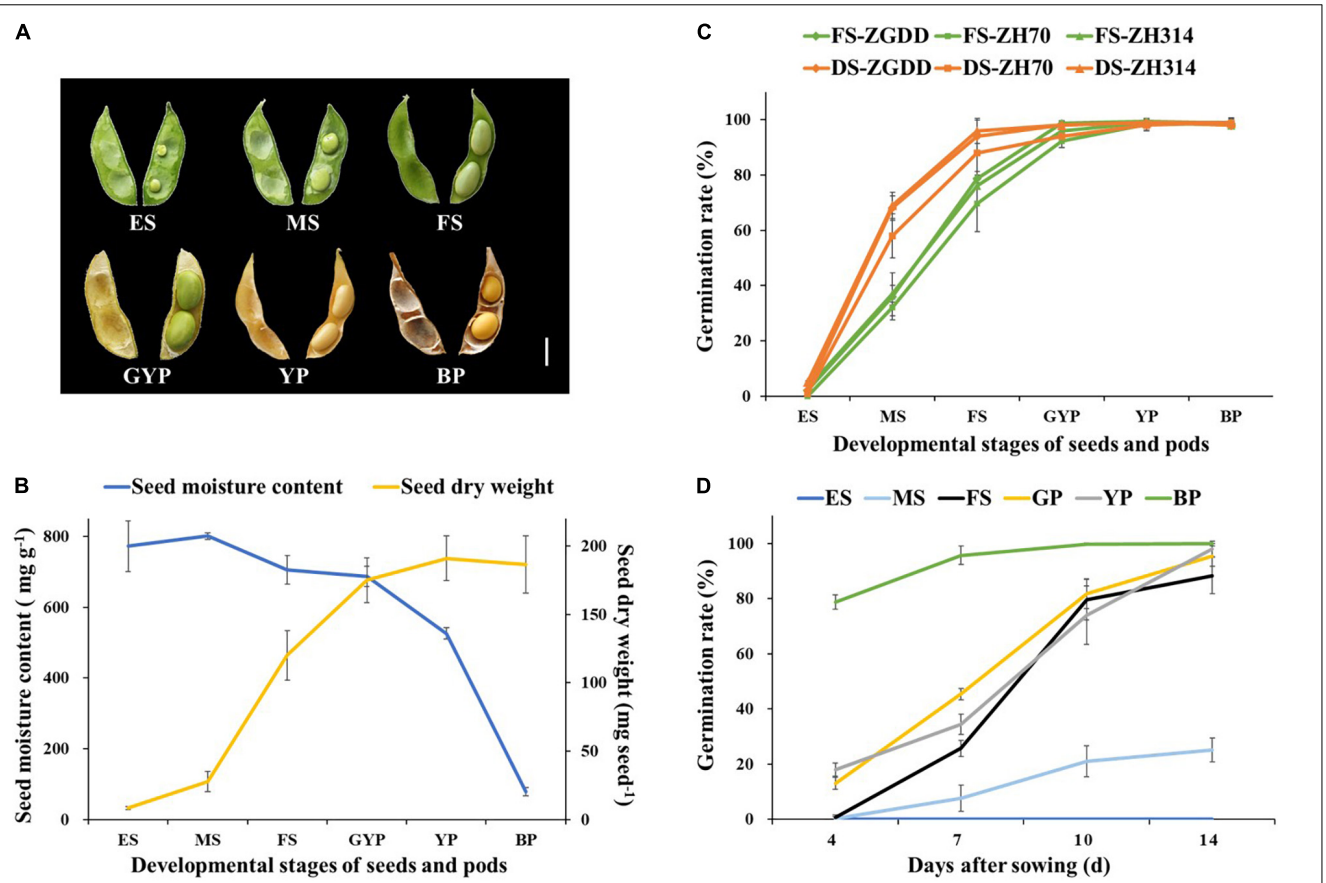


FIGURE 2 | The developmental status and the germination rate of seeds harvested at different reproductive stages. **(A)** Images of pods and seeds harvested at different reproductive stages. **(B)** The moisture content and dry weight of seeds harvested at different reproductive stages. **(C)** The comparison of germination rate between the freshly sown seeds (FS) after shelling and the desiccated seeds (DS). **(D)** The germination rate of the fresh seeds in different days after sowing. The seeds of soybean (*cv*. Heihe27) were sown immediately after shelling. Developmental stages of seeds and pods: ES, early seed pod; MS, medium-seed pod; FS, full-seed pod; GYP, green-yellow pod; YP, yellow pod; BP, brown pod. Scale bar = 1 cm. Values are means ± SD ($n \geq 5$).

8, 2018, and the plants reached R1 at 24.9 ± 1.1, 22.5 ± 1.2, and 25.0 ± 1.3 DAE, respectively. They began maturity at 84.6 ± 2.9, 83.6 ± 3.2, and 86.2 ± 3.8 DAE, respectively, and were harvested by fresh-pod picking on November 1, 2018 (80 DAE). In summary, two generations were accomplished from May 9 to November 1, 2018 (**Figure 1B**) in hybrid progenies from Mohe, Ganhe, and Suihua located in the north part of northeast China. However, the hybrid progenies from Changchun and Shenyang did not complete two generations in summer of Beijing. None of the second generation of those materials reached beginning maturity stage (R7) and the pod has not developed well before frost (November 16).

The hybrid progenies from YHH were sown on May 9, 2018, in Sanya, reached R1 at 23.9 ± 3.0 and 26.2 ± 2.0 DAE, respectively (**Figure 1D**). They began maturity at 76.6 ± 5.5 and 85.7 ± 3.3 DAE and were harvested by fresh-pod picking on July 7, 2018 (68 DAE). The next generations of those materials were sown on August 9, 2018, reached R1 at 22.6 ± 1.7 and 21.5 ± 1.1 DAE. They began maturity at 75.2 ± 5.6 and 82.9 ± 2.1 DAE, and were finally harvested by fresh-pod picking on October 10, 2018 (74 DAE). In summary, all of the hybrid progenies from YHH completed two generations from May 9 to October 10, 2018, in Sanya (**Figure 1B**).

Fresh Seeding Method Shortens the Soybean Growth Cycle

In an attempt to further shorten the generation length of soybean, a fresh-pod–picking method was developed, which could save 1 month in each generation. We first tested the germination of seeds picked from different pod developmental stages (**Figure 2A**). The dry matter content of FS picked at R6 stage was more than 60%, and then reached the maximum dry weight in YP, as the water content began to drop sharply in GYP (**Figure 2B**).

As a result, the germination percentage of fresh and dried seeds increased with increasing seed maturity (**Figure 2C**). The germination rate of dried and fresh seeds harvested at the FS stage reached 88.0 ± 2.6 and 69.7 ± 10.3%, respectively. Furthermore, the difference between the two treatments was not statistically significant, indicating that the fresh seeds harvested at the FS stage have a great germination capacity. There was

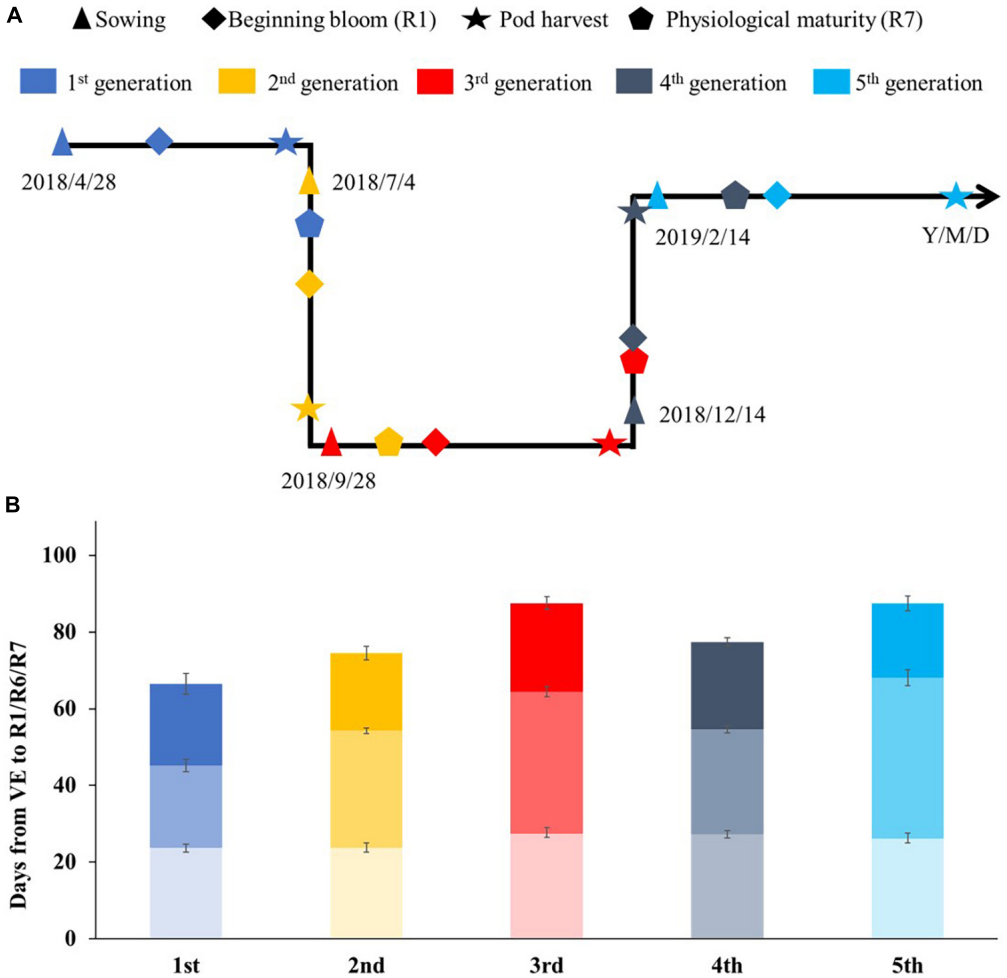


FIGURE 3 | The growth periods of soybean *cv*. Heihe27 under natural conditions (May to October) and glasshouse (November to April) in Beijing, 2018–2019. **(A)** Timeline of soybean development during summer nursery in Beijing and Hainan. **(B)** Developmental stages of Heihe27 planted in Beijing. VE, emergence; R1, beginning bloom; R7, beginning maturity.

no significant difference ($P > 0.05$) among different soybean genotypes. As seed maturity progressed to FS, seed germination significantly improved across all the varieties. Therefore, instead of drying seeds after fresh-pod picking, directly sowing fresh seeds harvested at the FS stage could save the drying time, which further advances the sowing time of the next generation by 7–10 days without decreasing the seed germination rate.

To evaluate the germination ability of fresh seeds detached from different developmental pods, we investigated the germination rate on days 4, 7, 10, and 14 after sowing. As shown in **Figure 2D**, the germination rate of fresh seeds harvested at the BP stage was 78.7 ± 2.6% on the 4th day after sowing (DAS) and reached the maximum on the 10th day. In the meantime, the germination rate of fresh seeds harvested at the GYP and YP stages were only 18.0 ± 2.4 and 13.0 ± 2.2% on the 4th DAS, and reached the maximum, 95.3 ± 3.7 and 98.0 ± 2.8% on the 14th day, respectively. The germination rate of fresh seeds harvested at the FS stage was almost 0 on the 4th DAS but reached the highest value of 88.3 ± 6.5% on the 14th DAS.

We further investigated how many generations can be obtained in 1 year using the fresh-seeding method. Super-early maturity variety Heihe27 was planted in the greenhouse. After recurrent planting, we advanced five generations from April 28, 2018, to May 1, 2019 (**Figure 3**). The average generation span was about 73 days.

Marker-Assisted Selection Advances the Soybean Breeding Program

After examining the haplotypes of maturity genes of *E1–E4* and the growth periods of the hybrid progenies (F_6) in different locations, 211 individual lines were selected for further analysis. We identified three alleles of *E1* (*e1-as*, *e1-fs*, and *e1-ns*), one allele of *E2* (*e2-ns*), three alleles of *E3* (*e3-fs*, *e3-ns*, and *e3-tr*), and four alleles of *E4* (*e4-new*, *e4-kes*, *e4-SORE-1*, and *E4*) in the 211 individual lines for phenotyping. Eighteen haplotypes of *E1–E4* were identified in the hybrid progenies (**Supplementary Table 3**). Based on the previous studies (Jiang et al., 2014;

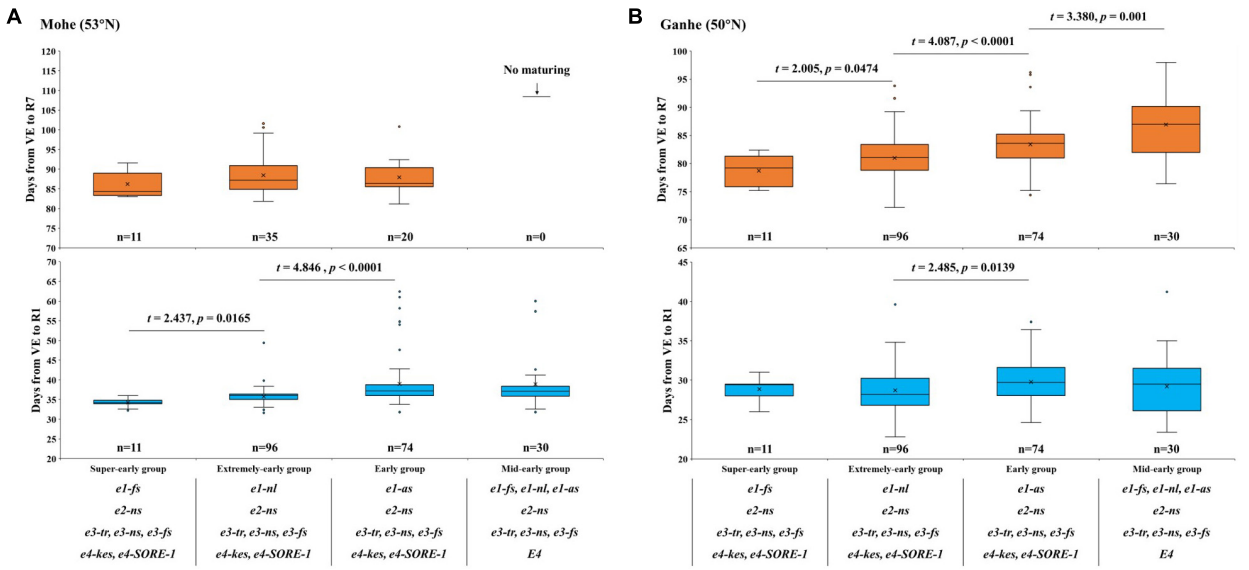


FIGURE 4 | Flowering time (days from VE to R1) and maturity time (days from VE to R7) of different E allele combinations in **(A)** Mohe (53°N) and **(B)** Ganhe (50°N). VE, emergence; R1, beginning bloom; R7, beginning maturity. The *t* value and *P* value for two-tailed *t*-test are shown above the box plot.

Li et al., 2017; Liu et al., 2020), we presumed a model to predict the growth period phenotype and maturity groups and divided the hybrid progenies into four groups: (1) super-early group: with the genotypes of *e1-fs/ e2-ns/ e3-tr*, *e3-ns*, *e3-fs/ e4-kes*, *e4-new*, and *e4-SORE-1*; (2) extremely early group: *e1-nl/ e2-ns/ e3-tr*, *e3-ns*, *e3-fs/ e4-kes*, *e4-new*, and *e4-SORE-1*; (3) early group: *e1-as/ e2-ns/ e3-tr*, *e3-ns*, *e3-fs/ e4-kes*, *e4-new*, and *e4-SORE-1*; and (4) mid-early group: *e1-fs*, *e1-nl*, *e1-as/ e2-ns/ e3-tr*, *e3-ns*, and *e3-fs/ E4*.

The phenotypic characterization of 211 hybrid progenies in Mohe showed that the flowering time of super-early hybrid progenies was significantly ($P < 0.05$) earlier than that of extremely-early hybrid progenies (**Figure 4A** and **Supplementary Figure 2**). The flowering time of super-early and extremely early hybrid progenies from the north part of NE was also significantly ($P < 0.05$) earlier than that of the early hybrid progenies from the middle or south part of NE. Except for the super-early hybrid progenies (11/11), the other three groups did not reach maturity normally: 37.2% (35/96) of the predicted extremely early hybrid progenies and 27.0% (20/74) of the predicted early hybrid progenies reached R7, while none (0/30) of the predicted mid-early hybrid progenies hardly mature normally in Mohe. Furthermore, the progression to R7 revealed that the predicted super-early hybrid progenies with the genotypes *e1-fs*, *e2-ns*, *e3-tr/ e3-ns/ e3-fs*, and *e4-kes/ e4-SORE-1* were suitable for cultivation in Beijicun of Mohe, the northernmost village of China, and the higher latitude regions.

The phenotypic characterization of 211 hybrid progenies in Ganhe Farm (**Figure 4B**) showed that the flowering time of extremely early hybrid progenies were significantly ($P < 0.05$) earlier than that of the early-group hybrid progenies. As expected, all of the predicted super-early hybrid progenies reached R7 in Ganhe, while more than 97.5% of the extremely early and early group hybrid progenies matured normally, showing that predicted extremely early and early group hybrid progenies were suitable for breeding selection in Ganhe. Additionally, the number of mid-early group hybrid progenies (66.7%) progressing to R7 in Ganhe revealed that the predicted mid-early group hybrid progenies were suitable for cultivation in Ganhe or the region further south.

DISCUSSION

Short daylength and high temperature are the superlative environments for accelerating the growth and development of short-day plants such as soybean (Mao et al., 2017). In

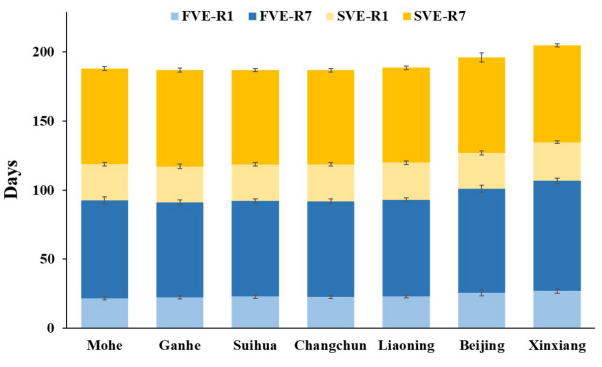
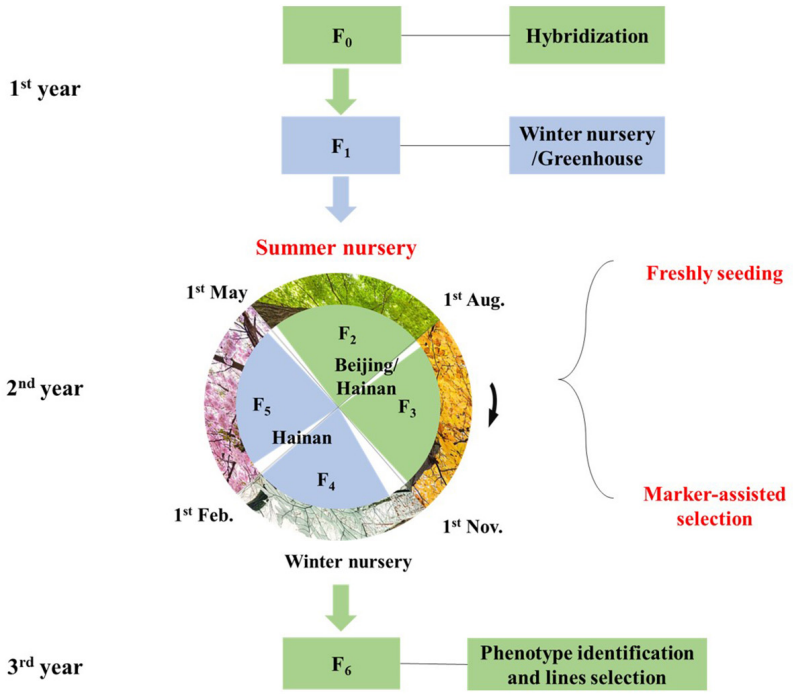


FIGURE 5 | Developmental stages of progenies of cross combinations from Northeastern Spring Planting Region and from Yellow–Huai–Hai Valleys Summer Planting Region planted in Hainan (Winter Nursery). F, first generation; S, subsequent generation; VE, emergence; R1, beginning bloom; R7, beginning maturity.



Maturity Group	Allele combinations of *E1-E4*	Predicted Suitable area
Super-early group	*e1-fs/ e2-ns/ e3-tr, e3-ns, e3-fs/ e4-kes, e4-new, e4-SORE-1*	≥ N53°
Extremely-early group	*e1-nl/ e2-ns/ e3-tr, e3-ns, e3-fs/ e4-kes, e4-new, e4-SORE-1*	N49°-N53°
Early group	*e1-as/ e2-ns/ e3-tr, e3-ns, e3-fs/ e4-kes, e4-new, e4-SORE-1*	N49°-N53°
Mid-early group	*e1-fs, e1-nl, e1-as/ e2-ns/ e3-tr, e3-ns, e3-fs/ E4*	≤ N49°

FIGURE 6 | Schematic representation of off-site speed breeding in soybean. The procedure of Speed Breeding System for soybeans from Northeastern Spring Planting Region and Yellow–Huai–Hai Valleys Summer Planting Region to fulfill four generations within 1 year (F_2–F_5) and seven generations (F_0–F_6) within 3 years.

China, winter nursery in tropical Hainan Island provided soybean breeders an opportunity to increase one or two additional generations during the off-season in north China (from November to April) (Wu, 1961; Kothari et al., 2011). In this study, we tried to speed up breeding of NE soybean by summer nursery in the YHH regions. As shown in the results, the daylength and temperature in Beijing (**Supplementary Figure 1**) were ideal for the rapid growth of hybrid progenies of super-early, extremely early, and early group from NE (**Figure 1**). However, the hybrid progenies classified as mid-early and late groups from NE could not attain two generations in Beijing. Therefore, in order to complete two generations in summer, the hybrids from these groups were recommended to be planted in lower latitude regions with shorter photoperiod and higher accumulated temperature, such as Anhui (29–34°N) or Jiangsu (30–35°N) in China. Our study also revealed that hybrid progenies from YHH can accomplish two generations in Hainan during summer. NE and YHH regions account for more than 75% of the soybean production in China. Soybean varieties in these two regions are within the maturity group (MG) IV, which is the main soybean genotype (Song et al., 2019) cultivated in North America and other mid- and high-latitude regions in the world. Thus, the speed-breeding methodology presented in our study has universal applicability. However, there was one limitation that the germination of the second generation was affected by heavy rains. Therefore, indoor seeding or drainage systems with deep trenches should be available to eliminate the influence of severe weather to the second generation.

In the prediction of the growth period in soybean hybrid progenies, *E1* and *E4* genotypes are major loci considered. We observed that genotypes with *e1-fs* and *e4* alleles are suitable for planting in Mohe or even higher latitude regions, while the genotypes with *e1-nl* or *e1-as* and *e4* alleles were suitable for planting at latitudes from 46°N to 52°N (such as Ganhe, Heihe, and Suihua). The genotypes with the *E4* dominant allele were suitable for planting in the area of latitude 46°N or even in the regions further south. We found that 100% of the super-early group materials could mature in Beijicun Village of Mohe, whereas about 37.2% of the extremely early group materials could mature in Beijicun. We observed that some lines with the same *E1–E4* alleles performed differently in the maturity stage, which might be caused by other genes, such as *FT1a/2a/5a/2b* and *PRR37* (Liu et al., 2017; Cai et al., 2018; Chen et al., 2020; Wang et al., 2020). Future studies should include the effect of these genes on the phenotypes.

To accelerate breeding, numerous methodologies were explored. The use of winter nursery doubled the rate of generation advancement (Gai et al., 2015), while artificial environment with varying photoperiod, temperature, or CO_2 concentration could achieve three generations in corn (Li et al., 2016); four generations in rice (Tanaka et al., 2016), legumes (Ochatt et al., 2002), and canola (Mobini and Warkentin, 2016; Yao et al., 2016); five generations in soybean (Nagatoshi and Fujita, 2019; Jahne et al., 2020); and six generations in wheat (Kothari et al., 2011) and cabbage (Williams and Hill, 2013). Biotechnologies such as the immature embryo culture or double haploid can shorten one generation time to 65–70 days in

soybean (Rosenberg and Rinne, 1987), 50–70 days in cotton (Wang et al., 2003), 39–55 days in barley (Zheng et al., 2013), 66–80 days in sorghum (Rizal et al., 2014), 48–61 days in oat (Liu et al., 2016), and 48–56 days in canola (Tanaka et al., 2016). These approaches with the help of artificial greenhouse were costly and scale limited.

In this study, the soybean materials from the north part of NE China went through two generations from May to November before the frost in the natural condition of Beijing and were then immediately sent to (November) winter nursery for the next two generations at Nanbin Farm of CAAS in Sanya, Hainan province (**Figure 5**). We established a speed-breeding system integrating off-site generation advancement and fresh-seeding method, under natural conditions with the accomplishment of at least four or more generations in 1 year (**Figure 6**). In this study, we summarized the operating procedures as follows:

(1) The hybrid progenies from the north part of NE were planted in the summer of YHH regions such as Beijing, seeded in early May, and the plants were expected to blossom in early June and harvested in late July. Similarly, the hybrid progenies from the middle and south parts of NE and that from YHH could be planted in Sanya, Hainan province in summer, seeded in early May, and the plants were expected to blossom in early June, and harvested in late July.
(2) After the plants reach the full-seed stage (R6), fresh pods with the fastest growth in the lower part of the plants were picked and sowed immediately after separation from pods.
(3) The second generation of hybrid progenies from north part of NE were sown in early August in YHH regions, and harvested in early November. Similarly, the second generation of hybrid progenies from middle and south parts of NE and that from YHH could be sown in early August in Sanya, Hainan province, and harvested in late October.
(4) During the above process, maturity groups and suitable planting area of each individual line could be predicted by identifying *E1–E4* alleles, which could further save the time of phenotypic identification in the target region.

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

TH and SS designed and managed the research project. YF, ES, SF, HZ, XS, SQ, MK, and TH conducted the field experiment. YF, TW, LW, and SY conducted the genotype identification. CW and WH provided advice on experimental implementation. YF, TH, LW, and ES took the lead in the manuscript preparation and all authors are qualified for authorship and were involved in drafting and revising this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.717077/full#supplementary-material

Supplementary Figure 1 | Day length and ambient temperature in Beijing and Sanya during soybean summer nursery in 2018. The day length data were downloaded from the website https://sunsetsunrisetime.com/sun. The ambient temperature data in Beijing and Sanya were from the website http://lishi.tianqi.com/beijing/index.html and http://lishi.tianqi.com/hainanqu/index.html.

Supplementary Figure 2 | Flowering time (days from VE to R1) and maturity time (days from VE to R7) of different *E* allele combinations. Flowering time and maturity time of **(A)** different *E* allele combinations, **(B)** *E1* allele, **(C)** *E3* allele, and **(D)** *E4* allele in Ganhe. VE, emergence; R1, beginning bloom; R7, beginning maturity. One-way ANOVA was used for the statistical analysis ($P < 0.05$).

Supplementary Table 1 | The sources and maturity group of parents.

Supplementary Table 2 | The primer sequences used in this study.

Supplementary Table 3 | The phenotype and haplotype combinations of *E1-E4* in hybrid progenies. "NA" means these materials cannot reach R7 until the first frost came.

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Genotypic Variability in Architectural Development of Mungbean (*Vigna radiata* L.) Root Systems and Physiological Relationships With Shoot Growth Dynamics

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Selection for root system architectures (RSA) to match target growing environments can improve yields through better adaptation to water and nutrient-limiting conditions in grain legume crops such as mungbean. In this study, the architectural development of root systems in four contrasting mungbean varieties was studied over time to explore their relationships to above-ground growth and development. Key findings suggested that early maturing mungbean varieties were characterized by more rapid root elongation rates and leaf area development, resulting in more vigorous root and shoot growth during early growth stages compared with a late maturing variety. The early maturing varieties also showed root morphological traits generally adapted to water-limited environments, such as deeper, longer and lighter roots. Early maturing varieties more rapidly colonized the top 10–20 cm of the soil profile during early growth stages, whereas the later maturing variety developed less prolific but 20–50% thicker roots in the same profile layers in later stages of crop growth. The diversity of root characteristics identified in these commercial varieties suggests that there are opportunities to combine desirable root traits with maturity types to target different production environments. Examples include deeper, longer, and thinner roots for crops to exploit deep profile reserves of water and nutrients, and thicker and shallower root systems for crops grown in shallow soils with stratified nutrient reserves and/or more favorable in-season rainfall.

Keywords: intact root growth, root system architecture, rooting depth, phosphorus acquisition, maturity type

INTRODUCTION

Mungbean (*Vigna radiata* [L.] Wilczek) is an economically important tropical grain legume crop that has the potential to play a key role in managing soil fertility as a nitrogen-fixing legume in crop rotation systems (Araujo et al., 2015; Foyer et al., 2016; Singh et al., 2016a,b). There is increasing interest in growing a higher frequency of grain legume crops in broadacre grain cropping systems,

especially mungbean and chickpea (*Cicer arietinum*), due to strong market demand and high commodity prices. In addition to these economic incentives, grain legumes can also deliver multiple benefits through a smaller environmental footprint, improved stock and human health, reduced use of synthetic nitrogen (N) due to biological N fixation, reduced soil pathogen populations and provide an option to increase plant-based dietary intake of minerals, vitamins and fiber (Parida and Das, 2005; Arnoldi et al., 2014; Vaz Patto et al., 2014). However, the reliability of mungbean production and its profitability in crop rotations needs to be improved if the strong market demand for mungbean is to be met. There are numerous challenges to reliable mungbean production in the major growing regions of India and Australia, especially abiotic factors in interaction with changing climatic conditions (Beebe et al., 2011; Basu et al., 2016; Singh et al., 2016a). Limited availability of water and poor soil fertility are widespread, resulting in poor crop growth and unprofitable mungbean yields (Araujo et al., 2015).

Plant growth and development are dependent on root morphology and root system architecture (RSA) that facilitate the acquisition of water and nutrients. Under adverse soil or environmental conditions, RSA could be a critical factor in determining profitable crop production (Lynch, 1995; de Dorlodot et al., 2007; Ye et al., 2018). The spatial distribution of roots in soil can influence the extent and timing of access to water and nutrients, thus impacting yield potential (Lynch, 1995; de Dorlodot et al., 2007; Hammer et al., 2009; Liang et al., 2017; Ye et al., 2018). Modifications to root systems may therefore improve adaptation to water- and nutrient-limiting conditions that are constraining yields in mungbean crops. However, genetic improvement of crop root systems requires knowledge of the intra-species variability in key root parameters and RSA and how these are controlled genetically (O'Toole and Bland, 1987). An understanding of the relationships between RSA and plant productivity is also necessary before effective breeding and management strategies can be developed (Gowda et al., 2011; Henry et al., 2011; Joshi et al., 2017). Despite its importance, few studies have explored the potential for including RSA as a selection strategy in crop improvement programs. Lawn and Rebetzke (2006) identified substantial variation for traits of potential agronomic, adaptive or taxonomic interest among 115 accessions of mungbean, mainly from Australia, West Timor, Papua New Guinea and India. However, genetic variation in RSA and relationships with plant growth and development in mungbean are largely unknown (Pratap et al., 2013, 2014; Singh et al., 2016b).

While RSA plays an important role in water and nutrient acquisition and plant growth, most studies that have characterized root architectural parameters represent a snapshot at a specific time (Manschadi et al., 2008; Singh et al., 2012, 2016b; Uga et al., 2013). Observations made at maturity cannot fully explain the relationship between RSA and plant growth and yield accumulation, as root systems are known to be plastic in nature and can interact dynamically with soil physical, chemical and biological factors at different stages of a growing season (Lynch, 1995; Wu et al., 2016; Chen et al., 2017). Additionally, most studies have used destructive techniques such as soil coring or trench wall methods to describe the RSA at the end of the experiment. These techniques are time consuming and tedious and cannot quantify the intact root growth patterns in terms of rate of growth, branching, spatial distribution and occupancy of the soil volume over time (McCully, 1995; Chen et al., 2017). Development of roots occurs in synchrony with shoot growth (Wang et al., 2006), so characterization of RSA over time is important to understand the interactions between RSA and shoot growth dynamics. The impact of differences in RSA established during vegetative growth will be maintained during reproductive growth in determinate species. However, in semi-determinate to indeterminate species like mungbean and other tropical/subtropical grain legumes, root growth occurs during both vegetative and reproductive phases, and so measurement of RSA at the end of an experiment cannot identify when RSA differences were likely to be affecting the critical growth stages of the plant. Multiple destructive samplings at critical growth stages are therefore needed to study the dynamic nature of root growth patterns relative to shoot growth and yield parameters.

Field excavation, trenches and soil coring have been used to quantify root growth and RSA in field studies (Trachsel et al., 2011; Vansteenkiste et al., 2014), but these methods are labor intensive and information on the actual root architecture is often lost. Recent advancements in the technologies used to measure static and dynamic root growth have included non-invasive methods where plants are grown in artificial gel media (Manschadi et al., 2008; Hargreaves et al., 2009), CT scans/X-ray micro-tomography (Hochholdinger, 2009; Mooney et al., 2012; Mairhofer et al., 2013) and MRI of intact soil cores (Schulz et al., 2013).

However, these techniques are only successful for very young plants (a few days to a couple of weeks old) growing in controlled conditions and exhibiting simple branching patterns. Minirhizotrons have also been used to non-destructively measure root growth, with this technique permitting tracing or imaging of intact roots on a transparent surface of a growth chamber (Singh et al., 2012; Downie et al., 2015). However, despite the confined rooting volumes used in such systems, roots seen on the transparent surface typically represent only *ca.* 20% of the total roots of a plant. Tracing and analysis of root images collected over time from these systems is very time consuming and there is often not much success in differentiating contrasting root systems (Singh et al., 2010, 2011, 2012).

The objectives of this study were therefore to develop a technique to study the intact root system of mungbean plants as they grow, using four contrasting mungbean varieties to characterize the morphological and architectural development of intact root systems. The novelty of this study was to understand the relationships between growth of the entire root system with the growth and development of the above-ground plant components during a growing season. This contrasts with most studies that explore these relationships at a single time point at the end of an experiment or growing season. A subsequent study uses these same varieties to explore responses to different phosphorus fertilizer application strategies in terms of RSA, plant growth and nutrient acquisition.

MATERIALS AND METHODS

Plant Material

The experiment compared four commercial mungbean varieties from Australia, with contrasting growth characteristics and maturity classes. All varieties were produced by the National Mungbean Improvement Program, Queensland Department of Agriculture and Forestry (DAF), Australia:

Jade-AU (3511-9 × VC 2768A, released in 2013)—a mainstay variety for Australia, producing large shiny seeds; a variety that retains green leaf area until harvest, and so can respond to residual soil water and nitrogen.

Berken (released 1975, direct introduction from the USA, no pedigree history)—older variety with low yield potential and highly susceptible to plant diseases; a more determinate growth pattern that is characterized by canopy senescence during pod filling and at grain maturity.

Celera II-AU (M 773 × OAEM58-62, released 2015)—a small seeded, short statured variety with distinguishing leaf morphology; resistant to the bacterial disease halo blight, caused by *Pseudomonas savastanoi* pv. *Phaseolicola.*

Putland (Berken × CPI20141, released 1991)—a small-seeded, photoperiod sensitive variety that produces large biomass.

The varieties Jade, Berken and Celera II are classified as early maturing (50–60 days), while the variety Putland is characterized by a much longer growing season (75–85 days) that is influenced by photoperiod. Within the early maturing varieties, Berken is slightly earlier than the Celera II (Lawn, 1979).

Experimental Site and Unit

The experiment was conducted in a temperature-controlled glasshouse at The University of Queensland, Brisbane, Australia (27°23′S, 153°06′E). Purpose-built root observation chambers were constructed from perspex sheets, with chamber dimensions being 60 cm high, 40 cm wide and 3 cm thick. Transparent perspex (8 mm thick) sides were used to enable viewing and scanning of roots. The perspex sheets were screwed to the metal frame of the chamber and the back of the chambers was lined with black plastic to allow easy removal at harvest. The chambers were wrapped in silver insulation to prevent exposure of the roots to light and to minimize fluctuations in soil temperature (**Figure 1**).

Each chamber was filled with 9 kg air dried soil (Vertosol—Isbell, 1986), which was collected in bulk from the top 15 cm layer of the soil profile from research fields on the Gatton Campus (Queensland, Australia). This soil was characterized by a clay content of 35–40%, pH_{CaCl2} 8.0, electrical $conductivity_{1:5soil-water}$ 0.4 dS/m, 24 mg Cl/kg soil, organic carbon 0.6%, Nitrate-N 79 mg/kg soil, Bicarbonate extractable (Colwell) P 18 mg/kg soil and a Cation Exchange Capacity of 51.7 cmol(+)/kg with 8.8% exchangeable sodium. Soil was not assessed as being deficient in any macro or micronutrients. The soil was air dried in the sun and then crushed to 5 mm size with a jaw crusher before being thoroughly mixed to provide a homogenous growing medium. The bulk density of the packed soil in the root observation chamber was estimated to be approximately 1.25 Mg/m^3. A complete liquid fertilizer (Peters Professional Water-Soluble Fertilizer Hydro-sol, ScottsSierra Horticultural Products Co., Marysville, OH, USA) was added to the soil before planting to ensure nutrients were non-limiting.

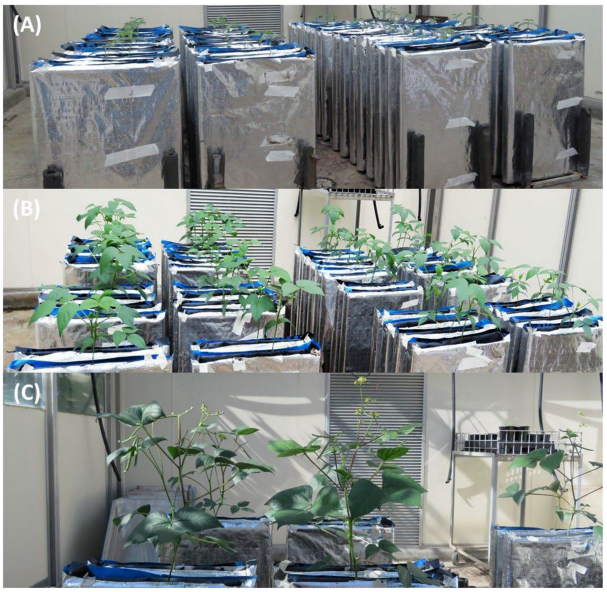

FIGURE 1 | Mungbean plants growing in root chambers in a temperature-controlled glasshouse with four replications. **(A)** Before harvest 1, **(B)** harvest 2, and **(C)** harvest 3.

Experimental Design

The treatments consisted of four varieties sampled at each of four harvest dates spaced 10 days apart commencing 20 days after emergence and designed to cover key vegetative and reproductive growth stages. Four replicate root chambers of each combination of variety and harvest date were laid out in a split plot design with harvest dates deployed as the main plots and varieties as the subplots (**Figure 1**).

Growing Conditions

Soil in each chamber was saturated and drained to reach field capacity before sowing, with the wetting up process typically taking 2–3 days. Once drained, three seeds of one of the four contrasting varieties were sown in each chamber and gradually thinned to one established plant 4 days after germination. The chambers were arranged on a stand that gave a plant-to-plant spacing of 20 cm, and chambers were watered from the top every 10 days to return the soil to field capacity and avoid development of water stress.

Measurements

Before each harvest, plant height (from base to top of the stem) and total number of branches (at the nodes) were recorded. Plants were then destructively sampled by collecting the shoot of each plant above the base of the stem and separating into stem, leaf and pod fractions (pods were present in the last two harvests of the early maturing varieties only). The total number of leaves,

number of fully expanded leaves, and leaf area (using a LICOR Planimeter (Li-3000 leaf area meter) were recorded.

After removing the shoot, chambers were saturated with water overnight, after which each chamber was laid flat and the top perspex plate was removed. Purpose-built plywood pinboards that matched the chamber dimensions and fitted with 3 cm long black nails positioned in a 2 × 2 cm grid were then placed on top of the exposed soil (**Figure 2**). The moist soil allowed the nails to be easily pushed into the soil of the chamber, while preserving most of the intact root system architecture. The pinboards plus soil and roots were then held erect while the soil was washed from the pinboard using a very fine, low pressure water spray to minimize disturbance of the intact root system (**Figure 2**). The total number of nodules on the root system was recorded, the length of the tap root was manually measured with a ruler and the diameter of the tap root 1 cm below the soil surface was measured with a digital caliper. The washed root system was then imaged with a digital camera (Canon, SX720 HS) mounted on a tripod and the images were converted to high-contrast black and white images using Adobe Photoshop software. Images were initially cropped to the same size and then image adjustment and threshold tools were used to convert the image into black and white. The average root angle of the first and second lateral branches was determined using "openGelPhoto.tcl" (www.activestate.com/activetcl), free software that calculates the angle of individual roots relative to the vertical plant (for example, Joshi et al., 2017). After imaging, the roots were stored in 70% ethanol in a cold room (4°C) for later manual measurements of the tap root length, number of nodules and total root dry weights.

Dry matter of each plant part (stem, leaf, pods and roots) was obtained after drying in a dehydrator for four days at 70°C. Development of leaf area, root surface area, top mass (shoot + pods), shoot mass (leaf + stem) and root mass were quantitatively determined for each growth stage, and these data were also expressed as values relative to the maximum value recorded for each parameter during the experiment. While this was typically the last harvest (H4) for parameters relating to mass, other parameters sometimes achieved their maxima earlier in the experiment (e.g., H3 for leaf area). The relative values were primarily used to contrast patterns of above and below ground growth and development for a variety, and between varieties.

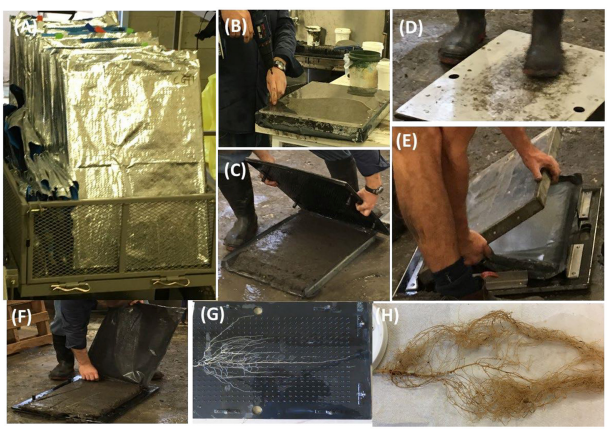

FIGURE 2 | Recovering intact root system architecture. **(A)** Root chambers brought in the soil laboratory, **(B)** screws are removed from the perplex panels, **(C)** top panel is removed so that pin board can be inserted, **(D)** pinboard is inserted with pressure, **(E)** panel of other side is removed, **(F)** black plastic holding the soil is removed, **(G)** a washed intact root system on the pinboard, and **(H)** a washed root system after storing in the ethanol for further analysis.

Intact Root Characteristics

Images of intact roots were analyzed using WinRhizo™ Pro 2019 software (https://regent.qc.ca/assets/winrhizo_software.html). The image was acquired from the camera by electing the "origin" setting in the software, and the following settings were chosen—image resolution was 600 dpi, the root background was changed to gray scale and calibration was performed by marking the length and width of the image (60 × 40 cm). The saved calibration was loaded onto each image before the start of the analysis. Before loading the calibration, the root diameter classes were changed into 10 different widths with a width interval of 0.25 mm. The total root length (cm), total root surface area (cm^2), root surface area in top and bottom 30 cm of the chambers, mean root diameter (mm) and the number of root tips, forks and crossings were determined from image analysis. The specific root mass (g/cm) was determined from total root weight divided by total root length. Images of intact roots were also analyzed based on vertical distribution within the root chamber, with the top 30 cm and bottom 30 cm analyzed separately for later growth stages (i.e., H3 and H4) when roots had started to reach the bottom of the root chambers. These analyses were conducted to determine whether varieties differed in RSA of the shallow (top 0–30 cm) and deeper (30–60cm) parts of the soil profile.

Statistical Analysis

Analysis of variance was performed using Genstat windows 18th edition (VSN International, 2015). A split plot treatment structure was used in the analysis, with harvest times as the main plots and varieties as the subplots. Least significant differences of means at 5% probability were used to compare differences between varieties for the various parameters.

RESULTS

An analysis of variance table showing the significance of main effects (varieties and harvest) and their interactions on selected plant parameters is presented in **Table 1**, with a more complete presentation of a wider set of parameters provided in **Supplementary Table 1** (ST1). Varietal differences were highly significant ($P < 0.001$) for the majority of all parameters. However, there were also many highly significant interactions ($P < 0.001$) between varieties and time of harvest, indicating rates of growth and development varied significantly between varieties as the development of the plant progressed from early growth toward pod formation and grain filling. These interactions were explored in detail for above ground biomass and leaf area accumulation (**Table 1**), and subsequently in an examination of

TABLE 1 | Leaf area development and accumulation of above and below ground biomass in four mungbean varieties with contrasting maturities.

Harvests	Varieties	Leaf area (cm^2)	Shoot dry weight (g)	Pod dry weight (g)	Root dry weight (g)	Root: shoot ratio
Harvest 1	Jade	37.5a	0.14a	–	0.07a	0.51f
	Putland	20.0a	0.09a	–	0.02a	0.20ab
	Berken	39.3a	0.18a	–	0.07a	0.4de
	Celera II	33.3a	0.12a	–	0.042a	0.36d
Harvest 2	Jade	251.4c	1.12bc	–	0.39b	0.34d
	Putland	175.4b	0.67b	–	0.25b	0.38d
	Berken	259.4c	1.05bc	–	0.35b	0.34d
	Celera II	210.8b	0.83bc	–	0.28b	0.34d
Harvest 3	Jade	489.9e	5.59	–	1.06c	0.19a
	Putland	642.4g	4.67de	–	1.17c	0.25bc
	Berken	425d	5.26f	–	0.84c	0.16a
	Celera II	608.5g	5.14f	–	0.91c	0.18a
Harvest 4	Jade	388.2d	4.27d	4.75a	1.18c	0.28bc
	Putland	812.4h	9.4g	–	2.22d	0.23b
	Berken	485.8e	4.48de	5.82b	0.82c	0.24b
	Celera II	576.6f	3.97d	6.28c	0.92c	0.23b

*Mean values for each parameter and harvest date are accompanied by letters to indicate significant differences from the variety*harvest date interaction.*

the relationship between above and below ground growth and development.

Root System Architecture (RSA) and Root Morphology

Visual records of growth and development of intact root systems for the experiment duration are presented in the **Figure 3**, while root morphometric data (from manual and WinRhizo measurements) are presented in the **Figure 4**. At early growth stages (H1 and H2), the early maturing varieties Jade and Berken, and to some extent Celera II, showed more vigorous root growth and root branching deeper in the root chamber, whereas the late maturing Putland showed relatively slow root growth and development (**Figures 3**, **4**). Visually, after 20 days of growth at H1 the tap root of the early maturing varieties had effectively reached the bottom of the chamber (i.e., 60 cm), whereas the late maturing Putland had only reached 50 cm deep at that time (**Figure 3**). However, in the later stages of growth all varieties showed similar tap root lengths of between 60 and 70 cm, with no significant differences between them (**Figure 3**). Early elongation and proliferation of the second and third order lateral roots was significantly more rapid for the early maturing varieties at H1 and H2 (**Figure 3**), but this trend was reversed in the mid and later growth stages (H3 and H4), when the later maturing Putland showed significantly more root growth. Visually, Putland and to some extent Celera II, grew relatively thicker roots in the surface soil, whereas Jade and Berken showed more root branching in the deeper layers (**Figure 3**). Jade recorded the greatest root surface area in the bottom layer, whereas Putland had the most root surface area in the top layers (see **Supplementary Table 1**). Jade also had greater root surface area in the bottom than the top 30 cm of the chambers, while Putland and to some extent Celera II showed an opposite trend. Berken had similar root surface areas in the top and bottom chamber (see **Supplementary Table 1**).

Key root morphometric measurements such as the total root length (**Figure 4A**) and root surface area (**Figure 4B**) supported the visual observations shown in the **Figure 3**, with the differences in root growth between varieties at different growth stages of particular interest. The early maturing varieties Jade and Berken, and to a lesser extent Celera II, showed greater root growth (length and surface area) early in the season (H1 and H2—**Figures 4A,B**) than Putland, with this trend reversed in later growth stages (H3 and H4).

Number of root tips, forks (indicating root branching patterns) and crossing (overlapping) were also quantified with WinRhizo. These root parameters were closely related to each other, so only the number of root tips have been presented (see **Supplementary Table 1**). Putland and Berken showed around 37% fewer root tips than Jade and Celera II. On average, the number of root tips increased *ca.* 11-fold (from 1,100 to 13,300) as plants aged. Specific root mass (root mass/root length) of the late maturing Putland was 20% to 50% greater than the three early maturing varieties, with Celera II and Berken showing lower specific root mass than Jade (**Supplementary Table 1**).

Root growth angle appeared to increase from H1 and H4 in all varieties, with Jade showing a lower root growth angle than the Celera II (see **Supplementary Table 1**). The number of nodules also increased over time in all varieties, ranging from 3 to 9 $plant^{-1}$, but there were no significant varietal differences (see **Supplementary Table 1**).

Development of Roots and Shoots

Relationships between shoot and root growth parameters were constructed for individual varieties, because of contrasting growth patterns evident between early and late maturing varieties (**Table 1**; **Figures 5**, **6**). Since the key root morphological

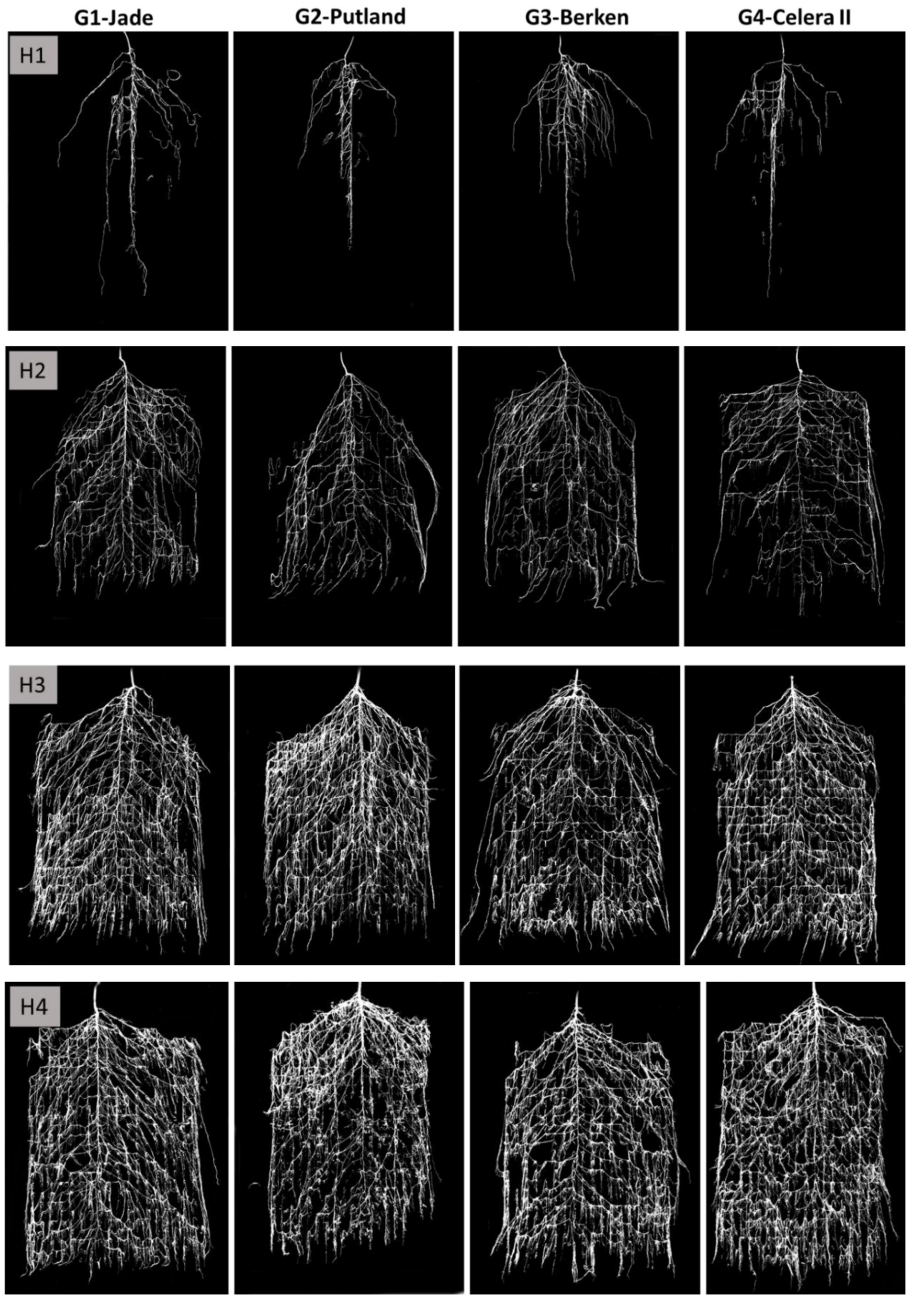

FIGURE 3 | Intact root growth images on the pinboard (after processing with adobe photoshop) at four harvests (H1, H2, H3 and H4) for mungbean varieties Jade, Putland, Berken and Celera II. These images were analyzed with WinRhizo for key root traits measurements.

parameters such as the total root length and root surface area were highly correlated with each other, and the root surface area (product of root length and root diameter) showed slightly better relationships with the shoot parameters, we used the development of root surface area (**Figure 4B**) to compare with the development of leaf surface area (**Table 1**). The relationship was curvilinear over time (H1 to H4) for all varieties, with root surface area increasing with leaf area until H3, after which root surface area did not increase further for any variety (data not presented). Leaf area also did not increase between H3 and H4, except for Putland. The relative development of both root surface area and leaf area are presented in the **Figure 5**, with values for each harvest expressed relative to the maximum value recorded for each parameter (shown in **Supplementary Table 1**).

A similar analysis was conducted for aboveground and belowground dry matter accumulation (**Table 1**), with the former considered as a whole (i.e., leaf, stem and pods where these were present) or simply as vegetative material (leaf and stem).

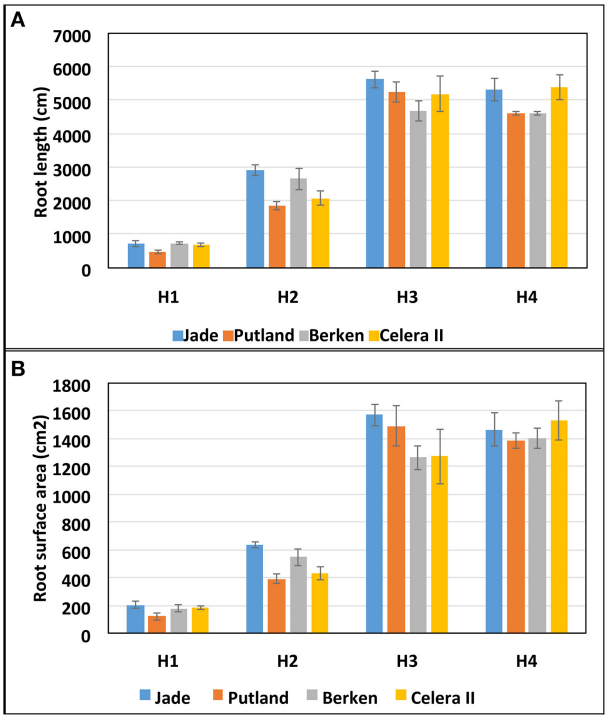

FIGURE 4 | Root morphometric measurements **(A)** total root length and **(B)** root surface area at four harvests (H1, H2, H3, and H4) for four mungbean varieties (Jade, Putland, Berken and Celera II). Bars represent standard error of means.

Significant interactions occurred between varieties and growth stages for above ground shoot mass, but not for the belowground root mass (**Table 1**). During the early growth stages, shoot mass was higher for the early maturing varieties than the late maturing Putland, which was consistent with the more rapid development of leaf area (**Figure 5**). All three relatively early maturing varieties had greater shoot mass than the late maturing Putland at H3 (**Table 1**). However, between H3 and H4 Putland accumulated >80% more shoot mass compared with the other varieties. In contrast to Putland, while other varieties did not grow any more shoot mass, or shoot mass appeared to decrease slightly during this period, these varieties instead accumulated biomass in pods. When these results were considered in relative terms (**Figure 6**), all varieties showed similar patterns of total above ground dry matter accumulation but different patterns of below ground dry matter accumulation, with root DM no longer increasing in any of the earlier maturity lines beyond 40 DAE (**Figure 6**). The contrast between total above ground dry matter and vegetative dry matter production is illustrated in **Figure 6**, with the latter showing that accumulation of vegetative dry matter and root dry matter were tightly correlated in all varieties.

DISCUSSION

The primary aim of this study was to observe and understand the dynamic nature of root growth and root system architecture (RSA) and its relationship with shoot growth (vegetative and reproductive) over time for mungbean varieties differing in maturity times. In this study, the homogenous soil profiles in the root chambers, adequate supplies of water and nutrients and semi-controlled experimental conditions allowed varietal differences in patterns of root and shoot growth to be expressed without the confounding biotic and abiotic effects commonly encountered in field studies.

Phenological Effects on Root Growth Dynamics and RSA

Differences in RSA between the late maturing variety Putland and the early maturing varieties Jade, Berken and Celera II were significant, and also differed between growth stages. The early maturing varieties showed more rapid root growth, developing denser and more prolific root systems at greater soil depths earlier in the crop life cycle (i.e., H1 and H2—**Figure 3**). In contrast, the later maturing Putland developed greater root length and relatively thicker roots in the surface soil during the later growth stages (i.e., H3 and H4). The observed visual differences were consistent with the image analysis of intact RSA and morphometric measurements made using WinRhizo PRO. Similar findings have also been recorded for sorghum (Singh et al., 2012) and rice (Uga et al., 2013), with early maturing varieties exhibiting relatively enhanced root growth and development in deeper soil layers during early growth stages compared to later maturing varieties. These studies also found that the late maturing lines had greater root lengths and thicker roots in the topsoil than in the deeper soil layers. It could be hypothesized that the root thickening and an increase in the root surface area for the late maturing variety was an artifact of growing in a restricted soil volume, and that root length and surface area would have continued to increase in a larger pot. However, root thickening and an increase in root surface area in Putland occurred only in the surface soil (**Figure 3** H3–H4; **Supplementary Table 1**), whereas early maturing varieties indicated relatively greater root surface area in the bottom section of the soil profile (for example, Jade, **Supplementary Table 1**). This suggests that the observed thickening and increase in the root surface area in the surface soil by Putland may not be due to restricted soil volume, and this is further supported by the observation of continuous and statistically significant increases in the number of root tips from H1 to H4 (**Supplementary Table 1**).

Root length, root surface area, number of root tips, root collar diameter and specific root mass also changed over time for each variety (**Table 1**; **Supplementary Table 1**). Growth of these parameters differed between early and late growth stages, reflecting the dynamic nature of root growth and RSA in mungbean. Chen et al. (2017) also reported root vertical angles changing with depth while phenotyping the dynamics of wheat (*Triticum. aestivum*) RSA in the field over time. Similarly, Hund et al. (2009) reported a linear trend in the axial root length at the early growth stages or seedling stage for maize (*Zea mays*), whereas Barraclough and Leigh (1984) reported a curvilinear trend in the root growth pattern until flowering for wheat. The dynamic nature of root system development

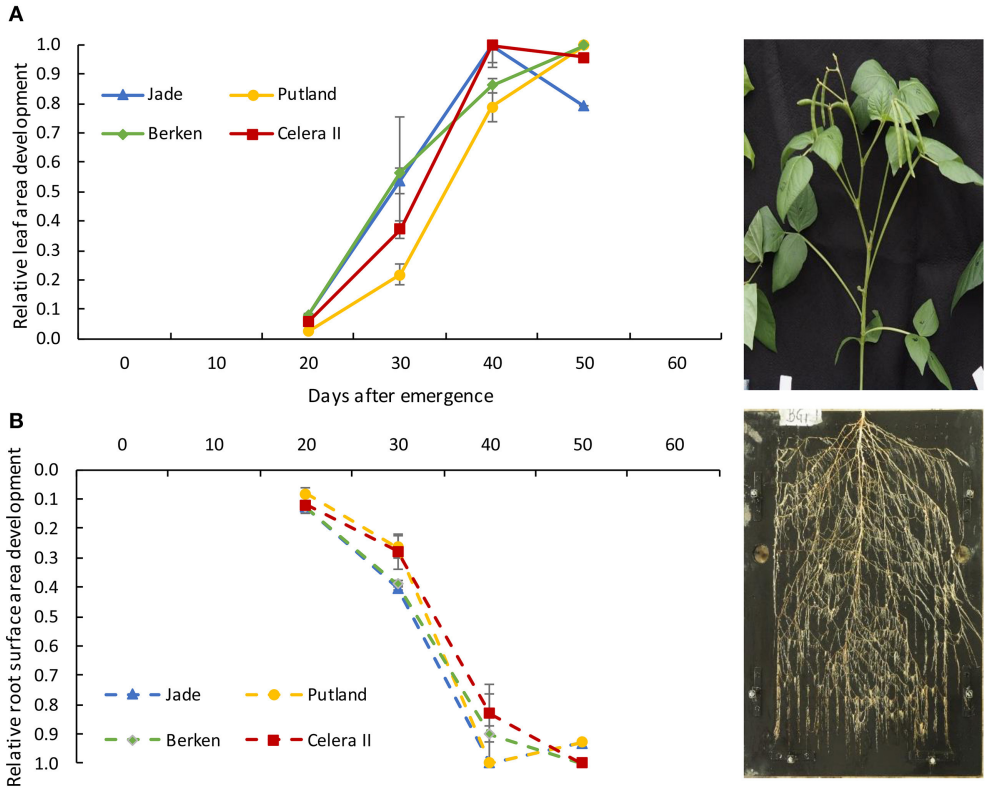

FIGURE 5 | Relative development of leaf area **(A)** and root surface area **(B)** from 20 days to 50 days of plant growth for four varieties; Jade, Putland, Berken and Celera II. Error bars indicate +/- standard error of means. The photograph represents the figures for the directional growth of shoot **(A)** and root **(B)**.

observed in our studies was linked to changes in the shoot and reproductive growth observed over the course of this experiment (as discussed later).

Early maturing varieties showed greater total root length and root mass at early growth stages (**Figure 4**; **Table 1**). However, differences in the total root length were less pronounced at the mid to late growth stages compared with that of Putland, which recorded large increases in the root mass from H3 to H4. Variable growth of these two key root morphological traits (root mass and length) resulted in a lower specific root mass (mass/length) for the early maturing varieties than the late maturing variety (see **Supplementary Table 1**). Lower root mass per unit length suggested a lower carbon requirement for root construction in the early maturing varieties compared to Putland, and the lighter roots in the early maturing types was also reflected in a significantly lower root mass compared with Putland at H4 (**Table 1**). The lighter roots of early maturing types have been identified as a preferred ideotype for root systems that have potentially reduced construction and maintenance costs in other studies (Lenochova et al., 2009; Zhu et al., 2010; Lynch, 2013). The root systems in these early maturing mungbean varieties, therefore, were not only characterized by the establishment of a deeper root system earlier in the growing season, with the potential to extract more water from deeper profile layers, but were able to achieve this at a lower C cost.

Wasson et al. (2012) advocated for greater root length densities at depth and reduced density in the topsoil to favor deep soil water extraction, and so the early maturing mungbean varieties in this study would potentially seem well-adapted to such conditions—at least during early growth stages. Conversely, the relatively thicker and more prolific surface root development in the later maturing Putland could provide better anchorage to support the vigorous shoot growth and larger biomass that accumulated during mid to late growth stages, and would be more effective in utilizing smaller rainfall events that wet only the top soil layers. Such root systems may also have a better horizontal spread and greater water extraction at a distance from the plant row, as observed for shallow rooting sorghum (*Sorghum bicolor*) lines (Singh et al., 2012). Greater nutrient acquisition has also been associated with increased soil exploration by roots in surface layers, especially in the case of immobile nutrients such as phosphorus (Bonser et al., 1996; Lynch and Brown, 2001). Whilst our study is not able to address the potential for improved nutrient foraging by the greater allocation of root biomass to surface soil layers with Putland, it is important to note that the increased root mass was not accompanied by an equivalent increase in root surface area (**Figure 4B**) in that part of the profile. This would be an important factor influencing the efficiency of recovery of nutrients like phosphorus, where diffusive supply over short distances is a key factor determining

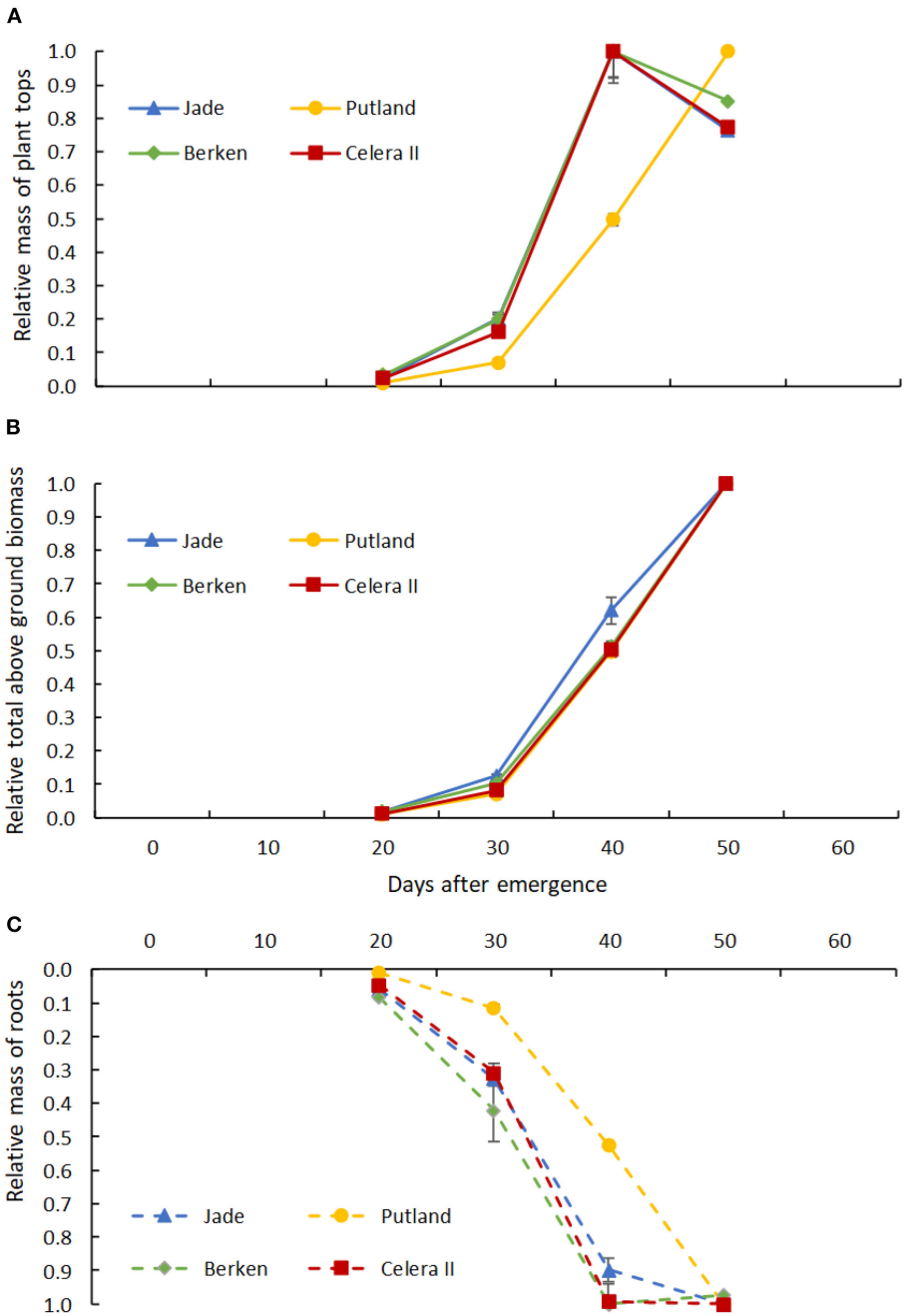

FIGURE 6 | Relative mass of **(A)** plant tops (leaf + stem) and relative mass of **(B)** aboveground biomass (leaf + stem + pods) to relative mass of root **(C)** from 20 days to 50 days of plant growth for four varieties; Jade, Putland, Berken and Celera II. Error bars indicate +/− standard error of means.

nutrient acquisition. Singh et al. (2012) noted that shallower rooting sorghum genotypes with wider root growth angle and thicker roots in the surface soil extracted more water from the surface layer in a drying soil. Shallower root systems are also more adapted to relatively shallower soil profiles and wider row spacing configurations (Singh et al., 2012). However, under terminal drought conditions shallow rooting varieties like Putland may underperform compared with deeper rooting early maturing varieties (Jade, Berken and Celera II), which would appear to be more suited to deeper soil profiles and narrow row spacing configurations.

The early maturing variety Jade showed relatively narrower root growth angle in this study. Root growth angle has been noted to be the key indicator of a deep or shallow rooting genotype (Singh et al., 2011; Uga et al., 2013; Chen et al., 2017) and plays a major role in determining RSA. However, while

root growth angle is primarily governed by plagiogravitropism (Nakamoto, 1994), it can also be influenced by other factors such as soil strength and soil water, (Nakamoto, 1993, 1994; Trachsel et al., 2011) soil temperature (Tardieu and Pellerin, 1991) and soil nutrition, especially phosphorus. Evidence from different crops has indicated that genotypes with narrow root growth angles are not only deep rooting, but they also tend to grow and develop more rapidly in both above and below-ground components, leading to early flowering and maturity. This characteristic was initially noted for genotypes of rice (Uga et al., 2013) and sorghum (Singh et al., 2012). The root morphological traits such as deeper and more prolific root development that were observed in early maturing mungbean varieties in this study have been linked to adaptation to water-limited environments in sorghum (Ludlow et al., 1990; Tsuji et al., 2005; Singh et al., 2012), rice (*Oryza sativa*) (Ekanayake et al., 1985; Kato et al., 2006; Uga et al., 2013), and maize (Hund et al., 2008).

Interactions Between Phenological Development and Plant Growth in Mungbean Varieties

The early maturing varieties Jade and Berken showed more rapid dry matter accumulation in both tops and roots during the early growth stages (i.e. H1 and H2—**Table 1**), slightly faster than Celera II and significantly more than Putland, with these differences consistent with more rapid leaf area development during that time (**Table 1**). To support this vigorous root and shoot growth, early maturing varieties also showed a greater dry matter production per unit leaf area, indicative of an increase in efficiency of resource capture or use efficiency.

Differences in biomass production between varieties had largely disappeared by H3 and were not evident at all at H4—even though the dry matter constituents now differed between varieties due to the addition of pods in Jade, Berken and Celera II (**Table 1**). The relative accumulation of above and below ground dry matter was strongly correlated in Putland, which exhibited solely vegetative growth during the study, but not in the earlier maturing varieties in which accumulation of root dry matter had effectively ceased after H3 (40DAE—**Figure 6A**). The contrast between relative accumulation of total and vegetative above ground dry matter (**Figure 6**) illustrated the significant impact of commencement of pod development on growth of other plant parts, both above and below ground, in the earlier maturing varieties. Pod establishment from 40 DAE resulted in the complete cessation of vegetative and root growth in the early maturing varieties, while growth of both components continued unabated in Putland. The photoperiod sensitivity of the variety Putland may have contributed to the extended vegetative phase in this study, as daylengths >13 h would likely have contributed to delays in the onset of flowering and subsequent reproductive development". Nevertheless, major changes in assimilate distribution patterns were triggered by the early establishment of the pods as sinks for assimilate, with these changes having significant implications for both the ability to efficiently exploit stored soil water deeper in a soil profile, and for optimum planting configurations (row spacings and plant densities) in varieties with differing phenology. Both Nord and Lynch (2009) and Lynch (2013) have previously highlighted that the transition from vegetative to reproductive growth has important implications for soil resource acquisition.

The Relationship Between Root and Shoot Growth Dynamics

Rapid expansion of leaf and root surface areas are critical to the establishment of structural frameworks needed to capture resources for subsequent crop growth (**Figures 5**, **6**), with the strong positive correlation between these indicators of above and below-ground resource capture noted in many other species. For example, Grieder et al. (2014) noted positive relationships between leaf area and root length and rooting depth for maize genotypes. The vigorous root growth and more rapid rates of root extension seen in the early maturing mungbean varieties in our study are consistent with acquisition of sufficient water and nutrients to be able to support the rapid shoot growth observed in these varieties. This synchrony of resource capture is essential to the rapid development of a competitive crop canopy consistent with the rapid phenological advancement during shorter growing seasons.

Poorter et al. (2009) reported close relationships between the rate of photosynthesis and shoot growth, and as noted by Lynch (2013), shoot characteristics that enhance the conversion of water or nitrogen to carbon and energy in photosynthesis will also permit greater root growth, and hence greater soil resource acquisition. This was illustrated for sunflower by Aguirrezabal and Tardieu (1996), who reported that root extension rate was related to photosynthetic photon flux density and leaf area development. An improved carbon assimilation rate was able to support increased rates of root elongation, increased root branching and overall greater root length (Aguirrezabal et al., 1993), subsequently increasing the water and nutrient uptake. While the rate of photosynthesis was not determined in our mungbean study, the greater dry matter production per unit leaf area during early growth stages was consistent with higher photosynthetic rates in the early maturing mungbean varieties. Arai-Sanoh et al. (2014) suggested that a high flux of cytokinins, mostly synthesized in the roots and root tips, could contribute to the high photosynthetic rate. Two of the three early maturing varieties in our study showed greater number of root tips (**Supplementary Table 1**).

The relationship between root growth and reproductive growth and/or yield parameters is complex, as both are influenced by biotic and abiotic factors. Watt et al. (2013) were able to relate a rapid seedling root screen with the rooting depth in vegetative growth stages, but not for the reproductive growth stage. Our mungbean study did observe that increased rates of deeper root development during early growth stages were correlated with early flowering and podding characteristics in the varieties studied. Conversely, the early onset of reproductive growth and pod establishment had very strong impacts on partitioning of carbohydrate to root growth and root dry weight during later growth stages. This would result in an erosion of

the benefit of rapid root establishment in the earlier maturing varieties, given that later maturing lines like Putland could continue to establish more roots over an extended vegetative period. The relative benefits of these contrasting phenologies and patterns of root development will differ between production environments and will also likely impact on the optimum agronomic management strategies.

CONCLUSIONS

Knowledge of the relationship between various root traits and plant productivity is necessary to effectively target management and breeding strategies to improve crop productivity. Our studies indicated that early maturing mungbean varieties were characterized by a combination of traits that contributed to more vigorous root and shoot growth during early growth stages than in a later maturing variety. Traits such as the deeper, longer and lighter roots found in these varieties would be expected to confer better adaptation to water-limited environments, although the rapid onset of reproductive growth and the cessation of subsequent root growth may limit the impact of these traits in the field. In contrast, the later maturing variety exhibited relatively thicker roots in the topsoil layers that could provide a better anchorage to support the larger plants with presumably a greater abundance of maturing pods that an extended growing season would facilitate, in the absence of other constraints.

While this study was conducted under conditions of adequate water and nutrient supply, the differences in observed root traits between varieties would suggest differential adaption to environments where water and/or nutrients may be suboptimal during the growing season. Mungbean crops are generally grown in marginal environments with limited soil moisture, and late maturing varieties with limited "in-crop" seasonal rainfall may have to survive on stored moisture in deeper layer of soil. However, in the wet seasons, crops may not use much of the deeper profile moisture at all, therefore, the type of the root system that will provide a water advantage will be entirely dependent on the growing seasons targeted and soil depth. Although our findings are based on a limited number of genotypes and there is a need for broader examination of variation, but we hypothesize that root systems that developed on the late maturing variety may be better adapted to relatively shallower soil depths, surface stratified nutrient reserves and wider row spacing configurations, whereas the narrower and deeper rooting observed in the early maturing varieties may be more suited to deeper soil profiles with more uniform nutrient distributions and narrow row configurations.

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

VS contributed to the conceptualization of the research, experimental set up, data collection, result analysis, and writing of the main manuscript text. MB contributed to the conceptualization and improved the text, and also led the project. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.725915/full#supplementary-material

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REVIEW
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Chickpea (*Cicer arietinum* L.) as a Source of Essential Fatty Acids – A Biofortification Approach

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Chickpea is a highly nutritious pulse crop with low digestible carbohydrates (40–60%), protein (15–22%), essential fats (4–8%), and a range of minerals and vitamins. The fatty acid composition of the seed adds value because fats govern the texture, shelf-life, flavor, aroma, and nutritional composition of chickpea-based food products. Therefore, the biofortification of essential fatty acids has become a nutritional breeding target for chickpea crop improvement programs worldwide. This paper examines global chickpea production, focusing on plant lipids, their functions, and their benefits to human health. In addition, this paper also reviews the chemical analysis of essential fatty acids and possible breeding targets to enrich essential fatty acids in chickpea (*Cicer arietinum*) biofortification. Biofortification of chickpea for essential fatty acids within safe levels will improve human health and support food processing to retain the quality and flavor of chickpea-based food products. Essential fatty acid biofortification is possible by phenotyping diverse chickpea germplasm over suitable locations and years and identifying the candidate genes responsible for quantitative trait loci mapping using genome-wide association mapping.

Keywords: chickpea (*Cicer arietinum* L.), essential fatty acids, biofortification, nutritional breeding, fourier transform infrared spectroscopy

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INTRODUCTION

Chickpea (*Cicer arietinum*) is a self-pollinating diploid (2n = 2x = 16) pulse crop with a 738 Mbp genome (Varshney et al., 2013). Chickpea primarily extended from *Cicer reticulatum* Ladizinsky approximately 11,000 years ago (Zohari and Hopf, 2000; Kerem et al., 2007), a variable wild species that originated in several regions of southeastern Turkey (37.3–39.3°N, 38.2–43.6°E; Kerem et al., 2007). Chickpea presently has 44 species, of which 35 are perennial, and nine are annual. Chickpea has two market classes—*kabuli* and *desi*—based on seed morphology (Knights and Hobson, 2016). Kabuli has become popular in Western markets as hummus and canned and raw seeds for salads and soups, whereas desi seeds are split and consumed in Southeast Asia as "channa dal."

Chickpea consumption is popular in many regions around the globe, mainly due to its high nutritional quality. The chickpea seed matrix is comprised of carbohydrates (50–58%), protein (15–22%), moisture (7–8%), fat (3.8–10.20%), and micronutrients (<1%; Jukanti et al.,

2012; USDA, 2021). Chickpea carbohydrates include a range of prebiotic carbohydrates, including sugar alcohols, fructooligosaccharides, raffinose family oligosaccharides, inulin, and resistant starch (Peterbauer and Richter, 2001; Johnson et al., 2020), which modulate the gut microbiome and improve human gut health (Roberfroid et al., 2009). The mean protein content in chickpea is nearly 18% [(kabuli: 18.4% (16.2–22.4%); desi: 18.2% (15.6–21.4%)], which is higher than lentil and field pea (Upadhyaya et al., 2016). Chickpea is rich in lysine and arginine and low in sulfur (S)-containing amino acids such as cysteine and methionine (Jukanti et al., 2012). Moreover, chickpea is a rich source of minerals, including iron (Fe), zinc (Zn), and selenium (Se).

The United Nations established Sustainable Development Goals to end global hunger and malnutrition by 2030 (United Nations, 2021a,b). Biofortification or conventional breeding with modern biotechnology to enhanced micronutrient concentrations in staple food crops has been vital to combat global hunger and malnutrition. To date, many staple food crops have been biofortified with micronutrients, and cultivars were released to these vulnerable populations globally (Harvest Plus, 2021). Chickpea is a target candidate pulse crop for mineral and vitamin biofortification (Thavarajah and Thavarajah, 2012; Vandemark et al., 2018, 2020; Kumar and Pandey, 2020). During the last decade, several global research foundations have attempted to develop Fe-, Zn-, and Se-enriched chickpea cultivars to combat micronutrient malnutrition or "hidden hunger" (Thavarajah and Thavarajah, 2012; Vandemark et al., 2018). Biofortified chickpea provides 5.2–6.0 mg of Fe, 2.5–5.3 mg of Zn, and 15.3–56.3 mg of Se in a 100-g serving, representing a significant portion of the recommended daily allowance (RDA) of these essential elements (Thavarajah and Thavarajah, 2012; Ray et al., 2014; Vandemark et al., 2018). A 100-g serving also provides 125–159 mg of magnesium (Mg), 93–197 mg of calcium (Ca), 0.7–1.1 mg of copper (Cu), 732–1,126 mg of potassium (K), and 263–370 mg of phosphorus (P; Thavarajah and Thavarajah, 2012). Chickpea is also a significant source of carotenoids; beta-carotenoid is the most abundant, followed by canthaxanthin and xanthophyll (Thavarajah and Thavarajah, 2012). Vitamins such as folic acid, tocopherols, and vitamin B complex (B_2, B_5, and B_6) are also found in chickpea (Jukanti et al., 2012). Overall, chickpea is a rich source of prebiotic carbohydrates, protein, and several micronutrients, and these components have already been incorporated into global chickpea biofortification programs (Thavarajah and Thavarajah, 2012; Vandemark et al., 2020). However, fat composition is the least-studied nutritional trait of chickpea, and genetic advancement studies are required to advance fatty acid biofortification.

Fats, which provide the storage energy required for seed germination (Nelson and Cox, 2008), occupy a minor proportion of the chickpea seed matrix compared to other nutrients. Chickpea is not an oilseed crop but has a higher fat content than other pulse crops (Jukanti et al., 2012). Sterols, tocopherols (phytosterols), and lipids are components of fat found in chickpea (Jukanti et al., 2012). The fatty acids in chickpeas—polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), and saturated fatty acids (SFAs)—mainly originate from the lipids. These are essential fatty acids (EFAs; ω-6 and ω-3 PUFAs), vital for humans in the biosynthesis of hormones and maintaining cellular integrity (Di Pasquale, 2009). Consequently, chickpea consumption can benefit human health by providing important fatty acids. This review focuses on global chickpea production, biofortification, the function of fats and benefits to human health, chemical analysis of EFAs, and possible breeding targets to optimize ω-6 and ω-3 fatty acids chickpea.

CHICKPEA PRODUCTION

Chickpea ranks third in the global production of pulses at ~11.6 million tons *per annum*, 80% of which is desi and the remaining 20% is kabuli (Merga and Haji, 2019). Chickpea is grown in nearly 57 countries worldwide in varying climatic and growing conditions (Merga and Haji, 2019). India was the leading global chickpea producer in 2019, followed by Turkey, Russia, Myanmar, Pakistan, and Ethiopia (FAOSTAT, 2020; **Table 1**). In great part due to India's large-scale production, Asia dominated global chickpea production in 2019 compared to the Americas (83.4 vs. 6.1%, respectively; FAOSTAT, 2020). In the last two decades, the harvested area has correlated with chickpea production, and both generally show an increase over time (except for lower production in 2015 and 2019; FAOSTAT, 2020). Notably, India has lower yields than smaller producers such as Ethiopia and Mexico (FAOSTAT, 2020), resulting in its position as the world's largest chickpea importer despite its large-scale production (Merga and Haji, 2019). During the last 2 years, India's imports increased from 0.19 MT in 2018 to 0.37 MT in 2019, possibly due to the lower yields in 2019 (9.93 MT) than in 2018 (11.3 MT).

BIOFORTIFICATION

Malnutrition is a persisting global calamity that is prevalent mainly in Africa and South Asia. It exists in three aspects: undernutrition (stunting, wasting, and underweight), obesity, and malnutrition associated with micronutrient deficiency (hidden hunger). The World Health Organization (WHO)

TABLE 1 | Global chickpea production and mean grain yields in 2019 (FAOSTAT, 2020).

Country	Production (MT)	Yield (kg/ha)
India	9.93	1,041
Turkey	0.63	1,217
Russia	0.51	918
Myanmar	0.49	1,316
Pakistan	0.45	474
Ethiopia	0.45	2084
United States of America	0.28	1730
Australia	0.28	1,069
Canada	0.25	1,614
Mexico	0.20	2,117

estimates over 2 billion people suffer from hidden hunger (Ritchie and Roser, 2017). At the same time, 150.8 million, 50.5 million, and 38.3 million children aged below 5 years are stunted, wasted, and overweight, respectively (Ritchie and Roser, 2017; Global Nutrition Report, 2018). South Asian women and school children are highly vulnerable to malnutrition. One-third of women of reproductive age are anemic and show higher susceptibility to obesity than men (Global Nutrition Report, 2018). Plant breeding and agronomical practices introduced in the 1960s during the green revolution primarily combatted global hunger, especially through large-scale cereal production, providing the necessary calories or proteins to these vulnerable populations (Thavarajah et al., 2014; Roorkiwal et al., 2021). However, the consumption of cereals contributed to hidden hunger or micronutrient malnutrition in most developing nations (Roorkiwal et al., 2021). Micronutrients mediate human physical and mental development and further serve as cofactors of enzymes that catalyze biochemical reactions in the body, modulating human physiology and growth (White and Broadley, 2005; Malik and Maqbool, 2020).

Several global approaches have been implemented to increase the bioavailability of nutrients in staple food crops (Welch and Graham, 2004; White and Broadley, 2005; Thavarajah and Thavarajah, 2012). However, technological, socio-economical, financial, and demographical constraints are challenges with nutrient fortification programs. Biofortification is breeding crops to optimize micronutrient concentration and bioavailability, enriching their nutritional value to combat hidden hunger (Garcia-Casal et al., 2017; Roorkiwal et al., 2021). Biofortification has three strategies: agronomic (fertilizing the soil or foliar application), conventional breeding approaches, and molecular technologies (Garcia-Casal et al., 2017). Biofortification is a convenient approach to combat 'hidden hunger,' primarily due to low financial investment, tendency to penetrate demographic barriers benefitting rural populations, and provision of germplasm to farmers at zero marginal expenditure during early investment (Bouis and Saltzman, 2017). In 2015, crops were biofortified for vitamin A (fleshy-orange sweet potato, cassava, and orange corn), Fe (beans and millet), and Zn (rice and wheat) by officially releasing the germplasm of biofortified varieties in 30 countries, further expanding trials and official breeding in more than 50 countries (Birol et al., 2015). However, biofortification attempts have been less frequent in pulses than in cereals (rice, wheat, and corn) during the present decade (Kumar and Pandey, 2020).

Current biofortification efforts in chickpea are focused on enriching micronutrients and reducing antinutrient factors (Sreeramaiah et al., 2007; Jukanti et al., 2012; Thavarajah and Thavarajah, 2012; Vandemark et al., 2020). Agronomic approaches such as fertilizer application (including foliar spraying) and genetic engineering (GE) have been attempted on chickpea to enrich minerals, such as Fe, Zn, and Se (**Table 2**, Poblaciones et al., 2014; Khalid et al., 2015; Pal et al., 2019, 2021). Soil and foliar application of Zn and urea can increase chickpea's Zn and Fe content (Pal et al., 2019).

TABLE 2 | Biofortification methods for chickpea.

Nutrient	Approach	Positive response	References
Selenium (Se)	Foliar application	Seed Se	Poblaciones et al., 2014
Iron (Fe)	Soil application of Plant growth-promoting rhizobacteria	Soil and seed Fe	Khalid et al., 2015
Zinc (Zn)	Foliar application with Zn fertilizer	Seed Zn	Shivay et al., 2015; Pal et al., 2019
Boron (B)	Seed coating	Nodulation, yield	Hussain et al., 2020
Fe and Zn	Conventional breeding/selection/ backcrosses	Seed Fe and Zn	Singh et al., 2021

A combined application of Fe, Zn, and urea (in a tank mix) can increase Fe and Zn concentrations in chickpea seeds and positively influence grain yields and protein levels (Pal et al., 2021). Two separate studies indicate inoculating Zn-solubilizing bacteria (*B. altitudinis*) and rhizobacteria at chickpea planting increase seed Zn and Fe concentration in low Zn soils (Khalid et al., 2015; Kushwaha et al., 2021). Transgenic approaches have also been used for Fe biofortification in chickpea. For example, overexpression of the nicotamine synthase 2 (CaNAS2) and ferritin (GmFER) genes in chickpea increased seed Fe concentration (Tan et al., 2018). However, the above transgenic approach has not demonstrated any significant outcome for conventional chickpea breeding programs. Although biofortification significantly focuses on micronutrients, the techniques followed can be applied to other macro nutritional traits (Garcia-Casal et al., 2017; Roorkiwal et al., 2021). Linoleic acid (LA; ω-6) is the most abundant (essential) fatty acid in chickpea, while α-linolenic acid (ALA; ω-3), the other essential fatty acid, is far less available in the seed (Jukanti et al., 2012). ALA is known for its human health benefits (Simopoulos, 2002, 2006, 2016). Thus, breeding chickpea to enrich the seed in ALA is important; however, the quantitative nature of these genetic traits makes chickpea breeding much complicated than for traits controlled by a single gene.

CHICKPEA LIPIDS

In chickpea, lipids persist as storage and membrane molecules. Storage lipids are triacylglycerols (TAGs), which are suspended as oil droplets (oily phase) on the cell cytosol serve as primary sinks of fatty acids (including EFAs; Nelson and Cox, 2008; Cagliari et al., 2011). TAGs are the most abundant neutral lipid in desi-type chickpea and typically serve as biosynthetic precursors and energy supplements during seed germination (Zia-Ul-Haq et al., 2007; Jukanti et al., 2012; Weselake et al., 2021). The general structure of TAGs includes a glycerol group esterified with three fatty acids, either similar or different (**Figure 1A**). Chickpea also has phospholipids

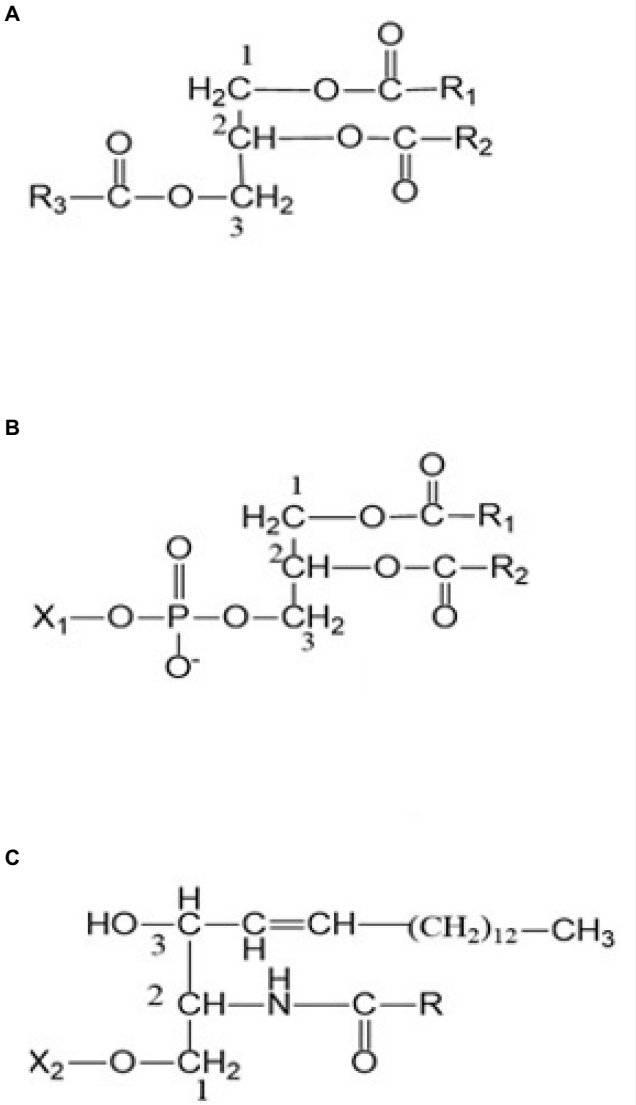

FIGURE 1 | (A) A triacylglycerol (TAG) **(B)** a phospholipid, and **(C)** a sphingolipid. R, R_1, and R_2 are alkyl or alkenyl groups attached to ester carbonyls. X_1: H, ethanolamine, choline, serine, glycerol, or phosphatidylcholine functional groups. X_2: H, phosphocholine, glucose, or oligosaccharide functional groups.

(glycerophospholipids; **Figure 1B**), sphingolipids (**Figure 1C**), glycolipids (galactolipids and sulpholipids; **Figure 2**), and phytosterols as membrane lipids (Clemente et al., 1998; Zia-Ul-Haq et al., 2007; Michaelson et al., 2016). Both storage and membrane lipids contribute to the total chickpea fat composition. The majority of the fatty acids in chickpea are originated from the storage lipids (TAGs), which are the most abundant neutral lipids in seeds (Jukanti et al., 2012). Chickpea has a general fat content of 3.8–10.2%, which is higher than other pulse crops (e.g., lentils, red kidney beans, etc.; Jukanti et al., 2012); the fat content also varies with market class, with ranges from 3.4–8.8% and 2.9–7.4% for kabuli and desi, respectively (Yadav, 2007).

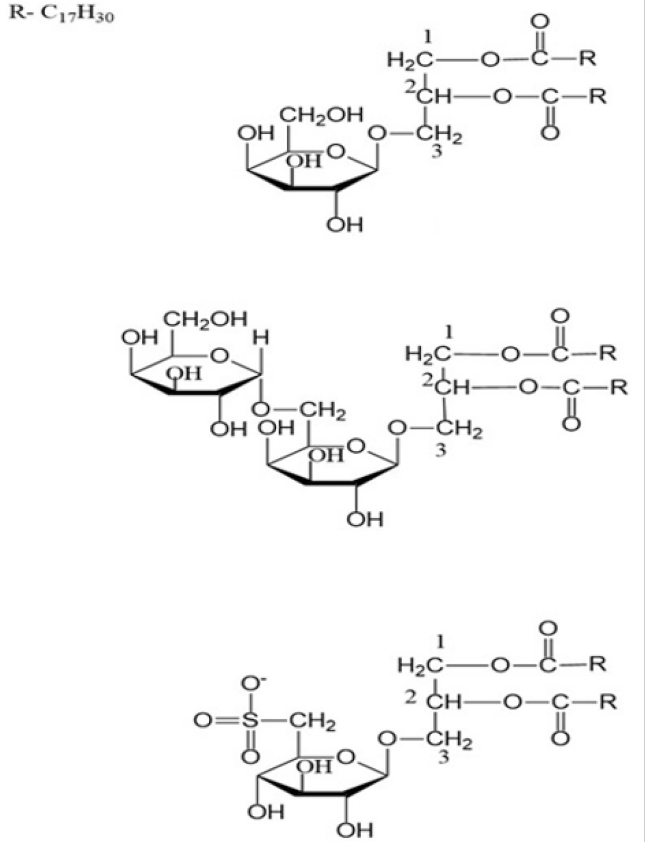

FIGURE 2 | Some typical structures of galactolipids.

FATTY ACIDS AND EFAS

Typically, fatty acids are long-chain hydrocarbon molecules with an attached carboxylic acid group. In chickpea, fatty acids mainly originate from TAGs (Zia-Ul-Haq et al., 2007; Jukanti et al., 2012) as previously indicated and are classified as saturated (with double bonds) or unsaturated (no double bonds) based on the bonding nature (Fahy et al., 2005; Rustan, 2005; **Figure 3**). Unsaturated fatty acids are divided into PUFAs and MUFAs. LA (ω-6) and ALA (ω-3) are PUFAs (Innis, 1991), while oleic acid (OA; ω-9) is a MUFA. LA and ALA are EFAs because they are not synthesized in humans (animals) and must be supplemented from the diet, while OA is not (because animals produce it; Warude et al., 2006) but serves as a precursor for LA. The enzymes to convert OA to LA and then LA to ALA (12-desaturase and 15-desaturase, respectively) exist in plants (Warude et al., 2006; Lee et al., 2016, i.e., chickpea). Within total chickpea fats, 66% are PUFAs, 19% are MUFAs, and 15% are SFAs. Both market classes have considerable amounts of LA (kabuli: ~51.2%, desi: ~61.62%) and OA (kabuli: ~32.6%, desi: ~ 22.31%), which are generally higher than for other edible pulses such as lentils (LA: ~44.4%, OA: ~20.9%), beans (LA: ~46.7%, OA: ~28.1%), and peas (LA: ~45.6%, OA: ~23.2%; Wang and Daun, 2004). Chickpea also contains palmitic acid (kabuli: ~9.41%, desi: ~9.41%) and ALA (kabuli: ~2.69%, desi: ~3.15%; Wang and Daun, 2004; Jukanti et al., 2012).

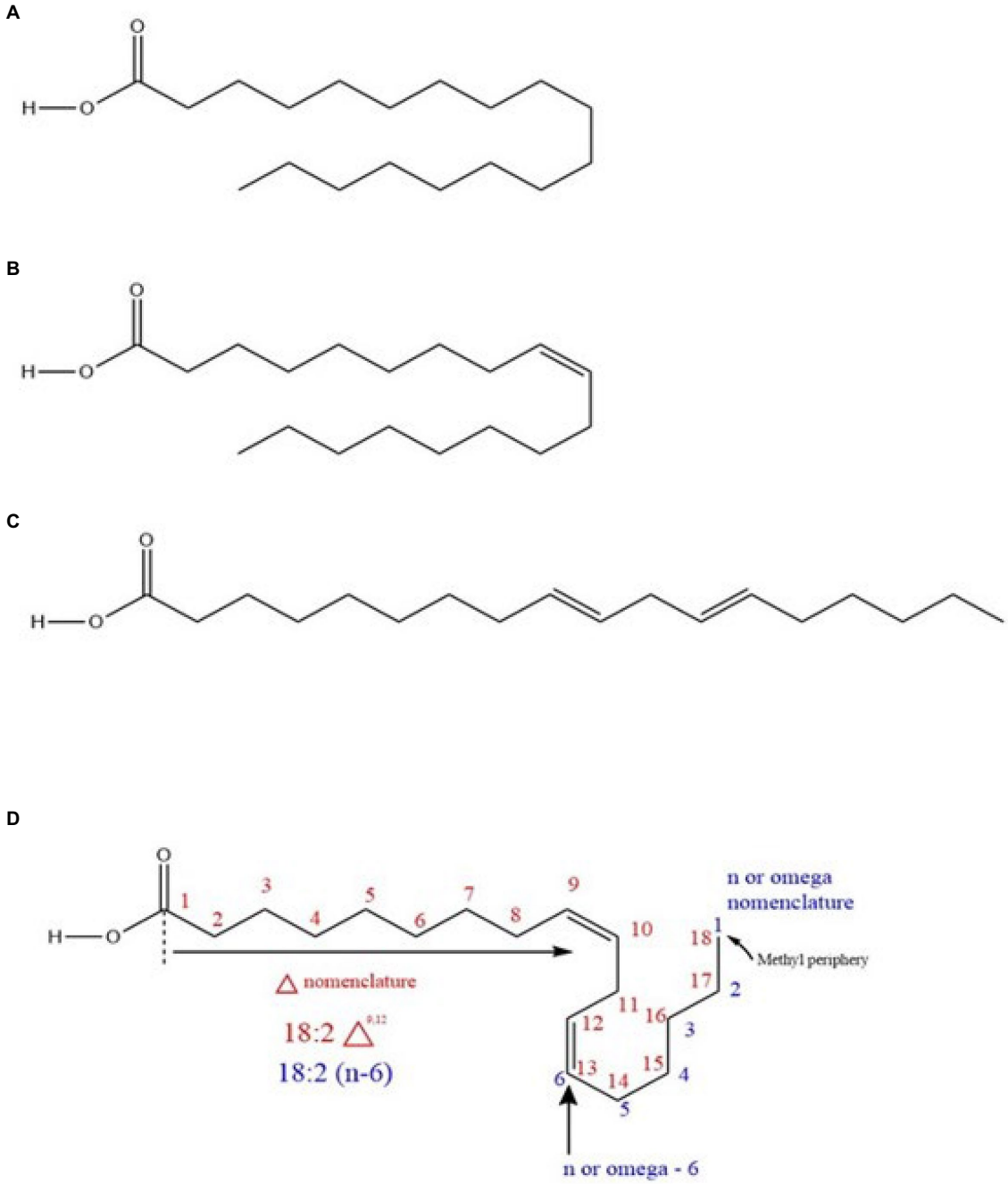

FIGURE 3 | (A) A saturated fatty acid (stearic acid) **(B)** a monounsaturated fatty acid (MUFA; oleic acid) **(C)** a polyunsaturated fatty acid (PUFA; trans linoleic acid), and **(D)** nomenclature system of a PUFA (cis linoleic acid).

FATTY ACIDS AND HUMAN HEALTH BENEFITS

A chickpea-based diet provides significant EFAs; the consumption of unsaturated vs. saturated fats can help maintain healthy cholesterol levels and reduce obesity and diabetic conditions (Kaur and Prasad, 2021). Furthermore, the presence of ALA in a chickpea-based diet reduces angiotensin-converting enzyme inhibition, which contributes to antihypertensive effects (Ogawa et al., 2009; Kaur and Prasad, 2021). Once EFAs are ingested, LA is metabolized to arachidonic acid (AA, an ω-6 EFA). In contrast, ALA is metabolized into eicosapentaenoic acid (EPA, an ω-3 EFA) and docosahexadecaenoic acid (DHA, an ω-3 EFA). AA and EPA undergo further biosynthesis to prostanoids and leukotrienes (de Caterina et al., 2007). These metabolites have several beneficial physiological effects on humans (Singh, 2005).

Metabolites with an ω-6 origin enhance platelet aggregation, while those of ω-3 origin are anti-inflammatory (Singh, 2005). The ω-6/ω-3 fatty acid ratio is an important indicator of the impact of EFAs on human health (Simopoulos, 2002). This ratio is a disease-controlling parameter, where the optimum range is 1–4:1 or 1–5:1 (Simopoulos, 2002, 2006; Singh, 2005). This value ranges from 1–2:1 for optimum health benefits for combating obesity (Simopoulos, 2016). However, in Western countries, this value ranges from 15–16.7:1 due to the low levels of ω-3 fatty acids in diets and comparatively high proportions of LA consumption (Simopoulos, 2002). Yet, no studies regarding the true impact of chickpea on this disease controlling parameter (ω-6/ω-3 ratio) and human metabolism have been published.

Chickpea based diet has a positive effect on diabetes and obesity. Adiponectin is a hormone that prevents type two diabetes and atherosclerosis (Achari and Jain, 2017). A randomized cross-over clinical trial with diabetic patients ($n = 32$) served with a chickpea diet (substituting two servings of red meat) increased levels of adiponectin in all patients (Mirmiran et al., 2019; Acevedo Martinez et al., 2021). Additionally, a study with diabetic rats has demonstrated reduced blood glucose and triglyceride levels upon feeding 400 mg/kg of aqueous and methanol-based doses of chickpea diets (Yagi and Yagi, 2018). Another clinical study (n = 30; men =17 and women = 13) reported that body weights, systolic blood pressure, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and total cholesterol reduced with a diet rich in chickpea and other legumes (Gupta et al., 2017). The above changes were significantly comparative to a diet restricted with legumes (Gupta et al., 2017). The effects of chickpea on obesity have been further studied using rats for 8 months. Their study has included a fatty diet as control and control with 10% (w/w) chickpea. The results indicated a 35% increment in HDL whereas a 23% decrement in LDL with an overall 30% reduction in LDL/HDL ratio (Gupta et al., 2017). The efficacy of a chickpea-based diet on diabetes and obesity needs further investigation with extensive clinical studies for the long term. Few studies indicated that nutritional responses in pulses may have been due to its high levels of low digestible carbohydrates, proteins, micronutrients, and low in anti-nutrients such as phytic acid, amylase inhibitors and lectins (Thavarajah and Thavarajah, 2012; Gupta et al., 2017).

THE IMPACT OF FOOD PROCESSING ON FATTY ACIDS

The fatty acid composition of chickpeas is sensitive to food processing. Cooking can increase the fat content in both kabuli and desi varieties (Wang et al., 2010), but pressure cooking can reduce the levels of the four main fatty acids in chickpea flour (Rajni et al., 2012; **Table 3**). Furthermore, food processing affects the quality and quantity of chickpea EFAs, as unsaturated fatty acids are directly exposed to oxygen and other reactants leading to auto-oxidation (Damodaran and Parkin, 2017). In particular, PUFAs are highly susceptible to auto-oxidation because they have more double bonds, any one of which could react with oxygen radicals (Damodaran and Parkin, 2017). Alkyl radicals with a PUFA origin are the major reactants that initiate PUFA depletion. High-temperature conditions in food processing could further increase these food quality-degrading reactions. Heat can significantly decompose the radicals formed (hydroperoxyl radicals) and multiply PUFA depletion (Damodaran and Parkin, 2017). The alterations depicted in **Table 3** result from such chemical changes while cooking (Rajni et al., 2012; Damodaran and Parkin, 2017). The presence of certain minerals (especially Fe) and isoenzymes such as lipoxygenase in raw chickpea (Halliwell and Gutteridge, 1990; Sanz et al., 1992; Girotti, 1998; Damodaran and Parkin, 2017) may catalyze EFA depletion during storage. Lipoxygenase mainly contributes to depleting ALA and LA, initiating hydroperoxide formation (Damodaran and Parkin, 2017). As a result, storage conditions must inhibit lipoxygenase in the chickpea to preserve the food quality and enhance the shelf life. Another impact of auto-oxidation is forming volatile aldehydes and unsaturated by-products with rearranged double bonds (trans fats; Damodaran and Parkin, 2017). Trans fat formation from PUFAs could occur due to unsaturated double bond cleavage and rearranging during higher temperature food processing. Volatile compounds (aldehydes) formed due to storage and food processing deplete the quality and aroma, leading to rancidity (Damodaran and Parkin, 2017), while trans fats are detrimental to human health. However, no studies related to rancidity and trans fats originating from chickpea foods are available in the literature. Future studies are required to understand these fatty acid concentrations after processing, cooking, and storage.

FATTY ACID ANALYSIS

Fatty acid extraction procedures and analytical instrumentation are essential for the accurate quantification of EFAs. Fatty acid profiles are measured using gas chromatography (GC) paired with a flame ionization detector (FID) or a mass spectrometer (MS; Laakso and Hiltunen, 2009; Chiu and Kuo, 2020). The FID is a universal detector, which creates signals for organic molecules (due to C-H bond cleavage), but fails in molecular identification (qualitative analysis; Skoog et al., 2018). Accordingly, MS is the most superior detection method for qualitative and quantitative analysis of fatty acids by GC. The

TABLE 3 | Fatty acid composition of raw and processed chickpea (Rajni et al., 2012).

Treatment	Fatty acid (%)			
	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid
Raw seed	9.7	27.9	57.3	1.6
Boiling	10.8	33.4	51.3	trace
Pressure cooking	9.6	27.7	56.3	1.6
Roasting	10.1	28.2	50.1	1.2

advantage of a mass-based detector is the ability to run a selective ion monitoring (SIM) analysis for all analytes (Sleeman and Carter, 1997). The SIM mode enables quantification irrespective of two analytes having close retention times. FID detection requires tedious efforts in terms of temperature programming to obtain entirely resolved chromatograms with minimal errors. Therefore, MS with SIM is the most appropriate and convenient method for fatty acid quantification and identification (Sleeman and Carter, 1997; Dodds et al., 2005). However, a major drawback of GC–MS techniques is the analysis time, cost, and labor. A short analysis time with high throughput is ideal for collecting data to screen fatty acids in breeding populations before advancing to varietal development stages.

Fourier-transform infrared (FTIR) spectroscopy measures the infrared spectrum of absorption or emission of a solid, liquid, or gas (Sindhu et al., 2015) and is a suitable technique to reduce the analytical time, cost, and labor but preserve high throughput. FTIR data models validated with GC–MS methods are robust tools to quantify fatty acids for high-throughput plant breeding research (Gómez-Caravaca et al., 2013). Non-destructive sample preparation and the application of hand-held FTIR devices in the field will enhance future chickpea breeding to select for fatty acid-rich accessions without the need for an analytical laboratory.

The electromagnetic spectrum's IR region is less energetic compared to the ultraviolet (UV)-visible region. Consequently, IR energy induces molecular vibrations rather than electronic excitations. The midsection of the IR (mid-IR, MIR) region has the most fundamental resonant frequencies that cause distinct molecular vibrations (Skoog et al., 2018). Consequently, FTIR utilizes MIR energy to generate signals based on molecular vibrations for qualitative and quantitative analysis. The working window of FTIR is 500–4,000 cm^{-1}, wherein signals due to functional group vibrations occur mainly between 1,500 and 4,000 cm^{-1} (functional group region; Lohumi et al., 2015). For fatty acids (**Figure 3**), the C=O (carbonyl), C=C (unsaturated double bonds), and C-H bonds undergo distinct oscillations in the functional group region. Therefore, based on the signal intensities (C=C and C-H signal ratios), fatty acids can be characterized by the degree of saturation and chain length, followed by quantification (Meiklejohn et al., 1957; Rabelo et al., 2015). Carbonyl stretching (~1742–1750 cm^{-1}) is one of the most distinct signals and is strongly applicable to fatty acid quantification (Yang et al., 2005; Rabelo et al., 2015). FTIR is a powerful tool for fatty acid analysis. NIR (near-IR spectroscopy) is another common tool in plant breeding programs used in parallel with FTIR technology (Downey, 1999). NIR technology is also available with added Fourier-transformation technology (i.e., FTNIR; Skoog et al., 2018). The primary difference between FTNIR and FTIR techniques is the working window. The FTNIR range (4000–12,820 cm^{-1}) is beyond the MIR range employed in FTIR, and the molecular vibrations occur as overtones and combined bands in the NIR range (Yang et al., 2005; Lohumi et al., 2015; Rabelo et al., 2015). FTIR and FTNIR have both been used for total fat analysis in food and seed composition analysis, and each method has advantages and disadvantages. Generally, calibration models for total fat based on FTNIR are better than those based on FTIR (Yang et al., 2005; Oliveira et al., 2006); however, FTIR is more informative than FTNIR due to its well-resolved spectral signals and because it provides better qualitative insight (Lohumi et al., 2015). In addition to fat analysis, NIR spectroscopy has been used in routine seed composition analysis for moisture, protein, starch, kernel hardness, color, and seed viability (William and Norris, 2001; Kusumaningrum et al., 2018; Skoog et al., 2018). FTNIR spectroscopy fits well with quantitative measurements of compounds with functional groups containing C-H, N-H, and O-H bonds based on NIR vibrational overtones (Skoog et al., 2018). Furthermore, the qualitative identification of functional groups using NIR is not optimal due to low resolution (William and Norris, 2001). Overall, the FTIR technique is unique and accurate with good resolution as a high-throughput tool to measure individual nutritional trails with low concentrations.

BREEDING APPROACHES

Current chickpea breeding is mainly focused on grain yield, disease resistance, and nutritional quality traits, including protein, minerals, prebiotic carbohydrates, and environmental stresses (Wang et al., 2017; Vandemark et al., 2018, 2020). Seed yield can be positively or negatively correlated with chickpea agronomic traits. For example, Toker (2009) shows chickpea seed yield is positively correlated with biomass ($r = 0.975$), harvest index ($r = 0.935$), plant height ($r = 0.853$), number of branches ($r = 0.797$), and pods per plant ($r = 0.675$) but negatively correlated with seed weight ($r = -0.660$) and ascochyta blight infection ($r = -0.872$). Wang et al. (2017) show positive correlations between seed protein concentration, plant height, and days of maturity and negative correlations between seed protein concentration, grain yield, and seed size. The concentrations of minerals, including K, P, and Zn, in chickpea seeds are influenced by genotype, location, and genotype×location interaction (Vandemark et al., 2018). Chickpea prebiotic carbohydrate concentrations vary across location, year, and genotype (Vandemark et al., 2020). Chickpea grain yield is negatively correlated with several prebiotic carbohydrates, including verbose ($r = -0.80$), stachyose ($r = -0.77$), sorbitol ($r = -0.66$), and mannitol ($r = -0.65$; G. Vandemark et al., 2020). Overall, grain yield is negatively correlated with most nutritional traits, including protein content, certain prebiotic carbohydrates, and minerals (Vandemark et al., 2018, 2020).

Heat, drought, and cold stresses are the common abiotic stresses affecting chickpea production worldwide (Jha et al., 2014). Plant lipids are linked to increased cold and heat tolerance in food crops. Fats alleviate environmental stresses by changing their PUFA composition in chloroplast lipids (Nelson and Cox, 2008). Drought stress generally increases LA and decreases ALA concentrations in response to desaturase enzymes (Yordanov et al., 2000). Lipids, including phospholipids and glycolipids, help chickpea plants withstand cold stress during the winter (Vigh et al., 1998). Desaturation of fatty acids is positively correlated with preventing cell lysis at colder temperatures

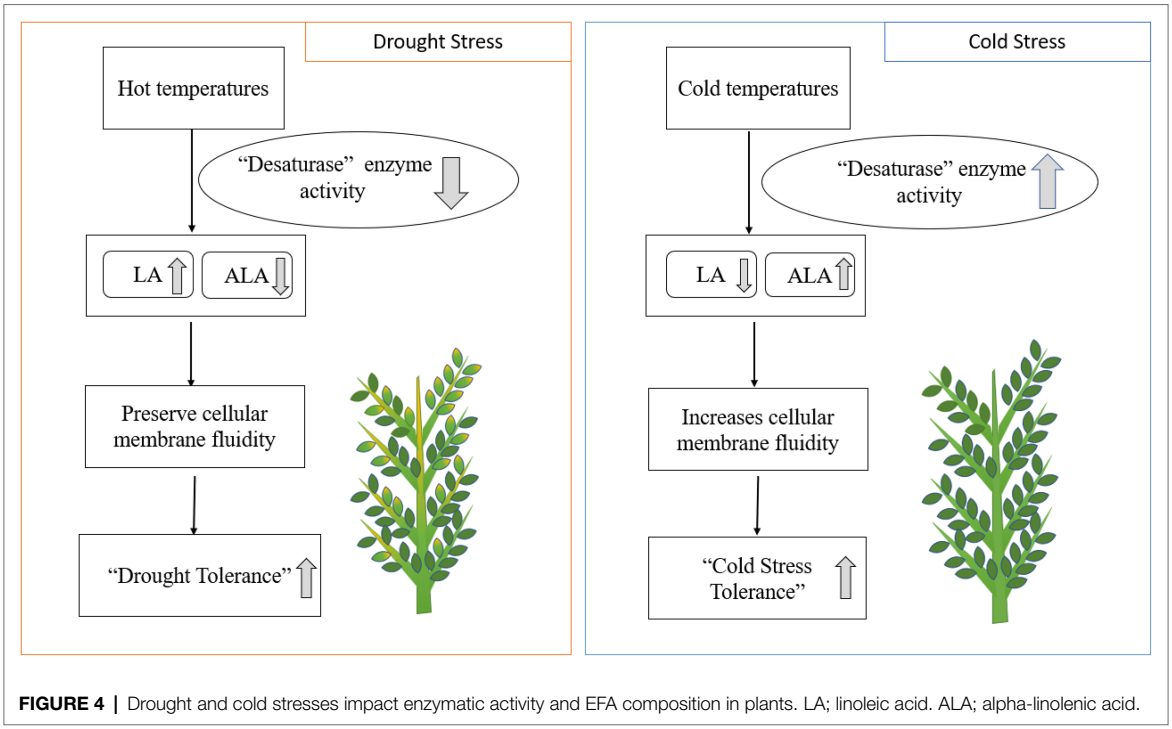

FIGURE 4 | Drought and cold stresses impact enzymatic activity and EFA composition in plants. LA; linoleic acid. ALA; alpha-linolenic acid.

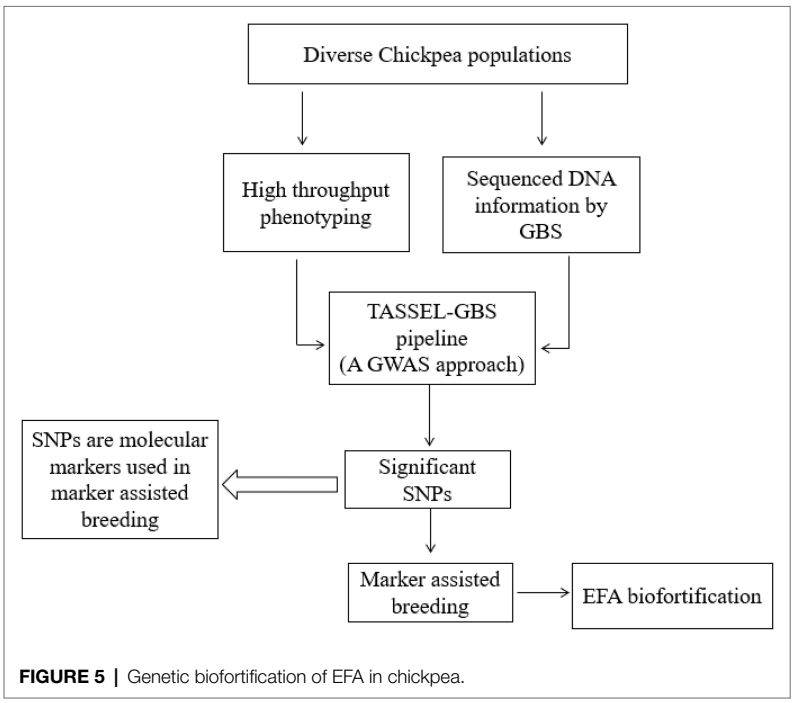

FIGURE 5 | Genetic biofortification of EFA in chickpea.

(Bakht et al., 2006; Shah et al., 2013). The increase in double bonds in PUFA chains contributes to plant cell membrane fluidity, increasing cold tolerance due to freezing point depression (Vigh et al., 1998). Increased ALA and reduced LA levels in chickpea leaves during cold stress indicate fatty acid desaturation at low temperatures (Bakht et al., 2006). Higher double bond indices (DBI) in extracted leaf fats at negative LT_{50} (lethal temperatures) values indicate higher levels of unsaturated fats at lower temperatures (a significant negative correlation, $r < 0$; Bakht et al., 2006; **Figure 4**). Genomic and gene-editing technology may enhance PUFA desaturation and accelerate breeding efforts to develop chickpea cultivars resistant to cold stress (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000; Bakht et al., 2006). PUFA-induced mutations in chickpea have revealed higher PUFA (LA) content leads to improved cold stress tolerance (Shah et al., 2013). Mutant desi genotypes (CM72/02

and CM137-01) and mutant genotypes of desi×kabuli introgression can also tolerate sustained cold stress (Shah et al., 2013).

Integrating traditional breeding and biotechnology approaches would benefit the development of chickpea cultivars resilient to climate change. Planting time and growing conditions also affect fatty acid composition in chickpea seeds, with OA and LA concentrations higher in chickpea planted in the fall than in the spring (Gül et al., 2008). Interactions between genotype and planting date can significantly affect the concentration of α and β tocopherols and palmitic acid, OA, and LA concentrations; ALA concentration is positively correlated with LA concentration and negatively correlated with OA and tocopherol concentrations (Gül et al., 2008). Nine candidate genes related to fats have been identified in soybean using quantitative trait loci (QTL) mapping (Nian and Cheng, 2020). A single gene associated with a lipid synthesizing and storage enzyme named diacylglycerol O-acyltransferase has also been identified in chickpea (Verma et al., 2015). Detailed QTL mapping studies on candidate genes associated with essential fatty acids in chickpea have not been reported.

CONCLUSION AND FUTURE PROSPECTS

Chickpea is a highly nutritious pulse crop rich in protein, prebiotic carbohydrates, fat, and a range of micronutrients. Chickpea is a rich source of EFAs, phytosterols, TAGs, and phospholipids. TAGs are the most dominant neutral lipid in chickpea. PUFAs, MUFAs, and SFAs are esterified within the lipids and bind to TAG's glycerol end or a phospholipid. The most dominant PUFA in chickpea is LA, followed by OA (MUFA) and ALA. LA is an ω-6 EFA, whereas ALA is an ω-3 EFA. The consumption of diets with an ω-6/ω-3 ratio of 4 to 5 is recommended for better human health. The ratio of EFAs from a chickpea diet and related human health benefits have yet to be studied using large clinical trials. EFA traits have not been extensively studied in chickpea breeding. Optimizing EFA levels in chickpea should be feasible by applying the genetic and transgenic approaches followed in chickpea biofortification for micronutrients. FTIR and FTNIR techniques should be incorporated into breeding programs to screen breeding populations; FTIR within the functional group region will assist qualitative and quantitative fatty acid analysis. Future genome-wide association studies are needed to develop marker-assisted breeding approaches for improving chickpea nutritional traits. Genome mapping studies could support the identification of corresponding QTLs and candidate genes associated with fatty acid biosynthesis (**Figure 5**). In general, chickpea produces LA (2.87 g/100 g) and ALA (0.112 g/100 g), but the cultivar information is not available (USDA, 2021). So far, human clinical studies have been published to confirm the impact of the prevailing chickpea EFA composition on human health. Percent recommended daily allowance (%RDA) for LA (ω-6 fatty acid) is not published; however, the %RDA of ALA (ω-3 fatty acid) for adult men and women is 1.6 and 1.1 g, respectively (Hjalmarsdottir, 2019). Future chickpea breeding strategies should address the safe, adequate increase of these essential fatty acids for human health. Future genomics and plant breeding advancements will also enhance chickpea's EFA concentrations and other nutritional traits and improve human health.

AUTHOR CONTRIBUTIONS

AU is a doctoral graduate student working on this project with DT; they created the hypothesis, objectives, outline the draft, and wrote the manuscript. LT, PT, WB, ES, and GV edited and added discipline-specific feedback. All authors contributed to the article and approved the submitted version.

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Conventional and Molecular Breeding Tools for Accelerating Genetic Gain in Faba Bean (*Vicia Faba* L.)

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Faba bean is a cool-season grain legume crop, which is grown worldwide for food and feed. Despite a decrease in area under faba bean in the past, the interest in growing faba bean is increasing globally due to its high seed protein content and its excellent ecological service. The crop is, however, exposed to diverse biotic and abiotic stresses causing unstable, low grain yield. Although, sources of resistance to main diseases, such as ascochyta blight (*Ascochyta fabae* Speg.), rust (*Uromyces viciae-fabae* (Pers.) Schroet.), chocolate spot (*Botrytis fabae* Sard.) and gall disease (*Physioderma viciae*), have been identified, their resistance is only partial and cannot prevent grain yield losses without agronomical practices. Tightly associated DNA markers for host plant resistance genes are needed to enhance the level of resistance. Less progress has been made for abiotic stresses. Different breeding methods are proposed, but until now line breeding, based on the pedigree method, is the dominant practice in breeding programs. Nonetheless, the low seed multiplication coefficient and the requirement for growing under insect-proof enclosures to avoid outcrossing hampers breeding, along with the lack of tools such as double haploid system and cytoplasmic male sterility. This reduces breeding population size and speed of breeding hence the chances of capturing rare combinations of favorable alleles. Availability and use of the DNA markers such as vicine-convicine (vc^-) and herbicide tolerance in breeding programs have encouraged breeders and given confidence in marker assisted selection. Closely linked QTL for several biotic and abiotic stress tolerance are available and their verification and conversion in breeder friendly platform will enhance the selection process. Recently, genomic selection and speed breeding techniques together with genomics have come within reach to accelerate the genetic gains in faba bean. Advancements in genomic resources with other breeding tools, methods and platforms will enable to accelerate the breeding process for enhancing genetic gain in this species.

Keywords: *Vicia faba*, conventional breeding, synthetic cultivars, marker-assisted selection, genomic selection, biotic and abiotic stresses

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INTRODUCTION

Faba bean (*Vicia faba* L.) is a cool-season grain legume cultivated throughout the world for human consumption and animal feed. Its high protein content (25–37%) (Duc et al., 1999; Warsame et al., 2020) makes it a highly valuable grain for both food and feed purposes. Among pulses it occupies sixth place in terms of production after common bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L.), cowpea [*Vigna unguiculata* (L.) Walp], and lentil (*Lens culinaris* Medik.) (FAOSTAT, 2021). Faba bean fits well in cereal-based cropping systems as a rotational crop that enhances soil fertility while breaking the cycle of biotic stresses associated with parasitic weeds and other pathogens. Its nitrogen fixation capacity is one of the highest among legumes, fixing nitrogen even in the presence of high levels of nitrogen in the soil (Herridge et al., 2008) and leaving a significant residue that reduces the need for application of inorganic *N* fertilizer in subsequent crops (Hauggaard-Nielsen et al., 2009).

No extant wild relative capable of producing fertile progeny when crossed with *V. faba* has been found and thus genetic diversity available for breeding purposes is limited to the cultivated genepool (Cubero, 1974; Duc et al., 2010). Recent archaeological findings suggest pre-domestication ancient form of faba bean existed about 14,000 years ago in el-Wad (Mount Carmel, Israel) (Caracuta et al., 2016). Closely related *Vicia* species such as *V. narbonensis* L., *V. galilea* L, and *V. johannis* L. have different numbers of chromosomes (Raina and Rees, 1983), protein profiles compared to *V. faba* (Ladizinsky, 1975), their DNA content is about half of that found in faba bean (Chooi, 1971), and they cannot be crossed with faba bean (Cubero, 1973; Maxted et al., 1991; Duc et al., 2010). Hybrid inter specific embryos were observed between *V. faba* and *V. narbonensis*, and *V. faba* and *V. johannis*, but probably post zygotic barriers stopped their further development and no viable seed could be formed (Ramsay and Pickersgill, 1986; Zenkteler et al., 1998; Wijaya, 2003). Although both *V. johannis* and *V. narbonensis* have resistance to aphids (*Aphis fabae* Scop) and chocolate spot (*Botrytis fabae* Sard), and tolerance to frost (Birch et al., 1985), these traits cannot be transferred to faba bean because of crossing barriers.

However, there is a high degree of genetic diversity in the current gene pool (Duc et al., 2010). Despite having a close genetic similarity within eco-geographical regions, differences exist across wide geographical regions. For example, accessions from China are markedly different from African, European and Asian counterparts (Wang H. et al., 2012). Within China, the spring sown faba beans were different from the winter sown types and they also differed from other Asian accessions which were close to the African and European accessions (Zong et al., 2009). However, Zong et al. (2010) later found the Chinese spring accessions resembled more to the African and European accessions than the Chinese winter types. Although four types of faba bean—*major*, *minor*, *equina* and *paucijuga* were described earlier, there is no reproductive barrier among them. The *paucijuga* type is considered as the most primitive extant faba bean lineage (Cubero, 1973; Cubero and Suso, 1981).

The primary center of origin of faba bean is likely to be the Mediterranean basin from where it spread to the Nile Valley, Central and Eastern Asia, and much later South America (Cubero, 1973; Duc et al., 2010). Faba bean is grown as a staple food crop in low rainfall areas in northern Africa (Morocco, Algeria and Tunisia), high rainfall areas in Ethiopia and Eritrea and under irrigation in Sudan and Egypt (north-east Africa) and in the highlands of the South American Andes. These are traditionally faba bean growing areas, but the farming systems have now shifted to monoculture of cereals, resulting in poor soil health, land degradation, increasing greenhouse gases through the use of chemical fertilizer, emergence of new pests and diseases, declining response to farm inputs and yield stagnation (World Bank Group, 2019). As a result, faba bean production in these countries has declined leading to increased cereal mono-cropping and these countries have become importers of faba bean (FAOSTAT, 2021). For example, Egypt, Morocco and Sudan imported faba bean worth US$343 million in 2019 from Australia and Canada (FAOSTAT, 2021). The import in west Asia is about US$69 million.

The global area under faba bean cultivation has almost halved from 5.4 million ha in 1961 to 2.6 million ha in 2019, but its productivity has increased from 0.9 t/ha to 2.1 t/ha in the corresponding years (FAOSTAT, 2021). Reasons for this decline are complex, but include competition from soybean, abandonment in areas where parasitic weeds have become endemic, and yield instability, as the crop is sensitive to many biotic and abiotic stresses. Although significant progress has been made in tackling biotic stresses, research on overcoming abiotic stresses is limited. To date reportedly <50% of potential yield has been achieved in faba bean (Duc, 1997; Mulugeta et al., 2019).

Genomic research in faba bean lags behind other major grain legumes, hampered by its gigantic genome size of 13.1 Gb and lack of investment in underpinning research compared to other crops. Pulses may be considered as research-neglected orphan crops and amongst pulses, faba bean has received comparatively less attention compared to pea, common bean, chickpea, lentil or even cowpea; it is an orphan of the orphans. There is a need to tap on the discoveries made in genetics and genomics research to make significant improvement of this crop. The main breeding objectives are acceleration of genetic gain for improving yield, quality traits and host plant resistance to insect pests and diseases. Traditionally, phenotypic selection has been the major contributor to genetic progress, but with the current advancement of DNA marker technology, phenotypic selection has been enhanced with the use of with marker assisted selection (MAS). DNA markers tightly linked to traits with major effect and high heritability have been discovered so far. However, often traits such as yield are complex and affected by environmental factors and by many other traits, making MAS less effective. Genomic selection based on prediction of breeding values, as a natural extension of MAS, may become available for faba bean as more high throughput dense markers and DNA data sets come available. A recent review by Khazaei et al. (2021) documents the current availability of genomic resources for faba bean. Liu et al. (2019) reported that the efficiency of genomic selection was markedly higher than phenotypic selection and the efficiency

would increase further if genomic selection was accompanied by speed breeding.

Gene editing is developing as a new breeding approach in many crops, thus, assisting to accelerate genetic gains. No CRISPR/Cas9 system has been reported for faba bean yet. The absence of an annotated reference genome for this crop poses challenges for the application of CRISPR/Cas gene editing, particularly with the design of specific gRNA-targeted genes of interest (Bhowmik et al., 2021). However, with recent gene discoveries for quality traits such as vicine-convicine (v-c) (Björnsdotter et al., 2021) and seed coat tannins (Gutierrez and Torres, 2019; Gutierrez et al., 2020), this technology may be used to advance our understanding of gene function and accelerate development of new cultivars with reduced anti-nutritional factors. The aim of this review is to highlight species-specific advantages, restrictions and limitations, progress made to date in applied genetics and crossbreeding and what needs to be done to markedly accelerate genetic gains in faba bean.

FABA BEAN KEY BREEDING OBJECTIVES

Breeding for Resistance to Foliar Diseases

Ascochyta blight (*Ascochyta fabae* Speg.), chocolate spot (*Botrytis fabae* Sard.) and rust [*Uromyces viciae-fabae* (Pers.) Schroet.] are major fungal diseases of faba bean and sources of genetic resistance are available in germplasm collections. These diseases can lower the grain yield by 35 to 90% (Hampton, 1980; Díaz-Ruiz et al., 2009) and 50 to 90% (Gorfu and Yaynu, 2001; Beyene et al., 2016), 30 to 68% (Rashid and Bernier, 1991; Marcellos et al., 1995), respectively. Resistance to these diseases was identified in International Center for Agriculture in the Dry Areas (ICARDA) in the 1980s (Hanounik and Robertson, 1988, 1989) and these genetic resources were later used in faba bean breeding programs globally (Sillero et al., 2010; Temesgen et al., 2015; Adhikari et al., 2016; Maalouf et al., 2016). Despite their use in breeding programs, there is limited understanding of their host-pathogen interactions. A high level of resistance to these diseases has not been found in the germplasm and the resistance is multigenic.

Ascochyta blight is a devastating disease in many countries including Europe, Canada, the Middle East and Oceania (Sudheesh et al., 2019). Sources of resistance to the disease have been reported, but the mode of resistance seems to be complex (Kohpina et al., 2000; Román et al., 2003; Díaz-Ruiz et al., 2009). For example, Kohpina et al. (2000) reported a major dominant gene for resistance in ILB 752, but minor genes in NEB 463. Modern varieties in Australia, such as Farah, Nura, PBA Rana, PBA Samira and PBA Amberley are moderately resistant. In Australia, two pathotypes of the fungus have been identified causing more concern to the breeders. With the availability of closely linked molecular markers (Avila et al., 2004; Kaur et al., 2014; Atienza et al., 2016; Sudheesh et al., 2019), it should be possible to pyramid multiple QTLs and enhance the level of resistance.

Chocolate spot is a serious disease which can cause up to 90% yield losses (Gorfu and Yaynu, 2001; Beyene et al., 2018) in favorable conditions because of its ability to grow on dead tissues in a short latent period (1–3 days). The disease can appear at any growth stage when the environmental conditions are conducive, but if it appears in flowering time, it can cause a complete crop failure. Some resistant lines were reported earlier (Bouhassan et al., 2004), and more lines have been found recently from ICARDA source (Beyene et al., 2018). Many varieties have been developed using ICARDA source of resistance in Ethiopia, Egypt, Sudan, Tunisia, China, Mexico and Australia. For example, Ethiopian researchers have released several high-yielding varieties with partial resistant to chocolate spot, such as Moti, Gebelcho, Gora, Obsie and Walki (Maalouf et al., 2019). Similarly, Australia has released Icarus and PBA Amberley as moderately resistant varieties (Pulse Australia, 2021). Although resistant varieties to the disease have been developed in Australia and Ethiopia, no information is available on whether Australian varieties will be effective against chocolate spot if they were grown in Ethiopia and vice versa.

A moderate level of resistance is available in the germplasm (Bouhassan et al., 2004; Beyene et al., 2018; Khazaei et al., 2018a; Maalouf et al., 2019), but to date no reliable DNA markers have been reported for this disease. Without availability of such markers, chocolate spot has been the most difficult disease to breed mostly because screening for the disease in field condition is unreliable. Faba bean is mostly grown in dry environments as a rain fed crop and the opportunity for its screening can occur only in humid conditions which occur seldom. Therefore, to date, improvement in host plant resistance to this disease has been very slow. An average reduction of 0.27% infection per year over 30 years was reported in Ethiopia (Tolessa et al., 2015) suggesting a coordinated and concentrated effort is needed for breeding resistance to this disease.

Rust is another important disease of faba bean causing 30–68% yield losses (Rashid and Bernier, 1991; Marcellos et al., 1995). Several sources of resistance to rust have been reported (e.g., Rashid and Bernier, 1986, 1991; Avila et al., 2003; Adhikari et al., 2016) but the level of resistance is only partial. At least three resistance genes, *Uvf1*, *Uvf2* and *Uvf3* have been identified (Avila et al., 2003; Adhikari et al., 2016; Ijaz et al., 2021b). *Uvf2*, located on chromosome III and *Uvf3* on chromosome V, are dominant and independent genes, but their relationship with *Uvf1* is not yet known (Ijaz et al., 2021b). Both genes can be tagged using KASP markers for marker-assisted selection. This should allow pyramiding of genes for enhancing the level of resistance.

Faba bean gall caused by *Physioderma viciae* (You et al., 2021) is a relatively new disease that is currently widespread in Ethiopia. It is not caused by *Olpidium viciae* Kusano as suggested earlier. Recent findings in Ethiopia indicate the availability of partial resistance in existing faba bean germplasm (Yitayih and Azmeraw, 2017; Wondwosen et al., 2019; Kassa et al., 2020). Molecular based research such as identifying quantitative trait loci (QTL) for host plant resistance has not yet been undertaken for faba bean gall.

Host plant resistance breeding is further complicated by the presence of pathogen variability in all three of the major diseases; rust (Herath, 1997; Ijaz et al., 2021a), chocolate spot (Hanounik and Maliha, 1986; Gorfu, 1996), and ascochyta blight (Stoddard et al., 1999). Despite having a moderate level of resistance in selected germplasm, little effort has gone into developing

reliable DNA markers that can be used by breeders as part of selection strategies.

Viral Diseases

The most prevalent of the many viral diseases than can affect faba bean are Broad bean mottle virus (BBMV), Broad bean stain virus (BBSV), Bean leaf roll virus (BLRV), Bean yellow mosaic virus (BYMV), Alfalfa mosaic virus (AMV), Faba bean necrotic yellows virus (FBNYV) and True broad bean mosaic virus (TBBMV) (Saxena, 1991; Bond et al., 1994; van Leur et al., 2006). Most viral diseases are not host specific and can infect various plant species, thereby allowing them to survive and multiply easily by-passing from one species to another throughout the seasons.

The typical mode of virus disease transmission is through seed and/or insect vectors such as aphids. Non-persistently transmitted viruses, such as BYMV and Pea seed borne mosaic virus (PsbMV) can be transmitted through seed, however, the seed transmission in faba bean is almost negligible in Australia (van Leur et al., 2006). Once a plant is infected by a virus, it cannot be cured, so prevention is the only strategy for controlling these diseases. The major virus affecting faba bean production in North Africa and West Asia is FBNYV which causes up to 90% yield loss in Egypt (Kumari and Makkouk, 2007). In Australia, where virus diseases cause sporadic losses, BYMV, BLRV and PsbMV are the most prevalent viruses. In 2020, however, BYMV caused up to 70% grain yield losses in northern New South Wales (van Leur, pers. comm.), thereby making it a larger risk than fungal diseases that can be controlled by fungicides. Newer cultivars, such as "PBA Nasma" and "PBA Nanu" in Australia are resistant to BLRV, but no effective resistance has been found for BYMV yet.

Parasitic Weeds

Orobanche and *Phelipanche* (broomrapes) species are root-parasitic plants that can devastate faba bean crops in Mediterranean Europe, West Asia and North Africa, thus resulting a drastic reduction in the crop area (Gressel et al., 2004; Khalil et al., 2004; Maalouf et al., 2011; Rubiales et al., 2016). These parasitic weeds lack chlorophyll and functional roots, and are completely dependent on the host plant. Among the several broomrape species that can infect faba bean, *Orobanche crenata* Forsk. and *O. foetida* Poir. are the most damaging and widespread weeds (Pérez-de-Luque et al., 2010; Rubiales and Fernández-Aparicio, 2012; Rubiales et al., 2014). These weeds are continuing to expand their ecological range to Ethiopia and Sudan forcing farmers to abandon faba bean cultivation in many parts of the country (Abebe et al., 2013).

However, there are some encouraging results with the finding of genotypes resistant to *Orobanche* (Cubero, 1994; Maalouf et al., 2011; Rubiales et al., 2014). The first significant finding of resistance was the identification of the family 402 derived from the cross Rebaya 40/F216 made at ICARDA (Cubero, 1994). Since then, different elite lines with large seed and Orobanche resistance were developed from ICARDA leading to the release of Orobanche resistant cultivars, such as Giza 402, Cairo 843, Misr1, and Misr3 in Egypt, Najah and Chourouk in Tunisia and Ashengie in Ethiopia (Maalouf et al., 2016, 2018). Similarly, Rubiales et al. (2014) found several accessions—V-1268, V-1302, V-1301, V-268, V-231, V-319 and V-1272 along with cultivar Baraca with resistance to both to *O. crenata* and *O. foetida* across many locations. In addition to *Orobanche*, another stem parasitic weed, dodder (*Cuscuta species*) is starting to become a serious problem in faba bean and other legumes in West Asia and North Africa. Although none of the above lines have complete resistance to *Orobanche*, they will certainly help to reduce the burden of parasitic weeds in faba bean. Use of partially resistant cultivars with the application of one to two sub lethal dose of glyphosate at flowering stage is the most practical method to reduce yield loss and weed seed bank over time (Zahran et al., 1980; Rubiales and Fernández-Aparicio, 2012).

Breeding for Abiotic Stresses

Heat, drought, frost and water logging are major abiotic stresses affecting the faba bean productivity. A comprehensive review on abiotic tolerance for grain legumes has been presented by Toker and Mutlu (2011). While the first two factors affect the crop globally, the latter two are ecologically and geographically specific to local environments. Waterlogging is a problem in limited geographical areas such as the high rainfall regions that are dominated by vertisols in Ethiopia, and the irrigated Nile River basin in Egypt. The effect of frost on vegetative growth has been widely studied in Europe (Arbaoui et al., 2008; Link et al., 2010; Sallam et al., 2015). Research on the effect of frost on the reproductive structures is not reported, as the crop in that region flowers when frost does not occur. However, this could be a future problem as the crop expands to northern latitude in continental climates. Furthermore, tolerance to seedling frost is not related to frost after flowering. Link et al. (2010) identified several accessions with superior frost tolerance, such as Côte d' Or, Hiverna, ILB3187, ILB2999, ILB14, ILB345. Promising winter hardy and frost tolerant faba beans might be found in the Hindu Kush area because materials from these regions are adapted to frost early in the vegetation phase (Olszewski, 1996). Inci and Toker (2011) found three accessions of faba bean from Turkey with freezing tolerance (**Supplementary Table 1**) and they also noted that wild relatives of faba bean, *viz. V. narbonensis* L. and *V. montbretii* Fisch. et C. A. Mey were more frost tolerant than faba bean. No comparative studies are conducted on frost damage in Australia, but in severe cases the losses can be as high as 60% (Maqbool et al., 2010). Frost is not a severe problem in southern Australia as the crop is acclimatized and hardened due to the much colder environment during the vegetative stage. It is, however, a problem in northern New South Wales and southern Queensland where the daytime temperatures are relatively warm, and night frost occurs, exposing plants to sudden sub-zero temperatures (Maqbool et al., 2010; Alharbi and Adhikari, 2020). Several frost tolerant genotypes at the vegetative stage, such as 11NF010a-2, PBA Warda, PBA Nasma and PBA Nanu were identified in Australia (Alharbi, 2018).

Heat stress in faba bean during the vegetative period can retard plant growth and development, but it is particularly harmful at the reproductive stage, causing reduction in pollen growth and viability that results in significant yield loss (Bishop

et al., 2016a). Extreme heat is the major threat to faba bean production in southern Egypt, Sudan, the Ethiopian lowlands, and in northern New South Wales and southern Queensland in Australia. Research conducted under high temperatures (above 35°C) have identified some heat tolerant genotypes, but wider testing for confirmation is still needed (Maalouf et al., 2019).

Low rainfall and variable soil moisture in dry areas are the major reasons for low and unstable grain yield of faba bean, especially where the crop is grown under rain fed conditions in the Mediterranean basin, East and North Africa, West Asia and Australia (Siddique et al., 2001). Drought reduces pollen viability and germination, but recent findings showed the female reproductive tissue was more sensitive to drought than the male part (Muktadir et al., 2021). Development of early maturing cultivars has been a breeding strategy to escape terminal drought. Although genotypic variation for drought tolerance has been documented (Abdelmula et al., 1999; Amede et al., 1999; Link et al., 1999; Khazaei et al., 2013; Muktadir et al., 2021), underlying mechanisms and selection methods for screening germplasm are not fully developed, thereby slowing the progress toward drought-tolerant cultivars. However, some characteristics, such as leaf-level carbon isotope discrimination, stomatal conductance and canopy temperature can be used as selection criteria for drought tolerance in faba bean and can be used for screening large sets of germplasm for drought tolerance under field trials (Khan et al., 2007; Muktadir, 2009).

Soil acidity is among the common problems limiting faba bean production in Ethiopia (Asefa et al., 2010; Jida and Assefa, 2014). It is associated with toxicities of hydrogen ion, aluminum, and manganese, and deficiency of calcium, molybdenum and phosphorus in the soil (Chen et al., 1993). Soil acidity also adversely affects survival, growth and nitrogen-fixation efficiency of Rhizobia (Graham, 1992; Zahran, 1999). Generally, *Rhizobium* strains vary markedly in their acid tolerance and ability to modulate on acid soils and some acid tolerant Rhizobia strains have been identified (Chen et al., 1993; Del Papa et al., 1999; Asefa et al., 2010; Jida and Assefa, 2014), but a higher acid tolerance of the bacteria does not mean a better symbiotic performance under acidic conditions. Therefore, both acid tolerance and symbiotic effectiveness are needed to improve nitrogen fixation, but these traits are not necessarily linked (Howieson et al., 1988). Belachew and Stoddard (2017) identified certain faba bean accessions, mostly from Ethiopia that were tolerant to acid soils (**Supplementary Table 1**) while Jida and Assefa (2014) identified certain Rhizobia tolerant to highly acidic soils in Ethiopia indicating evolutionary co-existence of symbiosis in faba bean under acid soil. Soil salinity is another significant problem facing agricultural production mostly in semi-arid agriculture systems causing 12-50% yield loss (Farooq et al., 2017). Genotypic variation for salinity tolerance exists in faba bean (Tavakkoli et al., 2012) and several QTL for ionic concentrations for sodium, potassium and chlorine were described by Asif and Paull (2021) for the first time, but these are yet to be verified.

In summary, there is evidence of availability of genetic resources for heat, drought frost, acidic soil and salinity tolerance and there are efficient methods for phenotypically screening against these traits (Stoddard et al., 2006; Zhou et al., 2018). However, there is a lack of efficient genetic methods of integrating these resources into effective breeding programs for developing cultivars with tolerance to these stresses. Major sources for resistance to biotic and abiotic stresses in faba bean are presented in **Supplementary Table 1**.

FABA BEAN BREEDING METHODS

The principal methods used in the development of virtually all modern faba bean cultivars include mass selection, sophisticated methods of recurrent selection, and conventional methods of cultivar development such as phenotype-based pedigree selection, single-seed descent line breeding, or development of synthetic cultivars (**Figure 1**). For these methods, parental lines are chosen on the basis of their pedigree and their phenotypic attributes.

Faba bean is a partially allogamous species, and in principle, is amenable to line breeding, population breeding, and hybrid breeding. The degree of outcrossing of faba bean varies widely among genotypes (10–70%) and is highly affected by the environment (Link, 1990; Ederer and Link, 1993; Suso et al., 1999, 2001; Gasim et al., 2004), including the degree of heat stress (Bishop et al., 2016b), which is one of the major challenges of breeding the crop. Outcrossing is a poorly heritable character and is markedly influenced by the inbreeding coefficient of a genotype (Link, 1990; Brünjes and Link, 2021) and by the type of insect pollinators. Moreover, faba bean genotypes markedly differ in their ability to spontaneously self-fertilize, spontaneous meaning without access of their pollinators, which are primarily honeybees (*Apis mellifera*) and bumblebees (*Bombus hortorum* and other *Bombus* spp.) (Link, 1990; Torres et al., 1993; Bishop et al., 2020). Thus, a calamity occurs if breeders multiply pure lines under insect-proof enclosures. These genotypes fully depend upon insect pollinators and do not set seed in their absence without mechanical tripping of flowers. Auto fertility is defined as the ability of a plant to self-fertilize and hence set seed without being tripped (Drayner, 1956; Torres et al., 1993). Genetic variability for auto fertility has been reported in faba bean, and higher levels of auto fertility were reported in F1 hybrids than in inbred lines (Link, 1990; Bishop et al., 2020). Australian bred cultivars are highly auto fertile as their original genetic resources trace back to ICARDA germplasm that were mostly auto fertile. Furthermore, they are initially grown and selected under insect-proof enclosures. Depending on the level of outcrossing, faba bean breeding may be performed under conditions of controlled selfing in insect-proof cages or, with less control and in case of little outcrossing, based on developing lines under open field conditions (Gharzeddin et al., 2019).

Hybrid vigor (heterosis) is pronounced in faba bean. Heterozygous F1-hybrids exceed the yield of their homozygous parents by 40 to 70% (Zeid et al., 2004; Dieckmann and Link, 2010). Early approaches to hybrid breeding using cytoplasmic male sterility (CMS) system in faba bean trace back to David Bond in Cambridge, UK in 1957 and Pierre Berthelem in Rennes, France in 1967 (Pfeiffer et al., 1993; Link et al., 1997). To date, hybrid breeding is not established in this crop due to

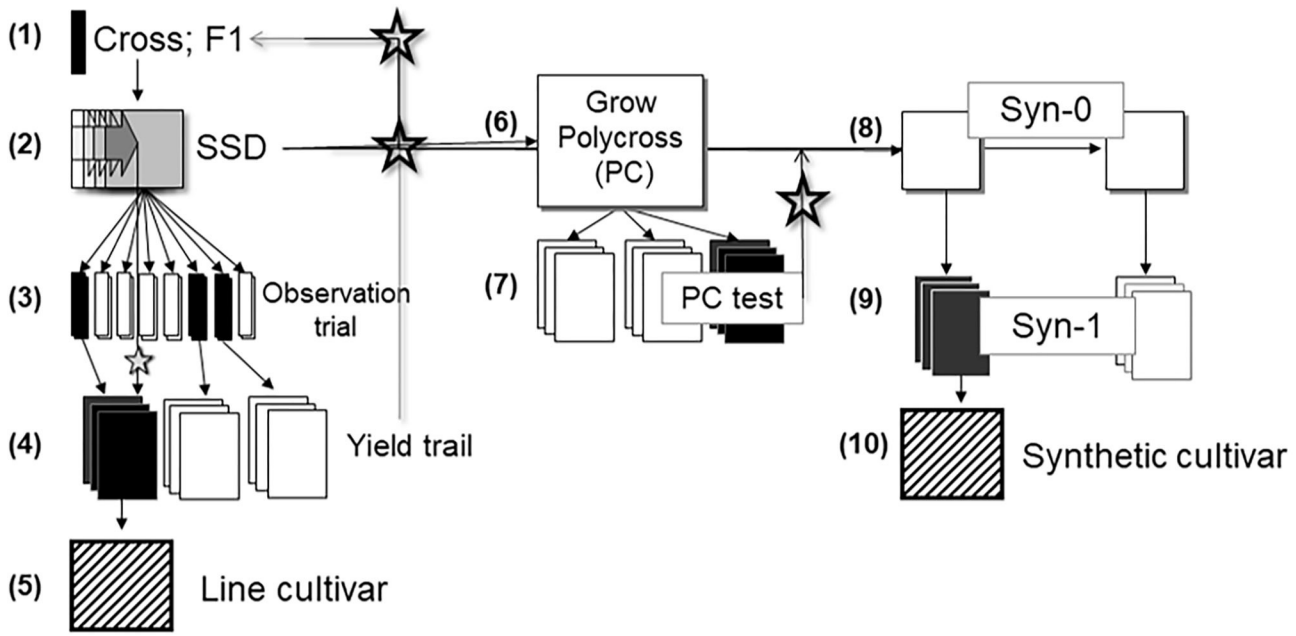

FIGURE 1 | Phenotypic-based breeding scheme in faba bean. (1) Making crosses, (2) Run SSD (single seed descent) until about F7, (3) Multiply SSD F8 individuals in open field and apply phenotypic selection on *per se* performance, (4) Test open pollinated offspring in replicated yield trials and identify best SSD lines as new crossing parental lines, (5) Identify and multiply the best SSD line(s) and release as a line cultivar or take the best lines as parents for a polycross, (6) Grow a polycross from selected SSD lines, (7) Run yield trial (PC test) and identify offspring of best SSD lines, (8) Grow best-predicted Syn-0 from best SSD lines, (9) Grow and test Syn-1, (10) Identify and multiply best synthetic cultivars. Star symbol indicates that information stems from field trials, seed from maintenance of SSD lines is taken for new crosses, for polycross and for Syn-0, respectively.

instability of the available CMS systems. An alternative to hybrid breeding is developing synthetic cultivars (i.e., a population breeding method). Breeding line cultivars and synthetic cultivars (or composite cultivars) are widely used products for faba bean improvement.

Hybrid Breeding

There are three major obstacles to faba bean hybrid breeding. First, an operational CMS system must be available. The available CMS systems in faba bean have shown an insufficient level of male sterility. The pollen sterility is not very "deep" and not reliable, and frequent, spontaneous reversion to fertility occur (Link et al., 1997; Maalouf et al., 2019). The second obstacle is the availability of appropriate pollinators. Pollinators often "steal" nectar, removing it without placing pollen on the stigma of the flowers (Marzinzig et al., 2018). Pollinator insects, if gathering pollen, learn to avoid the pollen-free mother plants, thus limiting seed set in hybrid seed production (Bond, 1989a,b; Duc, 1997; Marzinzig et al., 2018; Brunet et al., 2019). Lastly, the low propagation coefficient of faba bean (Link and Stützel, 1995) requires three or more generations of seed increase from the manually tended, single row level of propagation to reach the seed quantity required for certified seed production. Thus, all operational steps and tools for hybrid seed production, including the reliability of the CMS system, must be flawlessly conducted to be successful.

Given the lack of CMS and gametocides, breeders have employed monogenic traits to support or realize hybrid seed production. These traits include recessive and dominant nuclear-genetic pollen sterility (Duc et al., 1985), testa color, hilum color, cotyledon color, flower color, and other plant morphological characteristics as markers. In addition, even seed size was used, although this is a multigenic trait (Link, 2009). Breeders have shown high levels of creativity over the decades for faba bean hybrid breeding but a breakthrough has yet to occur (Bond et al., 1966; Berthelem and Le Guen, 1967, 1974; Berthelem, 1970; Bond, 1989a,b; Link et al., 1994; Link, 1998; Duc and Stoddard, 2018).

Line Breeding

Cultivars released via line breeding undergo strict selection intensity. One (the best) single line is developed into a cultivar, compared to two lines required to produce a hybrid, or more than three lines to develop a synthetic cultivar. Line cultivars can be developed faster than synthetic cultivars, since the latter need lines as components. Line cultivars are more widely accepted based on rules of distinctiveness, uniformity, and stability than synthetic cultivars. The single seed descent (SSD) procedure as proposed in **Figure 1** may be substituted by using F_2-derived lines (i.e., partial bulks) at F_3 or advanced generations to test, select and hence release new cultivars, however, such cultivars can be relatively heterogeneous. This approach is focused on short breeding cycles and relies on high seed multiplication rates.

Oftentimes, F_5-derived or F_6-derived lines are chosen as the basis for a new line cultivar and its maintenance breeding. Whichever approach is used for making lines, there is a need to multiply them under controlled self-fertilization or isolation: whether it is to initiate the multiplication as a line cultivar or to enter into the polycross or into a Syn-0 (**Figure 1**). The observation trial may be, focusing on synthetic cultivar breeding (see below), arranged as a soft version of a polycross (Ederer and Link, 1992a,b), with several rather than one plant per label and hence a reduced number of replicates; or, as top cross, with few replicates, and with half or less of the area devoted to a joint, constant pollinator genotype.

The low propagation coefficient of faba bean limits the breeding and especially the yield testing, compared to other crops. For example, one faba bean plant produces just enough seed for planting a 1 m^2 of a field plot (about 20–40 seeds). Hence, one individual plant of an SSD pipeline, after controlled self-fertilization, gives just enough seed for a small observation trial; harvesting that plot would give enough seed for a test in about 20 to 40 m^2 plot. This could be a trial in one location with two replicates; or two locations with one replicate including some checks in an augmented *p*-rep design (Moehring et al., 2013). A further point in conventional faba bean breeding is to optimize choice of parents. Focusing on line cultivars, basically half of the genetic variance of the breeding germplasm resides between the lines that result from the crosses that—potentially—can be made. The other half of the genetic variance lingers between these crosses, highlighting the importance of choice of parents (Falconer and Mackay, 1996) and therefore, it is of high importance to eliminate entire crosses that promise poorer performing lines than other crosses before investing too much in testing their offspring. Even if several crosses could be rejected as late as the observation trials in step 3 (**Figure 1**), then more plots of the more promising crosses could be tested in step 4. If, instead of SSD as in **Figure 1**, a pedigree-type of selection is installed, then as early as in generation F3 and F4, an observation trial or yield trial would allow to judge the yield potential of the entire cross (i.e., the entire family) and, as described above, eliminate inferior crosses (families). A corresponding marker investment should be made to select between crosses, before making them and after having made them.

Synthetic Breeding

In synthetic breeding, usually four or more founder lines serve as components of a cultivar. The cultivar can, hence, be "re-synthesized" from these lines at any time. Selecting these inbred lines based on their *per se* performance (**Figure 1**, step 3) is probably suboptimal because the synthetic population will not be as highly inbred as its components. The purpose of choosing synthetic breeding instead of line breeding is indeed to exploit heterosis as much as possible for agronomic performance, i.e., to decrease inbreeding (Ghaouti et al., 2008; Ghaouti and Link, 2009). Inbred lines as synthetic components should hence be selected mainly for their so-called varietal ability. This parameter is associated with general combining ability and breeding value (Cockerham and Weir, 1984; Ederer and Link, 1993). The varietal ability of a candidate line is approximately realized by the performance of its polycross-progeny or top-cross progeny (Maalouf et al., 1999). To assess, in addition to this, the line's *per se* performance, and even more to assess their degree of cross-fertilization and their paternal success, this all might allow to better predict the performance of the potential synthetics. Yet these additional parameters might not be worthwhile; assessing them might not be the optimum allocation of breeders' budget (Edere and Link, 1992; Ederer and Link, 1993; Brünjes and Link, 2021). *Per se* yield of lines and yield of their polycross progenies were correlated and had a genetic correlation coefficient (r_G) = 0.51 (Fleck and Ruckenbauer, 1989). Ghaouti et al. (2008) reported a slightly higher correlation ($r_G = 0.63$) between inbred lines and their polycross progenies for yield performance. Due to this, and because only slight corrections can be realized if *per se* performance is available in addition to polycross-progeny data, yield-testing of the lines may not be adequate (Ederer and Link, 1993). Yield testing of the lines themselves is expensive, since seed has to be multiplied under conditions of controlled selfing, whereas seed production for yield testing of progenies (from polycross or from top cross) is cheaper.

The terms polycross and top cross need not be taken literally. First, both options may serve a very similar purpose. A top cross may be easier to be conducted than a polycross. The pollinator (tester) of a top cross might be sown in strips, such as with 50% or less of the field area, the candidates would mainly cross-pollinate with the common pollinator, according to their individual extent of outcrossing.

The discrepancy between the line's *per se* performance and the performance of their open pollinated offspring (polycross-progenies; top cross-progenies) is caused by several reasons (beyond the deviations of randomness in the outcrossing that may come from compromises in the experimental and field lay-out; from pollinator behavior; from differences in paternal outcrossing success of the lines; Brünjes and Link, 2021). The major source of this discrepancy is General combining ability, which is a component of the polycross/top cross progenies' performance, and this is line-specific. The same applies for the degree of self-fertilization of the candidate lines and the paternal outcrossing success. Consequently, the level of inbreeding and actual composition of the progenies is line-specific. Yet, and moreover, the seed for inbred lines is produced in cages, seed for open-pollinated progenies is produced in open field, hence in different environmental conditions. Thus, when comparing performance of lines with that of their open-field derived progenies, seed size and seed quality and thus the resulting crop stands are expected to differ for non-genetic reasons (Bond and Pope, 1984).

Ederer and Link (1992a,b) simulated the relative importance of *per se* yield of lines, of general combining ability of lines, and of the degree of cross-fertilization of lines for the performance of synthetics (Syn-1, Syn-2 and Syn-3). The coefficient of determination (R^2) for *per se* yield as an independent variable varied from 0.41 to 0.64 (depending on the number of components and Syn generation). The R^2 of general combining ability as an independent variable ranged accordingly from 0.63 to 0.75. For the degree of cross-fertilization, the simulation R^2 was between 0.02 and 0.17,

with the higher values for Syn-1 and for four instead of eight components.

Link et al. (1991) analyzed phenotypic data from 36 spring faba bean lines along with nine synthetic populations (each derived from a quartet with four founders) across 12 environments in south Germany from 1986 to 1990. Their results showed that for days to anthesis, days to maturity, thousand seed weight, plant height and lodging, the R^2 of the averages of nine line quartets and the results of their nine synthetic populations was on average 0.84 (half of the associations were $R^2 > 0.90$). The R^2 for the association between the nine line quartets with yield of their synthetic populations was only 0.90 for Syn-0, 0.62 for Syn-1 and 0.48 for Syn-2. This decline through the synthetic generations very often follows the increasing difference in heterogeneity and heterozygosity between the (quartets of the) homozygous lines and the successive synthetic generations.

These considerations support to the notion that additional assessment of the *per se* performance of lines and hence a focus on the difference between *per se* performance and polycross/top cross-progeny performance may not be worthwhile, but rather all budgets may be devoted to field-phenotype the open-field derived progenies of the candidate lines.

It is not self-evident whether several synthetic populations have to be developed and tested, to select between them; or whether only those synthetics should actually be constructed to be further submitted to the official trials. Selecting between lines first based on observation trials (**Figure 1**) and then based on data of their polycross/top cross-progenies, then selecting between predicted (but not yet realized) synthetic cultivars may help focus resources on only the very-best predicted synthetic population(s) (Ederer and Link, 1992a,b, 1993; Schill et al., 1992).

Mutagenesis in Faba Bean

Novel mutations on certain faba bean genes will enrich its diversity for breeding. Such mutants may be induced by chemical mutagenesis or irradiation. Physical (e.g., gamma-ray) and chemical (e.g., ethyl methane sulphonate-EMS) mutagens have demonstrated their usefulness in faba bean mutation induction (Abdalla, 1982; Duc, 1995; Khursheed et al., 2018, 2019; Nurmansyah et al., 2019, 2020). Ion beam irradiation has also been reported as an effective and unique technique for inducing mutations in faba bean (Khazaei et al., 2018b). The first mutagenesis on faba bean was reported by Sjödin (1971) with the description of determinate mutants, *ti1* and *ti2* which significantly reduced the number of flowering nodes giving the plant type as "topless." Later, van Norel and Hoogendoorn (1989) reported a dwarf mutant, *dw1*, which reduced the internode length by almost 50%. Ramsay et al. (1991) reported a gene for reduced vicine-convicine content, *vcr*, and Duc (1995) found five nodulation mutants through EMS mutagen where one of them was a super modulating mutant (*f32*) giving 3-5 times more nodules than the normal type. Bhatia et al. (2001) has given a detailed description of 265 varieties developed through induced mutation on grain legumes including 13 in faba bean genotypes. A number of imazapyr resistance mutations through EMS were identified in South Australia (Mao et al., 2019) that resulted in the development of the first imidazolinone herbicide resistant variety, PBA Bendoc in Australia. Recently morphological diversity of faba bean mutant populations was explored (Nurmansyah et al., 2019, 2020). Mutation on grain legumes including faba bean was reviewed by Huyghe (1998). A number of mutation breeding projects across the world have proven the efficiency of mutagenesis in grain legumes to broadening the genetic variation (e.g., Micke, 1984; Tadege et al., 2009; Horn et al., 2016; Raina and Khan, 2020).

Seeking mutants that increase resistance against the root parasitic weeds broomrape and stem parasitic dodders, both being increasingly a problem in the Mediterranean basin, will promote faba bean production in this region; resistance of faba bean against herbicides that control these weeds would especially be useful and is a sought-for trait (Rubiales and Fernández-Aparicio, 2012; Fernández-Aparicio et al., 2016; Rubiales et al., 2016; Mejri et al., 2018). Similarly, mutants that decrease pod wall thickness will increase grain yield. Inbred lines harboring new diversity such as increased resistance or improved quality, accruing from recombination-fueled transgression or from mutagenesis (Bhatia et al., 2001) may be used at step (1) in **Figure 1**. Currently 20 faba bean mutant varieties have been developed from various countries which are listed at IAEA (International Atomic Energy Agency, 2021) mutant variety database (https://bit.ly/3yVEReM) that might be used in breeding programs. Mutagenesis combined with biotechnology tools may accelerate releasing novel faba bean germplasm and improved cultivars.

Speed Breeding

Speed breeding to shorten breeding cycles is a must have tool in any breeding programs for the purpose of increasing genetic gains per year. Significant progress has been made toward shortening the reproductive cycle and hence the overall process of cultivar development (see below) as a tool for accelerating the breeding of pulse crops (*reviewed by* Cazzola et al., 2021). For example, Samineni et al. (2020) reported that up to seven generations/year can be obtained in chickpea without applications of chemical treatment. Recently an *in vivo* speed breeding protocol, for the first time, for faba bean has been reported by Mobini et al. (2020). This can be a valuable tool for developing diverse germplasm and improved cultivars in a relatively short time span. They reported that application of cytokinin or cold treatment could increase pollen viability and seed setting, thus consequently decreasing the length of the breeding cycle in faba bean by 22 days from seed to seed. Additional reduction in faba bean generation time as part of a reliable speed breeding protocol may be obtained by implementing accelerated faba bean development to the flowering stage by regulating light and temperature. Recently, this has been achieved in Australia where five generations per year can be obtained (Janine Croser, 2021, pers. comm). Speed breeding can accelerate development of faba bean lines and may be integrated with other cutting-edge breeding tools such as marker assisted breeding and genomic selection based on estimated breeding values (Bhatta et al., 2021). However, due to the tiny amount of seed produced on a typical speed-bred plant and the expense of maintaining controlled environments, speed

breeding protocols are only suited to the primary cross/backcross and inbreeding generations of a breeding scheme, pending further research to extend the applications of speed breeding. All steps toward phenotyping of agronomic performance rely on considerable seed increase, probably in open pollination conditions. Phenotypic evaluations must eventually be carried out under "natural" conditions.

MARKER-ASSISTED SELECTION AND DNA MARKER AVAILABILITY

Marker-assisted selection is an indirect selection process using genetic markers that are tightly linked to traits of interest but are difficult or costly to phenotype. Selection on the basis of a DNA markers can be done for a fixed and predictable cost, whereas phenotypic selection may require either a dedicated, expensive or destructive screen, and deliver results that are still in part depending on the interactions of the genotypes with the actual conditions of such a test. A good example that illustrates how DNA markers could be implemented in faba bean breeding is the case of vicine and convicine (v-c), the main anti-nutritional factors limiting faba bean seed usage. The v-c causes favism in pre-disposed humans and lowers feed conversion efficiency in monogastric animals and therefore removal of v-c by genetic means is unequivocally desirable. Some decades ago, a natural low v-c variant was identified, and shown to be controlled by a single gene (Duc et al., 1989). This gene was mapped to the tip of faba bean chromosome 1 and a diagnostic SNP assay for use in selection was developed (Khazaei et al., 2015, 2017). It is now known that low v-c faba bean cultivars carrying the vc^{-} gene are safe for favism sufferers. All elements are in place for straightforward foreground selection of low v-c genotypes, thereby bypassing the need for expensive, time-consuming and destructive biochemical analysis of seeds (Khazaei et al., 2019a; Tacke et al., 2021). The vc^{-} marker and another marker for a herbicide (imidazolinone) tolerance (Mao et al., 2019) are being used routinely in faba bean breeding programs in Australia. Very recently, the biosynthetic pathway of v-c was uncovered (Björnsdotter et al., 2021). Similarly, robust DNA markers for low seed coat tannins *zt1* (Webb et al., 2016; Gutierrez and Torres, 2019) and *zt2* (Gutierrez et al., 2020; Zanotto et al., 2020), growth habit (*Vf_TFL1*, Avila et al., 2006, 2007) and rust resistance genes (*Uvf1* and *Uvf2*, Ijaz et al., 2021b) have been developed. Furthermore, closely linked DNA markers have been reported for host plant resistance to ascochyta blight (Avila et al., 2004; Kaur et al., 2014; Atienza et al., 2016; Sudheesh et al., 2019; Faridi et al., 2021), however, their verification and availability in breeder friendly format is yet to come. Detailed information on available QTLs and DNA markers for adapting to biotic and abiotic stresses in faba bean was recently reviewed by Khazaei et al. (2021). Faba bean breeding efforts would benefit greatly from development of effective DNA markers for improving resistance to chocolate spot.

GENOMIC SELECTION

Low-cost genome-wide genotyping and high-throughput phenotyping platforms open up the further possibility of speeding up selection for complex traits. Genomic selection by definition focuses on multigenic traits and is rather a black-box approach than the attempt to analyze and follow-up the genetic variation locus-by-locus and candidate-gene-wise. Genomic selection has been shown to be a useful tool in recurrent selection within base populations of allogamous crop species that are constructed as a synthetic population (Müller, 2017). Accuracy values for grain yield are typically lower than heritability values (or their square roots) from a series of yield trials. Yet, genomic predictions are conducted for individuals. Breeding values for grain yield or for agronomic performance, genomically estimated for single plants, is tremendously more precise than heritability of such single plants' performance. This allows genomic selection to be implemented based on single plants, and it especially allows markedly shortening of breeding cycles; the time-consuming phase of developing and phenotyping inbred lines can be eliminated from the selection and recombination phase of breeding and can be shifted to the cultivar-making phase. Genomic selection has much to offer faba bean breeding, provided that a highly cost-effective genome-wide genotyping platform becomes available.

Genomic selection may accelerate faba bean breeding by reaching a cross-to-cross cycle length of 6 months to 1 year, thereby increasing genetic gain per unit time. One possible implementation of genomic selection that would work well in faba bean is the two-part breeding strategy proposed by Gaynor et al. (2017) with application of genomic selection mainly in the first (population improvement) component of the scheme. The breeding population, i.e., the breeding germplasm, has to be genetically elite and diverse. Due to the partial allogamy, the individuals are more or less heterozygous. This population is genotyped, with a very high number of (representative) individuals. Genotyping is conducted on seeds instead of plants (chipped seed, Mills et al., 2020) and genotypic data are used to predict line *per se* performance or varietal ability (see above). Selection is applied accordingly, between chipped seeds. Then only the selected chipped seed is sown as a new generation of the breeding population, and is directly allowed to propagate further according to its natural, partially allogamous mode, under open pollination, without any intermittent phase of inbreeding or multiplication or phenotyping. A large, representative sample of offspring (seed) from this population is again genotyped, genomic estimated values are predicted and selection is again applied; and so forth. It is possible in this way, even without the benefit of speed breeding, to achieve two cycles per year. This rapid turnover may be termed "recurrent genomic selection." The process of creating inbred lines from the most promising, selected individuals is organized as a separate pipeline, which may be termed "cultivar development" or "product development" (Gaynor et al., 2017). Phenotypic data to steer and adjust the training of the algorithm for the genomic prediction

TABLE 1 | Theoretical composition of a partial allogamous population Cockerham and Weir, 1984, with 20% cross-fertilization of non-inbred individuals and 50% cross-fertilization of homozygous individuals (linear relationship between cross-fertilization and inbreeding; Link, 1995; Brünjes and Link, 2021).

Parameter	Hybrids	Intermediate generations			Highly inbred	
	F1	F2	F3	F4	F5	F5>
Inbreeding coefficient	0.000	0.500	0.750	0.875	0.938	>0.968
Frequency in population	0.339	0.271	0.176	0.101	0.055	0.057

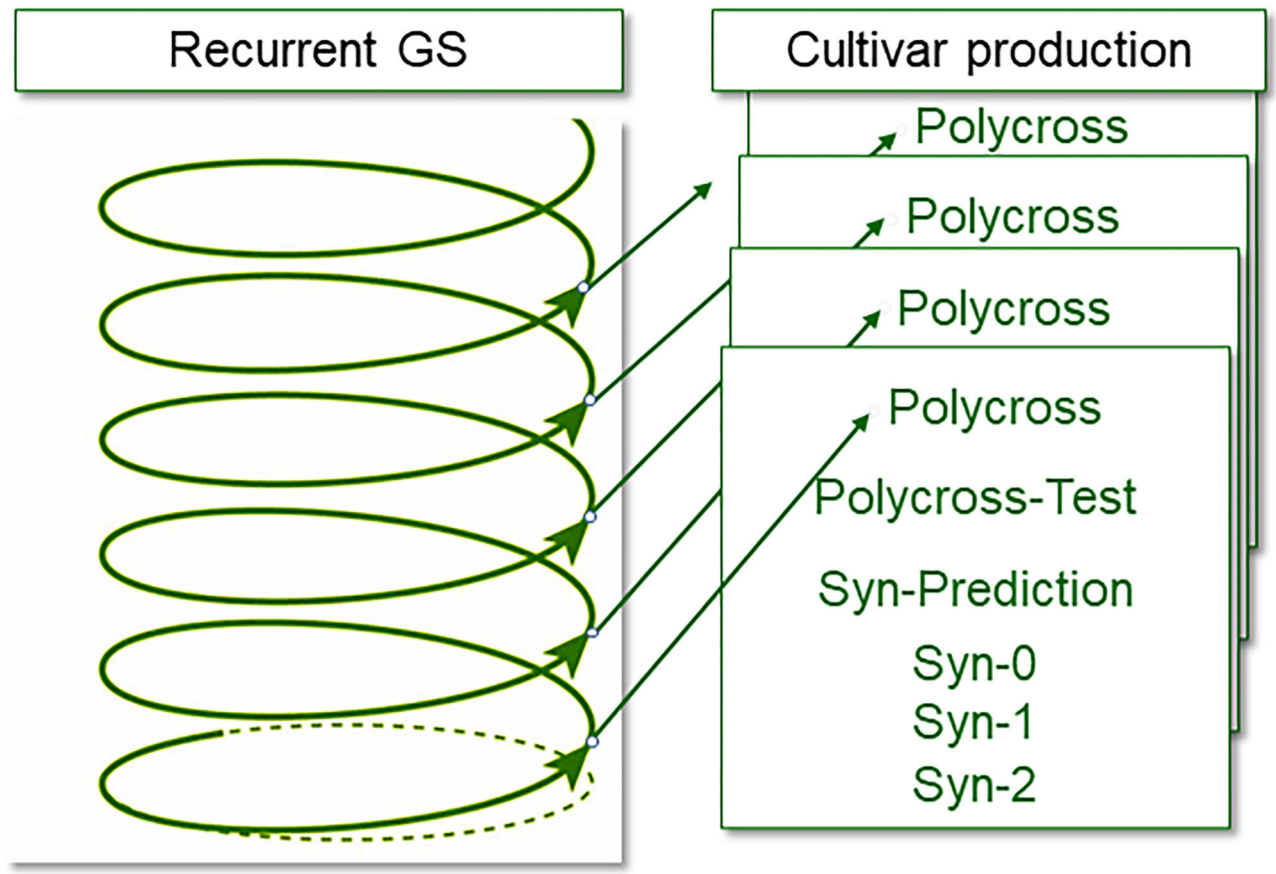

FIGURE 2 | Separation of recurrent genomic selection and recombination (based on single plants of a breeding population) from the actual cultivar development. Selection in the cultivar production is partly genomic data and partly phenotypic data (adapted from Gaynor et al., 2017).

is gathered during cultivar development (either for line or synthetic cultivars).

The partially allogamous nature of faba bean offers the recombination that is necessary for the fast-cycling recurrent selection and the self-fertilization that is required for the production of inbred material for cultivar development synchronously and at zero cost (**Table 1**). To enter the cultivar development with inbred lines, the only task is—via genotyping—to identify the inbreeding levels of the individuals of the breeding population (the population under recurrent genomic selection, **Figure 2**, **Table 1**) and find the more inbred ones. With genomic selection, the individuals in this fast-cycling population require genotyping anyway, and the inbred levels of the individuals accrue as a side product. The inbred individuals that enter the cultivar development pipeline transfer the level of genetic improvement that is realized in the recurrent population improvement. During cultivar development, genomic selection and phenotypic selection will both be applied. Selection will be based on an index value from phenotypic data and genomic estimated data. The scheme would be very similar to conventional line breeding and synthetic breeding. The maintenance breeding would immediately start as an inbred individual enters the cultivar development.

PHENOTYPIC RECURRENT SELECTION OF POPULATIONS

The proposed two-part genomic selection strategy adapted for faba bean as outlined above has not yet been implemented in practice. Utilizing currently available genotyping platforms (individual KASP assays or high density Axiom array), the cost of eliminating a genetically inferior individual plant by genome-wide genotyping is still beyond the means of most private or public breeding programs. However, what if phenotypic selection were performed on the basis of individual plant seed yield? This approach has been avoided in the past as an individual plants agronomic performance e.g., for yield, is considered to have low heritability, and in the context of heterogeneous crop stands, to be confounded by selection on the probably correlated trait of competitive ability, requiring elaborate honeycomb designs to circumvent (reviewed in Fasoula and Tokatlidis, 2012). However, selection on a single plant basis can be conducted at a very low cost per genotype evaluated, since only the top 5-10% of the population would need to be individually threshed and weighed, the rest being eliminated by eye or by recording the weight of the unthreshed plant requiring just seconds of evaluation time, and with none of the time required to inbreed and bulk. In fact, rapid recurrent selection based on phenotype has been empirically tested recently, with promising results. Tagkouli (2020) set up a faba bean polycross population with 22 founding cultivars and lines, using captive bumblebee colonies to maintain high outcrossing rates in each generation, and selecting individual plants with the highest seed yield in each of three successive field seasons in the UK (Reading) and two seasons in Ireland (Carlow). The respective populations selected were reciprocally tested across sites and their yield performance compared to elite spring varieties. Although no differences in these trials passed the thresholds for statistically significant differences, the thrice-selected Reading selected population outperformed all earlier selection generations as well as all elite checks in the Reading trial whereas this result was partly reversed in the reciprocal trial in Ireland. Detailed study of the Reading population performance in Reading showed this genetic gain was associated with a marked increase in hundred seed weight, faster emergence and canopy closure and a marked increase to very high levels of heterozygosity as judged by genome-wide genotyping of the selected individuals at each selective generation. Furthermore, signatures of purifying selection were detected at a number of specific loci, suggesting that the rapid turnover of highly recombined, highly heterozygous populations enabled rapid adaptation to environment through enrichment of alleles that confer selective advantage concurrently with optimizing levels of hybrid vigor in the population.

This observation that hybrid vigor is the major factor determining population performance was also noted by Ghaouti and Link (2009), who showed that average polycross progeny yield was greater than either the average of the inbred polycross parents ($n = 18$), or indeed the predicted yield of the best possible four-component synthetic that could be drawn from the 18 inbred lines, so this outcome of rapid recurrent selection is not surprising. In fact, where the natural environment exercises sufficient selection, mass selection alone can result in rapid adaptation of populations, as shown recently by Landry et al. (2017) for the case of development of winter-hardy faba bean populations for the US Pacific North-West.

The question that remains to be investigated is the relative efficiency and cost-benefit of genomic selection over phenotypic selection.

GENETIC GAIN

In the absence of a reference genome assembly for faba bean, high-density genetic maps and high-throughput approaches such as transcriptome analysis have been used for enrichment of genomic resources (Khazaei et al., 2021). At the writing of this article various cooperative reference genome assembly efforts are concurrently underway. The development of a reference genome will provide a foundational resource for faba bean research to accelerate genetic gain. Speeding up the translation of genomic knowledge into plant breeding would lead to accelerated genetic gain. By integrating genomic knowledge with other breeding tools and platforms will enable to accelerate the breeding process for enhancing genetic gain for faba bean. Translational research will require coordination and understanding between laboratory-based and field-based scientists that can work as a bridge between fundamental research and practical plant breeding.

The major pulse crops have not experienced expanded production and consumption at a uniform rate over the past 50 years. Lentil production has expanded at more than 10% annually, chickpea and dry bean at 2 to 3%, and pea at <1%; while faba bean production declined sharply until 1992 and it was practically recuperated in 2019 due to the productivity increases from <1 to 2.1 t ha^{-1} while the human population has increased at 2.4% annually (Khazaei et al., 2019b; FAOSTAT, 2021). Now that environmental and nutritional concerns combined with demand for plant protein have gained considerable momentum on a global scale, the improvement and expansion of yield potential, sustainable production stability and food industry quality of faba bean are more important than ever before.

THE FUTURE OF FABA BEAN BREEDING

Faba bean is a biologically and genetically unique crop that has much to offer both in terms of its nutritional density and agro ecological role in reducing fertilizer requirements and supporting pollinator populations. Agricultural policy makers and funders should be aware that there are many enticing opportunities to accelerate genetic gain in faba bean.

The conquering of the bloated 13 Gb diploid genome at last (fabagenome.dk) affords a timely opportunity to develop cheap genotype-by-sequencing approaches already common in other species. This is because low cost, low complexity skim sequencing requires a robust and complete reference genome assembly to map short reads to, whereas until now, the only successful genotyping approaches have required targeting of each individual

locus. Cheap genotyping-by-sequencing (GBS) should usher in a new era of genomic selection; however, hurdles are not just on the genotyping side. Modeling that takes the mixed mating system into account and develops population-and site-specific estimates of outcrossing rate, seed multiplication rate and trait heritability's is needed to ensure that genomic selection gives significant and cost-effective increases in the rate of genetic gain.

Burgeoning genome knowledge can be exploited in other arenas. A facile, high efficiency transformation system would enable gene editing of a growing list of genes whose function is relatively well-understood in related legumes at least, if not in faba bean. Clearly, there remains uncertainty over the timescale over which gene edited crops could become widely accepted, but we should take the long-term view and fund the underpinning work necessary to bring forward case studies by which the cost-benefit analysis can be done for specific applications. Meanwhile, classical mutagenesis, newly invigorated by cheap re-sequencing technology (Wang H. et al., 2012), may soon allow *in silico* mutagenesis to become a reality.

The ability to obtain an allelic series of mutants in specific target genes such as CENH3 either via gene editing or mutagenesis (Jacquier et al., 2020) should enable the production of doubled haploid inducer lines, which would immediately impact the rate of genetic gain, possibly even more than "speed breeding," while a cytoplasmic male sterility system, naturally occurring or transgenic, would enable the topic of hybrid breeding to be revisited. Up to this point, our pre-breeding research wish list could have applied to virtually any crop. However, faba beans' unique characteristics mean that we cannot simply adopt a generic blueprint for high-tech breeding technologies (i.e., from maize) without regard for the biological specificities of the crop.

The mutualistic relationship of faba bean with its insect pollinators needs more detailed study, both to understand better how natural pollinator populations can be nurtured to enhance long-term yield stability in the face of climate change (Bishop et al., 2016a), and to use pollinator-assisted outcrossing more effectively as a breeding and seed propagation tool (Tagkouli, 2020).

A related, longer-term objective relates to understanding genetic control of pollinator attraction and pollinator function traits on which the efficiency of pollinator services depend (Suso et al., 2016; Bailes and Glover, 2018; Bailes et al., 2018). Such an understanding would underpin the opposite but non-mutually exclusive goals of breeding for optimal interaction with a specific pollinator or breeding for complete pollinator independence (i.e., conversion of faba bean to a fully autogamous mating system).

Finally, the genetic control of the symbiotic relationship with rhizobia to develop nitrogen-fixing nodules also merits further investigation as it is clear there is unexplored variation in the rhizobial population for nodulation efficiency and nitrogen fixation rate and an unexplored variation in the faba bean population for strain selectivity, all of which needs to be understood in the context of highly variable soil physical and chemical properties and the resident soil microbiome.

CONCLUSIONS AND FUTURE PERSPECTIVES

In spite of the fact that faba bean has demonstrably the highest nitrogen fixation capability among grain legumes, high protein content increasing interest in faba bean *per se*, its production has not been expanded. Now that the environmental consequences of crop production and food systems are under intense scrutiny, faba bean is set to become an important source of plant protein. Increasing faba bean production will have impact on cropping systems that include other pulse crops. For example, faba bean can be used to extend temperate crop rotations that include field pea and lentil which are increasingly at high risk of yield loss due root rot caused by *Aphanomyces euteiches* (Karppinen et al., 2020). Demand for plant-based protein for food systems is rapidly expanding, and faba bean is an excellent source of protein flour because of its bland flavor and light color. The seed size of faba bean can be reduced without affecting yield potential, thereby lowering the costs of production. Seed shape can be changed from flat to round, making it more amenable to mechanical seeding systems that are widely used for soybean, allowing the seed distribution uniformity that will maximize yield, reduce seeding costs, and potentially reduce the spread of diseases that affect the canopy. Reducing seed size has the potential to reduce the overall costs of breeding per unit of genetic gain, and also reduce the overall costs of both breeding and commercial production for breeding programs targeting the protein extraction industry. All of these goals can be achieved more quickly by a coordinated breeding approach that focuses on maximizing genetic gain using a combination of newly available genetic technologies and focusing on breeding objectives that go beyond the traditional market place for faba bean.

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

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.744259/full#supplementary-material

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REVIEW
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Constraints and Prospects of Improving Cowpea Productivity to Ensure Food, Nutritional Security and Environmental Sustainability

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Providing safe and secure food for an increasing number of people globally is challenging. Coping with such a human population by merely applying the conventional agricultural production system has not proved to be agro-ecologically friendly; nor is it sustainable. Cowpea (*Vigna unguiculata* (L) Walp) is a multi-purpose legume. It consists of high-quality protein for human consumption, and it is rich in protein for livestock fodder. It enriches the soil in that it recycles nutrients through the fixation of nitrogen in association with nodulating bacteria. However, the productivity of this multi-functional, indigenous legume that is of great value to African smallholder farmers and the rural populace, and also to urban consumers and entrepreneurs, is limited. Because cowpea is of strategic importance in Africa, there is a need to improve on its productivity. Such endeavors in Africa are wrought with challenges that include drought, salinity, the excessive demand among farmers for synthetic chemicals, the repercussions of climate change, declining soil nutrients, microbial infestations, pest issues, and so forth. Nevertheless, giant strides have already been made and there have already been improvements in adopting sustainable and smart biotechnological approaches that are favorably influencing the production costs of cowpea and its availability. As such, the prospects for a leap in cowpea productivity in Africa and in the enhancement of its genetic gain are good. Potential and viable means for overcoming some of the above-mentioned production constraints would be to focus on the key cowpea producer nations in Africa and to encourage them to embrace biotechnological techniques in an integrated approach to enhance for sustainable productivity. This review highlights the spectrum of constraints that limit the cowpea yield, but looks ahead of the constraints and seeks a way forward to improve cowpea productivity in Africa. More importantly, this review investigates applications and insights concerning mechanisms of action for implementing eco-friendly biotechnological techniques, such as the deployment of bio inoculants, applying climate-smart agricultural (CSA) practices, agricultural conservation techniques, and multi-omics smart technology in the spheres of genomics, transcriptomics, proteomics, and metabolomics, for improving cowpea yields and productivity to achieve sustainable agro-ecosystems, and ensuring their stability.

Keywords: cowpea productivity enhancement, indigenous legume, *Vigna unguiculata*, nutritious human food, the largest producer status, smart biotechnological approaches, protein-rich fodder for livestock

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INTRODUCTION

With the world population expected to increase by up to 70% by 2050, the global community is faced with the constraint of providing safe and secure food supplies to an increasing number of people. The human population is projected to reach the 9.8 billion mark by 2030, bringing immense challenges in feeding the global populace (Tian et al., 2016; Pais et al., 2020). This will be a huge task, especially for the African continent, to handle in an era of climatic change and a growing population that will double by the year 2050 (Adedeji et al., 2020). Not only is the task of coping with this high human population growth rate in terms of the conventional agricultural production system daunting; it is also not environmentally/ecologically sustainable (Roell and Zurbriggen, 2020). In addition to the burgeoning human population, other factors that are posing threats to improvement in agricultural productivity include among others, climatic change, the loss of fertile agricultural land to urbanization, the challenges of phytopathogens and pests, abiotic challenges; high levels of salinity, and drought. Therefore, there is an urgent need to devise novel and workable solutions to achieve sustainable means of enhancing productivity in terms of agro-products and their nutritional composition.

Cowpea [*Vigna unguiculata* (L.) Walp.] is an annual leguminous crop that is grown throughout the world, but it is grown mainly in semiarid regions. Cowpea is a diploid, having $2n = 2x = 22$, with the size of its genome consisting of approximately 620 million base pairs (Lonardi et al., 2019). In terms of its importance, this indigenous African legume is economically, nutritionally, and environmentally the foremost crop that serves as a source of essential human dietary nutrients and as, a means of providing fodder for livestock. It also presents with other multi-functional traits, including the maintenance of the soil – ecology balance through nitrogen fixation in that it facilitates a symbiosis with nodulating bacteria (Ravelombola et al., 2017). Cowpea is of strategic importance to Africa in terms of the large quantities that can be produced and is, therefore, an important component in the economy (Walker et al., 2016). Having originated in Africa, cowpea is now grown worldwide in 100 countries (Singh, 2014; Gonçalves et al., 2016). The cowpea yield in 2020 was estimated to be in the region of 9.8 million, while by 2030, the projected yield is expected to rise to 12.3millon tons (Boukar et al., 2016). Cowpea is indeed a multi-faceted crop, providing revenue for millions of smallholder farmers, as well as for traders who sell the nutritious grain. By providing essential protein, minerals, and vitamins, it serves in most African countries, as a means of balancing the diet, thereby providing a cheaper means for accessing the necessary dietary nutrients and for positively influencing the well-being and health of the populace. In addition, all of its components are valuable as nutrients (Gonçalves et al., 2016) – the leaves, pods, and seeds are nutritionally high in protein, with less fat, and are used extensively as the vegetable component in diets. In both the urban and rural settlements in most African countries, women generate income by trading in processed cowpea food and snacks. Cowpea is also important in livestock production, where the plant's leaves and vines are dried and used as fodder/feed supplements in livestock husbandry. Cowpea is a key resource for a large number of people in the developing world, mainly in the arid/semi-arid tropical regions of the world (Muñoz-Amatriaín et al., 2017). Cowpea dry grain contains 23–32% protein and essential amino acids (Carvalho et al., 2017). Also, the green cowpea seeds, fresh and immature pods, and leaves contribute vegetable sources for human consumption (Gerrano et al., 2017, 2019). Its fresh leaves are used as vegetables, the haulms (cowpea pod walls, stems, and leaves) are used as livestock fodder, providing dietary nutrients for animals, and as income for the farmers (Kebede and Bekeko, 2020). Cowpea is highly prized as a source of food, for fodder in livestock feeds, and an important but cheaper means of improving and boosting soil fertility through biological nitrogen fixation. As important as it is in human nutrition, cowpea is equally useful in providing the necessary energy and protein in livestock production. More so, owing to its adaptation to different climatic conditions and its ability to grow in a less-fertile soil environment, it is highly appreciated as forage and a potential fodder crop for the future (Alemu et al., 2016). It is a key leguminous crop in the arid and tropical regions of Africa, Asia, and Latin America (Xiong et al., 2016). Cowpea is relished as a source of nutritious food and a variety of snacks that provide humans with cheaper proteins, thereby enhancing food security (Agbogidi and Egho, 2012; Muranaka et al., 2016). Cowpea is a vital source of beneficial micronutrients, proteins, amino acids, antioxidants, vitamins, and minerals, with immense therapeutic and nutritional security benefits (Jayathilake et al., 2018; Olabanji et al., 2018; Gondwe et al., 2019; Irondi et al., 2019; Owade et al., 2020). It is often used in mixed cropping systems to offer the multi-functional benefits of a nutritious grain, as a fodder crop, and as a means to improve soil fertility (Agza et al., 2012; Belay et al., 2017). Importantly, it is useful in agro-ecological conservation. It is used mainly as an inter-crop with other food crops to boost soil fertility and add nutrients to degraded soil through its nitrogen-fixing property (REGO et al., 2015). It is postulated that cowpea can fix about 337 kg N. ha-1 (Yahaya, 2019). The average nitrogen addition/contribution to the soil during the cowpea growth and development phase is in the range of 40–80 kg N. ha-1 and sometimes up to 200 kg N. ha-1 (Meena et al., 2015). Also, it is useful as a cover crop or an erosion-preventing crop; it helps in suppressing weeds; and also aids in the retention of moisture (Das et al., 2018). Another key advantage of cowpea production is that when used as an inter-crop with other crops, it induces the growth of beneficial soil microorganisms and reduces the use of synthetic agrochemicals (Bukovsky-Reyes et al., 2019; Sun et al., 2019). In terms of importance, cowpea production contributes significantly to economic productivity and environmental sustainability in Africa (Martins et al., 2003; Olajide and Ilori, 2017; Ovalesha et al., 2017; Cardona-Ayala et al., 2020).

The main cowpea-producing countries of the world are in sub-Saharan Africa, that is the Sudano-Sahelian vegetation region (Boukar et al., 2019). Nigeria has the highest production output, followed by Niger and Burkina Faso, in that order. In

terms of the metric ton production levels of cowpea grain, Nigeria is the largest producer in the world (FAO, 2020).

The productivity of cowpea in different countries differs in terms of the production output per area cultivated as highlighted in **Table 1**. However, despite all of the mentioned benefits of cowpea production in Africa in terms of the economies of scale, agri-food/nutritional benefits, and environmental stability influences, its productivity output is limited, and its status as an underutilized leguminous crop persists. The challenges militating against improved cowpea productivity in Africa include the following: climatic change and its adverse consequences on crop productivity include the issue of infrequent and erratic rainfall arising from, among others, drought and aridity issues, the decline in soil nutrients, the excessive use of synthetic chemicals, low-yielding seed cultivars, and infestations of pests and microbial pathogens (Rascovan et al., 2016; Afutu et al., 2017). Diverse strategies have been deployed by researchers in an attempt to breed cowpea for productivity enhancement. These strategies span through the initial selected germplasm collection from cowpea wild relatives and its natural population for desired genetic traits in order to create an improve cowpea genotypic varieties with agronomic traits and morphology through conventional hybridization and progeny cross-breeding techniques. These earlier breeding research techniques contributed to the development of many improved cowpea accession lines in the germplasm. However, significant barriers of improving cowpea varieties through the conventional breeding techniques like the challenges of intraspecific and interspecific crossing, genetic variation, genotype-by environment interaction, among others still persist. The advent of molecular tools such as RAPD, AFLP, ISSR, and other assisted marker selection genotypic breeding, was a milestone that led to genetic gains in cowpea productivity improvement. The advantages that are associated with this DNA molecular tools include: they are highly reproducible, cost-effective, and also it can deploy in the analysis of a large number of samples having genetic differences. Moving ahead, the advancement in molecular biology techniques that span genomics, proteomics, transcriptomics, and metabolomics, means cowpea-breeding research could now encompass assessing gene regulation and expression patterns for both abiotic and biotic resilient cultivars. These advance molecular technologies have been deployed to discern genotypic diversity existing in cowpea genome globally. Also, these advanced techniques have help cowpea breeders through genetic engineering to select desired gene traits and transfer across genetic barriers for cowpea improvement.

In summary, diverse technological tools have been deployed by researchers for cowpea-breeding enhancement, spanning the past, the present and future prospects that include [markers systems, genetics maps, high-throughput genotyping, and quantitative trait loci (QTL) analysis]. In addition, mutation breeding, tissue culture, reverse genetics, clustered regularly interspaced short palindromic repeats (CRISPR) technologies are being apply for genetic gain in cowpea. In spite of this progress, major efforts are still needed for cowpea productivity improvement because cowpea plant is a diploid with a very narrow genetic makeup and also, it reproduces through means of self-pollination. Therefore, to overcome this gap, innovative research efforts that transverse different continents are still required toward breeding cowpea for enhanced productivity. For Africa to leverage its position as the foremost producer of this vital indigenous legume, the continent must look ahead at ways of improving productivity by closing the gaps in yield and by limiting the constraints to cowpea productivity in an agro-ecologically sustainable way. Therefore, this review highlights the constraints of cowpea production in Africa, and also gives

TABLE 1 | Production output and productivity of cowpea by some selected countries in the world, excluding Brazil as (adapted from Faostat, 2020).

S/N	Country	Production in tons	Yield per hectare	Area harvested	Inference on production	Inference on productivity
1	Nigeria	2,606,912	9,137	2,853,097	1st	7th
2	Niger	2,376,727	4,035	5,889,677	2nd	18th
3	Burkina Faso	630,965	4,826	1,307,336	3rd	12th
4	Ghana	215,350	19,862	11,898	4th	2nd
5	Tanzania	202,865	4,096	30,366	5th	6th
6	Cameroon	185,832	4,043	258,898	6th	9th
7	Kenya	179,399	4,367	11,154	7th	10th
8	Mali	157,739	3,767	160,412	8th	11th
9	Myanmar	136,411	11,425	119,398	9th	4th
10	Sudan	104,667	2,678	333,638	10th	17th
11	Mozambique	89,356	5,545	284,451	11th	20th
12	Democratic Republic of Congo	72,726	4,432	95,803	12th	15th
13	Senegal	60,422	6,889	260,408	13th	19th
14	Malawi	42,456	13,515	159,345	14th	13th
15	United States	23,632	4,296	169,279	15th	1st
16	China	15,652	8,876	209,371	16th	5th
17	Madagascar	13,000	8,907	14,596	17th	8th
18	Uganda	12,439	9,750	208,059	18th	16th
19	Sri Lanka	11,180	11,770	9,499	19th	3rd
20	South Africa	4,871	10,360	15,108	20th	14th

an overview into the way these challenges can be circumvented through the deployment of smart biotechnological techniques/applications and insights concerning mechanisms of action for implementing eco-friendly biotechnological techniques, such as the deployment of bio inoculants, applying climate-smart agricultural (CSA) practices, agricultural conservation techniques, and multi-omics smart technology in the spheres of genomics, transcriptomics, proteomics, and metabolomics, for improving cowpea yields and productivity to achieve sustainable agro-ecosystems, and ensuring their stability.

PRODUCTION CONSTRAINTS

The production of cowpea in Africa, the epicenter of this foremost indigenous legume, is carried out mainly by subsistence farmers. The production output of these smallholder farmers is limited by diverse constraints that lead to low agronomic yields/productivity. The average yield of cowpea in Africa is about 600 kg/ha, which is still below its estimated optimum potential yield of 1,500–2,500 kg/ha (Kamara et al., 2018). Numerous constraints limit the improvement of cowpea yield and productivity in Africa. These limiting factors can broadly be termed as abiotic/biotic stresses and climatic variations, and they have had a huge influence on the overall productivity of cowpea grains and fodder vegetables that are produced in the different cowpea-producing nations of the world, and particularly in Africa.

Abiotic Stresses

Drought

Drought is a major challenge/constraint to achieving worldwide food security and production enhancement. Drought adversely affects plant growth at all developmental stages, impairing the morphology of the plant and the biochemical and physiological processes operating in the planted crops. These aspects subsequently affects, among others, the uptake of vital nutrients for plant growth and the ability of the seeds to germinate and of the plant to photosynthesize (Fahad et al., 2017; Lamaoui et al., 2018). Drought stress has negative consequences on the vitality and vigor of seeds and impairs seedling growth (Hatzig et al., 2018). The optimum growth/developmental stages in planted crops are adversely affected by drought, as observed in a decline in the rate of germination, seedling emergence and growth; impairments in vegetative growth, cell division and elongation; with mitotic processes also being affected (Farooq et al., 2009).

Drought stress can adversely affect the functioning of vital enzymes. Among other influences, the flowering stage of the plant could be negatively affected, as also the photosynthetic rate and the assimilate partitioning process. All these conditions eventually reduce the planted crop yields (Anjum et al., 2011).

Drought also impairs the proper functioning of the plant cell by producing oxidative damaging reactive species (ROS), which destroy plant lipids and proteins (You and Chan, 2015). Drought leads to adverse influences on the growth, development, and reproduction ability of planted cowpea, which limits the yield and productivity of the planted crops (Verbree et al., 2015; Daryanto et al., 2017; Ravelombola et al., 2018a).

Numerous studies have been done and are also on-going due to the enormity of drought stress challenges on cowpea productivity enhancement. In a study by Cui et al. (2020), they evaluated cowpea drought tolerance potentials at seedling stage. The experiment was done using a total of 36 cowpea-breeding lines in a completely randomized manner under drought stress conditions. Their results revealed that four (4) Arkansas cowpea breed lines are drought-tolerant, and they ranked better in terms of chlorophyll, healthiness and lodging score when compared to the other 32 genotypes. Therefore, these four cowpea breed lines could be further exploited in cowpea-breeding improvement. Also, in a study to highlight the constraining effects of drought stress on above-ground traits in cowpea plant, (Ravelombola et al., 2018a) assessed drought stress induced changes in 17 above ground traits in 30 cowpea genotypes at the seedling stage of growth for 28 days. Their findings showed that cowpea genotypes PI293568, PI349674, and PI293469 are slow to wilting, better adapted to drought, while the other susceptible genotypes are fast to wilt, the chlorophyll content is lower, and they undergo senescence faster too. The three (3) cowpea drought-tolerant genotype could be exploited further for advanced breeding.

More so, in a comprehensive study of drought tolerance response in cowpea plant (Carvalho et al., 2019) used four (4) cowpea genotypes to determine their physiological, biochemical and molecular response under water-limiting stress conditions. The output from this study highlighted the importance of stomata conductance, photosynthetic parameters, compatible solutes like anthocyanin and proline, as well as increase in enzymatic activity of reactive oxygen species scavenging enzymes like catalase, superoxide dismutase, glutathione reductase, guaiacol peroxidase. This study also characterized the drought gene expression profile of the four cowpea genotypes. Thirteen drought related genes were profile, and some of the genes were expressed higher than others under drought stress. The hallmark of the study was that cowpea genotype Cp5051 was the most drought tolerant due to a higher expression of drought-tolerant marker genes *VuHsp17.7* and *VuCPRD14*.

Salinity

Soil salinity is a major abiotic constraint to plant productivity. Salinity adversely impacts the metabolic and physiological processes in plants. Statistical report stipulated that upward of over 45 million hectares of agricultural soil are affected by this problem and that climatic change, as well as current irrigation practices, will exacerbate this situation (Munns and Gilliham, 2015; Parihar et al., 2015).

In addition, salinity stress negatively influences the rate of plant growth. The adverse influence of salinity reduces the fresh and dry weight of plants, while other vegetative growth traits are also adversely impacted (El-Beltagi et al., 2013; Mohamed et al., 2018).

Salinity stress leads to extensive damage in the adductive capacity of planted crops. It reduces lipid peroxidation and leads to the production of destructive oxidation species (ROS), that in turn causes damage to the key plant biomolecules (Ghonaim et al., 2021).

Salinity stress ultimately reduces yields and the productivity of planted cowpea crops, thereby affecting the goal of achieving enhanced global food security (Da Silva Sá et al., 2017; Ravelombola et al., 2018b).

Research into the impact of salinity on cowpea has indicated that it impairs cowpea seed germination, its vigor, and growth (Zahedi et al., 2012; Mini et al., 2015).

In order to evaluate salinity stress impact on cowpea cultivars (Ravelombola et al., 2019), investigated a simple protocol that could be deployed to assess the response of 30 cowpea genotypes to salinity constraint at seedling growth stage in a greenhouse experiment that profile 14 above ground traits response to sodium chloride (NaCl)-induced salinity. The findings indicated that relative salinity tolerance (RST) of cowpea genotype PI255774, all the plants were completely dead, while PI582438 performed best and the leaves were all green and had higher chlorophyll content. The outcome of this study validated simple protocol of assessing chlorophyll content and leaf injury for assessing salinity at seedling stage in cowpea. Also, in a study to investigate further the utility of chlorophyll content as a means of assessing salinity tolerance in cowpea seedling over time, (Dong et al., 2019) investigated how 24 different cowpea genotypes responded to salinity induced stress by monitoring the chlorophyll changes over a period of 24 days using a split-plot design. The results indicated the importance of genotype and the timing in relation to cowpea seedling response to salinity stress. Also, the chlorophyll content of the cowpea salt-tolerant cultivar was higher at day 24 of the experiment, while all the cowpea salt sensitive plant were dead at the end of the 24 days. In addition, salinity induce stress could further predispose cowpea cultivars to viral infestation. In their study, (Varela et al., 2019) assessed the consequences of exposing a cowpea severe mosaic virus (CPSMV)-resistant genotype to salinity induced stress. The results signify that vital protein pathways were altered, and there was proliferation of the (CPSMV), leading to the cowpea genotype changing from resistant to susceptible.

Heavy Metals

Heavy metals pose serious environmental constraints for and can adversely impact on plants and humans when the former bioaccumulate in plants and ultimately reach human beings *via* the food chain (Sidhu, 2016).

Heavy metals pose environmental and public health threats when they are discharged as by-products of industrial processes in the form of effluents (Wuana and Okieimen, 2011). Heavy metals, such as cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), chromium (Cr), and antimony (Sb), affect plant productivity and plant yields.

Heavy metals adversely affect the metabolic processes of the plant during the course of its growth and development. Heavy metals negatively influence the germination of seeds, while the vegetative growth rate (leaf, shoot, and root) is also impaired. Plants are adversely affected by heavy metals, as in the case of various physiological and biochemical processes such as the rate of photosynthesis, the uptake of nutrients, vital enzymatic reactions, as well as in the case of emergence of ROS (Azevedo and Rodriguez, 2012; Tiwari and Lata, 2018).

Research reported by Asagba et al. (2019) detailed the impact of Nickel toxicity on cowpea germination and other biochemical parameters. The investigation on phytotoxicity of nickel at varying concentration on cowpea seedling growth rate, length, fresh weight, as well as Ca^{2+} ATPase activity was assessed. The results indicated toxic impact of this heavy metal on cowpea seedling agronomic and biochemical parameters.

Also, in a study by Ogunkunle et al. (2020) that applied co-inoculation of arbuscular mycorrhizal fungi (AMF) and nano-TiO_2 to reduced oxidative stress and bioaccumulation of Cd in cowpea, it was reported that the total chlorophyll of the cowpea plant, as well as different reactive oxygen species enzymes were impacted negatively due to Cd induced toxicity.

Temperature Stress

As an abiotic stress factor, temperature in the case of low temperatures (chill stress) and high temperatures (heating stress) is a potential constraint in limiting the productivity and yield of planted crops globally. Temperature is a key abiotic parameter that influences the growth and development of plants (Hatfield and Prueger, 2015).

High temperatures limit the photosynthetic rate of the plant. The vegetative growth parameters and the metabolic activities of the plant are also adversely affected. Also, emergence, maturity/ripening, harvesting time (length of period/stage), and plant yield are affected (Prasad et al., 2008; Shah et al., 2011). Likewise, low-temperatures (chilling stress) adversely influence plant metabolic activity and negatively impact the growth/development of plants (Tian et al., 2011). Low temperatures (chilling stress) also negatively affect the germination rate, seedling emergence, and the vigor of the plant, so that the productivity of the plant is ultimately reduced (Abbas, 2012).

In a study on the impact of elevated temperature on the agronomic growth parameters and the nutritional status of cowpea at different growth phase, (Nevhulaudzi et al., 2020b) reported that there were differences in both the agronomic growth and nutritional parameters, and this is more pronounced at the flowering and pre-flowering stage.

Waterlogging Stress

Waterlogging stress affects the gaseous exchange in agricultural soil and negatively impacts crop productivity globally. It leads to an insufficient supply of oxygen to the plant roots and this in turn reduces the growth and development of the plant roots. It also leads to the inability of the plant to take up the necessary nutrients and nitrogen. Waterlogging affects the photosynthetic rate, reduces the vegetative agronomic growth rate of plants, leads to the senescence of leaves, and ultimately, negatively affects crop yield and productivity (Ren et al., 2014).

Higher or excessive soil water availability do not always favor cowpea growth. In a field study done in the Sudan

Savanna zone taking genotype environment interaction into focus, (Iseki et al., 2021) reported that excess water can inhibit the nitrogen-fixing capability of cowpea and lower its productivity.

Climatic Change Stress

Climatic changes in weather, as denoted by among others variability or fluctuations in the prevailing temperatures, rainfall, and the volume of greenhouse gases, are potentially limiting factors on various agro-input variables and ultimately affect the productivity of planted crops on a global scale (Awoye et al., 2017; Kukal and Irmak, 2018; Hounnou et al., 2019).

Climatic changes also adversely threaten the agri-food system at all scales: globally, nationally, regionally, and locally (Ajadi et al., 2011).

Climatic change negatively impacts agri-food input and output production systems because it influences the biotic and abiotic parameters of agricultural production. Hence, it affects planted crop yields (Challinor et al., 2014).

Changes in climatic conditions affect the biochemical, physiological, and metabolic activities of plants; the photosynthetic rate is affected, as are factors such as plant growth and development, and the rate of transpiration; there is also an imbalance in the elimination of CO_2, and a reduction in enzyme reactions; flowering may be affected, which could also lead to senescence (Srivastava et al., 2019). Predictive studies have forecast a reduction in food grain yields toward the later years of this current century (Pachauri et al., 2014), this hinging on expected extremes in global temperatures. Furthermore, most, if not all of the major food crops are adversely impacted by stress arising from heat at the different growth and developmental stages (Kaushal et al., 2016; Atlin et al., 2017). Global changes in climatic conditions have been found to adversely affect the health of humans, animal/livestock production, as well as planted crop productivity (Lesk et al., 2016; Mora et al., 2017).

In summary, many huge tasking constraints are militating against and slowing down the optimum yield production of cowpea in Africa. Some of these limiting challenges are highlighted in **Table 2**.

Biotic Stress

Worldwide, biotic stressors (roots and membrane pathogens) in large numbers lead to low productivity and low-quality agricultural products. Destructive pests and pathogens result in food insecurity on every scale – from the smallest to the largest thus leading to massive monetary losses on a global scale in terms of crop yield (Savary et al., 2019).

The main production constraints concerning biotic stress factors limiting cowpea productivity are exemplified by a wide range of organisms, including destructive pests; parasitic weeds, viral pathogens, bacterial pathogens, as well as fungal pathogens (Boukar et al., 2019).

Bacterial Diseases/Pathogens Affecting Cowpea Seeds, Plants, and Pods

A major constraint in limiting cowpea yields can be attributed to bacterial pathogens, which lead to massive crop losses of upward of 70% in the form of seed grain, pod, and fodder reduction (Agbicodo et al., 2010). Some of these destructive pathogens are transmitted *via* the seed (De Lima-Primo et al., 2015), while some are transmitted *via* the soil-borne route (Constantin et al., 2016). Some of the damaging symptoms of bacterial pathogen infestation in cowpea are brownish leaf spots, necrotizing and yellow halo leaf shapes, cracks noticeable on the stem, and pods filled with water, and blotch (Claudius-Cole et al., 2014). Among the most destructive bacterial pathogens of cowpea are members of the *Xanthomonas* genus (Shi et al., 2016; Durojaye et al., 2019).

Root-Knot Nematodes

Nematodes are responsible for huge losses in cowpea production and are also one of the constraints limiting improvements in cowpea production (Haegeman et al., 2012; Dareus et al., 2021). This they accomplish by impeding the uptake of water and nutrients. Also, nematodes limit cowpea growth and development by interfering in the pathways towards cell differentiation and in the transportation of auxin (Gheysen and Mitchum, 2011). *Meloidogyne javanica* and *Meloidogyne incognita* are the two major nematodes destroying cowpea (Oliveira et al., 2012).

Fungal Diseases/Pathogens Associated With Cowpea

Fungal pathogens are the topmost destructive agents/phytopathogens of planted crops globally (Fisher et al., 2012). Very many species of different genera of fungi destroy cowpea in the field and during the post-harvest stage. Furthermore, seed and soil-borne fungal pathogens have been implicated in the loss of cowpea production that sometimes rises to 100% (Mbeyagala et al., 2014). Notable fungal pathogens of cowpea include *Rhizoctonia solani*, *Colletotrichum* spp., *Fusarium oxysporum*, *Macrophomina phaseolina*, and *Sclerotium rolfsii* (Adegbite and Amusa, 2010; Pottorff et al., 2014).

Viral Diseases/Pathogens Associated With Cowpea

Viral pathogens can adversely impact cowpea productivity; some of these have been linked in some cases to cowpea losses of up to 100% (Nsa and Kareem, 2015). Their destructive mechanisms that negatively affect cowpea include the reduction they cause in the population/growth and development of Rhizobium, thereby reducing the necessary root nodulation in cowpea (Taiwo et al., 2014). Up to 40 viruses adversely affect cowpea yields globally. Some of the most devastating viral pathogens of cowpea are the cowpea aphid-borne mosaic virus (CABMV), cowpea wild mottle virus (CPMMV), and CPSMV (Boukar et al., 2013; Odedara and Kumar, 2017).

Parasitic Weeds

Parasitic weeds cause serious losses in cowpea production/yields (Li and Timko, 2009; Horn et al., 2015; Omoigui et al., 2017). Eliminating these weeds in the course of cowpea production is difficult because they could be dormant in the soil for upward of 20 years (Kamara et al., 2014). The major parasitic weeds

TABLE 2 | Highlight of constraints limiting productivity enhancement of cowpea plant in major producing nations of the world.

Productivity constraint	Crop of interest	Bioactive roles of stressors	References
Biotic limitation involving Cowpea Severe Mosaic Virus	*Vigna unguiculata*	The chlorotic lesion, mosaic formation, and necrosis	Oliveira et al., 2020
Combine abiotic stressors of CO_2, High temperature and UVB irradiation	*Vigna unguiculata*	Vegetative and reproductive growth stage impaired adversely	Singh et al., 2010
Drought stress	*Vigna unguiculata*	Reduction in vegetative biomass Photosynthesis, transpiration, and stomatal conductance	Cardona-Ayala et al., 2020
Abiotic limitation involving heavy metals (Chromium)	*Vigna unguiculata*	Adverse impact on nodulation and biological nitrogen fixation	Miranda et al., 2014
Biotic constraint caused by Legume Pod Borer (*Maruca vitrata* Fabricius) (LPB)	*Vigna unguiculata*	Complete crop failure due to feeding on all parts of cowpea	Sodedji et al., 2020
Biotic constraint caused by *Aplosporella hesperidica*	*Vigna unguiculata*	Adverse impact on cowpea leading to collar rot symptoms	Deepika et al., 2020a
Biotic constraint caused by *Fusarium equiseti*	Vigna unguiculata	Negative impact on cowpea resulting in root rot symptoms	Li et al., 2017
Biotic constraint caused by *Fusarium oxysporum*	*Vigna unguiculata*	Negative impact on cowpea resulting in stem and root rot symptoms	Shrestha et al., 2016b
Biotic constraint caused by *Fusarium proliferatum*	*Vigna unguiculata*	Negative impact on cowpea resulting in stem and dry root rot symptoms	Shrestha et al., 2016a
Biotic constraint caused by Singly and Interactive effects of cowpea mosaic viruses	Vigna unguiculata	Negative impact on Rhizobium nodulating ability	Taiwo et al., 2014
Biotic constraint caused by *Rhizoctonia solani*	*Vigna unguiculata*	Negative impact on cowpea resulting in collar rot and web blight symptoms	Vavilappalli and Celine, 2014
Biotic constraint caused by *Helminthosporium vignicola*	*Vigna unguiculata*	Negative impact on cowpea resulting in leaf spot disease symptoms	Sahoo and Beura, 2019
Biotic constraint caused by *Epicoccum nigrum*	*Vigna unguiculata*	Negative impact on cowpea resulting in leaf spot disease symptoms	Deepika et al., 2020b
The abiotic constraint of Drought on cowpea Landrace (A55)	*Vigna unguiculata*	Reduction in net productivity and photosynthetic ability	Gomes et al., 2020
The abiotic constraint of high temperature	*Vigna unguiculata*	Adverse impacts on physiology biochemistry and breeding traits in cowpea plant	Jha et al., 2020
Biotic constraint caused by *Dactuliophora mysorensis* sp. nov	*Vigna unguiculata*	Zonate leaf spot disease	Deepika et al., 2020c
Biotic constraint caused by *Nigrospora sphaerica*	*Vigna unguiculata*	Leaf spot disease	Deepika et al., 2021
The abiotic constraint of high salinity	*Vigna unguiculata*	Adverse impacts on chlorophyl content and eventual death	Dong et al., 2019
The abiotic constraint of high-temperature stress	*Vigna unguiculata*	Adverse impacts on plant development, with severe damage to vegetative and reproductive growth stages of cowpea	Barros et al., 2021
The abiotic constraint of combined high salinity and temperature stress	*Vigna unguiculata*	Adverse impacts on plant development, with the germination and vigor of cowpea plant, impaired	Nunes et al., 2019
Climate change limitation involving temperature and Relative humidity	*Vigna unguiculata*	Adverse impacts on the yield and development of cowpea plant as well as reduction in evapotranspiration	Cavalcante Junior et al., 2016
Biotic constraints caused by *Diplodia seratia*	*Vigna unguiculata*	Wilt and necrosis adverse effects on cowpea	Swilling et al., 2020

that adversely affect the enhancement of cowpea production in Africa are *Striga gesnerioides* and *Alectra vogelii* (**Figure 1**).

LOOKING AHEAD BEYOND THE CONSTRAINTS FOR COWPEA PRODUCTIVITY ENHANCEMENT WITH SUSTAINABLE BIOINOCULANTS AND SMART BIOTECHNOLOGICAL TECHNIQUES

It is worth re-emphasizing that the challenge of attaining enhanced cowpea productivity on a sustainable level is not merely a single limitation. Rather, it is a diversity of limitations requiring a high level of multi-tasking.

However, there are also multiple smart, and sustainable agro-biotechnological techniques that could be deployed in a sustainable manner to achieve improvements in cowpea productivity and production outputs. Elements of this technology, which is geared towards maximizing eco-friendliness and guaranteeing an improvement in safer agro-biotechnological productivity, are briefly listed, and their associated mechanisms of action are also explained.

1. The sustainable deployment of bio-inoculants (biofertilizers and biostimulants) to serve as an alternative to synthetic chemicals
2. The sustainable deployment of biological antagonists in the form of biopesticides to tackle pests in the field and during the post-harvest storage stage

3. The deployment of CSA practices as an adaptive technology option to mitigate the effects of climate change on the vulnerabilities of crop production
4. The deployment of smart and advanced biotechnological applications, such as metabolomics, transcriptomics, proteomics, and genomic-breeding tools for the improvement of cowpea varieties, which would possess the desired traits, such as drought tolerance, favorable salinity stress-tolerant levels, high yields, resistance to high temperatures and thermotolerance, resistance to disease, and a high potential for nodulation.
5. The application of conservation practices in agriculture

Sustainable Deployment of Bio-Based/ Microbial Resources as Alternative to Synthetic Agrochemicals

Microbial-based formulations have proved to be an effective alternative to the use of synthetic agrochemicals in crop production. These natural, eco-friendly and sustainable bioformulants are categorized as biopesticides, biostimulants, and biofertilizers.

To minimize crop losses and improve productivity, natural microbial-based formulations have been successfully deployed in agro-ecological crop production. The salient features of these resources are that they are cheaper, renewable, easy to handle, and more importantly, safe for human beings and the living environment (Kour et al., 2020; Castaldi et al., 2021).

To meet up with the challenge of feeding the rapidly increasing global population, there is a need to increase crop productivity. One popular means of solving the problem of global food insecurity is by boosting agricultural outputs/productivity through the application of synthetic agro-fertilizers.

Conventionally, synthetic agrochemicals are applied as inputs to intensify agricultural production systems. Various fertilizers, fungicides, herbicides, and pesticides are thus used in large-scale crop production systems. Initially, the advent of the chemical fertilizer was widely accepted because it helps to increase agricultural productivity and to solve global food consumption issues (Liu et al., 2015; Duan et al., 2016). However, the indiscriminate use of chemical fertilizers has led to air and groundwater pollution, which, mainly in the case of the latter has led to the eutrophication of water bodies (Vanlauwe et al., 2014). Also, the long-term effect of using chemical fertilizers results in bio-magnification and bio-accumulation in living organisms which have in their turn had negative impacts on the soil environment and ultimately on human and animal health (Calderón et al., 2017).

Therefore, the increasing concern of consumers and governments for food safety issues, has led stakeholders to explore newer ecologically and environmentally-friendly methods to replace or supplement the current chemical-based practices in agriculture. In fact, the use of bio-pesticides, bio-herbicides, and bio-insecticides has emerged as a promising alternative to chemical pesticidal products (Ahirwar et al., 2020).

Also, (Nicolopoulou-Stamati et al., 2016) reported that the use of chemicals in the form of pesticides, insecticides, and herbicides could affect the quality of the plant products and thus adversely affect human and animal health.

However, the search for environmentally and agro-ecologically sustainable alternatives to these synthetic agrochemicals has led to the deployment of quite an array of diverse forms of microorganisms being applied to function as biofertilizers, biostimulants, biopesticides, and plant growth promoters. Hence, they are being used to enhance a diversity of crop growth in numerous countries around the world, especially in the developing

FIGURE 1 | Microbial diseases of cowpea: **(A)** cowpea seed beetle, **(B)** yellow mosaic virus infected cowpea, **(C)** cowpea halo blight, **(D)** bacterial blight, **(E)** anthracnose, **(F)** cowpea mosaic diseased leaf, **(G)** bacterial bean blight, and **(H)** powdery mildew.

and emerging world (Igiehon and Babalola, 2017; Alori and Babalola, 2018; Omomowo and Babalola, 2019).

Different groups of microorganisms constitute different types of association with different host plants in the form of endophytic, epiphytic, and rhizospheric associations (Yadav, 2021). Thus, based on these associations, scientists have formulated bio-inoculants to solve the food security problem in an eco-friendly way.

Diverse terminologies have been used to qualify these metabolically and physiologically important microbial forms. They are known under terms such as biocontrol agents (BCAs), and are referred to as agriculturally beneficial microorganisms, e.g., arbuscular mycorrhizal fungi (AMFs), which are sometimes referred to as, among others, plant growth-promoting rhizobacteria, plant growth-promoting fungi (PGPFs), and plant growth-promoting bacteria (PGPBs). A lot of research in the field of applying microbial inoculants to different planted crops has been conducted by scientists and is still ongoing (Igiehon et al., 2019; Chaudhary et al., 2021; Chen et al., 2021). These beneficial species help to control or suppress plant diseases caused by pathogenic bacteria and fungi through different antagonistic mechanisms in that they produce antifungal and antibacterial compounds or feed as parasites on them (El-Sharkawy et al., 2018).

To solve the problem of food safety and the increasing concerns in respect of the environment in an eco-friendly manner, the use of biofertilizers, biopesticides, and biostimulants is gaining the necessary attention in the agricultural sector (Oleńska et al., 2020). Based on plant-microbial associations, the utilization of viable and sustainable microbiota or their groupings has long been established as a means to improve agricultural productivity, and is in fact on an upward rise (Chukwuneme et al., 2020; Adeleke and Babalola, 2021; Fasusi et al., 2021).

More importantly, with the advent of next generation sequencing technological availability and cheaper cost, research efforts in the field of metagenomics, metabolomics, proteomics, transcriptomics and genomics have revolutionize the prospects of applying plant growth-promoting microbiota as bioinoculants that are deployed as biofertilizer, biopesticides and biostimulants for the improvement of planted crops. With the advent of these advanced biotechnological techniques, researchers have elucidated studies on the root microbiome as the hidden treasure that possesses immense potential to revolutionize the strategies for improving plant growth, as well as abating biotic and abiotic constraints in plants (Mathur and Roy, 2021).

These root-associated microbiomes are known as prolific producers of phytohormones, mainly auxins, cytokinin, and ethylene as well as enzymes like the 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) and exopolysaccharides that help plants in inducing systemic resistance to both biotic and abiotic stressors. Newer and effective techniques have been deployed in isolating and characterizing root associated microbiome, and applying them as bioinoculants in improving the growth and development of planted crops (Liu et al., 2020; Romano et al., 2020).

The root microbiome consists of an enormous number of beneficial microbes such as plant growth-promoting rhizobacteria (PGPRs), fungal and bacterial endophytes and mycorrhizal fungi (Yu et al., 2019).

Metabolites that are secreted by this microbiota are associated with marked influences on plant growth promotion, response and mitigation to biotic and abiotic stressors. These bioactive metabolites include ACC deaminase, gibberellic acid (GAs), indole acetic acid (IAA), exopolysaccharides, melatonin, volatiles, and cytokinins (Jones et al., 2019; Qu et al., 2020).

It is anticipated that root exudates influence the rhizospheric microbial community and that analysis of the root microbiome signifies ecosystem functioning (Williams and de Vries, 2020). Therefore, a lot of research effort abound on exploration of the root microbiome as reservoir of novel microbial isolates and genes that may be beneficial as biofertilizers, biopesticides, and biostimulants in an era of climate change.

Plant growth-promoting rhizobacteria (PGPR) strains are able to produce IAA, solubilize phosphate, induce ACC deaminase, and chelate iron by producing siderophore. Therefore, their application is an effective means of alleviating stress in planted crops (Etesami and Jeong, 2018). The PGPR strains achieve improvement in the growth and tolerance of planted crops through the accumulation of compatible solutes like proline or glycine betaine, by enhancing the secretion of bioactive metabolites, as well as through inducing the expression of plant growth beneficial genes.

Recently, the Metabolomics profiling of *Sorghum bicolor* that was primed with PGPR isolates (*Bacillus* and *Pseudomonas*) and exposed to drought stress, induced systemic tolerance in the plants (Carlson et al., 2020).

Also, proteomic analyses of *Medicago truncatula* that was inoculated with *Sinorhizobium* sp. and exposed to drought stress, led to the upregulation of JA pathway and downregulation of ethylene biosynthesis which are vital for improved drought tolerance (Staudinger et al., 2016).

In addition, the inoculation of *Trichoderma* and *Pseudomonas* in rice plants that was subjected to drought stress induced the overexpression of antioxidative enzymes and the phenylpropanoid biosynthesis pathway, as well as other key drought responsive genes (Singh et al., 2020).

PGPR remains a promising option for improving crop drought resistance, as reveal in a transcriptomics study by Morcillo et al. (2021) applying the bioinoculant B. megaterium TG1-E1 on different tomato cultivars under drought conditions. The findings reveal several key mediators of TG1-E1-induced transcriptional regulation in tomato plants, including transcription factors, stress signaling components and regulators, and putative regulators of cell wall organization. Also, analysis of the metabolites indicated the presence of important compounds that include ethanolamine, amino acid, sugars, and pinitol, which aided in TG1-E1-triggered plant drought resistance.

By using high-throughput RNA-sequencing techniques (Thomas et al., 2019), characterized differentially expressed genes (DEGs) in rice roots upon inoculation with *A. brasilense*. The findings reveal plant growth promotion impacts, pathways and genes that are involved in the plant-microbe interactions.

Furthermore, in a study by Zhang et al. (2020) using culture independent 16S rRNA gene amplicon sequencing and culture-dependent functional analyses of *Alhagi sparsifolia* rhizosphere and root endospheric microbiome, identify key endophytic bacterial taxa and their genes facilitating drought resistance in wheat. Through comparative genomics analysis, a drought resistance-promoting strain was characterized, as well as the mechanisms deployed in colonization and enhancement of drought resistance in wheat was elucidated.

Deployment of Climate-Smart Agricultural Practices for Improving Productivity

One of the major challenges faced by humanity over the ages has been the task of tackling in a sustainable way environmental degradation and the consequences of climate change which are more pronounced in the case of natural ecosystems (Sarkar et al., 2020). The effects of climate change are more pronounced in agro-ecosystems because the sum total of all agricultural activities takes place on them and that is why they are the most vulnerable of all of the natural ecosystems (Dubey et al., 2020).

The deployment of ecologically and environmentally unfriendly practices such as the excessive intensification of agricultural practices on the land, the indiscriminate use of agrochemicals, such as pesticides, herbicides and fertilizers, as well as the consequences of anthropogenic activities, such as like urbanization, deforestation, industrialization, and the burning of fossil fuels, collectively result in greenhouse gas (GHG) emissions and the ultimate disruption of the agro-ecological balance (Lawrence and Vandecar, 2015; Dubey et al., 2016). To meet up to the challenges posed by the high consumption levels of a rapidly growing population has proved to be a huge task. This is especially true for the developing world where, under the changing climatic conditions, there is a need to adopt strategies and practices that are socially, economically, and ecologically acceptable in the management of our natural resources (Abhilash et al., 2016; Sarkar et al., 2017). Climate-smart agriculture presents various innovative practices that can be adopted to meet the global food demand while concomitantly mitigating the effects of unfavorable climatic conditions on the production of climatically vulnerable crops. CSA is based on existing knowledge, technologies, and sustainable agriculture (FAO, 2015) and presents an integrated approach to managing cropland, livestock, forests, and fisheries in order to achieve food security, reduced greenhouse gas emissions and to contribute to other development goals in the face of climatic changes (Palombi and Sessa, 2013; **Figure 2**).

According to Kumar et al. (2019), some of the CSA practices and technologies are able to mitigate the effects of climate change on the agro-ecosystem, to boost agricultural production and to reduce the effects of GHGs. They include the use of quality seeds and the planting of well-adapted crops, effective biodiversity management, and integrated pest management systems, efficient water management, sustainable land and soil management to ensure increased crop production, and sustainable and efficient mechanization.

Other CSA mitigation practices include low-input sustainable agriculture (LISA) practices, which focus on safe farming and that incorporate local knowledge of the farming system, and in so doing, produce abundant, nutritious, profitable food products without causing negative effects to both the natural agro-ecosystem and human health (Najafabadi et al., 2012). According to Sarkar et al. (2015) indigenous technical knowledge (ITR) concerns the knowledge that local people have gathered through their interactions with nature and that has allowed them to adopt mitigating measures to counter the effects of climate change and thus to boost their crop production.

Also, simulation model studies are vital tools that can be used to conduct studies of different agro-ecological regions in order to implement sustainable agricultural measures, to achieve effective and maximum production levels (Sarkar et al., 2020). Organic farming also goes a long way to reducing the effect of GHG emissions (Rakshit et al., 2010).

Importantly, (Cammarano et al., 2020) used the Agricultural Model Intercomparison and Improvement Project (AgMIP) as a tool that, in the face of the prevailing drought problem in the northeastern area of Free-state, South Africa, incorporated data about climate change, crops and the economy to provides and implement adaptation strategies to improve and increase the production of maize in this region. Likewise, (Ishikawa et al., 2020) used the farmers' participatory varietal selection (FPVS) method to collect information from local farmers in the southern regions of Burkina Faso, in West Africa. They used the collected data to gather information on how to breed and select newly improved drought-resistant cowpea seeds for maximum production, which would prove to be economically and socially beneficial.

Prospects of Advanced Multi-omics Biotechnological Techniques for Improving Cowpea Productivity

In this modern era, where there is a notion of smart biotechnological techniques that can turn around the immense challenges of optimizing agricultural system outputs productivity, the multi-omics biotechnological tools are usually the game-changer. These multifaceted biotechnological techniques encompassing genomics, transcriptomics, proteomics, and metabolomics offer great prospects for improving crop protection, crop yields/productivity, and for ensuring nutritional food sources that are safe and secured for human consumption.

Through the application of the techniques of genomics, transcriptomics, proteomics and metabolomics, plant breeding has improved, and biotic and abiotic stress-resistant and resilient crop cultivars have been developed, thus leading to the production of better-quality crops.

Multi-omics biotechnological tools encompass a knowledge of analytical chemistry, computational biology, and bioinformatics analysis, as well as other thematic areas of biology, to facilitate a systematic approach to research studies, which would then lead to crop production and productivity enhancement.

Metabolites, proteins, and genes are specific components that are targeted and researched to improve crop cultivars and to better understand their growth characteristics.

These smart biotechnological techniques are advanced, concise, precise, and valuable tools that can be specifically targeted for improving crops. In fact, they are vital tools for sparking the latest green revolution in agricultural productivity. They can be used to introduce genes, proteins, or metabolites of interest with good traits to improve and intensify the productivity of planted crops. Thus, fewer agro-resource inputs would then be necessary in agricultural systems to attain better agro-product outputs.

Multi-omics biotechnological tools can be deployed to reveal key information on (plants and microbes). Furthermore, these tools could be applied to orchestrate metabolic and physiological changes, and also in genetic engineering for crop improvement (Chassy, 2010). Multi-omics techniques can also be used in breeding transgenic crops with specific key agronomic traits (Ahmad et al., 2012).

The multi-omics biotechnological tools, namely genomics, transcriptomics, proteomics, and metabolomics, are inter-woven techniques, that are closely linked and that can be applied to overcome the daunting challenges of feeding the burgeoning global population in this era of climatic vulnerabilities. They can also be deployed to consolidate the foremost producer status of the African continent in that they are able to enhance cowpea productivity and production.

Genomics

Genomics is the foremost pioneer omics that is presented as an advanced biotechnological technique and that uses genes and the genome transformation of plants and microbes for molecular breeding in order to establish improved crop cultivars. Genomics techniques are fast and precise, and can be selectively used to highlight the functional genes of desired traits for the improvement of a plant. Specifically, genomics techniques can

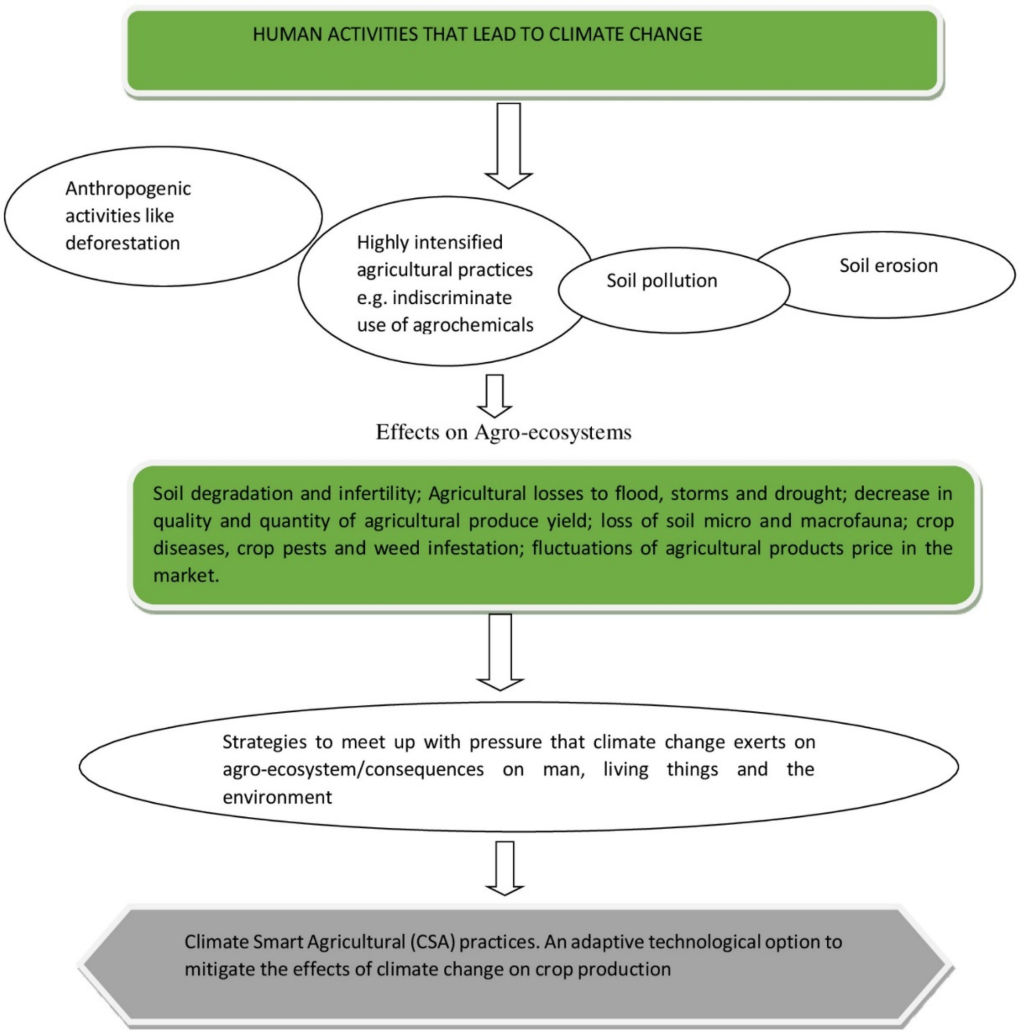

FIGURE 2 | Schematic highlighting of the different pressures exerted by climatic change and CSA as a mitigating practice to improve agricultural production.

be applied in the modification of genes in that they add genes to a plant, or by using RNAi, they knock down genes from a plant, in so doing, accomplish phenotypic traits of interest faster than the conventional plant-breeding method does. In the quest to enhance cowpea productivity, genomics-based smart biotechnology techniques have been deployed to breed improved cowpea cultivars. In such cases, the focus is on looking at the whole genome in terms of genotypic diversity and fingerprinting for cultivar improvement traits (Xu et al., 2017; Wu et al., 2018; Seo et al., 2020).

Molecular-based approach has been deployed towards improving cowpea cultivars using molecular markers and genomic-breeding techniques. An authenticated cowpea genetic resource is the foundation for efficient breeding and conservation. Genotypic diversity assessment is done by using both phenotypic and molecular traits characterization.

Research efforts at genetic breeding of cowpea cultivars using these DNA markers have been investigated by Kolade et al. (2016); Chen et al. (2017a).

SNPs are the preferred markers in genotypic assessment studies due to their wide distribution in the genome and they are highly efficient (Nkhoma et al., 2020).

Following advancement in plants genome resources, molecular markers are now widely deployed in genetic variability assessment, molecular breeding, and DNA fingerprinting (Su et al., 2018).

Among the genomic-breeding research effort, the Illumina Cowpea iSelect Consortium Array (Muñoz-Amatriaín et al., 2017) was an important landmark. This great research effort led to the development of a minicore (referred to as the "UCR Minicore") which composed of 368 domesticated cowpeas selected from a larger set of _5000 accessions comprising the UC Riverside cowpea collection.

This array contained 51,128 SNPs derived from whole genome sequences (WGS) of 37 diverse cowpea accessions. Single nucleotide polymorphism (SNPs) is distributed uniformly in cowpea genome and indicates variation in genes of cowpea. Thus, they provide an ideal resource for cowpea molecular breeding and new variety protection. SNPs are vital genomics techniques for assessment of key traits in cowpea like constructing genomic linkage map, for QTL, for the detection as well as assessing germplasm genetic diversity (Paudel et al., 2021).

Also, the majority of the international institute of tropical agriculture (IITA) minicore collection (298 accessions) was genotyped using genotypic base sequencing (GBS) with 2,276 SNPs, this identified three major subpopulations (Fatokun et al., 2018), but showed dispersion of West and Central African accessions across the three subpopulations.

Another giant stride in the progress of cowpea genomics study was achieved by using next generation sequencing advancement (Lonardi et al., 2019) to authenticate the whole genome of an improved cowpea genotypes, thus providing a key resource that is crucial to deciphering the morpho-physiological response of cowpeas.

Building on this developments and report of full SNP data release for the UCR Minicore, numerous follow up studies has been investigated for more focus cowpea research, that include studies on pattern of seed coat (Herniter et al., 2019), color of seed coat (Herniter et al., 2018), size of seeds (Lo et al., 2019), resistance to bruchid infestation (Miesho et al., 2019), plant herbivore resistance (Steinbrenner et al., 2020) and pod shattering (Lo et al., 2021).

With better comprehension of genomic basis of variation, genome-wide association studies (GWAS) studies have been highlighted in cowpea for pod length (Xu et al., 2017), root architecture (Burridge et al., 2017), cowpea plant improvement traits, as well as the flowering period (Muñoz-Amatriaín et al., 2021). All these findings are appreciated because cowpea genetic diversity assessment is necessary for strengthening breeding programs in order to develop high yielding dual-purpose cultivars with good grain and fodder yields.

Transcriptomics

Transcriptomics is a vital biotechnological technique that makes for a comprehensive understanding of genomics functionality (Valdés et al., 2013). Transcriptomics regulates the expression of genes in the context of biotic and abiotic stresses. Transcriptomics is a dynamic technique that expresses genes at any given time and under different circumstances.

With the advancement of functional genomics, the identification of novel genes having vital functions in plant growth/development and adaptation to stressful conditions have been characterized for crop cultivars (Zhang et al., 2017). Also, RNA expression profiling is important in understanding plant functionality.

Transcriptomics as a part of multi-omics biotechnological techniques have led to the detection of novel genes useful in response to both biotic and abiotic stresses in plants.

Transcriptomics approaches utilizes high-throughput sequencing platforms to generate enormous useful transcript data through techniques such as RNA sequencing, microarray and serial analysis of gene expression (SAGE) to elucidate non-coding and coding RNAs expression profiles to plant biotic and abiotic stresses (Cramer et al., 2011; Santos et al., 2018).

Several factors like the duration and extent of stress conditions, determines the adaptability and tolerance of a plant to stresses. However, experimental design, handling of tissue samples, isolation of RNA and stability of RNA also play major role in any transcriptomic analysis (Gokce et al., 2020).

The characterization of different parts of the cowpea plant through transcriptomics has been carried out in studies that express the diverse genes essential for cowpea growth and development. The stress-resilient genes have also been characterized and their role in the overall improvement of cowpea has also been highlighted (Yao et al., 2016; Chen et al., 2017b; Amorim et al., 2018; Spriggs et al., 2018).

Proteomics

Proteins are a vital constituent of plants. Large quantities of protein are responsible for the key functional roles that plants perform. As a smart biotechnological technique, proteomics entails the expression of functional characteristics, structural features, and the translation/manifestation of beneficial traits in plants. Another important attribute of the proteomics technique

is that it can be used to better elucidate a pesticide's mode of action, its mechanisms, and its biodegradation. The outputs/benefits that can be derived by applying proteomics include the authenticity of the food product, the assurance of food security that it represents and the sustainability of energy that the food product offers to consumer, as well as the maintenance of an environmental balance (Agrawal et al., 2012; Landim et al., 2017).

Proteomics as a key branch of "omics" technology aims at investigating protein's structure, function, as well as their interactions with other proteins and other components, including the modifications arising from these interactions through the use of analytical techniques.

Proteomics approach involves analysis and the elucidation of functional expression of proteins in order to understand biological processes (Iwamoto and Shimada, 2018; Chen and Weckwerth, 2020).

Proteins are vital components of all biological process. To fully comprehend the response of plants to biotic and abiotic stresses, proteomics studies must be assessed, along with other multi-omics technology (Gokce et al., 2020). Changes in gene expression influences appropriate response in protein composition/abundance and affect cellular functions.

Proteomics studies are assessed using spectroscopic method usually by mass spectroscopy (MS)-based technology. This is done by MALDI-TOF MS or with liquid chromatography mass spectrometry (LC–MS) techniques. Proteomics studies have led to the characterization of different stress response proteins in planted crops under stress conditions (Rathi et al., 2016; Kosová et al., 2018; Matamoros and Becana, 2021).

Metabolomics

Metabolomics is an advanced and powerful smart biotechnique that identifies functionally active metabolites, their roles, and the diverse biochemical processes that the metabolites play in plant genotypes and phenotypic expressions (Führs et al., 2009; Aliferis and Chrysayi-Tokousbalides, 2011). Metabolomics tools can be deployed in identifying and monitoring physiological responses in plants and the metabolic pathways or linkages arising from the biotic and abiotic stress exerted upon plants. In fact, these tools are able to enhance crop development and improve plant health (Dixon et al., 2006; Goufo et al., 2017).

In a study on drought response of three cowpea landraces using leaf physiological and metabolites profiling assessment, (Gomes et al., 2020), used gas chromatography time of flight mass spectrometry (GC-TOF-MS) and reported that cowpea landrace A116 genotype drought response was best with the accumulation of 14 bioactive metabolites that included proline, valine, and rhamnose and raffinose, isoleucine, fucose, urea, alanine, sucrose, and putrescine.

Also, in a study on metabolites (polyphenols and carotenoids) in *V. unguiculata* sprouts by Yeo et al. (2018), investigated using high-performance liquid chromatography (HPLC), electrospray ionization-mass spectrometry (ESI-MS), gas chromatography–mass spectrometry (GC–MS), and gas chromatography, 39 hydrophilic compounds were identified and quantitated. Thus, the study provides a new approach for enhancing the carotenoid and phenylpropanoid production of *V. unguiculata*.

Metabolomics as a powerful omics-based approach can be applied as a tool to explore different aspects in plant breeding, the regulatory mechanisms related to plant growth and development (including those related to crop productivity and performance), adaptation to biotic and abiotic stresses, nutritional improvement, and selection of cultivars for agriculture. Metabolomes are simply metabolites (both secondary and primary) having low molecular weight (usually <1,500 Da), including their precursors and intermediates of the corresponding biosynthetic pathways. Such compounds are considered the end products of gene expression and protein activity, modulating processes between the genome and environment and indicating the functional status of the organism. Moreover, they are an indispensable part of the plant metabolism, influencing all biological processes, such as plant biomass and architecture, and those involved in plant defense or adaptation to biotic and abiotic stresses (Sharma et al., 2021).

In a comprehensive study on cowpea osmoregulation response under drought stress, (Goufo et al., 2017) investigated and provided a detailed metabolic profile of a broad range of primary and secondary metabolites in cowpea, including elemental solutes using (leaves and roots). Their findings revealed that the mechanisms deploy in modifying cowpea metabolism response to water deficit is through interplay between the shikimate and arginine/proline pathways, leading to three drought-responsive metabolites, namely galactinol, proline, and quercetin 3-O-6"-malonylglycoside.

In a study aimed at identifying metabolic responses and key factors associated with Mn tolerance using Mn-tolerant and Mn-sensitive genotypic cultivars; (Führs et al., 2012) reported that manganese tolerance is a consequence of genotypic/constitutive higher concentrations of metabolites detoxifying manganese and reactive oxygen species.

Agricultural Conservation Practices for Crop Productivity Enhancement

Agricultural conservation practices are simple and cost-effective techniques for achieving sustainable productivity enhancement in planted crops. This technique is based on the use of a limited number of natural resources as inputs. Crop rotation, mixed farming methods, intercropping, the manual tillage of the soil, and the use of crop residues to reduce soil moisture loss through mulching are some of the methods employed. These simple, cost-effective techniques, using a limited number of resources as inputs, ultimately lead to crop productivity enhancement.

However, in order to effectively enhance crop productivity, it is necessary to find effective ways of adapting to climate change and the vulnerability it imposes on crops and farmers. The objective should always be to mitigate the adverse impacts of climate change on the environment (Lipper et al., 2014).

Conservation agriculture improves the quality of the soil – biologically, physically, and chemically, and thus ultimately makes an impact on the crop production outputs, with both positive and sustainable effects (Basavanneppa et al., 2017).

In addition to improving crop yields and achieving sustainability, conservation agriculture also augments microbial diversity and enhances microbial functionality (Yadav et al., 2017).

Conservation agriculture is increasingly being promoted as an adaptive climate-smart agricultural technique that can minimize the adverse effects of synthetic agrochemical usage in agricultural systems that generally lead to poor and depleted soil fertility (Pretty and Bharucha, 2014).

As an agroecological system tool, conservation agriculture can lead to enhanced crop productivity, the diminished use of agro-resource inputs, environmental sustainability, and advance the income generation potential of farmers (Prasai et al., 2018; Pariyar et al., 2019).

Conservation agriculture helps in enhancing soil fertility and in reducing the cost of the associated inputs. The application of conservation practices improves soil water conservation and soil moisture, minimizes runoff, reduces moisture losses through evaporation, boosts the biological properties of the soil, and enhances crop productivity (Hossain et al., 2015).

The beneficial effects of conservation agriculture on crop productivity can be classified into three main categories:

1. Conservation agriculture provides agronomic growth benefits and enhances soil health.
2. The sustainability of the environment and the soil and the sociological benefits of the agricultural production system are enshrined.
3. Conservation agriculture can lead to enhanced economic benefits and also improve efficiency in the agricultural sphere.

In a nutshell, conservation agricultural practices enhance the quality of planted crops, improve the fertility of the soil, and ultimately provide both socioeconomic and environmental benefits in a sustainable manner (Bell et al., 2019; Calcante and Oberti, 2019).

The applications of bio-based, renewable, agro-ecologically balanced, and advanced smart biotechnological techniques in achieving improvements in the productivity of cowpea and a few selected crops of economic importance are presented in **Table 3** as effective sustainable alternatives for crop improvement.

TABLE 3 | Sustainable deployment of bioinoculants and smart biotechnological techniques for the productivity enhancement of cowpea and some selected food crops.

Beneficial microbial inoculants	Crop of interest	Bioactive roles of inoculants	References
Co-inoculation of Bradyrhizobia strains	*Vigna unguiculata*	Growth improvement of cowpea	Do Nascimento et al., 2021
Mutant of Glomus sp. and Trichoderma harzianum (AMF60+TH)	*Vigna unguiculata*	Used for growth promotion and biocontrol of powdery mildew disease of cowpea	Omomowo et al., 2018
Mutant strains of Glomus versiforme and Trichoderma harzianum	*Vigna unguiculata*	Used for growth promotion and biocontrol of Cercospora leaf spot disease of cowpea	Omomowo et al., 2020
Genome-wide association studies (GWAS)	*Vigna unguiculata*	Enhancement of drought tolerance of cowpea	Ravelombola et al., 2021
Chitin-binding protein studies (CBV)	*Vigna unguiculata*	Toxic influence and reduction in larval mass and length of *Callosobruchus maculatus* (Cowpea weevil)	Ferreira et al., 2021
Genome-wide association studies (GWAS), meta-analysis and Sequence homology combination	*Vigna unguiculata*	Identification of candidate genes for cowpea seed size enhancement	Lo et al., 2019
Synergistic effects of co-inoculation with different AMF isolates and *Sinorhizobium meliloti*	*Vigna unguiculata*	Enhancement of above ground biomass production and nitrogen content	Kavadia et al., 2021
QTL mapping using recombinant inbred line (RIL) and transcriptome analysis	*Vigna unguiculata*	Identification of candidate genes for root-knot nematode resistance (Rk) in cowpea	Santos et al., 2018
SSR typing for diversity assessment and nitrogen fixation potentials	*Vigna unguiculata*	Identification of SSR marker for nitrogen fixation and other symbiosis-related traits	Mohammed et al., 2020
Synergistic influence of *Trichoderma* and *Bradyrhizobia* on cowpea growth improvement	*Vigna unguiculata*	Enhancement of cowpea growth biomass and photosynthetic pigments	Mendes et al., 2020
Proteomic approaches using miRNAs and Argonaute genes in response to CPSMV stress	*Vigna unguiculata*	Detection of miRNAs and genes that elicits a response to CPSMV	Martins et al., 2020
Transgenic cowpea plant response to *Maruca vitrata* legume pod borer	*Vigna unguiculata*	Improvement in the prevention of damage caused by pod borer due to genetically engineered cowpea	Kumar et al., 2021a
Deployment of Entomopathogenic fungi together with intercropping in managing *Aphis craccivora* infestation of cowpea	*Vigna unguiculata*	Reduction in the damage caused by an aphid infestation of cowpea	Mweke et al., 2020
Deployment of conservation agricultural practices of no-tillage and planting of cover crops	*Vigna unguiculata*	Improvement in soil carbon and nitrogen nutrient concentration, as well as good adaptation to water stress	Guzzetti et al., 2020
Deployment of yeast isolates in controlling *Rhizoctonia solani* infestation in cowpea	*Vigna unguiculata*	Effective in the biocontrol of damping-off and stem rot of cowpea plants caused by *R. solani*	De Tenório et al., 2019
Deploying encapsulated *Pseudomonas libanensis* in alleviating cowpea drought stress	*Vigna unguiculata*	Encapsulation of the beneficial microbe highlighted its positive impact on managing drought stress in cowpea	Souza-Alonso et al., 2021

(Continued)

TABLE 3 | Continued

Beneficial microbial inoculants	Crop of interest	Bioactive roles of inoculants	References
Application of embryonic axis explants for efficient regeneration, transformation, and genome editing of cowpea	*Vigna unguiculata*	CRISPR/Cas was used successfully to develop transgenic cowpea plantlet	Che et al., 2021
Application of *Bacillus subtilis* Dcl1in cowpea plant as growth enhancer, biocontrol, and abiotic stress abatement agent	*Vigna unguiculata*	Improvement in cowpea growth, biotic and abiotic stress effectors	Jayakumar et al., 2021
Deployment of MgO nanoparticles in enhancing cowpea growth and controlling nematode infestation	*Vigna unguiculata*	Improvement in cowpea growth and control of root-knot nematode infestation	Tauseef et al., 2021
Deploying *Bacillus sp.* Fcl1as pesticide toxicity alleviating and growth-promoting impact on a cowpea plant	*Vigna unguiculata*	Improvement in cowpea growth and also toxicity alleviating effects of pesticide	Juby et al., 2021
Application of *Bacillus cereus* NDRMN001 and *Kosakonia sp.* MGR1 to improve cowpea growth and remediate heavy metal toxicity	*Vigna unguiculata*	Enhancement in the growth characteristics of cowpea plant and also the remediation of heavy metal toxicity	Narayanan et al., 2021
Inoculation of *Bradyrhizobium* and salicylic acid effects in mitigating water stress deficit in cowpea plant	*Vigna unguiculata*	Effective in the improvement of cowpea growth, proline content, superoxide dismutase, and ascorbate peroxidase	De Andrade et al., 2021
Inoculation using *Bradyrhizobium* BR3267 with phosphorus and potassium fertilizer improves cowpea growth	*Vigna unguiculata*	The combined inoculant treatment was effective in increasing cowpea yield and growth parameters	Emmanuel et al., 2021
Interactive influence of *Bacillus subtilis* that were co-inoculated with mine water on the physiological and nutritional growth enhancement of cowpea	*Vigna unguiculata*	*Bacillus subtilis* co-inoculated with mine water, sequester heavy metals, and improve nutritional content and growth of cowpea	Nevhulaudzi et al., 2020a
Influence of inoculation using dark septate endophytic fungi on cowpea productivity under salinity stress	*Vigna unguiculata*	Improvement in nutritional content and photosynthetic rate of cowpea plant	Farias et al., 2020
Application of indigenous mycorrhizal and nano-$Ti0_2$ in reducing cowpea oxidative stress and Cd uptake	*Vigna unguiculata*	There was a reduction in both the Cd metal uptake and oxidative stress of cowpea due to co-inoculation treatment	Ogunkunle et al., 2020
Response of field-grown cowpea to inoculation with *Bradyrhizobium*	*Vigna unguiculata*	Improvement in agronomic growth parameters of cowpea plant due to bioinoculant treatment	Ayalew et al., 2021
Seed inoculant treatments using rhizobacteria and mycorrhizal improve the growth and nutrition of cowpea under water stress	*Vigna unguiculata*	Improvement in growth and nutritional content of cowpea due to mycorrhizal and rhizobacteria application *via* seed coating	Rocha et al., 2020
Inoculation with Rhizobia strains and AMF species	*Glycine max*	Yield and nutrient improvement of soybean	Igiehon et al., 2021
Inoculation with *Rhizobium* and Mycorrhizal Fungi species	*Glycine max*	Yield improvement of soybean under drought stress	Igiehon and Babalola, 2021
Inoculation with *Trichoderma* Isolates	*Glycine max*	Biocontrol of destructive nematode of soybean	De Oliveira et al., 2021
Bacillus sp. PS2 and PS10	*Zea mays*	Plant growth and yield enhancement of Maize	Chaudhary et al., 2021
Mixed inoculation of *Bacillus cereus* BI-8 and *Bacillus subtilis* BI-10	*Zea mays*	Plant growth and nutrient yield enhancement of Maize	Fouda et al., 2021
Azotobacter chroococcum	*Zea mays*	Soil health improvement and nutrient yield enhancement of Maize	Song et al., 2021
Application of different Microbial inoculants	*Wheat*	Improvement in wheat growth and soil microbiome diversity	Chen et al., 2021
Inoculation with endophytic fungi *Nectria haematococca*	*Green gram*	Growth and nutritional improvement of Green gram	Muthukumar and Sulaiman, 2021
Inoculation with Potassium solubilizing B*acillus cereus*	*Potato*	Growth and yield improvement of potato	Ali et al., 2021
Application of different Arbuscular Mycorrhizal fungi	*Cicer arietinum L*	Improving Arsenic metalloid tolerant and yield of chickpea	Garg and Cheema, 2021
Bacillus spp	*Pearl Millet*	Used as a biocontrol agent for fungal pathogens affecting Pearl millet	Kushwaha et al., 2020
Bacillus subtilis	*Oryza sativa*	Biocontrol agent for control of fungal disease of rice	Kumar et al., 2020
Bacillus pumilus strain JPVS 11	*Oryza sativa*	Improving growth/yield and salinity tolerance in rice	Kumar et al., 2021b
Inoculation with *Piriformospora indica*	*Oryza sativa*	Improving yield and arsenic tolerance in rice	Ghorbani et al., 2021
Single and co-inoculation with mycorrhiza	*Phaseolus vulgaris*	Improving yield and nutrition of snap bean	Beltayef et al., 2021
Inoculation with single and co-inoculation with AMF and PSB	*Zea mays*	Improvement in productivity of maize	Pacheco et al., 2021
Inoculation with *Funneliformis mosseae*	*Triticum aestivum L.*	Improving wheat productivity and enhancing soil health	Duan et al., 2021
Single and co-inoculation with *Piriformospora indica* and *Pseudomonas putida*	*Triticum aestivum L*	Enhancement in growth and nutritional status of wheat	Abadi et al., 2021

MECHANISTIC PHYSIOLOGICAL PROCESSES/ACTIONS INVOLVED IN THE DEPLOYMENT OF SMART BIOTECHNOLOGICAL TECHNIQUES TO IMPROVE COWPEA PRODUCTIVITY

Owing to the application of smart biotechnological techniques, diverse physiological and metabolic modes of activities are involved in improving the productivity outputs of cowpea. This can be achieved through direct and indirect modes of action such as those involved in, among others, directly supplying nutrients to plants, suppressing phytopathogens through the production of plant growth effectors, regulating the hormonal balance of plants, triggering various immune responses, and through the secretion of vital proteins (Santos Villalobos et al., 2018; Villarreal-Delgado et al., 2018). An overview is presented in **Figure 3**.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Yes! Daunting and herculean are the constraints that almost all African nations face in terms of improving their crop yields and productivity in the light of the current global challenges. These are aggravated by the global pandemic, climatic change, and a burgeoning population growth rate. However, a concerted effort directed at achieving the sustainable development goals of reducing poverty and eliminating hunger and malnutrition is what is called for. The first priority is to strive for an improvement in the agricultural system. The use of agro-ecologically balanced improvement techniques remains the surest way to achieve this. The constraints of low yields and the limited productivity of the cowpea, a valuable indigenous African legume at the forefront on the continent in terms of its potential as a food product, were highlighted in this review. The prospect of circumventing and overcoming these constraints is in fact a very real possibility. An essential requirement would be the use of viable tools. These would include the deployment of sustainable, ecosystem-friendly smart biotechnological tools: the application of bioinoculants, climate-smart agricultural practices, agricultural conservation techniques, as well as advanced multi-omics biotechnological tools for the improvement in cowpea yields and productivity enhancement. However, there are research gaps that still need to be worked upon to ensure success. Several collaborative efforts should be directed at building the capacity of plant breeders, agronomists, biotechnologists, and other allied stakeholders in the agri-food value chain in Africa to embrace these sustainable biotechnological techniques. Further research efforts should be directed at attaining specific functional traits in cowpea plants, in order to develop locality adaptive and climate-specific traits – the latter in response to climatic vulnerabilities and other external stressors – all for the benefit of the planted cowpea crop. Furthermore, efforts should also be directed at exploring an integrative and holistic approach to systematic biology that would combine systemic knowledge in the field of multi-omics biotechniques, genetic engineering tools, precision agricultural practices, techniques in genome editing technology (CRISPR/Cas), synthetic biology, bio-computational technology, as well as the emerging field of agro-nanobiotechnology for the improvement of the cowpea crop. The use of a synthetic microbial consortium, (SYNCOMs) should be deployed to the field to vigorously phenotype cowpea cultivars that are trait-specific and can be grown as a crop adapted to a niche environment, and favored by most cowpea producing marginal communities in Africa (**Figure 4**).

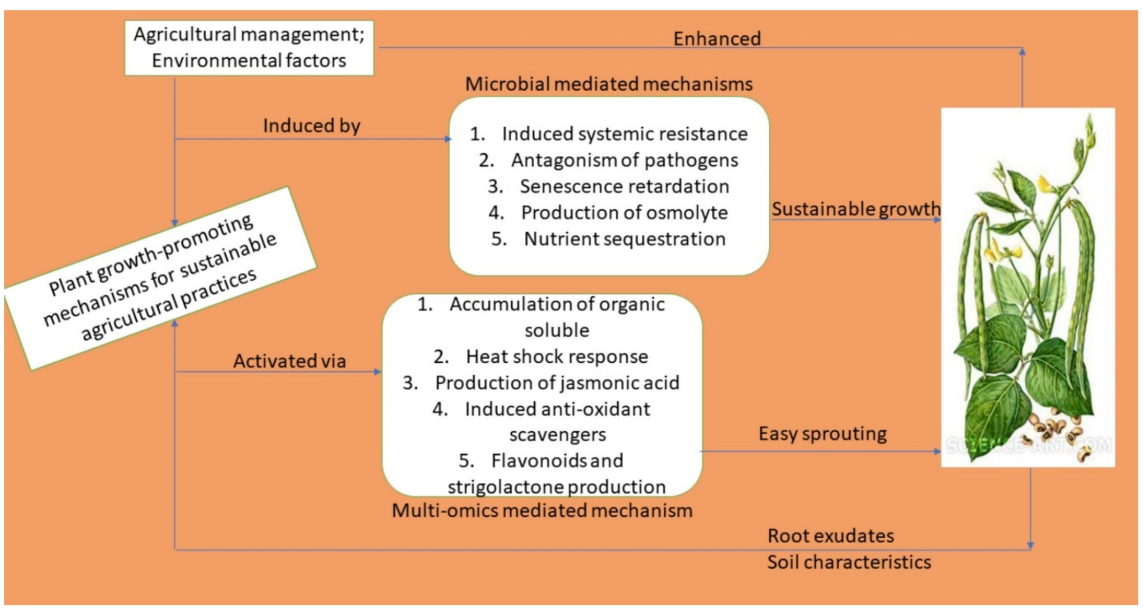

FIGURE 3 | Mechanisms of action of smart biotechnological techniques deployed in cowpea productivity enhancement.

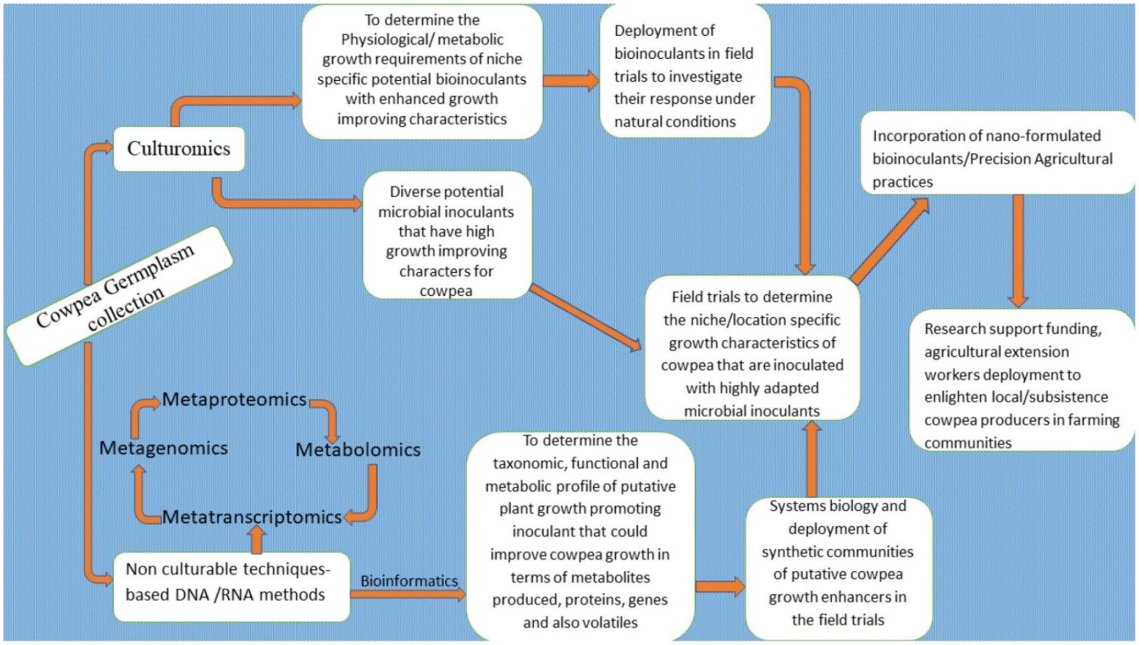

FIGURE 4 | Holistic approach and futuristic perspectives for improving cowpea productivity enhancement in Africa and consolidating the continent's foremost producer status.

There is, however, a need to integrate socioeconomic policy into this sound biotechnological know-how system in order, to reach a balance, as well as a guaranteed and steady flow of the necessary financial support for the associated research efforts. Attention should also be directed to developing a policy of backward integration to achieve positive and sustainable results in the context of improving and enhancing the productivity and yields of cowpea, a key leguminous crop that is considered to be of great importance in Africa.

AUTHOR CONTRIBUTIONS

OOB conceptualized the topic. OIO conducted the literature search and undertook the drafting and writing of the manuscript, while the final editing was carried out by OOB. The authors made substantial intellectual contributions to the work and agree to be accountable for the content of the work.

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Evidence for the Application of Emerging Technologies to Accelerate Crop Improvement – A Collaborative Pipeline to Introgress Herbicide Tolerance Into Chickpea

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Accelerating genetic gain in crop improvement is required to ensure improved yield and yield stability under increasingly challenging climatic conditions. This case study demonstrates the effective confluence of innovative breeding technologies within a collaborative breeding framework to develop and rapidly introgress imidazolinone Group 2 herbicide tolerance into an adapted Australian chickpea genetic background. A well-adapted, high-yielding desi cultivar PBA HatTrick was treated with ethyl methanesulfonate to generate mutations in the *ACETOHYDROXYACID SYNTHASE 1* (*CaAHAS1*) gene. After 2 years of field screening with imidazolinone herbicide across >20 ha and controlled environment progeny screening, two selections were identified which exhibited putative herbicide tolerance. Both selections contained the same single amino acid substitution, from alanine to valine at position 205 ($A_{205}V$) in the AHAS1 protein, and KASP^{TM} markers were developed to discriminate between tolerant and intolerant genotypes. A pipeline combining conventional crossing and F_2 production with accelerated single seed descent from $F_{2:4}$ and marker-assisted selection at F_2 rapidly introgressed the herbicide tolerance trait from one of the mutant selections, D15PAHI002, into PBA Seamer, a desi cultivar adapted to Australian cropping areas. Field evaluation of the derivatives of the D15PAHI002 × PBA Seamer cross was analyzed using a factor analytic mixed model statistical approach designed to accommodate low seed numbers resulting from accelerated single seed descent. To further accelerate trait introgression, field evaluation trials were undertaken concurrent with crop safety testing trials. In 2020, 4 years after the initial cross, an advanced line selection CBA2061, bearing acetohydroxyacid synthase (AHAS) inhibitor tolerance and agronomic and disease resistance traits comparable to parent PBA Seamer, was

entered into Australian National Variety Trials as a precursor to cultivar registration. The combination of cross-institutional collaboration and the application of novel pre-breeding platforms and statistical technologies facilitated a 3-year saving compared to a traditional breeding approach. This breeding pipeline can be used as a model to accelerate genetic gain in other self-pollinating species, particularly food legumes.

Keywords: mutation, imidazolinone, sulfonylurea, marker-assisted selection (MAS), accelerated single seed descent, sparse phenotyping

INTRODUCTION

Achieving food security under increasingly hostile environmental conditions[1] requires rapid innovation across all sectors involved in food production (Varshney et al., 2021). Effectively harnessing pre-breeding tools will be key to improving the rate of genetic gain in crop and horticultural species. For more than two decades, tools such as diagnostic markers for trait selection have improved the efficiency of plant breeding programs (Eagles et al., 2001; Xu et al., 2017). More recently, techniques to accelerate lifecycle turnover using modified single seed descent have been proposed to further truncate breeding pipelines in a range of species, including food legumes (Ochatt et al., 2002; Mobini et al., 2015; Croser et al., 2016; Mobini and Warkentin, 2016; Ribalta et al., 2017; Watson et al., 2018; Hickey et al., 2019; Cazzola et al., 2021). Missing from the literature is evidence of how these complementary established and emerging pre-breeding techniques can be combined and harnessed in a genetic improvement program to reduce the time to variety release. For food legumes, genetic improvement programs are generally in the public domain and under-resourced compared to cereals and oilseeds. As a result, breeding programs need to be agile to maximize efficiency involving collaboration with public pre-breeding at other institutions. Here, we present a case study of cross-institutional implementation of a compressed breeding pipeline to deliver an improved chickpea (*Cicer arietinum* L.) cultivar bearing a mutagenesis-derived gene for herbicide tolerance, a critical need of Australian grain growers for sustainably managing their farming system and improving production.

Among emerging pre-breeding techniques, the reduction of generation cycle time is the most cost-effective way to increase the rate of genetic gain (Cobb et al., 2019). Rate of genetic gain is measured by the breeders equation (Lush, 1943) where generation cycle time is the denominator and thus any time-saving innovation is beneficial to the overall rate of gain (Li et al., 2018). For chickpea, an accelerated single seed descent (aSSD) platform (modified from Croser et al., 2016) has been applied within the Australian breeding program on a commercial scale since 2015. Based on the principles of single seed descent (Goulden, 1939; Brim, 1966), plants are grown under controlled conditions designed to reduce vegetative biomass partitioning and prioritize reproductive speed. A combination of photoperiod extension, late-spring temperatures, light wavelength optimization, and precocious germination technology enable 50 to 60-day lifecycle completion year-round for chickpea, irrespective of the field flowering phenology (Croser et al., 2016; Atieno et al., 2021). Harnessing out-of-season accelerated generation turnover in combination with complementary tools such as marker-assisted selection (MAS) enables rapid identification and culling of lines not homozygous for a desired trait. The combined aSSD-MAS approach is thus a highly efficient method to rapidly introgress novel traits and target downstream breeding program resource allocation and forms a key role in the compressed breeding pipeline described here for chickpea.

Chickpea is grown across more than 14 million ha worldwide (Bulti and Haji, 2019). India is the largest producer and Australia, with up to one million ha under cultivation, comes in second (FAOSTAT, 2021). Expansion of the Australian chickpea production area has been driven by improved varieties. Such varieties deliver up to 85 kg of nitrogen per hectare (Herridge et al., 1995) and provide a financially viable crop to disrupt cereal fungal diseases such as "take all" (caused by *Gaeumannomyces graminis var. tritici*) and "crown rot" (caused by *Fusarium pseudograminearum*) (Kirkegaard et al., 2008). A major barrier to further uptake of chickpea is its poor competitiveness against common weed species and lack of in-crop weed control options. Australian producers wanting to incorporate chickpea into rotations are also deterred by its sensitivity to commonly used acetohydroxyacid synthase (AHAS)-inhibitor herbicides (Group 2), popular in Australian farming systems due to their broad-spectrum efficacy. The use of AHAS inhibitors to control weeds in other crops can prevent the inclusion of chickpea in a rotation for 2 years or longer in dry environments with a soil pH $>$ 6.5 (Hollaway et al., 2006).

The problem of susceptibility to residual herbicides is unlikely to be overcome by new herbicide chemistries due to regulatory and cost barriers. As a result, the development of herbicide-tolerant (HT) pulse crops is seen as a sustainable management option to enable existing herbicides to be used in new ways (Rüegg et al., 2007). Group 2 herbicide tolerance has been identified in Canadian chickpea germplasm (Thompson and Tar'an, 2014), however, these varieties are not adapted to Australian growing conditions. Mutagenesis has been particularly successful in developing tolerance to AHAS-inhibitor herbicides (Tan et al., 2005; Green, 2014), and the trait is now available in crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), canola (*Brassica napus* L.), barley (*Hordeum vulgare* L.), lentil (*Lens culinaris* Medik.), Sorghum (*Sorghum bicolor* (L.) Moench.), and sunflower (*Helianthus*

[1]https://www.ipcc.ch/ar6-syr/

annuus L.) (Tan et al., 2005; Tuinstra and Al-Khatib, 2008; Lee et al., 2011). AHAS inhibitors target the AHAS enzyme which catalyzes the first step of branched chain amino acid synthesis. Target-site tolerance is well described in weed and crop species and can be conferred by single amino acid residue substitutions at any of eight conserved sites in the AHAS protein (Duggleby et al., 2008). For chickpea, development of target-site tolerance to AHAS-inhibitor herbicides and incorporation of the trait into a well-adapted genetic background suited to Australian production would increase in-crop weed control options and reduce plant-back restrictions from soil residues of herbicides applied in previous crops.

If herbicide tolerance can be identified there remains the challenge to introgress the trait as quickly as possible into a well-adapted genetic background, retaining other key production traits such as resistance to Ascochyta blight (caused by *Ascochyta rabiei*). Rapid introgression of any trait is reliant on access to techniques to speed up conventional breeding timelines, as well as a method for statistically assessing superior genotypes among low seed numbers produced by rapid generation cycling techniques. Much of the challenge associated with selection of superior genotypes for yield is due to the magnitude of the genotype by environment interaction (GEI) (Cullis et al., 1996, 2010). The genetic value of genotypes for grain yield is predicted using data obtained from variety trials grown across multiple environments being representative of the target production environments. A multi-environment trial (MET) is a collection of variety trials conducted over a range of geographic locations and years and a growing body of literature illustrates the advantages in analyzing yield data from MET datasets using a factor analytic linear mixed model (FALMM) (e.g., Oakey et al., 2007, 2016; Beeck et al., 2010; Gogel et al., 2018). While FALMM has traditionally been used for large MET field datasets, a major advantage of this approach is the ability to accommodate incomplete MET data, i.e., not all varieties grown at all environments in a model-based approach. Here, the FALMM statistical approach is adapted to identify elite lines despite low seed numbers coming from aSSD and other controlled environment screening techniques, without compromising the speed of advanced line progression through the breeding pipeline.

Within the context of access to well-established protocols to improve breeding efficiency in chickpea, we set out to determine the feasibility of integrating, across public institutions, established pre-breeding and emerging breeding tools into a single, compressed crop-improvement pipeline. We describe the use of mutagenesis to develop a non-GM herbicide tolerance trait beneficial within the farming system. We provide a case study outlining the progression, in 4 years, from initial cross to Australian National Variety Trials (NVT) of herbicide-tolerant chickpea breeding line CBA2061 using a combination of innovative platforms: accelerated homozygosity, marker-assisted selection and advances in applied statistics optimal for achieving selection targets. We outline the effectiveness of efficient yield evaluation designs to fast-track entry into advanced breeding trials. In doing so, we provide evidence of a successful, comprehensive, and collaborative approach to accelerate genetic gain in chickpea which can be modified for trait introgression in other self-pollinating species.

MATERIALS AND METHODS

Breeding Approach, Germplasm, and Locations

Research activities and field trials as shown in **Figure 1** were undertaken across five publicly funded organizations (**Table 1**). Germplasm was provided by SARDI and the NSW DPI breeding program (**Table 2**).

Seed Mutation and Selection of Genotypes With Tolerance to Acetohydroxyacid Synthase-Inhibitor Herbicides

In 2013, a sample of c. 100,000 seeds (M_0) of PBA HatTrick were treated at SARDI with ethyl methanesulfonate (EMS) following the method of Mao et al. (2019). All seed multiplication and herbicide selection trials were undertaken at a site near Paskeville, South Australia. In 2013/14, the seed was field-bulked. In 2015, 1500 kg of M_3 seed was sown across 20 ha and screened with imazapyr herbicide at 300 g active ingredient (a.i.) ha^{-1} (Unimaz® 250 g L^{-1} imazapyr, UPL Australia Ltd). Six weeks after herbicide application, M_3 phenotypic selections were made based on the absence of visual herbicide damage symptoms such as leaf chlorosis, necrosis, or plant stunting. Selections were transplanted prior to flowering into 13.5 L plastic pots and grown in a shade house with hand-watering until harvest of M_4. For progeny testing, 10 M_4 seeds per selection were sown into 0.47 L plastic pots and at the 4–5 node stage, hand-sprayed with 37.5 g a.i. ha^{-1} of imazapyr to confirm herbicide tolerance (lethal rate for PBA HatTrick determined by preliminary pot test – data not shown). Herbicide damage was visually assessed 21 days after treatment (DAT).

In 2016, 80 kg of the same M_2 seed was sown across 1 ha and screened with 105 g of imazapic + 35 g of imazapyr a.i. ha^{-1} (Onduty® 525 g kg^{-1} of imazapic + 175 g kg^{-1} of imazapyr, BASF Australia Ltd). The M_2 selections were transplanted to 13.5 L pots as described above and M_3 seeds were harvested. Ten seeds from each selection were progeny-tested using 13 g of imazapic + 4.4 g of imazapyr ha^{-1} (lethal rate for PBA HatTrick determined by preliminary pot test – data not shown). Herbicide damage was visually assessed 21 DAT.

Herbicide Dose Response Experiment

The two mutant lines D15PAHI002 (identified in 2015) and D16PAHI001 (identified in 2016) were confirmed with KASP™ markers to contain the $A_{205}V$ mutation. In 2018, D15PAHI002, D16PAHI001, and cv. PBA HatTrick were grown in 0.47 L pots in a randomised complete block design (RCBD) with four replicates. Plants were compared for their response to increasing rates of two AHAS-inhibitor herbicides imazapyr and chlorsulfuron, representing Group 2 families imidazolinone and sulfonylurea. Herbicide treatments were applied at the 4–5 node growth stage

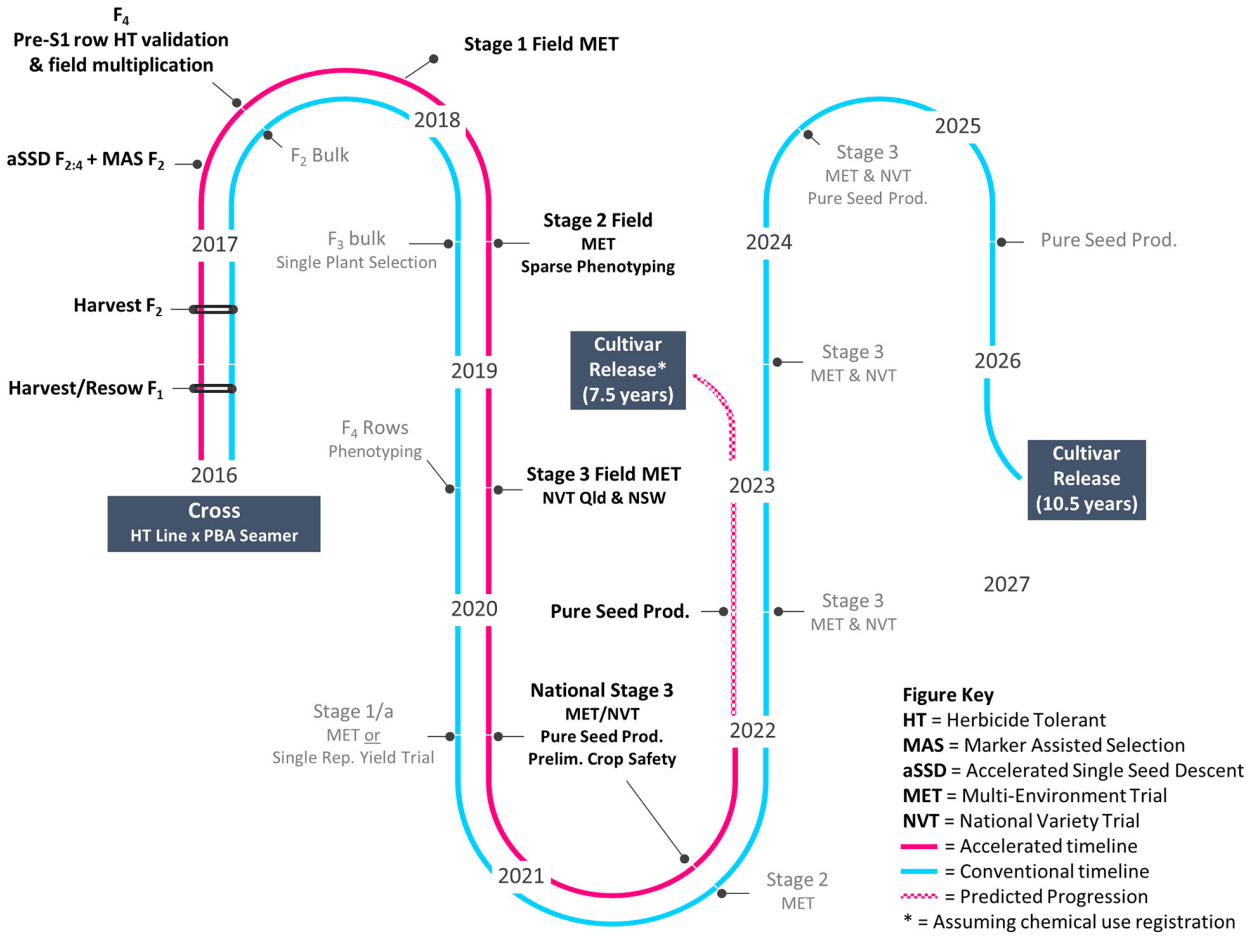

FIGURE 1 | Accelerated vs. conventional trait introgression timeline.

according to Mao et al. (2019). For each sample, above ground biomass was harvested 21 days after treatment (DAT), oven-dried at 60°C for 48 h, and dry weight was recorded. Data were analyzed using non-linear log-logistic regression models with the DRC package 3.0–1 in R v3.2.2.27 (R Core Team, 2020) as per Mao et al. (2019).

Candidate Gene Information and Marker Development

Genomic DNA (gDNA) was extracted from the progeny of the two mutant lines and PBA HatTrick using a modified CTAB DNA extraction protocol (Doyle and Doyle, 1987). Primers described by Thompson and Tar'an (2014) were used to amplify the *CaAHAS1* gene. PCR reactions were purified using Nucleofast filter plates (Macherey-Nagel, Duren, Germany) and Sanger-sequenced. Sequence reads were compared against the CDC Frontier *CaAHAS1* sequence (Varshney et al., 2013) and single nucleotide polymorphisms (SNPs) were identified using Geneious 10.2[2]. Translated coding sequences were aligned to the *Arabidopsis thaliana* AHAS1 amino acid sequence (GenBank accession NM_114714.3). The chickpea AHAS1 protein mutations are referred to by their homologous positions in the *A. thaliana* sequence.

Introgression of Target-Site Tolerance to Acetohydroxyacid Synthase-Inhibitor Herbicides Into Adapted Germplasm

Crossing between mutant M_5 line D15PAHI002 and cv. PBA Seamer was undertaken at NSW DPI in a temperature-controlled glasshouse in March 2016. A key component to accelerated trait delivery was the provision of D15PAHI002 to the breeding program in early 2016, prior to full trait confirmation and we report only results related to derivatives of D15PAHI002. Parental lines were grown, and homozygosity of the *CaAHAS1* HT allele in parent plants was confirmed using MAS. Crossing was undertaken similar to that described by Kalve and Tadege (2017). Hybrid F_1 seeds were grown and $n = 46$ F_2 lines were produced. Two further populations were developed within the pipeline; D15PAHI002 crossed with a high-performing desi with Ascochyta blight resistance (D1007 > 11F2TMWR2SS007) and a high-performing kabuli (K15195 > F103). Additional F_2 seeds

[2]https://www.geneious.com

TABLE 1 | Collaborating institutions, locations, and roles.

Institution	Role in accelerated breeding pipeline
South Australian Research and Development Institute* (SARDI)	Group 2 HT† development (SARDI_UoA) KASP marker development and application CE†† Ascochyta blight screening Crop safety field trials
University of Adelaide (UoA)	HT trait development (SARDI_UoA) Crop safety field trial design/interpretation
New South Wales Department of Primary Industries* (NSW DPI)	Pulse Breeding Australia** lead – hybridization, yield and herbicide field trials, herbicide selection screening. Field Ascochyta blight and Phytophthora root rot (Tamworth, NSW and Warwick, QLD) screening. Herbicide validation trials.
University of Western Australia* (UWA)	Accelerated single seed descent
University of Wollongong (UoW)	Biometric designs for yield trials and MET analysis of sparse phenotyping

**Pulse Breeding Australia (PBA) partners. †Herbicide tolerant, ††controlled environment. **Pulse Breeding Australia (PBA) chickpea program was a collaboration between Australian state-based departments of agriculture and The Grains Research and Development Corporation (GRDC).*

developed from these cross combinations were split with one set (total $n = 186$, including the 46 lines from D15PAHI002 × PBA Seamer) couriered to UWA for accelerated single seed descent (aSSD) $F_{2:4}$ while the other ($n = 790$) was kept at NSW DPI for Group 2 herbicide screening.

Accelerated Single Seed Descent (aSSD) $F_{2:4}$

F_2 seeds ($n = 186$ individuals) were received at UWA in October 2016 for accelerated single seed descent (aSSD) as applied in Atieno et al. (2021). Two weeks after emergence, leaves were sampled and couriered to SARDI for MAS. Plants homozygous for the HT trait were retained and staked, and immature seeds were removed at physiological maturity, *c.* 18 days after flowering. In-pod immature seeds were dried in seed envelopes in a *c.* 3 cm bed of orange indicator silica gel for 5–7 days at 25°C. At 8% seed moisture, measured with an active water meter (Rotronics) and converted to a seed moisture reading based on a moisture sorption isotherm for chickpea (Menkov, 2000), seeds were resown to soil and grown under the same conditions as the F_2 generation. The F_4 seed were left for an additional 14 days post-physiological maturity on the parent plant and lines were couriered to NSW DPI in March 2017 for further multiplication and evaluation.

Perlite-Based Controlled Environment Phenotyping for Tolerance to Acetohydroxyacid Synthase-Inhibitor Herbicides

A second set of F_2 ($n = 790$ individuals) seeds were phenotyped for HT in a plastic house at NSW DPI. The F_2 seedlings and parental checks were grown in slotted plastic trays containing perlite and, at the two-leaf growth stage, submerged for 10 s in a 2 ppm Imazapyr solution (Rotary Max® 240). Trays were drained and not watered for 24 h. At 48 h after treatment, plants displaying yellowing or wilting were discarded and tolerant plants were transplanted into 3.8 L pots. Leaves were sampled and couriered to SARDI for MAS. Homozygous HT plants were grown to maturity and F_3 seeds were harvested.

Marker-Assisted Selection Protocol

A KASP™ marker for the SNP identified in *CaAHAS1* was designed using the Kraken™ software package (LGC Biosearch Technologies, Middlesex, United Kingdom), and its reliability

TABLE 2 | Genotypes, pedigree, and use of germplasm.

Genotype* Seed source	Pedigree	Use
PBA HatTrick NSW DPI	Descendant of a Jimbour/ICC14903 cross	M_0 seed for mutation. Field and CE† studies.
D15PAHI002 SARDI	PBA HatTrick mutant with tolerance to HRAC Group 2 herbicide	Mutation line, identified at M_3 Female parent of CBA2061
D16PAHI001 SARDI	PBA HatTrick mutant with tolerance to HRAC Group 2 herbicide	Mutation line, identified at M_3
PBA Seamer NSW DPI	Descendant of a 98081-3024/PBA HatTrick cross	Pollen parent of CBA2061 Field and CE† studies.
CBA Captain NSW DPI	Australian cultivar, descendant of a CICA0910/D06314 > F3BREE2AB014 cross	Field and CE† studies.
Kyabra NSW DPI	Australian cultivar, descendant of an Amethyst//94631/Barwon//Lasseter/940-26//946-31/Norwin//8507-28H//Amethyst//T1069/8507-28H//946-31 cross	Field and CE† studies.
Genesis090* NSW DPI	Australian cultivar, descendent of FLIP82-150C/FLIP83-48C	Field and CE† studies.
PBA Boundary NSW DPI	Australian cultivar, descendant of JIMBOUR/ICC3996	Field studies.
PBA Seamer NSW DPI	Australian cultivar, descendent of 98081-3024/PBAHATTRICK	Field and CE† studies.
PBA Slasher NSW DPI	Australian cultivar, descendent of HOWZAT/ICC3996	Field and CE† studies.
PBA Magnus NSW DPI	Australian cultivar, descendent of FLIP97-159C/MACARENA//GENESIS114	Field and CE† studies.
CBA2061 NSW DPI	Advanced breeding line derived from cross between M_4 D15PAHI002 × PBA Seamer National Variety Trial entry	Prospective HT cultivar.

**Kabuli-type cultivar, all other germplasm: desi-type. † Controlled environment.*

was confirmed on parental DNA samples. Genomic DNA was extracted and diluted to ∼5 ng μL^{-1}, and ∼25 ng was used in KASP™ reactions using the LGC Genomics SNP Line system (LGC Biosearch Technologies, Middlesex, United Kingdom). Genotype calls were reported back to researchers at UWA and NSW DPI to select F_2 lines homozygous for the HT allele for further progression through the aSSD pipeline.

Herbicide Trait Confirmation, Yield, and Agronomic Evaluation

Line D15PAHI002, and 14 derivative HT sister F_4 aSSD lines were sown into a trait confirmation row trial at NSW DPI in September 2017 and compared for herbicide response to conventional chickpea varieties PBA Seamer, PBA Slasher, PBA Magnus, and Genesis090. Imazapyr 240 g L^{-1} (Rotary Max® 240) was applied at the 6–8 node stage at a rate of 200 g ha^{-1}. Tolerance was visually assessed 4 weeks after application, with symptoms progressing rapidly in warm spring conditions. In December 2017, lines were hand-harvested and machine-threshed for subsequent yield evaluation.

Seeds of HT line CBA2061 from the 2017 trait confirmation row trial were sown in a Stage 1 MET dataset and subsequent yield evaluation trials (**Table 3**; **Figure 1**). CBA2061 was assessed for agronomic suitability and yield against regionally specific cultivars CBA Captain, PBA HatTrick, PBA Boundary, PBA Seamer, and Kyabra. At each evaluation stage, plots were assessed for early vigor, flowering, maturity, plant height, and lodging. Lines were also assessed for resistance to Phytophthora root rot (caused by *Phytophthora medicaginis*) as per Bithell et al. (2021), and to locally aggressive isolates of Ascochyta blight (caused by *Ascochyta rabiei*) in controlled environment and field nurseries. Grain yield measurements were recorded at physiological maturity for each plot.

Crop Safety Evaluation

In 2020, dryland field trials to assess crop safety of D15PAHI002, D16PAHI001, and CBA2061 against cv. PBA HatTrick were conducted at two sites, Riverton and Turretfield, South Australia. At both sites, field trials were arranged in a RCBD with three blocks. Herbicide treatments were applied at the 5-node growth stage using a shrouded plot sprayer with flat fan nozzles at a 100 L spray volume ha^{-1} applied at 1 ms^{-1} and 220 kPa.

TABLE 3 | NSW DPI breeding program yield trials, National Variety Trials (NVT), and pure seed initiation for prospective cultivar CBA2061.

Year	2018	2019	2020	2021
Stage	1 (F3:5)	2 (F3:6)	3	3
Objective	Seed bulk, MET*	MET	MET, NVT**	MET, NVT Pure seed initiation
Number of sites	2	7	9	20
Site locations	Northern NSW, Southern QLD		Northern NSW, southern and central QLD	National

*Multi-environment yield trial, **national variety trial.

At Riverton, Trial 1 compared the response to application at the 5-node stage of 0, 0.5, 1, and 2 times the field use rate of 24.8 g ha^{-1} of imazamox + 11.3 g ha^{-1} of imazapyr recommended for imidazolinone-tolerant wheat (*Triticum aestivum* L.), faba bean (*Vicia faba* L.), and lentil. Trial 2 compared the response to simulated herbicide residue conditions by "incorporated by sowing" (IBS) treatments of 4.2 g a.i. ha^{-1} of metsulfuron methyl (Ally® 600 g kg^{-1} of metsulfuron-methyl FMC Australia) and 9 g a.i. ha^{-1} of chlorsulfuron (Glean®). At Turretfield, the response of CBA2061 and cv. PBA HatTrick were compared at 0, 1, and 2 times the field use rate of 24.8 g ha^{-1} of imazamox + 11.3 g ha^{-1} of imazapyr applied at the 5-node growth stage.

Herbicide damage scores were based on degree of chlorosis and plant stunting per plot and were taken at maximum herbicide expression *viz*. 8 weeks after treatment for post-emergent herbicide application and 16 weeks after treatment for IBS herbicide application. Grain yield measurements were recorded at physiological maturity. Results were analyzed using linear mixed models with the ASReml package (Butler et al., 2018). Additional site-specific extraneous fixed and random terms were included as required, and residual errors for each site were modeled using spatial methods. Residual maximum likelihood (REML) methodology was used for variance parameter estimation.

Factor Analytic Linear Mixed Model Statistical Approach for Multi-Environment Trial Evaluation

The approach of Smith et al. (2021a) was used to construct a MET dataset designed to maximize the amount of direct data on genotypes under consideration for selection in the current year (Cullis et al., 2020). Rather than generating separate designs for each trial, we implemented a new class of design, suited to Stage 1 (S1) trials. These designs are incomplete MET (IMET) designs. A comprehensive account of these methods is in preparation. Early versions of the ideas have been presented[3]. All designs were generated using the R package (R Core Team, 2020) OD (Butler and Cullis, 2018).

RESULTS

Mutation and Selection of Herbicide-Tolerant Plant Material

Following the mutagenesis of cv. PBA HatTrick with EMS, field screening across a 20 ha site for tolerance to Group 2 herbicide imazapyr resulted in the identification and confirmation of 14 putative tolerant selections. In 2015, M_4 seeds were harvested from 13 of the 14 selections. Progeny testing confirmed putative tolerance of one M_5 mutant line, D15PAHI002, and this line was provided to NSW DPI in 2016 and entered the accelerated breeding timeline (**Figure 1**). At 21 DAT with imazapyr, the 12 other selections were found to be severely damaged and did not yield seed.

[3]https://documents.uow.edu.au/content/groups/public/@web/@inf/@math/documents/doc/uow269164.pdf

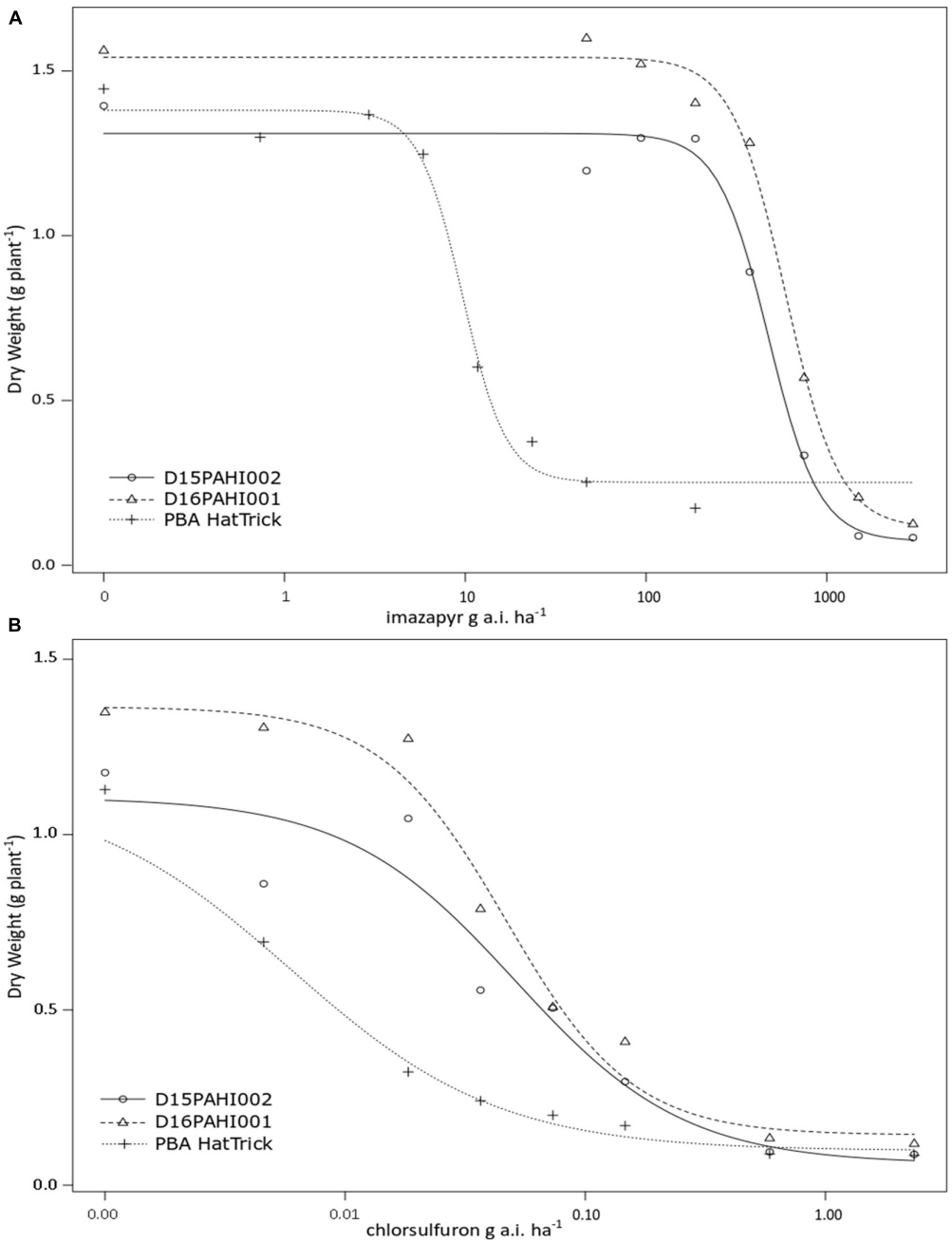

FIGURE 2 | Plant dry weight response at 21 days after treatment to increasing rates of imazapyr **(A)** or chlorsulfuron **(B)** of two mutant lines D15PAHI002 and D16PAHI001 and reference check; the mutant's background cv. PBA HatTrick.

In 2016, a 1 ha field screen for tolerance to imazapyr and imazapic, a common herbicide mix used in Australia, resulted in five further putative Group 2-tolerant selections. Of the three selections that yielded M_3 seeds and were progeny-tested, one M_4 line, D16PAH001, progressed to further evaluation. The remaining selections were severely damaged and did not yield seed.

In-pot controlled environment dose response trials demonstrated the mutant lines D15PAHI002 and D16PAHI001 and cultivar PBA HatTrick had typical herbicide response curves based on dry weight response to increasing rates of imazapyr and chlorsulfuron (**Figures 2A,B**). The mutant lines had a high level of tolerance to imazapyr. In response to imazapyr, the resistance factor (RF) for D16PAHI001 was significantly higher than for D15PAHI002 (**Table 4**). However, resistance of both was far higher, 51-fold and 39-fold, respectively, than cv. PBA HatTrick. Overall tolerance of both lines to chlorsulfuron was four to five times lower than tolerance to imazapyr (**Table 4**).

TABLE 4 | Estimated parameters – including resistance factor (RF) and 50% growth reduction (GR_{50}) values comparing the response of two mutant lines D15PAHI002 and D16PAHI001 to control cv. PBA HatTrick to the imidazolinone herbicide imazapyr or sulfonylurea herbicide chlorsulfuron.

Herbicide family	Active ingredient	Biotype	Upper limit	Slope	GR_{50}	RF
Imidazolinone	Imazapyr	PBA HatTrick	1.83 (0.52) ‡	1.39 (0.07) ‡	11.38 (2.14) ‡	–
		D15PAHI002	3.11 (0.92) †	1.31 (0.06) ‡	476.46 (59.43) ‡	41.89 (9.45) ‡
		D16PAHI001	2.95 (0.94) †	1.54 (0.06) ‡	590.11 (64.29) ‡	51.88 (11.27) ‡
Sulfonylurea	Chlorsulfuron	PBA HatTrick	1.00 (0.44) †	1.13 (0.10) ‡	0.01 (0.00) †	–
		D15PAHI002	1.23 (0.60) †	1.11 (0.09) ‡	0.05 (0.02) ‡	8.56 (4.44) *
		D16PAHI001	1.66 (0.50) ‡	1.36 (0.07) ‡	0.05 (0.01) ‡	7.8 (3.56) *

*Data were analyzed in the DRC package in R Studio. SE for parameter estimates in parentheses, GR_{50} is the herbicide application rate required to reduce the response of plants to 50%, RF is the resistance factor (GR50 mutant/GR50 control cultivar). Level of significance: *P = 0.10, †P = 0.05, ‡P = 0.01.*

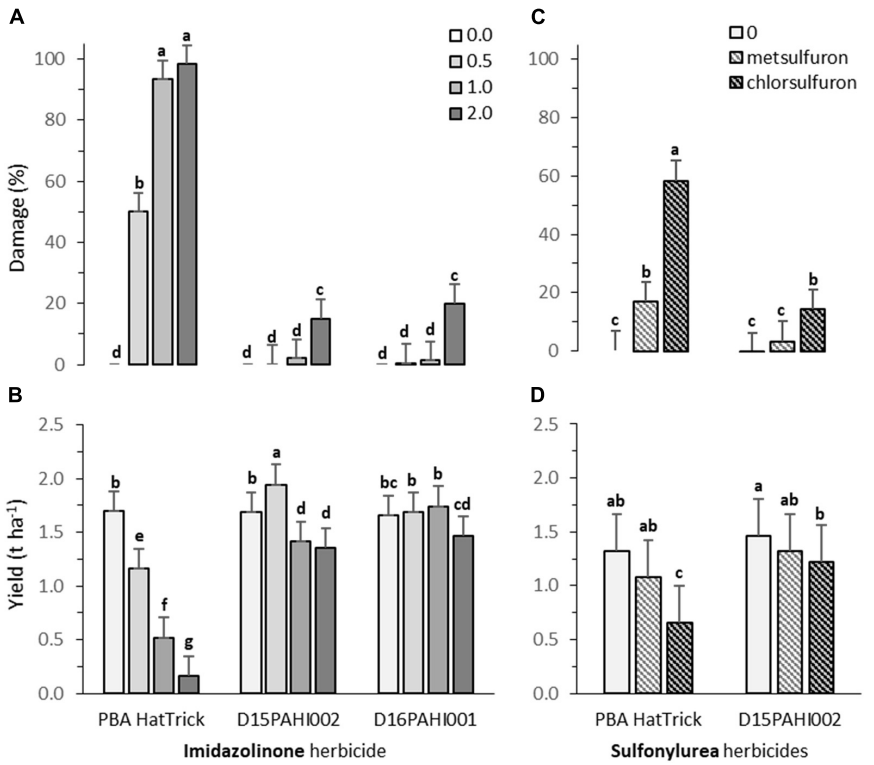

FIGURE 3 | Plant damage (%) and yield (t ha^{-1}) for cultivar PBA HatTrick and derivative mutants DH15PAHI002 and D16PAHI001 selected for tolerance to Group 2 imidazole herbicide following **(A,B)** post-emergent (5-node stage) applications of herbicides, at 0, 0.5, 1.0, and 2.0 times the recommended field use rate of 24.8 g a.i. ha^{-1} of imazamox + 11.3 g a.i. of imazapyr; and **(C,D)** for line DH15PAHI002 and PBA HatTrick, incorporated by sowing (IBS) applications of two Group 2 sulfonylurea herbicides at their recommended rate, 4.2 g a.i. ha^{-1} of metsulfuron and 9.0 g a.i. ha^{-1} of chlorsulfuron, in the 2020 trials at Riverton, South Australia. Plant damage was scored at 8 weeks after post-emergence spray treatment **(A)** or 16 weeks following herbicide incorporated by sowing (IBS) treatment **(C)**. In each graph, bars indicate means and whiskers indicate LSD ($\alpha = 0.05$); different letters (a-g) represent significantly different means.

Crop Safety Evaluation

Lines D15PAHI002 and D16PAHI002 exhibited a high level of imidazolinone tolerance and no damage at the recommended field application rate prescribed for other AHAS inhibitor-tolerant crops (24.8 g of imazamox + 11.3 g of imazapyr ha^{-1}, **Figure 3A**). Line D15PAHI002 had *c.* 20% yield reduction at this recommended rate and yield was not further reduced when the rate was doubled. Line D16PAHI001 had equivalent yields in the control treatment and at 2× the recommended rate, *c.* 11% less than for the 0.5× and 1× rates (**Figure 3B**). By comparison, cv. PBA HatTrick exhibited high levels of damage at both the 1× and 2× field rates with c. 70% yield reduction at the 1× rate and >90% yield reduction at the 2× rate (**Figures 3A,B**). In the current 2021 season, further crop safety field trials are underway at three locations to confirm this identified level of field HT has transferred to CBA2061.

Following sowing into simulated residues of metsulfuron herbicide, no significant plant damage was observed 16 weeks after sowing in line D15PAHI002 compared with 17% damage observed in PBA HatTrick (**Figure 3C**). For chlorsulfuron

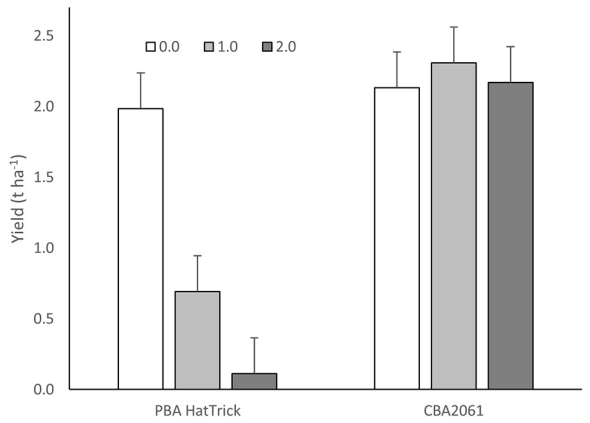

FIGURE 4 | Yield response of CBA2061 compared to cv. PBA HatTrick following application of the recommended field use rate of 24.8 g a.i. ha^{-1} of imazamox + 11.3 g a.i. ha^{-1} of imazapyr and twice the recommended rate in the 2020 field trial at Turretfield, SA. Bars indicate means and whiskers indicate LSD ($\alpha = 0.05$).

residues, moderate damage of *c.* 14% was observed in D15PAHI002 in contrast to *c.* 58% in PBA HatTrick (**Figure 3C**). The metsulfuron application rate of 4.2 g a.i. ha^{-1} was insufficient to cause yield reduction in either genotype under the seasonal and soil conditions for this site (**Figure 3D**). However, for chlorsulfuron residue treatment, yield was moderately reduced for D15PAHI002 (av. 1.5 to 1.2 t ha^{-1}) and substantially reduced (av. 1.3 to 0.7 t ha^{-1}) for PBA HatTrick (**Figure 3D**).

Trait Introgression and Acceleration

Line D15PAHI002 was the first putative HT selection to be identified by SARDI and was crossed opportunistically by NSW DPI with cv. PBA Seamer in 2016 (**Table 1**). PBA Seamer was selected for its semi-erect plant type, wide adaptation, good yield stability, and moderate resistance to Ascochyta blight and Phytophthora root rot. Hybrid seeds from this cross and two others, D15PAHI002 × D1007 > 11F2TMWR2SS007 and D15PAHI002 × K15195 > F103, were grown in the glasshouse at NSW DPI and split into two sets.

Grown under aSSD conditions, the first set of F_2 seeds (n = 186) flowered within 23–28 day of sowing and immature seeds were removed at 18 days after flowering. Taking T_b to be 0°C for chickpea (Lake et al., 2016), growing degree days from sowing to harvest ranged within 874 to 981 days. Following MAS, a total of 43 lines were recorded as homozygous for the HT trait and were resown to the following generation. The F_4 seeds were left to fully mature on the plant and 7–24 seeds from each line were returned to NSW DPI on July 23, 2017 for further multiplication and evaluation. The total generation cycle time was *c.* 60 days for F_2 and *c.* 72 days for F_3 to allow for seed maturation on the mother plant.

The second set of F_2 seeds (n = 790) was grown and evaluated for Group 2 HT in a temperature-controlled plastic house at NSW DPI. Within the perlite screen, herbicide-susceptible F_2 individuals and checks displayed yellowing or wilting 48 h after herbicide submergence treatment. The surviving plants (*c.* 1–35% of the F_2 seedlings from each population) were transplanted and grown to maturity. This alternative pathway took two and a half years longer than the aSSD MAS pathway, with field-based single plant selections at F_3 and single row multiplication required prior to yield trial entry.

Full-length coding sequences and translated amino acid sequences were generated for the *CaAHAS1* gene length for the tolerant D15PAHI002 line (GenBank accession OK078878) and the PBA HatTrick cultivar (GenBank accession OK078877). The D15PAHI002 and D16PAHI001 single amino acid substitution from alanine to valine at position 205 ($A_{205}V$) is identical to that described by Thompson and Tar'an (2014) (NCBI ref. seq. XM_004501646.3). KASP™ markers were developed for the SNP and used to genotype the aSSD and perlite-selected F_2 seedling sets. Among the F_2 HT plants selected at NSW DPI, 46% were homozygous for the *CaAHAS1*-tolerant allele, the rest were heterozygous. At UWA, following MAS, only homozygous *CaAHAS1*-tolerant plants were processed to F_4 resulting in substantial cost and time saving.

Herbicide Trait Confirmation

The line D15PAHI002 and the 14 aSSD-derived D15PAHI002 × PBA Seamer progeny lines including CBA2061, previously confirmed to be homozygous for the *CaAHAS1*-tolerant allele, were visually assessed in-crop and exhibited no sign of herbicide damage. The non-tolerant check cultivars Genesis090, PBA Magnus, PBA Seamer, and PBA Slasher showed symptoms of severe herbicide damage. The aSSD-derived lines were hand-harvested, threshed, and seed-utilized for further field evaluations.

Field Evaluation of Advanced Breeding Lines

Following treatment with 49.5 g ha^{-1} of imazamox + 22.5 g ha^{-1} of imazapyr, twice the recommended field use rate, CBA2061 gave an equivalent yield to untreated PBA HatTrick (**Figure 4**). Plants began exhibiting herbicide effects 2 weeks after treatment with maximum herbicide expression at 6 weeks and plant recovery at 8 weeks. By harvest, CBA2061 had recovered from any herbicide effect and showed no yield reduction regardless of herbicide application rate. The yield of PBA HatTrick was reduced by 68% at the single rate herbicide application and almost to nil at the double rate.

Multi-environment trial evaluation of CBA2061 against five locally adapted cultivars demonstrated their agronomic characteristics were similar in the northern growing region (southern QLD and northern NSW). CBA2061 is an early flowering line with mid-season maturity suited to the winter farming system requirements of this growing region. The line has a semi-erect plant type with sufficient plant height and lodging tolerance to indicate good harvestability. Disease data showed a moderate and acceptable level of resistance to Ascochyta blight and Phytophthora root rot comparable to conventional cultivars PBA HatTrick and PBA Seamer. CBA2061 is an angular-shaped

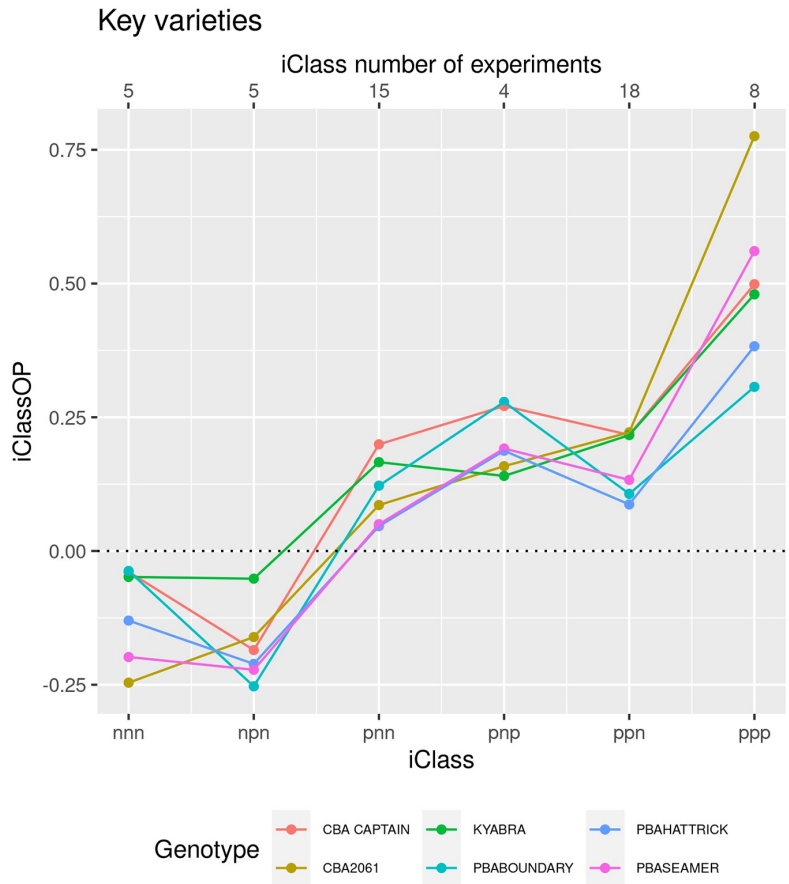

FIGURE 5 | iClass interaction plot for the breeding line CBA2061 and comparison with five regionally adapted cultivars.

desi chickpea with medium sized Jimbour-type seed and good milling quality similar to PBA HatTrick and PBA Seamer.

Field Evaluation of Yield Performance

The FALMM was fitted in a one-stage approach to permit model validation and examination in a statistically rigorous manner (as per Lee et al., 2006). FALMM was fitted to the desi north 2020 MET dataset along with an example of the application of the interaction class methodology for summarizing the results of the model fit (Smith et al., 2021b). There was a total of 55 environments and 99 trials in this MET dataset. Ancestral information was available on 5530 genotypes, with 4882 genotypes in the dataset. The mean inbreeding coefficient of the genotypes with data was 0.7621. A factor analytic (FA) model of order two and one was fitted to the additive GEI effects and the non-additive GEI effects, respectively. The percentage variance accounted for by the fit of the FA (2, 1) model was 77.9% with each of the three factors accounting for 35.7, 28.0, and 14.2%, respectively.

To obtain a graphical display of the crossover GEI in the MET dataset, a set of iClasses was created (as per Smith et al., 2021b) from the concatenated set of two-level factors, one for each factor and each with two levels [positive (p) and negative (n)], representing the sign of the loadings. For the desi dataset there were eight iClasses which formed the set of environments for which there was minimal crossover GEI between environments within the same iClass. The overall performance (OP) of each variety was the average of the E-BLUPs of the common variety effects from the latent FA regression model. The iClass Interaction Plot provides a metric for ordering the crossover GEI, as adjacent pairs of iClasses differ in the highest order factor so reflect the least amount of between iClass crossover GEI (**Figure 5**). Moving across iClasses from left to right in **Figure 5** demonstrates the lack of GEI amongst the highly related (except for Kyabra) genotypes. All related genotypes had a very high OP with common parents and grandparents. Kyabra exhibited a slightly different GEI to the other genotypes, while the breeding line CBA2061 had almost an identical pattern of GEI to PBA Seamer but had a slight yield advantage in iClasses "ppn" and "ppp."

DISCUSSION

A conventional, self-pollinated species breeding approach combined with aSSD, MAS, and sparse phenotyping platforms

facilitated progression in 4 years (2016–2020) of the case study herbicide-tolerant chickpea genotype CBA2061 from cross to National Variety Trials (NVT). It is expected that cultivar release will occur 3.5 years earlier than through a conventional breeding pipeline (**Figure 1**). The accelerated approach facilitated rapid introgression of the mutation event $Ala_{205}Val$ into well-adapted cv. PBA Seamer and accelerated advancement of the resulting Group 2-tolerant genotype CBA2061. Subject to successful registration with chemical companies, CBA2061 will be an important rotation tool in the cropping system to control broadleaf weeds and enable the diversification of broadleaf herbicide groups across the Australian farming system. The $Ala_{205}Val$ mutation, as previously described in a Canadian chickpea genotype by Thompson and Tar'an (2014), confers high-level tolerance to the imidazolinone herbicides in-crop and moderate level tolerance to soil residues of the sulfonylurea herbicides. The mutant-derived HT advanced breeding line CBA2061 exhibits high levels of crop safety with no damage or yield reduction at 1× or 2× the recommended field use rate of the imidazolinone herbicides. As a result, the crop is expected to be extended into areas where difficult to control weeds cause issues and will diversify herbicide options and reduce the risk of both herbicide resistance in weeds and residual herbicide damage.

Mutating an elite, high-yielding background (cv. PBA HatTrick) was a key part of the breeding strategy to ensure any advantageous mutation would be within an appropriate and current adaptive background for the target production regions. The alternative HT introgression pathways of crossing locally adapted material with previously identified HT Canadian chickpea material (Thompson and Tar'an, 2014), or mutation of a local, but less adapted parental background, would have led to further crossing and evaluation stages and extended the breeding pipeline timeframe. Further, the strategy of crossing putative HT lines immediately after field selection while controlled environment and field HT confirmation experiments were still underway resulted in derivatives being available at the time of HT trait confirmation for D15PAHI002. Breeding resources were then preferentially allocated to ensure rapid progression of the D15PAHI002 × PBA Seamer progeny through the pipeline. This contrasts with the conventional approach of progeny screening, dose response experiments and field validation research undertaken separately for up to 2 years prior to providing germplasm and knowledge to breeders. The second mutation line identified in 2016, D16PAHI001, had the same mutation but gave higher yield in HT crop safety trials. This line has also been crossed with regionally adapted breeding material and its derivatives are currently under evaluation within the accelerated pipeline. The coordination between pre-breeding and breeding partners aimed at rapid introgression of this HT trait stands as an example of how publicly funded organizations can work together to improve the rate of genetic gain in a time efficient manner for a critical industry trait.

Among parameters in the breeder's equation, cycle time is the easiest to understand, cheapest to manipulate, and the most powerful parameter for increasing genetic gain (Cobb et al., 2019). Cycle time, or generation interval, involves recycling breeding material back into the crossing block as quickly as a breeder can determine that a genotype is above average in breeding value for a desired quantitative trait. Rapid generation advancement, widely known as "speed breeding" (Watson et al., 2018), has been predominantly reported in Fabaceae species for recombinant inbred line production for molecular mapping and QTL discovery (Lulsdorf and Banniza, 2018; Uz Zaman et al., 2019; Atieno et al., 2021; Dadu et al., 2021; Taylor et al., 2021). Another practical use of accelerated life cycling is to achieve rapid out-of-season turnover of promising breeding germplasm. Cycling from $F_{2:4}$ out of season gives breeders access to germplasm that can be screened in-season and in GEI trials for beneficial agronomic traits, saving a year in a conventional breeding pipeline. One constraint is the low seed number resulting from intensive growth under controlled conditions, designed to prioritize reproductive growth over vegetative biomass. Using a FALMM approach enabled field selections based on predicted values, and adapting the MET designs for Stage 1 trials allowed lines to progress through yield evaluation despite limited seed availability.

Combining established MAS with emerging aSSD techniques provided further time-saving in the breeding pipeline. Integrating platforms such as aSSD and MAS with conventional breeding programs is not straightforward and requires plant breeder trust in quality assurance practices of the organizations handling the germplasm on their behalf and capacity available within platforms at time points suited to the breeding program. MAS allows for rapid, accurate, and cost-effective screening of large numbers of plants in breeding programs minimizing the use of herbicide bioassays. Combining molecular markers with markers and bioassays for other traits like disease resistance simplifies and accelerates the stacking of multiple traits. In this case study of HT chickpea, the MAS technology reduced the time from cross to cross allowing for the continual introgression of desirable traits into elite lines for improved agronomic fit and yield potential for industry. Implementing the marker assays at the F_2 generation reduced the total number of lines in aSSD by 75%, and MAS of perlite screen survivors eliminated heterozygous "escapes" ensuring only homozygous tolerant cross progeny were progressed and the efficient allocation of downstream breeding program resources toward priority germplasm.

Our findings confirm the practical feasibility of combining pre-breeding approaches into a pipeline to achieve rapid genetic improvement for a qualitative trait, herbicide tolerance. We note that a quantitatively inherited trait may require modification of this approach to account for the complexity of minor gene controls, e.g., genetic selection. A conventional breeding approach (**Figure 1**) would take 7 years to achieve a similar outcome for this qualitative trait introgression through a bulk pedigree method with single plant selection at $F_{3/4}$. Pulses receive far less support for pre-breeding efforts when compared with cereal crops. Pulse Breeding Australia (PBA), commissioned from 2006 to 2019, was a publicly funded breeding initiative of The Grains Research and Development Corporation, state-based Agriculture Departments and Universities that delivered chickpea, pea (*Pisum sativum* L.), lentil, faba bean, and lupin (*Lupinus angustifolius* L.) varieties to Australian growers. PBA linked pulse breeding programs with a network of pre-breeding projects, enabling efficient adoption of emerging germplasm and pre-breeding tools within breeding programs in a co-ordinated

manner. This joint research venture facilitated relationships among involved institutions to speed up trait delivery from pre-breeding to industry. The pipeline presented is an excellent applied example of (1) the benefit of investment by industry and government in novel pre-breeding tools and (2) demonstrable integration of such tools to accelerate genetic gain and delivery of improved varieties to producers. To achieve the output of a HT chickpea in NVT after 4 years has required collaborators to share a willingness to take risks, communicate well, and exchange breeding material and expertise in a manner which acknowledges the research and efforts of all participants. We expect this breeding approach can and will be used to accelerate genetic gain in any self-pollinated species with access to similar technologies.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

TS, LM, CP, KH, BC, JC, WE, and FR were chief investigators on the projects contributing data to this manuscript. DM, LM, DB, FR, ND, and SM undertook HT, aSSD, MAS, and data analysis of HT experiments. BC conceived and undertook statistics related to breeding program MET and FALMM. FO coordinated the pre-breeding and breeding projects providing data to this manuscript. JC, ND, DM, SM, KH, and JL wrote the first draft of the manuscript. All authors contributed to the manuscript revision and editing and approved the submitted version of the manuscript.

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Association Mapping for Yield Attributing Traits and Yellow Mosaic Disease Resistance in Mung Bean [*Vigna radiata* (L.) Wilczek]

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Mung bean [*Vigna radiata* (L.) Wilczek] is an important short-duration grain legume widely known for its nutritional, soil ameliorative, and cropping system intensification properties. This study aims at evaluating genetic diversity among mung bean genotypes and detecting genomic regions associated with various yield attributing traits and yellow mosaic disease (YMD) resistance by association mapping. A panel of 80 cultivars and advanced breeding lines was evaluated for 10 yield-related and YMD resistance traits during *kharif* (monsoon) and summer seasons of 2018–2019 and 2019–2020. A total of 164 genome-wide simple sequence repeat (SSR) markers were initially screened, out of which 89 were found polymorphic which generated 317 polymorphic alleles with an average of 3.56 alleles per SSR locus. The number of alleles at each locus varied from 2 to 7. The population genetic structure analysis grouped different genotypes in three major clusters and three genetically distinct subpopulations (SPs) (i.e., SP-1, SP-2, and SP-3) with one admixture subpopulation (SP-4). Both cluster and population genetic structure analysis categorized the advanced mung bean genotypes in a single group/SP and the released varieties in other groups/SPs, suggesting that the studied genotypes may have common ancestral history at some level. The population genetic structure was also in agreement with the genetic diversity analysis. The estimate of the average degree of linkage disequilibrium (LD) present at the genome level in 80 mung bean genotypes unveiled significant LD blocks. Over the four seasons, 10 marker-trait associations were observed significant for YMD and four seed yield (SY)-related traits *viz*., days to flowering, days to maturity, plant height, and number of pods per plant using the mixed linear model (MLM) method. These associations may be useful for marker-assisted mung bean yield improvement programs and YMD resistance.

Keywords: association mapping, yield attributing traits, *Vigna radiata*, SSR, linkage disequilibrium, MLM

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INTRODUCTION

Mung bean [*Vigna radiata* (L.) Wilczek], also known as green gram, is an annual herbaceous self-pollinated pulse crop having diploid chromosome number $2n = 2x = 22$ (Karpechenko, 1925). It has a small genome size of 543 Mbs (Kang et al., 2014), which makes it a valuable model for advancing the understanding of genetic diversity and genome evolution. It is an important food legume in Asia

and parts of Africa and America (Pratap et al., 2020). As a short-duration crop (55–70 days from sowing to maturity), it can be grown across seasons in varying cropping systems and crop rotations (Malik, 1994). It plays an important role in sustaining soil fertility by improving the physical and biological properties of the soil. In association with *Bradyrhizobium* bacteria, it fixes atmospheric nitrogen in the soil (Joshi et al., 2003). Mung bean is highly nutritious and an inexpensive source of easily digestible high-quality protein, amino acids, lipids, fat, fiber, ash, and carbohydrates and provides 334–344 kcal energy (Srivastava and Ali, 2004; Day, 2013; Choudhary and Suri, 2014; Singh et al., 2018). Besides, mung bean seeds have no anti-nutritional factors such as trypsin inhibitors, phytohemagglutinin, and tannin (Chen et al., 2003). Currently, the realized average productivity of mung bean is well below the economic level. The major reasons for stagnancy in its productivity are limited genetic variability, negative impact of high genotype × environment interaction (GEI), and susceptibility of the existing cultivars to various biotic and abiotic stresses, ultimately leading to yield instability (Chauhan et al., 2010; Pratap et al., 2019a). Modern crop breeding has further resulted in an increase in the genetic uniformity among the mung bean cultivars leading to further erosion of the genetic diversity.

Assessment of genetic diversity is a step of paramount importance and is a prerequisite for improvement in any crop. The estimation of genetic diversity is valuable in the selection of diverse and compatible parental genotypes. This helps to generate segregating progenies with maximum genetic variability and also in the introgression of desirable traits from diverse or wild germplasm into the commercial cultivars to broaden their genetic base (Barrett and Kidwell, 1998; Saravanakumar et al., 2004; Sangiri et al., 2007). The most important agronomic and economic trait in crop plants is yield, which is a function of multiple interacting component traits, controlled by multiple loci with a largely ambiguous genetic basis. To launch a breeding program for the improvement of plant genotype with a desirable combination of traits, complete information regarding the association of these traits with yield as well as detailed information on the genetic mechanism controlling various traits is important.

Molecular studies provide more reliable data than morphological and physiological data (Rahman et al., 2011) owing to the lack of environmental influence. DNA markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR) and single nucleotide polymorphisms (SNPs) have commonly been used for genetic diversity studies in plants. Among these, SSR markers are reported to be highly reliable due to their high degree of polymorphism, multi-allelic nature, reproducibility, codominance, locus specificity, abundance, and capacity of wide genome coverage (Powell et al., 1996) when compared with other DNA markers. These have been widely used in various crop species as potent tools for evaluation of genetic diversity (Somta et al., 2008), quantitative trait locus (QTL) mapping, genome-wide association study (GWAS) (Bohra et al., 2014), and marker-assisted selection (MAS) (Kumar et al., 2011; Pratap et al., 2017). Association analysis is a high-resolution method for genetic mapping using existing germplasm and their phenotypic information for the trait concerned (Flint-Garcia et al., 2003) and helps to understand the genetic basis of a complex trait like yield. It permits a survey of a wide range of alleles at each locus, detection of marker-trait associations at the whole genome level, and identification of elite alleles for significantly associated loci. Marker-trait association study has the advantage over conventional QTL mapping (Atwell et al., 2010) since it considers natural populations with more recombination events and mutations which might have occurred over multiple generations. On contrary, QTL mapping uses constructed biparental mapping population with limited recombination allowing detection of QTL in limited resolution. This creates a hindrance in the implementation of MAS in breeding programs, especially where linkage drag is a problem. Therefore, association study offers a higher mapping resolution of traits (Addington et al., 2011) and can overcome hindrance in the adoption of MAS in breeding programs (Mackay and Powell, 2007). This study aims to evaluate the genetic diversity and marker-trait associations in a panel of commercial mung bean cultivars and advanced breeding lines using SSR markers for genetic dissection of important SY-related traits along with yellow mosaic disease (YMD) resistance in order to expedite genetic improvement.

MATERIALS AND METHODS

Plant Materials

The plant materials for this study comprised 80 diverse mung bean genotypes including 46 released cultivars recommended for cultivation in different agro-climatic zones in India and 34 advanced breeding lines developed at Chaudhary Charan Singh Haryana Agricultural University (CCS HAU), Hisar, India. The salient features and pictorial representations of the released cultivars are available elsewhere (Pratap et al., 2019b; Project Coordinator's Report, 2020), whereas the advanced breeding lines are currently at different stages of multilocation evaluation for the possible release of the best ones as commercial cultivars.

Phenotypic Evaluation

The genotypes were evaluated for yield traits and reaction to YMD caused by *Mung bean yellow mosaic India virus* (MYMIV, identity of causal virus established in other studies) in four seasons under field conditions during *Kharif* (Monsoon) and summer seasons of 2018–2019 and 2019–2020 at the Pulses Research Area of the Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar, which is situated at a latitude of 29°10′N, 75°44′E, 215.2 m above msl. Each genotype was sown in a plot of three rows of 4 m length in two replications following a randomized complete block design. All the recommended agronomic practices for the experimental location were adopted to raise a robust crop. The genotypes were observed for yield-related traits *viz*., days to flowering (DF), days to maturity (DM), plant height (PH) in cm, pod length (PL) in cm, 100-seed weight (SW) in g, reaction to YMD, number of branches (NB) per plant, number of pods (NP) per plant, number of seeds (NS) per pod, and SY per plant in g. All these quantitative traits were

measured in each plot on five randomly selected plants. Disease scoring for the YMD was performed 45 days after sowing (DAS) following Ahmed (1985) on a 0–9 scale (**Supplementary Table 1**). The correlation, mean values, SE, SD, and range were estimated for all the quantitative characters using IBM SPSS version 26.1 software.

Genotyping the Mapping Panel

Young leaves were collected from all mung bean genotypes at the two-leaf stage for total genomic DNA extraction using the cetyltrimethylammonium bromide (CTAB) method as suggested by Saghai-Maroof et al. (1984) with minor adjustments. Extracted DNA quality was estimated by agarose gel electrophoresis (0.8%), and the quantity of DNA was determined using a NanoDrop spectrophotometer. Each DNA sample was normalized to a concentration of 50 ng/μl for use in PCR. PCR amplifications were carried out with 15 μl reaction mixture including 10× *Taq* buffer with 15 mM of $MgCl_2$, 2.5 mM of dNTPs, 1 U of *Taq* DNA polymerase (GeNei Bangalore), 50 ng of template DNA, and 10 μmol of forward and reverse primers [Integrated DNA Technologies (IDT), Inc., United States] in an Applied Biosystem Thermocycler. The amplification conditions were programmed as initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, primer specific annealing at 45–55°C for 1 min, primer extension at 72°C for 1 min, and final extension at 72°C for 7 min. PCR products were resolved by using 3% agarose gel electrophoresis in 1× TBE buffer. Fragments were visualized under UV trans-illuminator and documented using BIO-RAD Gel Doc^{TM} XR, United States, and alleles from each genotype were scored manually. A total of 164 SSRs from different *Vigna* species, namely, adzuki bean (Wang et al., 2004), common bean (Blair et al., 2013), cowpea (Li et al., 2001), and mung bean (Kumar et al., 2002; Somta et al., 2009; Pratap et al., 2016; Suman et al., 2019; Singh et al., 2020) used in this study are listed in **Supplementary Table 2**.

Statistical Analysis

Statistical analysis such as mean, range, two-way ANOVA (Panse and Sukhatme, 1964), genotypic coefficient of variance (GCV), phenotypic coefficient of variance (PCV), broad-sense heritability, and genetic advance as percentage of mean was calculated for the 10 studied traits using INDOSTAT software[1].

Genetic Diversity Analysis

The allelic data of 89 polymorphic SSRs were scored in the form of base pairs (bp) and subjected to statistical analysis using GenAlEx version 6.51b2 to calculate the total number of alleles (Na), effective allele frequency (Ne), Shannon information index (*I*), observed heterozygosity (Ho), expected heterozygosity/genetic diversity (He), genetic differentiation indices, pair-wise population Nei genetic identity, and analysis of molecular variance (AMOVA) (Peakall and Smouse, 2006). The polymorphic information content (PIC) was calculated following Botstein et al. (1980) as $PIC = 1 - \Sigma (P_{ij})^2$, where, P_{ij} denotes the frequency of *i*th allele of a *j*th locus summed across all alleles revealed by *j*th locus primer. Genotypic data of 89 polymorphic markers were used to generate distance-based weighted neighbor-joining (WNJ) dendrogram tree using DARwin 6[2]. The codominant allelic data were run at 30,000 bootstraps to draw the phylogenic tree and later, it was used as the robust signal for explaining the genetic diversity of released and advanced genotypes of mung bean.

Population Structure Analysis

Population structure and the number of subpopulations (SPs) were determined using STRUCTURE software version 2.3.4 (Pritchard et al., 2000; Falush et al., 2007). The admixture model and correlated allele frequency model were selected to estimate the number of subgroups present in the association panel. Initially, 10 runs for the value of *K* ranging from 2 to 10 were conducted with a burn-in period of 100,000 followed by 200,000 Markov Chain Monte Carlo (MCMC) iterations. Then, the STRUCTURE HARVESTER web version 0.6.94 tool was used for obtaining the optimum *K* value determined by plotting the LnP (D) value against *K* (Earl and vonHoldt, 2012) which is based on the approach of Evanno et al. (2005).

Association Analysis

Association analysis was conducted to reveal the marker-trait association using TASSEL software version 2.1 (Bradbury et al., 2007). General linear model (GLM) with Q matrix generated through STRUCTURE and mixed linear model (MLM) with kinship matrix (K) generated through TASSEL along with the Q matrix were used to extract information on the association of the markers with YMD and yield-related traits. The QQ plot was generated using R package (qqman).

RESULTS

Genetic Variability and Correlation

ANOVA revealed highly significant mean squares for all the traits across four environments *viz*., *Kharif* (2018), *Kharif* (2019), Summer (2019), and Summer (2020) as well as in *Kharif* and Summer pooled over environments (**Supplementary Table 3**). Very less difference between PCV and GCV estimates was observed (**Supplementary Tables 4a–c**), and the GCV and PCV were categorized as low (<10%), moderate (10–20%), and high (>20%). Among the studied traits, YMD (41.03 and 43.39%) and the NB per plant (21.68 and 22.05%) had high GCV and PCV, respectively. High heritability (>60%) was recorded for all the traits with ranging from 97.69% in seed size to 65.95% for the NS per pod. The magnitude of genetic advance as percentage of mean was high (>20%) for YMD (79.89%), NP (43.91%), and SS (24.84%). Moderate genetic advance (10–20%) was observed for PH (18.65%), SY (17.60%), NP (16.01%), and PL (15%), whereas low genetic advance (<10%) was recorded for DM (7.56%), DF (6.13%), and NS (5.94%). High GCV, heritability, and genetic advance were observed for YMD and NP, while low GCV, high heritability, and low genetic advance were recorded for DF, NS, and DM.

[1]https://www.indostat.org

[2]http://darwin.cirad.fr/

Correlation of traits estimated using the pooled phenotypic data of all seasons revealed that the SY was positively associated with PL, NB, NP, and NS and negatively associated with YMD. DF showed a positive correlation with DM, PH, and YMD. DM showed a positive association only with DF. PH exhibited a significant positive association with DF, YMD, and NB. A positive correlation of PL was observed with SW, NS, and SY, whereas NB showed a significant positive association with PH, NP, and SY. SW was found positively correlated with PL and negatively correlated with PH and NB. YMD was observed to be positively associated with DF and PH, whereas negatively correlated with PL, NP, NS, and SY (**Table 1**).

Allelic Diversity

A total of 89 polymorphic SSRs were used to assess the genetic diversity among released cultivars and advanced breeding lines of mung bean. Most of the primer pairs amplified with varying allele sizes and ranged between 100 and 310 bp. All the polymorphic primer pairs generated 317 polymorphic alleles with an average of 3.56 alleles per SSR locus. The number of alleles at each locus (Na) varied from 2 (BMD-18, SSR-1AC127, SSR-1AC188, GMES1823, PVag003, PVag005, VR039, CP00361, CP5096, CEDG15, CEDG24, CEDG60, CEDG70, CEDG116, CEDG290, DQ9393, DQ469293, MBSSR008, PvM22, VM27, VR023, and VR032) to 7 (BM146, CEDG115, and GMES035). The number of effective alleles varied from 1.02 (CEDG290 and VR023) to 4.49 (BM146) with an average of 1.82. Shannon's information index value varied from 0.07 to 1.63. The fixation index ranged from −0.93 to 1, and total 80 SSR loci showed the fixation index value 1. Heterozygosity was observed in nine SSR loci which ranged from 0.01 (CEDG41) to 0.97 (BMD-26) with an average of 0.05. The expected heterozygosity ranged from 0.02 (CEDG290 and VR023) to 0.78 (BM146) with an average of 0.38. The PIC value of SSRs varied from 0.02 (CEDG290 and VR023) to 0.96 (CEDG305) with an average of 0.43. The maximum PIC value was recorded for the marker CEDG305 (0.96) followed by DMBSSR080 (0.95), X62 (0.93), CEDG147 (0.93), DMSSR199 (0.91), and CP10667 (0.90) (**Table 2**).

Cluster-Based Genetic Diversity

The WNJ analysis (**Figure 1**) distributed 80 genotypes into three major clusters (A–C). Among these clusters, cluster C was the biggest one accommodating 50 (62.5%) genotypes followed by cluster A with 20 (25%) genotypes and cluster B with 10 (12.5%) genotypes.

Cluster A could be further subgrouped into two subclusters, namely, AI and AII, both these subclusters consisted of released varieties only. Subcluster AI (10 varieties) had seven released varieties developed at the ICAR-Indian Institute of Pulses Research, Kanpur (ICAR-IIPR), two at Rajasthan Agriculture Research Institute (RARI), Durgapura, and one at ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi. Subcluster AII (10 varieties) consisted of six released varieties developed at CCS HAU, Hisar, two at RARI, Durgapura and two at Rajasthan Agriculture University, Regional Research Centre (RAU RRS), Ganganagar.

Cluster B accommodated nine released varieties and one advanced genotype which could be further grouped into three subclusters, namely, BI, BII, and BIII. Subcluster BI (five genotypes) accommodated four released varieties, i.e., one each from Bhabha Atomic Research Centre (BARC), Trombay; Anand Agricultural University (AAU), Anand; CCS HAU, Hisar, and Dr. Panjabrao Deshmukh Krishi Vidyapeeth (PDKV), Akola and one advanced genotype from CCS HAU, Hisar. Subcluster BII consisted of two released varieties, i.e., one each of PDKV, Akola and Agriculture Research Station, Lam (ARSL), Andhra Pradesh. Subcluster BIII comprised of three released varieties, two developed at Banaras Hindu University (BHU), Varanasi and one at Govind Ballabh Pant University of Agriculture and Technology (GBPUA&T), Pantnagar.

Cluster C (50 genotypes) could be further divided into five subclusters, from CI to CV. Subclusters CI and CII accommodated 15 and 12 advanced breeding lines from CCS HAU, Hisar, respectively. Subcluster CIII consisted of a total of 16 genotypes including 11 released varieties developed at different centers [1 each developed at Tamil Nadu Agricultural University-National Pulses Research Centre (TNAU-NPRC), Vamban; Tamil Nadu Agricultural University (TNAU), Coimbatore; Chandra

TABLE 1 | Correlation coefficients for various quantitative characters in mung bean.

	DF	DM	PH	PL	SW	YMD	NB	NP	NS	SY
DF	1									
DM	0.492**	1								
PH	0.353**	0.095	1							
PL	−0.230*	−0.048	−0.08	1						
SW	−0.174	−0.078	−0.332**	0.739**	1					
YMD	0.223*	0.030	0.254*	−0.385**	−0.194	1				
NB	0.121	−0.095	0.276*	−0.227*	−0.422**	−0.024	1			
NP	−0.182	−0.155	−0.183	0.100	−0.042	−0.689**	0.418**	1		
NS	−0.151	0.046	0.083	0.525**	0.166	−0.493**	0.108	0.342**	1	
SY	−0.152	−0.045	−0.098	0.305**	0.056	−0.737**	0.315**	0.708**	0.507**	1

**5% level of significance; **1% level of significance.*

DF, days to 50% flowering; DM, days to maturity; PH, plant height; PL, pod length; SW, 100-seed weight; YMD, yellow mosaic disease; NB, number of branches per plant; NP, number of pods per plant; NS, number of seeds per pod; SY, seed yield per plant.

TABLE 2 | Details of polymorphic markers along with their allelic diversity and PIC.

Locus	Na	Ne	*I*	Ho	He	uHe	*F*	PIC
BM212	4.000	1.328	0.519	0.000	0.247	0.248	1.000	0.247
BM1	5.000	4.227	1.508	0.000	0.763	0.768	1.000	0.832
BM146	7.000	4.494	1.631	0.000	0.778	0.782	1.000	0.793
BMD-12	6.000	3.661	1.419	0.000	0.727	0.731	1.000	0.727
BMD-23	4.000	2.156	0.880	0.000	0.536	0.540	1.000	0.537
BMD-48	4.000	1.397	0.572	0.000	0.284	0.286	1.000	0.286
BMD-5	3.000	1.469	0.597	0.113	0.319	0.321	0.648	0.319
BMD-29	3.000	1.838	0.758	0.000	0.456	0.459	1.000	0.525
BMD-35	3.000	1.253	0.391	0.000	0.202	0.203	1.000	0.202
BMD-47	3.000	1.958	0.838	0.463	0.489	0.492	0.055	0.499
BMD-13	3.000	1.354	0.490	0.000	0.261	0.263	1.000	0.277
BMD-6	5.000	2.401	1.044	0.000	0.583	0.587	1.000	0.846
BMD-18	2.000	1.161	0.266	0.000	0.139	0.140	1.000	0.144
BMD-31	5.000	1.409	0.650	0.000	0.290	0.292	1.000	0.292
BMD-26	3.000	2.023	0.726	0.975	0.506	0.509	−0.928	0.506
CEDG115	7.000	3.313	1.387	0.000	0.698	0.703	1.000	0.879
CEDG147	5.000	2.658	1.180	0.000	0.624	0.628	1.000	0.913
CEDG220	6.000	1.407	0.656	0.000	0.289	0.291	1.000	0.297
CEDG244	6.000	1.527	0.749	0.000	0.345	0.347	1.000	0.345
CEDG254	5.000	3.302	1.339	0.000	0.697	0.702	1.000	0.720
CEDG256	3.000	2.696	1.036	0.825	0.629	0.633	−0.311	0.660
CEDG293	5.000	1.544	0.685	0.000	0.353	0.355	1.000	0.353
CEDG295	5.000	2.297	0.970	0.700	0.565	0.568	−0.240	0.572
CEDG296	6.000	1.665	0.860	0.000	0.399	0.402	1.000	0.415
CEDG305	5.000	2.143	1.067	0.000	0.533	0.537	1.000	0.956
CEDG048	3.000	1.701	0.720	0.000	0.412	0.415	1.000	0.412
CEDG053	3.000	1.414	0.509	0.000	0.293	0.295	1.000	0.293
CEDG071	4.000	2.546	1.067	0.000	0.607	0.611	1.000	0.705
CEDG073	3.000	1.594	0.628	0.000	0.373	0.375	1.000	0.373
CEDG088	6.000	2.775	1.220	0.000	0.640	0.644	1.000	0.890
CEDGAT009	5.000	3.604	1.362	0.000	0.723	0.727	1.000	0.773
CP1038	5.000	3.397	1.308	0.000	0.706	0.710	1.000	0.710
CP10667	4.000	2.982	1.227	0.000	0.665	0.669	1.000	0.902
DMSSR001	4.000	2.402	1.089	0.413	0.584	0.587	0.293	0.589
DQ345305	5.000	1.262	0.481	0.000	0.208	0.209	1.000	0.208
SSR-1AC127	2.000	1.190	0.297	0.000	0.160	0.161	1.000	0.167
SSR-1AC188	2.000	1.051	0.117	0.000	0.049	0.049	1.000	0.049
GMES162	3.000	1.569	0.635	0.000	0.363	0.365	1.000	0.398
GMES1823	2.000	1.311	0.400	0.000	0.237	0.239	1.000	0.237
GMES035	7.000	4.020	1.580	0.000	0.751	0.756	1.000	0.753
PVag003	2.000	1.161	0.266	0.000	0.139	0.140	1.000	0.144
PVag005	2.000	1.536	0.533	0.000	0.349	0.351	1.000	0.349
PVat001	3.000	1.569	0.635	0.000	0.363	0.365	1.000	0.398
PvM03	3.000	2.256	0.884	0.938	0.557	0.560	−0.684	0.561
PvM13b	5.000	1.972	0.936	0.000	0.493	0.496	1.000	0.538
SSR1AC-177	4.000	1.977	0.885	0.000	0.494	0.497	1.000	0.498
VR013	5.000	2.707	1.222	0.000	0.631	0.635	1.000	0.693
VR015	4.000	1.428	0.579	0.000	0.300	0.302	1.000	0.300
VR016	5.000	2.126	1.023	0.000	0.530	0.533	1.000	0.560
VR037	3.000	1.164	0.314	0.000	0.141	0.142	1.000	0.144
VR021	3.000	1.481	0.547	0.000	0.325	0.327	1.000	0.360
VR039	2.000	1.105	0.199	0.000	0.095	0.096	1.000	0.098
VrD1	3.000	2.506	0.994	0.000	0.601	0.605	1.000	0.864
X49	3.000	2.477	1.000	0.000	0.596	0.600	1.000	0.647

(Continued)

TABLE 2 | (Continued)

Locus	Na	Ne	*I*	Ho	He	uHe	*F*	PIC
X62	3.000	1.785	0.724	0.000	0.440	0.442	1.000	0.930
X65	4.000	1.366	0.560	0.000	0.268	0.269	1.000	0.273
AF35050	3.000	1.165	0.318	0.000	0.142	0.142	1.000	0.143
CP00361	2.000	1.190	0.297	0.000	0.160	0.161	1.000	0.167
CP5096	2.000	1.250	0.352	0.000	0.200	0.201	1.000	0.212
CEDC055	4.000	1.659	0.790	0.000	0.397	0.400	1.000	0.397
CEDC033	3.000	1.165	0.318	0.000	0.142	0.142	1.000	0.142
CEDG100	5.000	1.849	0.916	0.000	0.459	0.462	1.000	0.459
CEDG013	3.000	1.490	0.576	0.000	0.329	0.331	1.000	0.329
CEDG15	2.000	1.051	0.117	0.000	0.049	0.049	1.000	0.049
CEDG24	2.000	1.568	0.548	0.000	0.362	0.364	1.000	0.362
CEDG035	3.000	1.490	0.576	0.050	0.329	0.331	0.848	0.329
CEDG41	3.000	1.078	0.177	0.013	0.073	0.073	0.828	0.073
CEDG60	2.000	1.406	0.464	0.000	0.289	0.291	1.000	0.289
CEDG70	2.000	1.438	0.483	0.000	0.305	0.307	1.000	0.305
CEDG97	4.000	1.813	0.886	0.000	0.448	0.451	1.000	0.454
CEDG116	2.000	1.078	0.160	0.000	0.072	0.073	1.000	0.072
CEDG136	5.000	1.883	0.876	0.000	0.469	0.472	1.000	0.942
CEDG150	3.000	1.106	0.227	0.000	0.096	0.097	1.000	0.096
CEDG185	3.000	1.497	0.597	0.000	0.332	0.334	1.000	0.334
CEDG267	3.000	1.349	0.466	0.000	0.258	0.260	1.000	0.277
CEDG290	2.000	1.025	0.067	0.000	0.025	0.025	1.000	0.025
DMSSR080	3.000	1.967	0.856	0.000	0.492	0.495	1.000	0.947
DMSSR199	4.000	1.945	0.819	0.000	0.486	0.489	1.000	0.908
DMSSR043	3.000	1.316	0.442	0.000	0.240	0.242	1.000	0.240
DQ9393	2.000	1.406	0.464	0.000	0.289	0.291	1.000	0.289
DQ469293	2.000	1.503	0.517	0.000	0.335	0.337	1.000	0.335
VR022	3.000	1.349	0.466	0.000	0.258	0.260	1.000	0.258
J01263	4.000	1.397	0.572	0.000	0.284	0.286	1.000	0.284
MBSSR008	2.000	1.503	0.517	0.000	0.335	0.337	1.000	0.335
PvM22	2.000	1.568	0.548	0.000	0.362	0.364	1.000	0.362
VM27	2.000	1.133	0.234	0.000	0.117	0.118	1.000	0.117
VR023	2.000	1.025	0.067	0.000	0.025	0.025	1.000	0.025
VR032	2.000	1.311	0.400	0.000	0.237	0.239	1.000	0.237
BMD8	3.000	1.291	0.453	0.000	0.225	0.227	1.000	0.233
Mean	3.562	1.825	0.694	0.050	0.376	0.378	0.905	0.427

Na, number of alleles; Ne, number of effective alleles; I, Shannon's information index; Ho, observed heterozygosity; He, expected heterozygosity; uHe, unbiased expected heterozygosity; F, fixation index; PIC, polymorphic information content.

Shekhar Azad University of Agriculture and Technology (CSAUAT), Kanpur; BARC, Trombay; Odisha University of Agriculture and Technology (OUAT), Berhampur; 2 developed at Punjab Agricultural University (PAU), Ludhiana; 4 at GBPUA&T, Pantnagar; and 5 advanced genotypes developed at CCS HAU, Hisar. Subcluster CIV (five genotypes) comprised three released varieties of ICAR-IARI, New Delhi and one each of RARI, Durgapura, and PAU, Ludhiana. Subcluster CV consisted of two genotypes, i.e., one advanced genotype of CCS HAU, Hisar and one released variety of PAU, Ludhiana (**Table 3**).

Population Genetic Structure

Population genetic structure was used to analyze the structure of the population in the context of genetic diversity and the relatedness of the individuals within the group. Delta *K* value was used to estimate the significant number of SPs in all genotypes at the molecular level (**Figure 2**) by Evanno table. Population structure categorized the 80 cultivars and advanced mung bean genotypes into three genetically distinct SPs, namely, SP1 (marked by red), SP2 (green), and SP3 (blue) along with admixture group SP4 (mixture of colors) (**Figure 3**). Genotypes with *Q* values $\geq$0.7 were considered pure, while genotypes having <0.7 scores were considered admixture. Out of 80 genotypes, 68 (85%) resembled their hierarchy, and 12 (15%) were observed as the admixture form. The maximum number of genotypes (30) were grouped in SP3, followed by SP2 (20), SP1 (18), and SP4 (12).

The 18 genotypes in SP1 consisted of one released variety each developed by AAU, Anand, CCS HAU, Hisar, and BARC and fifteen advanced genotypes developed at CCS HAU, Hisar.

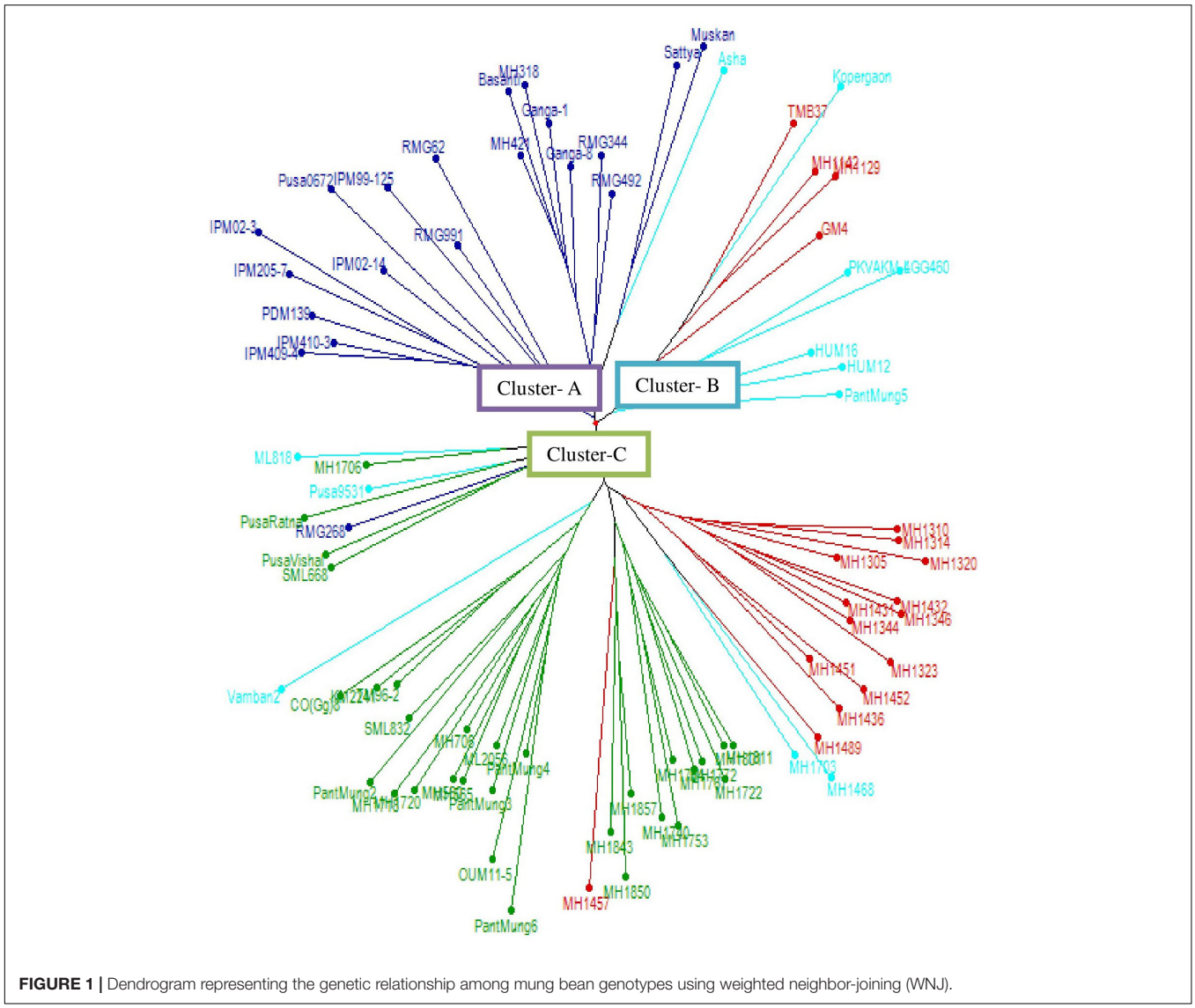

FIGURE 1 | Dendrogram representing the genetic relationship among mung bean genotypes using weighted neighbor-joining (WNJ).

SP2 accommodated 20 (25%) released varieties developed at CCS HAU, Hisar (5); RAU RRS, Ganganagar (2); RARI, Durgapura (5); ICAR-IIPR, Kanpur (7); and ICAR-IARI, New Delhi (1). SP3 comprised of 30 (37.5%) genotypes which included one variety each of TNAU, Coimbatore; OUAT, Berhampur; BARC, Trombay; and CSAUAT, Kanpur; 3 of PAU, Ludhiana; 4 of GBPUA&T, Pantnagar; 2 of ICAR-IARI, New Delhi; and 17 advanced genotypes developed at CCS HAU, Hisar. SP4 consisted of 10 released varieties and two advanced genotypes (15%). One released variety each belonged to CCS HAU, Hisar; GBPUA&T, Pantnagar; PAU, Ludhiana; ICAR-IARI, New Delhi; TNAU-NPRC, Vamban; and ARSL, Andhra Pradesh and two varieties each of BHU, Varanasi, and PDKV, Akola and the two advanced genotypes from CCS HAU, Hisar (**Table 3**).

Genetic Diversity Within Subpopulations

The number of alleles per locus ranged from 2.43 (SP4) to 2.72 (SP2), and the number of effective alleles varied from 2.29 (SP2) to 2.66 (SP3) per locus. Shannon's index minimum mean value was observed for SP4 (0.57) and maximum for SP3 (0.61), and the number of private alleles varied from 0.11 (SP4) to 0.30 (SP2). The mean value of expected heterozygosity ranged from 0.33 (SP4) to 0.35 (SP1 and SP3), and unbiased expected heterozygosity was slightly higher (0.36) for SP1 and SP3 and minimum for SP2 (0.34) (**Table 4**). For better visualization, genetic diversity within SPs is represented graphically (**Figure 4**). The genetic differentiation indices among the population (Fst) ranged from 0.001 (between SP1 and SP2, SP1 and SP3, SP2 and SP3, and SP2 and SP4) to 0.008 (between SP3 and SP4) (**Table 5**). The pair-wise Nei genetic identity value varied from 0.90 (SP1 vs. SP2) to 0.95 (SP1 vs. SP3) (**Table 6**). The differences within and among the groups studied from AMOVA analysis revealed that 7% of molecular variance was present among four SPs, 80% among individuals, and 13% of the total variation was observed within individuals (**Table 7**).

TABLE 3 | Grouping of released and advanced breeding lines based on weighted neighbor-joining and population genetic structure.

Genotypes	Sub population/color code	WNJ clustering	Pedigree	Source
Asha	Admixture	AII	K 851 × L 24-2	CCS HAU, Hisar
HUM 12	Admixture	BIII	HUM 5 × DPM 90-1	BHU, Varanasi
HUM 16	Admixture	BIII	Pusa bold1 × HUM 8	BHU, Varanasi
LGG 460	Admixture	BII	Lam M2 × ML 267	ARS, Lam
MH 1468	Admixture	CI	MH 318 × AKM 9904	CCS HAU, Hisar
MH 1703	Admixture	CI	IPM 02-17 × MH 521	CCS HAU, Hisar
Kopergaon	Admixture	BI	CO 5-KM 2 × MG 50-10 (G)	Maharashtra
ML 818	Admixture	CV	5145/87 × ML 267	PAU, Ludhiana
Pant Mung 5	Admixture	BIII	Selection from VC 6368	GBPUA&T, Pantnagar
PKV AKM-4	Admixture	BII	BM 4 × PS 16	PDKV, Akola
Pusa 9531	Admixture	CIV	Selection from NM 9473	IARI, New Delhi
Vamban 2	Admixture	CIII	VGG 4 × MH 309	NPRC, Vamban
GM 4	Red (SP1)	BI	GM-3 × Pusa 9333	AAU, Anand
MH 1129	Red (SP1)	BI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1142	Red (SP1)	BI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1305	Red (SP1)	CI	MH 98-1 × MH 565	CCS HAU, Hisar
MH 1314	Red (SP1)	CI	MH 3-18 × Pusa 0672	CCS HAU, Hisar
MH 1315	Red (SP1)	CI	MH 3-18 × Pusa 0672	CCS HAU, Hisar
MH 1320	Red (SP1)	CI	MH 421-S-14-3	CCS HAU, Hisar
MH 1323	Red (SP1)	CI	MH 3-18 × AKM 99-4	CCS HAU, Hisar
MH 1344	Red (SP1)	CI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1346	Red (SP1)	CI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1431	Red (SP1)	CI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1432	Red (SP1)	CI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1436	Red (SP1)	CI	Muskan × BDYR 2	CCS HAU, Hisar
MH 1451	Red (SP1)	CI	MH 98-1 × Pusa 0672	CCS HAU, Hisar
MH 1452	Red (SP1)	CI	MH 98-1 × Pusa 0672	CCS HAU, Hisar
MH 1457	Red (SP1)	CII	MH 98-1 × MH 565	CCS HAU, Hisar
MH 1489	Red (SP1)	CI	MH 318 × Pusa 0871	CCS HAU, Hisar
TMB 37	Red (SP1)	BI	Kopergaon × TARM-2	BARC, Trombay
CO(Gg) 8	Blue (SP3)	CIII	COGG 923 × VC 6040	TNAU, Coimbatore
KM 2241	Blue (SP3)	CIII	Samrat × PDM 54	CSAUAT, Kanpur
MH 1706	Blue (SP3)	CV	IPM 02-17 × MH 565	CCS HAU, Hisar
MH 1718	Blue (SP3)	CIII	KM 2241 × MH 521	CCS HAU, Hisar
MH 1720	Blue (SP3)	CIII	IPM 02-19 × MH 565	CCS HAU, Hisar
MH 1722	Blue (SP3)	CII	Pusa 0672 × MH 521	CCS HAU, Hisar
MH 1740	Blue (SP3)	CII	IPM-409-4 × MH 318	CCS HAU, Hisar
MH 1753	Blue (SP3)	CII	MH 421 × IPM 205-7	CCS HAU, Hisar
MH 1754	Blue (SP3)	CII	MH 421 × IPM 205-7	CCS HAU, Hisar
MH 1767	Blue (SP3)	CII	MH 534 × MH 318	CCS HAU, Hisar
MH 1772	Blue (SP3)	CII	VGG-rt-1 × Sattya	CCS HAU, Hisar
MH 1801	Blue (SP3)	CII	IPM 02-17 × MH 521	CCS HAU, Hisar
MH 1811	Blue (SP3)	CII	Sattya × IPM 409-4	CCS HAU, Hisar
MH 1843	Blue (SP3)	CII	LGG 460 × Sattya	CCS HAU, Hisar
MH 1850	Blue (SP3)	CII	Sattya × IPM 409-4	CCS HAU, Hisar
MH 1857	Blue (SP3)	CII	Sattya × MH 318	CCS HAU, Hisar
MH 560	Blue (SP3)	CIII	Asha × BDYR 1	CCS HAU, Hisar
MH 565	Blue (SP3)	CIII	Asha × BDYR 1	CCS HAU, Hisar
MH 706	Blue (SP3)	CIII	MH 96-1 × BDYR 2	CCS HAU, Hisar
ML 2056	Blue (SP3)	CIII	ML 1165 × ML 1191	PAU, Ludhiana
OUM 11-5	Blue (SP3)	CIII	Mutant of Dhauli	OUAT, Berhampur
Pant Mung 2	Blue (SP3)	CIII	Mutant of ML-26	GBPUA&T, Pantnagar
Pant Mung 3	Blue (SP3)	CIII	LN 294-8 × L 80	GBPUA&T, Pantnagar
Pant Mung 4	Blue (SP3)	CIII	T 44 × UPU 2	GBPUA&T, Pantnagar

(Continued)

TABLE 3 | (Continued)

Genotypes	Sub population/color code	WNJ clustering	Pedigree	Source
Pant Mung 6	Blue (SP3)	CIII	Pant M 2 × AMP 36	GBPUA&T, Pantnagar
Pusa Ratna	Blue (SP3)	CIV	VC 6368 × ML 267	IARI, New Delhi
Pusa Vishal	Blue (SP3)	CIV	Selection from NM 92	IARI, New Delhi
SML 668	Blue (SP3)	CIV	Selection from NM 94	PAU, Ludhiana
SML 832	Blue (SP3)	CIII	SML 302 × Pusa bold1	PAU, Ludhiana
TM 96-2	Blue (SP3)	CIII	Kopergaon × TARM-2	BARC, Trombay
Basanti	Green (SP2)	AII	Asha × PDM 90-1	CCS HAU, Hisar
Ganga-1	Green (SP2)	AII	Local selection from Kaluwala	RAU RRS, Ganganagar
Ganga-8	Green (SP2)	AII	K 851 × Pusa 105	RAU RRS, Ganganagar
IPM 02-14	Green (SP2)	AI	IPM 99-125 × Pusa bold2	IIPR, Kanpur
IPM 02-3	Green (SP2)	AI	IPM 99-125 × Pusa bold2	IIPR, Kanpur
IPM 205-7	Green (SP2)	AI	IPM 02-1 × EC 398889	IIPR, Kanpur
IPM 409-4	Green (SP2)	AI	PDM 288 × IPM 03-1	IIPR, Kanpur
IPM 410-3	Green (SP2)	AI	IPM 03-1 × NM 1	IIPR, Kanpur
IPM 99-125	Green (SP2)	AI	PM 3 × APM 36	IIPR, Kanpur
MH 318	Green (SP2)	AII	Asha × BDYR 1	CCS HAU, Hisar
MH 421	Green (SP2)	AII	Muskan × BDYR 2	CCS HAU, Hisar
Muskan	Green (SP2)	AII	PDM 116 × Gujarat-1	CCS HAU, Hisar
PDM 139	Green (SP2)	AI	ML 20/19 × ML 5	IIPR, Kanpur
Pusa 0672	Green (SP2)	AI	11/395 × ML 267	IARI, New Delhi
RMG 268	Green (SP2)	CIV	R 288-8 × J 781	RARI, Durgapura
RMG 344	Green (SP2)	AII	Mung selection-1 × J-45	RARI, Durgapura
RMG 492	Green (SP2)	AII	Mutant of RMG 62	RARI, Durgapura
RMG 62	Green (SP2)	AI	R 288-8 × China mung	RARI, Durgapura
RMG 991	Green (SP2)	AI	RMG 268 × UPM 98	RARI, Durgapura
Sattya	Green (SP2)	AII	PDM 116 × Gujarat-1	CCS HAU, Hisar

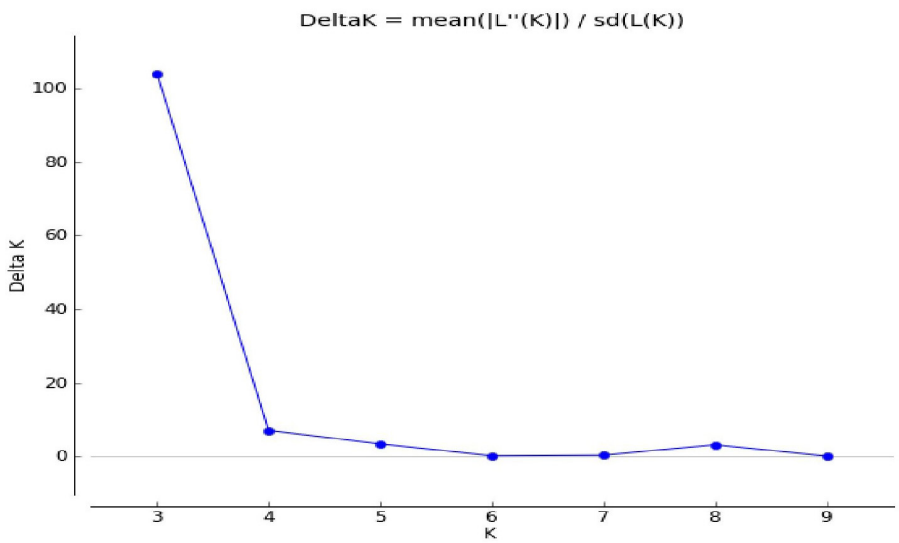

FIGURE 2 | Population estimation using LnP (D) derived Δ*k* (*K* = 2–10).

Linkage Disequilibrium

Significant linkage disequilibrium (LD) blocks were observed in the genome-wide LD analysis as demonstrated by triangle heat plots for pair-wise LD between SSR using TASSEL software (**Figure 5**). The R^2 value between marker pairs ranged from 0.1 to 0.49 (between VR039 and SSR188). The R^2 value above 0.1 between marker pairs was considered to be in LD, and there were 75 marker pairs having the R^2 value above 0.1. The marker BMd23 had the highest LD with 16 markers (i.e., BMd35, BM212, BMD6, CEDC55, CEDG185, CEDG70, CP1038, DMSSR199,

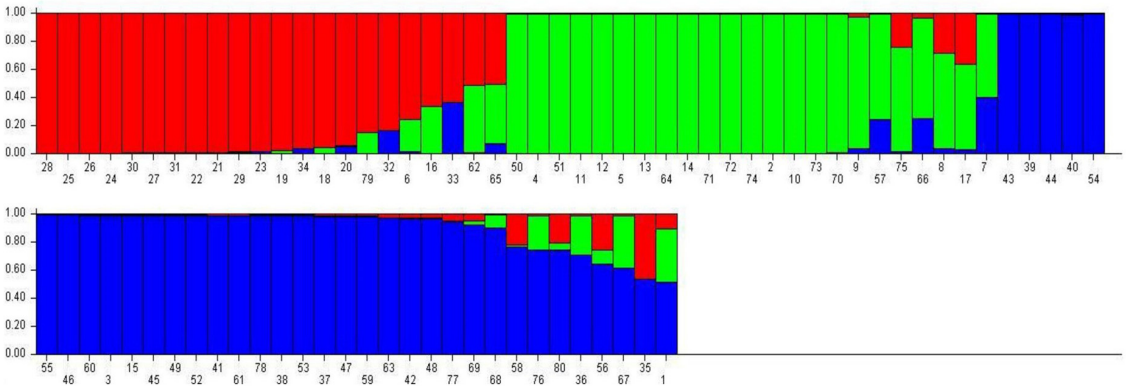

FIGURE 3 | Bar graph representing population genetic structure of mung bean genotypes performed by admixture method in STRUCTURE.

TABLE 4 | Genetic diversity and mean allelic pattern across subpopulations of mung bean genotypes.

Population	Sub-population 1		Sub-population 2		Sub-population 3		Sub-population 4	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Na	2.640	0.114	2.719	0.114	2.674	0.109	2.427	0.115
Na frequency ≥ 5%	2.607	0.114	2.292	0.097	2.663	0.109	2.427	0.115
Ne	1.744	0.072	1.709	0.072	1.741	0.075	1.712	0.072
I	0.604	0.041	0.595	0.039	0.612	0.039	0.573	0.043
No. private alleles	0.169	0.043	0.303	0.065	0.124	0.035	0.112	0.034
No. LComm alleles (≤25%)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
No. LComm alleles (≤50%)	0.326	0.055	0.315	0.052	0.382	0.059	0.213	0.047
He	0.349	0.023	0.338	0.022	0.349	0.022	0.334	0.024
uHe	0.358	0.024	0.344	0.022	0.358	0.022	0.348	0.025

Na, no. of different alleles per locus; Ne, no. of effective alleles per locus; I, Shannon's index; He, expected heterozygosity; uHe, unbiased expected heterozygosity.

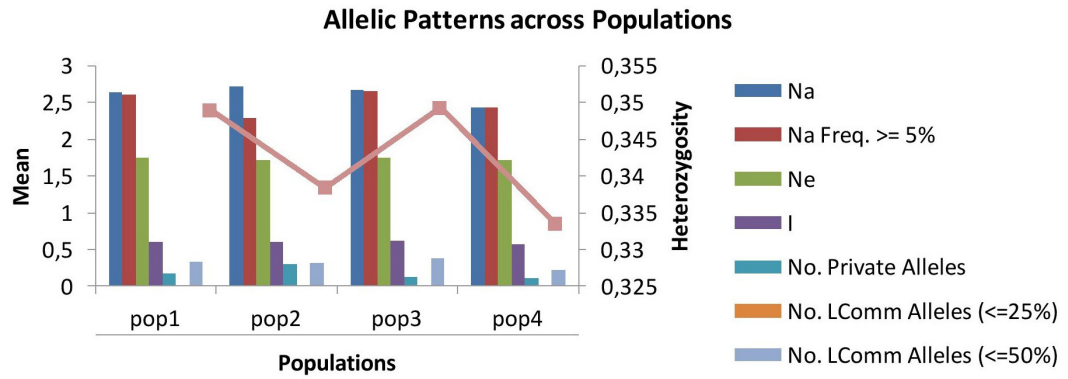

FIGURE 4 | Graphical presentation of allelic patterns across the subpopulation.

DQ469293, DQ9393, GMES035, PVag005, PVM22, SSR1AC-177, VR015, and X49) followed by DQ469293 which had LD with 8 markers (i.e., X49, SSR1AC-177, DQ9393, CP10667, CEDG70, CEDG41, BMd35, and BMd23).

Association Analysis

Marker-trait association study was conducted using the mean values of all the SY-related traits based on a phenotypic evaluation over four environments and the allelic data of 89 polymorphic SSRs. A total of 38 marker-trait associations were observed to be significant for yield-related traits and YMD resistance by the generalized linear model (GLM-Q) at a corrected *p*-value of ≤0.0005 (Benjamini and Hochberg, 1995; **Supplementary Table 5**). Eleven marker-trait associations were found significant using the most accepted maximum likelihood model (MLM-Q + K) for four yield-related traits, namely, DF, DM, PH, and NP, and also for YMD resistance at *p*-value ≤0.01 (**Table 8**). This association explained high phenotypic variation, i.e., 41.55%

TABLE 5 | Pairwise population *F*st values.

Population	SP1	SP2	SP3	SP4
SP1	0.000	0.001	0.001	0.006
SP2		0.000	0.001	0.001
SP3			0.000	0.008
SP4				0.000

TABLE 6 | Pair-wise population matrix of Nei genetic identity.

Population	SP1	SP2	SP3	SP4
SP1	1.000			
SP2	0.902	1.000		
SP3	0.952	0.944	1.000	
SP4	0.949	0.913	0.948	1.000

TABLE 7 | Analysis of molecular variance.

Source	df	SS	MS	Est. Var.	%
Among population	3	232.160	77.387	1.228	7
Among individual	76	2262.053	29.764	13.760	80
Within individual	80	179.500	2.244	2.244	13
Total	159	2673.713		17.232	100

df, degree of freedom; SS, sum of square; MS, mean sum of square; Est. Var., estimated variance; %, percentage of variance.

through GLM and 13.57% through MLM. The maximum number of markers exhibited association with PH (five, i.e., DMBSSR043, CEDG97, DQ9393, CEDG295, and CEDG88) followed by YMD (two, i.e., J01263 and CEDG220). One MTA each for DF (VR022), DM (BM146), and NP (BMd12) in different seasons was identified from the MLM approach. In both GLM and MLM approaches, a total of four MTAs were found to be common across seasons for NB associated with BMd12, PH with DMBSSR043 and CEDG97, and DM associated with BM146. The marker BMd12 associated with NB expressed consistently in *kharif* (monsoon) 2018 and 2019. Similarly, CEDG88 associated with PH was identified consistently in the summer seasons during both years. CEDG97 and DQ9393 associated with PH were identified in *kharif* 2019 and pooled over *kharif* data. VR022 associated with DF was consistently identified in *Kharif* 2018, pooled data of *kharif* as well as the pooled data of *kharif* and summer (**Figure 6**). The MTA study also revealed the presence of pleiotropic markers in mung bean, i.e., a single marker associated with more than one trait, such as BMd12 associated with NP, PL, SW, and YMD, and CEDG97 associated with PL, PH, and NS. Likewise, the markers DMBSSR001, CP1038, VR021, BMd35, CP5096, BM146, DQ9393, and CEDG220 were also associated with different traits.

DISCUSSION

Despite many research efforts undertaken for mung bean genetic improvement during the last few decades, its productivity still falls short of acceptable levels. The major reasons for stagnancy in its productivity are insufficient genetic variability, poor harvest index, high influence of GXE interaction, and susceptibility of many of the available cultivars to various biotic and abiotic stresses (Nair et al., 2019; Pratap et al., 2021), which ultimately result in yield instability. In addition, genetic improvement through breeding efforts is slow due to inadequate utilization of genomic resources and a dearth of trait-linked molecular markers to undertake molecular breeding for accelerated crop improvement. Molecular markers, owing to their environmental independence, are important tools to estimate the genetic variation present in the germplasm. These also have an advantage in the breeding program as these can be used to adjudge the presence or absence of a particular gene/allele or genomic segments contributing to the trait expression. Therefore, this study was conducted with a panel of 80 released varieties and advanced breeding lines of mung bean for the purpose of estimating the genetic diversity using molecular markers and detecting loci associated with yield attributing traits and YMD resistance by association analysis. At the phenotypic level, a considerable amount of variability was observed among the mung bean genotypes for all the studied characters. Furthermore, very less difference between PCV and GCV estimates was observed which indicated the inherent nature of variability and lesser influence of environmental factors on the expression of these traits.

Selection for yield may be effective if all the traits that directly or indirectly affect the yield are considered during selection. In-depth prior knowledge of the magnitude and direction of the association among the characters is imperative for operating an efficient selection program in crop plants. In the present investigation, SY was found positively associated with PL, NB, NP, and NS and negatively associated with YMD. The present results with respect to yield attributes are in consonance with the findings of Saeed et al. (2007), Win et al. (2009), Kumar et al. (2010), Khajudparn and Tantasawat (2011), Zaid et al. (2012), and Baisakh et al. (2016). A negative correlation between yield and YMD resistance was also reported by Alam et al. (2014) and Anuradha et al. (2019).

Initially, 164 SSRs from different *Vigna* species (adzuki bean, cowpea, mung bean, and common bean) were selected (Pratap et al., 2015; Suman et al., 2019; Singh et al., 2020). SSRs from related species, namely, cowpea (Li et al., 2001), common bean (Blair et al., 2013), and adzuki bean (Wang et al., 2004) could be easily cross transferred to mung bean in earlier studies. In this study also, most of the primer pairs from related species were amplified with varying allele sizes ranging between 100 and 310 bp and, therefore, exhibited their potential across closely related *Vigna* species (Pratap et al., 2015). All polymorphic primer pairs generated 317 polymorphic alleles with an average of 3.56 alleles per SSR locus, and the number of alleles at each locus (Na) varied from 2 to 7 which is consistent with earlier studies (Sangiri et al., 2007; Shrivastava et al., 2014; Singh et al., 2020). Heterozygosity was observed in nine SSR loci which ranged from 0.01 to 0.97 with an average of 0.05. The expected heterozygosity ranged from 0.02 to 0.78 with an average of 0.38. Therefore, this study suggests the existence of ample genetic diversity among the released and advanced mung bean genotypes used, and this may be useful for the selection

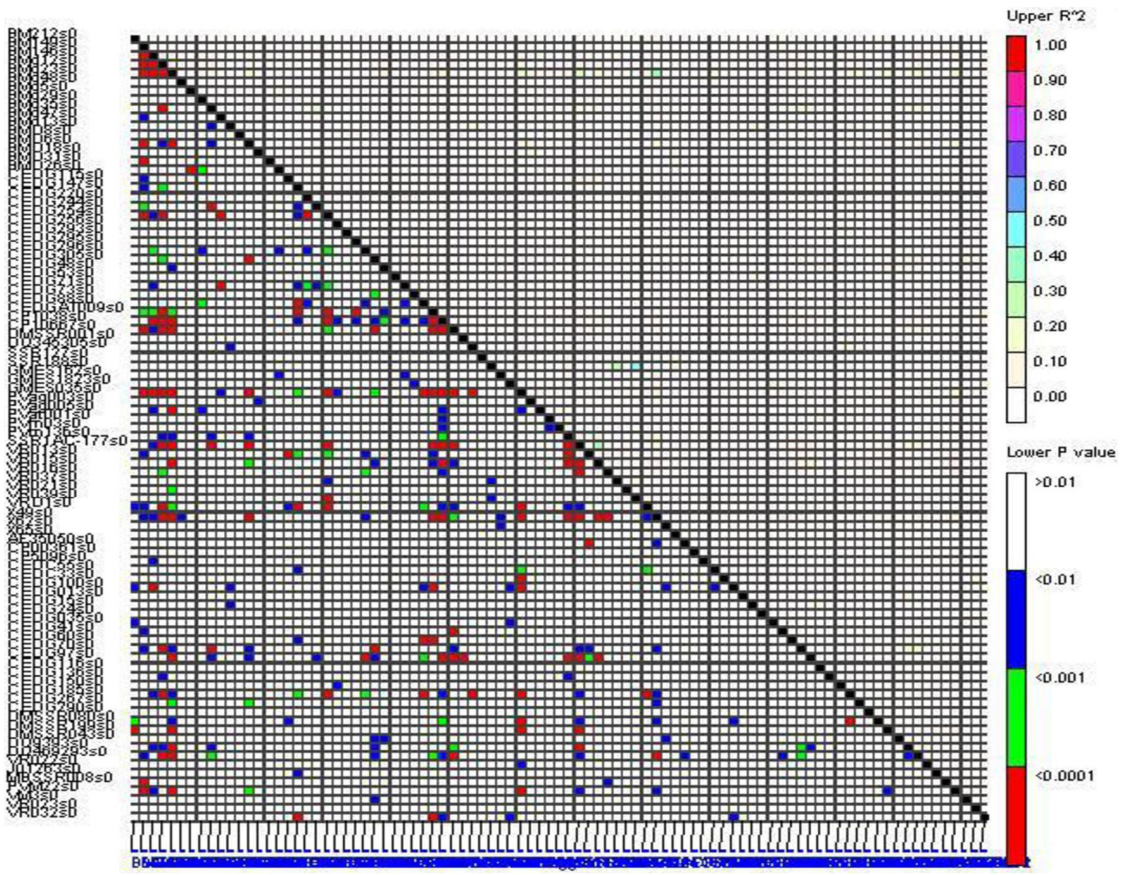

FIGURE 5 | Triangle heat plot showing pairwise locus combination in mung bean genotypes.

of genotypes for hybridization programs directed toward mung bean improvement. The genotypes found highly diverse at the molecular level are expected to exhibit more heterotic effects in F_1 generation, and the information generated in this study could be considered valuable for developing heterotic pool in mung bean.

The wide range of PIC values of SSRs indicated that the markers used in this study were ample to explore the genetic diversity among studied genotypes. The PIC value obtained in this study using *Vigna*-species-specific SSRs is in accordance with earlier studies (Tangphatsornruang et al., 2009; Lestari et al., 2014; Shrivastava et al., 2014; Pratap et al., 2015; Markam et al., 2018; Suman et al., 2019; Singh et al., 2020). Pratap et al. (2015, 2021) recorded maximum PIC value for J01263, VR0163, VR0338, and SSR-IAC-177 (0.89) followed by BMD-12 (0.88), and in this study also, the BMD-12 marker locus revealed high genetic variation (PIC 0.73) among different mung bean varieties. A total of 30 primers were observed to have a PIC value of $\geq$0.5 and 32 primers having above-average PIC value suggesting that the highly polymorphic SSRs would be a valuable resource for assessing the mung bean genetic diversity and QTL mapping studies.

The WNJ analysis distributed 80 genotypes into three major clusters (A, B, and C). Among these, cluster C was the major cluster comprising all the advanced genotypes while the other two clusters consisted of all released varieties developed at different institutes. The subclusters AI and AII consisted of released varieties only. In an earlier study, Suman et al. (2019) assessed the genetic diversity of 18 mung bean genotypes, and the dendrogram based on SSR data grouped the mung bean cultivars IPM 02-14 and PDM 139 (developed at ICAR-IIPR, Kanpur) in the same cluster and HUM 12, HUM 1, and HUM 16 (developed at BHU, Varanasi) and few other varieties in another similar cluster. Lestari et al. (2014), Chen et al. (2015), Pratap et al. (2016), and Kaur et al. (2018) also reported similar clustering results in mung bean. Most recently, Pratap et al. (2021) in their analysis of 41 released varieties and elite lines of mung bean also reported grouping of all the varieties developed at IIPR after the year 2000 in a single cluster.

Population genetic structure categorized all 80 mung bean genotypes into three genetically distinct SPs along with the admixture class as observed in WNJ analysis. Pratap et al. (2021) also grouped 41 mung bean elite lines in 3 SPs. Noble et al. (2018) also determined four SPs in the cultivated mung bean germplasm genotyped with integrated DArT and genotyping-by-sequencing (GBS) methodology. Qin et al. (2017) studied 338 genotypes of cowpea from different geographic regions of the world and found 3 SPs. Reddy et al. (2020) and Singh et al. (2020) also employed released varieties, advanced breeding lines, and exotic genotypes

TABLE 8 | Significant marker-trait associations identified from MLM (Q + K) approach in different environments.

Kharif-2018					*Kharif*-2019					*Kharif*-pooled				
Trait	**Locus**	**Allele**	***p*-Value**	R^2	**Trait**	**Locus**	**Allele**	***p*-Value**	R^2	**Trait**	**Locus**	**Allele**	***p*-Value**	R^2
DF	VR022	175	0.0098	3.18	DM	BM146	285	0.0071	13.4	DF	VR022	175	0.0096	2.7
NP	BMd12	180	6.6E−05	2.95	NP	BMd12	195	0.0014	2.79	PH	CEDG97	110	0.0063	6.5
PH	DMSSR043	200	0.0043	13.6	PH	CEDG97	110	0.00049	10.3	PH	DQ9393	210	0.0088	2.9
YMD	J01263	180	0.00015	4.15	PH	DQ9393	210	0.0082	3.41	Summer-pooled				
YMD	CEDG220	170	0.0034	4.58	PH	CEDG295	190	0.01	3.4	PH	CEDG88	160	0.01	7.9
Summer-2019					**Summer-2020**					***Kharif*-summer-pooled**				
PH	CEDG88	160	0.0049	6.61	PH	CEDG88	160	0.0095	9.53	DF	VR022	175	0.01	2.7

MLM, mixed linear model.

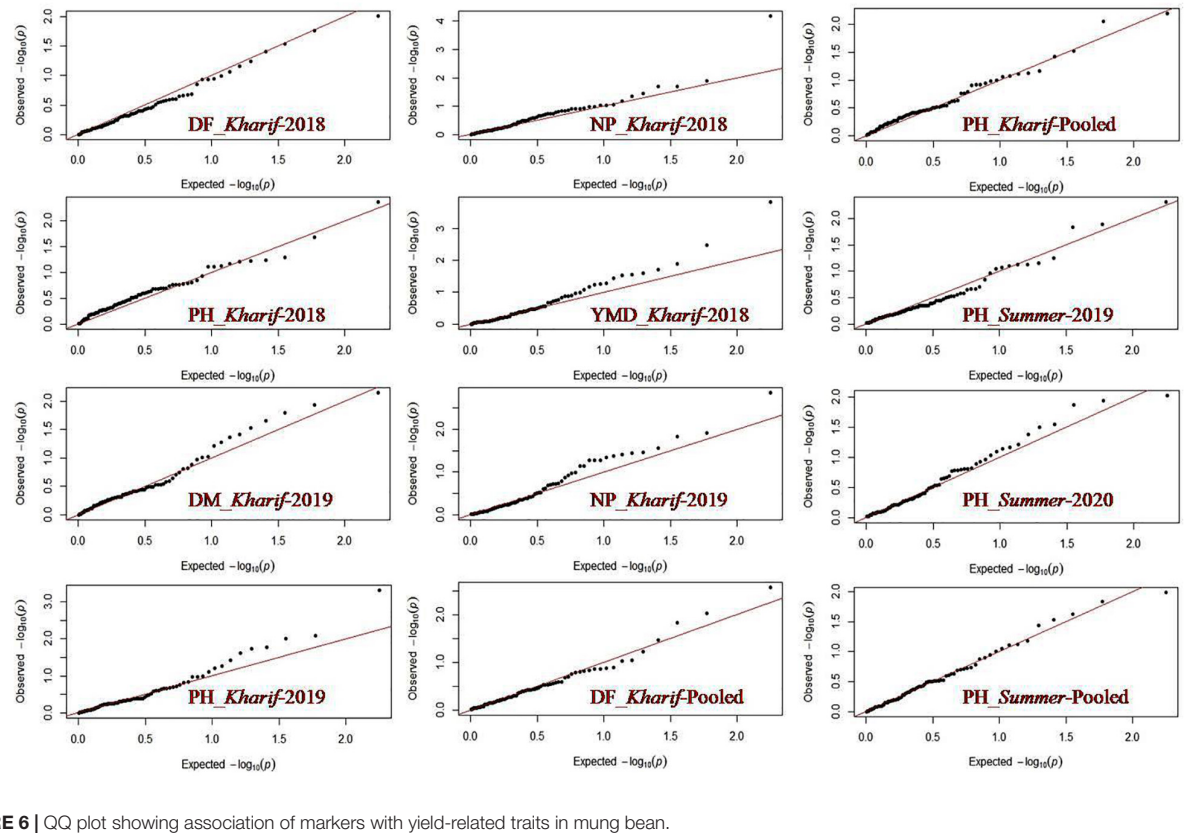

FIGURE 6 | QQ plot showing association of markers with yield-related traits in mung bean.

of mung bean and reported that the released varieties grouped together in one SP as also identified in this study. It is noteworthy that in the present investigation, both the cluster analysis and the population genetic structure categorized the genotypes in a similar manner as all advanced breeding lines were grouped into a single cluster or SP, while the released varieties developed at different institutes were categorized in different clusters or SPs. This study suggests that all the advanced genotypes and released varieties of mung bean from different institutes might have a certain degree of common ancestral history; therefore, population genetic structure was in agreement with genetic diversity analysis.

Association mapping is a powerful tool used for dissecting complex traits based on LD. It exploits historical and evolutionary recombination present in an unstructured population to map QTLs in higher resolution (Flint-Garcia et al., 2003). A significant and true marker-trait association can be utilized for MAS to improve breeding efficiency in terms of time and cost (Pratap et al., 2017; Singh et al., 2020). Significant LD blocks were observed in the genome-wide LD analysis with 80 SSR genotypic data, and a similar pattern of LD in different *Vigna* species was reported (Galeano et al., 2012; Xu et al., 2012; Noble et al., 2018; Kumar et al., 2019; Reddy et al., 2020). Ten significant marker-trait associations for yield-related traits and YMD resistance were identified over the four different environments along with their pooled data using the most accepted maximum likelihood model. However, few associations were consistently expressed across seasons.

Singh et al. (2018) reported five molecular markers (CEDG044, CEDG256, cp05325, GMES0214, and VrD1) to be associated with 100-SW, three (CEDG166, VrD1, and MBSSR238) with the NP/plant, and two markers (CEDG056 and GMES0214) with the NS/pod in mung bean following QTL mapping based on single marker analysis in a recombinant inbred line (RIL) mapping population developed from the cross between MYMIV susceptible cultivar Sonali and resistant wild relative of mung bean (*V. radiata* var. *sublobota*). In our study, six markers, namely, VrD1, CEDG044, cp05325, GMES0214, CEDG166, and CEDG056 reported by Singh et al. (2018) as associated with different traits, had been employed but none of them could be found associated with any of the studied traits. This disagreement could be primarily due to the difference in the mapping population and the approach followed in the earlier study. Furthermore, the single-marker analysis used in the earlier study is not considered a robust approach to map quantitative traits and many times results in the spurious association. The limited number of recombination events in the biparental mapping population results in mapping QTLs in larger genomic intervals than the association mapping. These QTL flanking markers might not be associated with traits when employed in association mapping due to the existence of high recombination events which might break the linkage between earlier-associated markers with traits.

A number of earlier studies claim that YMD resistance in mung bean and other *Vigna* crops is governed by one or two quantitative genes. However, a few recent QTL and association mapping studies indicate that resistance is governed by multiple genes (Singh et al., 2018, 2020). In this study, five MTAs (BM146, BMd12, BMD26, CP1038, and CP5096) from GLM and two MTAs (CEDG220 and J01263) from the MLM approach were identified for YMD resistance. Singh et al. (2020) reported 14 and 12 MTAs linked with MYMIV resistance following GLM and MLM methods, respectively. Among these, the marker CP1038 common in both the studies was also identified in *kharif* 2019 and pooled over *kharif* data in this study. Besides the association of CP1038 with YMD, its association with PH and SY was also observed, and therefore, this genomic segment is considered to be pleiotropic. Furthermore, BM212 shown to be associated with MYMIV resistance by Singh et al. (2020) has a trait association with DM in our study. Singh et al. (2018) reported four QTLs linked with MYMIV resistance based on single marker analysis in a RIL mapping population developed from susceptible cultivar Sonali and resistant wild relative of mung bean (*V. radiata* var. *sublobota*) but none of them were found to be associated with YMD resistance in this study.

Few studies on mapping quantitative traits in mung bean following the association mapping approach have been reported till date in traits such as seed coat color (Noble et al., 2018), seed mineral content (Wu et al., 2020), MYMIV resistance (Singh et al., 2020), salinity tolerance (Breria et al., 2020), and phosphorus use efficiency (Reddy et al., 2020; **Supplementary Table 6**). However, this is the first report identifying MTAs for yield-related traits along with YMD resistance in mung bean. Nonetheless, a comparatively less number of MTAs was identified in this study which could be due to less number of markers deployed, and therefore, this warrants examining more markers, especially the mung bean-specific markers which have been developed in the last 3–4 years. This study not only identifies MTAs for various yield attributing traits but also validates the marker associated with YMD resistance identified in earlier studies. Therefore, this study would help in fine mapping of common YMD resistance loci identified across different studies and would eventually help in improving mung bean varieties for YMD resistance following fast track and precise molecular breeding with linked markers. Furthermore, the markers for yield-related traits would also be helpful in fast-track breeding for mung bean improvement utilizing these after validation across different populations.

CONCLUSION

The population genetic structure analyses grouped the 80 mung bean genotypes into three major clusters and three genetically distinct SPs with one admixture SP based on 89 genome-wide polymorphic SSRs. This generated 317 polymorphic alleles with an average of 3.56 alleles per SSR locus. Both, i.e., cluster analysis and genetic population structure, categorized the advanced mung bean breeding genotypes in a single group/SP and the released varieties in other groups/SPs suggesting that the studied genotypes may have common ancestral history at some level. The genetic population structure was in agreement with the genetic diversity analysis. A total of 38 and 10 marker-trait associations for yield-related traits and YMD resistance by GLM and MLM methods, respectively, were identified as significant, and one SSR marker CP1038 associated with YMD resistance was validated. These associations may be useful in marker-assisted mung bean improvement programs in future after validation of the markers in biparental mapping populations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

VR, RY, RS, and AP designed the experiment and wrote the manuscript. VR, APn, and GK conducted field and laboratory experiments and generated the data. VR, RY, PSS, and AP analyzed the data and interpreted the results. RY, AP, and RS supervised the research. RY, AP, PSS, and RS reviewed the manuscript. All authors read the manuscript and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.749439/full#supplementary-material

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Agro-Morphological Characterization of Lentil Germplasm of Indian National Genebank and Development of a Core Set for Efficient Utilization in Lentil Improvement Programs

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Lentil (*Lens culinaris* Medik.) is one of the major cool-season pulse crops worldwide. Its increasing demand as a staple pulse has led to the unlocking of diverse germplasm collections conserved in the genebanks to develop its superior varieties. The Indian National Genebank, housed at the Indian Council of Agricultural Research (ICAR)-National Bureau of Plant Genetic Resources, New Delhi, India, currently has 2,324 accessions comprising 1,796 indigenous and 528 exotic collections. This study was conducted to unveil the potential of lentil germplasm by assessing its agro-morphological characteristics and diversity, identifying trait-specific germplasm, and developing a core set. The complete germplasm set was characterized for two years, i.e., 2017–2018 and 2018–2019, and data were recorded on 26 agro-morphological traits. High phenotypic variability was observed for nine quantitative and 17 qualitative traits. A core set comprising 170 accessions (137 Indian and 33 exotic) was derived based on the characterization data as well as geographical origin using a heuristic method and PowerCore software. This core set was found to be sufficiently diverse and representative of the entire collection based on the comparison made using Shannon–Weaver diversity indices and χ^2test. These results were further validated by summary statistics. The core set displayed high genetic diversity as evident from a higher coefficient of variance in comparison to the entire set for individual traits and overall Shannon–Weaver diversity indices (entire: 1.054; core: 1.361). In addition, the total variation explained by the first three principal components was higher in the core set (70.69%) than in the entire collection (68.03%). Further, the conservation of pairwise correlation values among descriptors in the entire and core set reflected the

maintenance of the structure of the whole set. Based on the results, this core set is believed to represent the entire collection, completely. Therefore, it constitutes a potential set of germplasm that can be used in the genetic enhancement of lentils.

Keywords: *Lens culinaris*, characterization, core-set, genetic diversity, trait-specific germplasm

INTRODUCTION

Lentil (*Lens culinaris* Medik.; 2n = 2 × = 14) is a diploid, self-pollinating, and cool-season pulse that is grown worldwide (Kumar and Gupta, 2020). It is regarded as one of the founder crops of neolithic agriculture (Zohary and Hopf, 1973). In addition, the global productivity of lentils has increased from an average yield of 806 kg ha^{-1} to 1194.6 kg ha^{-1} during the last two decades (FAOSTAT, 2021). In India, lentil ranks as the second-most important winter pulse crop after chickpea. In 2019, 1.23 million tons of lentil was produced in India, with a mean productivity of average yield of 901 kg ha^{-1} (FAOSTAT, 2021). However, India's current yield of lentils is considerably lower than that of several other countries because of the poor yield of cultivars.

Poor seedling vigor, low biomass, delicate stem, low harvest index, lodging, less conversion of flower to the pod, and climate-induced stresses are the primary yield-reducing factors in lentils (Erskine et al., 2009). In addition, the narrow genetic base or limited parentage of modern varieties has emerged as a major concern for lentil improvement. Consequently, the potential genetic gains in lentil productivity could not be achieved. Genebanks are the source of genes exhibiting valuable traits that can be utilized not only to develop superior varieties but also those with tolerance to stresses induced due to changing climate (Díez et al., 2018). Therefore, it is essential to search genebanks and identify novel germplasm for its use in lentil-breeding programs to enhance cultivar productivity and resilience to climate change. The efficient use of genetic diversity in the varietal development program is an effective method to enhance yield gains and cope with the emerging climate-induced stresses.

To identify novel accessions corresponding to the genes with desired traits, it is prudent to characterize the germplasm collections conserved in the genebanks. However, the characterization of extensive collections by plant breeders is time- and resource-consuming, genebank curators have developed a concept of a core set from entire collections that are characterized at once to identify the targeted germplasm and use it efficiently (Frankel and Brown, 1984; Brown, 1989; van Hintum et al., 2000). The core set refers to a minimum set of germplasm that captures the entire range of genetic variability of any crop, with minimum repetitiveness. Being smaller in size and diverse in nature, the core set can be efficiently used as a kickoff point to enhance genetic gains, including the use of phenomics and genomics tools in less time. Globally, legume germplasm curators have developed core sets in soybean (Oliveira et al., 2010), cowpea (Mahalakshmi et al., 2007), pigeonpea (Reddy et al., 2005), groundnut (Upadhyaya et al., 2003), chickpea (Upadhyaya et al., 2001), lentil (Simon and Hannan, 1995; Tullu et al., 2001), and lablab bean (Vaijayanthi et al., 2015) to enhance the germplasm use. The Indian Council of Agricultural Research-National Bureau of Plant Genetic Resources (ICAR-NBPGR) has developed core sets for Indian mustard (Nanjundan et al., 2021), wheat (Phogat et al., 2020), chickpea (Archak et al., 2016), wild lens (Singh et al., 2014), brinjal (Gangopadhyay et al., 2010), and mungbean (Bisht et al., 1998). Therefore, the aim of the present study was to develop a core set of cultivated lentil germplasm conserved in Indian national genebank based on agro-morphological characterization and diversity indices for accelerating utilization of germplasm in lentil breeding programs.

MATERIALS AND METHODS

Plant Material

The study material consisted of 2,324 cultivated lentil germplasm accessions comprising 1,796 indigenous collections (ICs) and 528 exotic collections (ECs). The indigenous accessions were collected from different lentil-growing areas in India after exploring several crops from 1976 to 2017. Geo-coordinates of each collection site were mapped using geographic information system (GIS) tools (**Figure 1**). Geo-referenced maps were prepared with WGS84 datum and geographic projection system using GIS tools (Semwal et al., 2014). The germplasm was characterized during the winter season of 2017–2018 and 2018–2019 at Research Farm of ICAR-NBPGR located at a latitude of 28°38′N and longitude of 77°10′ E and an altitude of 228.61 m above the mean sea level. The soil of the location was sandy loam type having an optimal pH range. Lentil germplasm accessions were sown under natural field conditions. The recommended package of practices for growing lentil was followed. To meet the crop's balanced nutrient demand, 20 kg ha^{-1} nitrogen and 40 kg ha^{-1} phosphorus was applied as a basal dose before sowing. One presowing irrigation was given to ensure proper germination in the field, while irrigation was also given at the pod formation stage. The seeds were treated with a mixure of thiram (2g) and carbendazim (1 g) per kg of seed. Manual weeding was completed at 25–30 and 45–50 days after sowing. The experimental design consisted of an augmented block (Federer and Raghavarao, 1975) with four popular checks, namely, DPL62, IPL316, IPL526, and L4717. The experiment was replicated in 24 blocks. A hundred accessions were sown per block uniformly in 23 blocks, whereas, the 24^{th} block was incomplete with 24 accessions. The accessions were sown in paired rows of two m length, with a row-to-row distance of 30 cm. The detailed timeline of the experiment is shown in **Figure 2**.

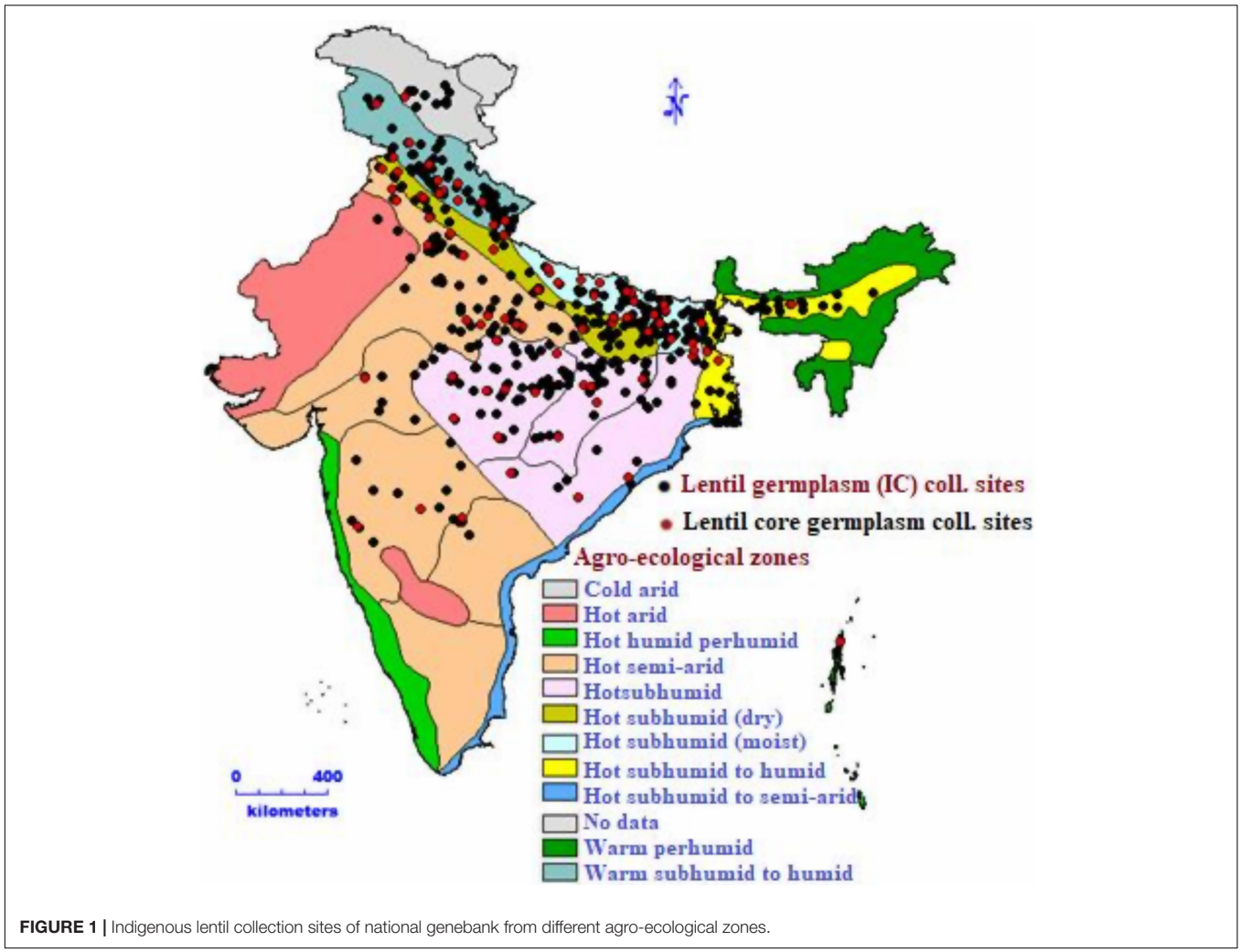

FIGURE 1 | Indigenous lentil collection sites of national genebank from different agro-ecological zones.

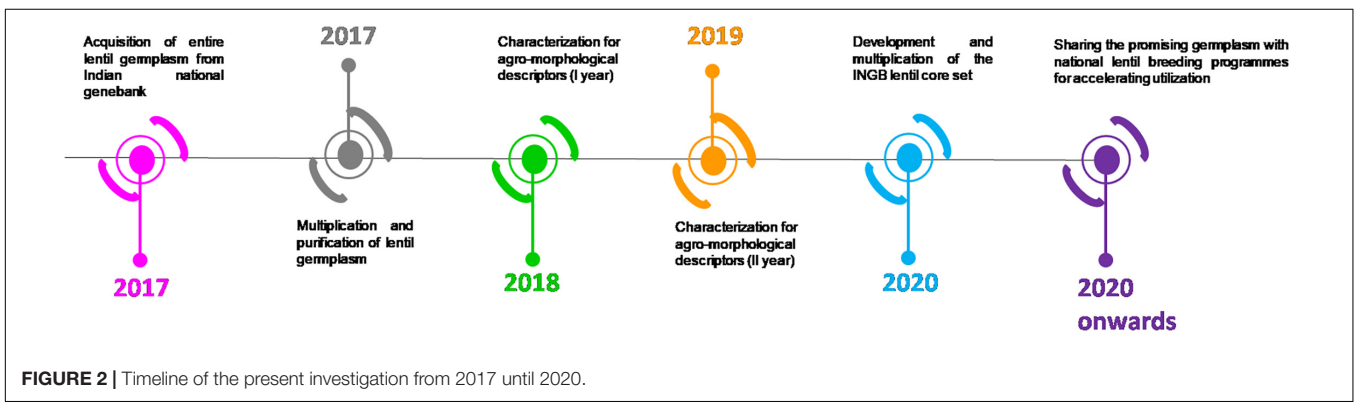

FIGURE 2 | Timeline of the present investigation from 2017 until 2020.

Agro-Morphological Descriptors Used in the Experiment

Twenty-six agro-morphological descriptors (nine quantitative and 17 qualitative) were selected for phenotypic characterization using descriptor lists of lentil (IBPGR, 1985; Mahajan et al., 2000) (**Table 1**). The qualitative characters were (1) early plant vigor (EPV), (2) seedling stem pigmentation (SSP), (3) growth habit (GH), (4) leaf color (LC), (5) leaf pubescence (LP), (6) leaflet size (LS), (7) flower ground color (FGC), (8) tendril length (TL), (9) biomass score (BS), (10) lodging score (LoD), (11) pod pigmentation (PP), (12) pod dehiscence (PD), (13) pod shedding (PS), (14) seed shape (SS), (15) seed coat color (SCC), (16) pattern of seed testa (PST), and (17) cotyledon color (CC). The quantitative traits included (1) days to 50% flowering (DF), (2) days to 80% maturity (DM), (3) plant height (PH), (4) number

TABLE 1 | Descriptors used for agro-morphological characterization of lentil germplasm of Indian national genebank.

S. No.	Descriptors	Code	Stage of observation	Descriptor state
Qualitative traits				
1	Early plant vigor	EPV	25 DAS	Poor (1), Good (2), Very Good (3)
2	Seeding stem pigmentation	SSP	45DAS	Absent (0), Present (1)
3	Growth habit	GH	50% flowering	Erect (1), Semi-erect (3), Horizontal (5)
4	Leaf color	LC	50% flowering	Green (1), Light green(2), Pigmented (3)
5	Leaf pubescence	LP	50% flowering	Absent (0), Slight (3), Medium (5), Dense (7)
6	Leaflet size	LS	On lower flowering nodes	Small (3), Medium (5), Large (7)
7	Flower ground color	FGC	Open flower in the morning	White (1), Yellow (2), Red (3), Purple (4)
8	Tendril length	TL	Pod filling	Rudimentary (1) Prominent (2)
9	Biomass score	BS	Mid pod filling	Low (3), Medium (5), High (7)
10	Lodging score	LoD	Mid pod filling	Absent (0), Present (1)
11	Pod pigmentation	PP	Near to maturity	Absent (0), Present (1)
12	Pod dehiscence	PD	Scored a week after maturity	Absent (0), Low (3), Medium (5), High (7)
13	Pod shedding	PS	Scored a week after maturity	None (0), Low (3), Medium (5), High (7)
14	Seed shape	SS	Post-harvest	Flattened (1), Globose (2)
15	Seed coat color	SCC	Post-harvest	Yellow (1), Green (2), Brown (3), Pink (4), Gray (5), Black (6)
16	Pattern of seed testa	PST	Post-harvest	Absent (0), Dotted (1), Spotted (2), Marbled (3), Complex (4)
17	Cotyledon color	CC	Post-harvest	Yellow (1), Orange (2), Green (3)
Quantitative traits				
18	Seed diameter (mm)	SD	Post-harvest	Average of five replications
19	Seed thickness (mm)	ST	Post-harvest	Average of five replications
20	Days to 50% flowering	DF	50% flowering	Plot basis
21	Secondary branches per plant	SBP	Maturity	Average of five replications
22	Plant height (cm)	PH	Late pod filling stage	Average of five replications
23	Pods per plant	PPP	Maturity	Average of five replications
24	Seeds per pod	SP	Maturity	Average of five replications
25	Days to 80% maturity	DM	80% maturity	Plot basis
26	100-seed weight (g)	SW	Post-harvest	Average of five replications

secondary branches per plant (SBP), (5) seed diameter (SD), (6) seed thickness (ST), (7) pods per plant (PPP), (8) seeds per pod (SPP), and (9) 100-seed weight (SW).

Development and Validation of Core Collections

A statistical software, PowerCore, was used to constitute a core collection based on the characterization data generated using 26 different descriptor traits (Kim et al., 2007). Continuous variables were classified into different categories based on Sturges' rule (Sturges, 1926) ($K = 1 + \log_2 n$), where n and K mean the number of observed accessions and number of classes, respectively. The number of classes can be modified. The modified heuristic algorithm was implemented to identify and select accessions of core collections using the strategy of advanced maximization or allelic richness. Subsets created using PowerCore represented all observation classes with the least allelic redundancy, ultimately ensuring a highly reproducible entry list. Core sets were extracted with different combinations of variables to assess their efficacy using PowerCore and using qualitative traits, quantitative traits, a combination of quantitative and qualitative traits, and a combination of quantitative and qualitative traits and passport data. Another method, known as principal component score strategy (PCSS), was used to extract the core (Noirot et al., 1996). This method uses principal component analysis (PCA) to eliminate collinearity between variables and sample individuals based on their cumulative relative contribution. The Newman–Keuls procedure was followed to compare the means of the entire collection and developed core sets (Newman, 1939; Keuls, 1952) for descriptor traits. The similarity of distribution frequencies in the core and entire sets was evaluated using the χ^2 test. The Shannon–Weaver diversity index (SDI) was used to compare the representativeness of the whole set and the germplasm selected as core entries.

Data Analysis

The data recorded for nine quantitative traits were first analyzed separately for each year, after which a combined analysis was performed using the general linear model (PROC GLM) method of SAS 9.3 for augmented design. The accessions were considered random and year as a fixed effect. Levene's test was performed to assess the homogeneity of error variances before performing the combined analysis (Levene, 1960). Descriptive statistics were estimated for the complete set and the core collections separately. Frequency distribution for different classes of qualitative traits was obtained using Excel, and histograms for quantitative traits were drawn using IBM statistical package for the social sciences (SPSS) statistics (version 20.0). Variability in descriptor traits between ICs and ECs was compared using boxplots developed using Statistical Analysis Software-JMP 14 software. Hierarchical cluster analysis was performed using the Euclidean distance matrix following Ward's minimum variance method for accession number grouping and estimating genetic relationships. Core sets were extracted using PowerCore and the PCS strategy. The principles laid by Oliveira et al. (2010) were used to calculate the range retention percentage. In addition, the distribution of homogeneity for each of the descriptor traits was analyzed using the χ^2 test. For the quantitative traits, classes were formed based on Sturges' formula. The observed number of accessions in the core set in each class was determined and was tested against the expected number of accessions using the χ^2test. Further, the SDI (H') was computed as a measure of phenotypic variability using the phenotypic frequencies of quantitative and qualitative traits (Shannon and Weaver, 1949) in the entire and different core sets extracted using the following formula.

$$H' = -\sum_{i=1}^{n} \text{pi. ln pi}$$

where p_i is the proportion of germplasm accessions in the i^{th} class of an n-class characteristic and n is the number of phenotypic categories for a character. Phenotypic correlation coefficients (r) among descriptors in the core collection ($Core^d$) and entire set were estimated. Moreover, the contribution of different descriptor traits to multivariate polymorphism and conservation of contribution in the core collection were assessed based on PCA. The SAS 9.3 program was used to estimate the correlation and PCA (SAS Institute, 2012).

RESULTS

Agro-Morphological Characterization of Lentil Germplasm

The actual germplasm collection sites of lentils in India (**Figure 1**) were presented using geo-referenced map. These maps revealed that most of the lentil germplasm were collected from Uttar Pradesh, Bihar, Uttarakhand, and Madhya Pradesh. Geo-referenced map showed that lentils were collected from Western Himalayas (Kashmir, Himachal Pradesh, and Uttarakhand) to Eastern Himalayas (Assam and Arunachal Pradesh). It was collected from high-altitude areas in north (Leh) to low-lying areas of eastern part of India (West Bengal). Collections from dry areas of Rajasthan to wet areas of North-east Himalayas indicated that India has sufficient diversity in its collections. Indian lentil collections showed the unique distribution pattern having representation from four global biodiversity hotspots, the Himalayas, the Western Ghats, the Indo-Burma region, and the Sundaland (Includes Nicobar group of Islands). All 2,324 accessions and four checks were characterized under the agro-climatic region in the north-western Indian conditions for two consecutive years (2017–2018 and 2018–2019). The experimental field view and seed coat color diversity are presented in **Figure 3**.

Characterization Using Qualitative Descriptor Traits

The frequency distribution of the entire set was not normal and varied among traits. In certain cases, a particular trait was predominant, whereas it was dispersed in other cases (**Figure 4**). The majority of the accessions (2,061) showed purple pigmentation on the stem at the seedling stage, whereas leaf color was recorded as green. A slight to medium leaf pubescence was prominent, whereas 48 accessions were glabrous. EPV of most of the accessions (2,044) was good, whereas 275 accessions were highly vigorous. Prominent tendril was

FIGURE 3 | (A) Field view of agro-morphological characterization of lentil germplasm and **(B)** seed coat diversity.

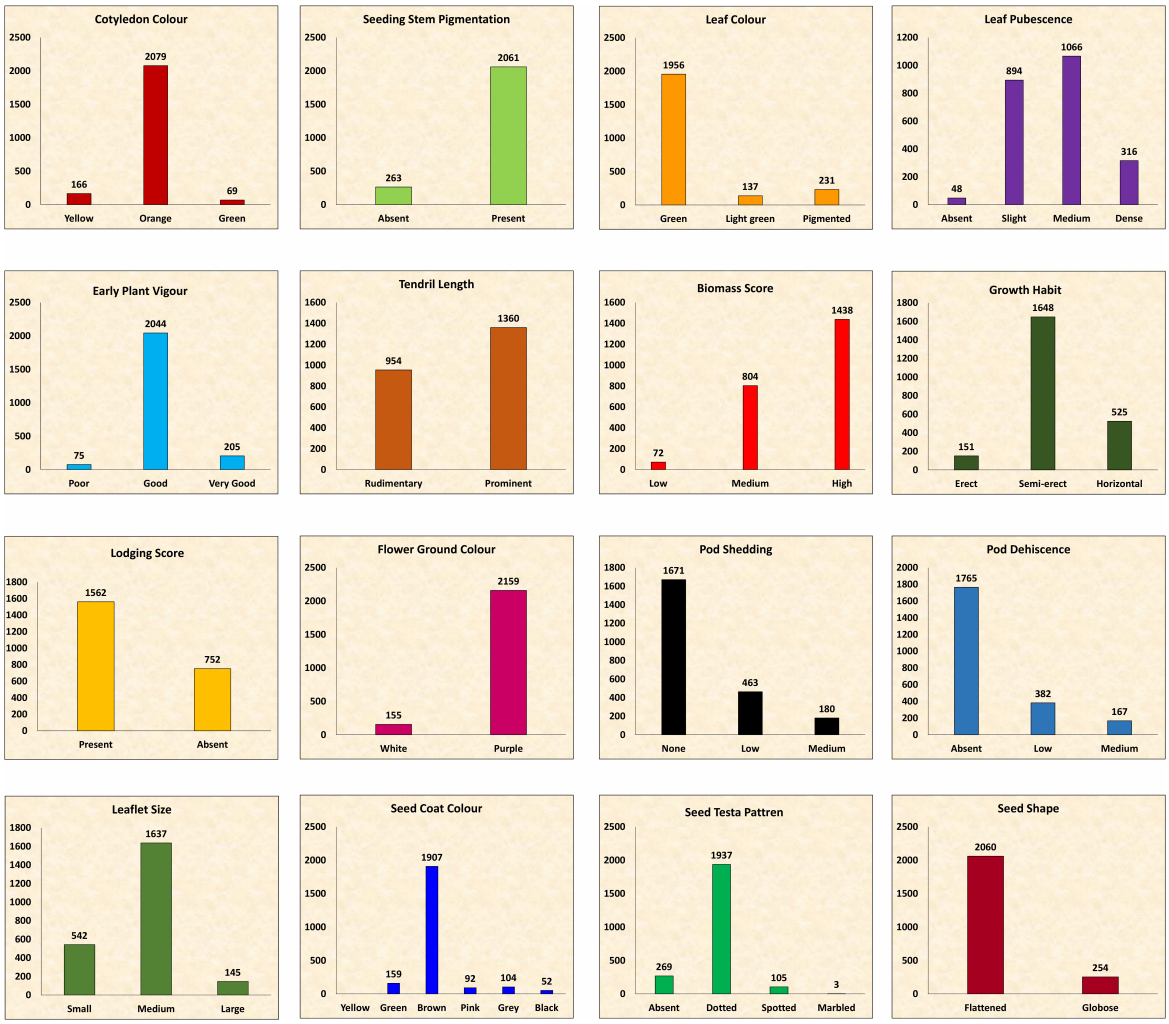

FIGURE 4 | Frequency distribution of entire lentil germplasm for qualitative traits.

recorded in 1,360 accessions, whereas 954 accessions showed rudimentary tendril. High biomass was observed at the mid pod filling stage in 1,438 accessions, whereas 72 accessions showed low biomass. Semi-erect plant growth was more prominent (1,648 accessions), and horizontal growth was recorded in 525 accessions, whereas the growth in 151 accessions was erect. Around 67% of the accessions showed lodging at the time of pod filling. The maximum number of accessions (2,159) were of purple flower ground color. After a week of maturity, medium pod shedding was recorded in 180 accessions, whereas most of the accessions (1,671) showed no pod shedding. Pod dehiscence was absent in 1,765 accessions, whereas low to medium pod dehiscence was noted in 549 accessions. Pod pigmentation was absent in the majority of the accessions, whereas brown seed coat color and dotted testa pattern were prominent. A flattened seed shape was observed in 2,060 accessions, whereas 254 accessions showed the globose seed shape. There was substantial variation in the cotyledon color with a maximum of orange type.

Agronomic Evaluation Using Quantitative Traits

The results revealed significant variations as evident from the mean, range, and coefficient of variations (**Supplementary Table 1** and **Figure 5**). The days to 50% flowering and maturity ranged from 51 to 123 and 93 to 140 days, respectively, whereas 100-seed weight varied from 0.75 to 7.6 g. The average seed thickness and seed diameters were 2.42 and 4.18 cm, respectively. The mean plant height and the number of secondary branches were 35.34 and 28.64 cm, respectively. The coefficient of variation ranged from low (<10.17%; days to flowering, seed thickness, days to maturity, and number of seeds per pod) to high (>17.34%; the number of secondary branches, 100-seed weight, number of pods per plant, and seed diameter) for key descriptor traits. In addition, substantial variation in the range for the number of pods per plant, number of secondary branches per plant, 100-seed weight, and earliness was observed. The least variation was

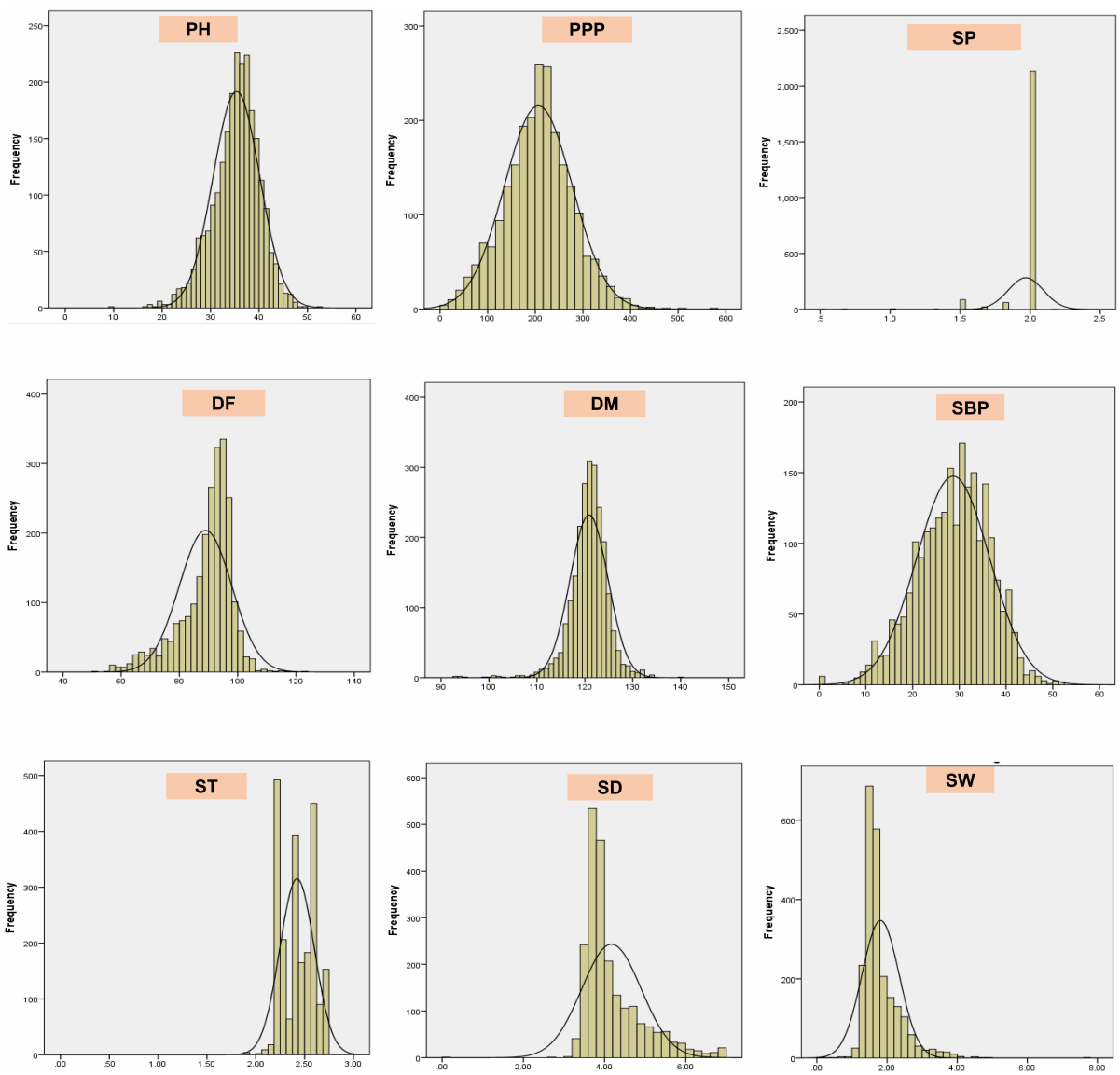

FIGURE 5 | Frequency distribution plots showing the variability of nine quantitative traits in the entire lentil germplasm conserved in Indian national genebank.

observed in the number of seeds per pod, with the majority of the accessions with two seeds per pod in studied accessions. ICs showed earliness, small seeds, and low biomass as compared to the ECs. The days to 50% flowering, days to 80% maturity, and seeds per pod were negatively skewed, indicating that a majority of the accessions had higher mean values for these traits and days to 80% maturity and seeds per pod showed high kurtosis values with a relatively higher peak. Boxplot analysis revealed a comparison of trait distribution between exotic and indigenous accessions. The average performance for plant height, days to 50% flowering, pods per plant, and number of secondary branches was lower in ECs than in ICs. However, the mean value of seed weight was higher in ECs than in ICs (**Figure 6**).

Clustering of Lentil Germplasm Accessions

Grouping several accessions into certain homogenous clusters assists in selecting diverse parents. This allowed the accurate comparison among all probable pairs of individuals and brought together gene constellations, yielding desired progenies through crossing between different parents. Hierarchical cluster analysis with Ward's method of minimum variance was applied to assess genetic diversity. Based on nine quantitative traits, phenotypic relationships among lentil accessions were determined using Euclidean distances. The Indian national gene bank (INGB) lentil accessions were grouped into 12 clusters with a varied number of accessions in each cluster (**Figure 7** and **Supplementary Table 2**). A list of core set accessions

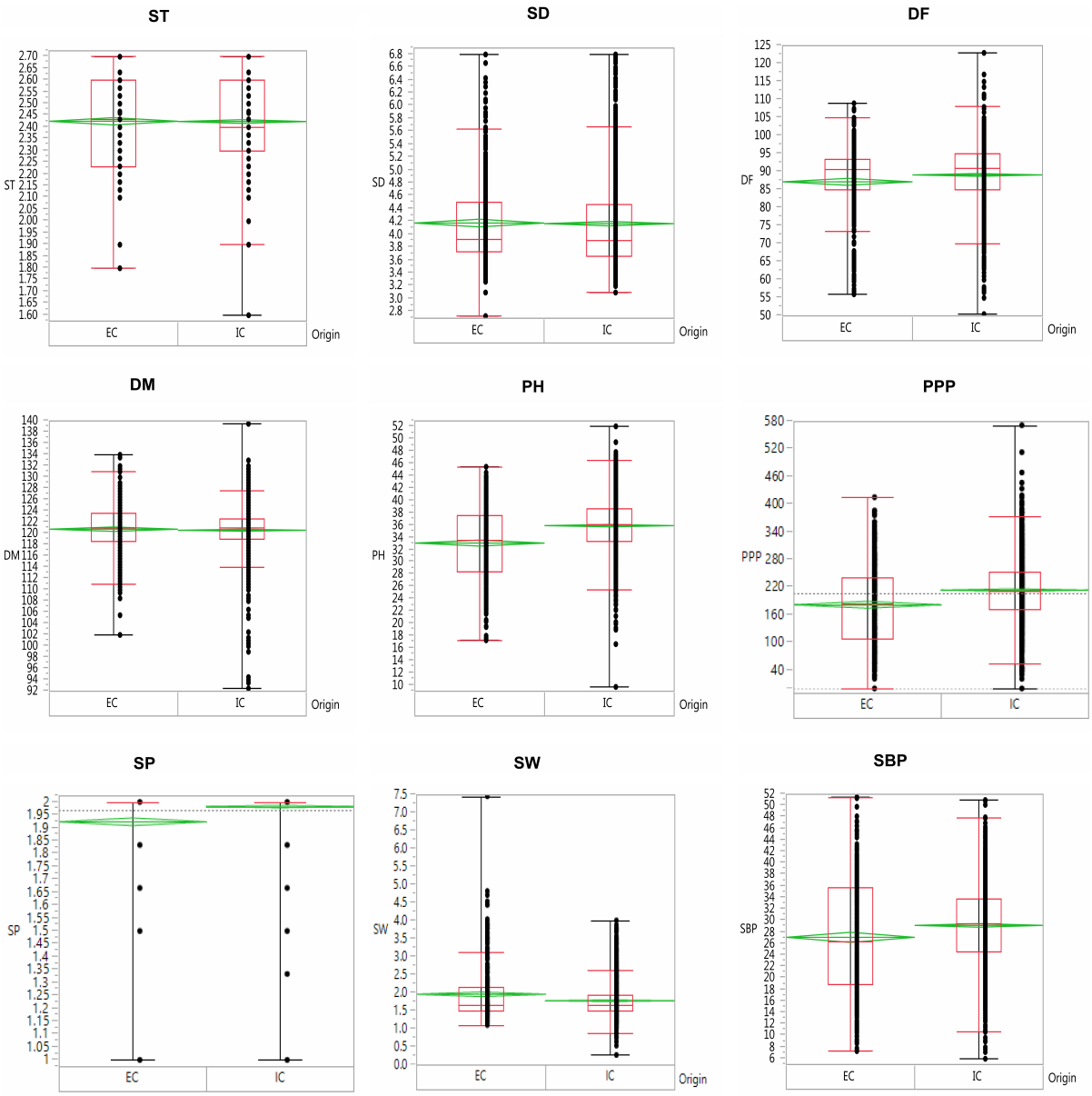

FIGURE 6 | Boxplot depicting the variability of traits in the indigenous collection (IC) and exotic collection (EC) of the entire lentil germplasm conserved in Indian national genebank.

with cluster number is given in **Supplementary Table 3**. The cluster also showed heatmap distribution of accessions, showing trait variability in different clusters represented by different colors. Cluster II was the largest with 393 accessions followed by Clusters III and IV with 382 and 253 accessions, respectively. Cluster II grouped 320 ICs and 73 ECs accessions; whereas Cluster XI was the smallest with only 38 accessions (24 ECs and 14 ICs). The cluster mean values of studied traits showed that Clusters XI and XII included extra-early accessions, such as IC241529, IC241531, and IC241532, whereas early to medium maturing accessions were grouped into Clusters VI and VII. Late flowering accessions such as IC73690, IC73692, IC381127, and IC398044 grouped in cluster I. Proportionately, exotic accessions were relatively dominant in Clusters X, XI, and XII, and indigenous were dominant in Clusters II to IX. Cluster XII comprised all exotic accessions from Syria. Cluster V contained accessions having very tall plant types (IC329110, IC398793, and IC59038, a high number of pods per plant (IC78387 and IC78398), and a high number of secondary branches (IC148333, IC16453). The accessions such as EC223214 and IC241543 grouped in cluster XII had highest mean value for seed weight (3.239 g) whereas cluster III had accessions with mean value of 1.523 g.

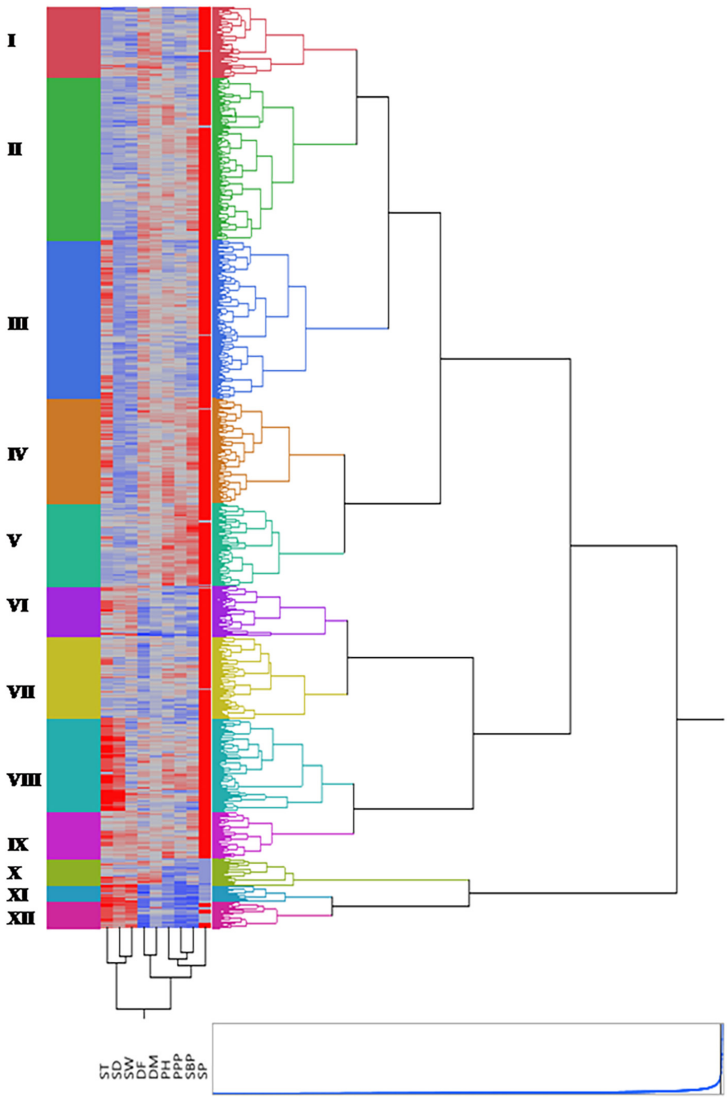

FIGURE 7 | Dendrogram along with heatmap of entire germplasm generated by performing hierarchical cluster analysis. Twelve clusters are represented by different colors. The two-dimensional heatmap is represented by columns and rows. Each column represents different quantitative trait and each row an accession. The higher the trait value, the brighter is the red and similarly, lower the trait value, brighter is the blue color, respectively.

Identification of Trait-Specific Germplasm

Potential accession numbers for unique traits were identified from characterization datasets and validated over the years and locations at NBPGR, New Delhi, The International Center for Agricultural Research in the Dry Areas (ICARDA), Amlaha, and NBPGR, Regional Station, Ranchi. Some of them are depicted in **Figure 8**. The accession IC317520 was identified with a unique seed morphotype having extended funiculus. A total of 50% of plants with accessions such as IC241532 and IC241529 exhibited very early flowering (51 days) relative to all checks used in the study. The accession EC267615 displayed a novel type of erect plant architecture, indicating its suitability for mechanical harvesting. Pod setting in this genotype was observed at more than 15 cm above the ground. The accessions IC199461 and IC201716 had a high number of secondary branches (>50) and pods per plant (>400) relative to all checks used in the study. The accession EC499760 was of bold seeded type (100-seed weight, 7.1–7.83 g) and was imported from the United States of America (USA). A novel and unique multiflower (MF) germplasm accession, IC241473, was identified the first time that formed up to 16 flowers per peduncle (FPP) at multiple flowering nodes. In addition, this accession showed fasciation of the main stem.

Development and Validation of Core Set

The core sets were extracted using combinations of different datasets using the PowerCore software. Additionally, the PCSS was used to develop the core sets. These core sets were designated

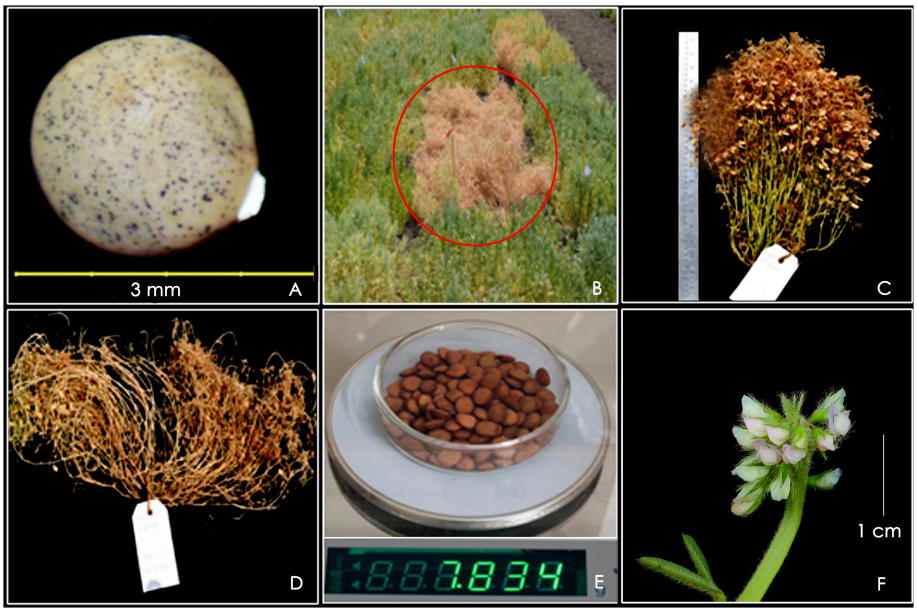

FIGURE 8 | Trait-specific lentil germplasm, **(A)** IC317520: unique seed morphotype with an intact funiculus; **(B)** IC241532: early maturing accession (circled in red) relative to surrounding germplasm; **(C)** EC267615: suitable to mechanical harvesting; **(D)** IC199461: high number of secondary branches; **(E)** EC499760: bold seeded type with high 100-seed weight; **(F)** IC241473: multiflowering germplasm.

as Core[a], Core[b], Core[c], Core[d], and Core[e] when generated using qualitative traits, quantitative traits, a combination of both traits, a combination of both traits and passport data, and PCSS. A total of 170 accessions were selected based on the trio of qualitative and quantitative traits and passport data using PowerCore (**Table 2** and **Supplementary Table 3**). A total of 67 accessions were selected when the data of only qualitative variables were used, whereas 71 accessions and 79 accessions were selected, respectively, when data of only quantitative characteristics and both variables were used for the analysis. Accessions with the maximum relative contribution corresponding to 10% representation were used as the criteria to select the core; thus, 221 accessions were selected based on the PCS strategy. Relative SDI was estimated for both quantitative and qualitative traits in entire lentil accessions and in core sets developed using a different strategy that revealed adequate diversity. The lentil germplasm was more diverse ($H' > 0.5$) for all descriptors, except for EPV, SSP, FGC, PP, SS, CC, and SP. The average SDI for quantitative traits was higher than qualitative traits. The SDIs were consistently higher in the core sets derived from different datasets than in the entire set, signifying an enhanced representation of the available diversity in each core set. PowerCore yielded a better representation of diversity than PCSS. The average SDIs for all traits revealed that Core[d] (1.361, 170 accessions) was more diverse, followed by Core[b] (1.340, 71 accessions) and Core[a] (1.302, 67 accessions) (**Table 2**).

Qualitative traits in the INGB collections were represented in the core sets developed by all strategies, except the core developed by quantitative method and PCSS strategy, indicating that the Core[a], Core[c], and Core[d] captured the allelic richness of the entire INGB collections. The range of quantitative descriptors was classified into 12 groups for frequency distribution and χ^2 analysis based on PowerCore. Frequency distribution analysis indicated the homogeneity of distribution of several traits by Core[a](10), Core[b](13), Core[c](11), Core[d] (18), and Core[e](9) (**Table 3**). Thus, the core set developed by combining qualitative and quantitative traits and passport data well-represented the structure of the entire lentil germplasm. The results showed that the variability existing in the entire set was well-symbolized in the core collection. Differences in the means of the entire collection and Core[d] were insignificant for quantitative descriptors, except days to flowering and seed weight. In addition, the range retention percentage was 100, capturing a complete range of diversity, including all extremes (**Table 4**).

Correlation and Principal Component Analysis

The lentil germplasm accessions recorded significant and positive correlations between seed thickness and seed diameter, equally in both entire set and core set (r = 0.539) and between days to 50% flowering and days to 80% maturity (r = 0.582 in the entire set, r = 0.731 in the core set). Plant height was positively correlated with days to 50% flowering (i = 0.378), days to 80% maturity (r = 0.287), pods per plant (r = 0.496), seeds per pod (r = 0.521), and number of secondary branches (r = 0.542) and negatively with seed thickness (r = –0.034) and seed diameter (r = –0.234) in the core set. Similar trends were recorded in the entire lentil collection. The number of secondary branches was positively correlated with days to 50% flowering, days to 80% maturity, plant height, pods per plant, and seeds per pod

TABLE 2 | Comparison of the Shannon–Weaver diversity index (SDI) of the entire lentil collections to lentil core sets derived from different datasets and strategies.

Traits	Entire	Core[a]	Core[b]	Core[c]	Core[d]	Core[e]
Leaflet size	0.744	0.812	0.780	0.805	0.828	0.682
Growth habit	0.770	1.110	0.959	0.928	1.080	0.082
Early plant vigor	0.441	0.739	0.783	0.756	0.817	0.302
Seeding stem pigmentation	0.357	0.678	0.600	0.588	0.671	0.309
Leaf color	0.548	0.939	0.872	0.856	0.916	0.258
Leaf pubescence	1.069	1.113	1.119	1.115	1.099	1.148
Flower ground color	0.256	0.619	0.525	0.458	0.597	0.404
Tendril length	0.689	0.731	0.696	0.718	0.749	0.643
Pod pigmentation	0.016	0.121	0.085	0.082	0.140	0.004
Pod dehiscence	0.706	0.662	0.639	0.786	0.695	0.617
Pod shedding	0.770	0.799	0.689	0.871	0.809	0.622
Seed shape	0.378	0.381	0.395	0.504	0.387	0.264
Seed coat color	0.670	1.040	0.871	0.927	1.006	0.775
Pattern of seed testa	0.571	0.918	0.849	0.759	0.895	0.650
Biomass score	0.854	1.177	1.121	1.071	1.161	0.908
Lodging score	0.632	0.668	0.682	0.677	0.688	0.651
Cotyledon color	0.288	0.585	0.628	0.441	0.606	0.544
Seed thickness	1.667	1.643	1.909	1.811	1.942	1.684
Seed diameter	1.570	1.656	1.841	1.729	1.889	1.749
Days to 50% flowering	1.691	2.332	2.259	2.060	2.230	1.901
Days to 80% maturity	1.358	1.664	1.993	1.708	1.928	1.609
Plant height	1.726	2.118	2.215	2.035	2.180	1.780
Pods per plant	1.825	1.886	1.953	1.904	1.973	2.051
Seeds per pod	0.375	0.873	1.056	0.714	1.003	0.599
100-seed weight	1.466	2.124	2.128	1.855	2.126	1.832
No. of secondary branches	2.121	2.201	2.266	2.267	2.263	2.120
No. of accessions	2,324	67	71	79	**170**	221
Average SDI (Quantitative traits)	1.553	1.833	1.958	1.787	**1.948**	1.703
Average SDI (Qualitative traits)	0.574	0.770	0.723	0.726	**0.773**	0.521
Average SDI (Overall)	1.054	1.302	1.340	1.257	**1.361**	1.112

Core[a], Core[b], Core[c], Core[d], and Core[e] referred to core sets generated using qualitative traits, quantitative traits, a combination of both traits, a combination of both traits and passport data, and principal component score strategy (PCSS). Bold values indicating the selected core set with maximum diversity (highest SDI value in Core[d]).

TABLE 3 | Chi-square test for comparison of frequency distribution for descriptor traits studied in lentil core sets derived from different datasets and strategies.

Traits	Core[a]	Core[b]	Core[c]	Core[d]	Core[e]
Leaflet size	2.209^{ns}	3.009^{ns}	2.118^{ns}	2.629^{ns}	81.484*
Growth habit	12.281*	12.680^{ns}	12.195*	7.216*	743.761*
Early plant vigor	24.694*	20.834*	29.412*	22.345*	108.249*
Seeding stem pigmentation	11.724^{ns}	12.546^{ns}	12.518^{ns}	14.739^{ns}	1538.154*
Leaf color	15.716*	13.723*	18.720*	19.387*	502.182*
Leaf pubescence	4.385^{ns}	4.359^{ns}	4.552^{ns}	2.403^{ns}	4.359^{ns}
Flower ground color	10.897*	7.337^{ns}	10.941*	8.043*	9.627*
Tendril length	1.379^{ns}	0.233^{ns}	1.198^{ns}	1.641^{ns}	4.481*
Pod pigmentation	1.065*	1.063^{ns}	1.406^{ns}	1.061^{ns}	0.006^{ns}
Pod dehiscence	4.156^{ns}	4.195^{ns}	1.782^{ns}	0.562^{ns}	2.207^{ns}
Pod shedding	2.099^{ns}	5.461*	2.172^{ns}	3.698*	8.700*
Seed shape	3.694^{ns}	6.628^{ns}	6.622^{ns}	3.541^{ns}	6.447^{ns}
Seed coat color	11.690^{ns}	15.299^{ns}	15.238^{ns}	13.443^{ns}	113.237*
Pattern of seed testa	15.958*	14.860^{ns}	19.354*	8.335^{ns}	25.439^{ns}
Biomass score	11.031^{ns}	12.283^{ns}	16.299^{ns}	15.155^{ns}	2.187^{ns}
Lodging score	26.989^{ns}	27.143^{ns}	35.654^{ns}	23.490^{ns}	0.835^{ns}
Cotyledon color	10.287*	14.811*	14.292*	5.529^{ns}	27.990*
Seed thickness	235.628*	135.150*	94.694*	20.108^{ns}	146.220*
Seed diameter	69.870*	92.813*	81.421*	18.659^{ns}	229.906*
Days to 50% flowering	96.521*	77.337*	116.731*	34.433*	24.360*
Days to 80% maturity	52.890*	36.112*	39.905*	19.101^{ns}	24.250*
Plant height	24.272*	34.940*	36.027*	18.916^{ns}	17.800^{ns}
Pods per plant	58.418*	81.329*	53.987*	33.097^{ns}	55.711*
Seeds per pod	9.737*	12.215*	12.572*	19.969*	9.888*
100-seed weight	38.426*	47.352*	52.075*	77.273*	74.607*
No. of secondary branches	41.033*	53.699*	40.724*	28.447^{ns}	26.303^{ns}

*Core[a], Core[b], Core[c], Core[d], and Core[e] referred to core sets generated using qualitative traits, quantitative traits, a combination of both traits, a combination of both traits and passport data, and principal component score strategy (PCSS). *P < 0.05.*

negatively with 100-seed weight, both in the core collection and entire set (**Figures 9**, **10**).

Principal component analysis revealed the association among different descriptors and their contribution toward variability. The first three PCA components provided a realistic summary of the data and explained 70.69% of the total variation in the core set. The first principal component (PC1) accounted for 39.36% of the total variation, whereas PC2 and PC3 accounted for 17.73% and 13.60% variance, respectively. These values were comparable with those of the entire lentil collection, explaining 68.03% of the variation (PC1:37.49%; PC2:16.62%, and PC3:13.91%). The negative variation in PC1 was mainly contributed (>50%) by days to 50% flowering, days to 80% maturity, plant height, number of secondary branches per plant, and pods per plant and 100-seed weight in the entire and lentil core set (**Supplementary Table 4** and **Supplementary Figure 1**).

DISCUSSION

Genebanks are reservoirs of germplasm variability containing vital genes. These play a crucial role in crop-breeding programs (Qiu et al., 2013; Sandhu and Singh, 2021). Efficient and effective use of genebank collections in crop improvement depends on a thorough understanding of the existing genetic variability, knowledge of the genes present in individual accessions, and their value for use (Ortiz, 2002). Characterization and evaluation are the most crucial activities of plant genetic resources (PGR) management (Jones, 1984). Its scope has increased due to the increasing emphasis on conservation. The use of genebank accessions through characterization and evaluation has resulted in the development of genetic stocks, breeding lines, and commercial varieties (**Figure 11**; Carvalho et al., 2013).

TABLE 4 | Mean, range, and CV in entire and core set of lentil germplasm.

Traits	Mean			Minimum	Maximum	Minimum	Maximum	CV (%)	
	Entire	Core[d]	Significance level	Entire		Core[d]		Entire	Core[d]
ST	2.42 ± 0.01	2.41 ± 0.01	Ns	1.60	2.70	1.60	2.70	6.96	7.68
SD	4.18 ± 0.02	4.19 ± 0.06	Ns	2.73	6.80	2.73	6.80	17.41	18.38
DF	88.96 ± 0.19	85.43 ± 1.02	*	51.00	123.00	51.00	123.00	10.17	15.41
DM	120.88 ± 0.08	119.50 ± 0.51	Ns	93.00	140.00	93.00	140.00	3.28	5.55
PH	35.34 ± 0.10	34.04 ± 0.51	Ns	9.70	52.13	9.70	52.13	13.64	19.41
PP	206.38 ± 7.48	183.48 ± 6.73	Ns	20.83	571.33	20.83	571.33	34.56	47.69
SP	1.97 ± 0.01	1.92 ± 0.02	Ns	1.00	2.00	1.00	2.00	6.38	12.47
SW	1.91 ± 0.01	1.99 ± 0.06	**	0.75	7.6	0.75	7.6	28.50	39.50
SBP	28.67 ± 0.16	26.91 ± 0.73	Ns	6.00	51.50	6.00	51.50	27.33	35.16

ST, seed thickness; SD, seed diameter; DF, days to 50% flowering; SBP, secondary branches per plant; PH, plant height; PP, pods per plant; SP, seeds per pod; DM, days to 80% maturity; SW, 100-seed weight.
**Significant at 5% level.*
***Significant at 1% level.*

Agro-Morphological Characterization of Lentil Germplasm

Geo-referenced maps of India revealed that most of the Indian accessions were from the Gangetic plains and eastern part of India belonging to Uttar Pradesh (254), Bihar (203), Uttarakhand (181), and Madhya Pradesh (96). These are the most populated regions of India, with the massive scope of pulse production in their rice fallow areas (Chowdhury et al., 2020). The majority of the germplasm was collected from hot subhumid (dry and moist) to hot subhumid agro-ecological zones of India. In contrast, the ECs, conserved in INGB, represented all six continents, primarily Asia.

The major steps in crop improvement are assessing the variability for desired traits and their utilization in breeding programs (Kumar et al., 2015). Previous researchers adopted the piecemeal approach to characterize the agro-morphological traits in lentil germplasm collections (Gautam et al., 2013; Jha et al., 2014; Kumar and Solanki, 2014). However, in this study, the entire genebank collections representing all agro-ecological zones were characterized in one go. Significant differences among lentil accessions based on agro-morphological characterization indicated their potential for use in current and future lentil breeding programs. The results showed significant variations in certain traits such as days to flowering, seed thickness, days to maturity, number of secondary branches, 100-seed weight, number of pods per plant, and seed diameter. The wide range of variability obtained was attributable to the diverse collection assessed, involving native and introduced germplasm originating from diverse geographical origins with different genetic makeup (Phogat et al., 2020).

Lentil is a less vigorous grain legume that faces competition from weeds during the early growth stage. Lentil germplasm with increased vigor can dominate the weeds, ultimately enhancing the yield (Sharma et al., 2018). The previous researchers reported that EPV and rapid canopy development as key traits for escaping drought at the terminal stage because these traits lead to the early onset of maturity (Sarker et al., 2005; Kumar et al., 2012). The majority of exotic accessions had light-green leaves, whereas indigenous varieties were dominated by green to dark green canopy. In addition, the purple pigmentation was observed to be disappeared with the increase in the ambient temperature. Similarly, in subterranean clover, Nichols et al. (1996) reported prominence of purple pigmentation during winter, which is appeared with the arrival of spring. Wide variations were recorded in leaf pubescence, ranging from the absence of pubescence to dense pubescence. Low or moderate leaf pubescence has promoted tolerance to aphids (Kumari et al., 2009). However, Tripathi et al. (2020) indicated that pubescence on the pod is a unique feature of cowpea, a marker-trait for insect resistance. Variation was also reported in leaflet size, tendril length, flower ground color, pod shedding, pod dehiscence, testa color, and seed shape.

Elias et al. (1979) suggested that the protein quality is affected by seed coat color in beans. Diversity in seed color among accessions may provide valuable genetic resources for biofortified lentils. Orange cotyledons are preferred by Indians, which is also reflected in Indian genebank collections (Singh et al., 2014). These agro-morphological descriptors can facilitate the differentiation of distinct phenotypic classes and are used as diagnostic keys for taxonomic delineation (Pundir et al., 1985; Gore et al., 2019). The mode of inheritance of these traits can be investigated using the principles of classical genetics. This mega characterization program showed that most of the Mediterranean germplasm were found with low biomass and poor yield. The direct use of these accessions appeared difficult in Indian conditions, leading to the ubiquitous selection of Indian germplasm with high biomass and better yield traits.

The entire lentil germplasm was more diverse for quantitative traits such as number of secondary branches per plant, pods per plant, plant height, days to 50% flowering, seed thickness and qualitative traits, leaf pubescence, biomass score, pod shedding, growth habit, leaflet size, and pod dehiscence. Plant height (cm) in the INGB lentil core set ranged from 9.70 to 52.13 with mean value of 34.04, while it was ranged from 15 to 40 cm with mean value of 23 cm in the USDA core set sown

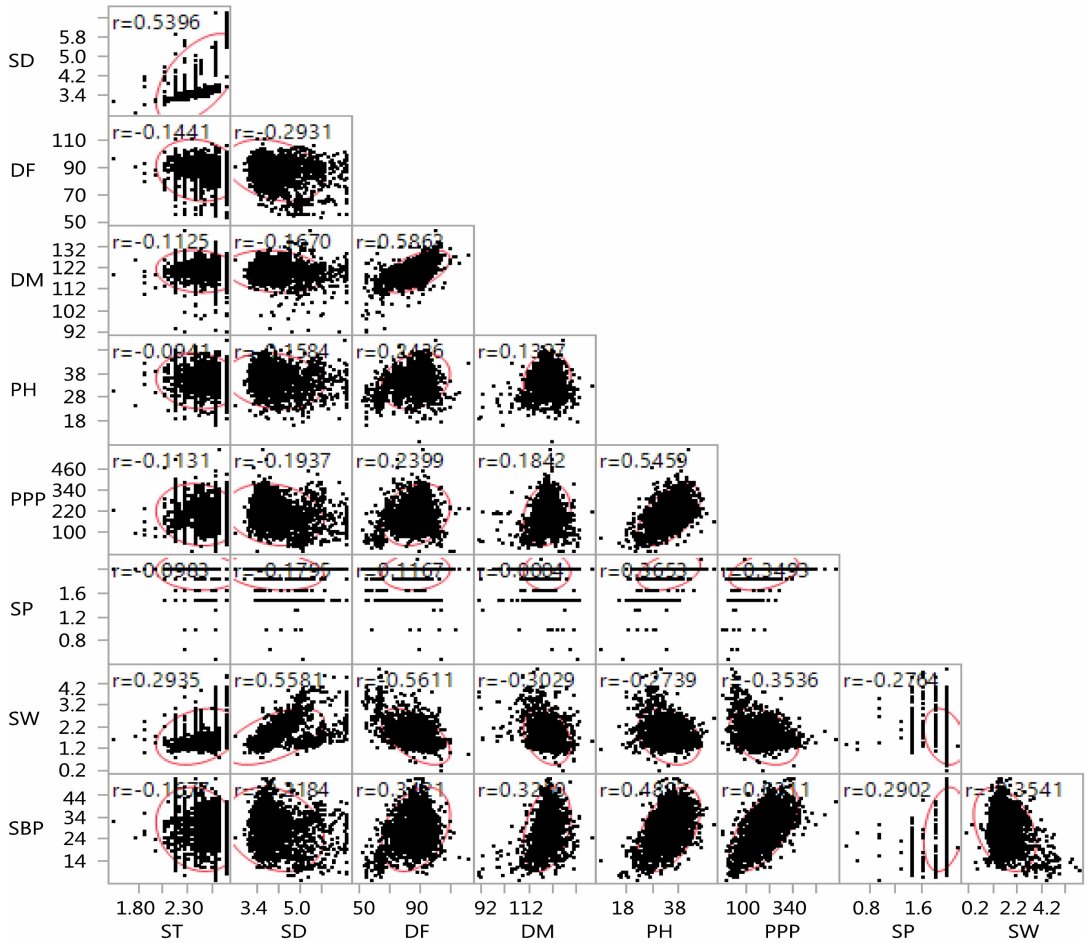

FIGURE 9 | Scatter plot matrix showing the correlation between nine agro-morphological traits of the entire lentil germplasm conserved in the Indian national genebank.

at Pullman, Washington, United States, in 1996 (Tullu et al., 2001). An average value of days to flowering and maturity is lower in the USDA lentil core set than the INGB lentil core set (Tullu et al., 2001). The grouping of germplasm in different clusters allowed us to study their genetic diversity, whereas the heatmap depicted the trait diversity. Germplasm accessions belonging to diverse clusters can be used in cross-breeding programs and recombination studies; for example, Clusters XI comprised early maturing accessions such as IC241529, IC241531, and IC241532 (less than 100 days), and cluster X comprised late maturing accessions such as EC267554, EC267615, IC258265, and EC440747 > 125 days. Similarly, Clusters XI had accessions with low plant height IC241488, IC241501, IC241529, and IC241531 (<20 cm) and cluster V had accessions with larger plant height, IC329110, IC398793, and IC59038 (>50 cm). The accessions such as EC223214, IC241543 (with high 100-seed weight) grouped in cluster XII and may be crossed with accessions with low seed weight IC361467, IC521438 (<1.4 g) from cluster III for genetics and mapping purposes.

Identification of Trait-Specific Germplasm

The ultimate aim of germplasm characterization is to support crop-breeding programs (He and Li, 2020; Guerra-García et al., 2021). Similarly, the present work unfolded the treasure of lentil collections conserved in the NGB. The accession IC317520 collected from Rajasthan was identified as a unique seed morphotype with intact funiculus at maturity (Tripathi et al., 2019). This was reported for the first time and is registered as a genetic stock (INGR19072) for its utilization in lentil breeding programs. An Intact funiculus may provide resilience to drought stress, resulting in high yields. This trait may help to gain area in rice-fallow areas of eastern India, where the crop is sown at conditions of conserved moisture (Lamichaney et al., 2021; Tripathi et al., 2021). Rice–wheat and rice–rice systems in the Indo-Gangetic plains dominate South Asia's cereal systems (Kumar et al., 2013). A substantial reduction in the acreage under pulses is attributed to the expansion of high-yielding wheat and boro rice in northern India and eastern to north-eastern India, respectively. This trend of pushing out pulses is likely to continue

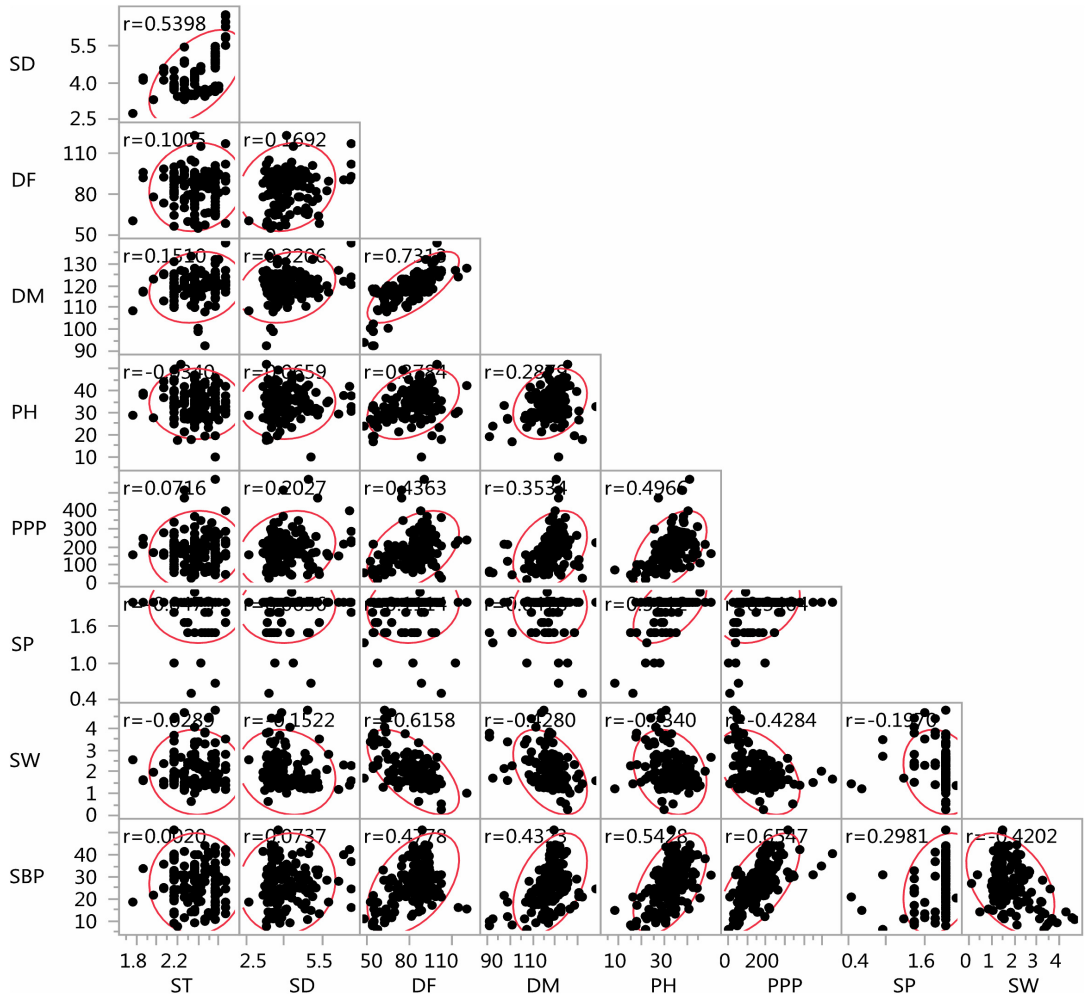

FIGURE 10 | Scatter plot matrix showing the correlation between nine agro-morphological traits of the Indian national genebank lentil core set.

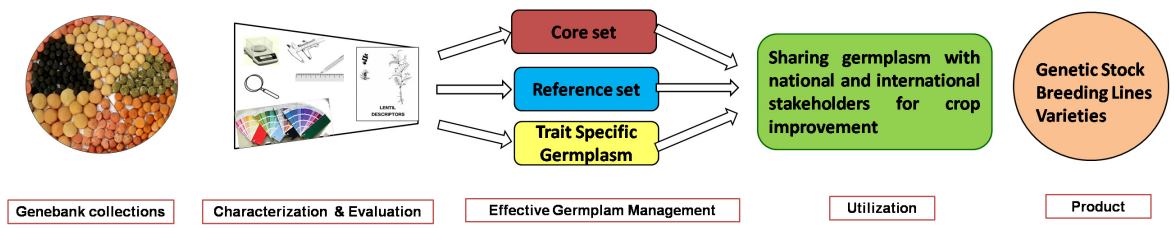

FIGURE 11 | Flowchart depicting the stepwise utilization of Indian national genebank lentil collections.

unless extra-early varieties that fit into these cropping systems are developed (Erskine et al., 1993; Shrestha et al., 2006). Similarly, in the rice-fallow areas of South Asia, the top soil layer generally dries at the harvesting stage, making it impossible to sow the next crop. Under such conditions, extra-early lentil germplasm can be used as donors to breed early maturing varieties to fit into the cropping system and convert these mono-cropped areas into double-cropped areas, thereby increasing the production of lentils under rice-based systems. In a previous study, a lentil accession with 59 days to flower was reported (Kumar and Solanki, 2014). However, the present experiment discovered accessions, IC241529 and IC241532, flowered in 51 days. The number of pods per plant and the number of secondary branches are the primary quantitative descriptor traits that can be used as kickstarting points for selecting high-yielding germplasm. Further, the seed size and shape of lentils are important traits because they affect the market class, cooking time, and the quality and yield of milled lentils (Fedoruk, 2013). Indigenous

and exotic accessions are broadly classified into small- and large-seeded types, respectively. Descriptor traits, such as tall, erect, top pod-bearing habits, thick stems, synchronous maturity, lodging tolerance, and non-shattering reported contributing to the development of varieties with machine harvestability (Kumar et al., 2013). Genetic variability for these traits was recorded in the INGB lentil germplasm. In an earlier study, Idleb-2 variety was released for machine harvesting (El-Ashkar et al., 2004). Identified germplasm, EC267615, may provide avenues for breeding machine harvestable varieties in India. Multiflowering accessions can be identified with the expression of three or more flowers at one or multiple flowering nodes (Gaur and Gour, 2002; Benlloch et al., 2015; Sanwal et al., 2016; Devi et al., 2018). In several legumes, previous researchers have documented multiflowering clusters for a few flowering nodes in *Cicer* (up to nine FPP) (Gaur and Gour, 2002), *Pisum* (up to five FPP) (Devi et al., 2018), and *Lens* (up to seven FPP) (Mishra et al., 2020). However, this study found a unique multiflowering germplasm accession, IC241473, in cultivated lentils, formed up to 16 FPP at multiple flowering nodes. This trait can fulfill the aim of genebanks to work in the direction of conservation and utilization. Trait specific germplasm (TSG) developed in this study were shared with the National Agricultural Research System (NARS) partners under the material transfer agreement for utilization in the varietal development programs.

Development of Core Collection

Globally, the first and foremost reason behind the limited use of conserved germplasm by plant breeders is huge and uncharacterized germplasm collections in certain genebanks (Ortiz et al., 2008). Characterized germplasm and smaller subsets can enhance germplasm use in trait discovery (Dutta et al., 2015). Core sets are subsets of large and diffused germplasm collections, containing selected accessions that represent the genetic variability of the entire set and acts as the entry point to the entire collection for trait-specific evaluation (Ortiz et al., 1998, 1999; Huamán et al., 1999; Upadhyaya et al., 2003). PowerCore is a fast and reliable tool for extracting a core set with a higher percentage of diversity than other methods used (Kim et al., 2007; Zhang et al., 2011; Dutta et al., 2015).

A subsampling procedure based on 26 agro-morphological descriptors and geographical origin data resulted in the selection of 170 germplasm accessions as the lentil core set, whereas only 79 accessions were sampled without passport information. Therefore, the inclusion of stratification into the geographical origin and places should be a choice for the sampling procedure. The INGB lentil core set amounted to 7.31% of the entire collection (2,324 accessions) conserved in the INGB, New Delhi. Similarly, Archak et al. (2016) selected 1,103 accessions (7.5%) as the chickpea core set from 14,651 accessions conserved in the INGB. As expected, the INGB lentil core set comprised 80.58% accessions belonging to Indian origin. However, only 12.89% (37 accessions) of the Indian accessions contributed to a lentil core set developed for agro-morphological descriptor traits by United States Department of Agriculture (USDA) (Tullu et al., 2001). The USDA lentil core consists of 12% of the 2,390 lentils (Simon and Hannan, 1995; Tullu et al., 2001). The maximum representation of germplasm accessions in INGB lentil core collections was from India (137), followed by Syria (22), indicating the rich diversity of cultivated lentils in India. A total of 95.88% of the core set belonged to Asian countries, including 15.29% from other Asian countries (Syria [mainly from ICARDA], Bangladesh, Israel, and Pakistan). As lentil is the main crop of Asia (Laskar et al., 2019), the INGB core set can be used as a diversity hub to meet the present and future challenges of the Asian lentil breeding program. Simon and Hannan (1995) also reported the utility of the legume cores in chickpea, lentil, and pea developed by USDA for directing users toward desirable germplasm from targeted geographic regions, and facilitating users at the preliminary stages of germplasm evaluation.

While sampling for a core set, the phenotypic associations in the core set need to be preserved for maintaining coadapted genetic complexes (Ortiz et al., 1998) and efficient utilization of germplasm (Upadhyaya et al., 2001). Nine traits were studied and 36 comparisons were performed between the pairs of characteristics. All trait combinations maintained similarity in correlation coefficient value between the entire INGB collection and the INGB core set. Earlier studies on the development of core in various crops such as chickpea (Upadhyaya et al., 2001; Archak et al., 2016), eggplant (Gangopadhyay et al., 2010), and wheat (Phogat et al., 2020) also reported the preservation of trait association in the core set. The strong correlation among some of the traits, such as days to 50% flowering and days to maturity (r = 0.52 in the entire collection, r = 0.731 in core set), and secondary branches per plant and pods per plant (r = 0.63 in the entire collection, r = 0.65 in core set), showed that future germplasm characterization might use days to 50% flowering and secondary branches per plant during preliminary evaluation. This trait is easier to measure than other traits, such as days to maturity and pods per plant. Similar observations were reported by Upadhyaya et al. (2003). Examination of the entries' spatial distribution and explaining the variance through PCA were also reported as an exploratory criterion for evaluating the core set in the study of Bisht et al. (1998) and Wang et al. (2008). In the present study, the first three PCs collectively explained 70.39% of the total variation in the core sets and 68.03% in the entire set. Germplasm accessions were majorly distributed in one large group and one small group in both the complete set and the core collection of INGB lentil germplasm.

CONCLUSION

The present study was based on the characterization of 2,324 accessions of cultivated lentils that resulted in the development of a core set of 170 accessions. The core set possessed a high diversity and allelic richness for major descriptor traits as revealed by the summary statistics, chi-square test, and SDI. The lentil core set described in this study represents the variability existing in the germplasm conserved in the INGB and provides insights into the traits for earliness, seed size, growth habit, and other vital agronomic traits. Identified and validated TSGs could serve as a potential source of novel alleles and genes. Characterization data of INGB lentil germplasm and developed multipurpose

core set may serve as a valuable resource for lentil workers and open the avenues for germplasm utilization in the selection, mapping, genomics, and trait discovery to attain sustainable lentil production under climate changing regime.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KT and AK planned and designed the research. KT, PG, AKS, GC, RBh, RBa, and RM assisted in data recording. JK, KT, and DM analyzed the data. NS, VG, and PG contributed to the materials. DS and PG performed the passport data analysis and geo-referenced map. KT and PG prepared the manuscript. AS, GM, JR, and HD edited the manuscript. KS provided the necessary support for inter-institutional collaboration. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.751429/full#supplementary-material

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