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Disease and pest resistance in legume crops

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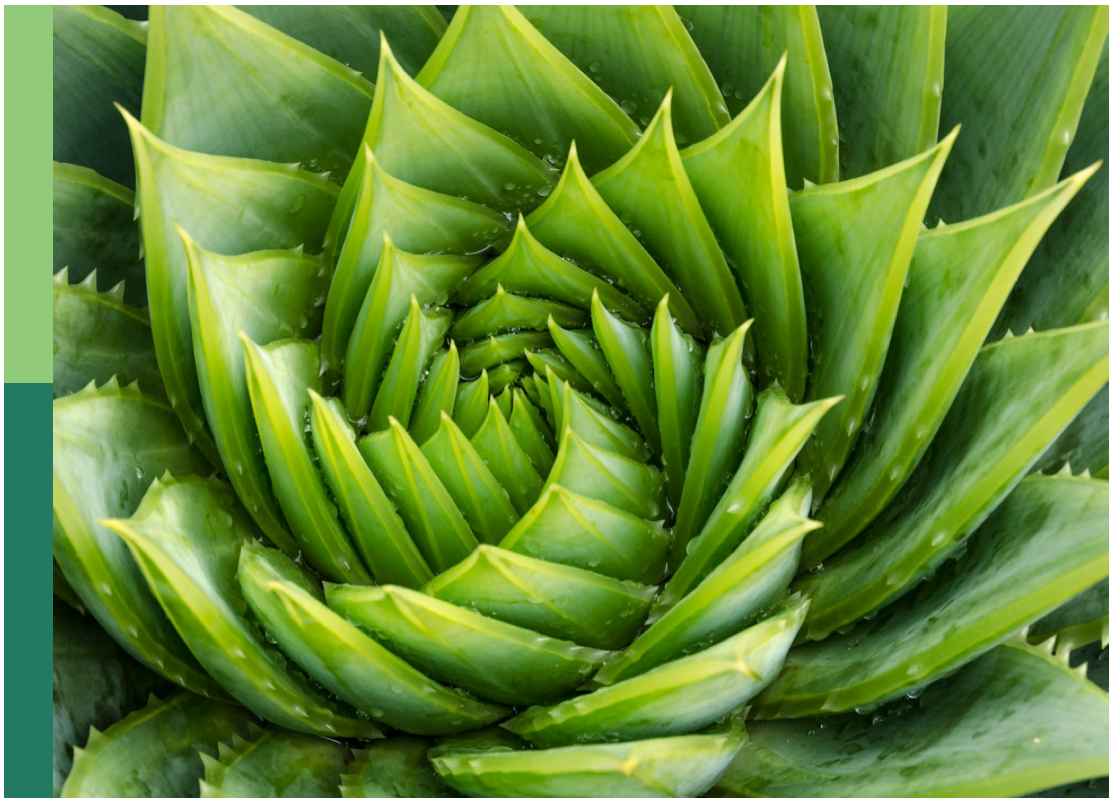

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Disease and pest resistance in legume crops

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Table of contents

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Editorial: Disease and pest resistance in legume crops

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Editorial on the Research Topic
Disease and pest resistance in legume crops

In the past decade, food legumes that show potential increase in production are chickpea, mungbean, blackgram, soybean, lentil, chickpea and faba bean. Besides that, common bean keeps being a crucial staple food. However, production of these food legumes is significantly hampered by both biotic and abiotic stresses (Nair et al., 2019; Boufleur et al., 2021). Major diseases of legumes include powdery and downy mildews, *Botrytis* grey molds, root rots, Ascochyta blights, anthracnoses, rusts, wilts, bacterial blights and mosaic diseases. In addition, damages caused by nematodes, parasitic weeds, and chewing/sap-sucking insects like pod borers/whitefly add to this long list of constraints for legume production.

Plant breeding programs aim to develop pest and disease-resistant varieties. To accurately characterize germplasm, it is crucial to understand how genetics and host-pathogen/pest interactions work (Rubiales et al., 2015). In addition, identifying novel alleles, inter-crossing and backcrossing strategies can enrich breeding germplasm through the use of wild relatives of crops. Alternatively, selection for disease and pest resistance is through high throughput field phenotyping. Genomic technologies can enable the identification and characterization of resistance genes and the functional characterization of their products (Mukankusi et al., 2019).

The objectives of the Research Topic on *Disease and Pest Resistance in Legume Crops* were to compile advances in research towards managing diseases and pests through various omics approaches, mechanism underlying host-pathogen interactions, germplasm characterization using modern genomics and phenomics tools, and implementation of novel approaches for disease and pest resistance in legumes that can be used in crop improvement. The topic received a tremendous response from scholars, with nine accepted articles contributed by 59 authors from worldwide.

Martins et al. investigated the genetic architecture of grass pea resistance to *Uromyces pisi* through a genome-wide association approach. The authors reported six single-nucleotide

polymorphism (SNP) markers linked with disease severity, signifying that partial resistance is oligogenic, located on chromosomes 4 and 6. After mapping with pea reference genome, (Mukankusi et al., 2019) proposed 19 candidate genes encoding for leucine-rich repeat, NB-ARC domain, and TGA transcription factor family, among others, which might help in understanding the molecular mechanisms of quantitative resistance to rust in grass pea.

Yang et al. reported that cinnamic acid enhances wilt in faba bean (*Vicia faba* L.) caused by *Fusarium oxysporum* f. sp. *fabae* by increasing activity of cell wall degrading enzymes and content of lignin in the stem produced by pathogen. To mitigate this problem, they found that intercropping of faba bean with wheat reduced the occurrence of wilt by decreasing the activity of cell wall degrading enzymes.

Joshi et al. revealed field pea (*Pisum sativum* L.) resistance against Ascochyta blight, caused by *Peyronellaea pinodes* and *Didymella pinodella*. Their study reports varieties with high levels of resistance against both pathogens and susceptible variety to be used as a susceptible check in disease screening program. They also found that in resistant genotypes, accumulation of hydrogen peroxide was lower compared to susceptible genotype.

Macrophomina phaseolina (Tassi) Goidanich causes dry root rot and blight diseases in many legumes and ashy stem blight (ASB) in common bean. In this context, Viteri et al. identified major quantitative traits loci (QTLs) and SNP markers associated with ashy stem blight resistance. Two SNPs, Chr03_39824257 and Chr03_39824268 were identified as the strongest markers associated with resistance to this disease and the drought sensitive gene *Phvul.003G175900* was recognized as one candidate for ASB resistance in the recombinant inbred lines (RIL).

Another study on genetic mapping and inheritance of resistance of common bean against anthracnose was carried out by Gomes-Messias et al.. Their findings revealed that anthracnose resistance in BRSMG Realce (an Andean bean [*Phaseolus vulgaris* L.] cultivar) is controlled by a major resistance gene, i.e., *Co-Realce* located on chromosome Pv04, flanked by SNP markers, snp1327 and snp12782 at a distance of 4.48 cM apart each other. Thus, a selection efficiency of 99.2% makes these SNPs suitable for marker-assisted selection (MAS).

Ferreira et al. reported two transgenic whitefly-tolerant common bean lines with an intron-hairpin construct to induce post-transcriptional gene silencing against *Bemisia tabaci vATPase* (*Bt-vATPase*) gene, with stable expression of siRNA. When compared to non-transgenic controls, insects fed on the transgenic line Bt-22.5 expressed 50% less *Bt-vATPase*. Whitefly-tolerant transgenic elite common bean cultivars can be developed contributing to the management of whitefly and viral diseases in common bean.

Taboada et al. review of literature revealed that white mold incited by *Sclerotinia sclerotiorum*, angular leaf spot by *Pseudocercospora griseola*, and web blight and root rot by *Rhizoctonia solani* were the major fungal diseases threatening common bean production in Argentina. Morpho-molecular features of about 200 isolates of these pathogens are discussed in this review along with screening of common bean genotypes under controlled and field conditions.

Parihar et al. reviewed genomics breeding strategies for major biotic stresses in Pea (*Pisum sativum* L.). Several QTLs and genetic markers associated with genes controlling resistance to pea diseases available for marker-assisted breeding are summarised in this review. In the long run, a judicious combination of conventional and cutting-edge omics-based breeding strategies will enhance genetic gain and optimize the development of biotic stress-resistant cultivars in order to sustain pea production in changing climates.

Roy et al. published a systematic review on breeding approaches for disease resistance in lentil (*Lens culinaris* Medik). They summarised the major genetic resources of lentil, disease screening methods and molecular markers associated with disease resistance that can be used in MAS program after further genetic validation in different genetic backgrounds. Roy et al. also focuses on mutation breeding, and recent interventions in omics technologies including CRISPR/Cas9 technology for improving disease resistance in lentil with advantages and limitations.

Research contributions to this Research Topic highlight the multiple dimensions of disease and pest resistance in legumes. In addition, the topic also covers disease screening techniques, the role of conventional and omics-based breeding approaches in improving yield limitation caused by major pests and diseases, and progress toward making legume varieties more resilient to disease or pest outbreaks under the shadow of climate change.

Author contributions

All authors contributed to the review of manuscripts and preparation of this editorial. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Association Mapping of *Lathyrus sativus* Disease Response to *Uromyces pisi* Reveals Novel Loci Underlying Partial Resistance

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Uromyces pisi ([Pers.] D.C.) Wint. is an important foliar biotrophic pathogen infecting grass pea (*Lathyrus sativus* L.), compromising their yield stability. To date, few efforts have been made to assess the natural variation in grass pea resistance and to identify the resistance loci operating against this pathogen, limiting its efficient breeding exploitation. To overcome this knowledge gap, the genetic architecture of grass pea resistance to *U. pisi* was investigated using a worldwide collection of 182 accessions through a genome-wide association approach. The response of the grass pea collection to rust infection under controlled conditions and at the seedling stage did not reveal any hypersensitive response but a continuous variation for disease severity, with the identification of promising sources of partial resistance. A panel of 5,651 high-quality single-nucleotide polymorphism (SNP) markers previously generated was used to test for SNP-trait associations, based on a mixed linear model accounting for population structure. We detected seven SNP markers significantly associated with *U. pisi* disease severity, suggesting that partial resistance is oligogenic. Six of the associated SNP markers were located in chromosomes 4 and 6, while the remaining SNP markers had no known chromosomal position. Through comparative mapping with the pea reference genome, a total of 19 candidate genes were proposed, encoding for leucine-rich repeat, NB-ARC domain, and TGA transcription factor family, among others. Results presented in this study provided information on the availability of partial resistance in grass pea germplasm and advanced our understanding of the molecular mechanisms of quantitative resistance to rust in grass pea. Moreover, the detected associated SNP markers constitute promising genomic targets for the development of molecular tools to assist disease resistance precision breeding.

Keywords: genome-wide association study (GWAS), grass pea, partial resistance, natural variation, rust

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INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is a cool-season legume crop with considerable economic importance, particularly in the developing nations of India, Bangladesh, and Ethiopia (Dixit et al., 2016; Das et al., 2021). This species is seen as a promising source of calories and proteins, and its resilience to adverse abiotic constraints has great potential for expansion in drought-prone and

marginal areas (Vaz Patto and Rubiales, 2014; Rubiales et al., 2020). Grass pea accessions can be classified into two main ecotypes, mostly considering their seed morphological features: one ecotype with accessions with larger and light-colored seeds usually originated from Mediterranean countries, and the other ecotype with accessions with smaller and dark-colored seeds, mostly from Asian countries (Przybylska et al., 2000). The genetic structure analysis was performed using molecular markers of a worldwide germplasm collection of grass pea accessions (Sampaio et al., 2021a). Moreover, further phenotypic characterization revealed that accessions from the Mediterranean region showed higher resistance to *Fusarium oxysporum* Schl. f. sp. *pisi* Snyd. and Hans. (fusarium wilt causal agent in grass pea), as compared with the remaining geographic origins (Sampaio et al., 2021b). These findings highlight the importance of assessing the grass pea germplasm natural diversity for a more educated utilization and conservation of grass pea genetic resources.

Resistance to pests and diseases is an important feature of grass pea (Vaz Patto and Rubiales, 2014) as is for most crops, including legumes (Rubiales et al., 2015). One example of such biotic threats, with a devastating impact on a wide range of legume crops worldwide, is the rust disease (Sillero et al., 2006). Several rust species can infect the Fabaceae family, the majority belonging to the genus *Uromyces* (Link.) Unger (Rubiales et al., 2011). These are biotrophic leaf pathogens, dependent on the infected host cells to remain viable throughout the infection process for successful colonization and completion of the life cycle (Panstruga, 2003; Martins et al., 2020). Both monogenic and polygenic resistances to rust have been reported in common bean, soybean, and faba bean among others, with the identification of loci controlling resistance and the development of closely linked molecular markers for selection breeding (Avila et al., 2003; Miklas et al., 2006; Hyten et al., 2007; Garcia et al., 2008; de Souza et al., 2011; Childs et al., 2018; Ijaz et al., 2021). However, limited information is available on rust resistance in most legumes. Hypersensitive resistance has been reported in lentil for instance (Rubiales et al., 2013), which might suggest monogenic resistance, but genetic analyses are not available so far. In other legumes such as pea or chickpea, hypersensitive resistance has not been identified in spite of thorough searches, although variation for partial resistance (Barilli et al., 2009a; Sillero et al., 2012) and associated QTL have been reported (Madrid et al., 2008; Barilli et al., 2010, 2018). Additionally, a few transcriptomic and proteomic studies on different legume-rust pathosystems indicated that rust induced important molecular changes on a general battery of plant defenses such as pathogenesis-related transcription factors (Madrid et al., 2008; *Medicago truncatula* Gaertn.—*Uromyces striatus*), reactive oxygen species-detoxifying enzymes (Castillejo et al., 2010; *M. truncatula—U. striatus* J. Schröt.), and the phenylpropanoid pathway (Barilli et al., 2015; pea—*Uromyces pisi*).

Previous studies showed that rust in grass pea is mainly attributed to *U. pisi* ([Pers.] D.C.) Wint., the causal agent of pea rust (Vaz Patto and Rubiales, 2009). Partial resistance, characterized by a compatible plant–pathogen interaction (non-hypersensitive response) and reduced disease severity (sensus Parlevliet, 1979), was frequently observed in an Iberian grass pea germplasm collection in response to *U. pisi* infection. Further histological evidence revealed that this partial resistance was attributed to restriction in haustorium formation, reduction in haustorium number per colony, and intercellular growth of infection hyphae (Vaz Patto and Rubiales, 2009).

The genetic architecture of partial resistance is often described to be associated with several loci each with variable effects on the resistance response observed to a pathogen (St. Clair, 2010). This, allied to the observable reduction on pathogen colonization and consequently reduced selective pressure imposed on the pathogen, contributes to increased durability and stability of partial resistance (Pilet-Nayel et al., 2017). This is particularly important for pathogens with a high risk of breaking down resistance genes due to the coexistence of sexual and asexual reproduction systems and with an effective air dispersal, such as rust pathogens (McDonald and Linde, 2002).

A more detailed characterization of the genetic architecture of partial resistance to rust in grass pea is still lacking. This may be in part attributed to the still limited genomic resources available in this species, hampering more efficient exploitation of previously identified resistant grass pea accessions as sources of favorable alleles in breeding for improved resistance. The few efforts taken to revert this unfavorable situation resulted in the development of a high-throughput transcriptome assembly of grass pea accessions (Almeida et al., 2014) and the closely related *Lathyrus cicera* L. accessions (Santos et al., 2018) against *U. pisi* infection. Results from the study by Almeida et al. (2014) revealed that differences among grass pea partially resistant and susceptible accessions were mostly related to the regulation of phytohormones signaling pathways, expression of pathogenesis-related (PR) genes such as the mildew resistance locus O (*mlo*), chitinases involved in fungal cell wall degradation, and genes involved in the production of secondary metabolites with antimicrobial activity. The highlighted pathogenesis-related mechanisms provided a valuable preliminary overview on the resistance mechanism activated in grass pea against rust infection to be further validated with results from other approaches such as genetic mapping.

The availability of cost-effective, high-throughput genome-wide single-nucleotide polymorphism (SNP) genotyping platforms is very relevant, especially in underutilized crops like grass pea (Kamenya et al., 2021). The development of a robust set of molecular markers for a particular species allows fine mapping of linked genomic loci controlling important traits through a genome-wide association study (GWAS). GWAS is a powerful methodology for harnessing the natural variation occurring in plant germplasm collections to dissect the allelic variants controlling complex traits (e.g., partial resistance to biotic stress). This approach has proven successful in better characterizing the response of a worldwide diverse grass pea germplasm collection (the same targeted in this study) against biotic constraints, namely, fusarium blight (Sampaio et al., 2021a). Although the absence of a fully assembled grass pea

reference genome imposed some challenges to the interpretation of GWAS results, a comparative approach with the pea reference genome (Kreplak et al., 2019), highly macrosyntenic to grass pea (Santos et al., 2021), has shown to be a reliable strategy to propose candidate genes.

In this study, we searched for novel resistance sources against *U. pisi* in a comprehensive germplasm collection of 182 grass pea accessions representative of worldwide diversity. Furthermore, we studied the genetic architecture of grass pea partial resistance to rust infection, through a GWAS as the first step for a more efficient precision breeding. To achieve this, the phenotypic response of the worldwide grass pea collection of accessions inoculated with *U. pisi* was combined with a previously generated high-throughput SNP markers screening, and significant SNP-trait associations were detected.

MATERIALS AND METHODS

Phenotypic Data

Plant Materials and Pathogen Isolates

The disease reaction of a worldwide germplasm collection of 182 grass pea accessions was evaluated in response to *U. pisi*. This germplasm collection was the same as described by Sampaio et al. (2021a) to identify genomic regions controlling resistance for fusarium wilt in grass pea. Two main ecotypes have been defined in grass pea related to their seed morphological traits, mostly composed of accessions with Mediterranean (big and light-colored seeds) or Asian origin (small and dark-colored seeds) (Przybylska et al., 2000). Therefore, as a proxy to this ecotype classification, the germplasm collection was classified based on their seed color (89 accessions with light seed color, and 93 accessions with dark seed color), seed size (52 accessions with large seeds and 130 accessions with small seeds), and geographical origin (91 accessions with European origin, one accession with Canadian origin, one accession with Brazilian origin, 60 accessions with Asian origin, 13 accessions with Ethiopian origin, 10 accessions with North African origin, and six accessions with unknown origin) (**Supplementary Table 1**). Seed size classification was assigned based on the weight (g) of 100 seeds (below 18 g accessions were considered small, and over 18 g accessions were considered large).

Seeds from the grass pea accessions and the susceptible control pea cv. 'Messire' were surface sterilized and germinated as described earlier (Sampaio et al., 2021b). Following this, seedlings were transferred to 0.5 L pots (one plant/pot) containing 1:1 sand and peat mixture and maintained under controlled conditions (12 h light 22°C/12 h dark 20°C photoperiod, 60% relative humidity, and 200 $\mu mol/m^2$ s of illumination). Experiments related to the phenotypic evaluation of the germplasm collection were carried out at the facilities of Consejo Superior de Investigaciones Científicas- Instituto de Agricultura Sostenible (CSIC-IAS), Córdoba, Spain.

The monopustular isolate UpCo-01 of *U. pisi* used in the inoculation assay was kept at −80°C at the Institute for Sustainable Agriculture-CSIC, Córdoba, and was multiplied on plants of the susceptible *Pisum sativum* cv. 'Messire' before use.

Rust Inoculation and Disease Response Assessment

Grass pea accessions were analyzed using a randomized complete block design. Three independent inoculation assays with *U. pisi* were performed on the 20-day-old whole grass pea plants (3–5 seedlings per accession, per inoculation assay). Inoculations with *U. pisi* were conducted by dusting 2 mg of rust spores/plant diluted with pure talc (1:10, w:w), with the help of a small manual dusting device (Vaz Patto and Rubiales, 2009). Pea cv. 'Messire' plants were included as susceptible checks in every inoculation assay. Inoculated plants were incubated for 24 h at 20°C and 100% humidity in complete darkness. Following incubation, spore germination was checked under a microscope, and plants were transferred back to the growth chamber where they were originally maintained. Disease severity (DS) and infection type (IT) were assessed 12 days after inoculation. DS was visually scored as the percentage of leaf area covered by rust pustules. IT was estimated based on the scale of Stakman et al. (1962), where IT 0, no symptoms; IT 1, necrotic flecks with minute pustules barely sporulating; IT 2, necrotic halos surrounding small pustules; IT 3, chlorotic halos surrounding pustules; IT 4, well-formed pustules with no associated chlorosis or tissue necrosis.

Phenotypic Data Analysis

Phenotypic data were subjected to residuals inspection to evaluate normality (quantile–quantile plot, QQ), the presence of outliers, and homogeneity of variance (residuals vs. fitted values). Since the residual's variance followed a normal distribution, no data transformation was applied.

A linear mixed model was applied for the DS trait, DS = *accessions* + *inoculation assay* + *accessions.inoculation assay*, where *accessions* is the genotypic term, *inoculation assay* (1–3) corresponds to the three independent inoculation assays, and *accession.inoculation assay* corresponds to the interaction between accessions and the independent inoculation assays. In a first step, best linear unbiased predictors (BLUPs) were obtained while fitting the model with all terms as random. A restricted maximum likelihood (REML) was applied to estimate the variance components of the linear mixed model and broad-sense heritability [VHERITABILITY procedure in Genstat software, according to Cullis et al. (2006)]. Following this, the best linear unbiased estimates (BLUEs) for each accessions were estimated while setting the term *accession* as fixed and the terms *accessions.inoculation assay* and *inoculation assay* as random. A Wald test for the significance of the fixed effects was performed using the generated BLUEs dataset.

A linear mixed model was used to estimate how much of the variation of accessions' response to *U. pisi* infection could be explained by geographical origin or seed morphology (seed color and size). The following linear mixed model was applied to investigate differences among seed origin classes: DS = *seed origin* (fixed term) + *inoculation assay* + *accessions* (random terms).

A similar model was used to estimate how much of the accessions' variance was explained by *seed size* or *seed color*, replacing *seed origin* with each of these terms. A Wald test was performed to test the significance of the fixed effects. Fisher's multiple-comparison tests were applied to the means of DS scores, at P-value ≤ 0.05. All analyses were performed using the Genstat software, 20th edition (VSN International, 2021).

Genotypic Data

Association Mapping Analysis

A GWAS was performed with GenStat software in the mixed-model framework, fitting SNP markers as fixed terms and accessions as random terms, using REML (Malosetti et al., 2007). Adjusted means (BLUEs) of the DS scores from the 182 grass pea accessions were tested for association with a previously available genotypic dataset.

Both the genotypic datasets constituted 5,651 SNP markers after quality control, and the pea genome marker positions previously described by Sampaio et al. (2021a) were retrieved to perform the present association analysis. This genotypic dataset was obtained from two high-throughput genotyping-by-sequence providers [Dart-Seq^{TM} genotyping (Kilian et al., 2012) and BGI, Beijing Genomic Institute, Beijing, China] using genomic DNA extracted from young leaves. Physical positions of SNP markers were assigned based on the pea reference genome v1a (Kreplak et al., 2019) as the most phylogenetic closely and highly syntenic-related species (Santos et al., 2021) with a better assembled sequenced genome.

Three linear mixed models, as described in the study of Sampaio et al. (2021a), were tested to control for false-positive SNP-trait associations: a naïve model, not accounting for population structure or family relatedness (Phenotype = SNP + *Error*); a model accounting for population structure (Q), using 15 principal components from the principal component analysis (PCA) (*Phenotype* = Q + *SNP* + *Error*); and a model accounting for familial relatedness (K), using kinship matrix K (*Phenotype* = *SNP* + *Accession* + *Error*), with *Accession* random effects structured following a kinship matrix K (Malosetti et al., 2007).

The principal components to account for the population structure among accessions and the kinship matrix to account for familial relatedness among accessions were previously calculated by Sampaio et al. (2021a) and retrieved to use in the present analysis. These calculations were made using a total of 1,058 SNP markers, evenly distributed across the pea genome, corresponding approximately to 1 SNP per megabase pair (Mbp).

The most appropriate model was selected following the inspection of the inflation factor value and quantile–quantile (QQ) plots of the P-values with the least deviations from the null hypothesis. The observed $-\log_{10}(P\text{-value})$ of each SNP marker was plotted against their assigned pea chromosomal position, based on comparative mapping with the pea reference genome v1a (Kreplak et al., 2019), to generate a Manhattan plot. Significant SNP-trait associations were detected at a threshold of $-\log_{10}(P\text{-value}) = 3.5$. This threshold was established taking into consideration two aspects: the size of the association panel used and the background noise of the Manhattan plots. Similar criteria were already described in other works with comparable or slightly smaller panel sizes and a similar number of markers, focusing on partial resistance traits (Tessmann and Van Sanford, 2018; Basile et al., 2019; Leitao et al., 2020) to avoid losing potentially interesting regions while applying a conservative type of adjustment such as Bonferroni correction [$-\log_{10}(P\text{-value}) = 5.053$]. Moreover, adjusted P-values according to the Benjamin and Yekutieli false discovery rate procedure (Benjamini and Yekutieli, 2001) were calculated considering an $\alpha = 0.2$ and k (number of LD blocks per chromosome) = 3,007, to control for type I errors caused by multiple testing. The effect of the minor frequency SNP allele was estimated in relation to the most frequent reference allele.

Allelic Variant Frequency on the Single-Nucleotide Polymorphisms Associated With the Trait of Interest Within Seed Classes

Frequencies of the favorable allele contributing to resistance, of each SNP marker detected as associated with the DS trait in response to *U. pisi* inoculation, were calculated by counting the number of accessions with a given seed color (dark or light), seed size (small or large), and geographical origin (Canadian, Brazilian, Asian, Ethiopian, European, North African or with unknown origin) that had the favorable allelic variant and divided by the total number of accessions with the same seed trait or geographical origin.

Linkage Disequilibrium and Candidate Gene Identification

Linkage disequilibrium (LD) per chromosome was estimated as the squared coefficient of correlation between marker pairs (r^2), after correction for population structure using the principal component scores from Eigenanalysis as implemented in Genstat software and described by Sampaio et al. (2021a). For this calculation, all grass pea markers with an assigned position (3,180 SNP markers) on the pea reference genome were considered. LD decay was visualized for each chromosome while plotting r^2 against the physical mapping distance in Mb. The LD decay threshold ($r^2 = 0.2$) was used to estimate the average genetic distance for which markers were considered to be no longer correlated. Accordingly, the distances to which LD decayed to the r^2 threshold for the chromosomes where SNP-trait associations were detected are the following: 0.074 Mb for chromosome 4 and 0.14 Mb for chromosome 6.

A genomic window for each SNP marker location significantly associated with the trait measured was established by subtracting and adding the average genetic distance considering the respective chromosomal LD decay. The physical boundaries of each chromosomal LD block (for which LD $r^2 > 0.2$) were used as query positions on the pea reference genome v1a (Kreplak et al., 2019) to retrieve the list of candidate genes mapped within those boundaries. Candidate genes for the response to *U. pisi* were considered if they contained a significantly associated SNP or were in LD with a significantly associated SNP marker. Annotation of the candidate genes was given by

the JBrowse tool available at https://urgi.versailles.inra.fr/Species/Pisum. Candidate gene functional characterization was obtained using the Mercator v2.0 (Schwacke et al., 2019).[1]

Candidate Gene Relative Expression Analysis by Reverse-Transcribed Quantitative PCR

Plant Material, RNA Extraction, and cDNA Synthesis

Three partially resistant grass pea accessions (i.e., PI165528 DS = 20%, PI283566 DS = 12%, and PI577183_A DS = 23%) and two susceptible accessions (i.e., PI283574 DS = 30% and PI221467_B DS = 38%) to *U. pisi* infection were selected for gene expression of candidate genes Psat6g006240 and Psat4g145320 (harboring the SNP markers detected as associated with the disease response), plus the candidate gene Psat6g010840 (in LD with SNP2174 and SNP2175). Three plants per accession (biological replicates) per time-point were inoculated as described earlier (see the "Materials and Methods" section). Leaves were collected from non-inoculated plants at 0 h after inoculation (HAI) and from inoculated grass pea plants at different time points (e.g., 12 HAI, 24 HAI, and 48 HAI). Leaves were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Total RNA was extracted from 100 mg of frozen leaves grounded to a fine powder in liquid nitrogen using a mortar and a pestle. RNA was isolated using the GeneJet Plant RNA Purification Kit (ThermoFisher Scientific, Vilnius, Lithuania) and treated with DNase I Kit (Ambion, Austin, TX, United States). RNA quantification was performed using Qubit 2.0 Fluorometer (Life Technologies, NY, United States) with Qubit RNA BR Assay Kit (ThermoFisher Scientific, Waltham, MA, United States). RNA purity was assessed by wavelength ratios measurement (260/280 and 260/260 nm) using Nanodrop ND-2000C spectrophotometer (ThermoFisher Scientific, Waltham, MA, United States). cDNA was synthesized from 500 ng of total RNA from each sample using the iScript cDNA Synthesis Kit (Biorad, Hercules, CA, United States).

Primer Design

Specific primers were designed for the target candidate genes using the gene sequence obtained with the JBrowse tool[2] as a template. The Primer3Plus tool[3] (Boston, United States) was used for primer design, with the default setting for RT-qPCR optimal conditions, and primer specificity was assessed using the Primer-BLAST NCBI tool. Specific primers were designed in the 3′ intra-exonic regions and synthesized by STABVida (Caparica, Portugal). Primer sequences can be found in **Supplementary Table 2**.

Quantitative Reverse-Transcribed Quantitative PCR Assay

Relative gene expression of target candidate genes among partially resistant and susceptible grass pea accessions was analyzed by RT-qPCR on a Light Cycler® 480 System, using the LighCycler 480 SYBR Green I Master protocol. As reference genes β-tubulin, photosystem I P700 apoprotein, y-tubulin, helicase, and histone H2A.2, previously described as reference genes for *Lathyrus* spp. (Almeida et al., 2014; Santos et al., 2018), were tested. Using the geNorm and NormFinder packages from GenEx v.5 software (MultiD, Goteborg, Sweden), histone H2A.2 and *y*-tubulin were selected as reference genes for the following gene expression assays.

Reverse-transcribed quantitative PCR (RT-qPCR) was performed for each of the three biological replicates per accession and time-point assessed (non-inoculated, 12 HAI, 24 HAI, and 48 HAI). Thermo cycling reactions were carried out following the described conditions: denaturation step at 90°C for 5 min; 45 cycles of amplification for 10 s at 95°C; 10 s at 60°C, and 10 s at 72°C. A melting curve was performed to detect non-specific PCR products or contaminants. A non-template control without cDNA was included for each primer mix to detect possible contaminations.

Relative expression values of each candidate gene were normalized to both reference genes using as calibrator the relative expression values of the non-inoculated (0 HAI) samples of the most susceptible accession to PI221467_B, following the Pfaffl (2001) method (2001). Fold change data were transformed into a logarithmic scale (base 2). A two-way ANOVA was conducted to inspect for differences between accessions and time-points per candidate gene. The *post hoc* Tukey's multiple comparison tests were used for means comparison at P-value ≤ 0.05.

RESULTS

Continuous Variation of Resistance Response in Grass Pea Response to *Uromyces pisi* Inoculation Might Be Related to Geographical Origin but Not to Ecotype Classification

All grass pea accessions showed a compatible interaction with *U. pisi*, with no associated macroscopically visible necrosis on the leaf surface (IT 3-4). In spite of this high IT, a continuous variation was observed in terms of disease severity, from 10 to 45%, with an average of 28.2 ± 6.3%, with significant differences detected among accessions (P-value ≤ 0.05, Wald test). The majority of the studied accessions were moderately susceptible (DS > 25%) to the *U. pisi* isolate used in this study (**Figure 1**). None of the grass pea accessions exhibited complete resistance (total absence of symptoms). Reduced DS scores (DS < 25%), in spite of high infection type (e.g., IT 4 and IT 3), were detected in 35% of the germplasm collection, hinting for partial resistance. Broad-sense heritability of grass pea DS for *U. pisi* was 0.89 (**Supplementary Table 3**).

Accessions with Ethiopian origin were shown to be the most susceptible (higher DS values) to rust infection ($P \leq 0.05$). Moreover, when accessions were classified based on seed color (dark or light), dark seed color accessions were the most susceptible. As for accessions classified by seed size,

[1] https://www.plabipd.de/portal/mercator4

[2] https://urgi.versailles.inra.fr/Species/Pisum

[3] https://primer3plus.com

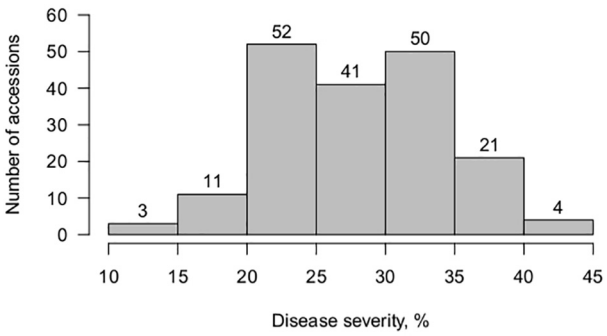

FIGURE 1 | Frequency distribution of *Uromyces pisi*-induced disease severity (DS) scores (%) in a worldwide collection of 182 grass pea accessions.

no differences were observed among larger or smaller seeds (**Supplementary Table 4**).

Grass Pea Partial Resistance to *Uromyces pisi* Is Controlled by Multiple Loci

Adjusted means calculated from the DS scores of 182 accessions were tested for SNP-trait associations with the previously generated high-quality 5,651 SNP markers, 2,471 of which had no known chromosomal position based on comparative mapping with pea reference genome v1a (Kreplak et al., 2019).

As previously described for the grass pea association panel understudy (Sampaio et al., 2021a), a clear genetic structure was detected, which, if not accounted for, could lead to the detection of false-positive associations. For this reason, SNP-trait associations were tested comparing a linear mixed model not accounting for any structure (naïve model) with models considering either population structure (Eigenanalysis) or kinship relationship among accessions (*K* matrix). Following an inspection of inflation factor values (near 1 is indicative of a better-fitting model) (**Supplementary Table 5**) and Q–Q plots (**Supplementary Figure 1**) of the *P*-values with the least deviations from the null hypothesis of the models tested, the model accounting for population structure (Eigenanalysis) was selected as the most appropriate. Results described hereafter were obtained with this model.

A total of seven SNP markers were significantly associated with the response to *U. pisi* inoculation (measured by DS) using a threshold of $-\log_{10}(P\text{-value}) = 3.5$ (**Figure 2**). Six of the associated SNP markers were located in chromosomes 4 and 6, while the remaining SNP markers had no known chromosomal position. SNP2145, located on chromosome 6, had the strongest association with the DS trait [$-\log_{10}(P\text{-value}) = 4.262$]. Each of the associated SNP markers explained only a portion of the observed phenotypic variance (6.5–8.1%). The SNP markers that explained the biggest proportion of phenotypic variation were SNP2174 (7.8%), SNP2175 (7.8%), SNP2145 (8%), and SNP1323 (8.1%) (**Table 1**).

A negative effect on grass pea DS of the variant allele in relation to the most frequent allele was detected for the majority of the SNP-trait associations, the exception to this being SNP5649. Given that DS is a measure of susceptibility, a negative effect of the variant allele suggests that the presence of the mentioned allele promotes increased resistance to rust infection.

Accessions With European and North African Origin Are Promising Sources of Allelic Variants Contributing to Partial Resistance

According to the accessions' geographical origin, favorable alleles of the significantly associated SNPs, contributing to partial resistance against *U. pisi* infection, showed higher frequencies in accessions with North African, European, and with unkown origin (**Figure 3**). A different situation was observed for SNP5649, presenting a higher frequency of favorable alleles in accessions with Asian, Ethiopian, and American (Canadian and Brazilian) geographical origins.

When accessions were classified based on their seed color (light or dark) and seed size (large or small), we observed that allelic frequencies of accessions with light-colored and larger seeds matched being the same observed for accessions characterized with dark-colored and smaller seeds (**Figure 4**). For

TABLE 1 | List of single-nucleotide polymorphism (SNP) markers significantly associated [$-\log_{10}(P\text{-value}) = 3.5$] with grass pea response to *Uromyces pisi* infection.

SNP ID	Pea Chr	$-\log_{10}(P\text{-value})$	*P* value	Adjusted by *p*[a]	Reference allele	Variant allele	Frequency[b]	Effect[c]	V_{QTL}/V_G[d] %
SNP1323	4	3.655	2.2×10^{-4}	1.4×10^{-4}	C	G	0.4444	−2.0284	8.1
SNP1385	4	3.860	1.3×10^{-4}	1.7×10^{-4}	A	G	0.0675	−3.5784	6.5
SNP1402	4	4.012	9.7×10^{-5}	1.7×10^{-4}	C	T	0.0915	−3.2601	7.1
SNP2145	6	4.262	5.4×10^{-5}	2×10^{-4}	A	C	0.1234	−3.0442	8
SNP2174	6	4.064	8.6×10^{-5}	1.8×10^{-4}	A	G	0.2532	−2.2660	7.8
SNP2175	6	4.064	8.6×10^{-5}	1.8×10^{-4}	A	C	0.2532	−2.2660	7.8
SNP5649	Unmapped	3.771	–	–	G	A	0.057	3.9820	6.8

For each SNP locus, the chromosomal position through comparative mapping with pea genome v1a, the effect of the variant allele, and the proportion of genotypic variance explained are shown.

[a] *Calculated according to Benjamin and Yekutieli procedure.* [b] *Frequency of the variant allele.*

[c] *Effect of the variant allele.*

[d] *Proportion of the genotypic variance explained by each of the significantly associated SNPs [V_{QTL} = 2Freq (1 − Freq) effect2; V_G = Estimated variance of the genotypic component].*

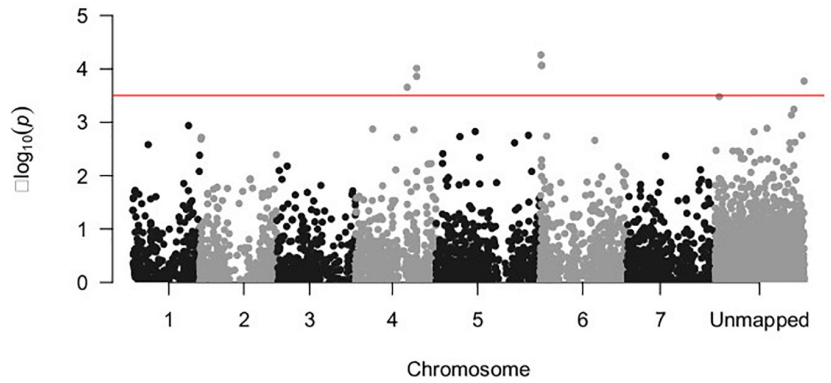

FIGURE 2 | Manhattan plot depicting the $-\log_{10}(P\text{-value})$ vs. chromosomal position of 5,651 SNP markers associated with the disease response of a grass pea collection of 182 accessions infected with *U. pisi*. The red line shows the threshold $-\log_{10}(P\text{-value}) = 3.5$ for the detection of significantly associated genomic regions.

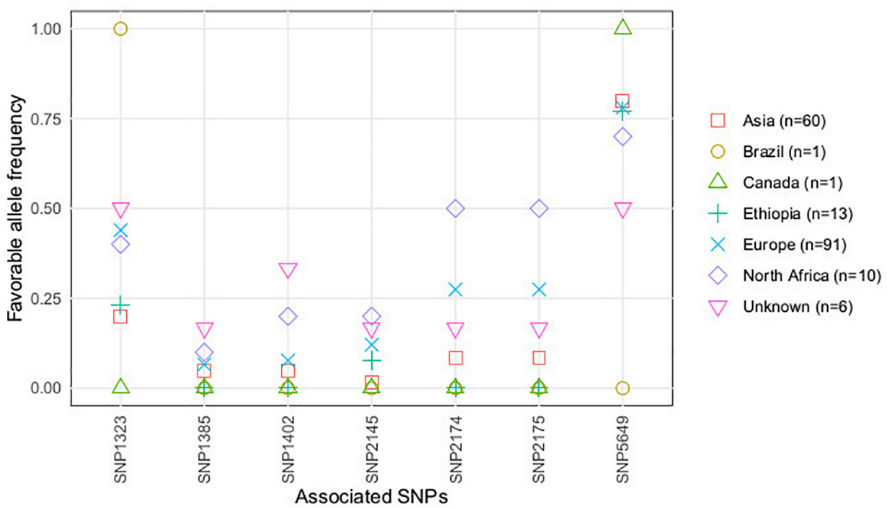

FIGURE 3 | Favorable alleles' frequency (conferring resistance) of the SNPs associated with *U. pisi* DS, based on the grass pea accessions classified by seed size (large, small) and seed color (light, dark).

SNP1323, SNP2174, and SNP2175, favorable allele frequencies were highest for accessions with light and larger seeds, whereas for the remaining SNPs almost no differences were detected between the seed color and seed size classes of accessions.

For the majority of the associated SNPs' favorable alleles (conferring resistance), a decrease in allele frequency was observed in accessions with increasing DS scores (**Supplementary Figure 2**). SNP5649 was the exception to this, presenting the highest frequency of the favorable allele conferring partial resistance, still present in accessions with a DS score over 40%.

Partial Resistance Candidate Genes Are Involved in a Diverse Array of Cellular Functions

Considering an LD decay threshold of $r^2 = 0.2$, the associated SNP markers SNP1385 and SNP1402 were in LD. The same was observed for the SNP2174 and SNP2175, sharing the same chromosomal region. The chromosomal locations of the significantly associated SNP markers detected through GWAS were inspected to propose candidate genes using comparative mapping with pea reference genome v1a (Kreplak et al., 2019). The genomic window selected to search for candidate genes was established considering the LD decay of the chromosome where the SNP-trait association was detected. Candidate genes harboring, or in LD, with associated SNP markers with known chromosomal position were identified following SNP marker sequence alignment against the pea genome. Accordingly, a total of 19 candidate genes were highlighted. A list with all the candidate genes can be found in **Supplementary Table 6**. Except for SNP1385 and SNP1402, all SNP markers significantly associated with the disease response to *U. pisi* were mapped within genes. Moreover, considering the degree of chromosomal LD block around each SNP marker identified,

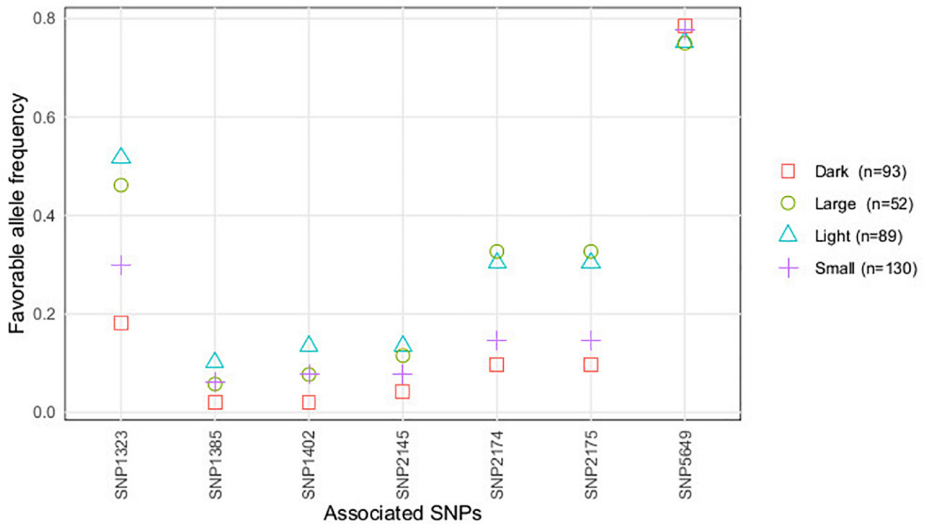

FIGURE 4 | Favorable allele's frequency (conferring resistance) of the SNPs associated with *U. pisi* DS, based on the grass pea accessions classified by geographical origin.

we achieved a mapping resolution to the gene level in 50% of the cases (2 LD blocks where a single candidate gene was identified).

Considering the multiple candidate genes linked to the detected SNP-trait associations, we will restrict the candidate genes description to those that were detected harboring the most strongly associated SNP marker and with biological relevance in the context of disease resistance. The strongest significant association [SNP2145 on chromosome 6, $-\log_{10}(P\text{-value}) = 4.262$] was mapped within the protein-coding gene Psat6g006240, coding for the GTP1/OBG GTP-binding protein family signature. In LD with SNP2145, the candidate gene Psat6g006320 was identified. This gene codes for an NB-ARC domain, the key regulator of the activity of resistance (R) proteins. SNP2174 and SNP2175 (chromosome 6), also among the strongest significant associations [$-\log_{10}(P\text{-value}) = 4.064$], were located within the coding sequence of Psat6g010800, a gene coding for the multidrug and toxin extrusion (MATE), and required for the transport of secondary metabolites and phytohormones, among others. Considering the LD decay in the chromosome where these SNP markers were mapped, a leucine-rich repeat (LRR) domain, known structural features of the majority of plant R proteins, was identified as a candidate gene.

Besides the identified candidate genes linked to the regions where the strongest SNP-trait associations were detected, an additional candidate gene putatively involved in disease resistance pathways was identified. On chromosome 4, SNP1323 was located within the gene Psat4g145320. This gene codes for a seed dormancy control protein, functionally annotated as the basic leucine zipper (bZIP) TGA transcription factor (TF), known to regulate the expression of pathogenesis-related (*PR*) genes.

Reverse-Transcribed Quantitative PCR Results Highlight Psat6g006240 (GTP1/OBG GTP-Binding Protein Family Signature) and Psat6g010840 (Leucine-Rich Repeat) as Putative Candidate Genes Differentially Expressed Among Grass Pea Accessions With Contrasting Rust Infection Responses

Relative gene expression of Psat4g145320 (bZIP TGA transcription factor) and Psat6g006240 (GTP1/OBG GTP-binding protein family signature) selected as candidate genes harboring significantly associated SNP markers, and Psat6g010840 (leucine-rich repeat) in LD with SNP2174 and SNP2175, were analyzed by RT-qPCR in phenotypically contrasting grass pea accessions (partial resistant vs. susceptible). Expression analysis of the candidate gene Psat6g010800 was not conducted given the struggle found in designing specific primers, and thus, no gene expression data are presented for the mentioned gene. Patterns of gene expression at different time-points (non-inoculated, 12 HAI, 24 HAI, and 48 HAI) among grass pea accessions with contrasting disease responses are presented in **Supplementary Figure 3**.

Regarding candidate gene Psat6g006240, we detected a significant gene downregulation in the susceptible accession (PI283574), as compared with the partially resistant accessions (e.g., PI283566, PI577138_A, and PI165528). In the susceptible accession, candidate gene downregulation was observed by 12 HAI (fold change > 1), culminating at 48 HAI with a fold change of 2, whereas in the partially resistant accessions, gene expression was unchanged throughout the infection process (fold change < 1) (**Supplementary Figure 3A**). In the candidate

gene Psat6g010840, differences in relative gene expression were observed while comparing the partially resistant (PI577138_A and PI165528) and the most susceptible accession (PI221467_B). In the partially resistant accessions, we observed a constitutive relative gene expression throughout the time-points investigated, whereas in the susceptible accession, a continuous increase in relative gene expression was detected, culminating at 48 HAI with a 1-fold change (**Supplementary Figure 3B**). As for the remaining candidate gene (Psat4g145320), no significant differences were detected among the phenotypically contrasting grass pea accessions.

DISCUSSION

Although considered a model species for a more sustainable agriculture due to its resilience to different stresses, little is known about the genetic basis of disease resistance, particularly to rust infection, in grass pea. This has restricted an efficient use of existing sources of resistance in grass pea precision breeding. To address this knowledge gap, we targeted through a GWAS a comprehensive germplasm collection of 182 grass pea accessions representative of worldwide diversity, revealing different sources of partial resistance to *U. pisi* infection. Several identified associated genomic regions suggested an oligogenic control of rust resistance in grass pea and allowed the proposal of potential candidate genes.

A continuous variation in the disease response to *U. pisi* was observed on the grass pea collection of accessions characterized here. This variation encompassed a wide range of responses from partially resistant to highly susceptible accessions. The most frequently observed phenotype was a compatible interaction (IT 3, IT 4), not associated with hypersensitive reaction, as it has been previously described on a more regional collection of Iberian grass pea accessions (Vaz Patto and Rubiales, 2009), and fitting the partial resistance definition (Parlevliet, 1979). In other legume-rust pathosystems, incomplete non-hypersensitive type of resistance was also the most frequently observed. In a very similar manner, a continuous distribution of disease response to *U. pisi* was observed in a pea germplasm collection, and the partially resistance sources identified were non-hypersensitive (Barilli et al., 2009b). This was also the case in chickpea (*Cicer arietinum* L.)—*Uromyces ciceris-arietini* Grognot (Sillero et al., 2012).

Moreover, the described natural variation of grass pea response to *U. pisi* resulted in the identification of seven significantly associated SNP markers, distributed by at least two different chromosomes. This demonstrates that the analyzed variation is controlled by multiple loci. This oligogenic nature of resistance to rusts has been often described in cool season legumes (Barilli et al., 2010, 2018; Rai et al., 2011; Childs et al., 2018). The partial resistant phenotypes of the grass pea described here, allied to the polygenic basis of resistance mapped in this study, are particularly interesting in the context of promoting more durable and stable crop protection in plant breeding for pathogen control, particularly for rust pathogens that, due to their effective air dispersal and co-existence of sexual/asexual reproduction strategies, are among the pathogens with increased risk of breaking down the effectiveness of resistance genes (McDonald and Linde, 2002).

Each of the significantly associated SNP marker identified here explained a fraction of the total genotypic variation, ranging from 6.5 to 8.1%. The multiple SNP-trait associations identified, allied to the reduced effect of each SNP marker on the trait measured, are consistent with the partial and quantitative nature of the grass pea response to *U. pisi*. Considering the high broad-sense heritability (0.89), this suggests that additional molecular components of disease resistance to *U. pisi*, explaining the remaining genetic variance, remained to be identified. It could be that a myriad of common variants of smaller effects (as usually occurs in polygenic traits, St. Clair, 2010) are contributing to the measured variance, but they were not uncovered by this association mapping approach. Another factor that could explain this missing heritability could be related to the occurrence of additional causal rare variants that could further contribute to the genotypic variance in the association panel (Eichler et al., 2010). However, given the adopted minor allele frequency (<5%) threshold for SNP data quality control, due to the limited statistical power to detect their contribution to the natural phenotypic variation (Sham and Purcell, 2014), rare allelic variants were excluded from the association mapping analysis.

Several of the significantly associated SNP markers detected here were located within or in LD with *a priori* candidate genes putatively involved in disease resistance pathways. The identification of such biologically relevant candidate genes further strengthens the usefulness of the GWAS approach to better understand the genetic basis of partial resistance to *U. pisi* in grass pea. For instance, in LD with SNP2145 [the strongest association with the DS measured, $-\log_{10}(P\text{-value}) = 4.262$] we identified an NB-ARC domain coding gene (Psat6g006320). This domain is a signaling motif shared by the nucleotide-binding leucine-rich repeat (NB-LRR) protein family, regarded as key R proteins conferring resistance to a wide variety of plant pathogens (Tameling and Joosten, 2007). Structure-function analysis on the NB-ARC domain highlighted their regulatory role in controlling NB-LRRs' activity (Van Ooijen et al., 2008). Another important common domain among several R proteins, LRR coding gene was also identified (Psat6g010840) in LD with SNP2174 and SNP2175. This domain is believed to provide recognition specificity for pathogen-derived elicitors (Tameling and Joosten, 2007). Although often associated with the expression of complete resistance, *R* genes have also been detected as co-localized with QTL controlling partial resistance in other pathosystems (Kump et al., 2011; Fukuoka et al., 2015; Raboin et al., 2016). Previous studies have hypothesized that functional polymorphisms on R proteins can alter disease resistance in a quantitative manner and contribute to continuous phenotypic variation among accessions in an association panel (Fukuoka et al., 2015). Contrasting with its role in disease resistance, our RT-qPCR results of the Psat6g010840 showed an induced gene expression upon infection with *U. pisi* in the susceptible accession (PI221467_B), culminating at 48 HAI (1-fold change).

As for the partially resistant accessions (PI577138_A and PI65528), the candidate gene showed a significantly lower constitutive expression. This suggests that Psat6g010840-induced expression might be contributing to disease susceptibility upon rust infection in grass pea, which seems to contradict their involvement in plant immunity as described above. Another important protein family related to the significantly associated SNP markers (SNP1323) is the basic leucine zipper (bZIP) TGA transcription factor (TF), coded by the candidate gene Psat4g145320. These TFs are required for SA-dependent plant defense responses effective against biotrophic pathogens and known to regulate the expression of *PATHOGENESIS-RELATED* (*PR*) genes (Wang et al., 2016). Despite their biological relevance, we detected no obvious gene expression patterns among phenotypically contrasting grass pea accessions for this candidate gene.

Co-localized with SNP2145, we identified the candidate gene Psat6g006240 (coding for GTP1/OBG GTP-binding protein family signature), differentially expressed among grass pea accessions with contrasting phenotypes to *U. pisi* infection. RT-qPCR analysis on the mentioned candidate gene revealed a significant downregulation in the more susceptible accessions (PI283574) following *U. pisi* infection (fold change $>$ 1 from 12 HAI onward), whereas in the partially resistant accessions (PI283566, PI577138_A, and PI165528), gene expression remained unchanged throughout the time-points assessed (fold change $<$ 1). The basic functions of this subfamily of proteins in plants are not well described, particularly their putative function in contributing to disease resistance. Although this finding might hint at a possible role of the mentioned candidate gene in resistance to rust infection, a more detailed characterization, by functional validation and analysis of global transcriptomic profile, is still required.

Until this study, there was a lack of studies focusing on the identification of resistance genes effective against *U. pisi* infection in legumes overall. This situation compromises efforts to assess if the SNP-trait associations identified here could harbor *a priori* resistance genes with particular relevance for *U. pisi* infection, which could be mapped through comparative mapping with, for instance, the pea reference genome used in this study. Indeed in the case of pea, previous efforts to map genomic regions controlling *U. pisi* resistance were developed in the wild-related *Pisum fulvum* Sibth. and Sm. and resulted in the identification of three QTLs: *UpDSII* (assigned to pea chromosome 4), *UpDSIV*, and *UpDSIV.2* (assigned to pea chromosome 6) (Barilli et al., 2018). When analyzing the pea genomic position of the molecular markers mapped in the proximity of the mentioned QTLs, we identified several SNP markers included in our genotypic panel that were flanking the *UPDSIV* QTL. However, they were not detected as significantly associated with the DS presently measured.

Previous studies have highlighted the usefulness of comparative mapping with the pea reference genome to propose resistance loci in grass pea. This has been further supported by the recent work of Santos et al. (2021), describing a high linkage map synteny between grass pea and pea. Nonetheless, in this study, 1 of the 7 significantly associated loci was considered unmapped, due to lack of alignment with the pea reference genome (considering the threshold *E*-value $< 1 \times 10^{-5}$), hindering the proposal of a candidate gene. These constraints highlight the need for a fully assembled and annotated grass pea reference genome (still ongoing, Emmrich et al., 2020) to attain the full potential of GWAS in the identification of candidate genes of interest and to infer about the molecular components encoded by them. The release of a fully annotated grass pea reference genome would lead to a higher percentage of SNP markers with a known chromosomal position and to identify further candidate genes. Nevertheless, it was still possible to get extra evidence on the relevance of the unmapped associated SNP marker on the genetic control of the grass pea—*U. pisi* pathosystem. Indeed, the marker sequence of the unmapped associated SNP marker identified herein (SNP5649) had high sequence homology with one contig found differentially expressed in the RNA-Seq transcriptomic comparison between resistant and susceptible grass pea accessions against *U. pisi* inoculation (Almeida et al., 2014). This contig (a423210_6) was detected to be over-expressed in the resistant accession as compared with the susceptible (fold change $>$ 1), but unfortunately with no functional annotation yet assigned.

Previous genetic diversity studies on the association panel analyzed here have structured the collection in two genetic clusters: one mostly consisting of accessions with lighter and larger seeds, mainly from Europe and North Africa, while darker and smaller seed accessions, predominantly from Asia, composed a second cluster (Sampaio et al., 2021b). In this study, we observed that dark-colored seed accessions, with Ethiopian origin, were the most susceptible against *U. pisi*, as compared with the remaining accessions. Accessions originated from North Africa with light and large seeds had a high frequency of the favorable alleles for the majority of the associated SNPs. This observation reveals that the mentioned grass pea accessions could constitute a promising source of resistant alleles, especially for breeding efforts focused on varieties with similar seed morphological types. Accessions with Canadian and Brazilian origin also presented high frequencies of the favorable allele for SNP1323 and SNP5649; however, these results might be overestimated given the fact that these geographic groups were composed of only one accession each.

The results presented here highlight the usefulness of exploiting the natural variation in grass pea germplasm to reveal genomic regions controlling resistance to *U. pisi*. To the best of our knowledge, this is the first study on association mapping of partial resistance in grass pea *U. pisi* pathosystem. We observed a continuous range of disease responses in response to rust infection. Several newly resistant loci controlling partial resistance to *U. pisi* and putative *a priori* known and novel resistance genes were identified, suggesting an oligogenic basis; however, detailed functional characterization is needed to better describe the underlying molecular mechanisms. Nevertheless, the identified favorable SNP alleles constitute already important

genomic tools to assist precision resistance breeding initiatives in grass pea and phylogenetically related legume crops such as pea.

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

DM carried out the inoculation assays, analyzed the phenotypic data, performed the genome-wide association study and RT-qPCR experiments, and drafted the manuscript. DR participated in the inoculations and discussion of the results and revised the manuscript critically. MCVP coordinated the study and took part in the discussion, drafting, and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Genomics Enabled Breeding Strategies for Major Biotic Stresses in Pea (*Pisum sativum* L.)

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Pea (*Pisum sativum* L.) is one of the most important and productive cool season pulse crops grown throughout the world. Biotic stresses are the crucial constraints in harnessing the potential productivity of pea and warrant dedicated research and developmental efforts to utilize omics resources and advanced breeding techniques to assist rapid and timely development of high-yielding multiple stress-tolerant–resistant varieties. Recently, the pea researcher's community has made notable achievements in conventional and molecular breeding to accelerate its genetic gain. Several quantitative trait loci (QTLs) or markers associated with genes controlling resistance for fusarium wilt, fusarium root rot, powdery mildew, ascochyta blight, rust, common root rot, broomrape, pea enation, and pea seed borne mosaic virus are available for the marker-assisted breeding. The advanced genomic tools such as the availability of comprehensive genetic maps and linked reliable DNA markers hold great promise toward the introgression of resistance genes from different sources to speed up the genetic gain in pea. This review provides a brief account of the achievements made in the recent past regarding genetic and genomic resources' development, inheritance of genes controlling various biotic stress responses and genes controlling pathogenesis in disease causing organisms, genes/QTLs mapping, and transcriptomic and proteomic advances. Moreover, the emerging new breeding approaches such as transgenics, genome editing, genomic selection, epigenetic breeding, and speed breeding hold great promise to transform pea breeding. Overall, the judicious amalgamation of conventional and modern omics-enabled breeding strategies will augment the genetic gain and could hasten the development of biotic stress-resistant cultivars to sustain pea production under changing climate. The present review encompasses at one platform the research accomplishment made so far in pea improvement with respect to major biotic stresses and the way forward to enhance pea productivity through advanced genomic tools and technologies.

Keywords: biotic stresses, genomics, proteomics, marker assisted breeding, speed breeding

INTRODUCTION

Pea (*Pisum sativum* L.), being cultivated throughout the world, either for food, fodder, and feed, is considered an important winter season food legume (Rubiales et al., 2019; Parihar et al., 2020). Cotyledons' color of pea grains varies from yellow, green, and orange that are used in the human diet in different forms such as *dal, stew, chhola, vegetables, snacks, soup, chat,* and flour, while whole seeds are mainly used as animal feed (Mahajan et al., 2018; Singh et al., 2018). Nutritionally, pea seeds are considered to have about 21–33% protein and 56–74% carbohydrate, with an average iron, selenium, zinc, and molybdenum of about 97, 42, 41, and 12 ppm, respectively (Parihar et al., 2016, 2021). Therefore, it serves as an important ingredient in providing nutritional security for resources poor people in developing countries. Moreover, its consumption minimizes the risk of several chronic diseases such as diabetes (Marinangeli and Jones, 2011), subsides blood cholesterol levels (Ekvall et al., 2006), improves cardiovascular health (Singh et al., 2013), possesses cancer prevention attributes (Kalt, 2001; Steer, 2006), administers body weight, and improves gastrointestinal affairs (Fernando et al., 2010; Lunde et al., 2011).

It is being cultivated widely across many countries in the world (Parihar et al., 2021). Its worldwide cultivated area has increased from 6.58 to 8.09 mha and production from 10.44 to 16.21 mt since 2010. Canada, Russia, China, India, and the United States are the major pea-producing countries (Parihar et al., 2020); however, the United States shares the highest total production of pea (39.33%), followed by Europe (36.98%) and Asia (18.09%). At present, its average productivity is about 2.0 t/ha globally, which recorded an increase of about 36% in a decade (2007–2017), but the potential productivity of this crop is up to 5.0 t/ha in several countries including Netherland, Denmark, Belgium, Germany, and Finland harvests about 3.45–5.01 t/ha (Toker and Mutlu, 2011). However, countries such as India, China, Australia, and Myanmar are recording very low productivity of less than 2.00 t/ha (FAO, 2021). During the past few decades, the gain in yield of pea (15.3 kg/ha/year) is relatively low as compared to other crops, which could be majorly attributed to the least investment in the pea research program (Rubiales et al., 2019). Also, the susceptibility of a pea toward many abiotic/biotic stress is another reason for low productivity which becomes a serious threat to its sustainable productivity especially under changing climatic conditions (Parihar et al., 2020). The most devastating diseases that affect the productivity of pea are powdery mildew (PM), ascochyta blight (AB), rust (PR), wilt (FW), and root rots (Parihar et al., 2013; Mahajan et al., 2018), of which PM caused by *Erysiphe* pisi (DC.), *E. baeumleri* (Magnus) (U. Braun & S. Takam.), and *E. trifolii* (Grev.) has the potential of reducing seed yield by 25–80% (Warkentin et al., 1996; Ghafoor and McPhee, 2012). PR caused by *Uromyces viciae-fabae* (Pers.) J. Schröt. *or U. pisi* (Pers.) de Bary is reported to cause yield losses up to 30% (Barilli et al., 2010, 2018; Singh et al., 2015) while, AB, results due to a mixture of fungal species [*Ascochyta pisi* (Lib.), *Peyronellaea pinodes* (Berk. & A. Bloxam), *Phoma medicaginis* var. *pinodella* (L.K. Jones), *P. Koolunga* (Davidson), and *P. glomerata* (Corda) (Wollenw. & Hochapfel)], is one of the most complex and severe diseases worldwide (Bretag et al., 2006; Tran et al., 2014) with a potential of reducing grain yield by about 60% (Liu et al., 2016). Fusarium root rot (FRR) incited by *Fusarium solani* f. sp. *pisi* (W.C. Snyder & H.N. Hansen), which may occur in both dry and wet field conditions, reduces yield significantly (Porter, 2010). Similarly, fusarium wilt (FW) caused by *F. oxysporum* f. sp. *pisi* (W.C. Snyder & H.N. Hansen) has about 11 different races (Gupta and Gupta, 2019), of which races 1 and 2 are distributed widely affecting the productivity of pea significantly, whereas races 5 and 6 are sporadically distributed (Infantino et al., 2006; Bani et al., 2018). A disease caused by *Aphanomyces euteiches (*Drechsler*)* is common root rot (CRR) and is prevalent in the United States, Europe, and Canada causes wilting of the roots (Wicker et al., 2003; Pilet Nayel et al., 2005; Chatterton et al., 2015; Desgroux et al., 2016; Wu et al., 2018). Several insect pests such as pod borer complex [*Helicoverpa armigera* (Hübner), *Etiella zinckenella* (Treitschke), and *Polyommatus boeticus* L.], bruchid (*Bruchus pisorum* L.) pea leaf weevil (*Sitona lineatus* L.), leaf miners [*Chromatomyia horticola* (Goureau)], stem fly [*Melanagromyza phaseoli* (Vanschuytbroeck)], aphids [*Acyrthospihon pisum* (Harris)], and cut worms [*Agrotis ipsilon* (Hufnagel)] seriously reduce the yield of pea by affecting the crop growth (Sharma, 2000; Yadav and Patel, 2015; Yadav et al., 2019). Pod damage of about 40% has been observed in pea due to pod borer complex infestation (Dahiya and Naresh, 1993).

The development of resistant cultivars to the biotic and abiotic stresses is an outstanding tactic to enhance the productivity of any crop including pea. Therefore, knowledge of the genetics of disease and pest resistance is essentially required to breed the resistant/tolerant cultivars. In addition to this, genomic advances especially the accessibility of draft genome sequence of pea (Kreplak et al., 2019) have facilitated the identification of the genes responsible for disease and pest resistance/tolerance and also helped in uncovering the genetics of quantitatively inherited resistance of several major diseases and pests. Moreover, genomics has also facilitated modernizing the conventional breeding for rapid and precise development of resistant cultivars in crop plants including pea. Information on genetics, genomics, and breeding of biotic stress resistance in pea is scattered and only limited attempts were made to review the different aspects of biotic stress resistance (Fondevilla and Rubiales, 2012; Smýkal et al., 2012; Rubiales et al., 2015; Tayeh et al., 2015a). Recently, Mahajan et al. (2018) discussed the genetic improvement in pea in relation to biotic stresses; however, the information provided was largely related to legumes in general and in brief about pea. Thus, an effort is made through this review to make available the comprehensive information pertaining to genetic and genomic advancement at one platform as well as to share a futuristic road map using modern genomic and genetic tools in pea breeding that could aid the crop breeders in developing high-yielding multiple stress resilient pea cultivars.

CURRENT STATUS OF GENETIC RESOURCES

Genetic improvement in a target crop species requires availability and judicious exploitation of genetic resources. Globally, more than 98,000 pea accessions, comprised of advanced breeding lines (13%), landraces (38%), mutant stocks (5%), wild species (2.6%), and cultivars (34%), are available and conserved in diverse genebanks (Smýkal et al., 2015; Warkentin et al., 2015; Rubiales et al., 2019; Coyne et al., 2020). The National Institute for Agricultural Research (INRA), France, Australian Grains Genebank (AGG), N.I.Vavilov Research Institute of Plant Industry, Russia, US Department of Agriculture (USDA), United States, Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany, and International Center for Agricultural Research in the Dry Areas (ICARDA), Lebanon are the six leading active pea germplasm repositories in the world with about 8,839, 7,432, 6,790, 6,827, 5,343, and 4,596 accessions, respectively (**Figure 1**). The National Germplasm Repositories of various countries also hold a good number of pea accessions such as 4,558 accessions in Italy, 3,837 in China, 4,484 in India, 3,298 in the United Kingdom, 2,896 in Poland, 2,849 in Sweden, 2,311 in Ukraine, and 2,110 in Aberystwyth University, United Kingdom. Besides, seven other countries hold > 1,000 accessions of *Pisum* in their national germplasm treasury (**Figure 1**). Interestingly, the National Genebank of Israel possesses a collection of crop wild relatives (CWRs) such as *Pisum fulvum* and *P. sativum* subsp. *elatius* var. *pumilio*, which contributes to about 2% of the entire preserved germplasm (Smýkal et al., 2013, 2015; Warkentin et al., 2015). This share of CWR has accessions to *P. fulvum* (706), *P. s*. subsp. *elatius* (624), *P. s*. subsp. *sativum* (syn. *P. humile/syriacum*; 1562), and *P. abyssinicum* (540) (Smýkal et al., 2013). Besides CWR and cultivated accessions, 575 and 122 accessions of pea mutant stocks are also available at the John Innes Collection, the United Kingdom and the Institute of Plant Genetics Resources Collection, Bulgaria, respectively (Smýkal et al., 2015). A Targeted Induced Local Lesions in Genomes (TILLING) population of 9,000 lines (Coyne et al., 2020) and fast neutron generated deletion mutant resources (around 3,000 lines) are also available, which are being exploited to identify various developmental genes (Smýkal et al., 2015). Internationally, several web-portals have been developed using the database of pea genetic resources such as the European Cooperative Program on Plant Genetic Resources, Cool Season Food Legume Database, Genetic Resources Information Network and System-wide Information Network for Genetic Resources, and KnowPulse for keeping records and disseminating the information related to pea genetic resources.

Crop wild relatives that include *Pisum* species and subspecies are in general a source of countless fascinating traits including various yield attributing parameters (Mikić et al., 2013). Besides, it is a source of resistance to several biotic stresses, e.g., pea seed weevil (Clement et al., 2002, 2009), PM (Fondevilla et al., 2007b; Esen et al., 2019), PR (Barilli et al., 2010), AB (Jha et al., 2012), and broomrape (Fondevilla et al., 2005). The significance of CWR has been demonstrated by successfully introducing a novel dominant gene (*Er3),* responsible for resistance to *E. pisi* from *P. fulvum* (Sharma and Yadav, 2003; Fondevilla et al., 2008a). Moreover, some *P. fulvum* accessions were reported to show resistance against bruchid, broomrape, and *Mycosphaerella pinodes* and are subsequently being utilized in hybridization programs (Fondevilla et al., 2005; Coyne et al., 2020). Similarly, resistance to PR (Barilli et al., 2010, 2018) and AB (Fondevilla et al., 2005; Jha et al., 2012) has been observed in *P. fulvum*. Diversity for the *eIF4E* gene and novel alleles for virus resistance has also been identified from CWR (Ashby et al., 2011; Konečná et al., 2014). In a recent report, the relationship between neoplasm and pea weevil (*Bruchus pisorum* L.) damage was not established in F_1 and F_2 derived from the inter-subspecific crosses of *P. sativum* subsp. *sativum* (with neoplasm) and *P. sativum* subsp. *elatius* (without neoplasm) in field conditions (Sari et al., 2020).

Interestingly, the germplasm with the least commercial acceptance in terms of colored seed coat and flowers was accredited as a wonderful resistance source for root rot diseases (Grunwald et al., 2003; Weeden and Porter, 2007) and *Aphanomyces* (Hamon et al., 2011). Most significantly, the resistance to different biotic stresses can also be transferred from *Lathyrus species* that are harbored in the tertiary pea gene pool (Patto et al., 2007, 2009), preferably through the utilization of contemporary biotechnological techniques. Most recently, super-early progeny derived from an interspecific cross between *P. sativum* and *P. fulvum* flowered in 13–17 days and set pod in 18–29 days after emergence. Such progeny could be used as a complementary to "speed breeding," to generate more than six generations per year in an appropriate climate compartment (Sari et al., 2021). Significant contributions have been made toward the identification of resistant genetic resources for major biotic stresses in pea (**Table 1**), which might be utilized in breeding programs and further genetic analysis for the identification of new resistance genes.

CURRENT KNOWLEDGE ON GENETICS FOR DISEASE RESISTANCE

Knowledge of genes controlling disease resistance is important to accelerate the success of any breeding program (Shashikumar et al., 2010). Understanding gene action/effects operating in a particular breeding population helps to select a suitable parent for hybridization and breeding procedure for making genetic improvements of resistance against that disease (Sharma et al., 2013). Notably, the pea is acknowledged as the original model organism and was utilized in the finding of Mendel's laws of inheritance, which laid the foundation for modern plant genetics. In the recent years, inheritance has been studied for resistance attributes of disease in pea by several researchers (Lamprecht, 1948; Yarnell, 1962; Blixt, 1974; Gritton, 1980; Kalloo and Bergh, 1993; Kumar et al., 2006; Amin et al., 2010), and genes were identified and mapped using conventional gene mapping approaches. Varieties with inbuilt resistance are the most appropriate, competent, and economic strategies for

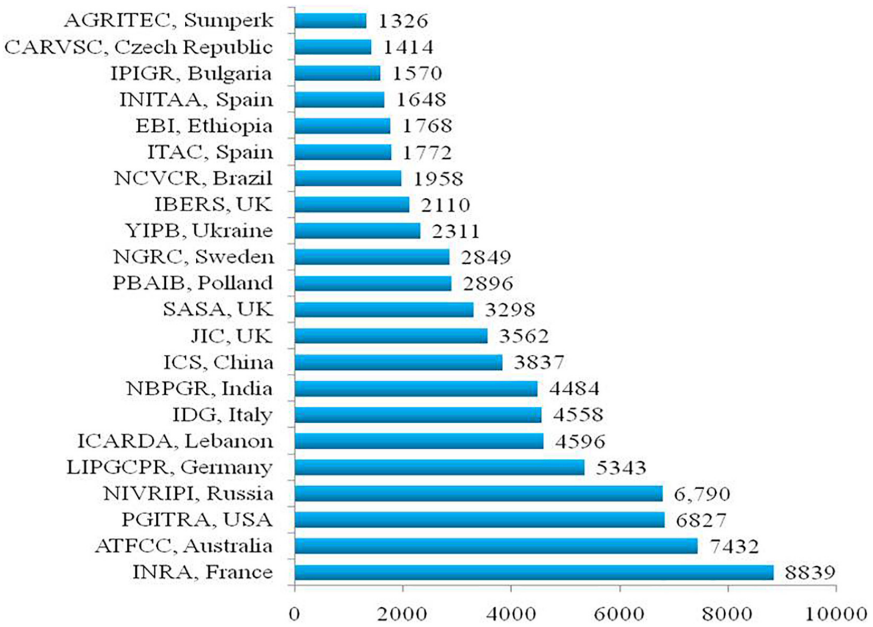

FIGURE 1 | Major pea germplasm holding organizations worldwide (Warkentin et al., 2015; https://www.genesys-pgr.org/).

tackling biotic stresses. Therefore, comprehensive efforts have been made to understand the inheritance of biotic stresses. Inheritance study for PM revealed that it is being operated by two recessive genes (*er1* and *er2*) and one dominant gene (*Er3*) (Fondevilla et al., 2007a). A recent report illustrated that PM resistance is operated *via er1* owing to the non-functioning of gene *PsMLO1* (Humphry et al., 2011). The gene *er2* is reported to provide complete resistance to PM but is efficient only in location-specific breeding (Tiwari et al., 1997; Fondevilla et al., 2006), while gene *Er3* confers resistance in *P. fulvum* (Fondevilla et al., 2007a, 2010).

With regard to PR resistance, it was reported to be operated by a single dominant gene (*Ruf*) (Tyagi and Srivastava, 1999); however, the polygenic nature of gene action (Singh and Ram, 2001) and partial dominance of a single gene in conjunction with minor and additive genes (2–3) (Singh et al., 2012) have also been found recently. A single dominant gene governs resistance toward races 1 and 2 of *F. oxysporum* f. *pisi*, pea enation mosaic virus, *F. solani* f. sp. *pisi,* brown root rot, bacterial blight, downy mildew, and other root rot diseases of pea, whereas a recessive gene regulates resistance to pea seed borne mosaic virus (*sbm*), yellow bean mosaic virus (*mo*), pea mosaic virus (*pmv*), and bean virus (Amin et al., 2010; Mohan et al., 2013). However, Davidson et al. (2004) reported downy mildew to be controlled by a single dominant gene and two complementary recessive genes. The nature of inheritance of AB and FRR resistance has been reported to be regulated by many genes (Kraft, 1992; Fondevilla et al., 2007b; Carrillo et al., 2014b; Jha et al., 2017). The pod resistance for pea weevil is quantitatively controlled whereas the seed resistance is operated by three (*pwr1*, *pwr2,* and *pwr3*) major recessive alleles (Byrne et al., 2008). The neoplasm appearance on pods is controlled by a single dominant gene and its expressivity is influenced by one or a combination of environmental factors (Sari et al., 2020).

EXPLOITATION OF GENETIC KNOWLEDGE THROUGH TRADITIONAL BREEDING APPROACHES FOR BIOTIC STRESS RESISTANCE

Numerous biotic stresses including FW, AB, PM, PR, FRR, and CRR are serious threats to pea production (Bohra et al., 2014). These diseases are reported to occur in a severe form in almost all the pea growing countries. Therefore, efforts have been made to exploit the available genetic knowledge of resistance through conventional breeding for these key biotic stresses for developing resistant cultivars (Fondevilla and Rubiales, 2012; Ghafoor and McPhee, 2012). To develop high yielding pea cultivars possessing PM resistance, three genes, namely, *er1*, *er2,* and *Er3* have been exploited successfully using conventional breeding approaches (Heringa et al., 1969; Fondevilla et al., 2007c). The *er1* gene has the highest existence in resistant pea accessions followed by the *er2* gene, which is harbored in restricted accessions (Tiwari et al., 1997). Therefore, the *er1* gene that provides resistance through the pre-penetration resistance mechanism has been largely exploited in most pea improvement programs worldwide (Fondevilla et al., 2006). PR is another serious disease, scattered across the countries where the pea is being cultivated. Resistance to PR has been reported to be polygenic (Singh et al., 2012) and oligogenic (Vijayalakshmi et al., 2005). AB or black spot disease is one of the most devastating diseases of peas causing yield setbacks of up to 60% (Xue et al., 1996; Liu et al., 2016). Being seed borne, the rate of transmission from seed to sapling

TABLE 1 | Potential resistance source of different biotic stresses in field pea.

Biotic stress	Germplasm/variety/wild relatives	Country	References
Powdery mildew	9057, 9370, 9375, 10609, 10612, 18293, 18412, 19598, 19611, 19616, 19727, 19750, 19782, 20126, 20152, 20171, It-96, No. 267, and No. 380	Pakistan	Azmat et al., 2012
	Medora, PS9910188, PS810765, PS810324, Stirling, PS0010128, PS8 10240, PS710048, PS810191, 3272, 3273, Lifter, Franklin, and Fallon	Pakistan	Nisar et al., 2006
	P. fulvum (P660-4)	Spain	Fondevilla et al., 2007b
	HFP4, EC598878, EC598538, EC598757, EC598704, EC598729, EC598535, EC598655, EC598816, EC381866, IC278261, IC267142, IC218988, IC208378, IC208366	India	Rana et al., 2013
	LE 25, ATC 823, KPMR-10, T-10, P-185,6533, 6587, 6588, JI 210, DMR 4, DMR 7, DMR 20	India	Ghafoor and McPhee, 2012
	HFP 9907 B, Pant Pea -42, VL Matar 42, IPFD 99-13, IPFD 1-10, IPF 99-25, Pusa prabhat, Ambika	India	Dixit and Gautam, 2015
	Highlight, AC Tamor, Tara, Mexique 4, Stratagem, JI 210, JI 1951, JI 1210, JI 2480	Canada	Tiwari et al., 1997
	Glenroy, Kiley, Mukta, M257-3-6, M257-5-1, PSI 11, ATC 1181	Australia	Liu et al., 2003
	GPHA-9, GPHA-19	Ethiopia	Assen, 2020
	JI2480	India	Katoch et al., 2010
Rust	IPF-2014-16, KPMR-936 and IPF-2014-13,	India	Das et al., 2019a
	PJ 207508, C 12, Wisconsin, DMR 3, Pant P 5, Pant P 8, Pant 9, HFP 8711 and HUDP 15, IPFD 1-10	India	Chaudhary and Naimuddin, 2000; Dixit and Gautam, 2015
	JP-4, FC-1, Pant P 11, HUDP 16, JPBB-3, HUP 14	India	Dhall, 2015
Downey mildew	Mukta, Snowpeak	Australia	Davidson et al., 2004
Pea seed-borne mosaic virus (PSbMV)	PI 193586, PI 193835	Ethiopia	Hagedorn and Gritton, 1973
Pseudomonas syringae pv. *pisi (*race 6, 8)	JI0130	Spain	Martín-Sanz et al., 2012
Pseudomonas syringae pv. *Pisi* (race 8)	Forrimax, JI2546, PI-277852, ZP1328, Cherokee, Corallo, Lincoln, JI2385, PM29, PM232, PM33, JI1829, ZP1282, ZP0104, ZP1301, ZP0123, ZP0168	Spain	Martín-Sanz et al., 2012
Mycosphaerella blight (*Mycosphaerella pinodes)*	CN 112432, CN 112441, CN 112513	Canada	Jha et al., 2012
	P. fulvum (P651), Radley	Spain	Fondevilla et al., 2005
Stem fly *(Melanagromyza phaseoli*)	P-4039, P-4107	India	Vishal and Ram, 2005
Leaf miner *(Chromatomyia horticola*)	P-4107	India	Vishal and Ram, 2005
Pea weevil (*Bruchus pisorum*)	*P. fulvum* (ATC113)	Australia	Hardie et al., 1995; Byrne et al., 2008
Pulse beetle (*Callosobruchus chinensis* L.)	*P. sativum* (ACP 11), *P. elatius* (AWP 442) *P. fulvum* (AWP 600, AWP 601)	Turkey	Esen et al., 2019
Fusarium root rot (*Fusarium solani* f. sp. *pisi)*	PI215766, PI244121	United States	Grunwald et al., 2003
	JI 1794 (*P. sativum* subsp. *elatius*).	United States	Hance et al., 2004
	PI125839, PI125840, PI175226, PI220174, PI223526, PI223527, PI226561 and PI227258	United States	Porter, 2010
Fusarium oxysporum f. sp. *pisi*	JI1412, JI1760 (*P. sativum* ssp.), P633 (*P. sativum* ssp. *arvense*), P42 (*P. sativum* ssp. *elatius*)	Spain	Bani et al., 2012, 2018

for *A. pisi* and *P. pinodes* is 40–100% (Maude, 1966; Xue, 2000), with an ability to remain viable on seeds for 5–7 years (Wallen, 1955). To date, the absolute resistant source for AB has not been identified; however, a prominent scale of resistance was found in accession (P651) of *P. fulvum*, which is being actively utilized in pea improvement (Wroth, 1998; Sindhu et al., 2014). The polygenic inheritance pattern of AB makes the development of resistant cultivars through conventional breeding very difficult. The FRR is considered a serious bottleneck in harnessing the full potential of a cultivar (Bisby, 1918; Jones, 1923). The condensed soil with a temperature of 18–24°C is the ideal thermal regime for the proliferation of FRR (Kraft and Boge, 2001). Unfortunately, complete resistance to this disease is yet to be explored; however, genetic sources carrying partial tolerance to this disease are

TABLE 2 | Available genetic maps for different biotic stresses in field pea (*Pisum sativum* L.).

S. No.	Population	Population size	Type of population	Markers	Marker type	Total map distance (cM)	References
Powdery mildew (*Erysiphe pisi*)							
1	Kaspa × Yarrum	106	RIL	821	SSR and SNPs	1910	Sudheesh et al., 2014
2	Kaspa × ps1771	106	RIL	852	SSR and SNPs	1545	Sudheesh et al., 2014
3	C2 × Messire	100	F2	720	RAPD/SCAR	–	Fondevilla et al., 2008a
4	Slow × JI1794	51	RIL	200	RAPD/RFLP	–	Timmerman et al., 1994
5	Almota × 88V1.11	111	F2	200	RAPD/RFLP	–	Timmerman et al., 1994
6	Lincoln/JI2480	111	F2	152	SSR	51.9	Katoch et al., 2010
7	Radley × Highlight	99	F2:3	416	RAPD/SCAR	–	Tiwari et al., 1998
8	PG 3^{HFP4} × PG 3	208	F2	633	RAPD/SCAR	–	Srivastava et al., 2012
9	Majoret × 955180	192	F2	315	SSR	49.9	Ek et al., 2005
10	Solara × Frilene-derived mutant	230	F2	585	ISSR, RAPDs, AFLPs	66.4	Pereira et al., 2010
11	Sparkle × Mexique	–	F2	–	RAPD/SCAR	–	Tonguç and Weeden, 2010
12	Bawan 6 × DDR-11	102	F2	9	SCAR/SSR	–	Sun et al., 2016
13	WSU 28 × G0004389	120	F2:3	20	SCAR/SSR	–	Sun et al., 2019
14	Bawan 6 × G0004400	119	F2:3	20	SCAR/SSR	–	Sun et al., 2019
15	G0001778 × Bawan 6	71	F2:3	5	SSR	–	Sun et al., 2016
16	Qizhen 76 × Xucai 1	91	F2	148	SSR	–	Sun et al., 2015
17	Xucai 1 × Bawan 6	161	F2	148	SSR	–	Sun et al., 2015
Rust (*Uromyces pisi, Uromyces fabae)*							
1	IFPI3260 × IFPI3251	94	F3	146	RAPDs and STSs	1283.3	Barilli et al., 2010
2	HUVP 1 × FC 1	136	RIL	153	SSRs, RAPD, and STSs	634	Rai et al., 2011
3	IFPI3260 × IFPI3251	84	RIL	12,058	DArT, SNP, SSR, and STS	1877.45	Barilli et al., 2018
Ascochyta blight (*Mycosphaerella pinodes*)							
	JI1089 × JI296	–	–	–	–	–	Clulow et al., 1991
1	Erygel × 661	174	F2	62	RFLP, RAPD	550	Dirlewanger et al., 1994
2	A88 × Rovar	133	RIL	96	RFLP, RAPD, and AFLP	1050	Timmerman-Vaughan et al., 2002
3	Carneval × MP1401	88	RILs	239	AFLPs, RAPDs, and STSs	1274	Taran et al., 2003
4	A26 × Rovar and A88 × Rovar	148	F2	99	RAPDs, RFLPs, AFLPs, and STSs	930	Timmerman-Vaughan et al., 2004
5	JI296 × DP	135	RIL	206	RAPD, SSR and STS	1061	Prioul et al., 2004
6	P665 × Messire	111	RIL	303	SSRs	1188.97	Fondevilla et al., 2008b
7	P665 × Messire	111	RIL	248	SSRs	1119.46	Fondevilla et al., 2011; Carrillo et al., 2014a,b
8	Alfetta × P651	51	RIL	10,985	SNPs (GBS)	86.3	Jha et al., 2017
9	Carerra × CDC Striker	134	RIL	3389	SNPs	1008.8	Gali et al., 2018
Fusarium root rot (*Fusarium solani f. sp. pisi)*							
1	Carman × Reward	71	RIL	213	Microsatellite marker (SSRs)	53.1	Feng et al., 2011
2	DSP (W6 17516) × 90–2131 (PI 557501)	111	RIL	10 gene based markers	CAPS and dCAPS	1323	Coyne et al., 2015
3	Baccara × PI 180693	178	RILs	914	SNPs	1073	Coyne et al., 2019
4	JI1794 × Slow	51	RILs	–	–	1289	Timmerman-Vaughan et al., 1996; Hance et al., 2004
5	Afghanistan"(*sym2*) × A1078-239	19		–	–	–	Weeden and Porter, 2007
6	CMG × PI220174	225	RILs	–	–	–	Weeden and Porter, 2007

(Continued)

TABLE 2 | (Continued)

S. No.	Population	Population size	Type of population	Markers	Marker type	Total map distance (cM)	References
Fusarium wilt (*Fusarium oxysporum*. f. sp. *pisi*)							
1	K586 × Torsdag	139	RILs	355	RAPD	1139	Laucou et al., 1998
2	"Lifter"/"Radley" Shawnee"/"Bohatyr	393, 187	RILs	13	CAPS, SSR	–	Jain et al., 2015
3	Shawnee × Bohatyr	187	RILs	272	RAPDs and SSRs	1716	McPhee et al., 2012
4	Green Arrow × PI 179449	80	RILs	72	TRAP	–	Kwon et al., 2013
Common root rot (*Aphanomyces euteiches*)							
1	Puget × 90–2079	127	RILs	324	AFLPs, RAPDs, SSRs, ISSRs, STSs, isozymes	1094	Pilet Nayel et al., 2002; Loridon et al., 2005; Hamon et al., 2013
2	Puget × 90–2079	127	RILs	324	AFLPs and RAPDs	1523	Pilet Nayel et al., 2005
3	Baccara × PI180693, Baccara × 552	356	RILS	224	SSRs, RAPD and RGA	1652	Hamon et al., 2011
	Baccara × PI180693	178	RIL	4620	SNPs	705.2	Hamon et al., 2011, 2013; Duarte et al., 2014; Tayeh et al., 2015a
4	DSP × 90–2131	111	RILs	168	RAPDs, RFLPs and SSRs	1046	Hamon et al., 2013
5	Pea-Aphanomyces collection	175		13,204	SNPs	NA	Desgroux et al., 2016
6	Pea accessions	266		14,157	SNPs	NA	Desgroux et al., 2018
7	MN313 × OSU1026	45		–	–	–	Weeden et al., 2000
Pseudomonas (*Pseudomonas syringae pv. pisi*)							
1	JI15 × JI399	77	RILs	151	RFLPs	1700	Ellis et al., 1992
2	Vinco × Hurst'sGreenshaft, Partridge × EarlyOnward	–	–	–	–	–	Hunter et al., 2001
3	JI281 × JI399	53	RILs	421	RFLPs	2300	Hall et al., 1997
4	P665 × Messire	111	RILs	248	RAPD, STSs, SSR, and EST	1188.58	Fondevilla et al., 2012
Broomrape (*Orobanche crenata*)							
1	P665 × Messire	115	F2	217	RAPD and STS	1770	Valderrama et al., 2004
2	P665 × Messire	111	RILs	246	RAPDs, STSs, ESTs	1214	Fondevilla et al., 2010
Pea weevil (*Bruchus pisorum*)							
1	Pennant × ATC113	270	RILs	155	SSRs	2686	Aryamanesh et al., 2014
2	P665 × Messire	108	RILs	6540	SNPs (DArTseq platform)	2503	Aznar-Fernández et al., 2020
Aphid (*Acyrthosiphon pisum*)							
1	*P. fulvum* IFPI3260 × *P. fulvum* IFPI3251	84	–	12,058	DArT, SNP, SSR and STS	1877.45	Barilli et al., 2020
Pea seed-borne mosaic virus (PSbMV)							
1	88V1.11 × 425	88	F2	–	RFLP, RAPD, allozyme	–	Timmerman et al., 1993

available in pea (Gretenkort and Helsper, 1993; Porter, 2010). Noteworthy, the majority of the colored flower accessions portrayed a good level of resistance to FRR as compared to white colored flower accessions (Grunwald et al., 2003). Also, the polygenic inheritance of this disease has made the development of resistant varieties more complicated (Muehlbauer and Kraft, 1973; Kraft, 1992). FW is another severe production menace scattered around the world caused by *Fusarium oxysporum*. f. sp. *pisi* and causes absolute yield loss under appropriate environmental circumstances (Aslam et al., 2019). The most favorable soil temperature for FW disease development is 23–27°C. In total, 11 different races of fusarium have been discovered considering its virulence (Gupta and Gupta, 2019); of them, races 1 and 2 have become cosmopolitan; on the contrary, races 5 and 6 are prevailing in some areas (Bani et al., 2018). Among these races, race 1 is considered the most devastating and dominating (Kraft and Pfleger, 2001). Being a soil-borne pathogen, it may outlast for a prolonged period below the ground without pea crop (Gupta and Gupta, 2019). McPhee et al. (1999) recognized resistance sources against races 1 and 2 and used them to breed resistant cultivars. Interestingly, one CWR accession (PI 344012) having resistance to races 1 and 2 has been identified. Knowledge of inheritance is vital for incorporating any attribute of interest in the targeted genotype. Therefore,

the inheritance pattern of resistance to *Fop* races 1, 5, and 6 have been studied and confirmed that it is monogenic with dominance in nature, while resistance to race 2 is regulated quantitatively (McPhee et al., 1999, 2012; Rispail and Rubiales, 2014; Bani et al., 2018). The monogenic dominant resistance is successfully introgressed in many pea cultivars (McPhee, 2003). The integration of quantitatively operated resistance in a targeted background is a cumbersome task wherein molecular markers can support significantly to accelerate the introgression process. For such traits, visual selection always remains long-lasting and labor exhaustive. Thus, modern genomic tools and techniques have paved a way for questing, utilizing, and choosing the naturally available sources of resistance against FW in pea (McClendon et al., 2002; Smýkal et al., 2012).

In pea under congruent circumstances particularly under excess moisture in the soil, CRR reduces grain yield significantly by severe damage to the root framework and subsequent wilting of the infected plant (Wu et al., 2018). Unfortunately, the existing old school disease management approaches such as crop rotation and seed treatments are incapable of controlling this disease completely, owing to the prolonged persistence of the pathogen in the form of oospores, which can contaminate crops at any phase. Consequently, resistant cultivar development has been advocated as an ultimate aim in the pea breeding scheme. Few accessions of pea having moderate resistance to CRR have been identified and subsequently used in breeding programs for developing cultivars (Pilet Nayel et al., 2002, 2005; Roux-Duparque et al., 2004; Moussart et al., 2007; Pilet Nayel et al., 2007; Hamon et al., 2011; McGee et al., 2012; Conner et al., 2013; Hamon et al., 2013; Lavaud et al., 2015). However, polygenic inheritance of this disease and its linkage with some objectionable attributes such as lengthy internodes, anthocyanin content, and delayed-flowering made it difficult to breed CRR-tolerant cultivars (Marx et al., 1972; Pilet Nayel et al., 2002).

TOWARD GENOMIC-BASED DISEASE AND INSECT-PEST RESISTANCE BREEDING

Mapping Gene/Quantitative Trait Loci Using Molecular Markers

Traditional gene mapping could not be used widely to map the genes/quantitative trait loci (QTLs) regulating disease resistance because of narrow variability and their polygenic inheritance pattern. Moreover, quantitatively inherited traits are highly influenced by environmental conditions; therefore, the DNA-based markers are widely exploited to map genes/QTLs regulating quantitatively inherited traits in pea. In this crop, DNA-based markers that include STMS (Haghnazari et al., 2005); ISSR (Lázaro and Aguinagalde, 2006), SRAP (Esposito et al., 2007), SNP (Duarte et al., 2014), IRAP (Smýkal et al., 2008a), RBIP (Smýkal et al., 2008b), EST-SSR (Teshome et al., 2015), and SSR (Handerson et al., 2014; Negisho et al., 2017; Mohamed et al., 2019) have been developed and successfully utilized to compute genetic variations. However, similar to other crop species, only SSR makers have become popular owing to their low cost, rapidness, polymorphism, and reliable (Snowdon and Friedt, 2004). More recently, next-generation sequencing has authorized the quick discovery of SNPs and the development of an array for genotyping in pea (Leonforte et al., 2013; Duarte et al., 2014; Sindhu et al., 2014). The initial linkage maps were developed in pea utilizing various molecular markers, which were further used in mapping genes/QTLs controlling biotic stress tolerance. The genes such as *er 1, er2, and Er3* and their alleles conferring resistance to PM have been mapped using different types of markers (**Table 2**). In pea, sequencing of cDNA belonging to *PsMLO1* has identified a new allele *er1-6* of gene *er1* that has been validated by a closely linked specific SSR marker (Sun et al., 2016). In addition to this, alleles, namely, *er1-8* and *er1-9* have been mapped using co-dominant functional markers and validated in pea (Sun et al., 2019). The single dominant gene controlling FW resistance has also been mapped using dominant and co-dominant markers (Jiang, 2013), which were not appropriate for marker-assisted selection (MAS) due to their poor linkage with gene and dominant nature. Thus, Jain et al. (2015) recently designed a co-dominant CAPS marker with 94% accuracy and found that it was helpful in the selection of resistance toward *F. oxysporum* race 1. QTL mapping has been followed for genes regulating partial or intricate inherited resistance and recognized major or minor QTLs for biotic stress tolerance in pea. For example, molecular mapping has identified one major gene (*Ruf*)/QTL (*Up1*, *Qruf)* and one minor QTL (*Qruf1*) for PR resistance (Vijayalakshmi et al., 2005; Barilli et al., 2010; Rai et al., 2011). However, markers associated with these genes/QTLs were not close enough (>5.0-cm distance) for utilization in MAS. Further validation of markers linked with QTL *Qruf* and *Qruf1* did not show complete discrimination between PR susceptible and resistant genotypes limiting their application for marker-assisted breeding (MAB) (Singh et al., 2015). However, high-density molecular maps based on SNP makers and the use of isogenic lines (NILs) and heterogeneous inbred family (HIF) populations have provided opportunities for fine mapping of the genes/QTLs and identified more closely linked makers for precise MAS (Mohan et al., 1997; Tuinstra et al., 1997). The SNP marker-mediated linkage mapping has identified three QTLs (*UpDSII, UpDSIV, and UpDSIV.2*) for PR resistance (Barilli et al., 2018). For AB resistance, various QTL mapping studies have recognized various genomic regions concerned with the regulation of resistance (**Table 3**; Timmerman-Vaughan et al., 2002; Taran et al., 2003; Fondevilla et al., 2008b). Recently, Jha et al. (2015) have identified SNPs within the linked genes, namely, *RGA-G3A* (RGA-G3Ap103) and *PsDof1* (PsDof1p308), which displayed a noteworthy relationship with AB resistance. Correspondingly in another report association of nine QTLs with resistance to AB has been reported in an interspecific population derived by crossing *P. sativum* (Alfetta) and *P. fulvum* (P651), of which, only QTLs *abIII-1* and *abI-IV-2* were found to be stable over the locations/years (Jha et al., 2016), which were further fine mapped in HIF populations (Jha et al., 2017). Furthermore, selective genotyping was done utilizing genotyping-by-sequencing (GBS)

in RILs recognizing eight novel SNP markers within the abI-IV-2 QTL with no extra SNPs in the QTL abIII-1. Similarly, several QTLs explaining phenotypic variation up to 53.4% for polygenic inherited FRR resistance have been recognized using SSR and SNP markers (Coyne et al., 2019). The genome-wide association study (GWAS) refined or validated the previously reported QTLs and identified new loci for resistance to *A. euteiches* (Desgroux et al., 2016), which identified 52 QTLs including six previously identified QTLs for its resistance. However, Desgroux et al. (2018) employed a comparative GWAS approach for resistance to *A. euteiches* in a large set of contrasting pea genotypes (266) using 14,157 SNP markers and identified 11 genomic intervals having significant association with resistance to *A. euteiches* and also confirmed numerous QTLs reported previously. One SNP marker, mapped to the major QTL *Ae-Ps7.6*, was linked with disease resistance and root system architecture, which can be employed in regular pea breeding programs to reduce root rot incidence in pea.

MARKER-ASSISTED SELECTION

A close association of markers with a trait of interest is the prerequisite of MAS, which identifies the target traits without assessing their phenotype in the early generation (Tayeh et al., 2015a). Both biparental and association mapping approaches have been utilized in the identification of closely associated markers with genes controlling disease resistance in pea. Such gene-linked markers control resistance to PM (Lakshmana Reddy et al., 2015), pea enation or seed borne mosaic virus (Swisher Grimm and Porter, 2020), FW (Jiang, 2013; Kwon et al., 2013), PR (Singh et al., 2015; Barilli et al., 2018), AB (Carrillo et al., 2014b; Jha et al., 2015, 2017), FRR (Coyne et al., 2019), and CRR (Lavaud et al., 2015; Desgroux et al., 2016) and are available for MAB. The marker-assisted backcrossing (MABC) has been successfully used for the introgression of QTLs for *Aphanomyces* root rot (ARR) resistance into several recipient genotypes (Hamon et al., 2013; Lavaud et al., 2015). During the recent years, efforts were made to identify markers closely linked with disease resistance genes. However, such markers are not being widely used in the MAB program for developing resistant cultivars due to their poor linkage with target traits. These efforts have proved the utility of MABC and MAS in pea improvement. Accessibility of the reference genome will pave the way toward finding the genes of interest and understanding the genetic background of individuals at the genome level by deploying molecular markers responsive to high-throughput genotyping.

GENOMICS FOR UNDERSTANDING THE COMPLEX GENETICS OF BIOTIC STRESS RESPONSE AND IDENTIFICATION OF CANDIDATE GENES

Resistance in the host plant can occur at different stages during compatibility interaction between pathogen and host. Therefore, many mechanisms, metabolic pathways, and proteins are involved in the host plant and pathogen compatibilities. Thus, many genes have to be expressed to control these metabolic pathways or proteins for completing the infectivity of the pathogen with the host plant. Functional knowledge of these genes can help to understand the genetics involved in host plant resistance, which can further be utilized to develop resistant cultivars against a disease. During the recent years, genomic advances have made it possible to know the candidate genes involved in plant resistance by analyzing transcripts of genes expressed during host–pathogen interaction.

Transcriptomics

Transcriptome analysis has been used to know functional genes responsible for resistance in host plants in many food legumes including pea. In pea, different approaches have been used to recognize the genes responsible for disease and pest resistance (Fondevilla et al., 2011). In the case of white mold [*Sclerotinia sclerotiorum* (Lib.) de Bary], 2,840 host expressed sequence tags (ESTs) (pea) and 996 pathogen ESTs (*S. sclerotiorum*) were identified manifesting exclusively amid the host–pathogen interface, of which about 10% of pea ESTs demonstrated their alliance with genes concerned to its defense against various biotic or abiotic stress, whereas about 9% of *S. sclerotiorum* ESTs exhibited their association with genes reguating pathogenicity or virulence (Zhuang et al., 2012). In another study, microarray analysis investigated gene expression alteration associated with contagion with *D. pinodes* in pea where 346 genes were found to be regulated differentially between resistant and susceptible response, which was responsible mainly for cell wall build-up, phytoalexin and phenylpropanoid metabolism, genes encoding pathogenesis-associated (PR) proteins, and detoxification processes (Fondevilla et al., 2011). The use of deepSuperSAGE identified 17,561 different UniTags, of which about 70% were known sequences from pea or other plants. Among these, 509 UniTags were differentially articulated (Fondevilla et al., 2014). A similar approach was adopted to identify the candidate genes controlling resistance to bacterial blight infection and found a set of about 651 UniTags that expressed differentially between the resistant and susceptible genotypes (Martín-Sanz et al., 2016). In another study, a transcriptome analysis was used to identify the genes and understand the resistance mechanism against *P. pisi* and *A. euteiches* and identified nearly 574 and 817 genes, respectively that were differentially articulated in response to *A. euteiches* contamination at 6 h post-inoculation (hpi) and 20 hpi, respectively, whereas 544 and 611 genes were expressed differentially against *P. pisi* at 6 and 20 hpi, respectively (Hosseini et al., 2015). These genes were associated with phenylpropanoid metabolism, strengthening of the cell wall, and hormonal (jasmonic acid, auxin, and ethylene) signaling (Hosseini et al., 2015). In a comparative transcriptome analysis, contrast responding genotypes to *E. pisi* infection have identified 2,755 transcripts suggesting altered gene expression between the susceptible and resistant genotypes. This study further identified glycolysis as the major pathway of ATP production during pathogen growth and identified genes responsible for putative receptor and regulatory sequences involved in the defense system of resistant genotypes (Bhosle and Makandar, 2021). This

TABLE 3 | Genomic region or markers associated with resistance to different biotic stresses in field pea (*Pisum sativum* L.).

Trait	Marker name and type	Gene/QTLs	Distance (cM)	Linkage group	References
Fusarium root rot (*Fusarium solani* f. sp. *Pisi*)	AA416/SSR, AB60/SSR	*QTL*	NA	VII	Feng et al., 2011
	CAPS/ dCAPS	*Fsp-Ps2.1, Fsp-Ps6.1, Fsp-Ps3.1, Fsp-4.1, Fsp-Ps7.1*	8.9–28.5	IIa, IIIb, VI, VII	Coyne et al., 2015
	Ps900203/SNP, Ps900299/SNP	*Fsp-Ps 2.1, Fsp-Ps3.2, Fsp-Ps3.3*	23.5–49.3	II, III	Coyne et al., 2019
Rust (*Uromyces fabae*)	SC10-82_{360}/RAPD, SCRI- 71_{1000}/RAPD	*Ruf*	10.8–24.5	–	Vijayalakshmi et al., 2005
	AA446/SSR, AA505/SSR, AD146/SSR, AA416/SSR	*Qruf, Qruf1*	7.3–10.8	VII	Rai et al., 2011
	AA121/SSR, AD147/SSR	*Qruf2*	6.0	I	Rai et al., 2016
Rust (*U. pisi*)	OPY11_{1316}/RAPD, OPV17_{1078}/RAPD	*Up1*	6–13.4	III	Barilli et al., 2010
	AD280/SSR, 3567800/ DArT, 3563695/DArT, 3569323/ DArT,	*UpDSII, UpDSIV, UpDSIV.2*	1.5–5.0	II, IV	Barilli et al., 2018
Fusarium wilt (*Fusarium oxysporum*. f. sp. *Pisi)*, race1	p254/RFLP	*Fw*	6.0	IV	Dirlewanger et al., 1994
	ACG :CAT_222/AFLP ACC :CTG_159/AFLP, Y15_1050/RAPD/	*Fw*	1.4–4.6	III	McClendon et al., 2002
	Y15_*999*/SCAR	*Fw*	–	III	Okubara et al., 2005
	AD134_213/SSR, AA5_225/SSR, AA5 _235/SSR, AB111_166/SSR, AD73/SSR, AB30/SSR AD85_178/SSR	*Fw*	2.5–12.3	III	Loridon et al., 2005
	Fw_Trap_480/SCAR, *Fw_Trap_340/SCAR*, *Fw_Trap_220/SCAR*	*Fw*	1.2	III	Kwon et al., 2013
	Aux1.SNP1, Hlhrep_SNP6, Hlhrep_SNP1, Cwi1_SNP3, Cwi1.SNP1, PPT2.SNP1, FVE.SNP6, PM34like.SNP2, ProteasB.SNP2, PFK_SNP1, Subt_SNP2, Sus3_SNP8, Trans_SNP1, TE002G22_SNP3	*Fw*	–	I, II, III, V, VI, VII	Cheng et al., 2015
	THO/CAPS, AnMtL6, Mt5_56, PR X1TRAP13, TC112650/SSR, TC112533/SSR	*Fw*	0.5–3.9	III	Jain et al., 2015
Fusarium wilt, race 2	PSMPSAD171/ SSR	*Fnw*	–	–	McPhee et al., 2004
	AC22_185/SSR, AD171_197/SSR, AB70_203/SSR, AD180_161/SSR, AB85-284	*Fnw 4.1, Fnw 3.1, Fnw 3.2*	–	3, 4	McPhee et al., 2012
Fusarium wilt, Race5	U693a/RAPD, T3_650/RAPD	*Fwf*	5.6–5.8	II	Okubara et al., 2002
	Aatp	*Fwf*	9.1	II	Coyne et al., 2000
Powdery mildew	p236/RFLP	*er-1*	9.8	VI	Dirlewanger et al., 1994
	OPD10_{650}/RAPD	*er-1*	2.1	VI	Timmerman et al., 1994
	ScOPD-10 $_{650}$*/SCAR*	*er-1*	3.7	VI	Rakshit, 1997
	OPL-6_{1900}/RAPD, Sc-OPE-16_{1600}/RAPD	*er-1*	2–4	VI	Tiwari et al., 1998
	Sc-OPO-18_{1200}/RAPD	*er-1*	0.0	VI	Tiwari et al., 1998
	ScOPD-10 $_{650}$*/SCAR*	*er-1*	3.4	VI	Janila and Sharma, 2004
	OPO-02_{1400}/RAPD, OPU-17_{1000}/RAPD	*er-1*	4.5–10.3	VI	Janila and Sharma, 2004
	PSMPSAD60/SSR, *PSMPSAA374/SSR*, *PSMPA5/SSR, PSMAD51/SSR*	*er-1*	10.4–14.9	VI	Ek et al., 2005; Loridon et al., 2005
	$SCW4_{637}$, $SCAB_{1874}$	*Er-3*	2.8	IV	Fondevilla et al., 2008a

(Continued)

TABLE 3 | (Continued)

Trait	Marker name and type	Gene/QTLs	Distance (cM)	Linkage group	References
	OPW04_637/RAPD, OPC04_640/RAPD, OPF14_1103/RAPD, OPAH06_539/RAPD, OPAG05_1240/RAPD, OPAB01_874,	*Er-3*	0.0–6.3	IV	Fondevilla et al., 2008a
	BA9/RAPD, Act2B/RAPD, OD15/RAPD, BC210/RAPD, BC483/RAPD, OB11/RAPD, BC407/RAPD	*er-1*	8.2	VI	Tonguç and Weeden, 2010
	OPX17_1400/ScX17_1400	*er-2*	2.6	III	Katoch et al., 2010
	$OPO06_{1100y}$/SCAR, $OPT06_{480}$/SCAR and AGG/CAA_{125}/SCAR, OPE161600/SCAR and A5420y/SSR	*er-1*	0.5–23.0	VI	Pereira et al., 2010
	OPB18/RAPD	*er-1*	11.2	VI	Nisar and Ghafoor, 2011
	$OPB18_{430}$	*er-1*	11.2	VI	Nisar and Ghafoor, 2011
	GIM-300/SmII/CAPS	*er1-5*	–	VI	Pavan et al., 2011
	$ScOPX04_{880}$/SCAR, $ScOPD\text{-}10_{650}$/*SCAR*	*er-1*	0.6–2.8	VI	Srivastava et al., 2012
	er1-1/AsuHPI-B/CAPS, *er1-4*/AgsI/CAPS, *er1-2*/MGB/STS, *er1-3*/XbaI/dCAPS, *er1-5*/HRM54/HRM	*er1-1, er1-4, er1-2, er1-3, er1-5*	–	VI	Pavan et al., 2013
	c5DNAmet; PSMPSAD60	*er-1*	8.1–15.4	VI	Sun et al., 2015
	AD60/SSR, c5DNAmet	*er-1*	8.1–15.4	VI	Sun et al., 2015
	c5DNAmet; PSMPSAD60	*er-1*	9.0–11.9	VI	Wang et al., 2015
	$ScOPD10\text{-}_{650/SCAR}$, $ScOPE16\text{-}_{1600/SCAR}$, PSMPSAD60/SSR, PSMPSA5/SSR, c5DNAmet,	*er-1*	4.2–26.2	VI	Sun et al., 2016
	InDel111–120	*er-1-7*	4.2	VI	Sun et al., 2016
	SNP1121/SNP	*er1-6*		VI	Sun et al., 2016
	AD60/SSR; c5DNAmet/SSR	*er1-6*	8.8–22.8	VI	Sun et al., 2016
	KASPar-er1-1, KASPar-er1-3, KASPar-er1-4, KASPar-er1-5, KASPar-er1-6, KASPar-er1-7, KASPar-er1-10, KASPar-er1-11	*er-1*	–	VI	Ma et al., 2017
	c5DNAmet, AA200/SSR, PSMPSAD51/SSR, OPX04-880/SSR,	*er-1*	3.5–12.2	VI	Sun et al., 2019
	KASPar-*er1*-8 and KASPar-*er1*-9	*er1*-8, *er1*-9	0.0	VI	Sun et al., 2019
Common root rot (*Aphanomyces euteiches*)	P393 /RFLP	-		IV	Weeden et al., 2000
	E7M4.251/AFLP, N14.950/RAPD, U326.190/RAPD, E3M3.167/AFLP	*Aph 1, Aph 2, Aph 3*	–	IVb	Pilet Nayel et al., 2002
	E7M4.251/AFLP, U370.900/RAPD, U326.190/RAPD, E3M3.167/AFLP	*Aph 1, Aph 2, Aph 3*	0–2.0	IVb	Pilet Nayel et al., 2005
	AF0164458, AA176, A08_2000, X03_1000, E12_1100	Total 135QTLS most stable QTLS (*Ae-Ps1.2, Ae-Ps2.2, Ae-Ps3.1, Ae-Ps4.1 and Ae-7.6*)	–	I, II, III, IV, V, VI, VII	Hamon et al., 2011
	X03_1000, AB70, A19_800, AF016458, AA430942, E8M2_280, IJB174, J14_850, AB122b	*27 Meta QTLs 2* MQTL-Ae25, MQTL-Ae26	–	I, II, III, IV, V, VII	Hamon et al., 2013
	AA446-486, PA8, AB23-376, AA430942, AB145-364, AD57-300, AA175-282, AB112-402, AD83, AC75-297, PD21-226	Ae-Ps7.6, Ae-Ps4.5, Ae-Ps2.2, Ae-Ps3.1, Ae-Ps5.1	–	II, III, IV, V, VII	Lavaud et al., 2015, Lavaud et al., 2016
	AA122, AA387, AB101	*52 QTLs* *Major QTLs* (*Ae-Ps4.4-4.5, Ae-Ps7.6*)	–	IV, VII	Desgroux et al., 2016
	Ps115429/SNP	*Ae-Ps7.6*	–	VII	Desgroux et al., 2018

(Continued)

TABLE 3 | (Continued)

Trait	Marker name and type	Gene/QTLs	Distance (cM)	Linkage group	References
Ascochyta Blight (*Peyronellaea pinodes)*	p227/RFLP, p105/RFLP, p236/ RFLP	QTL	–	IV, II	Dirlewanger et al., 1994
	c206/RFLP, M02-835/RAPD, sM2P5-234/SCAR M27/SCAR, J12-1400/RAPD, C12-680/RAPD, W17-150/RAPD, P346/RFLP, sY16-112/SCAR1 M2P2-193/AFLP sB17-509/SCAR, S15-1330/RAPD	*Asc1.1, Asc2.1, Asc3.1, Asc3.2, Asc4.2, Asc4.3, Asc5.1, Asc7.1, Asc7.2, Asc7.3*	–	I, II, III, IV, V, VII	Timmerman-Vaughan et al., 2002, 2004, 2016
	AFLP/RAPD/STS	*ccta2*,*cccc1*, *acct1*	–	II, IV, VI	Taran et al., 2003
	V03-1200/RAPD, PSm PSAA175/SRR, PSMPSAA 163.2/SSR, PSMPSAA399/SSR, G04-950/RAPD, E08-980/RAPD	*mpIII-1, mpIII-3, mpVa-1, mpVII-1, mpVI-1*	–	III, V, VI, VII	Prioul et al., 2004
	DRR230-b, PsDof1	*mpIII-1, mpIII-4*	–	III	Prioul-Gervais et al., 2007
	OPM6598/OPW5387, OPAI141353/OPW21157, OPAI141273/OPAI141353, OPRS4782, OPK6818, OPB111477	*MpIII.1, MpIII.2, MpV.1, MpII.1, MpIII.3, MpIV.1*	–	II, III, IV, V	Fondevilla et al., 2008b
	OPAI14_1353/AA175, OPAI14_1273/OPAI14_1353	Total 14 QTLS, and Major QTLs (*MpIII.3_DRl_06, MpIII.3_DS_06, MpIII.3_DRst_06*)	–	III	Fondevilla et al., 2011
	PsDof1p308/SNP, RGA-G3Ap103/SNP	-	–	III, VII	Jha et al., 2015
	PsC8780p118, PsC22609p103, PsC8031p219, PsC20818p367, PsC7497p542, PsC13000p248, PsC4701p407	*abI-IV-1, abI-IV-2, abI-IV-3, abI-IV-4, abIII-1,abVII-1, abI-IV-5, abIII-2, abVII-2*	–	I-IV, III, VII	Jha et al., 2016
	Sc33287_25420/SNP, Sc34405_60551/SNP, Sc33468_44352/SNP, Sc12023_67096/SNP	*abIII-1, abI-IV-2,* abI-IV-2.1, abI-IV-2.2	–	I-IV, III, VII	Jha et al., 2017
	PsC1846p336 - Sc5317_256613/SNP, Sc3030_71736 - PsC7000p195/SNP, Sc8865_149928 - Sc7388_112888/SNP	QTLs	–	IIIb	Gali et al., 2018
	sC8780p118/SNP	*QTL abIII-1*		III	Jha et al., 2019
Ascochyta Blight *(Didymella pinodes)*	OPM4_490/OPK6_887, agpl1_SNP2/MSU515_SNP3, OPZ10_576/Sugtrans_SNP3, sut1_SNP1/OPRS4_699	*MpII.1, MpIII.5, MpV.3, MpV.2*	–	II, III, V	Carrillo et al., 2014b
Pea common Mosaic virus	*p252*	*mo*	15.9	II	Dirlewanger et al., 1994
Pea seed-borne mosaic virus (PSbMV)	GS185/RFLP	*sbm-1*	8.0	II	Timmerman et al., 1993
	G05_2537/RAPD, L01_910/RAPD, P446/RFLP, sG05_2537/STS	*sbm-1*	4.0	II	Frew et al., 2002
Pea enation mosaic virus (PEMV)	CNGC, tRNAMet2	*En*	1.3–2.5	III	Jain et al., 2013
White mold **(***Sclerotinia sclerotiorum*)	Chr5LG3_562563492, Chr5LG3_568430003, Chr5LG3_568430003, Chr5LG3_569648908	13 QTLS	–	III	Mahini et al., 2020
Pea weevil (*Bruchus pisorum*)	3546831/DArT, 3551908/DArT, 3548194/DArT, 3552459/DArT, 3549249/DArT, 3549680/DArT,	*BpSl.I*, *BpSl.II* and *BpSl.III, BpLD.I*	–	I, II, IV	Aznar-Fernández et al., 2020
Pea Aphid (*Acyrthosiphon pisum)*	3568590/ DArT,3569349/ DArT, 3535012/ DArT,3536533/ DArT, 3535795/ DArT, 3537104/ DArT, 3568629/ DArT, 3536355/ DArT	*ApI, ApII, ApIII, ApIV.1, ApIV.2, ApV*	–	I, II, III, IV and V	Barilli et al., 2020
Pseudomonas syringae pv. Syringae	OPW5387/RAPD, OPJ121504/OPO61121	*Psy1 and Psy2*	–	III, VI	Fondevilla et al., 2012
Broomrape (*Orobanche crenata*)	STS P48	*Ocp1*	–		Valderrama et al., 2004
	OPM4_978, OPAE5_538, OPP4_479/OPE11_660, OPAA19_702	*n°br03_1, n°br03_2, n°br03_3, n°br04*	–	I, III, V and VI	Fondevilla et al., 2010

information of disease resistant candidate genes can further be utilized for the development of functional markers for MAB.

Proteomics

Disease and pest infestation trigger changes in the protein profile of the host plant. Knowledge of such protein profiles responsible for compatible interaction between host and pathogen can help in better understanding the host plant resistance mechanism at the molecular level. In addition to this, the abundance of specific proteins can be used as the markers for differentiating resistant and susceptible genotypes, which can be utilized in resistance breeding. Therefore, during the recent years, efforts have been made on proteomic analysis for diseases and pests in pea. Resistance to AB is a complex trait, and infection of this disease alters proteins and their abundance. First protein markers linked to AB resistance have been depicted utilizing resistant and susceptible genotypes. Subsequently, quantitative estimation of these proteins was done in a mapping population for the detection of putative protein markers linked with AB resistance and explored its possible use in breeding (Castillejo et al., 2020). This study eventually developed a group of potential protein markers for resistance to AB and advocated a molecular mechanism against AB resistance in pea. Previously, the proteomic approach identified changes in host proteins during infection of downy mildew in a susceptible cultivar of pea (Amey et al., 2008), of which the levels of eight proteins [PI176 (protein accession number P13239), ABR17 (protein accession number Q06931), glycine-rich RNA-binding protein (protein accession number P49311), cytosolic GAPDH (protein accession number P34922), chloroplastic GAPDH (protein accession number P12858), photosystem I reaction center subunit II (protein accession number Q9S7H1), ATP synthase epsilon chain (protein accession number P05039), and photosystem I iron sulfur center (protein accession number P10793)] increased significantly in the infected leaves of the susceptible plant. Identification of these proteins provided the base for the advancement to reveal molecular defense mechanisms to *P. viciae* infection (Amey et al., 2008). In another study, proteomic analysis of PM susceptible and resistant genotypes resulted in the identification of proteins concerned with photosynthetic activity and carbon metabolism, signal transduction functions, protein synthesis, and protein degradation, which aids in understanding the mechanisms of *E. pisi* resistance in pea (Curto et al., 2006). Similarly, in a recent study, proteomic analysis was done for PM isolates infecting susceptible pea cultivar and identified proteins involved in virulence and pathogenesis through signal transduction, secondary metabolite formation, and stress functions (Bheri et al., 2019). For understanding the resistance mechanism to *Acyrthosiphon pisum* (pea aphid), a serious pest of pea, proteomic analysis between contrasting genotypes identified the proteins mostly corresponding to amino acid metabolism, carbohydrate metabolism, folding or degradation, stress response, photosynthesis, signal transduction, and transcription or translation suggesting the role of different metabolic pathways in controlling resistance to this pest (Carrillo et al., 2014a). Thus, proteomic analysis has provided better insight into the molecular mechanism underlying disease and pest resistance in pea, and hence, it is further required to enhance the understanding of the molecular mechanism of quantitatively inherited diseases and pests resistance in pea.

FUTURE BREEDING STRATEGIES FOR DEVELOPING CULTIVARS RESISTANT TO BIOTIC STRESSES

Development of Functional Markers

Poor association of molecular markers with genes/QTLs controlling disease resistance has led to their limited use for MAS in pea breeding programs. Therefore, the development of the functional markers within targeted genes/QTLs controlling the disease resistance is important for this purpose. Earlier, few efforts have been made to develop functional markers for the *er1* gene controlling PM in pea (Sun et al., 2016, 2019). A functional co-dominant CAPS marker with 94% accuracy was found useful for the selection of resistance genes responsible for *F. oxysporum* race 1 (Jain et al., 2015). Furthermore, next-generation sequencing also assisted in developing functional SNP markers from genes/QTLs governing resistance to different diseases in pea. For example, SNP markers within two candidate genes (*PsDof1* and *RGA-G3A*) were identified for AB resistance (Jha et al., 2015). Association mapping with a large number of SNP markers developed through next-generation sequencing identified SNP marker, associated with a major QTL *Ae-Ps7.6* responsible for reducing ARR severity and root system architecture (RSA). Therefore, the identified genes for RSA could be utilized in improving ARR incidence in pea. Furthermore, the availability of a reference genome sequence of pea along with a high-throughput next-generation genotyping platform provides the opportunity to identify the candidate genes for targeted traits and development of functional markers linked with disease resistance genes for marker-assisted breeding in pea.

Toward Genomic Selection in Pea

For obtaining maximum genetic gain with more accuracy, genomic selection (GS) using molecular markers is a promising approach. This can help to improve biotic stress resistance, which is a primary breeding objective of the pea genetic improvement program. This approach is more useful for improving quantitatively inherited disease resistance in pea. It uses genome-wide molecular markers associated with resistance genes for predicting and selecting high breeding value lines. In a recent review, different models used in GS were discussed in detail; particularly, the use of multivariate GS models (MTGS) over single trait GS (STGS) was presented (Budhlakoti et al., 2019). Multi-trait GS (MTGS) methods may provide more accurate genomic-estimated breeding values (GEBVs). Several MTGS methods were used for GS, e.g., the multivariate mixed model approach (Jia and Jannink, 2012; Klápště et al., 2020), Bayesian multi-trait model (Jia and Jannink, 2012; Cheng et al., 2018), multivariate regression with covariance estimation (MRCE) (Rothman et al., 2010), and conditional Gaussian graphical model (cGGM) (Chiquet et al., 2017).

Jia and Jannink (2012) presented three multivariate linear models (i.e., GBLUP, Bayes A, and Bayes Cπ) and compared them with univariate models. Most of the successful events of the utilization of GS in biotic stress resistance were in cereal crops. In wheat, GS was used for three types of rust, Fusarium head blight, septoria tritici blotch, PMD, tan spot, and *Stagonospora nodorum* blotch (Budhlakoti et al., 2022). The genomic prediction accuracies for these diseases ranged from 0.14 to 0.85 (Daetwyler et al., 2010; Rutkoski et al., 2012; Mirdita et al., 2015; Juliana et al., 2019; Sarinelli et al., 2019). Similarly, in the case of rice, GS has been used in blast disease tolerance (Huang et al., 2019). In maize, GS has been used against *Stenocarpella maydis* causing ear rot (Dos Santos et al., 2016) and heavy infestation of Striga (Badu-Apraku et al., 2019). In the case of barley, for Fusarium head blight, the prediction accuracy was 0.72 (Lorenz et al., 2012; Sallam and Smith, 2016). Though limited reports of the use of genomic selection to improve biotic stresses in pea are available, efforts have been made to know the impact of the marker density, statistical method, and/or the training population size for evaluating genomic prediction accuracy using the number of seeds per plant, thousand seed weight, and flowering time. Such information provides opportunities for developing GS strategies (Tayeh et al., 2015b), which is important for biotic stress tolerance in pea.

Mining Allelic Variants for Resistance Genes

Breeding for improving a trait requires ample availability of diversity in germplasm for the targeted traits. In pea, a large collection of genetic resources is available, which are a reservoir of undiscovered allelic variants for many traits (Tanksley and McCouch, 1997; Smýkal et al., 2012). This large collection may have new resistant allele(s) of the gene(s) controlling disease incidence in pea. For mining such alleles from germplasm, there is a need to test the entire germplasm for their response following a specific screening protocol, which is not only time-consuming but also expensive. However, current genomic tools have provided an opportunity to uncover the allelic variation, especially for those monogenic traits for which candidate genes are already known (Robaglia and Caranta, 2006; Hofinger et al., 2011; Reeves et al., 2012). The use of such genomic tools increases the identification of allelic variants for resistance genes by screening the wild and cultivated germplasm in several crops (Bhullar et al., 2009). In pea, eukaryotic translation initiation factor 4E provides resistance against many potyviruses. Therefore, gene *eIF4E* encoding this factor has been used for the identification of allelic diversity among 2,803 pea accessions, which resulted in the identification of four *eIF4EA-B-C-S* variants, whose distribution was geographically linked, suggesting its independent evolution (Konečná et al., 2014). This study has opened an avenue of research for the identification of new allelic variants for complex diseases of a pea.

Toward Epigenetic Breeding

Transgenerational epigenetic variation, which transfers steadily to the next generation, becomes one of the important strategies for breeding climate-resilient cultivars in crop plants. These variations cause alteration in gene expression through DNA methylation or histone modification (Kumar et al., 2019). Identification or genome-wide mapping of epigenetic markers can help the breeder to manipulate epigenomic variability toward the development of climate resilient crop varieties. This epigenetic variation was detected in host plant resistance against a broad array of plant pathogens such as fungi, bacteria, viruses, nematodes, oomycetes, and herbivorous insects (Espinas et al., 2016; Ramirez-Prado et al., 2018; Alonso et al., 2019). For example, in soybean, methylome has been identified for compatible interaction of roots with cyst nematodes (Rambani et al., 2015). In pea, differences have been detected for methylations among plants, which were propagated through *in vitro* culture for a long time (Smýkal et al., 2007). Artificially induced and naturally occurring epigenetic variations controlling plant disease resistance were identified, and similar efforts are required to identify epigenetic variation responsible for polygenetically inherited disease resistance in pea. In pea, no potential genetic sources for resistance are available so far for many serious diseases, and hence, new epigenetic alleles can be generated using promising approaches such as induced gene-specific DNA methylation and epigenome editing (Zhi and Chang, 2021). Thus, epigenetic breeding has a great potential for improving disease resistance in pea.

Genome Editing

In pea, insect pests and diseases are the major yield-limiting factors and hence pose a substantial threat to food security globally. In recent years, genome editing or modification has revolutionized the functional analyses of genes and the introduction of new alleles for the trait of interest into commercial crop plants (Mushtaq et al., 2019). Different approaches of genome editing have been developed for this purpose; however, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (CRISPR-Cas9), meganucleases, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZNFs) are being used extensively for genetic improvement (Mushtaq et al., 2019). In crop plants, susceptibility (*S*) or resistance (*R*) genes have been considered eventual targets intended for escalating crop protection (Singh et al., 2016; Ren et al., 2017). These genes were identified as the best candidate for gene editing for conferring disease or pest resistance in a crop (Das et al., 2019b). In addition to this, editing of most conserved regions of multiple viral genomes using multiplex CRISPR/Cas9 system also helped in conferring disease resistance in various crops by interfering with their duplication and progress (Iqbal et al., 2016). In pea, the transcriptomic analysis provides elucidation of the genes and pathways concerned with disease or pest resistance. Moreover, the study of expression alteration, modification, and interaction of protein during the plant-pathogen interface provided knowledge of key proteins involved in pathogenesis. This information is a useful repository for editing or modification of the genome of a crop or realtered pathogen toward the development of resistant cultivars (Barakate and Stephens, 2016). In addition to this, genome editing can be used to alter epi-alleles

or to generate new epi-alleles involved in disease resistance (Latutrie et al., 2019).

Transgenic Technology

In pea, limited resistance sources are available among cross-compatible germplasm for several devastating diseases and insect pests such as FRR, CRR, PR, alfalfa mosaic virus, and bruchids. Therefore, transferring resistance genes from other non-cross-compatible species is one of the ways to develop resistant cultivars, possibly by developing transgenic plants. However, genetic transformation in pea is not easy when compared to other legume crops due to difficulties in transformation and plant regeneration (Svabova et al., 2005; Warkentin et al., 2015). Although, during the recent years, advances in biotechnology have made possible the development of transgenics in pea for diseases and insect pests. For example, transgenic lines with two chimeric genes encoding the coat protein (CP) of alfalfa mosaic virus (AMV) strain NZ1 have been developed and tested under green house and field conditions for improved AMV resistance in pea. However, results showed partial virus resistance of transgenic lines having genetically modified AMV CP sequences (Timmerman-Vaughan et al., 2001). In another study, two antifungal genes (chitinase and glucanase) for resistance to fungal diseases have been transferred using genetic transformation, and transgenic pea has been developed by stacking these genes (Amian et al., 2011). Weevils are the most devastating insect of food legumes including pea. Genetic resistance to this insect is not available currently in cross-compatible germplasm. However, a gene for alpha-amylase inhibitor-1 (αAI) has been identified in the common bean that completely protects from weevil destruction. This has been transferred through a genetic transformation in pea, and developed transgenic lines showed resistance to this pest. Moreover, αAI transgenic peas are found to be less allergenic than beans or non-transgenic peas in mice (Reiner et al., 2013).

In a more recent study, four antifungal genes, *1-3* β *glucanase* (G), *endochitinase* (C) (belonging to the PR proteins family), *polygalacturonase inhibiting proteins* (PGIPs) (P), and *stilbene synthase* (V), have been transformed for disease tolerance in European pea cultivars. This resulted in the development of transgenic lines having an individual antifungal gene or all four genes that were stacked through hybridization. However, the resistance of these transgenic lines against FRR was not consistent over the years in confined field trials probably due to lower relative gene expression in the roots (Kahlon et al., 2018). Although, these studies showed the possibility of developing transgenic pea against major diseases and insect pests. Thus, transgenic technologies have great promise but the economic

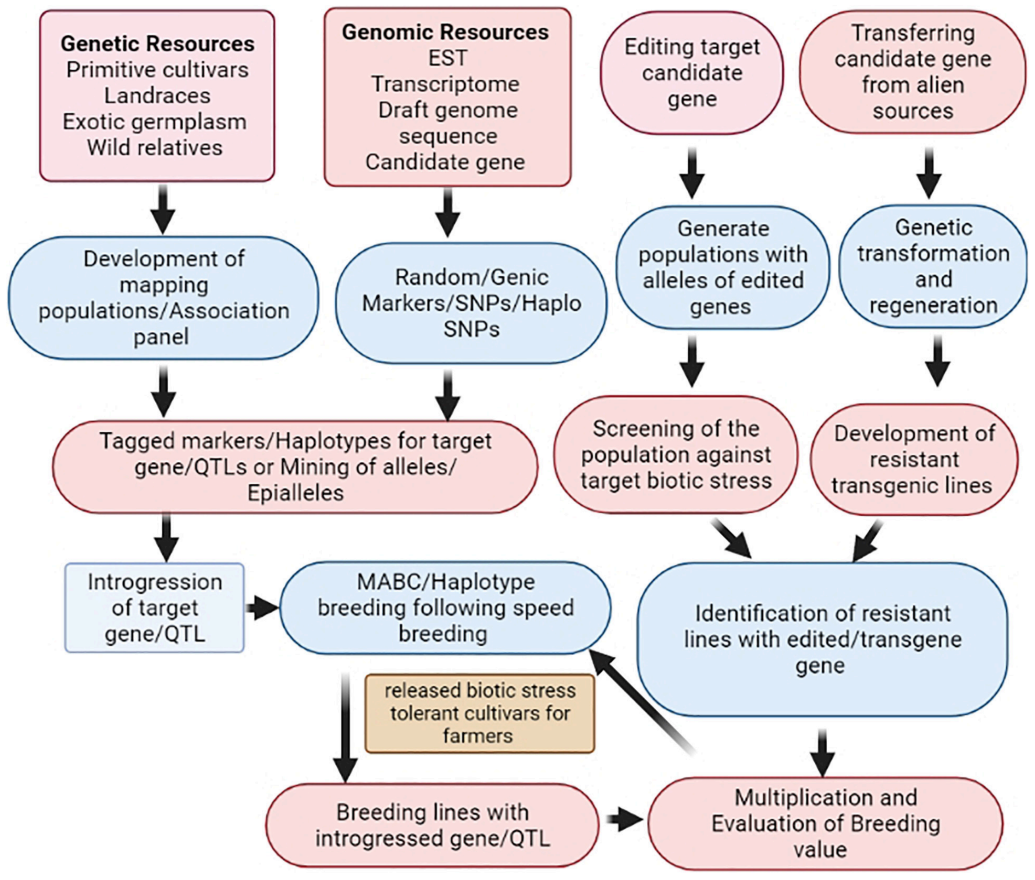

FIGURE 2 | Genomic-assisted breeding strategies for biotic stress tolerance.

benefits of genetically modified (GM) pea will need to surpass the regulatory costs, time, and labor involved in bringing a GM crop to market. In addition to this, more research experiments are required on issues associated with genetically modified crops, such as discrete changes in the molecular architecture, cellular function, and antigenicity of the expressed protein translated from the transferred gene in the transgenic plants. In pea, transgenic expression of a plant protein (alpha-amylase inhibitor-1) from the common bean, which is a non-native host of pea, led to the synthesis of a structurally modified form of this inhibitor. The effect of this modified protein has been studied in mice and found that non-native proteins in transgenic plants may lead to structural modification with altered immunogenicity (Prescott et al., 2005).

Speed Breeding

Environmental conditions play an instrumental role in making crop plants susceptible to biotic stresses. The changing environmental condition due to global warming provides opportunities for evolving new races and pathogens, which has significantly raised concern for meeting global food security. Therefore, there is an urgent need of developing resistant cultivars within a short period of time. However, present breeding approaches take several years to develop the resistant cultivars, and hence, the current improvement rate is inadequate to meet the future food demands. Elongated generation advancement time of crops is one of the key reasons for delay in the development of improved resistant cultivars against biotic stresses. Therefore, in recent years, speed breeding has emerged as a powerful tool for accelerating crop research and breeding as several workers have developed speed breeding protocols in pea for shortening the breeding time (Ghosh et al., 2018; Watson et al., 2018; Cazzola et al., 2020). These speed breeding techniques along with new biotechnological tools available in pea can accelerate the development of resistant cultivars against new emerging pathogens or races due to climate changes in the following way:

- Taking 4–5 breeding generations in a year could substantially reduce the time span to release a variety.
- Development of RIL mapping populations within a short period of time using speed breeding can help in the rapid identification of QTLs for disease resistance and their use in the breeding program for developing improved resistant cultivars.
- The MABC for introgression of QTLs/genes controlling disease resistance can be faster through speed breeding leading to the rapid development of improved and resistant cultivars.
- The amalgamation of speed breeding with other modern breeding and biotechnological techniques such as genome editing, genomic selection, and high-throughput genotyping has great potential for accelerating the genetic gain toward the development of biotic stress-tolerant cultivars.

CONCLUSION AND PERSPECTIVES

Pea is an important and exceptionally high-yielding cool season pulse crop in the world. Numerous biotic stresses are the key constraints in harnessing the full production potential of a pea, of which fungal diseases such as PM, FW, FRR, AB, CRR, and PR causing infection during different growth stages are devastating to the crop. Nevertheless, sincere efforts have been made to elevate the productivity and production of pea, but many more milestones are yet to be achieved for making it a resilient crop to upcoming challenges. Several major and minor genes/QTLs governing important biotic stresses in pea have been dissected and mapped using existing genomic tools, nevertheless, not utilized to a large extent in regular pea breeding programs. The reliable DNA markers flanking the genes/QTLs of interest could accelerate the introgression of resistance from the resistance sources using the genomic-assisted protocol to speed up the pea breeding program accomplishments more efficiently and precisely. Updated research efforts are warranted for the amalgamation of next-generation genomics and phenomics in pea improvement programs. The schematic diagram explains how different genomic approaches can be combined to accelerate the success of a pea breeding program (**Figure 2**). This figure also explains the combined use of genetic resources, genomic resources, and advanced biotechnological tools in the pea improvement program for the development of biotic stress-resistant cultivars. Underlying resistance mechanisms for AB, PM, and pea aphids have been elucidated using different pathogenic resistance proteins pertinent to the genes and pathways involved in pathogen resistance. However, more concentrated efforts are needed in the future on proteomic and transcriptomic analyses to untangle the disease and pest resistance mechanism in pea at the molecular level and to validate the sequencing results at the functional level for the identification of candidate genes controlling biotic stress resistance. This information will be certainly useful for editing or modification of crop genomes or realtered pathogens to develop resistant cultivars. Genome-wide association and genomic selection, which elucidate specific genetic variations at the genome scale, should be judiciously used for the identification of several gene(s)/QTLs exerting smaller effects on the biotic stress resistance. The transgenic technology should be exploited to let researchers utilize the variability existing outside the crop's primary/secondary gene pool and also offer an opportunity to conquer crossability constraints. In addition, induced gene-specific DNA methylation and epigenome editing can be exploited to generate new epigenetic alleles for different biotic stresses. Most recently, speed breeding or rapid generation advancement protocols developed for shortening breeding times (4–5 cycles/year) have emerged as a potent technology for accelerating genetic gain in pea. Though, several tools and technologies are in hand judicious use to reap the best of them is challenging, certainly, there is a huge scope to achieve new heights in productivity enhancement by breeding biotic stress-resistant pea cultivars.

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

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Cinnamic Acid Toxicity on the Structural Resistance and Photosynthetic Physiology of Faba Bean Promoted the Occurrence of Fusarium Wilt of Faba Bean, Which Was Alleviated Through Wheat and Faba Bean Intercropping

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Background: The pattern of intercropping wheat and faba bean is an effective means to alleviate continuous cropping obstacles.

Aim: To study the mechanism by which cinnamic acid promotes faba bean wilt and the mechanism by which intercropping alleviates this effect.

Methods: Hydroponics was used to study the effects of inoculation with or without *Fusarium oxysporum* f. sp. *fabae* (FOF) and the effect of addition of different concentrations of cinnamic acid on seedling growth, Fusarium wilt, stem cell wall degrading enzyme activity, lignin content, tissue structure of the stem and leaf photosynthesis in monocropping and intercropping systems following the inoculation of faba bean with FOF.

Results: Treatment with FOF significantly reduced the biomass and leaf photosynthesis of faba bean compared with the control. Microscopic observation showed that the xylem vessels of the stem were slightly thickened. Compared with FOF alone, the combination of FOF and cinnamic acid stress significantly increased the activity of cell wall degrading enzymes (CWDEs) produced by FOF in the stem and content of lignin in the stem. Microstructural observation showed that cell wall thickening of the xylem conduit, stratification, formation of a cavity and even caused the dispersion of tissue cell structure in the stem tissue of faba bean. Furthermore, the biomass and leaf photosynthesis of faba bean decreased significantly, and the occurrence of faba bean wilt increased. Compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatment significantly reduced the activity of CWDEs of FOF produced in faba bean stems and increased the lignin content. In addition, observation of the microstructure indicated that the tissue structural cell wall thickened after the stem had decreased, and the amount of colloidal substances and their containment decreased, causing a further decrease in tissue deformation, smaller

intercellular spaces, less divided layer cell damage, an increase in the aboveground biomass and leaf photosynthesis of faba bean and a decrease in the occurrence of faba bean wilt.

Conclusion: Cinnamic acid decreased the resistance of tissue structure and promoted the occurrence of wilt. Wheat and faba bean intercropping improved the resistance of tissue structure, which reduced the occurrence of wilt.

Keywords: cinnamic acid, faba bean, fusarium wilt, toxic action, defense capability, photosynthetic characteristics, intercropping

INTRODUCTION

As theworld's population continues to grow, the demand for food and cash crops also increases. Owing to the limitations in arable land, the area of arable land being added is reduced, and the continuous planting of the same crops on the same land has become the most common mode in intensive and large-scale agriculture and horticultural production (Alexandratos and Bruinsma, 2012; Zeng et al., 2020). However, continuous planting for many years can lead to continuous cropping disorders. Continuous cropping obstacles can cause weak plant growth, a reduction in yield, poor quality and an increase in soilborne diseases (Li et al., 2016; Gao et al., 2019; Zeng et al., 2020). Among them, the frequent occurrence of soilborne diseases has always been an intractable problem in actual production (Young, 1984; Grodzinsky, 1992). The root cause of soilborne diseases is that the number of soilborne pathogens exceeds a critical value (Elmstrom and Hopkins, 1981; Caperton et al., 1986). Most soilborne diseases are common in soybean (*Glycine max*) (Dhingra and Muchovej, 1979; Haddoudi et al., 2020), potato (*Solanum tuberosum*) (Rai, 1979), cowpea (*Vigna unguiculata*) (Aigbe et al., 1999), tomato (*Solanum lycopersicum*) (Nedumaran and Vidhyasekaran, 1981) and other field crops and cash crops. Soilborne pathogenic fungi can survive for several years or even decades in the soil in the absence of a host (Buruchara and Camacho, 2000; De Borbat et al., 2017). The number of soilborne pathogens is regulated by allelopathy (Wu et al., 2008a).

Allelopathy is the inhibition or promotion of chemicals, which are released into the environment by one plant to affect another (Rice, 1984). Autotoxicity is a special form of allelopathy in which plants produce toxic substances primarily through root secretion or the decomposition of residual roots, thereby inhibiting their own growth (Singh et al., 1999). Li et al. (2018) found that autotoxic substances were released into the rhizosphere soil of ginseng (*Panax ginseng*) and accumulated to some concentration, thus, inhibiting its growth. In recent years, increasing amounts of attention have been paid to allelopathic autotoxicity, which plays a key role in the occurrence of soilborne diseases (Huang et al., 2013; Li et al., 2014a; Zhang et al., 2020). Studies have shown that cinnamic acid secreted by asparagus (*Asparagus officinalis*) is considered as the primary toxin of asparagus roots, which can stimulate *Fusarium* spp. to infect asparagus and promote soilborne diseases (Peirce and Miller, 1993). The accumulation of autotoxic substances in flower rhizospheres aggravates the occurrence of soilborne peanut diseases (Li et al., 2013). Wu et al. (2008b) found that the activities of pectinase, cellulase, amylase and protease produced by *F. oxysporum* increased significantly after cinnamic acid was added to the culture medium of this fungus. The CWDEs can degrade the host tissue structure (Klechkovskaya et al., 1998; Pekkarinen et al., 2000; Yi and Wu, 2000; Aparna et al., 2009). The results showed that the autotoxic substances promoted the production of CWDEs by pathogens, which is an important way to promote the occurrence of soilborne diseases. It has also been suggested that allelopathic autotoxic substances promote disease occurrence by reducing the resistance of crops to pathogens (Nighjr, 1990). Under the stress of *p*-hydroxybenzoic acid, the degree of damage to strawberry root tissue structures was aggravated, which significantly increased the infection rate of *F. oxysporum* and promoted the occurrence of wilt (Qi et al., 2015). It has been reported that under cinnamic acid stress, the stomatal conductance and net photosynthetic rate of cucumber (*Cucumis sativa*) leaves decreased, which inhibited the photosynthesis of the leaves and further promoted the occurrence of cucumber Fusarium wilt (Ye et al., 2004). The results showed that the autotoxic compounds reduced the resistance of plants to pathogens by reducing the resistance of their tissues, cells, and photosynthesis, rendering the plants more susceptible to infection and increasing the incidence of diseases.

Currently, the prevention and control of soilborne diseases in agricultural production generally comprises physical prevention and control, chemical prevention and control, and other methods. Steam high-temperature disinfection of soil is a simple and effective method to prevent soilborne diseases (Katan, 1980). However, the disinfection of soil with steam easily causes secondary colonization and the mass enrichment of pathogenic microorganisms, resulting in negative effects on the subsequent growth of crops (Fenoglio et al., 2006). Chemical prevention and control primarily use various chemical agents to control soil pathogens. Mao et al. (2012) fumigated a cucumber nursery with 98% methyl isothiocyanate, which greatly reduced the number of *F. oxysporum* propagules in soil. However, these chemical methods not only eliminate pathogens in the soil but also kill beneficial microorganisms, aggravate environmental pollution and disrupt the soil microecological balance. All these control methods have their limitations. Therefore, the development of effective and environmentally friendly soilborne disease management strategies has been a key research focus (Chen et al., 2019). Breeding resistant varieties is considered the most direct and effective measure to combat wilt. Studies have shown that

the root exudates of resistant peanut (*Arachis hypogaea*) species ("quanhua-7") significantly reduced the number of spores and amount of spore germination compared with non-resistant peanut species ("guanhua-5") (Li et al., 2013). This could be an important route for disease-resistant varieties to inhibit the occurrence of wilt. Simultaneously, reasonable intercropping is a method of planting two or more crops together. In practical production, it is a green and efficient planting method and is often used to control soilborne diseases (Li et al., 2014b; Ren et al., 2016). Studies have shown that compared with pea and corn monocropping, intercropping of corn and pea increased the yield of pea and promoted the growth of corn (Hu et al., 2016). It has been reported that maize (*Zea mays*) and potato intercropping can effectively control the occurrence of potato Fusarium wilt (Autrique and Potts, 1987). Intercropping of *Atractylodes lancea w*ith peanut can reduce the incidence of root rot of continuously cropped peanut (Li et al., 2014b). The results showed that intercropping could effectively promote crop growth and inhibit the occurrence of Fusarium wilt. Currently, the mechanism of intercropping to alleviate soilborne diseases primarily focuses on the effects of intercropping on pathogen growth, rhizosphere microflora and community structure. It has been reported that the incidence of watermelon *F. oxysporum* in paddy and watermelon intercropping in dry farming decreased by decreasing the number of watermelon *F. oxysporum* and rhizosphere fungi and increasing the number of soil bacteria in the root zone (Ren et al., 2008). Intercropping with onion (*Allium cepa*), garlic (*A. sativum*) and cucumber changed the microbial community structure of cucumber soil and reduced the incidence of cucumber Fusarium wilt (Xiao et al., 2012). The results showed that intercropping could reduce the incidence of soilborne diseases by reducing the number of pathogens and improving the rhizosphere microflora and community structure. However, there have been few studies on how intercropping regulates the effects of pathogenic factors, such as CWDEs, tissue structure resistance and photosynthetic physiology in plants.

As one of the oldest crops in the world, faba bean (*Vicia faba* L.) is widely cultivated all over the world, providing a large amount of protein for humans and animals, and is valuable for medicine and health care (El Idrissi et al., 2020). However, continuous cultivation of faba beans frequently results in soilborne wilt (Stoddard et al., 2010). In Yunnan Province of southwest China, wheat is often grown with faba beans to control the bean wilt. Few studies have explored the mechanism of the occurrence of faba bean wilt owing to the synergistic effect of *F. oxysporum* and autotoxic substances and mitigation of this disease owing to the effect of intercropping systems. Our previous study showed that cinnamic acid is one of the primary autotoxic substances of faba bean, and its pathogenic mechanism has been studied from the ability of cinnamic acid to help faba bean become resistant to *Fusarium oxysporum* f. sp. *fabae* (FOF) and produce defense enzymes (Guo et al., 2020). However, the synergistic effects of FOF and cinnamic acid on pathogenicity and the resistance of faba bean tissue structure, as well as the alleviating mechanism of the wheat and faba bean intercropping system, are still unclear. Therefore, this study utilized a hydroponics experiment to determine the following: (1) the synergistic effect of FOF and cinnamic acid on the occurrence of faba bean wilt and the ability of wheat intercropping to mitigate the infection; and (2) the synergistic effect of FOF and cinnamic acid on promoting the occurrence of faba bean wilt and the potential mechanism of wheat and faba bean intercropping to effectively control the occurrence of faba bean wilt by reducing its pathogenicity and enhancing the resistance of faba bean tissue structure.

MATERIALS AND METHODS

Test Materials

Faba bean seeds of the resistant disease variety "89–147" and wheat variety "Yunmai 53" were purchased from the Yunnan Academy of Agricultural Sciences (Kunming, China) (Fabae Yu et Fang, FOF; *Fusarium oxysporum* Schlecht. f. sp *fabae* Yu et Fang) was isolated from an infected, continuously cropped faba bean plot. The spores were collected by filtration with four layers of gauze and diluted into a suspension ($\leq 1 \times 10^6$ CFU·mL^{-1}) for plant inoculation. The spores were cultured on potato dextrose agar (PDA) plates and incubated at 28°C for 7 days at a constant temperature.

A volume of 90 L of Hoagland Nutrient Solution configuration. The mass elements $CaCl_2{\cdot}6H_2O$ 135 g, KNO_3 45.9 g, $MgSO_4{\cdot}7H_2O$ 44.1 g, KH_2PO_4 12.6 g and trace elements H_3BO_3 257.4 g, $MnCl_2{\cdot}4H_2O$ 162.9 g, $ZnSO_4{\cdot}7H_2O$ 19.8 g, $CuSO_4{\cdot}5H_2O$ 7.2 g, $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$ 8.1 g were mixed in 50 L of distilled water in a 100 L plastic bucket. A total of 501.3 g of FeSO4·7H_2O was added to 7.2 L of distilled water and then boiled at 95°C in a water bath. A total of 670.5 g of Na_2-EDTA was added and evenly stirred. Once cooled to room temperature, the solution was transferred to a 100 L plastic bucket. Finally, 32.8 L of distilled water was added to the plastic bucket.

Experimental Design

The experiment was conducted in the glass greenhouse of Yunnan Agricultural University from September to December 2019. The experiment was conducted by hydroponics with nutrient solution in a multi-factor randomized design. Factor A was the inoculation treatment (without inoculation FOF: -F-0ca, inoculation FOF: +F+0ca). Factor B: four concentrations of cinnamic acid were added following the inoculation of FOF (inoculation FOF and 0 mg·L^{-1} cinnamic acid: +F+0ca, inoculation FOF and 50 mg·L^{-1} cinnamic acid: +F+50ca, inoculation FOF and 100 mg·L^{-1} cinnamic acid: +F+100ca, inoculation FOF and 200 mg·L^{-1} cinnamic acid: +F+200ca). Factor C was treated by two planting modes (faba bean monocropping: M, wheat and faba bean intercropping: I). Thus, the experiment had 10 treatments. Each treatment was conducted in triplicate, with each treatment and its replicates randomly allocated in the greenhouse, and the experiment was repeated three times.

A total of 300 full-sized uniform faba bean seeds and 200 full-sized uniform wheat seeds were treated in 10% (v/v) hydrogen peroxide (H_2O_2) for 30 min, germinated in the dark for 12 h in a saturated solution of $CaSO_4$ and dark porcelain discs for 48 h.

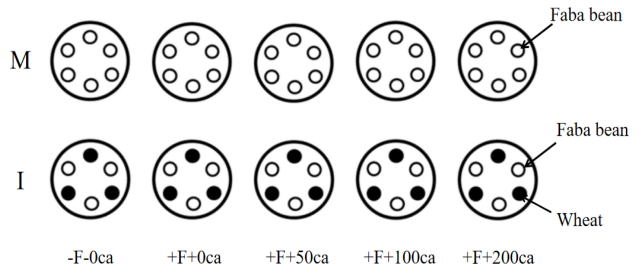

FIGURE 1 | Schematic diagram of hydroponic cultivation of nutrient solution in faba bean monocropping and wheat and faba bean intercropping. Ca, cinnamic acid; F, *Fusarium oxysporum* f. sp. *fabae;* I, intercropping; M, monocropping.

The germinated seeds were planted in sterile quartz sand soaked with water and watered daily at a set time. When the faba bean seedlings grew to 4∼6 true leaves and the wheat to three leaves, faba bean and wheat seedlings with the same amount of growth were selected and transferred into plastic basins (25 cm in upper diameter, 13 cm in lower diameter and 16 cm in height) filled with 2 L of Hoagland nutrient solution. Six faba bean seedlings were planted in each monoculture plastic basins (**Figure 1**). Simultaneously, three faba beans and three wheat plants were transplanted into each intercropping plastic basin (**Figure 1**). After 2 days of transplantation, 1×10^6 CFU·mL^{-1} FOF spore suspension and different concentrations of cinnamic acid were added near the roots of faba bean based on different treatments. One plastic basin was used for each treatment. There were a total of 30 pots with 135 faba bean and 45 wheat plants. All the faba bean and wheat plants were grown under natural light, 26/19°C day/night temperatures, and 70–85% relative humidity. A ventilation pump was used for 24 h in the pots. The pH of the nutrient solutions ranged from 5.7 to 7.1 (Asaduzzaman and Asao, 2012). The nutrient solution was replaced every 2 days, and a ventilation pump was used continuously in the incubator.

Measurement of Seedling Growth Parameters and Investigation of Faba Bean Wilt

Faba bean seedlings were transplanted for 45 days, and three faba bean plants with the same growth were selected from each treatment to measure the plant height and maximum leaf length and width. Each treatment was measured once and repeated three times independently.

The investigation of wilt was conducted 45 days after the transplantation of faba bean seedlings. In the faba bean monocropping treatment, one pot was investigated in each treatment; three faba beans were investigated in each pot, and each treatment was investigated once and repeated independently three times, with a total of nine plants. In the wheat and faba bean intercropping treatment, one pot was studied in each treatment; three faba bean plants were studied in each pot. Each treatment was investigated once and repeated independently three times, with a total of nine plants. The classification method of faba bean wilt was investigated using a 5-grade classification standard (Dong et al., 2016). Grade 0: asymptomatic; Grade 1: partial lesions or slight discoloration of the stem base or root (except for the primary root); Grade 2: diseased spots at the base of the stem or the main lateral root but not in patches; Grade 3: lesions, discoloration or decay appeared at one-third to one-half of the stem base or root, and the lateral roots were significantly reduced; Grade 4: the base of the stem was surrounded by disease spots, or most of the roots were discolored and rotten; Grade 5: the plants withered and died. The incidence and disease index were calculated as follows:

$$\text{Incidence} = \text{number of infected plants/total number of investigated plants} \times 100\%$$

$$\text{Disease index} = \Sigma(\text{number of diseased plants at all levels} \times \text{corresponding grade value})/(\text{highest value} \times \text{total number of investigated plants}) \times 100\%$$

Preparation and Determination of the Activity of Cell Wall Degrading Enzymes From Faba Bean Stems

One gram of fresh stem was ground with in a mortar on ice, and the extract was centrifuged at 5,000 rpm for 30 min at 4°C to collect the crude enzyme solution. This solution was boiled for 10 min and centrifuged at 5,000 rpm for 30 min to collect the supernatant as the inactivated enzyme solution. The cellulase activity was assayed as described by Bell et al. (1955) with slight modifications. The crude enzyme solution (0.1 mL) was mixed with 0.2 mL of 0.6% carboxymethyl cellulose in 0.05 mol·L^{-1} citric acid buffer at pH 4.8 and incubated at 50°C for 30 min. A volume of 1.0 mL of 3, 5-dinitrosalicylic acid (DNS) was immediately added and boiled for 5 min. After cooling, 0.7 mL of deionized water was added, and the absorbance was measured at 540 nm to determine the cellulase activity ($\mu g \cdot g^{-1} \cdot h^{-1}$). The inactivated enzyme solution was used as the control. The enzyme activity of each treatment was measured three times. Moreover, the activity of pectinase was assayed as described by Bell et al. (1955) with slight modifications. A solution of 0.2 mL of 0.25% polygalacturonic acid in 0.05 mol·L^{-1} citric acid buffer at pH 4.8 was added to 0.1 mL of the crude enzyme solution with 0.3 mL citric acid buffer and incubated at 50°C for 1 h. A volume of 1.8 mL of DNS was added, and the mixture was boiled for 5 min. After cooling, the absorbance was measured at 540 nm to determine the pectinase activity ($\mu g \cdot g^{-1} \cdot h^{-1}$). The inactivated enzyme solution was used as the control. Each treatment was measured once and repeated independently three times.

One gram of the stem was accurately weighed and ground in a mortar with 2 mL of 0.1 mol·L^{-1} phosphate buffer (pH 7.8) and a small amount of quartz sand on ice. A volume of 3 mL of the phosphate buffer was added and ground into a homogenate. The sample was centrifuged at 4,000 rpm at 4°C for 15 min, and the precipitate was discarded. The supernatant was collected, and the volume was brought to 10 mL with the phosphate buffer. The solution obtained was used as the crude enzyme solution.

A volume of 0.2 mL of the crude enzyme solution was mixed with 0.8 mL of activator (0.1 mol·L^{-1} phosphate buffer, pH 7.8, containing 20 mmol·L^{-1} Cysteine and 1.0 mmol·L^{-1} EDTA) and preheated in a 37°C water bath for 10 min. One mL of 1% casein phosphate buffer (0.1 mol/L, pH = 7.8) was added to this and preheated to 37°C for 10 min. Immediately, 3 mL of trichloroacetic acid (TCA) solution that contained 0.11 mol·L^{-1} TCA, 0.22 mol·L^{-1} sodium acetate, and 0.33 mol·L^{-1} acetic acid was added to this to stop the reaction (control, TCA was added first followed by the substrate casein, incubated stationary for 30 min, and centrifuged at 8,000 rpm for 10 min. The absorbance of the supernatant was measured at 275 nm to determine the protease activity (U·g^{-1}) (Guo et al., 2006). Each treatment was measured once and repeated independently three times.

Approximately 1 g of stem from one faba bean plant per treatment was ground in a mortar with a small amount of quartz sand and 2 mL of distilled water, and the homogenate was poured into a centrifuge tube with 6 mL of distilled water. The extract was placed at room temperature for 15–20 min and stirred every few minutes for complete extraction. The mixture was centrifuged at 3,000 rpm for 10 min, and the supernatant was brought to a constant volume with distilled water. It was then shaken to obtain the original amylase solution. Ten mL of the original amylase solution was diluted with distilled water to obtain the amylase dilution. Amylase stock solutions (1.0, 1.0, 1.0, 0, 0, and 0 mL) were placed in a water bath at 70°C for 15 min, cooled in running water, mixed with DNS reagent (2, 0, 0, 2, 0, and 0 mL, respectively) and incubated for 10 min in a 40°C water bath. To this, 1.0 mL of 1% starch solution was added and incubated for another 5 min at 40°C. Finally, DNS reagent (0, 2, 2, 0, 2, and 2 mL, respectively) was added to the tubes, shaken, placed in water for 5 min, cooled, and brought to a volume of 20 mL with distilled water. After shaking well, the absorbance was measured at 540 nm to determine the amylase activity (mg·g^{-1}·min^{-1}) (Li, 2000). Each treatment was measured once and repeated independently three times.

Extraction and Measurement of Lignin From Faba Bean Stems

The mercaptoacetic acid method was used as described by Bruce and West (1989). Two grams of one faba plant per treatment of fresh weight of stems were ground in 7 mL of 99.5% ethanol, centrifuged for 30 min at 10,000 *g* at 25°C and then precipitated at room temperature for 12 h. The precipitate was dried, and 50 mg was placed in a centrifuge tube. A volume of 5 mL of 2 N HCl and 0.5 mL of mercaptoacetic acid was steamed in a boiling water bath for 8 h and then cooled in an ice bath. Following centrifugation for 30 min at 10,000 *g* at 4°C, the precipitate was added to 2.5 mL of distilled water and centrifuged at for 5 min at 10,000 *g* and 4°C. The precipitate was suspended in 5 mL of 1 N NaOH and incubated at 25°C for 18 h, during which it was gently stirred several times and then centrifuged at 10,000 *g* for 30 min. The supernatant was removed, and 1 mL of concentrated HCl was added, precipitated at 4°C for 4 h, centrifuged for 30 min at 10,000 *g* to remove the precipitate, and then 3 mL of 1 N NaOH was added to dissolve the precipitate. The relative lignin content (mg·g^{-1}) was determined at 280 nm with NaOH as a blank control (Bruce and West, 1989). Each treatment was measured once and repeated independently three times.

Sample Preparation and Observation of the Stem Sections of Faba Bean

Paraffin sections were prepared as described by Wang et al. (2013). The sections of one faba bean plant per treatment were placed in xylene I for 20 min, xylene II for 20 min, anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, 75% alcohol for 5 min, rinsed with tap water, and placed in safflower dye for 1–2 h. The sections were washed slightly with tap water to remove the excess dye and decolorized with 50, 70, and 80% gradient alcohol. They were then put in solid green dye for 30–60 s, soaked with anhydrous ethanol in three tanks, and dehydrated. Finally, the slices were transparent in ethanol and xylene for 5 min. The slices were removed from the xylene and dried slightly. After neutral gum sealing, the slices were observed with a microscope (Eclipse CI; Nikon, Tokyo, Japan) (Wang et al., 2013). Four sections ($n = 4$) were made for each treatment and repeated three times, and each section was observed four times.

Determination of Photosynthetic Physiological Indices in Faba Bean Leaves

A total of 45 days after the faba bean seedlings were transplanted, the leaf gas exchange was measured from selected leaves obtained from the tip of the faba bean stem and harvested between 9:00 and 12:00. The leaves chosen were the second fully expanded leaves of 4–6 fully expanded compound leaves that were not damaged by pathogens. Data of gas exchange parameters, such as the leaf net photosynthetic rate (P*n* μmol CO_2·m^{-2}·s^{-1}), transpiration rate (T*i*, mmol H_2O·m^{-2}·s^{-1}), stomatal conductance (G*s*, mol H_2O·m^{-2}·s^{-1}), and intercellular carbon dioxide concentration (C*i*, μmol·mol^{-1}) were generated by a LI-6400 Portable Photosynthesis System (LICOR, Lincoln, NE, United States), an open-flow infrared gas analyzer adapted with light and temperature control systems for each leaf sample. The chlorophyll content (SPAD) was determined using an Fk-yl04 chlorophyll meter (Shandong Fangke Instrument Co., Ltd., China). The following conditions were maintained during the gas exchange assay: 25°C air temperature, 80 to 90% relative humidity, 400 μmol·mol^{-1} CO_2 concentration, and 1,000 μmol·m^{-2}·s^{-1}. Each treatment was measured once and repeated independently three times.

Data Processing and Statistical Analysis

SPSS 18.0 (SPSS, Inc., Chicago, IL, United States) was used to statistically analyze the data. Each dataset was tested for homogeneity of variance using a normal probability plot. A multi-factor way analysis of variance (ANOVA) was used to analyze the data. Least significant difference (*LSD*) was used to separate the means between the treatments, which were considered significant at $P \leq 0.05$. All the data are shown as the mean ± standard error.

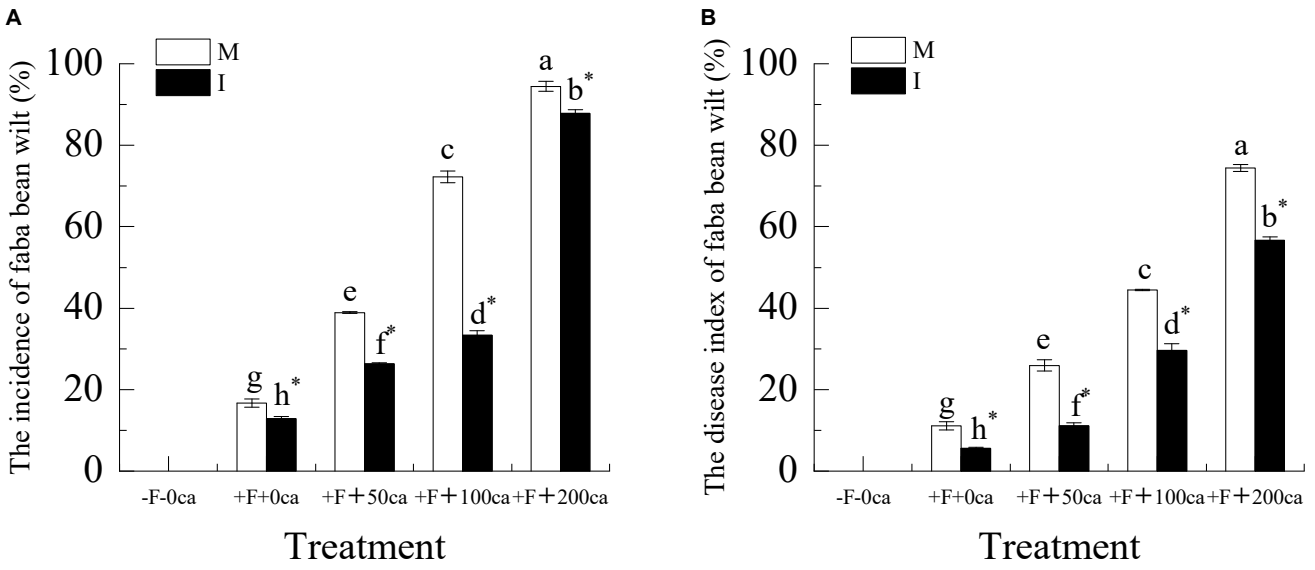

FIGURE 2 | Effects of FOF and cinnamic acid stress on faba bean wilt and the intercropping effect. **(A)** The incidence of faba bean wilt, **(B)** The disease index of faba bean wilt. Values in the figure are the mean ± standard error. Different lowercase letters after the data indicate a significant difference ($P < 0.05$). *Significant differences between monocropping and intercropping treatments with the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid; F, Fusarium wilt; FOF, *Fusarium oxysporum* f. sp. *fabae*; I, intercropping; M, monocropping.

RESULTS

Effects of FOF and Cinnamic Acid Stress on Faba Bean Wilt and the Intercropping Effect

As shown in **Figure 2** for the faba bean monocropping treatment, compared with the +F+0ca treatment, the treatments of F+50ca, +F+100ca and +F+200ca significantly increased the incidence of faba bean wilt by 133.35, 333.23, and 466.52%, respectively, and significantly increased the faba bean wilt disease index by 133.39, 300, and 570.02%, respectively.

Under the +F+0ca, +F+50ca, F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly decreased the incidence of faba bean wilt by 22.79, 32.23, 53.84, and 6.9%, respectively, and significantly decreased the faba bean disease index by 49.9, 57.15, 33.32, and 23.87%, respectively.

Effects of FOF and Cinnamic Acid Stress on the Growth of Faba Bean and Intercropping Effects

As shown in **Figure 3**, under the faba bean monocropping treatment compared with the -F-0ca treatment, the treatment of +F+0ca significantly decreased the plant height, maximum leaf length and maximum leaf width by 22, 16, and 19%, respectively,

Under the faba bean monocropping treatment, compared with the +F+0ca treatment, the treatment of +F+50ca resulted in a significant decrease in the maximum leaf length of faba bean by 16%. Compared with the +F+0ca treatment, the treatments +F+100ca and +F+200ca significantly decreased the plant height, maximum leaf length and maximum leaf width by 21 and 46, 26 and 30%, and 23 and 32%, respectively.

Under the +F+0ca, +F+ 50ca, F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly increased the plant height and maximum leaf length by 14, 20, 23, and 33%, respectively, and 11, 20, 27, and 9%, respectively. Under the +F+0ca and +F+ 200ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly increased the maximum leaf width by 25 and 20%, respectively.

Effects of FOF and Cinnamic Acid Stress on the Cell Wall Degrading Enzyme Activity of Stem of Faba Bean and Intercropping Effects

As shown in **Figure 4**, under the faba bean monocropping treatment, compared with the +F+0ca treatment, the treatments of F+50ca, +F+100ca and +F+200ca significantly increased the activities of pectinase in the faba bean stems by 325, 605.89, and 2,364.92%, respectively, significantly increased the activities of cellulase in the faba bean stems by 142, 226.57, and 308.64%, respectively, significantly increased the activities of protease in the faba bean stems by 19.06, 60.07, and 111.93%, respectively, and significantly increased the activities of amylase in the faba bean stems by 56.25, 72.91, and 485.41%, respectively.

Under the +F+0ca, +F+50ca, F+ 100ca and +F+200ca treatments, compared with the faba bean monocropping

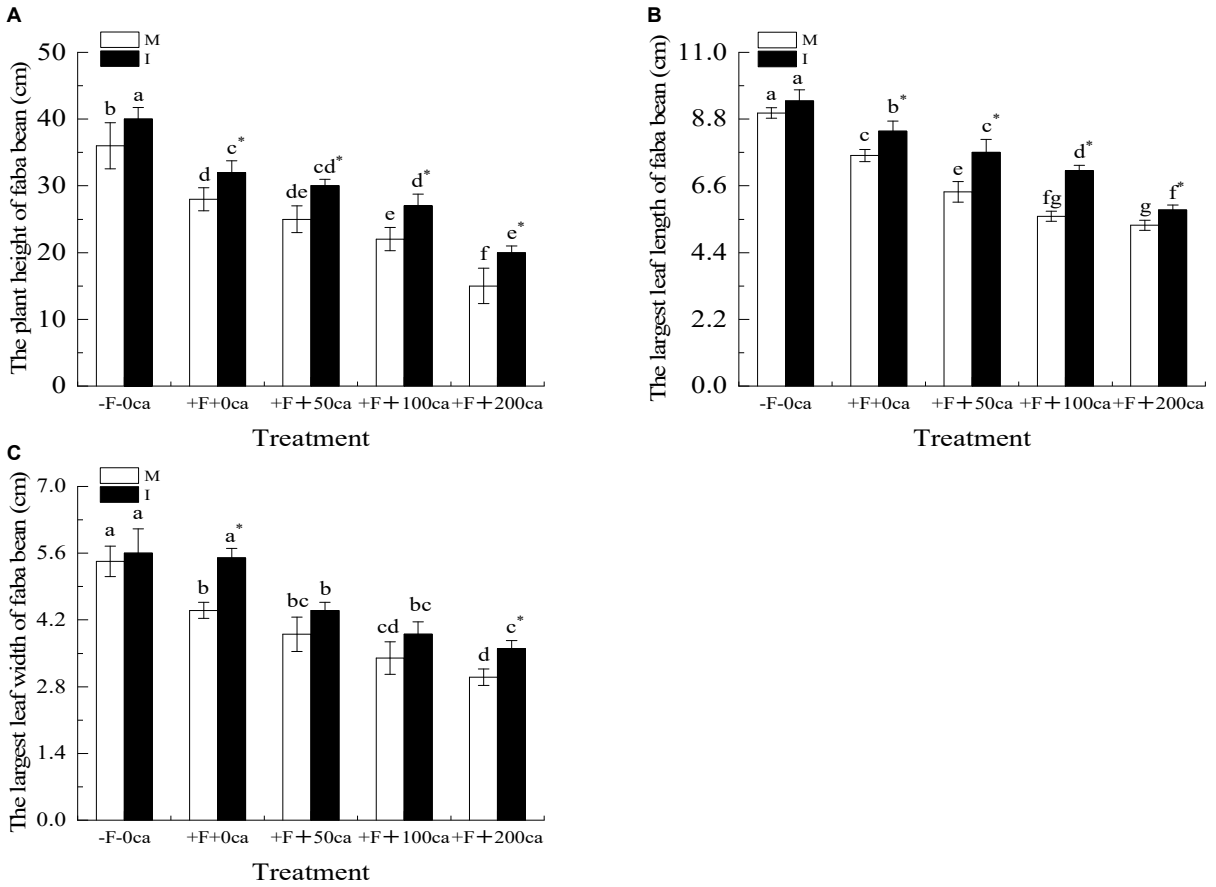

FIGURE 3 | Effects of FOF and cinnamic acid stress on the growth of faba bean (*Vicia faba*) and intercropping effect. **(A)** Plant height of faba bean, **(B)** The largest leaf length of faba bean, **(C)** The largest leaf width of faba bean. Values in this figure are the mean ± standard error. Different lowercase letters after the data indicate significant differences ($P < 0.05$). *significant differences between monocropping and intercropping treatments with the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid, FOF, *Fusarium oxysporum* f. sp. *fabae*; I, intercropping; M, monocropping.

treatment, the treatments of wheat and faba bean intercropping significantly decreased the activities of pectinase in the faba bean stems by 19.44, 30.47, 33.15, and 40.34%, respectively, significantly decreased the activities of cellulase in the faba bean stems by 25.07, 8.94, 16.05, and 18.26%, respectively, significantly decreased the activities of protease in the faba bean stems by 40.12, 32.96, 14.67, and 6.58%, respectively, and significantly decreased the activities of amylase in the faba bean stems by 62.5, 48, 32.53, and 57.29%, respectively.

Effects of FOF and Cinnamic Acid Stress on Lignin in Faba Bean Stems and the Intercropping Effects

As shown in **Figure 5**, under the faba bean monocropping treatment, compared with the -F-0ca treatment, the treatment of +F+0ca significantly increased the lignin content of faba bean by 75.5%. Compared with the +F+0ca treatment, the treatments of +F+50ca and +F+100ca significantly increased the lignin content in the faba bean stems by 17.94 and 63.98%, respectively. Compared with the +F+0ca treatment, the treatment of +F+200ca significantly decreased the lignin content in the faba bean stems by 24.41%.

Under the +F+0ca and +F+50ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly decreased the contents of lignin in the faba bean stems by 40.74 and 13.86%, respectively. Under the F+100ca and +F+ 200ca treatments, compared with the faba bean monoculture treatment, the treatments of wheat and faba bean intercropping significantly increased the lignin content in the faba bean stems by 7.71 and 165.35%, respectively. The results showed that in wheat and faba bean intercropping, the lignin content in stem of faba bean decreased significantly under the treatments of FOF and FOF with low concentrations of cinnamic acid after FOF inoculation.

Effects of FOF and Cinnamic Acid Stress on Tissue Structure in the Stems of Faba and Intercropping Effects

As shown in **Figure 6**, the production of paraffin sections enabled microscopic observation that showed that under faba bean

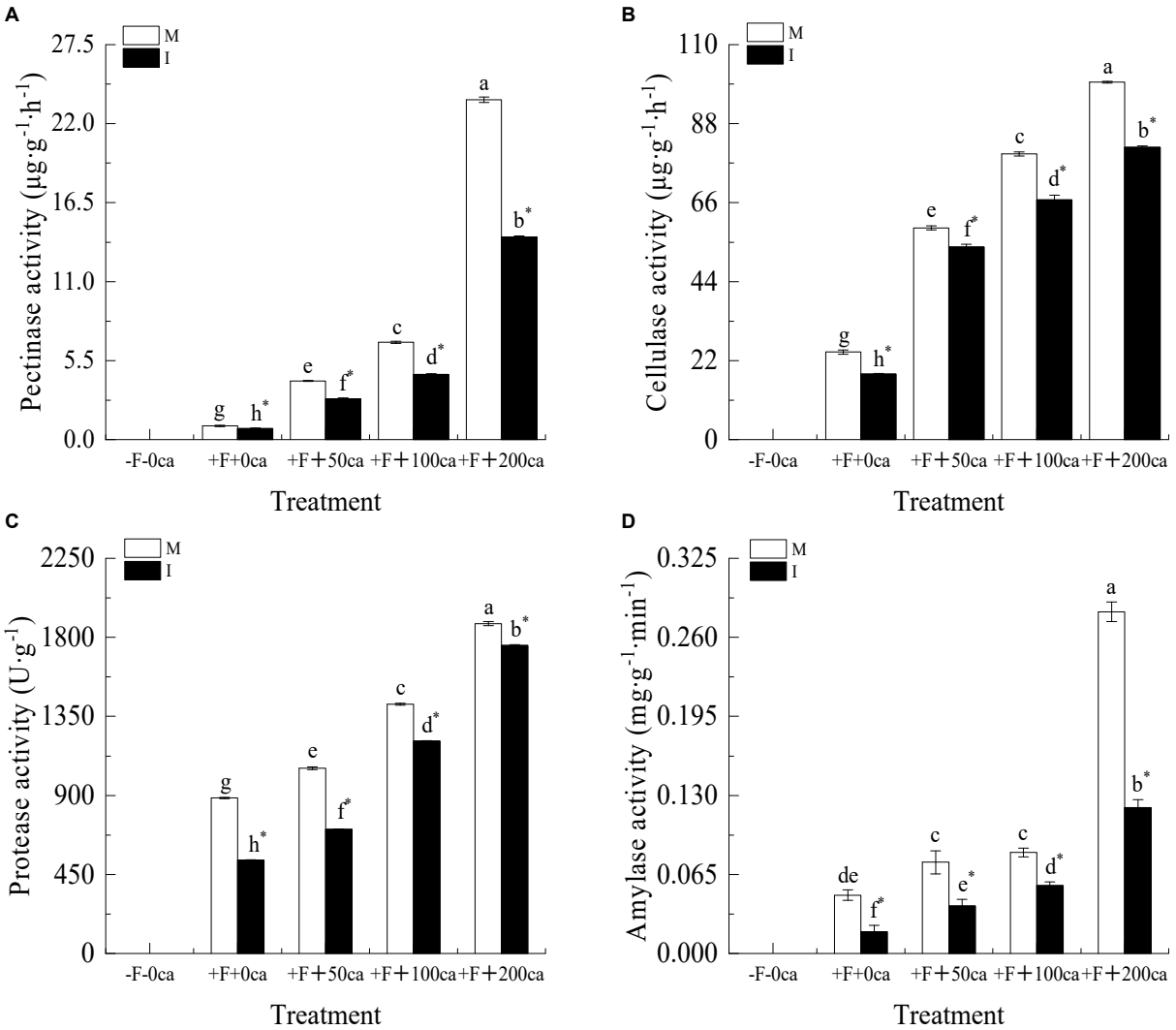

FIGURE 4 | Effects of FOF and cinnamic acid stress on stem cell wall degradation enzyme of faba bean and intercropping effect. **(A)** Pectinase, **(B)** cellulase, **(C)** protease, **(D)** amylase. Values in the figure are the mean ± standard error. Different lowercase letters after data indicate significant difference ($P < 0.05$). *Significant differences between monocropping and intercropping treatments with the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid; FOF, *Fusarium oxysporum* f. sp. *faba*; I, intercropping; M, monocropping.

monocropping treatment, compared with the -F-0ca treatment, the tissue structure of stems following treatment with +F+0ca was closely arranged and intact. However, the cell wall of conduit tissue was thickened. Compared with the +F+0ca treatment, the +F+50ca treatment resulted in stem duct tissue cells that were more thickened, and small amounts of gelatinous substances and inclusions appeared in the basic tissue cells of the cortex, and the cells twisted. Under the faba bean monocropping treatment, compared with the +F+0ca treatment, the +F+100ca treatment resulted in thickening in the ductal tissue cells of the stem, and the cells of cambium tissue showed colloid substances and inclusions. The basic tissue cells displayed colloid substances; inclusion of the cortex further increased, and the basic tissue cells of cortex partially broke. Under the faba bean monocropping treatment, compared with the +F+0ca treatment, in the +F+200ca treatment, the conduit tissue thickened and decreased; the cambium cells displayed many gelatinous substances and inclusions, and some cambium cells showed the phenomenon of a dividing cell layer with a hollow cavity. Many gelatinous substances and inclusions appeared in the basic tissue cells of the cortex, and many of the basic tissue cells of the cortex showed the phenomenon of broken cells and a cavity dividing cell layer. In addition, many tissue cells had died. The results showed that the faba beans after FOF inoculation added different concentrations of cinnamic acid, which thickened the catheter tissue stem cells, cambium tissue cells in colloidal material, inclusions and splinter cell layers in the cavity, cortex tissue cells in the colloidal material, basic contents and cell disruption and cavity splinter cell layer.

Under the -F-0ca treatment, compared with the faba bean monocropping treatment, there was no difference in the

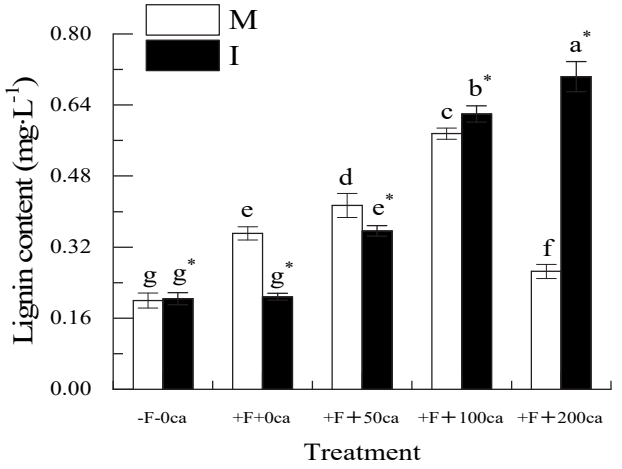

FIGURE 5 | Effects of FOF and cinnamic acid stress on lignin in the stems of faba bean (*Vicia faba*) and the effect of intercropping. Values in the figure are the mean ± standard error. Different lowercase letters after data indicate significant difference ($P < 0.05$). *Significant differences between monocropping and intercropping treatments at the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid; FOF, *Fusarium oxysporum* f. sp. *fabae*; I, intercropped; M, monocropped.

structures of stem tissues and cells between the wheat and faba bean intercropping treatments. Under the +F+0ca treatment, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments led to a lower degree of thickening of the conduit tissue cells that was less than that in the faba bean monocropping treatment. Under the +F+50ca treatment, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments resulted in additional thickening of the ductal tissue cells of faba bean stems, and the basic tissue cells of cortex were only distorted. No gelatinous substances and inclusions were found. Under the +F+100ca treatment, compared with the faba bean monocropping treatment, there were no inclusions in the cambium tissue cells and many cell contortions in the basic tissue cells of cortex of faba bean following the wheat and faba bean intercropping treatments. Under the F+200ca treatment, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly thickened the ductal tissue cells in the faba bean stems, and the cambium tissue cells did not exhibit the phenomenon of dividing cell layers in the cavity, while the basic tissue cells in the cortex only had a small amount of cell fragmentation and dividing cell layer cavity. The results showed that wheat and faba bean intercropping effectively increased the integrity of stem tissue structure.

Effects of FOF and Cinnamic Acid Stress on the Photosynthetic Physiology of Faba Bean Leaves and the Intercropping Effects

As shown in **Figure 7**, under the faba bean monocropping treatment, compared with the -F-0ca treatment, the treatment of +F+0ca significantly decreased the relative chlorophyll content of faba bean leaves by 7.05%.

Under the faba bean monocropping treatment, compared with +F+0ca, the treatment of +F+50ca did not significantly change the relative chlorophyll content of the faba bean leaves. However, treatments with +F+100ca and +F+200ca significantly decreased the relative chlorophyll content of faba bean leaves by 10.99 and 15.64%.

Under the -F-0ca, +F+0ca, +F+100ca and F+200ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly increased the relative chlorophyll content of the faba bean leaves by 3.89, 5.55, 5.60, and 7.12%, respectively. The results showed that wheat and faba bean intercropping could significantly increase the relative chlorophyll content of faba bean leaves.

As shown in **Figure 8**, under the faba bean monocropping treatment, compared with -F-0ca, the treatment of +F+0ca significantly decreased the T*i*, G*s* and P*n* of faba bean leaves by 9.38, 8.47, and 10.01%, respectively, and significantly increased the C*i* of faba bean leaves by 7.68%.

Under faba bean monocropping treatment, compared with the +F+0ca treatment, the treatments of +F+ 50ca, +F+ 100ca and +F+ 200ca significantly decreased the T*i* of faba bean leaves by 2.83, 8.79, and 12.2%, respectively, significantly decreased the G*s* of faba bean leaves by 34.88, 48.86, and 53.63%, respectively, significantly decreased the P*n* of faba bean leaves by 15, 24.49, and 38.12%, respectively, and significantly increased the C*i* of faba bean leaves by 4.61, 6.57, and 7.18%, respectively.

Under the -F-0ca treatment, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly decreased the C*i* of faba bean by 10.72% and increased the P*n* of faba bean by 17.76%. Under the +F+0ca, +F+50ca and F+100ca treatments, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping significantly increased the T*i* of faba bean leaves by 3.97, 3.21, and 5.28%, respectively, significantly increased the G*s* of faba bean leaves by 9.09, 21.55, and 3.96%, respectively, significantly increased the P*n* of faba bean leaves by 21.11, 19.57, and 18.27%, respectively, and significantly decreased the C*i* of faba bean leaves by 12.49, 7.13, and 3.2%, respectively. Under the F+200ca treatment, compared with the faba bean monocropping treatment, the treatments of wheat and faba bean intercropping of faba bean leaves significantly increased the G*s* by 27.16%.

DISCUSSION

In recent years, an increasing amount of attention has been paid to cinnamic acid, which plays a key role in promoting Fusarium wilt (Li et al., 2014a; Zhang et al., 2020). Research has shown that compared with the treatment that lacked cinnamic acid, exogenous treatment with cinnamic acid promoted the incidence of cucumber wilt by 214.4–266.8% (Ye et al., 2004). Tian et al. (2019) found that the exogenous addition of ferulic acid, a derivative of cinnamic acid secreted by strawberry (*Fragaria* × *ananassa*) roots, could improve the disease index

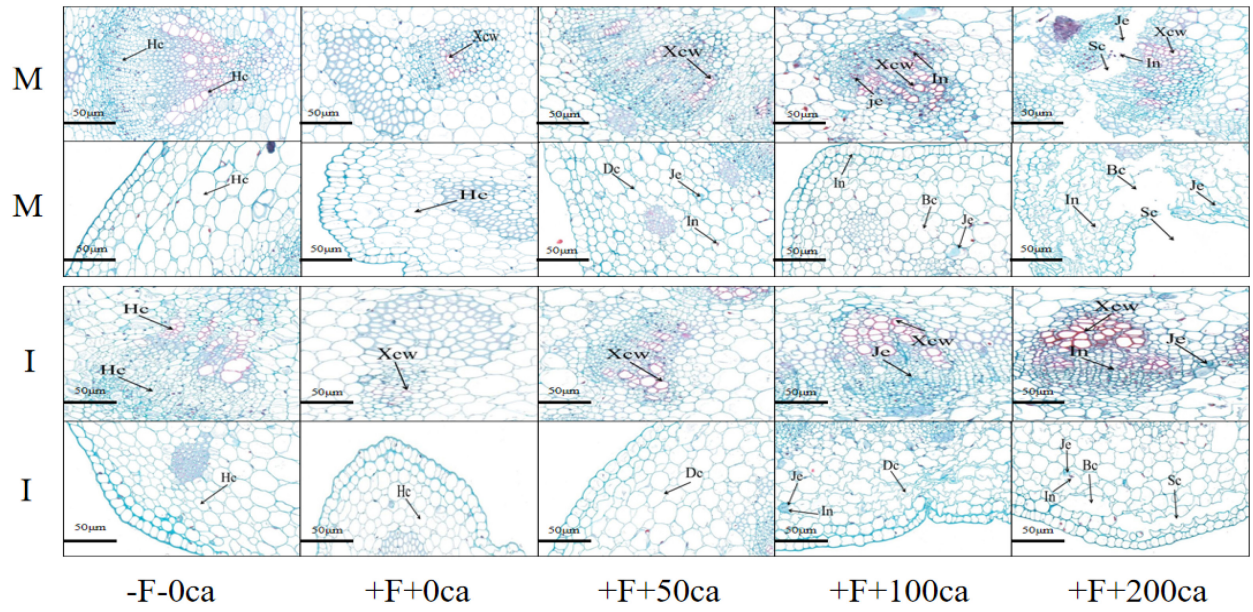

FIGURE 6 | Effects of FOF and cinnamic acid stress on tissue structure in the faba bean (*Vicia faba*) stems and the intercropping effect. Hc: Healthy cell, Xcw: Xylem cell wall, Je: Jelly cell, In: Inclusion, Dc: Deformed cell, Bc: Broken cell, Sc: Schismatic cell layer.

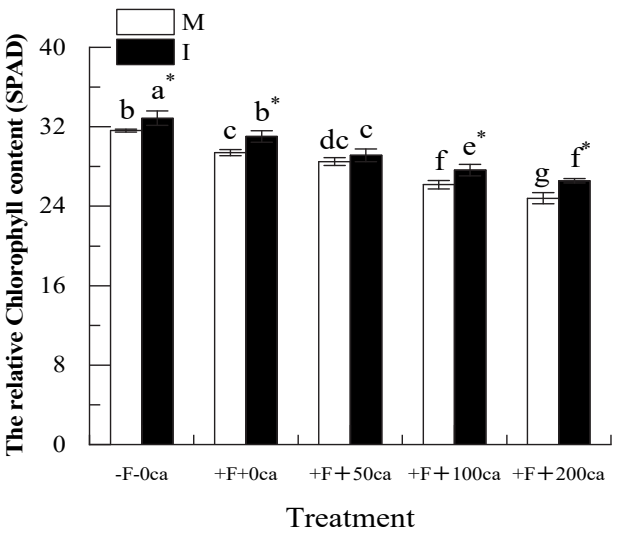

FIGURE 7 | Effects of FOF and cinnamic acid stress on the relative chlorophyll content of faba bean leaves and the intercropping effect. Values in the table are mean ± standard error. Different lowercase letters after data indicate significant difference ($P < 0.05$). *Significant differences between monocropping and intercropping treatments with the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid; FOF, *Fusarium oxysporum* f. sp. *fabae*; I, intercropping: M, monocropping.

of strawberry wilt by 37.03%. In this study, we obtained similar results to the study above. Under the faba bean monocropping treatment, compared with the +F+0ca treatment, the +F+50ca, +F+ 100ca and +F+200ca treatments significantly increased the incidence and disease index of faba bean wilt (**Figure 2**). These results indicated that cinnamic acid plays an important role in promoting the occurrence of faba bean wilt. Some studies have shown that reasonable intercropping is an effective measure for disease control. Compared with watermelon monocropping, wheat and watermelon intercropping significantly reduced the incidence of watermelon wilt (Xu et al., 2015). Studies have also shown that compared with the tomato monocropping treatment, tillering onion and tomato intercropping treatment can effectively reduce the incidence and disease index of Verticillium wilt in tomato by 35.58 and 19.83%, respectively (Fu et al., 2015). In this study, we obtained similar results to the previous study above. Under the +F+0ca, +F+50ca, +F+100ca and +F+200ca treatments, a comparison of the faba bean monocropping treatment with the wheat and faba bean intercropping treatments significantly decreased the incidence and disease index of faba bean wilt (**Figure 2**). The results showed that wheat and faba bean intercropping could effectively control the occurrence of faba bean wilt.

Allelopathic autotoxic substances can not only promote the occurrence of soilborne diseases but also directly inhibit the normal growth and development of crops. Studies have shown that compared with a lack of cinnamic acid, treatment with exogenous cinnamic acid can significantly reduce the aboveground and underground dry weight and the leaf area growth rate of cucumber plants (Li et al., 2017). In this study, we obtained results similar to the study by Li et al. (2017). Under the faba bean monocropping treatment, compared with the -F-0ca treatment, the +F+0ca treatment significantly reduced the plant height, maximum leaf width and maximum leaf length of faba bean. Compared with the +F+0ca treatment, the +F+50ca, +F+100ca and +F+200ca treatments significantly reduced the plant height, maximum leaf width and maximum

leaf length of faba bean even more (**Figure 3**). The results showed that cinnamic acid inhibited the growth of faba bean shoots. Studies have shown that intercropping can promote plant growth. Compared with cucumber monocropping, garlic and cucumber intercropping significantly increased the shoot and root biomass of cucumber (Xiao et al., 2013). In this study, we obtained results that were similar to those of Xiao et al. (2013). Under the +F+0ca treatment, the +F+ 50ca, +F+ 100ca and +F+ 200ca treatments, compared with faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly increased the plant height, maximum leaf length and maximum leaf width of faba bean (**Figure 3**). The results showed that wheat and faba bean intercropping could alleviate the synergistic effect of FOF and cinnamic acid and promote the growth of faba bean.

When host plants are infected by pathogens, CWDEs produced by plant pathogenic fungi are considered to be important pathogenic factors (Annis and Goodwin, 1997). Gharbi et al. (2015) found that the CWDEs secreted by *Verticillium dahliae* were closely related to the occurrence of *V. dahliae* in olive (*Olea europaea*), potato and sunflower (*Helianthus annuus*). Studies have shown that allelopathic autotoxic substances can improve the activity of pathogenic factors (CWDEs) secreted by pathogens. Wu et al. (2008b) found that the activities of pectinase (590%), cellulase (760%), amylase (2006%) and protease (27.0%) produced by *F. oxysporum* increased significantly after cinnamic acid was added to the culture medium of this fungus (Wu et al., 2008b). In this study, we obtained results similar to those of Wu et al. (2008b). Under the faba bean monocropping treatment, compared with the +F+0ca treatment, the +F+50ca, +F+100ca and +F+ 200ca treatments significantly increased the activities of pectinase, cellulase, protease and amylase produced by FOF in the stems of faba bean (**Figure 4**). The results showed that cinnamic acid promoted FOF to produce more active CWDEs in faba bean, improved its pathogenicity, and created favorable conditions for the further infection of faba bean. Studies have shown that different CWDEs require synergistic action during the pathogenic process of pathogens to effectively improve the disease risk of the host (Guo et al., 2019). In the pathogenic process of *Xanthomonas* on rice, different CWDEs need to be secreted to act synergistically and promote the occurrence of rice wilt (Ray et al., 2000). In this study, we concluded that FOF synergistically causes disease to faba beans by secreting cellulase, pectinase, protease and amylase and may cause more damage to faba beans. In this study, under the +F+0ca, +F+50ca, +F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly reduced the activities of pectinase, cellulase, protease and amylase in faba bean stems (**Figure 4**). This is similar to the results of Li C. X. (2019), who showed that the root exudates of wheat could reduce the activities of pectinase, cellulase, protease and amylase produced by *F. oxysporum* (Li C. X., 2019). The results obtained in this study could be because the wheat root exudates in the wheat and faba bean intercropping system reduced the activity of CWDEs produced by FOF in faba bean. The results showed that wheat and faba bean intercropping could reduce the virulence of FOF to faba bean by decreasing the activity of CWDEs produced by FOF in faba bean.

To successfully infect plants, pathogens must overcome the mechanism of host resistance formed during coevolution. The resistance of tissue structure is the first host defense of pathogens. This primarily refers to some components of the cell wall, stomatal special structure, small molecule resistant substances, proteins that destroy fungal cell permeability, and ribosome inactivation proteins among others. The resistance to tissue structure is related to the contents of cutin, lignin and lignin, and the changes of these components directly affect the innate resistance of plants. Lignin is a complex polymer that is found in the secondary cell wall of plants. It plays a crucial role in the solidification of cell walls and creates a non-degradable barrier for pathogens, thus, strengthening the protection of plants against biological stress (Bonawitz and Chapple, 2010; Moura et al., 2010). In this study, under the faba bean monocropping treatment, compared with the -F-0ca treatment, treatment with +F+0ca significantly increased the lignin content in faba bean stems (**Figure 5**). The possible reason is that the stress response of faba beans was activated following FOF inoculation. The +F+0ca, +F+50ca, +F+100ca and +F+200ca treatments showed a "low promoting and high inhibiting" effect on lignin content in the faba bean stems (**Figure 5**). Under the faba bean monocropping treatment, compared with the +F+0ca, the +F+50ca and +F+100ca treatments further increased the lignin content in faba bean stems (**Figure 5**). The possible reason was that the stress response of faba bean was further activated under 50 $mg{\cdot}L^{-1}$ and 100 $mg{\cdot}L^{-1}$ cinnamic acid stress, which promoted the increase in lignin synthesis. Under the faba bean monocropping treatment, compared with +F+0ca, the +F+ 200ca treatment significantly decreased the lignin content in faba bean stems (**Figure 5**). The possible reason is that when the concentration of cinnamic acid reaches 200 $mg{\cdot}L^{-1}$, the resistance of the tissue structure of faba bean is not enough to resist the damage of cinnamic acid, and the cell wall of the tissue defense mechanism cannot be thickened, thus, reducing the resistance of faba bean to FOF infection. These results indicate that cinnamic acid could inhibit lignin synthesis in faba bean stems and reduce resistance to FOF, which could be an important mechanism by which cinnamic acid promotes the occurrence of faba bean wilt. In this study, under the +F+0ca and +F+50ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly reduced the lignin content in the faba bean stems (**Figure 5**). Studies have shown that the root exudates of wheat can inhibit the activity of *F. oxysporum* that causes Fusarium wilt in watermelon (Lv et al., 2018). In this study, the probable cause was that wheat root exudates inhibited the activity of FOF in wheat and faba bean intercropping. Under the +F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly increased the lignin content in faba bean stems (**Figure 5**). In this study, the possible reason was that 100 $mg{\cdot}L^{-1}$ and 200 $mg{\cdot}L^{-1}$ of cinnamic acid promoted the pathogenicity (CWDEs) of FOF, and the stress response of faba beans was not enough to resist the damage

of FOF. Studies have shown that the root exudates of wheat can increase the content of lignin in watermelon (Li C. X., 2019). Wheat helped the faba bean to activate the resistance of tissue structure by exuding root exudates, and lignin synthesis increased substantially. The results showed that wheat and faba bean intercropping could reduce the damage of FOF and improve the resistance of tissue structure of faba bean. This could be an important mechanism for the effective control of faba bean wilt in wheat intercropping.

By observing the cell structures of plant tissue, we can study the changes in cell structure of plant tissue under stress, which can provide a cytological basis for plant injury. Studies have shown that pathogen invasion can promote the thickening of potato cell walls (Perry and Evert, 1983). In this study, we observed paraffin sections of plant tissue cells and found that under the faba bean monocropping treatment, compared with the -F-0ca treatment, the +F+0ca treatment resulted in a thickening of the xylem vessels of faba bean stems (**Figure 6**). This could be the stress response of faba bean to FOF under FOF stress. Further studies have shown that allelopathic autotoxic substances can destroy the tissue and cell structure of plants. Qi et al. (2015) found that under the stress of *p*-hydroxybenzoic acid, cells in the epidermis, subcutaneous and middle column of the strawberry root system, resulting in severe damage (Qi et al., 2015). In this study, under the faba bean monocropping treatment, compared with the +F+0ca treatment, the xylem vessels in faba bean stems became even thicker in the +F+50ca, +F+ 100ca and +F+ 200ca treatments, and the basic tissue cells of the cambium and cortex were invaded by gelatinous substances and inclusions (**Figure 6**). The degree of cell distortion, cell fragmentation, cell structure dispersion and even cell cavities was aggravated. The possible reason is that cinnamic acid promotes the production of high levels of CWDE activity by FOF, which leads to the leakage of a large amount of cell structures and lignin from the stem, and the breakdown of cell defense system (**Figure 6**). These results indicate that cinnamic acid can promote the pathogenicity of FOF, further aggravating the damage to stem tissue and cell structures, which could be one of the important mechanisms that enables cinnamic acid to promote the occurrence of faba bean wilt. In this study, under the +F+0ca, +F+ 50ca, +F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments cause a reduction in the amounts of gelatinous substances and inclusions in the tissue structure of faba bean stems; fewer cells were twisted and broken, and the degree of cavities in the divided cambium was reduced (**Figure 6**). Studies have shown that the root exudates of wheat can reduce spore germination, sporulation and mycelial growth of *F. oxysporum* f. sp. *niveum*, the causal agent of watermelon Fusarium wilt (Lv et al., 2018). In this study, we hypothesized that the root exudates of wheat in the wheat and faba bean intercropping inhibited the growth and reproduction of FOF and reduced the damage of FOF to faba bean.

After pathogens invade, they can cause the chlorosis of leaves and reduce the photosynthetic physiological characteristics of plants and promote the occurrence of diseases (Kim et al., 2010; Xie et al., 2020). Chlorophyll is the main pigment in plant photosynthesis, which can absorb, transfer and transform light energy. The level of pigment content in plant leaves directly affects the strength of plant photosynthesis (Mohsenzadeh et al., 2006). Gamliel et al. (1997) showed that the chlorophyll (21.19%) content decreased after potato was infected with Verticillium wilt. In this study, under the faba bean monocropping treatment, compared with the -F-0ca treatment, the +F+0ca treatment significantly reduced the relative chlorophyll phase content of faba bean leaves (**Figure 7**). Studies have shown that autotoxic substances can reduce the content of chlorophyll in plant leaves. Baziramakenga et al. (1994) found that the addition of cinnamic acid to soybean reduced the chlorophyll (27%) content in soybean leaves compared with the control. In this study, we obtained similar results. Under the faba bean monocropping treatment, compared with the +F+0ca treatment, the +F+50ca, +F+100ca and +F+200ca treatments significantly reduced the relative chlorophyll content of faba bean leaves (**Figure 7**). Studies have shown that compared with the treatment without *F. oxysporum* inoculation, the treatment with *F. oxysporum* f. sp. *cucumerinum* decreased the P*n* and G*s* of cucumber leaves and increased the C*i* (Ye et al., 2004). Studies have also shown that allelopathic autotoxicity can reduce plant photosynthesis and promote disease occurrence. Compared with the lack of cinnamic acid addition, the cinnamic acid treatment reduced the P*n* and G*s* of cucumber leaves, increased the C*i*, and promoted the occurrence of Fusarium wilt (Ye et al., 2004). In this study, we obtained similar results to those described above under the faba bean monocropping treatment, which compared with -F-0ca, the +F+0ca treatment, significantly reduced the T*r*, G*s* and P*n* of faba bean leaves but significantly increased the C*i* (**Figure 8**). Compared with the +F+0ca treatment, the +F+50ca, +F+100ca and +F+200ca treatments further significantly reduced the T*r*, G*s* and T*r* but significantly increased the Ci (**Figure 8**). The possible reason is that the factors that lead to the decrease in photosynthetic rate under adverse conditions primarily include stomatal and non-stomatal factors. Whether stomatal or non-stomatal factors are the primary reasons for the decrease in P*n* can be determined by changes in the G*s* and C*i* (Farquhar and Sharkey, 1982). If the G*s* decreases under stress, the C*i* should clearly decrease, and the P*n* should decrease. The change in direction of change in the C*i* and P*n* should be the same. The primary reason for the decrease in P*n* was the decrease in stomatal conductance. If the G*s* decreased while the C*i* remained unchanged or even increased, then the decrease in photosynthetic rate should be caused by non-stomatal factors, such as a reduction in the ability of mesophyll cells to assimilate compounds (Hartley et al., 2006). The direct cause of this non-stomatal factor could be the destruction of the chloroplasts of faba bean leaves, which resulted in a reduction in chlorophyll synthesis and a loss of the ability to assimilate CO_2. Finally, the photosynthetic physiology of faba bean decreases, caused the wilting phenomenon of faba bean leaves and causing the leaves to turn yellow. In this study, under the +F+0ca, +F+100ca and +F+200ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly increased the relative percentage content of faba bean leaves

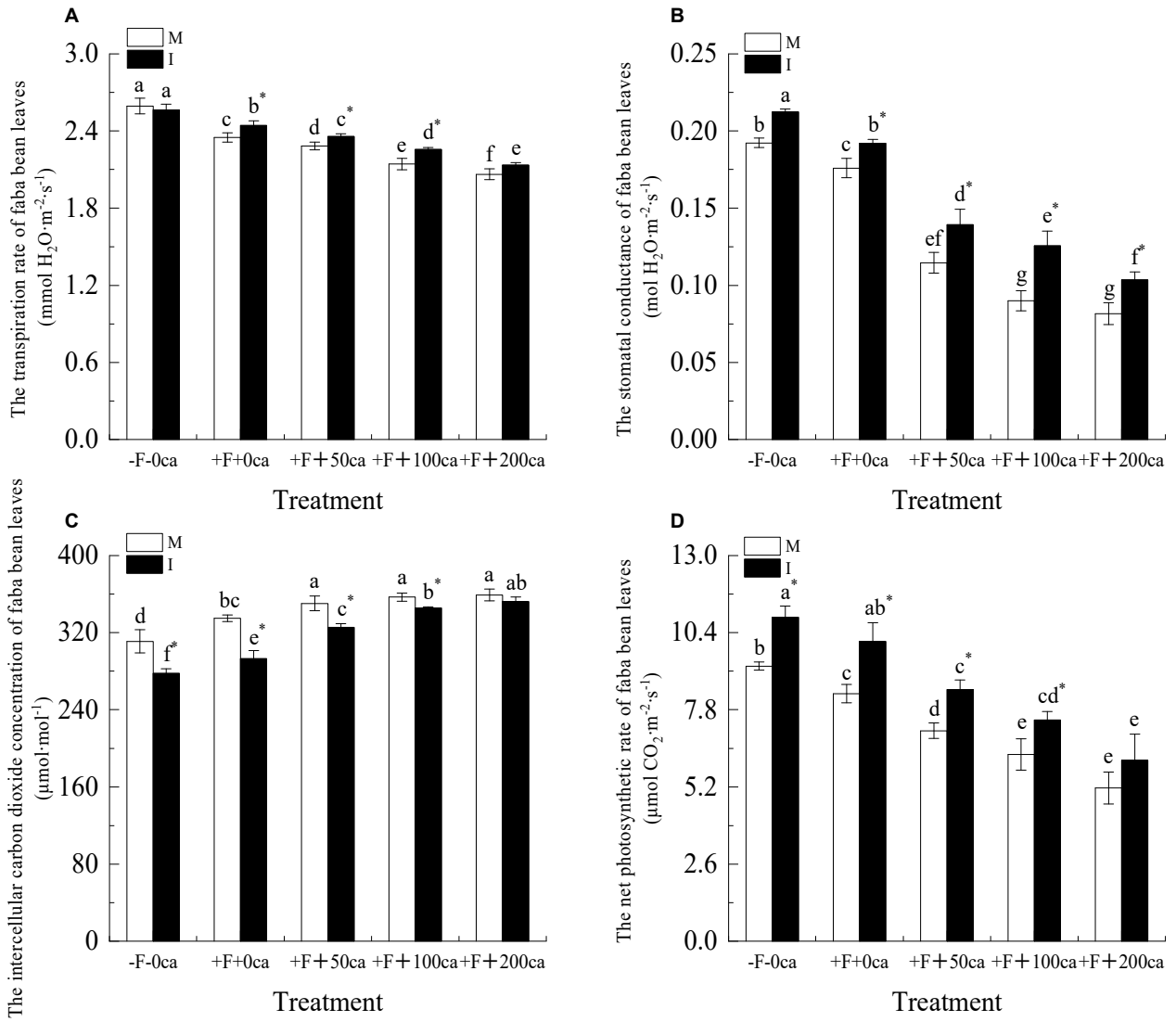

FIGURE 8 | Effects of FOF and cinnamic acid stress on the photosynthesis of faba bean (*Vicia faba*) leaves and intercropping effect. **(A)** Transpiration rate (T*i*), **(B)** Stomatal conductance (Gs), **(C)** Intercellular carbon dioxide concentration (C*i*), **(D)** Net photosynthetic rate (P*n*). Values in the table are mean ± standard error. Different lowercase letters after data indicate significant differences ($P < 0.05$). *Significant differences between monocropping and intercropping treatments with the same FOF and cinnamic acid levels ($P < 0.05$). ca, cinnamic acid; FOF, *Fusarium oxysporum* f. sp. *pisi*; I, intercropping; M, monocropping.

that are green (**Figure 7**). Under the +F+0ca, +F+50ca and +F+100ca treatments, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments significantly increased the T*i*, G*s*, and P*n* and decreased the C*i* (**Figure 8**). Studies have shown that the root exudates of wheat can inhibit the activities of pathogenic bacteria (Lv et al., 2018). In this study, we hypothesized that the possible reason was that the wheat root secretion in wheat and faba bean intercropping inhibited the activity of FOF, indirectly improving the ability of faba beans to defend themselves, reducing the energy requirement for faba beans to defend themselves, reducing the respiration of faba bean, and then reducing the intercellular carbon dioxide concentration in faba bean leaves. However, under the +F+200ca treatment, compared with the faba bean monocropping treatment, the wheat and faba bean intercropping treatments did not significantly change the T*i*, C*i* and P*n* of faba bean leaves (**Figure 8**). The possible reason is that the effect of wheat and faba bean intercropping could be limited. As we observed in a previous study, the tissue structure of stems was severely damaged under the dual stress of FOF and 200 mg·L^{-1} cinnamic acid. We hypothesized that the tissue and cell structure of faba bean leaves could be seriously damaged under the double stress of FOF and 200 mg·L^{-1} cinnamic acid, and the normal photosynthetic function would be lost. The results showed that wheat and faba bean intercropping could significantly increase photosynthesis and decrease the occurrence of faba bean wilt under a particular range of FOF and cinnamic acid stress.

The combined action of autotoxic substances and soilborne pathogens leads to the occurrence of serious soilborne diseases and the inhibition of plant growth in recent years (Li Y. et al., 2019). In our study, the occurrence of faba bean wilt was explained by examining the increase in the *in vivo* activity of the CWDEs of FOF and the reduction in the tissue resistance of faba bean. This shows that the occurrence of the faba bean wilt is a complex process. Wheat and faba bean can reduce the *in vivo* activity of CWDEs in FOF to improve the tissue resistance of faba bean and reduce the occurrence of wilt. We planted a resistant faba bean variety ("89–147") in soil where faba beans have been continuously cultivated for many years and found that faba bean wilt occurred. The incidence of faba bean wilt was reduced through the use of wheat and faba bean intercropping (Supplementary Data). The possible reason for this is that, in actual field production, the occurrence of faba bean wilt is owing to multiple factors. On the one hand, it could be that FOF can survive in continuous soil for many years. Alternatively, with the increase of its continuous cropping years, faba bean secretes autotoxic substances in the rhizosphere soil that continuously accumulate and aggravate the rhizosphere soil habitat. It works in concert with FOF. Moreover, the resistance of the host decreased. In the future, how to prevent the faba bean wilt caused by multiple factors should not be initiated from the perspective of a single control of pathogenic fungi but should be considered through a comprehensive control strategy. Therefore, we used disease-resistant varieties to improve the resistance of our hosts and improve the microecological environment of the rhizosphere by combining diversified planting (intercropping) to inhibit the growth of pathogenic fungi. A new control model of faba bean Fusarium wilt disease was developed from the combination of host resistance, rhizosphere microecology and pathogenic fungal interaction. The further application of this model will play an important role in sustainably and effectively controlling the occurrence and harm of faba bean wilt, protecting the ecological environment, improving the photosynthetic ability of faba bean, promoting the quality of faba bean products and increasing the income of farmers.

CONCLUSION

Cinnamic acid increased the activity of CWDEs secreted by FOF in the stems, reduced the resistance of tissue and cell structure of faba bean, created favorable conditions for FOF to infect faba bean, reduced photosynthesis in the leaves, and promoted the occurrence of faba bean wilt. Wheat and faba bean intercropping decreased the activity of CWDEs secreted by FOF in the stem and improved the resistance of tissue structure of faba bean, thus, enhancing the leaf photosynthesis of faba bean and reducing the occurrence of faba bean wilt.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WY conceived the original screening and research plans, designed the experiments and analyzed the data, and finished writing this thesis. YG assisted in the design of the experiment and proposed some suggestions for modification of this manuscript to WY. YL assisted in the data analysis to WY. YZ provided the technical assistance to WY. YD and KD supervised the experiments, agreed to serve as the author responsible for contact and ensures communication. All authors contributed to the article and approved the submitted version.

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Whitefly-tolerant transgenic common bean (*Phaseolus vulgaris*) line

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Common bean (*Phaseolus vulgaris* L.) is a staple food in Brazil with both nutritional and socioeconomic importance. As an orphan crop, it has not received as much research attention as the commodity crops. Crop losses are strongly related to virus diseases transmitted by the whitefly *Bemisia tabaci*, one of the most important agricultural pests in the world. The main method of managing whitefly-transmitted viruses has been the application of insecticides to reduce vector populations. Compared to chemical vector control, a more sustainable strategy for managing insect-borne viruses is the development of resistant/tolerant cultivars. RNA interference has been applied to develop plant lines resistant to the whitefly in other species, such as tomato, lettuce and tobacco. Still, no whitefly-resistant plant has been made commercially available to date. Common bean is a recalcitrant species to *in vitro* regeneration; therefore, stable genetic transformation of this plant has been achieved only at low frequencies (<1%) using particle bombardment. In the present work, two transgenic common bean lines were obtained with an intron-hairpin construct to induce post-transcriptional gene silencing against the *B. tabaci vATPase* (Bt-*vATPase*) gene, with stable expression of siRNA. Northern blot analysis revealed the presence of bands of expected size for siRNA in leaf samples of the line Bt-22.5, while in the other line (11.5), the amount of siRNA produced was significantly smaller. Bioassays were conducted with both lines, but only the line Bt-22.5 was associated with significant mortality of adult insects (97% when insects were fed on detached leaves and 59% on the whole plant). The expression of the Bt-*vATPase* gene was 50% lower (p<0.05) in insects that fed on the transgenic line Bt-22.5, when compared to non-transgenic controls. The transgenic line did not affect the virus transmission ability of the insects. Moreover, no effect was observed

on the reproduction of non-target organisms, such as the black aphid *Aphis craccivora*, the leafminer *Liriomyza* sp. and the whitefly parasitoid *Encarsia formosa*. The results presented here serve as a basis for the development of whitefly-tolerant transgenic elite common bean cultivars, with potential to contribute to the management of the whitefly and virus diseases.

KEYWORDS

dry bean, *Bemisia tabaci*, RNA interference, insect pest management, *vATPase*

Introduction

The whitefly *Bemisia tabaci* (Genn.; Hemiptera: Aleyrodidae) biotype MEAM1 is currently considered one of the most important crop pests worldwide, for several reasons, including its wide geographic distribution, in all continents, strong performance as a vector of plant viruses and ability to colonize several plant families. Moreover, this insect presents high adaptability to different environments and rapid selection of insecticide-resistant populations. Whiteflies are a threat to food security, especially for developing countries (De Barro et al., 2011). As a generalist insect, *B. tabaci* feeds on a wide range of host plants, including common beans, cotton, tomatoes and soybeans. For those reasons, in countries with a tropical climate, *B. tabaci* can be found in both cultivated areas and native vegetation throughout the year, placing this insect among the ten most invasive pests in the world (Chen et al., 2016). In addition to the direct constraint caused by feeding on the plant, the whitefly is responsible for the transmission of several plant viruses, which is considered the main damage associated with this insect in agricultural crops. *B. tabaci* is the exclusive vector of viruses from the genus Begomovirus.

Common bean (*Phaseolus vulgaris* L.) is a staple food in Brazil with nutritional and food security importance, as a relevant source of protein. It is also a crop of substantial impact on the Brazilian agribusiness, because it is produced in all regions of the country, in three cropping seasons per year, with a diversified use of technology. The majority of the common bean production in Brazil is carried out in small-holder farmers, providing employment and income to family producers. On the other hand, the crop is also produced by industrial farmers, in larger areas, with supplementary irrigation mainly in the Central Brazil growing area.

One of the main challenges of the crop is the high incidence of virus diseases, the most important ones transmitted by *B. tabaci*. Losses of up to 100% have been reported due to damages associated with the Begomovirus bean golden mosaic virus (BGMV; Souza et al., 2016). In addition to this virus, the whitefly transmits other viruses to common beans and soybeans, such as the Carlavirus cowpea mild mottle virus (CPMMV) and a recently reported Cytorhabdovirus (Alves-Freitas et al., 2019; Pinheiro-Lima et al., 2020). Currently, the main method of managing whitefly-transmitted viruses has been the intensive use of insecticides to reduce the vector population. However, the intense use of the same insecticide molecules, often not associated with other management techniques, has rapidly reduced the efficacy of insecticides and selected whitefly populations resistant to the majority of the active ingredients on the market, thus limiting the efficiency of chemical control. In recent years, there are no records of new insecticides to control this pest, which indicates a limitation in the development of new synthetic molecules. Furthermore, the excessive use of synthetic pesticides poses a risk to human health and to the environment, in addition to increasing production costs. It is not difficult to find reports of 20 applications per common bean crop season for the management of this insect pest (Souza et al., 2016).

A more sustainable strategy for pest management is the development of pest resistant/tolerant plant cultivars. Strategies for the development of commercial cultivars resistant/tolerant to whitefly-transmitted viruses have been developed, for example, the transgenic common bean cultivar BRS FC401 RMD, which is resistant to BGMV (Bonfim et al., 2007; Faria et al., 2016; Souza et al., 2016) and the tomato cultivar BRS Sena, tolerant to Bemogoviruses (Quezado-Duval et al., 2014). However, considering the plasticity of the "virus transmission ability" phenotype of *B. tabaci*, as well as its high adaptation to a wide range of environments and hosts, plant breeding for resistance to plant virus may contribute to the virus disease management, but not to the management of other viruses transmitted by this insect vector. As an efficient vector of plant viruses, even a single adult whitefly is capable of carrying and transmitting different species of viruses, acquired from mixed-infected plants.

Although there is no commercially available whitefly-resistant plant line yet, some reports show the development of whitefly-resistant plants by stable genetic transformation, such as tomato, tobacco and lettuce (Ibrahim et al., 2017; Pizetta et al., 2021; Xia et al., 2021), but not common beans. Our team has developed the first transgenic common bean cultivar in the world, resistant to BGMV, which has recently been made commercially available. Although some common bean cultivars have been reported to present tolerance to the whitefly through antixenosis (Silva et al., 2014, 2019; Hoshino et al., 2017; Jesus et al., 2021), using interfering RNA (RNAi) to silence important genes in the insect is also a promising strategy, because it can be more specific to the target insect and generally leads to high mortality. Silencing the insect *vATPase* gene (Bt-*vATPase*), using RNAi in Hemipteran insects has proven to reduce survival and to interfere in the development of juvenile stages, including *B. tabaci*

(Thakur et al., 2014; Ibrahim et al., 2017). The ATPase enzyme is part of the family of ATP-dependent proton pumps located in a variety of eukaryotic cell membranes. It is responsible for controlling pH in intracellular compartments and its activity affects several cellular processes, such as intracellular membrane transport, processing and transport of neurotransmitters, as well as regulating the entry of viruses and microorganisms. Here we report the development of the first transgenic common bean line with tolerance to the whitefly *B. tabaci*, by silencing the insect *vATPase* gene, using RNAi. We generated two transgenic lines and one of them was tolerant to the whitefly, causing significant mortality of adult insects. The next step will be to transfer the transgene to elite common bean lines for possible commercial use by farmers after biosafety studies.

Materials and methods

Insect colonies

The whiteflies *Bemisia tabaci* MEAM1 biotype used in the experiments were originated from a colony on common bean (*P. vulgaris*, cv. Pérola), kept under screenhouse conditions, at Embrapa Arroz e Feijão, Santo Antônio de Goiás, GO, Brazil (16° 28′ 00" S, 49° 17′ 00" W; 823 m asl), as previously described (Pizetta et al., 2021). To obtain age-synchronized adult insects, plants containing whitefly eggs laid for 2 h were isolated in insect cages, after removing the adults, until reaching the fourth larval instar. Adult insects used in the mortality experiments were collected 1 day after the onset of adult emergence.

A colony of the black aphid *Aphis craccivora* was obtained from bean plants collected at Embrapa Arroz e Feijão and maintained on common bean plants isolated in insect cages. The whitefly parasitoid *Encarsia formosa* was obtained from a colony maintained on whitefly nymphs fed on kale (*Brassica oleracea*) plants.

Genetic transformation

A partial sequence of 647 bp from the *B. tabaci v-ATPase* gene was cloned in sense and antisense orientations in the vector pSIU (Tinoco et al., 2010) generating pBtATPase, as previously described (Ibrahim et al., 2017), for genetic transformation of the common bean (Supplementary Figure S1). The ATPase interference cassette is under the control of the doubled 35SCaMV promoter with an enhancer sequence from the alfalfa mosaic virus (dCaMV35S) and the terminator is that of the *nopaline synthase* gene (*nos*). The selection gene used was the *Atahas*, with the complete promoter and terminator from *Arabidopsis thaliana*, conferring tolerance to the herbicide imazapyr. The RNAi construct will be referred to as *ΔATPase* from now on.

Genetic transformation of the common bean cultivar Olathe Pinto was performed as described (Aragão et al., 1996; Bonfim et al., 2007). Briefly, common bean seeds were surface disinfested in 70% ethanol (V/V) for 1 min, followed by immersion in 2.5% sodium hypochlorite for 10 min. Soon after, three washes were performed using sterile water. After the last wash, the seeds were soaked in sterile water for approximately 18 h. After this period, the seed embryonic axis was excised and their apical meristems exposed after the removal of the primordia of the primary leaves (plumule), with the aid of a stereoscopic microscope. Then, they were placed in sterile Petri dishes (60x15mm) containing MS medium amended with phytagel, with the apical meristem facing the center of the dish. Particle bombardment of DNA was performed using a particle accelerator as described by Sanford (1990), Klein et al. (1992). Embryos were transferred to plant tissue culture containers with selective culture medium containing 6-benzylaminopurine (BAP; 10 mg/l) and imazapyr (80 nm), which were kept in a growth chamber at 24°C and 16 h photoperiod. The explants that developed and were positive for the presence of *Atahas* gene by PCR were transferred to a container with sterile substrate, covered by a plastic bag that was gradually removed so that the explants could acclimate to the environment. After this process, they were transferred to pots with soil and fertilizer and kept in a greenhouse to complete the development and for PCR analysis. For that, DNA was isolated from leaf tissues as described (Dellaporta et al., 1983) and amplified by PCR with the following pair of primers: AHASP124F 5′ACTAGAGATTCCAGCGTCAC3′ and AHAS500CR 5′GTGGCTATACAGATACCTGG3′ for the detection of the selection gene *Atahas*. Thermal cycling conditions were denaturation at 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and 60°C for 30 min.

Progeny analysis

Segregation ratio was evaluated at the second and third generations (T_2 and T_3) of self-pollinated transformed plants, analyzing the presence of the *ΔATPase* by PCR, as described. Pearson's Chi squared (χ^2) was used to determine whether the observed segregation ratio was consistent with a Mendelian ratio of 3:1, at 95% level of confidence. Homozygous plants were used for the reported bioassays.

Production of *ΔATPase* siRNAs

Leaf samples from 10-day-old plants were collected in liquid nitrogen for total RNA isolation, using Trizol (Invitrogen), as recommended by the manufacturer. Non-transgenic plants with the same genetic background (cv. Olathe pinto) and the same age were used as controls. SiRNA analysis was performed as described (Bonfim et al., 2007; Pizetta et al., 2021), using a DNA probe corresponding to the *vATPase* PCR fragment, which was amplified using the primer

pair ATPXS1 (TTCTAGAGCTCTATCACACTATCTGAGT AC)/ATPSK1(GGTACCACTAGTGGGAAGTTTTTATCGTAG) labeled with α^{32}P dCTP and the DecaLabel DNA Labeling Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The bands were visualized with a fluorescent image analyzer (FLA-3000; Fujifilm).

Whitefly mortality assays

Two sets of bioassays were conducted for each common bean transgenic line, the first one using detached leaves and the other with the whole plant. To keep the detached leaves during the experiments, a bioassay system was developed using 50 ml Falcon tubes, containing 1.5 ml microtubes fixed to the bottom (Figure 1A). The detached leaves were accommodated with the petioles inside the microtubes containing water (Figures 1B,C) and the system was covered with voile fabric. Each replicate consisted of one detached leaf from an individual transgenic or non-transgenic plant ($n = 15$) and 20 two-day-old adult insects carefully collected from the colony with the aid of 15 ml Falcon tubes with one end opened (Figure 1D). The evaluations were carried out 5 days after the assembly of the assay, counting the number of live and dead adults on the leaves (Figure 1E), with the aid of an insect aspirator. This bioassay was repeated twice for each transgenic line.

For the experiments with whole plants, transgenic and non-transgenic plants ($n = 10$) with the primary leaves fully expanded were submitted to whitefly oviposition for 1 h. After that, adult insects were removed from the leaves and plants were kept on a growth chamber (25°C and 16 h:8 h light/dark photoperiod) during the insect development from egg to adult, for about 20 days. When the insects reach the fourth instar, plants were isolated in individual cages, made of plastic cups covered with voile fabric, to avoid the scape of adult insects (Figures 1F,G). 7 days after adult emergence, the numbers of live and dead adults and empty pupae were counted on each plant.

Gene silencing in insects

The expression levels of the *vATPase* gene in *B. tabaci* feeding on transgenic (T_3 generation; line Bt-22.5) and non-transgenic common beans were determined by qRT-PCR. About 150 two-day-old adult insects were transferred to transgenic and non-transgenic plants ($n = 3$) isolated in individual cages. After 48 h, insects were collected using an insect aspirator coupled to microtubes, which were immediately placed on liquid nitrogen.

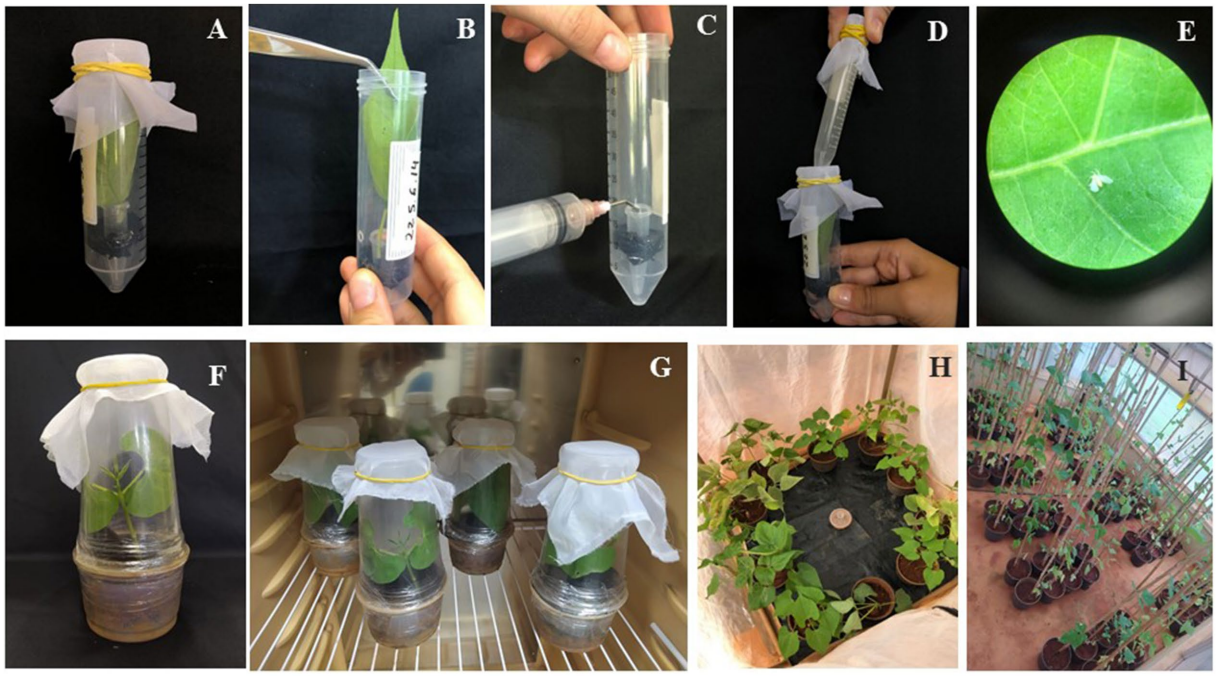

FIGURE 1
Whitefly mortality and preference assays. **(A)** Bioassay system developed to conduct the insect mortality assays with detached leaves of the transgenic common bean lines, with voile fabric covering the upper part of the tube; **(B)** Plant leaf being inserted into the bioassay system; **(C)** Microtube used to place water and maintain the leaf for the period of the evaluations; **(D)** Release of adult insects inside the Falcon tube; **(E)** Close-up photo of dead insect on GM bean leaf; **(F)** Plastic cup cage to isolate the plants; **(G)** Plants in the growth chamber for the whole-plant experiment; **(H)** Transgenic and non-transgenic common bean plants randomly distributed in a circle under a large voile cage, where insects were released in the center, for the preference assays and **(I)** Transgenic and non-transgenic common bean plants distributed in blocks in the greenhouse for the experiment during the common bean cycle.

Total RNA was isolated from ~150 adult insects/replicate, and 200 ng of RNA from each sample were used to synthesize the cDNA with the Promega GoScript Reverse Transcription System kit, according to manufacturer's instructions. PCR reactions were performed using the Step OnePlus real-time PCR system (Thermo Fisher Scientific) with SYBRGreen detection. Primers for the *vATPase* and *actin* genes were designed using the PrimerQuest tool (IDT Integrated DNA Technologies, Inc.), as described (Pizetta et al., 2021). The relative levels of *vATPase* transcription in the different RNA samples were normalized in relation to the *actin* gene, an internal standard. Quantitative assays were performed using three biological samples. The relative level of expression was calculated using the Livak method (Livak and Schmittgen, 2001).

Preference and oviposition assays

Pots with transgenic or non-transgenic plants ($n = 5$) were placed inside a large voile fabric cage (1.5 mL × 1.5 mW × 1.5 mH), in a circle, randomly distributed (Figure 1H). In the center of the cage, 400 adult whiteflies were released. The number of adult insects sitting on the primary leaves of the plants was counted 48 h later, using a small mirror to prevent the insects from leaving the plants due to the movement of the foliage. After counting the number of adults, one leave of each plant was removed and taken to the laboratory for egg counting under a stereoscopic microscope.

Effect of the transgenic common bean line on virus transmission by the whitefly

To evaluate the potential effect of silencing the *vATPase* gene in the whitefly on virus transmission by the insect, we conducted transmission assays with two economic important viruses of common beans: cowpea mild mottle virus (CPMMV), which has a mild effect on common beans, and bean golden mosaic virus (BGMV), which cause a severe mosaic and stunting on susceptible plants. Common bean plants cv. BRS Pérola, susceptible to both viruses, were used as the inoculum source. After an acquisition period of 24 h, viruliferous whiteflies were transferred to two individual cages, one of them containing a common bean plant from line Bt-22.5 and the other one containing a non-transgenic common bean Olathe plant. Insects were kept on these two treatments for 48 h. After that, 100 insects were transferred from each treatment to larger cages, containing 30 recipient plants (Olathe Pinto, non-transgenic) for a 24 h inoculation period. Then, all adult insects were manually removed from the plants, using an insect aspirator, and plants which were kept in cages for 25 days, for virus infection evaluation. Virus incidence was assessed by visual symptoms, using a 1–4 scoring scale, in which 1 = no symptom, 2 = light symptoms, 3 = moderate symptoms and 4 = strong symptoms (Arias et al., 2015). Virus detection was analyzed on symptomatic and asymptomatic plants ($n = 12$) by PCR and RT-PCR, using the primers CPMMV-F 5′ACGTCTCGAGCTGGAGTCAGTGTTTG3′/CPMMV-R (5′ACGTGAATTCTTACTTCTTAGCGTG3′) and BGMV_pAC1v 1978 (5′GCATCTGCAGGC CCACATYGTCTTYCCNGT 3′) / BGMV_ pAV1c715 (5′GATTTCTGCAGTTDATRTTYTCRT CCATCCA 3′).

Effect of the transgenic common bean line on two whitefly generations

A greenhouse experiment was carried out to simulate the effect of the transgenic line Bt-22.5 on the whitefly population in the field, because the field release has yet to be requested to the Brazilian National Biosafety Technical Commission (CTNBio). The experiment was carried out in a randomized block design. Transgenic and non-transgenic seeds, 48 of each, were sown in large plant pots and randomly distributed in six blocks inside the greenhouse, to mitigate the potential effect of spots with different light incidence. Each block was composed by 8 plants of each treatment (Figure 1I). When the plants had the two primary leaves fully expanded, kale plants hosting fourth instar nymphs of the whitefly were randomly distributed among the blocks, so that the emerging adults could freely move to the common bean plants. The whitefly-source kale plants were kept in the greenhouse for 2 days and then they were removed. The number of adult whiteflies sitting on the plants were sampled in 18 plants/treatment weekly. From the third week on, leaves from 18 different plants/treatment were randomly collected every week and analyzed in the lab, using a stereoscope microscope, to count the number of eggs, nymphs and empty pupae. Data were collected weekly, until the plants enter the R8 stage (pod filling), comprising 2.5 whitefly generations. Pods from three plants/treatment/block were harvested at the end of the plant cycle to evaluate the number of pods per plant, seeds per pod and mass of 100 seeds. Seed mass was corrected at 13% moisture. The mass of 100 seeds was estimated from the mass of seeds collected from three plants.

Bioassays with non-target organisms

To evaluate the potential effect of the transgenic common bean line Bt-22.5 on a non-target organism that feeds directly on bean leaves, with a feeding habit similar to the whitefly, we evaluated the reproduction of the black aphid *A. craccivora*. Five 4th instar nymphs of *A. craccivora* were carefully transferred to each primary leaf of transgenic (Bt-22.5) and non-transgenic (cv. Olathe Pinto) plants ($n = 4$), using a soft wet paintbrush. Leaves were isolated with individual little bags, made of voile fabric. Plants were then kept on a growth chamber at 25°C and 16 h light: 8 h dark photoperiod. 7 days later, the total number of aphids in each plant was counted using a stereoscope microscope. In another set of experiments, the whitefly parasitoid *E. formosa* was used to evaluate a potential

indirect effect of the transgenic common bean line to a beneficial non-target organism. Transgenic and non-transgenic common bean plants ($n = 10$, considering each primary leaf as a replicate), with their primary leaves fully expanded, were submitted to whitefly oviposition for 2 h. After that, the adults were removed and plants were isolated in cages. The apical leaves were pruned to avoid excessive plant growth. When the nymphs reached the 3rd instar, the plants were randomly distributed in a circle, in the middle of which a kale (*Brassica oleracea*) plant containing adults of *E. formosa* was placed, so that the parasitoids could move to the common beans to parasitize the whitefly nymphs. 2 days later, the adult parasitoids were manually removed from the common bean plants, using an insect aspirator. After 12 days, close to the parasitoid emergence, common bean leaves were collected to sample the number of parasitized nymphs and non-parasitized nymphs. These experiments were repeated twice and data from both experiments were analyzed together.

Additionally, during the greenhouse experiment to look at the effect of the transgenic line Bt-22.5 on the whitefly over two generations, a natural infestation of the leafminer *Liriomyza* sp. occurred, severely damaging the plants, because the common bean cultivar Olathe Pinto is highly susceptible to this insect pest and it was not possible to use insecticides without affecting the whitefly population. Then we included an assessment of the occurrence of the *Liriomyza* sp. larvae on the transgenic and non-transgenic plants, as another non-target insect species. The number of larvae was counted in three leaves per plant, and 3 plants/block ($n = 18$). The level of damage on the leaves was evaluated in the same leaves ($n = 18$), using a scoring scale from 1 to 4 (1 = no mining; 2 = a few mines in less than 20% of the leaflets, no defoliation; 3 = mines present in up to 50% of the leaflets, some defoliation leaflets; 4 = many mines in almost all the leaflets (90%) and defoliation of greater than 31%.), adapted from (Singh and Weigand, 1994).

Statistical analysis of the bioassays data

The homogeneity of variances was verified by the Levene test and data normality by the Shapiro–Wilk test. Means of normally distributed data were compared using the t test ($p < 0.05$). Non-parametric data was analyzed using the Wilcoxon test. In the preference assay, the number of eggs and adults was modeled using the GLM with Binomial Negative distribution. In the experiment to look at the whitefly generations on the common bean plants, the total number of adult insects, eggs, empty pupae and nymphs per treatment were analyzed using the above mentioned tests, considering block effects. For the scoring scales, the analysis was performed considering the frequency of each of the scoring scale per treatment. These frequencies were compared by the chi-square value and by a proportion test, where the null hypothesis indicated that the percentage of plants with a certain score was similar in the two treatments. All statistical analysis were performed using the R software (R CORE TEAM, 2019).

Results

Analysis of common bean transgenic plants

In 44 transformation attempts, 8,764 explants were subjected to particle bombardment for genetic transformation. Of these, only nine T_0 plants were positive for the presence of the *ΔvATPase* transgene, resulting in a low rate of transformed plants (0.1%), as expected (Russell et al., 1993; Aragão et al., 1996). From the nine T_0 plants, only two transmitted the transgene to the progeny (T_1). These two lines were named 11.5 and Bt-22.5. Among the 9 T_1 plants of the line 11.5 obtained, 7 plants were positive for the transgene, while for the line Bt-22.5, 3 of the 7 plants were positive. Seeds collected from individual self-pollinated T_1 plants were sowed for the progeny analysis of the T_2 (line Bt-22.5) and T_3 (line 11.5) generations ($n = 20$). Most of these lines did not segregate as expected (Table 1). However, all 20 plants from the progeny of the line Bt-22.5 were positive for the selection gene *Atahas*, indicating that this line was homozygous for the transgene *ΔATPase* (Table 1; Figure 2A). A similar pattern was observed for line Bt-22.5.6, in which, 19 of the 20 plants were positive for the transgene (Table 1).

Northern blot analysis revealed that plants from lines 11.5 and Bt-22.5 produced siRNA bands corresponding to the expected size range (Figure 2B). However, the siRNA band from line 11.5 was weaker than that of line Bt-22.5. No signal was observed for the non-transgenic control plants.

For the transgenic line Bt-22.5, no phenotypical difference was observed, compared to the non-transgenic plants. Additionally,

TABLE 1 Progeny analysis of T_2 and T_3 generations of transgenic common bean cv. Olathe Pinto lines (n=20).

Common bean line	Generation	Positive[a]	Negative[a]	χ^2	P[b]
11.5.1.3	T3	12	8	1.7	0.200
11.5.2.4	T3	8	12	11.3	0.001
11.5.3.5	T3	10	10	5.4	0.020
11.5.4.19	T3	9	11	8.1	0.005
11.5.5.12	T3	15	5	0.0	1.000
11.5.6.21	T3	10	10	5.4	0.020
11.5.7.23	T3	7	13	15.0	0.0001
Bt-22.5.6	T2	19	1	3.3	0.07
Bt-22.5.5	T2	20	0	5.4	0.020
Bt-22.5.2	T2	10	10	5.4	0.020

[a]Data are based on PCR analysis for detection of the ΔATPase transgene.
[b]Probability of the observed segregation fits the expected 3:1 Mendelian ratio at 95% confidence interval.

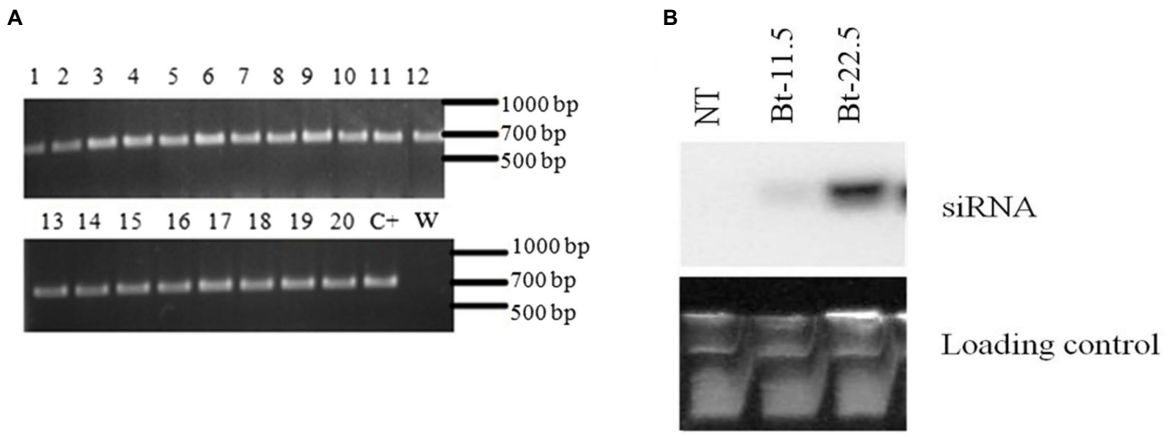

FIGURE 2
Analysis of common bean transgenic plants and relative expression of the *vATPase* gene in the whitefly *Bemisia tabaci*. **(A)** Progeny analysis of the transgenic common bean line Bt-22.5 for the marker gene *ahas*. Numbers 1 to 20 correspond to the 20 T_2 plants from seeds collected from plant Bt-22.5, C+ is the positive control and W is water, used as negative control. **(B)** Northern blot analysis for the detection of *Bemisia tabaci* vATPase small interfering RNA (siRNA) isolated from transgenic common bean lines Bt-11.5 and Bt-22.5. NT is the non-transgenic common bean cv. Olathe Pinto. SYBR Safe stained RNA served as loading control.

the number of pods per plant, seeds per pod and the mass of 100 seeds did not present significant difference between treatments (Supplementary Table S1).

Effect of the transgenic plants on the whitefly

Mortality of adult whiteflies was significantly higher in the transgenic common bean line Bt-22.5 both for the detached leaf and for the whole plant experiments (Figure 3), compared to the controls. The experiment with detached leaves resulted in a higher mortality (97%) than the experiments with the whole plant assays (59%; Figure 3). In contrast, when insects fed on detached leaves of the line 11.5, mortality was not different from that observed in the control plants (data not shown).

Expression of the *vATPase* gene in insects was significantly reduced when they fed on the transgenic common bean line Bt-22.5, less than half of the expression observed in insects that fed on the control plants (Figure 4). In the preference assay, a reduced proportion of adult whiteflies (27.6%) and eggs (25.9%) was observed, on average, on the transgenic plants compared to the number of insects sampled on the control plants (data not shown). In the experiment to look at the whitefly generations during the cycle of the common bean plants, 2.5 whitefly generations were evaluated. The total number of eggs, empty pupae and nymphs did not differ between transgenic and non-transgenic plants (Figure 5A). However, the total number of adult insects was significantly lower on the transgenic plants (Figure 5A). Accordingly, the average number of adults per treatment was significantly higher on the control plants in four of the eight sampling dates (Figure 5B). Remarkably, when the second-generation adults began to emerge (May 3rd and May 9th), the population increased significantly faster in the non-transgenic controls, until the number of adults almost coincided in the last sampling date.

Effect of the transgenic common bean line Bt-22.5 on virus transmission by the whitefly

Visual symptoms of virus disease were observed in 61.2% of the non-transgenic plants, while in the transgenic plants only 45.5% of the plants virus symptoms (non-significant; Table 2). For the plants that presented virus symptoms, the proportion of plants in each virus disease severity score was not different between treatments (Figure 6). Although many plants were asymptomatic, PCR analysis showed that the proportion of plants infected with CPMMV and BGMV did not differ between treatments (transgenic vs. non-transgenic; Table 2).

Non-target organisms

The reproduction of the black aphid *A. craccivora*, a non-target insect pest, was unaffected after feeding on the common bean transgenic line Bt-22.5 for 7 days ($p < 0.05$; Figure 7A). In the bioassays with the whitefly nymph parasitoid *E. formosa*, the number of parasitized whitefly nymphs in the transgenic plants was not significantly different from that of the non-transgenic plants (Figure 7B). Additionally, the average number of *Liriomyza* sp. larvae on the transgenic and non-transgenic plants was not significantly different (Figure 7C). Also, both transgenic and non-transgenic plants were similarly damaged by the leafminer

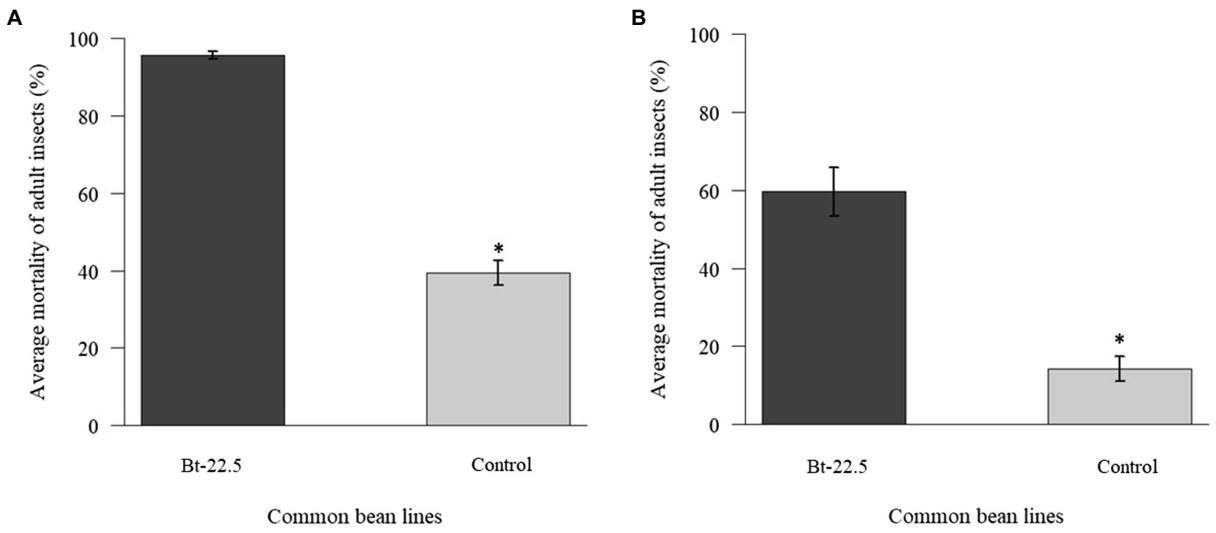

FIGURE 3
Mortality of adult whiteflies *Bemisia tabaci* after silencing their *vATPase* gene on the transgenic common bean line Bt-22.5. **(A)** Detached leaves (n=15) and **(B)** whole plant (n=10) experiments (*p<0.05).

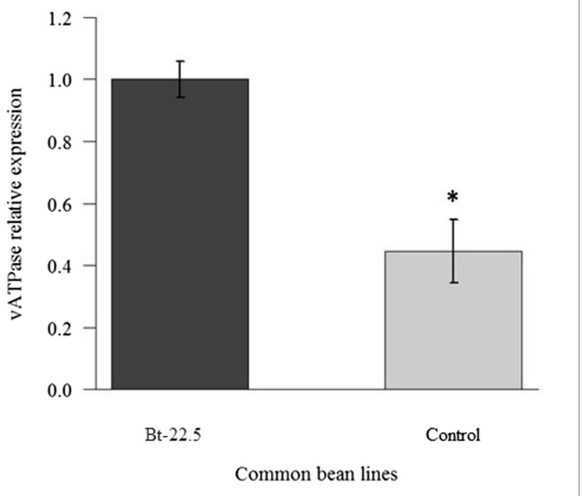

FIGURE 4
Relative expression of the transcripts of the *vATPase* gene in *Bemisia tabaci adults* after 48h of feeding on the transgenic common bean plants, determined by qRT-PCR (*p<0.05, n=3).

larvae, with no difference on the level of damage they caused on the plants (Figure 7D).

Discussion

Common bean is a staple food in Brazil, with social and economic importance. The crop is produced all over the country, in three growing seasons per year, which means that insect pests have a favorable environment to reproduce and keep high populations throughout the year. Whitefly management in the common bean crop is particularly relevant, because this insect is a vector of viruses that can severely impair crop yield and grain quality (Souza et al., 2016). Chemical control of the insect population has been the most used control method, although alternatives have been developed, such as biological control and virus resistant common bean cultivars (Faria et al., 2016; Souza et al., 2018; Sani et al., 2020; Silva et al., 2022). Common bean cultivars with tolerance to the whitefly through antixenosis have been reported, resulting in reduced number of eggs, nymphs and adults sitting on the plants, in field assays (Silva et al., 2014, 2019; Hoshino et al., 2017; Jesus et al., 2021). In spite of the identification of these sources of tolerance in the common bean germplasm, no common bean cultivar has been developed or registered for resistance to the whitefly, to date. Using RNAi to silencing genes in the insect might be a more specific and durable strategy, with potential to cause high insect mortality. The whitefly *B. tabac*i is a highly efficient vector of plant viruses, able to transmit numerous viruses from mixed-infected plants with different levels of efficiency. Some of these viruses are transmitted by the whitefly in a non-persistently manner, which means that the virus acquisition period is very short (1 to 3 min). Insect probing behavior plays a major part in this mode of transmission, meaning that even brief probes can be sufficient for a quick plant-to-plant spread of these viruses. Considering this insect-virus mode of interaction, a strategy aiming to cause insect mortality is more promising for virus disease management. Plant genetic transformation for gene silencing *via* RNAi is a viable option to achieve significant insect mortality rates and it has been successfully used to obtain stable transgenic whitefly-resistant plants, such as tomato and lettuce (Ibrahim et al., 2017; Pizetta et al., 2021; Xia et al., 2021), but not common beans, so far.

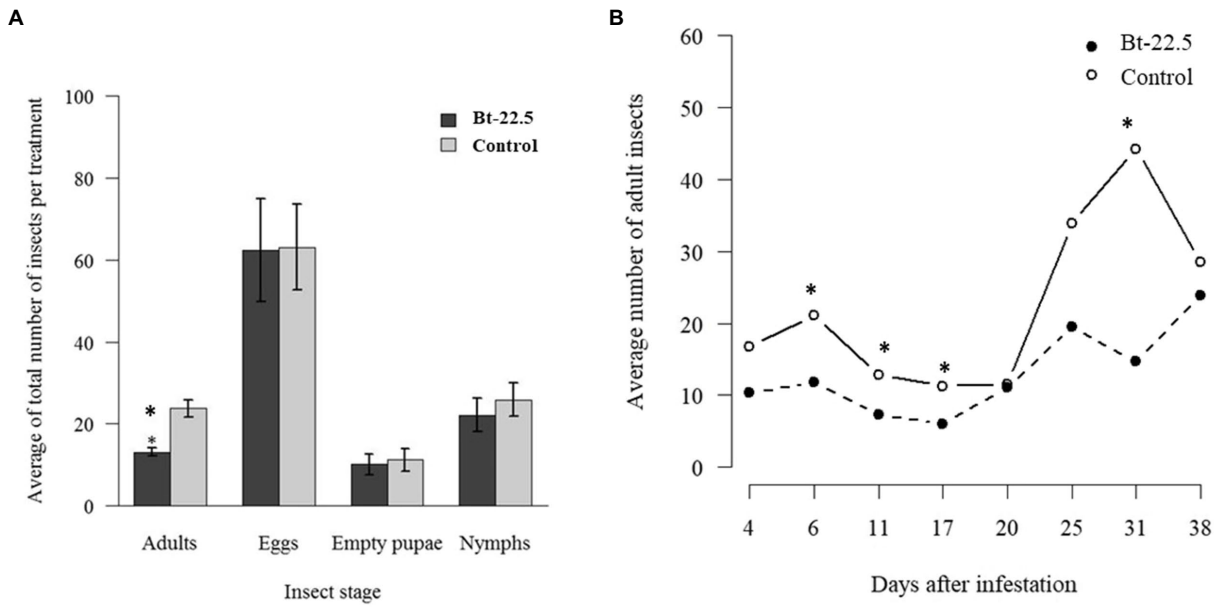

FIGURE 5
Development of the whitefly population during the cycle of the transgenic common bean plants. **(A)** Average of the total number of adult insects, eggs, empty pupae and nymphs per treatment; **(B)** Average number of adult insects per treatment in each sampling date (*p<0.05).

TABLE 2 Proportion of common bean cv. Olathe Pinto plants (non-transgenic) with virus symptoms, positive for BGMV and CPMMV by PCR, after inoculation by viruliferous whiteflies previously fed on the transgenic common bean line Bt-22.5 or on the non-transgenic plants for 48h.

Virus detection	Transgenic line Bt-22.5	Non-transgenic line	Value of p
Plants with virus symptoms	10/22 (45.5%)	21/34 (61.2%)	0.355544
BGMV$^+$ plants	2/12 (16.7%)	3/12 (25%)	0.932414
CPMMV$^+$ plants	12/12 (100%)	12/12 (100%)	NA

Our results show the development of the first common bean transgenic line with tolerance to the whitefly *B. tabaci*. Stable transformation of grain legumes has been considered a challenge (De Clercq et al., 2002). Because the common bean *P. vulgaris* is recalcitrant to *in vitro de novo* regeneration from callus, *Agrobacterium tumefaciens*-mediated genetic transformation of common beans is still difficult to achieve and therefore, the most reliable technique that made it possible obtaining a commercial cultivar of transgenic common bean, to date, was particle bombardment (Aragão et al., 1996; Faria et al., 2016). This technique presents a lower transformation rate, compared to *A. tumefaciens*-mediated genetic transformation of other plant species (De Clercq et al., 2002). Accordingly, in our experiments, only two stable transgenic common bean lines were obtained, that is, which passed the transgene to their progeny, representing an efficiency rate of 0.02%. From those, only one of them presented a significant amount of targeting siRNA, associated with a significant insect mortality and silencing of the target gene in the insects. These results are in agreement with the gene silencing ability of the other transgenic plant species, lettuce and tomato, that our team previously engineered using the same genetic construction (Ibrahim et al., 2017; Pizetta et al., 2021). In lettuce, silencing of the whitefly *vATPase* gene was associated with higher mortality, from 83.8–98.1% (Ibrahim et al., 2017), while in tomato, insect mortality was similar to our current results with the common bean line Bt-22.5, about 60% (Pizetta et al., 2021). Silencing of the *vATPase* gene has been reported as an efficient method to interfere with survival and development of the whitefly *B. tabaci* (Upadhyay et al., 2011; Thakur et al., 2014), although it seems to be more effective on adult insects. Significant mortality of 2nd instar nymphs was reported for the transgenic tomato, while the transgenic lettuce also delayed the whitefly development from nymphs to pupae (Ibrahim et al., 2017; Pizetta et al., 2021). In the current work, no significant difference in the survival or development of the whitefly young stages was observed on the transgenic common bean lines (data not shown). The other common bean line obtained in the current work, named 11.5, although positive for the transgene, produced a smaller amount of siRNA and did not cause significant insect mortality. This might be related to the DNA integration site, number of transgene copies and other inherent obstacles of plant genetic transformation. The transgenic plants did not present any other phenotypical difference from the non-transgenic plants.

Conducting mortality experiments with adult whiteflies is challenging because *B. tabaci* is a small, fragile and highly mobile insect, which makes it difficult to handle the insects without damaging their stylets, for example, when using an

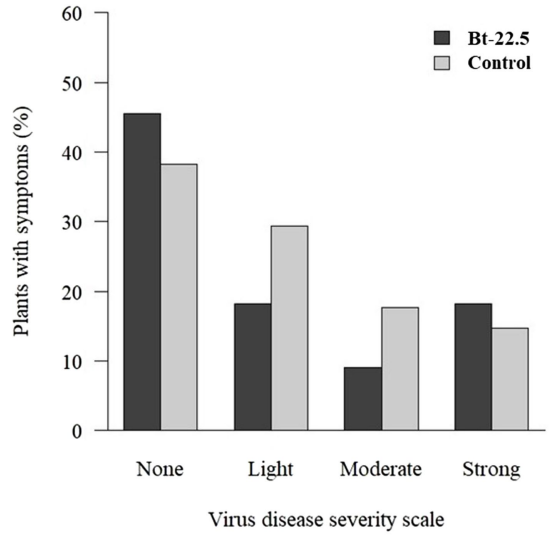

FIGURE 6
Proportion of transgenic and non-transgenic common bean plants with visual symptoms of virus disease, according to a scoring scale from 1–4, in which 1=no symptom, 2=light symptoms, 3=moderate symptoms and 4=strong symptoms (Arias et al., 2015).

insect aspirator. Also, it is difficult to visualize dead insects on the plant or in the soil because the tiny whiteflies disintegrate very quickly. Therefore, we tested different methods for the mortality experiments, such as using detached leaves, to reduce the space in which the dead insects would be located. However, the detached leaf experiments resulted in high mortality rates, which could be artificial. To check that, we developed another methodology to conduct the experiments using whole plants and minimal insect handling. For that, we exposed the plants to adult whiteflies for oviposition and then, we removed the adults and waited until the nymphs developed into a new generation of adults on the plants. This methodology has the advantage of minimally disturbing the insects while they develop from egg to adult on the transgenic plant, thus increasing the exposition time of insects to the transgene and also reducing the mortality due to random effects in the controls. Our results show insect mortality on the common bean line Bt-22.5 was higher on the experiment with detached leaves, in comparison with the experiment with whole plants. Even so, the mortality in the whole plant indicates a good level of tolerance to the insect, which can contribute to pest management, along with other management tools already available. Accordingly, in other studies the two methods of bioassays generally show a positive data correlation, although in some cases the responses point to more or less pronounced effects, depending on the target organism (Sharma et al., 2005; Michel et al., 2010; Miller-Butler et al., 2018). Also, for distinguishing resistant from susceptible genotypes, the two methodologies generally correlate well. This is in agreement with our observations, which show that the experiments with detached leaves were useful to select the most promising resistant lines for further confirmation with the whole plant methodology. Some variation can still be found in future experiments, depending on uncontrolled field and climate conditions.

In the preference assay, the number of adult insects and the number of eggs were reduced on the transgenic common bean line Bt-22.5, which is in agreement to the lower oviposition reported for the transgenic lettuce and tomato, genetically engineered with the same RNAi construct (Ibrahim et al., 2017; Pizetta et al., 2021). Corroborating this result, in the experiment to look at the whitefly generations during the cycle of the common bean plants, the total number of adults and the number of adults per sampling date were lower on the transgenic plants, suggesting that these plants contributed to reduce the whitefly population and the start of the second generation. A similar pattern was observed in the study with the transgenic lettuce resistant to the whitefly, although in that study, the number of insects from all stages were lower on the transgenic plants (Ibrahim et al., 2017). However, most studies on the development of transgenic plants in the literature do not report the effect of gene silencing over insect generations, or during the plant life cycle.

Regarding virus transmission, our results show that feeding on the transgenic plant did not affect the whitefly ability to transmit two viruses, in different modes (circulative and non-circulative). In fact, silencing the *vATPase* gene in the insect was not expected to affect virus transmission, because this gene has not been reported to be as relevant for the vectoring ability of Hemipterans as other genes, such as *HSP70*, *cathepsin B*, *cyclophilin B* and *α-glucosidase* (Götz et al., 2012; Chen et al., 2016; Pinheiro et al., 2017; Hasegawa et al., 2018; Kanakala et al., 2019; Lu et al., 2021). A transcriptomic study showed that the *vATPase* gene was not differentially expressed in whiteflies that acquired the tomato chlorosis virus (ToCV), compared with insects that fed on non-infected plants (Kaur et al., 2017). The variation in the level of virus symptoms in the plants that we observed in the present study are similar to natural infections in the field and may be explained by other factors, for example, environmental effects, number of insects feeding on each plant and viral load variation among insects.

Furthermore, the transgenic common bean line Bt-22.5 did not cause unexpected effects on the reproduction and development of three non-target organisms. Two of these insect species are also considered as insect pests: the black aphid *A. craccivora*, which has a feeding habit similar to the whitefly, and the leafminer *Liriomyza* sp. Our results are in agreement with the non-target assays conducted for the whitefly-resistant transgenic tomato lines with different non-target organisms, also insect pests, such as the green peach aphid *Myzus persicae*, the spider mite *Tetranychus urticae* and the tomato leafminer *Tuta absoluta* (Pizetta et al., 2021; Xia et al., 2021). This suggests that even for the organisms with similar *vATPase* gene sequences, silencing of the *B. tabaci vATPase* was specific to the target species. The other non-target organism evaluated is a beneficial insect, the parasitoid of whitefly nymphs *E. formosa*. Our results show that silencing the whitefly *vATPase* did not affect the ability of the parasitoid to reproduce in the whitefly nymphs.

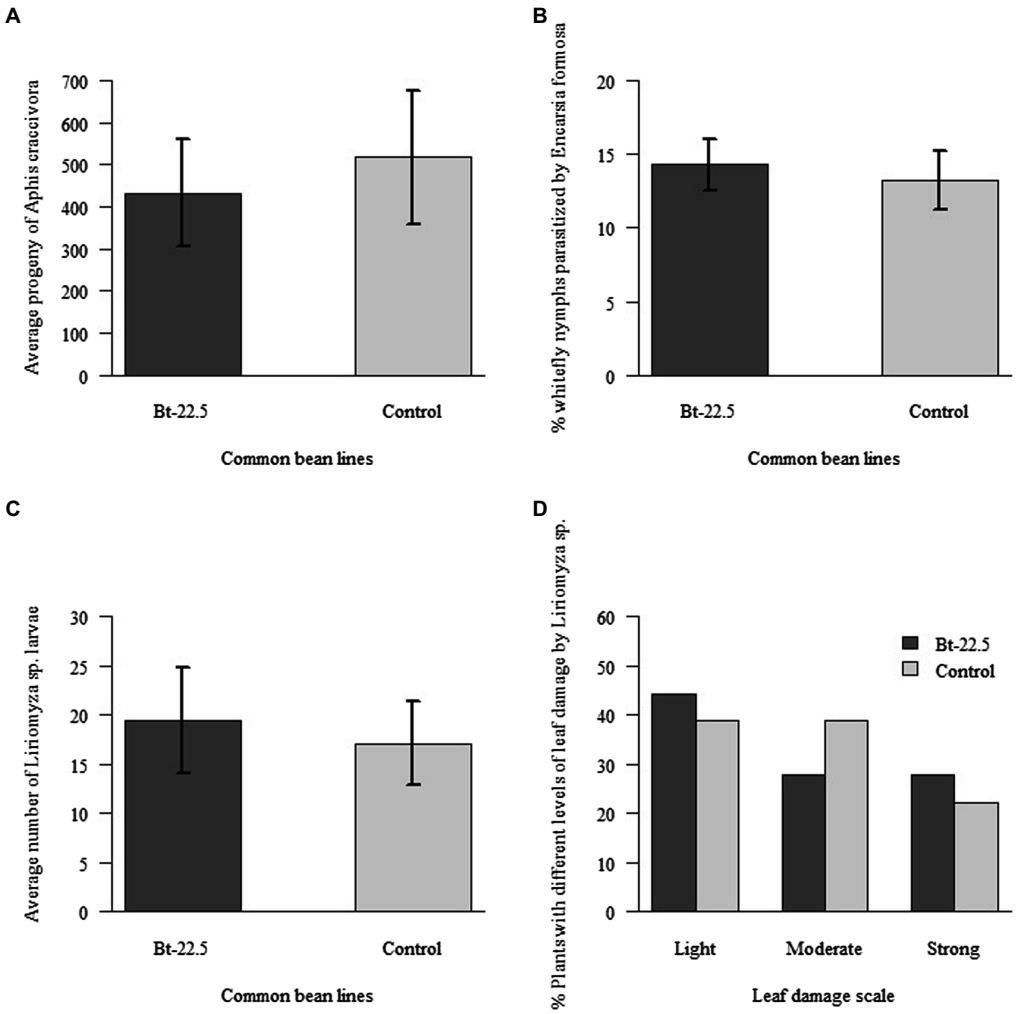

FIGURE 7
Development of non-target insects on plants of the transgenic common bean line Bt-22.5 expressing an RNAi construct for the silencing of the whitefly *Bemisia tabaci vATPase* gene, compared to the non-transgenic control: **(A)** Average progeny of the black aphid *Aphis craccivora* (*n*=3); **(B)** Average percent of whitefly nymphs parasitized by *Encarsia formosa* (*n*=3). **(C)** Average number of larvae of the leafminer *Liriomyza* sp. (*n*=18); **(D)** Percent of plants with different levels of leaf damage from *Liriomyza* sp.

In summary, our results show that the transgenic common bean line Bt-22.5 can contribute with the management of the whitefly, along with other management tools, with potential to reduce the need of numerous insecticide sprays. The next step will be crossing the line Bt-22.5 with elite genotypes from the Embrapa common bean breeding program, to introduce the whitefly tolerance into common bean genotypes along with other desirable agronomic traits, such as high yield, grain quality and multiple virus resistance (BGMV, BCMV and CPMMV), as it was recently reported (Silva et al., 2022).

Conclusion

Our results show the development of a stable transgenic common bean plant tolerant to the whitefly *B. tabaci* that can eventually be used as an additional management tool in Integrated Pest Management (IPM). Plant-mediated silencing of the *B. tabaci vATPase* gene conferred a reasonable level of whitefly-tolerance to the transgenic common bean line. The transgenic plants did not show any other phenotypical difference, nor negative effects on the evaluated non-target insect species. This transgenic common bean event represents a sustainable pest management strategy that might contribute to avoid the intensive use of insecticides and to reduce environmental and financial costs.

Data availability statement

The datasets generated for this study can be found in the Embrapa's research data repository, SIEXP [https://www.siexp.cnptia.embrapa.br/siexp-mweb/]. Transgenic seeds will be made available after the development and release of a new commercial cultivar.

Author contributions

PP, JF, FA, AF, JB, LH, and TS contributed to the conception and design of the study. JF, AF, GC, MM, FA, PP, and EF conducted the genetic transformation assays and *in vitro* regeneration of transgenic plants. AF, AZ, JB, PP, and JS contributed to the conduction of the bioassays with insects. AF, FA, CP, and PP conducted the siRNA quantification assays. JS, AF, and PP performed the statistical analysis and elaborated graphs and figures. PP, FA, LH, and TS contributed with research grant funding application and management. AF wrote the first draft of the manuscript. PP wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.984804/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Plasmid map displaying the organization of the whitefly *Bemisia tabaci vATPase* partial gene sequence cloned in sense and antisense, intercalated by intron 3 of the *malate synthase* gene of *Arabidopsis thaliana* for the production of siRNAs and the Atahas gene, with the complete promoter and terminator of *A. thaliana*, for the selection of transgenic plants, conferring resistance to the herbicide imazapyr.

SUPPLEMENTARY TABLE S1
Agronomic traits of the transgenic common bean line 22.5 and the non-transgenic control line.

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Characterization of fungal pathogens and germplasm screening for disease resistance in the main production area of the common bean in Argentina

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The common bean (*Phaseolus vulgaris* L.) is the most important grain legume in the human diet, mainly in Africa and Latin America. Argentina is one of the five major producers of the common bean in the world, and the main cultivation areas are concentrated in the northwestern provinces of this country. Crop production of the common bean is often affected by biotic factors like some endemic fungal diseases, which exert a major economic impact on the region. The most important fungal diseases affecting the common bean in Argentina are white mold caused by *Sclerotinia sclerotiorum*, angular leaf spot caused by *Pseudocercospora griseola*, web blight and root rot caused by *Rhizoctonia solani*, which can cause production losses of up to 100% in the region. At the present, the most effective strategy for controlling these diseases is the use of genetic resistance. In this sense, population study and characterization of fungal pathogens are essential for developing cultivars with durable resistance. In this review we report diversity studies carried out on these three fungal pathogens affecting the common bean in northwestern Argentina, analyzing more than 200 isolates by means of molecular, morphological and pathogenic approaches. Also, the screening of physiological resistance in several common bean commercial lines and wild native germplasm is reviewed. This review contributes to the development of sustainable management strategies and cultural practices in bean production aimed to minimize yield losses due to fungal diseases in the common bean.

KEYWORDS

white mold, angular leaf spot, web blight, Rhizoctonia root rot, *Sclerotinea sclerotiorum*, *Pseudocercospora griseola*, *Rhizoctonia solani*, fungal diseases

Introduction

The American continent is the center of domestication of many crops that are essential in the diet of human populations, such as maize (*Zea mays* L.), tomato (*Solanum tuberosum* L.), potato (*Solanum lycopersicum* L.), and common bean (*Phaseolus vulgaris* L.). The common bean is the dry grain legume most consumed in the world due to its high content of proteins, carbohydrates, fibers and minerals, being a main part of the diet of many countries in America and Africa (Broughton et al., 2003; Gepts et al., 2008). Domestication of the common bean occurred independently in two regions throughout the continent. Therefore two major gene pools, named Mesoamerican and Andean, are recognized in the population structure of the wild and the domesticated beans (Papa and Gepts, 2003; Papa et al., 2005, 2007; Rossi et al., 2009; Cortinovis et al., 2020; Tobar Piñón et al., 2021). Domesticated beans further diverged into genetically distinct races giving rise to the diversity of market types known today (Kwak and Gepts, 2009; Tobar Piñón et al., 2021).

Dry beans world production reached 27.5 million tons in 2020 (FAO, 2022). Argentina is among the top five common bean exporting countries and exports 90% of its production, supplying the crop to many Latin American countries (FAO, 2022). Bean production is located in the northwestern region of Argentina (NWA), comprising the provinces of Jujuy, Salta, Tucumán, Santiago del Estero and Catamarca. These regions are characterized by a great climatic and environmental heterogeneity, reaching a common bean production of 633.823 tons per year (FAO, 2022). Within this heterogeneous landscape, biotic stress is one of the main limiting factors for bean production (Basavaraja et al., 2020).

The common bean is affected by numerous diseases caused by fungi, viruses, bacteria and nematodes that affect production in different ways. To date, more than 200 diseases that cause significant losses in bean yield have been reported (Schwartz and Pastor Corrales, 1989; Assefa et al., 2019). Although NWA presents adequate conditions for common bean development, its production is constrained by different phytosanitary problems and the lack of disease resistance varieties. The main fungal diseases that affect bean production in the region are white mold [*Sclerotinia sclerotiorum* (Lib.) de Bary], angular leaf spot [*Pseudocercospora griseola* (Sacc.) Crous and U. Braun], web blight and Rhizoctonia root rot (*Rhizoctonia solani* Kühn). These are the most dispersed diseases in the different bean production areas in the country and are the most important due to the economic losses they cause (Vizgarra et al., 2011, 2012).

At the present, the most effective strategy for controlling these diseases is the use of genetic resistance. In this sense, population study and characterization of fungal pathogens are essential for developing cultivars with durable resistance. In this review we report diversity studies carried out on these three fungal pathogens affecting common bean in northwestern Argentina, analyzing more than 200 isolates by means of molecular, morphological and pathogenic approaches. Also, the screening of physiological resistance in several common bean commercial lines and wild native germplasm are covered in this review.

White mold

White mold (WM) caused by *Sclerotinia sclerotiorum* is one of the most destructive fungal diseases of the common bean worldwide (Boland and Hall, 1994). This necrotrophic fungus has a broad host range of more than 400 species in 75 plant families, including field crops, cereals, horticultural crops, trees, shrubs and several weed plants (Boland and Hall, 1994). Some of the major economic crops affected include dry bean, potato, soybean [*Glycine max* (L.) Merr.], sunflower (*Helianthus annuus* L.), canola (*Brassica napus* L.), lettuce (*Lactuca sativa* L.), carrot (*Daucus carota* L.), and pea (*Pisum sativum* L.) (Carpenter et al., 1999; Mert-Türk et al., 2007; Hemmati et al., 2009; Attanayake et al., 2013; Lehner et al., 2015; Abán et al., 2018; Panullo et al., 2018). In Argentina, WM has been detected in all bean production areas, reaching seed yield and quality losses up to 80–100% on susceptible common bean cultivars under favorable weather conditions (Singh and Schwartz, 2010). WM disease affects all aerial parts of plants regardless of the growth stages of the plant. Disease symptoms of WM typically begin with water-soaked lesions on leaves and stems (**Figure 1**). As the disease progresses, a thick white mycelium growth followed by hard black sclerotia is observed in internal and external tissues of the plant, which causes distal portions of the plant to wilt and then become necrotic (Steadman and Boland, 2005). Eventually, the plant will appear bleached in color, with plant parts showing shredded characteristics due to tissue breakdown (Purdy, 1979). Sclerotia can germinate myceliogenically to infect adjacent plant tissues and carpogenically via apothecia from which ascospores are dispersed within the crop. Sclerotia eventually fall to the ground as infected stems dry out and the host plant dies. These sclerotia serve as the primary source of inoculum of the disease (Bolton et al., 2006). The longevity of sclerotia in the soil varies from 1 year (Brustolin et al., 2016) to up to 8 years (Adams, 1979), making this pathogen extremely hard to control in the field. WM disease can also be spread by the movement of seeds contaminated and sclerotia mixed with seeds from one field to another, irrigation runoff water and wind-blown ascospores, which can travel a considerable distance of 3–4 km between fields (Cubeta et al., 1997; Steadman and Boland, 2005).

Sclerotinia sclerotiorum is a homothallic and haploid fungus that can reproduce asexually (clonally) by means of mycelium or sexually by means of self-fertilization or recombination (Attanayake et al., 2014) to produce apothecia with ascospores. However, sexual reproduction in haploid fungi is frequently

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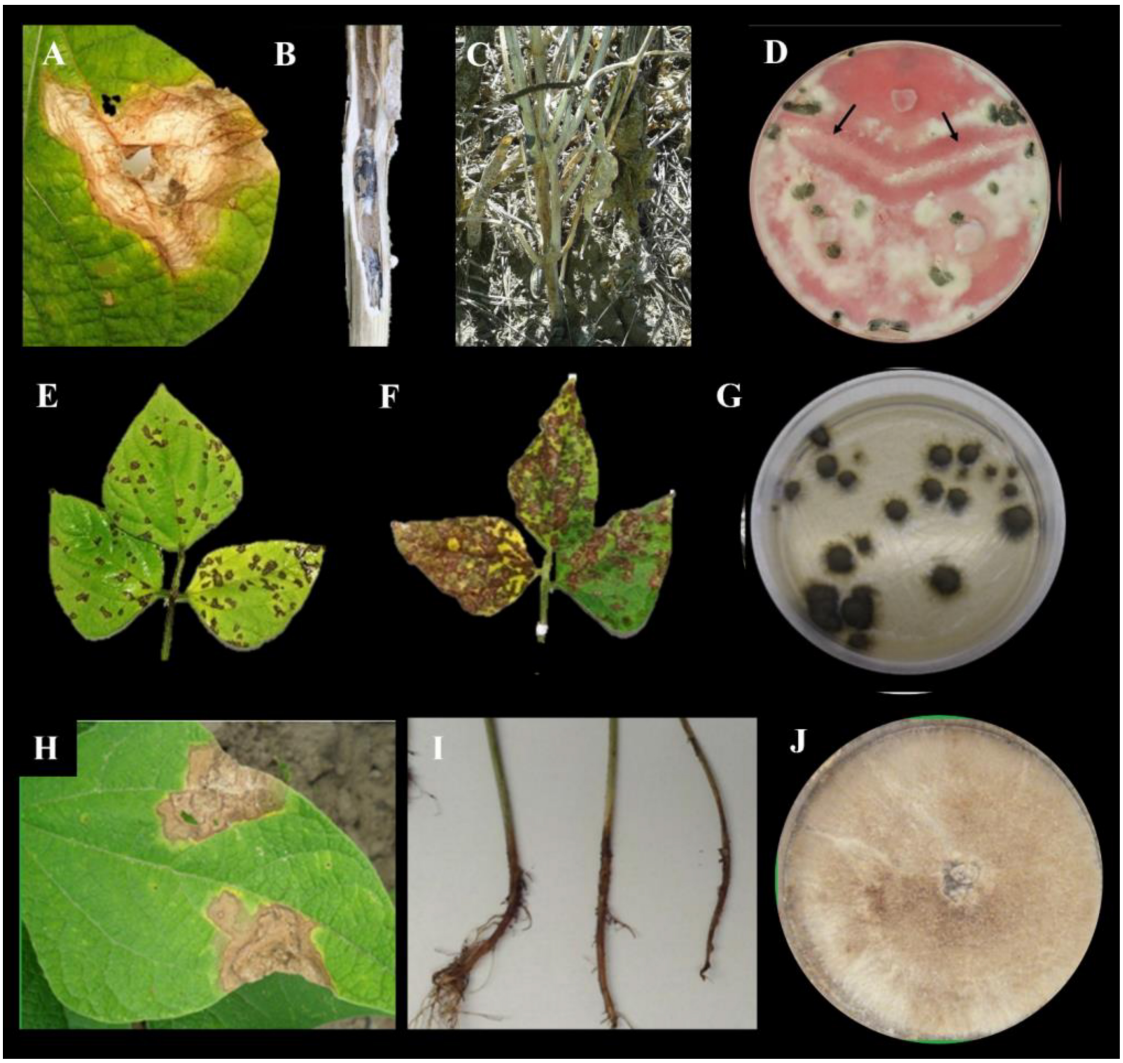

FIGURE 1
White mold symptoms on common bean **(A)** leaf and **(B)** stem. **(C)** Common bean cultivar showing white mold symptoms. **(D)** Mycelial compatibility test between three isolates of *Sclerotinia sclerotiorum*. The arrows indicate incompatible reactions with band of aerial mycelium in the interaction zone. **(E,F)** Common bean cultivars showing a susceptible reaction to *Pseudocercospora griseola*. **(G)** Andean *P. griseola* isolate. **(H)** Web blight symptoms on common bean leaf. **(I)** Rhizoctonia root rot symptoms on common bean. **(J)** *Rhizoctonia solani* isolates obtained from common bean seed and soil.

equivalent to clonal reproduction (Billiard et al., 2012) because the genetic exchange that exists is scarce and is not enough to break the predominant pattern of clonal population structure (Tibayrenc and Ayala, 2012). According to recent research, mycelial compatibility groups (MCGs) are useful as a rough measure of standing genotypic diversity but are not adequate to infer population genetic processes (Kamvar and Everhart, 2019; **Figure 1**). However, other studies have suggested taking into account the structure imposed by the MCGs in addition to a set of molecular markers in population analyses (Lehner and Mizubuti, 2017; Lehner et al., 2019; Silva et al., 2021). Over the past few years, the population structure of *S. sclerotiorum* has been extensively documented from different host crops and from different regions in the world (Atallah et al., 2004; Sexton et al., 2006; Hemmati et al., 2009; Ekins et al., 2011; Attanayake et al., 2013; Clarkson et al., 2013, 2017; Aldrich-Wolfe et al., 2015; Dunn et al., 2017; Panullo et al., 2018; Faraghati et al., 2022). In early studies, *S. sclerotiorum* populations exhibited a predominantly clonal population structure with low genetic diversity based on MCGs and DNA fingerprinting genotypes (Kohli et al., 1992; Cubeta et al., 1997; Hambleton et al., 2002). However, in subsequent studies, evidence of recombinant populations and mixed population structures with high rates of genetic variability have been reported using microsatellite (SSR) markers and linkage disequilibrium measures (Atallah et al., 2004; Sexton and Howlett, 2004; Hemmati et al., 2009;

Attanayake et al., 2014; Panullo et al., 2018). Whether a pathogen population is clonal or recombining is best addressed by studying the association of alleles among different loci through the index of association (I_A) (Smith et al., 1993; Milgroom, 1996) based on clone-corrected data to reduce the bias produced by overrepresented data and to increase sensitivity in the detection of recombination (Milgroom, 1996). Recombinant populations with high rates of genetic variability tend to have a high evolutionary potential and therefore are more likely to overcome host resistance. Thus, the presence of *S. sclerotiorum* recombinant populations in a particular region is of great interest to the delineation of strategies for WM management and crucial for breeders seeking to develop new resistant cultivars (Milgroom, 1996; McDonald and Linde, 2002).

Despite the fact that the bean crop is cultivated in many countries, the genetic diversity and population structure of *S. sclerotiorum* in common bean crops have only been analyzed in Brazil (Lehner et al., 2015, 2017, 2019; Silva et al., 2021), the United States (Kamvar et al., 2017) and Argentina (Abán et al., 2018, 2021). In Brazil, the first study using microsatellite markers analyzed 79 isolates and reported high genotypic variability among *S. sclerotiorum* isolates (Gomes et al., 2011). However, in a subsequent study using linkage disequilibrium measures, Lehner et al. (2015) reported that despite the relatively high genotypic diversity observed among isolates, the SSR loci were in linkage disequilibrium, and thus, the *S. sclerotiorum* population had a clonal genetic structure. These results were later supported in larger studies, where the pathogen population of Brazil not only remained clonal but also structured according to MCGs (Lehner et al., 2019; Silva et al., 2021). Silva et al. (2021) analyzed 238 isolates, and only 22 MCGs and 64 SSR haplotypes were found, with no association between SSR haplotypes and MCGs. Although their clonal lineages were widely distributed in space and persistent over time, evidence of some degree of outcrossing was detected (Silva et al., 2021). In the case of common bean fields in the United States, Kamvar et al. (2017) reported that *S. sclerotiorum* populations had a clonal population structure with low genetic diversity using MCGs and SSRs. In this study, 366 isolates were analyzed from production fields and WM screening nurseries from dry bean cultivars among different geographic locations in the United States (320), France (22), Mexico (18), and Australia (6). A total of 165 MLH and 87 MCGs were observed, with no relationship between SSR haplotypes and MCGs. In contrast to Brazil, the United States populations from dry bean fields were structured by region, and no evidence of structuring by MCGs was detected.

In Argentina, the molecular and morphological identification of 116 *S. sclerotiorum* isolates from the main common bean production area was reported by Abán et al. (2018). Morphological identification was confirmed by PCR amplification and sequencing of the rRNA ITS region, which presented 100% similarity compared to *S. sclerotiorum* sequences. In addition, a first approach of the mode of reproduction and population structure was analyzed by means of MCGs and URPs (Universal Rice Primers) molecular haplotypes (Abán et al., 2018). A total of 52 MCGs and 59 URP haplotypes were found. All the MCGs were location specific, while only 12% of the URP haplotypes were shared among locations. Moreover, most of the isolates were highly aggressive, while no variation among locations was observed. Based on measures of multilocus linkage disequilibrium, the occurrence of both clonal and sexual reproduction was suggested in *S. sclerotiorum* populations from common bean fields in northwestern Argentina (Abán et al., 2018). Since most population structure analyses are based on SSR markers, a later study based on microsatellite markers was performed (Abán et al., 2021). In this study, 109 isolates of *S. sclerotiorum* from six dry bean fields in the main production area of Argentina were analyzed using nine microsatellite loci. A total of 30 SSR haplotypes were identified, of which 18 haplotypes were unique. Population genetic structure analysis based on linkage disequilibrium analysis suggested the occurrence of both modes of reproductive behavior, with sexual recombination being the most frequent (Abán et al., 2021). The high levels of recombination and gene flow detected in this study highlighted the need for breeding programs to develop new cultivars resistant to WM.

The integrated management of the disease includes the use of resistant or tolerant cultivars, cultural practices, fungicide applications during the flowering stage, upright growth habit plants, wide row spacing in combination with low plant density (Vieira et al., 2012, 2022), and biological control by different antagonistic fungi, bacteria and organic amendments, which has been recently reviewed by Smolińska and Kowalska (2018). Regarding biological control, different native strains of the genus *Bacillus* with the potential to control WM on bean seeds and seedlings in NWA, was reported by Sabaté et al. (2018). To date, however, there are no known common bean cultivars with complete resistance and current biological control methods are rarely sufficient to completely reduce the population of the pathogen; thus, fungicide applications remain the most effective tool for disease control, but overuse and misuse of fungicides increase the risk of fungicide resistance emergence (McDonald and Linde, 2002). Moreover, populations with frequent outcrossing will have relatively higher levels of genetic diversity; thus, the risk of fungicide resistance emergence is increased (McDonald and Linde, 2002). Hence, the best strategy to minimize yield losses and reduce production costs in a sustainable farming context is the use of varieties with genetic resistance to WM. When evaluating genetic resistance to WM, physiological resistance and disease avoidance traits are considered for the selection of resistant genotypes. Both characteristics are quantitatively inherited, and resistance and avoidance QTLs have already been identified (Mkwaila et al., 2011; Pérez-Vega et al., 2012; Miklas et al., 2013;

Vasconcellos et al., 2017). A comparative map including 27 QTLs for WM resistance and 36 QTLs for disease-avoidance traits was developed by Miklas et al. (2013). Vasconcellos et al. (2017) identified 37 QTLs located in 17 loci, nine of which were defined as meta-QTLs. These are robust consensus QTLs representing effects across different environments, genetic backgrounds and related traits. Moreover, within the confidence interval for five of the meta-QTLs, candidate genes expressed under *S. sclerotiorum* infection, such as ethylene-responsive transcription factor, peroxidase, cell wall receptor kinase *COI1* and MYB transcription factor were found. These nine meta-QTLs are recommended as potential targets for molecular marker-assisted selection for partial resistance to WM in the common bean (Vasconcellos et al., 2017).

Currently, there are no commercial bean varieties available with WM resistance. In previous studies, however, low levels of resistance have been reported in genotypes of Mesoamerican origin (Ender and Kelly, 2005; Pascual et al., 2010; Mkwaila et al., 2011) and in wild beans (Terpstra and Kelly, 2008; Mkwaila et al., 2011), and high levels of resistance have been reported in genotypes of Andean origin (Maxwell et al., 2007; Singh et al., 2007; Pascual et al., 2010; Mkwaila et al., 2011; Soule et al., 2011; Pérez-Vega et al., 2012). In addition, higher levels of WM resistance have been introgressed from interspecific crosses with secondary gene pool *Phaseolus* species such as *P. coccineus*, *P. polyanthus*, and *P. costaricensis* (Schwartz et al., 2006; Singh et al., 2009, 2013, 2014).

In Argentina, the physiological resistance of 20 common bean accessions (cultivars and lines) was assessed at 7, 14, and 21 days post-inoculation with five genetically distinct isolates of *S. sclerotiorum* collected from the main common bean growing area of NWA (Aban et al., 2020). These isolates were previously characterized using URP and SSR molecular markers, MCGs and pathogenicity tests (Abán et al., 2018, Aban et al., 2020). Based on the modified Petzoldt and Dickson scale (Terán et al., 2006), all cultivars and lines were susceptible at the end of the assessment, except line A 195, which was resistant to WM against the five isolates tested and was significantly different from all accessions. Line A 195 is a registered WM-resistant germplasm (Singh et al., 2007) from the Centro Internacional de Agricultura Tropical (CIAT) in Colombia. In previous studies, line A 195 showed partial levels of resistance to different highly and weakly aggressive *S. sclerotiorum* isolates (Viteri et al., 2015), including one pathogen isolate (ARS12D) collected in Salta, Argentina in 2012 (Viteri et al., 2015). Regional common bean breeding programs aimed at obtaining broadly adapted cultivars with durable resistance to WM should account for the regional variation within a pathogen population to ensure the development and release of durable WM-resistant common bean cultivars. Line A 195 is a promising parental genotype to be used in regional breeding programs.

Angular leaf spot

Angular leaf spot (ALS), caused by the ascomycota fungus *Pseudocercospora griseola*, is one of the diseases that causes great economic losses to bean production (Schoch et al., 2009). This pathogen is an important etiological agent mainly in countries with subtropical and tropical climates, such as Brazil, Argentina, Bolivia and African countries (Guzmán et al., 1995; Pastor-Corrales et al., 1998; Vizgarra et al., 1999, 2011; Ploper et al., 2002, 2016; Espeche et al., 2018). In recent years, the incidence of the disease has increased, causing great economic losses, favored by the monoculture system and the narrow genetic base of the commercial bean varieties. In Argentina, yield losses in common bean crops range from 20 to 50% (Stenglein, 2007), and in other regions, such as Brazil and African countries, yield losses can reach up to 80% of the total crop production (De Jesus et al., 2007; Singh and Schwartz, 2010).

ALS disease is mainly destructive in warm and humid areas, affecting the yield and quality of bean seeds. Symptoms are visible on leaves and pods, which present angular brown interveinal spots and circular brown lesions, respectively (**Figure 1**). The spots on the leaves eventually coalesce, causing premature defoliation (Crous et al., 2006). The pathogen conidia are spread mainly by wind and water droplets. However, agricultural practices have a great influence on the spread of the disease, being carried by agricultural implements and contaminated seeds that facilitate pathogen transmission.

In Argentina, ALS is considered one of the most destructive and problematic diseases for bean production (Vizgarra et al., 2011, 2012, 2016; Espeche et al., 2018). In NWA ALS is a widely distributed fungal disease, particularly in the south of Salta and southeast of Catamarca, mainly in black bean cultivars and in seasons with above-average rainfall during the reproductive period of the crop (Ploper et al., 2016). Under high disease pressure, a substantial reduction in leaf area is observed and the photosynthetic capacity of bean plants decreases during grain filling, when the demand for photosynthates is the highest (**Figure 2**; Cole, 1966; Hagedorn and Wade, 1974; Schwartz and Galvez, 1980; Cardona Mejía et al., 1995).

Knowledge of the genetic variability of the pathogen population present in each crop-producing region is extremely important for the development of effective management strategies. The ALS pathogen is known for the wide virulence diversity exhibited by isolates from different locations. *P. griseola* pathotypes are defined based on the pathogenicity reaction to a set of 12 common bean differential genotypes (Pastor-Corrales and Jara, 1995; **Supplementary Table 1**). Based on their reaction to ALS differential cultivars, all *P. griseola* pathotypes (known as races) are separated into Andean and Mesoamerican pathotype groups that correspond to the two common bean gene pools, sustaining the coevolution of the pathogen with its common bean host (Guzmán et al., 1995; Mahuku et al., 2002a;

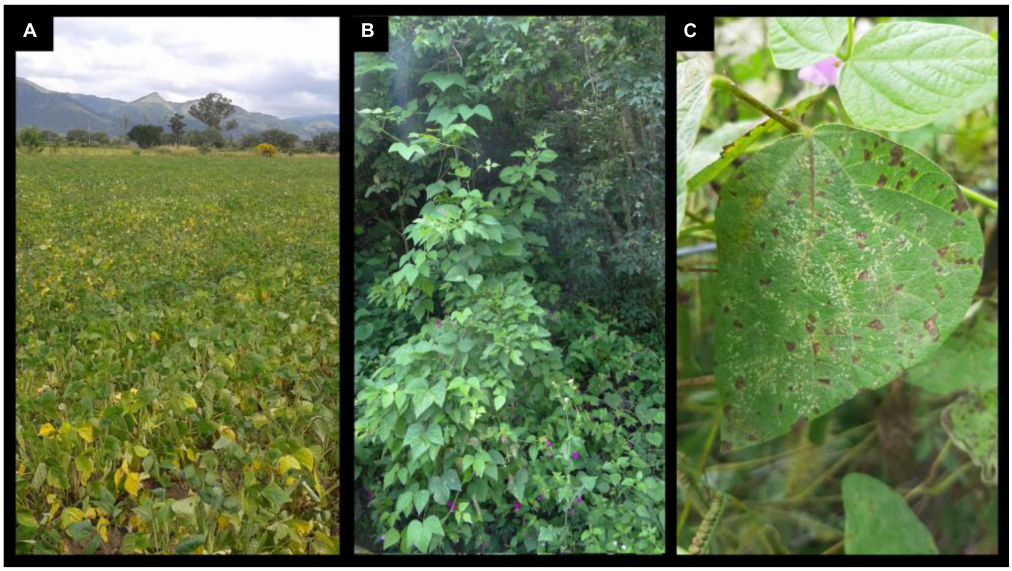

FIGURE 2
(A) Plants showing angular leaf spot symptoms in a common bean field in northwestern Argentina. **(B)** Argentinean wild bean exhibiting its characteristic indeterminate growth habit. **(C)** Wild bean showing angular leaf spot symptoms.

Stenglein and Balatti, 2006; Rezene et al., 2018). Isolates obtained from Andean cultivars were virulent only in Andean bean differential cultivars, which is why these races were called Andean, while isolates from Mesoamerican cultivars were virulent in Mesoamerican ones (Beebe and Pastor-Corrales, 1991; Mahuku et al., 2002a; Stenglein and Balatti, 2006). The existence of a third group of races, named Afro-Andean group, capable of infecting both Andean and Mesoamerican differential cultivars has been reported (Mahuku et al., 2002a,b; Wagara et al., 2004, 2011; Serrato-Diaz et al., 2020). The set of differential cultivars has been widely used throughout the world, allowing the comparison of *P. griseola* races between different localities, countries and even continents. The isolation and characterization of *P. griseola* in Argentina was first reported by Stenglein and Balatti (2006). In this study, 45 isolates collected within the main common bean production area in NWA were classified into 13 races based on the set of bean differential cultivars. Some races, such as 63–15 and 63–7, occurred more frequently than others with the coexistence of different races in certain areas of production (Stenglein and Balatti, 2006). The most pathogenic race was 63–63 reported in Zárate, Tucumán. Races that overcome the resistance of all differential cultivars have been reported in Argentina, Central America, Brazil, and Africa, suggesting the need to expand the number of differential cultivars to better identify these pathotypes (Stenglein and Balatti, 2006; Nay et al., 2019b). In this sense, new genotypes have been proposed as candidates to expand the standard set of differential cultivars (Nay et al., 2019b).

DNA sequence-based comparisons are of great importance to determine the diversity of a pathogen in a region and ensure the availability of an up-to-date barcode that provides meaningful information for plant health (Crous et al., 2013). The ITS region has been widely used by mycologists as a standard barcode, and ITS sequences are currently available for several fungal species identified in public databases (Begerow et al., 2010; Schoch et al., 2012; Rezaee Danesh and Demir, 2020). With respect to *P. griseola*, Aparicio (2020) reported the taxonomical identification of Argentinian pathotypes based on ITS sequences, differentiating the isolates of *P. griseola* f. *mesoamericana* from the isolates of *P. griseola* f. *griseola*, generating a phylogenetic tree similar to that previously obtained by Crous et al. (2006). In addition, polymorphic sites in the sequences of the ITS 1 and ITS 2 regions were identified, which are useful for the development of diagnostic specific oligonucleotides based on the single nucleotide polymorphisms (SNPs) detected.

Several molecular markers have been used to analyze ALS pathogen variability (Guzmán et al., 1999; Mahuku et al., 2002b, 2009; Stenglein and Balatti, 2006; Abadio et al., 2012; Ddamulira et al., 2014; Nay et al., 2019a). However, finding genetically accurate and operationally simple markers for the study of *P. griseola* variability is not an easy task (Mahuku et al., 2002b). In Argentina, high levels of genetic diversity were observed within the Mesoamerican and Andean groups of the fungus using dominant molecular markers (Stenglein and Balatti, 2006; Aparicio, 2020), in agreement with previous reports from Africa and Brazil (Mahuku et al., 2002b; Abadio et al., 2012). Molecular analyses of Argentinean *P. griseola* isolates performed

with RAPD and ISSR markers (Stenglein, 2007) significantly distinguished between the Mesoamerican and Andean isolates; however, unique band patterns or haplotypes were generated for less than 50% of the isolates analyzed. Guzmán et al. (1999) developed specific primers to identify *P. griseola* isolates of each gene pool. However, these specific primers were only efficient in differentiating isolates of Andean origin when used on Argentinean isolates (Aparicio, 2020), demonstrating the wide variability exhibited by isolates from different regions. On the other hand, URP markers were found to be useful tools to differentiate ALS pathogen isolates, being even more efficient than RAPD and ISSR markers (Aparicio, 2020).

Diversity studies of isolates from Argentina showed that *P. griseola* had great pathogenic variability (Stenglein, 2007; Aparicio, 2020). Although Mesoamerican isolates of *P. griseola* had greater genetic diversity than the Andean isolates (Wagara et al., 2004), Aparicio (2020) reported a greater diversity in the Andean group. This may be due to the introgression of genes from the Mesoamerican to Andean isolates, which was also suggested by Stenglein (2007), since in this region, both types of beans (Mesoamerican and Andean) are grown. Moreover, in the same leaf of a bean plant, isolates belonging to the Mesoamerican and Andean groups can be found (Guzmán et al., 1999; Stenglein and Balatti, 2006; Stenglein, 2007; Crous et al., 2013). Based on what is known about the coevolution between the gene pools of the host and the pathogen of the common bean and the high virulence and potential for overcoming resistance of the pathogen, Andean and Mesoamerican resistance gene pyramiding would be the most appropriate strategy to generate cultivars with durable ALS resistance (de Carvalho et al., 1998; Sartorato et al., 1999; Corrêa et al., 2001; Namayanja et al., 2006; Gonçalves-Vidigal et al., 2011, 2013; Vizgarra et al., 2011; Oblessuc et al., 2012, 2013, 2015; Goncalves-Vidigal et al., 2020).

To date, integrated management is the most widely used strategy for ALS management, which involves cultural methods (crop rotation, seed sanitation and adequate planting dates), chemical methods (fungicide use) and biological methods (resistant genotypes). Numerous studies agree that the most sustainable strategy to control ALS disease is the use of resistant cultivars. Many genotypes were evaluated in search of new sources of ALS resistance, including the identification of SNP markers to be used in breeding assisted selection and pyramidization of resistance genes (Singh and Schwartz, 2010; Nay et al., 2019b). The TUC 550 cultivar, was the first black bean cultivar with resistance to ALS in Argentina developed by the Estación Experimental Agropecuaria Obispo Colombres (EEAOC) from germplasm introduced from CIAT. This cultivar was released in 2010 and showed resistance to different races of the pathogen that were the most prevalent in bean cultivated areas (Vizgarra et al., 2018). These results highlight the importance of knowing the local variability of *P. griseola* isolates to generate genotypes adapted to the region and with durable resistance over time. Other cultivars, such as MAB 333 and MAB 336, introduced from CIAT reported high levels of resistance to angular leaf spot in field evaluations (Vizgarra et al., 2011). Recently, the TUC180 and TUC241 cultivars, that are red and cranberry type beans, were reported to be resistant to races 63–7 and 31–0 by Aparicio (2020). These genotypes are new potential parents for future combinations, considering that breeding for ALS resistance should be continuous because of the high pathogenic variability exhibited by the pathogen.

The identification of new resistance genes is a major goal for geneticists to broaden the common bean genetic base against the ALS pathogen, to understand the nature of defense genes and to define haplotypes for marker design to assist in breeding. Resistance to the ALS pathogen is largely conferred by single dominant resistance genes, named *Phg-1*, *Phg-2*, and *Phg-3*, but a quantitative nature of resistance that includes two major QTLs named *Phg-4* and *Phg-5* has also been reported (de Carvalho et al., 1998; Sartorato et al., 1999; Corrêa et al., 2001; Teixeira et al., 2005; Namayanja et al., 2006; Chataika et al., 2010; Gonçalves-Vidigal et al., 2011, 2013; Oblessuc et al., 2013, 2012; Keller et al., 2015; Nay et al., 2018, 2019a). The *Phg-1*, *Phg-4*, and *Phg-5* loci are from common bean cultivars of the Andean gene pool, whereas *Phg-2* and *Phg-3* are from beans of the Mesoamerican gene pool. The *Phg-1* locus mapped on chromosome Pv01 in the AND 277 cultivar (Gonçalves-Vidigal et al., 2011), the *Phg-2* locus mapped on chromosome Pv08 in México 54 cultivar and its allele *Phg-2*^{2} is present in BAT 332 (Sartorato et al., 1999; Namayanja et al., 2006), and the *Phg-3* locus mapped on Pv04 in the Ouro Negro cultivar (Corrêa et al., 2001; Faleiro et al., 2003; Gonçalves-Vidigal et al., 2013). On the other hand, the major QTL *Phg-4* mapped on chromosome Pv04 in the G5686 and CAL 143 cultivars (Mahuku et al., 2009; Oblessuc et al., 2012; Keller et al., 2015; Souza et al., 2016), and the QTL *Phg-5* mapped on Pv10 in the CAL 143 and G5686 cultivars (Oblessuc et al., 2012, 2013; Keller et al., 2015; Souza et al., 2016).

Currently, breeding is based on a few well-characterized single resistance genes that are easily transferred to elite commercial cultivars (Nay et al., 2019b). However, due to the wide virulence diversity of *P. griseola*, there is a high risk of losing this resistance. Therefore, new breeding strategies based on a broad diversity of qualitative and quantitative spectra of resistance genes are essential for the development of cultivars with durable resistance (Nay et al., 2019b).

Until a few years ago, most ALS resistance studies were based on biparental mapping populations with the identification of associated markers that were often polymorphic only in segregating populations from specific crosses. Currently, with the availability of a reference genome of common bean (Schmutz et al., 2014; Vlasova et al., 2016) and the development of high-throughput genotyping platforms (Hyten et al., 2010; Goretti et al., 2014; Gujaria-Verma et al., 2016; Raatz et al., 2019), genome-wide association studies (GWAS) have become an efficient and powerful tool for the discovery of novel ALS

resistance genes (Perseguini et al., 2016; Zuiderveen et al., 2016; Tock et al., 2017; Fritsche-Neto et al., 2019; Nay et al., 2019a; Vidigal Filho et al., 2020). Perseguini et al. (2016), using GWAS with 180 common bean accessions, identified QTLs controlling resistance to anthracnose and ALS diseases. A total of 11 SSRs and 17 SNPs associated with resistance to race 0–39 of *P. griseola* were detected. The authors reported three SNP markers, two located on chromosome Pv03 and one on Pv07, that were associated with both diseases. Nay et al. (2019a) conducted GWAS in a large common bean panel, which included the ALS most resistant genotypes available at CIAT, and tested it under greenhouse and field conditions at multiple sites in Colombia and Uganda. A major ALS resistance locus conferring resistance in all trials was detected on chromosome Pv08, coinciding with the previously characterized resistance locus *Phg-2* (Sartorato et al., 1999). The resistance locus *Phg-4* on chromosome Pv04 was effective against one particular pathotype. Moreover, DNA sequence-based clustering identified eleven functional haplotypes at *Phg-2*; one conferred broad-spectrum ALS resistance, and six showed pathotype-specific effects (Nay et al., 2019a). The authors highlighted the importance of ALS pathotype specificity for durable resistance management strategies in common bean. Fritsche-Neto et al. (2019) performed GWAS in 60 inbred elite lines from Brazil and evaluated them under field conditions, identifying one SNP associated with ALS resistance loci on chromosome Pv10 and two SNPs associated with anthracnose resistance loci on chromosome Pv02. Vidigal Filho et al. (2020) conducted a GWAS approach using 115 Brazilian accessions and reported SNP markers associated with resistance to race 31–23 of *P. griseola*, which mapped on chromosomes Pv02 and Pv04, whereas for race 63–39, SNPs were mapped on chromosomes Pv03, Pv06, and Pv08. Recently, de Almeida et al. (2021) performed GWAS and linkage mapping approaches to identify ALS resistance loci at different plant growth stages. Different QTLs were detected showing a different quantitative profile of the disease at different plant growth stages. The previously reported *Phg-1, Phg-2*, *Phg-4*, and *Phg-5* loci were validated, and a new QTL named ALS11.1^{AM} located at the beginning of chromosome Pv11 was reported (de Almeida et al., 2021). All these studies, based on high-throughput genotyping platforms and GWAS, revealed several resistance genes involved in the ALS response. Molecular markers cosegregating with these resistance loci and haplotypes represent a powerful tool for the development of superior varieties with improved levels of ALS resistance.

Domestication has narrowed the genetic diversity of common beans and, in recent decades, plant breeding has accelerated this process decreasing their potential to adapt to changing conditions of biotic and abiotic stress. Common bean wild relatives represent a particular source of variability for many genetically important traits and have been identified as a source of resistance to some biotic stresses, such as bruchids (Kornegay et al., 1993; Osborn et al., 2003), white mold (Mkwaila et al., 2011), common bacterial blight (Beaver et al., 2012) and web blight (Beaver et al., 2012). NWA represents the southern limit of the Andean gene pool of bean and is probably an area of domestication (**Figure 2**; Kwak and Gepts, 2009; Rodriguez et al., 2015). High levels of genetic diversity in Argentinean wild populations have been reported, suggesting that the Andean gene pool has a large genetic base in this region (Menéndez-Sevillano, 2002; Galván et al., 2006, 2010a). A high level of tolerance to *P. griseola* races was observed in wild beans from NWA with the identification of resistance gene analog sequences (Stenglein, 2007; Galván et al., 2010b). Recent studies based on 34 wild bean populations evaluated with three of the most widely distributed races in the main cultivation areas in Argentina was reported by Aparicio (2020). Resistant and tolerant genotypes were observed depending on the pathotype tested. Three wild genotypes resulted resistant to race 63-7, while the other six genotypes were tolerant. This wild germplasm represents new sources of Andean resistance genes and is of great interest to broader the genetic base of bean cultivars.

Web blight

Common bean web blight (WB), caused by the basidiomycete fungus *R. solani* Kuhn [teleomorph *Thanatephorus cucumeris* (Frank) Donk] is among the most economically important epidemics, given its level of dispersion in bean production areas in the humid tropics causing significant losses in seed quality and yield (Beaver et al., 2021). Web blight is a limiting factor in Argentina (Vizgarra et al., 2012; Spedaletti et al., 2016) and in other regions of Central America and the Caribbean (Gálvez et al., 1989; Godoy-Lutz et al., 2008; Mora-Umaña et al., 2013), Brazil (Alves de Sousa et al., 2014; Boari et al., 2020; Chavarro-Mesa et al., 2020), and Africa (Wortmann et al., 1998; Masangano and Miles, 2004). WB epidemics are favored by rainy weather, high relative humidity (>80%) and high-to-moderate temperature (30–20°C) (Gálvez et al., 1989). The WB fungus has a wide host range and the capacity to survive saprophytically as sclerotia and mycelium in the soil and on plant debris (Cardoso and Luz, 1981), limiting the effectiveness of crop rotation to control the disease. Rain drops are an important source of WB infection splashing soil particles containing mycelium and sclerotia of the pathogen. The basidial stage of the WB pathogen produce basidiospores which are disseminated and produce small circular lesions on the leaves in the canopy. Under humid and warm weather conditions, the lesions expand into irregularly shaped, water soaked lesions and coalesce giving a scalded appearance to infected plants (**Figure 1**; Godoy-Lutz et al., 1996).

WB pathogen identification resides on assigning *R. solani* isolates to anastomosis groups (AGs) based on the mycelial compatibility between them (Sneh et al., 1991; Carling, 1996). Currently, 15 AGs, with numerous subgroups, have been reported (Liu and Sinclair, 1993; Carling, 1996; Carling et al., 2002; Sharon et al., 2008), of which AG 1, AG 2, and AG 4 have been associated with common bean WB (Galindo et al., 1982; Gálvez et al., 1989; Tu et al., 1996; Godoy-Lutz et al., 2003, 2008; Yang et al., 2007; Dubey et al., 2014). Some of these AGs were further divided into intraspecific groups (ISGs) based on rDNA-ITS sequence analyses, epidemiological differences and cultural characteristics (AG 1-IA, AG 1-IB, AG 1-IE, AG 1-IF, AG 2-2IV, AG 2-2WB; Godoy-Lutz et al., 2003, 2008). Web blight isolates from different regions of Latin America and the Caribbean, where WB is endemic, have been identified by the analysis of rDNA-ITS sequences (Godoy-Lutz et al., 2003, 2008; Spedaletti et al., 2016). AG1 IE and AG-1 IF isolates have been reported as the most common and aggressive within the AG-1 complex, infecting common bean cultivars with moderate levels of resistance (Godoy-Lutz et al., 2008). However, isolates of AG 2-2WB associated with bean WB in Honduras, Costa Rica, Dominican Republic and Ecuador, have been reported (Godoy-Lutz et al., 2003, 2008; Mora-Umaña et al., 2013).

In Argentina, the molecular identification of *R. solani* causing WB in cultivated bean fields has been reported by Spedaletti et al. (2016). In this study 97 isolates recovered from bean plants showing symptoms of WB were identified as *R. solani* AG 2-2WB by means of specific primers and the phylogenetic analysis of rDNA-ITS sequences. Moreover, a great variability in virulence was observed among the isolates in a pathogenicity assay performed in black bean seedlings using colonized wheat grains as source of inoculum. Thirty-two percent of the isolates resulted as highly virulent on the basis of the disease reaction on foliar tissues and no correlation between virulence and geographical origin was detected. Moreover, a few isolates were aggressive on hypocotyls supporting previous observations (Godoy-Lutz et al., 1996; Valentín Torres et al., 2016). Isolates recovered from wild beans (*Phaseolus vulgaris* var. *aborigineus*) growing in the same area have also been identified as *R. solani* AG 2-2WB (Godoy-Lutz et al., 2003, 2008; Spedaletti et al., 2016).

The use of resistant cultivars is an important factor of an integrated management of WB disease. Beaver et al. (2021) recently reviewed the status of breeding for resistance to WB in common bean and although significant progress has been made, common bean cultivars with high levels of resistance to diverse AG groups are still lacking. There are cultivars that in some countries have moderate levels of resistance to WB while in other countries they are more susceptible to the disease (Poltronieri and Ferreira de Oliveira, 1989), emphasizing the fact that local pathogenic WB isolates, characterized by their anastomosis group, should be used in germplasm screening to allow for the identification of sources of genetic resistance (Beaver et al., 2021). Considering this, 23 common bean cultivars inoculated with two highly virulent AG 2-2 isolates collected in northwestern Argentina were evaluated for WB resistance by Spedaletti et al. (2017). Based on the disease incidence (DI) on foliar tissue, the Leales B30 and Leales CR5 cultivars, developed by the Instituto Nacional de Tecnologia Agropecuaria (INTA) from Argentina, were classified as resistant (1 = DI < 3) to both isolates. The identification of resistant varieties using isolates identified in the NWA region represents a significant contribution to breeding programs aimed at achieving elite cultivars with durable WB resistance.

Rhizoctonia root rot

Root rot (RR) caused by *Rhizoctonia solani* is among the major diseases affecting the common bean in Argentina and other bean growing areas worldwide (Abawi, 1989; Mathew and Gupta, 1996; Naseri and Mousavi, 2015), particularly in low soil fertility regions, with limited crop rotation and intensive seasonal bean production (Miklas et al., 2006). Rhizoctonia RR symptoms include sunken, reddish-brown lesions on seedling roots and stems (Abawi, 1989), resulting in young seedling damping-off (**Figure 1**; Reddy et al., 1993; Hagedorn, 1994). Yield losses, resulting in upward to 100%, have been reported (Abawi, 1989; Singh and Schwartz, 2010). *R. solani* is a soil-borne pathogen that spreads from plant to plant through the formation of mycelial bridges between roots and infested soil debris. The pathogen survives on seeds, facilitating long-distance and overwintering dispersal (Abawi, 1989; Schwartz et al., 2005).

Root and hypocotyl rot have been reported to be caused by isolates of *R. solani* AG 1, AG 2, AG 4, and AG 5 (Galindo et al., 1982; Abawi, 1989; Tu et al., 1996; Eken and Demirci, 2004; Nerey et al., 2010; Valentín Torres et al., 2016). Moreover, AG 4 has been reported to be the prevalent group associated with root and hypocotyl rot in Argentina and other common bean growing areas worldwide, such as Brazil, Cuba, Iran, Turkey and the Democratic Republic of the Congo (Muyolo et al., 1993; Meinhardt et al., 2002; Nerey et al., 2010; Haratian et al., 2013; Kiliçoğlu and Özkoç, 2013; Spedaletti et al., 2017). In Argentina, the presence of various *R. solani* AGs in seed and soil samples from bean fields naturally infested with RR has been reported (Spedaletti et al., 2017). Based on the variability in the rDNA-ITS sequence, most of the isolates (92%) were identified as *R. solani* AG 4, including AG 4 HG-I (20%) and AG 4 HG-III (26%). Moreover, great variability in virulence among the isolates was observed in a pathogenicity approach under controlled conditions toward bean seedlings, and four virulence categories were defined according to the disease reaction on root and foliar tissues. Considering that seed and soil-borne inoculum play a significant role in pathogen dispersal in the

region, the use of certified seeds free of sclerotia is essential to reducing the incidence of Rhizoctonia RR disease. *R. solani* AG 4 can affect other commercial crops that are grown in rotation with beans, such as maize and tobacco (Mercado Cárdenas et al., 2015). Mercado Cárdenas et al. (2015) identified *R. solani* AG 4 HG-I and AG 4 HG-III isolates obtained from tobacco plants with damping-off and sore shin symptoms in different localities in NWA. This highlights the importance of using non-host crops in rotational systems that may reduce root rot incidence, leading to improved control.

However, the most effective strategy for controlling Rhizoctonia RR is the use of resistant cultivars. Genetic resistance to *R. solani* has been reported to be controlled by major as well as minor genes with additive effects (Zhao et al., 2005; Oladzad et al., 2019). Thus, screening for resistance to this soil-borne pathogen is challenging since environmental factors can greatly affect phenotypic responses. Some studies on the identification of Rhizoctonia RR-resistant germplasm have been conducted in common bean (Muyolo et al., 1993; Peña et al., 2013; Adesemoye et al., 2018; Oladzad et al., 2019). Peña et al. (2013) identified genotypes with partial resistance to *R. solani* by screening 275 bean lines in a greenhouse assay. Conner et al. (2014) reported five partially resistant cultivars among 37 common bean lines from different market classes evaluated under field conditions. Recently, Oladzad et al. (2019) performed a wide-scale resistance screening across the Andean (ADP; $n = 273$) and Middle American (MDP; $n = 279$) diversity panels. These diversity panels consist of modern genotypes commonly used in production fields and have been developed to represent bean genetic diversity within each gene pool, facilitating genetic analyses (Cichy et al., 2015; Moghaddam et al., 2016). The Rhizoctonia RR resistance responses of 28 genotypes of the ADP and 18 of the MDP were similar or higher than that of the VAX 3 line used as a resistant control. These new sources of resistance to Rhizoctonia RR will be useful parents for common bean breeding programs. Moreover, a GWAS was performed to discover genomic regions associated with Rhizoctonia RR resistance using the ADP and MDP (Oladzad et al., 2019). This study provided evidence for the existence of one major QTL on Pv01 identified in the MDP and another major QTL on Pv02 in the ADP. These regions were associated with gene clusters encoding proteins similar to known disease resistance genes (Oladzad et al., 2019). This information will be useful to develop molecular markers to facilitate the introgression of Rhizoctonia RR resistance into elite cultivars.

Concluding remarks

Nowadays it is challenging to facilitate the improvement of crops with such global importance like the common bean while developing cultivars that meet the nutritional requirements of a constantly growing world population and that can also adapt to biotic and abiotic stresses, in the current conditions of climate change.

In this review we described the major fungal disease problems that affect common bean production with emphasis in Argentina. Significant advances have been made in pathogen identification and characterization supplying information on their variability, population structure and reproductive behavior in the main common bean production areas in the country. Furthermore, the selection of representative local isolates supported germplasm screening in regional common bean breeding programs for the development of cultivars with durable resistance.

Managing fungal diseases is complex, so these studies contribute to sustainable management strategies such as genetic resistant cultivars, chemical and biological control, and cultural practices aimed at minimizing yield losses due to WM, ALS, WB, and Rhizoctonia RR, in the region. This review assembled information about the best resistant sources of WM (line A 195), ALS (TUC550, MAB 333, MAB 336, TUC180, and TUC241) and WB (Leales B30 and Leales CR5) in Argentina, which is relevant considering that the use of genetic resistant cultivars is the most promising management tool with the most negligible environmental impact. Regarding Rhizoctonia RR, further germplasm screening based on the pathogen diversity observed in the region, should be performed for the identification of resistant genotypes. Moreover, wild bean populations growing in NWA represent a valuable source of new resistance genes to broaden the common bean genetic base against these pathogens. All these genotypes are being considerate as candidates to generate a diverse association panel for a GWAS approach, that will accelerate the identification of markers associated to the resistance genes and their use in bean improvement.

Author contributions

CA, GT, and MG contributed to the conception and design of these work and wrote the first draft of the manuscript. YS, MA, and EM wrote sections of the manuscript. GT edited the figures. GM and PO-B edited and revised the manuscript. MG and GM performed the funding acquisition. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Characterization of field pea (*Pisum sativum*) resistance against *Peyronellaea pinodes* and *Didymella pinodella* that cause ascochyta blight

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Ascochyta blight is one of the most destructive diseases in field pea and is caused by either individual or combined infections by the necrotrophic pathogens *Peyronellaea pinodes*, *Didymella pinodella, Ascochyta pisi* and *Ascochyta koolunga*. Knowledge of disease epidemiology will help in understanding the resistance mechanisms, which, in turn, is beneficial in breeding for disease resistance. A pool of breeding lines and cultivars were inoculated with *P. pinodes* and *D. pinodella* to study the resistance responses and to characterize the underlying resistance reactions. In general, phenotypic analysis of controlled environment disease assays showed clear differential responses among genotypes against the two pathogens. The released variety PBA Wharton and the breeding line 11HP302-12HO-1 showed high levels of resistance against both pathogens whereas PBA Twilight and 10HP249-11HO-7 showed differential responses between the two pathogens, showing higher resistance against *D. pinodella* as compared to *P. pinodes.* OZP1604 had high infection levels against both pathogens. Histochemical analysis of leaves using diamino benzidine (DAB) showed the more resistant genotypes had lower accumulation of hydrogen peroxide compared to susceptible genotypes. The digital images of DAB staining were analyzed using ImageJ, an image analysis software. The image analysis results showed that quantification of leaf disease infection through image analysis is a useful tool in estimating the level of cell death in biotic stress studies. The qRT-PCR analysis of defense related genes showed that partially resistant genotypes had significantly higher expression of *PsOXII* and *Pshmm6* in the *P. pinodes* treated plants, whereas expression of *PsOXII*, *PsAPX1*, *PsCHS3* and *PsOPR1* increased in partially resistant plants inoculated with *D. pinodella*. The differential timing and intensity of expression of a range of genes between resistant lines challenged with the same pathogen, or challenged with different pathogens, suggests that there are

multiple pathways that restrict infection in this complex pathogen-host interaction. The combination of phenotypic, histochemical and molecular approaches provide a comprehensive picture of the infection process and resistance mechanism of pea plants against these pathogens.

KEYWORDS

field pea, Ascochyta blight, controlled environment, DAB staining, gene expression, resistance responses

Introduction

Field pea (*Pisum sativum* L.) is the most commonly grown pulse globally with important production areas including Canada, Russia, China, USA and India (FAO, 2019) (http://www.fao.org/faostat/en/#rankings/countries_by_commodity_exports). In 2020, field pea was cultivated on over 8.1 million ha with production of 14.6 million tonnes. Annual production in Australia over the past five years has been approximately 280,000 MT per year (ABARES, 2022). It is one of the most important legume crops and serves as a good source of protein for both human and animal consumption. On average, seeds contain between 15-30% protein with water-insoluble globulins and water-soluble albumins forming major fractions (Robinson et al., 2019). Furthermore, the crop plays a critical role in farming systems where it can fix atmospheric nitrogen through symbiosis with rhizobium, thus helping to reduce the use of nitrogen fertilizers (Nemecek et al., 2008; Jensen et al., 2012), as well as being a disease break crop when used in rotation with cereals and oilseeds.

Ascochyta blight, commonly known as "black spot" in Australia, is one of the most devastating diseases of field peas. It is ubiquitous in nature and has been reported in most of the field pea growing countries and can cause yield losses of up to 60% in Australia (Bretag et al., 2006). Multiple pathogens cause this disease including *Asochyta pisi* Lib. (teleom. *Didymella pisi*) (Chilvers et al., 2009), *Ascochyta pinodes* (teleomorph: *P. pinodes* (Berk. & Blox), *D. pinodella* (L.K. Jones) Morgan-Jones & K.B. Burch, and *Ascochyta koolunga* (Davidson et al., 2009) and various combinations of these can form a disease complex. In Australia, more recently *Phoma herbarum* (Li et al., 2011) and *Phoma glomerata* (Tran et al., 2014) were also reported to be part of ascochyta blight disease complex. During the 1960s, breeding focused on developing lines resistant to *A. pisi*. This likely led to *P. pinodes* becoming the most prevalent and destructive pathogen (Tivoli and Banniza, 2007).

Cooler temperatures with wet and humid conditions are most conducive to disease development (Bennet et al., 2019). These pathogens mostly infect the aerial plant parts such as leaves, stem, flower and pods. Under favorable conditions *P. pinodes* infects both seedlings and adult plants and shows symptoms of lesions on leaves and stem, foot rot and the affected seeds show shrinking and dark discoloration (Ahmed et al., 2015). *D. pinodella* causes similar symptoms to *P. pinodes*, typically being less severe on aerial parts but more severe in the roots where foot rot can extend damage to below ground plant parts (Bretag et al., 2006). *D. pinodella* survives well in warmer climates and severity of foot rot is higher in plants grown at 28° C or higher (Linford and Sprague, 1927).

Agronomic and physiological practices have been deployed in attempts to control this disease. The use of fungicides, intercropping (Fernández-Aparicio et al., 2010), reduced canopy architecture and burial of infected debris (Schoeny et al., 2008) are some of the methods to reduce the severity of infection. These methods are not ideal as the use of fungicides and burial of infected debris can harm the environment, while a reduced canopy and intercropping may lead to lower yields.

Breeding genotypes for durable resistance is the most viable option albeit limited success has been reported due to non-availability of good levels of resistance in the germplasm and lack of good screening methods (Fondevilla et al., 2008; Adhikari et al., 2014). The differential response of genotypes against *P. pinodes* identified 22 pathotypes in Canada (Xue et al., 1998), 15 in Australia (Ali et al., 1978), and 6 in Germany (Nasir and Hoppe, 1991). The resistance against ascochyta blight may be stage specific as genotypes that were resistant at the seedling stage were not always resistant when plants were mature (Ali et al., 1978). The inheritance of resistance to *D. pinodella* showed that the variety "Kinnauri" carried a single dominant resistance gene (Rastogi and Saini, 1984). Among other reports there have been several studies of incomplete resistance against *P. pinodes* in field pea germplasm albeit higher level of resistance has been detected in other *Pisum* species (Wroth, 1998; Fondevilla et al., 2005). Resistance against *P. pinodes* is a complex trait governed by quantitative trait loci (Prioul-Gervais et al., 2007; Fondevilla et al., 2008) and incorporation of multiple loci from unadapted sources brings considerable risk of transfer of unwanted alleles. The identification of multiple pathogens, pathotypes and quantitative resistance loci highlights complexities in breeding for resistance. Therefore, knowledge of specific defense

responses against this pathogen can play an important role in developing strategies to improve germplasm responses to this disease.

The resistance reaction of plants against any pathogen involves a series of responses that can be either systemic or local and has been associated with cell death (Nasir et al., 1992), protein-cross linking in epidermal cell wall (Bradley et al., 1992), accumulation of hydrogen peroxide (H_2O_2) and peroxidase activity (Alvarez et al., 1998). The tight relationship between epidermal cell death and smaller lesion size has been demonstrated in *Pisum* spp. when inoculated with *P. pinodes* (Carrillo et al., 2013). As an antipathogen agent, H_2O_2 is one of the prominent reactive oxygen species (ROS) and plays a critical role in plant defense by creating a toxic environment resulting in the restriction of pathogen growth. Apart from this, H_2O_2 also plays a key role as a signaling molecule (Allan and Fluhr, 1997). The production of ROS can result in extensive damage to cells and may lead to cell death (Mansoor et al., 2022). This has been proposed as a mechanism for the development of a hypersensitive response upon pathogen recognition (Levine et al., 1994). The outburst of H_2O_2 was shown to have a critical role in stimulating salicylic acid synthesis ultimately leading to systemic acquired resistance (SAR) against *Alternaria solani* and *Verticillium dahliae* in potato (Wu et al., 1997). The interaction of H_2O_2 with other signaling molecules such as abscisic acid (Terzi et al., 2014) and ethylene (Yang, 2014) has been well characterized in mung bean, maize, and Arabidopsis respectively. Genes that were associated with jasmonic acid and ethylene signaling pathways were upregulated upon inoculation with *P. pinodes* in field pea (Fondevilla et al., 2011).

The regulation of defense related genes forms an integral part of the resistance mechanism and has been well characterized for various plant pathogens in field pea (Tran et al., 2018), cucumber (Pu et al., 2014), sunflower (Şestacova et al., 2016) and rice (Pan et al., 2014). The study of such genes also provides critical information about the molecules involved in plant-pathogen interactions. Previous studies have demonstrated the induction of various defense related genes such as polyphosphoinositide metabolism (Toyoda et al., 1992), phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Yoshioka et al., 1992) upon infection of field pea with *P. pinodes*. The elevated transcript levels of PAL and CHS were demonstrated in the presence of elicitors from *P. pinodes* (Toyoda et al., 1993). The *Hmm6*, which encodes 6a-hydroxymaackiain methyltransferase that catalyses the terminal step in biosynthesis of pisatin, a phytoalexin from pea tissue (Wu et al., 1997), showed a 10-fold induction at 48 hours post infection (HPI) compared to 2 HPI against *Aphanomyces euteiches* (Hosseini et al., 2015). The *PsOXII* gene which encodes a peroxidase, was upregulated three-fold while *hmm6* gene showed two times higher expression in the resistant line P665 than the susceptible variety Messire upon inoculation with *P. pinodes* (Fondevilla et al., 2011). Another ROS scavenging antioxidative enzyme ascorbate peroxidase (*APX1*) was shown to have a pivotal role in scavenging H_2O_2 as a result of pathogen attack (Creissen et al., 1994). Fusarium head blight infection in wheat caused a rapid increase in APX activity as early as 3 HPI (Spanic et al., 2017). The oxophytodienoic acid reductase I (OPR1), one of the genes associated with jasmonic acid (JA) biosynthesis, which is involved in the plant growth and development, showed significant induction in the shoots of wheat at 24 and 72 hours post treatment with methyl jasmonate (Liu et al., 2016).

Here, we have evaluated of the disease reactions of 16 field pea genotypes originated from Australia against two pathogens, *P. pinodes* and *D. pinodella*, that cause ascochyta blight and the underlying resistance response against those pathogens. There is little reported on the resistance responses against *D. pinodella*. This work describes the underlying resistance reactions against *P. pinodes* and *D. pinodella* through detection of H_2O_2 (histochemical) and quantification of defense related genes through quantitative RT-PCR (molecular) approaches in a time series manner.

Materials and methods

Preparation of plant materials and experimental setup

Two experiments were conducted to study the defense responses of field pea genotypes against two pathogens. Experiment 1 consisted of phenotypic screening of 16 genotypes that were chosen from previous knowledge of their reactions to infection with *P. pinodes*. Experiment 2 was conducted for the histochemical and molecular characterization of four resistant and susceptible genotypes.

In experiment 1, the genotypes were assessed for their reactions to *P. pinodes* and *D. pinodella* in the CE assay. Based on the disease scores of experiment 1 the four best and worst performing genotypes in terms of disease scores were selected for a histochemical analysis, and the top and bottom two genotypes in terms of scores were selected for molecular characterization against the same pathogens in experiment 2. The list of genotypes used in experiment 1 and experiment 2 are presented in Tables 1, 2 respectively. Pots with a diameter of 13.5 cm and a depth of 13.5 cm were filled with legume mix (Biogro, SA, Australia) and planted with three seeds per pot of each genotype. In the experiment 2, pots with a diameter of 7 cm and a depth of 16 cm were filled with the same potting mix and planted with one seed per pot. The leaf samples were harvested at four time points for molecular characterization.

Seedlings were grown in the CE with a day/night temperature of 24°/15° C with a 16:8 h light: dark cycle. When the seedlings were at the 3-4 node stage, they were transferred to

TABLE 1 The field pea genotypes used in the controlled environment experiment 1.

Sl. Nr	Genotypes	Status
1	05H161-06HOS2005-BOG09-2	Breeding line
2	PBA Butler	Cultivar
3	OZP1305	Breeding line
4	PBA Oura	Cultivar
5	OZP1408	Breeding line
6	09HP216-10HO2-3	Breeding line
7	10HP249-11HO-7	Breeding line
8	11HP028-12HO-3	Breeding line
9	11HP160-12HO-1	Breeding line
10	11HP302-12HO-1	Breeding line
11	PBA Wharton	Cultivar
12	11HP420-12HO-13	Breeding line
13	Kaspa	Cultivar
14	PBA Twilight	Cultivar
15	OZP1604	Breeding line
16	WAPEA2211	Breeding line

a growth chamber with a temperature of 15° C and 12 h each light and dark period for inoculation. The inoculation was carried out in custom made translucent tents that had pipe fittings to allow misting from a humidifier. The plants were inoculated with either the *P. pinodes* or *D. pinodella* pathogens. The plants were transferred to the CE 24 h prior to the inoculation to acclimatize to the growth conditions. The experimental setup in the CE is presented in Supplementary Figure S1.

Preparation of inoculum and inoculation

Isolates of *P. pinodes* (ID: Twilight) and *D. pinodella* (ID: MPA) were grown on Potato Dextrose Agar plates for 2-3 weeks. Conidia were harvested by flooding the plates with sterile distilled water and gently rubbing the surface of the agar with a glass spreader to loosen conidia. A hemocytometer was used to determine the spore count and the concentration was adjusted to 1 x 10^5 (Hwang et al., 2006) conidia per ml in sterile distilled water. Pulse penetrant (Nufarm, Victoria, Australia), a surfactant, was added at 0.06% to the prepared inoculum just before inoculation. Plants of the control treatment were sprayed with sterile distilled water mixed with 0.06% pulse penetrant. In experiment 1, the three plants in each of four replicated pots of each genotype that were to be inoculated were sprayed evenly with the inoculum and tents were closed to maintain humidity. All inoculated plants were scored seven days post inoculation using a continuous scale of 0 to 9 as described by Xu et al. (1996), where 0 represented no infection and a score of 9 represented 90-100% infection. The best performing genotypes from experiment 1 were selected to study the underlying resistance response against *P. pinodes* and *D. pinodella* in the time series of experiment 2, where one plant in each of three replicated pots of each genotype was evenly sprayed with the inoculum.

Detection of hydrogen peroxide localization using DAB staining

The accumulation of H_2O_2, a ROS, was detected through diaminobenzidine (DAB) staining. The methodology described here for *in situ* detection of H_2O_2 was adapted from Daudi and O'Brien (2012). In brief, at 72 and 96 HPI samples were taken per pathogen, genotype and replicate and placed in Petri dishes. DAB staining solution (1mg/ml) was prepared in sterile distilled water at pH 3. The solution was covered with aluminium foil due to its sensitivity to light. An aliquot of 25 µl of Tween 20 (0.05 v/v) together with 2.5 ml of 200 mM sodium phosphate solution were added to DAB to prepare 10 mM sodium phosphate DAB staining solution. The staining solution was applied to the Petri dish containing the leaf, ensuring that the leaf was completely immersed. The plates were placed in an opaque box and gently agitated on a shaker for 4 h at 100 rpm. Following incubation, the DAB solution was replaced by bleaching solution (ethanol:acetic acid:glycerol = 3:1:1) and placed in the water bath (90-95° C) for 15 min. This helps to remove chlorophyll but retains the brown precipitation caused by DAB reacting with H_2O_2. After the

TABLE 2 The field pea genotypes used in the controlled environment experiment 2.

Pathogen	Genotype	Group	Pathogen	Genotype	Group
Peyronellaea pinodes	05H161-06HOS2005-BOG09-2	Resistant	*Didymella pinodella*	10HP249-11HO-7	Resistant
Peyronellaea. pinodes	09HP216-10HO2-3	Resistant	*Didymella pinodella*	11HP302-12HO-1	Resistant
Peyronellaea. pinodes	11HP302-12HO-1	Resistant	*Didymella pinodella*	PBA Twilight	Resistant
Peyronellaea pinodes	PBA Wharton	Resistant	*Didymella pinodella*	PBA Wharton	Resistant
Peyronellaea pinodes	10HP249-11HO-7	Susceptible	*Didymella pinodella*	09HP216-10HO2-3	Susceptible
Peyronellaea pinodes	11HP160-12HO-1	Susceptible	*Didymella pinodella*	OZP1604	Susceptible
Peyronellaea pinodes	OZP1604	Susceptible	*Didymella pinodella*	PBA Oura	Susceptible
Peyronellaea pinodes	PBA Twilight	Susceptible	*Didymella pinodella*	WAPEA2211	Susceptible

required duration of the heat treatment, the bleaching solution was replaced by fresh bleaching solution and incubated at room temperature for 30 min. Samples were stored at 4° C overnight. The leaves were visualized the following day for DAB staining and digital photographs of the leaves were acquired.

Quantification of leaf disease infection through DAB staining

The digital images of DAB stained leaves were analyzed using ImageJ software (version 1.53c, https://imagej.nih.gov/ij/). The polygon selection tool was used to select the edges of the image followed by an inverse of the image. This selects the whole region of the image except the inversed image. The area outside of the leaf in the inversed image was filled to remove from subsequent analysis. A color threshold was applied to select the disease affected part by using HSB (Hue*Saturation*Brightness) color space. The resulting image was then converted to binary, and a mask was generated. The mask outlines the region of interest (ROI). The ROI manager tool was used to extract the pixel numbers of the disease affected part. Similarly, the total pixel numbers of the whole leaf without applying threshold were estimated. The percentage of the disease affected part was calculated using the pixel numbers of disease affected part and whole leaf.

Sample collection, extraction of RNA and synthesis of cDNA

The leaves of pea plants that were inoculated with *P. pinodes* and *D. pinodella* were harvested at 0, 24, 72 and 96 hours post infection (HPI). For each of the inoculated treatments three biological replicates were harvested in aluminium foil, snap frozen in liquid nitrogen and stored at -80° C until further use. The bench tops, glassware, pestles, and mortars were treated with RNasZAP™ (Sigma Aldrich, St. Louis, MO, USA). Total RNA was extracted from 100 mg of each of the treated leaves using Spectrum™ Plant Total RNA kit (Sigma Aldrich, St. Louis, MO, USA) as per manufacturer's protocol. The concentration of RNA was determined by a Nanodrop 2000 spectrophotometer (Thermal co., USA) and the integrity and quality were confirmed by loading on 1% agarose gel stained with SYBR safe. (Thermo Fisher Scientific, Carlsbad, CA). Contaminating DNA was removed by digesting the RNA sample with DNase I (RNase-free) (New England Biolabs, Ipswich, MA). The DNase I treated RNA and cDNA of random samples were confirmed for the absence of genomic DNA by performing a PCR with *PsGAPDH* primers that amplify an intron-exon-intron sequence of field pea glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Die et al., 2010; Fondevilla et al., 2011; Tran et al., 2018). The *PsHistone3* primers were used as PCR control along with *PsGAPDH* primers. A 500 ng aliquot of total RNA was used to synthesize cDNA using the Lunascript RT supermix system (New England Biolabs, Ipswich, MA).

Gene expression analysis using qRT-PCR

The qRT-PCR was performed on three biological and three technical replicates to study the real time expression of defense related genes. The CFX384 touch real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to perform the reaction. The 10 µl qRT-PCR reaction mix consisted of 5 µl of 2X Luna universal qPCR master mix (New England Biolabs, Ipswich, MA), 1 µl (10 µM) of each of the forward and reverse primers, 1 µl (1 µg) cDNA and remaining of nuclease free water. The primers used in the qRT-PCR are provided in the Table 3. The standard curve for each qRT-PCR primer pairs was generated by plotting logarithm of four step 10 fold dilutions (10^0, 10^{-1}, 10^{-2} and 10^{-3}) of starting pooled cDNA quantity and threshold cycle (Ct) values. The qRT-PCR reaction was performed using the following conditions: 95°C for 1 min, 39 two-step cycles each at 95°C for 15 s and 60°C for 30 s, with a plate read after each cycle and a final melting curve of 60–95°C for 5 s with an increment of 0.5°C per melt curve temperature and a plate read after each temperature step. The slope and the R^2 values of the standard curve were calculated. The efficiency (E) was calculated using the formula $E = (10^{(-1/\text{slope})}) - 1$. The qRT-PCR reactions were carried out in triplicate and included a no template control. The ΔCt of non-inoculated and inoculated samples for each GOI at each time points were used to calculate the ratio of relative mRNA levels using the formula as proposed by (Pfaffl, 2001),

$$R = \frac{\left(E_{target}\right)^{\Delta Ct_{target(control-sample)}}}{\left(E_{reference}\right)^{\Delta Ct_{reference(control-sample)}}}$$

where relative expression ratio, $E_{targert}$ and $E_{reference}$ are qRT-PCR efficiencies of the target and reference genes respectively, $\boldsymbol{\Delta Ct_{target}}$ is the difference in Ct values of control and sample for GOI and $\boldsymbol{\Delta Ct_{reference}}$ is the difference in Ct values of control and sample for reference gene. The geometric mean of the expression levels *PsHistone3*, an endogenous reference gene was used to calculate the normalization index (Fondevilla et al., 2011; Tran et al., 2018).

Statistical analyses

The two experiments were performed using a randomized complete block design. Experiment 1 and experiment 2 were conducted with four and three replicates of each genotype, respectively. The disease severity of genotypes was scored in the experiment 1 and the severity of disease in

TABLE 3 The RT-qPCR primers of defense related and reference genes used in this study.

	Name	Primer sequence	Amplicon (bp)	Reference
1	*PsOX11-F*	CTTGGAGGACCCACATGGAT	61	(Fondevilla et al., 2011)
	PsOX11-R	TTTGGCTTGCTGTTCTTGCA		
2	*PsApx1-F*	GGCACTCTGCTGGTACTTTTG	72	(Fondevilla et al., 2014)
	PsApx1-R	CGGCTTGGTGCTTAATTGTT		
3	*Pshmm6-F*	TTTGAACTTTGTTGGTGGAGATATG	80	(Fondevilla et al., 2011)
	Pshmm6-R	AATCATGCAGAACCCACTTGAGT		
4	*PsCHS3-F*	CCAAACTGTTAGGTCTTCGTCCAT	65	(Fondevilla et al., 2014)
	PsCHS3-R	GGCAAAACACCCTTGTTGGT		
5	*PsOPR1-F*	AAGTGAATGACAGAACCGATGA	60	(Fondevilla et al., 2011)
	PsOPR1-R	ATGGAAACCGACAGCGATT		
6	*PsHistone3-F*	GGAAGTATCAGAAGAGCACAGA	182	(Knopkiewicz and Wojtaszek, 2019)
	PsHistone3-R	AATGGCACAAAGGTTGGTATC		
7	*PsGAPDH-F*	GTGGTCTCCACTGACTTTATTGGT	156	(Die et al., 2010)
	PsGAPDH-R	TTCCTGCCTTGGCATCAAA		

1:5, defense related genes; 6:7, reference genes; F, Forward; R, Reverse; bp, base pairs.

each genotype in the experiment 2 was assessed using DAB staining. The data was analyzed using R statistical software (https://cran.r-project.org). To test the consistency of performance of the genotypes from two experiments, the association between the leaf damage digital area of experiment 2 and disease scores of experiment 1, was studied using Pearson's correlation (r). The disease scores and qRT-PCR fold change were analyzed by non-parametric Kruskal-Wallis rank-sum test and multiple comparisons among genotypes were performed using False Discovery Rate correction. The pathogen aggressiveness was determined by analyzing the disease scores of each pathogen using Wilcoxon-Mann-Whitney test.

Results

Performance of genotypes in the disease assay

In experiment 1, 16 genotypes were assessed for their performance against infection by *P. pinodes* and *D. pinodella*. In general, there was a differential response between genotypes for disease severity for both pathogens. The severity of infection ranged from 2 to 7 for *P. pinodes* whereas for *D. pinodella* the range was 1 to 5. Among the plants inoculated with *P. pinodes*, along with the released variety PBA Wharton, the breeding lines 11HP302-12HO-1, 05H161-06HOS2005-BOG09-2 and 09HP216-10HO2-3 showed significantly lower infection compared to the susceptible genotypes Kaspa, PBA Twilight, 11HP160-12HO-1 and OZP1604 (Figure 1A). In contrast the breeding lines 10HP-249-11HO-7 and 11HP302-12HO-1 showed significantly reduced infection compared to OZP1604, WAPEA2211 and PBA Oura upon inoculation with *D. pinodella* (Figure 1B). Additionally, the genotypes showed varied disease severity in response to the two pathogens. The genotype 10HP249-11HO-7 had lowest disease score of 1 against *D. pinodella* while the same genotype showed relatively higher disease score of 7 against *P. pinodes* (Figures 1A, B). The genotype OZP1305 had a low disease score of 1 against *D. pinodella* while moderately high disease score of 5 was observed against *P. pinodes*. Based on the differential responses of the genotypes against both pathogens, the best performing four resistant and four susceptible genotypes were selected to characterize the resistance against *P. pinodes* and *D. pinodella*. Overall, the disease analysis showed that *P. pinodes* infection occurred earlier and was more aggressive than *D. pinodella* with median scores of 6 and 3 respectively (Figure 1C).

Detection of H_2O_2 localization and quantification of leaf damaged digital area

The field pea leaves inoculated separately with *P. pinodes* and *D. pinodella* showed accumulation of H_2O_2 which was observed as dark-brown precipitates due to oxidation of DAB by H_2O_2 and peroxidase (Figure 2). H_2O_2 accumulation was observed in response to infection as early as 72 HPI but was more evident at 96 HPI. Higher level of H_2O_2 was observed in the susceptible genotypes compared to their partially resistant counterparts. The varying amount of H_2O_2 accumulation in the partially resistant genotypes demonstrated the differential response against *P. pinodes* (Figure 2A). The accumulation of H_2O_2 was evident wherever black spot symptoms were observed. Moreover, a greater level of H_2O_2 accumulation was observed in the leaves inoculated with *P. pinodes* compared to *D. pinodella* (Figures 2A, B) which verified the

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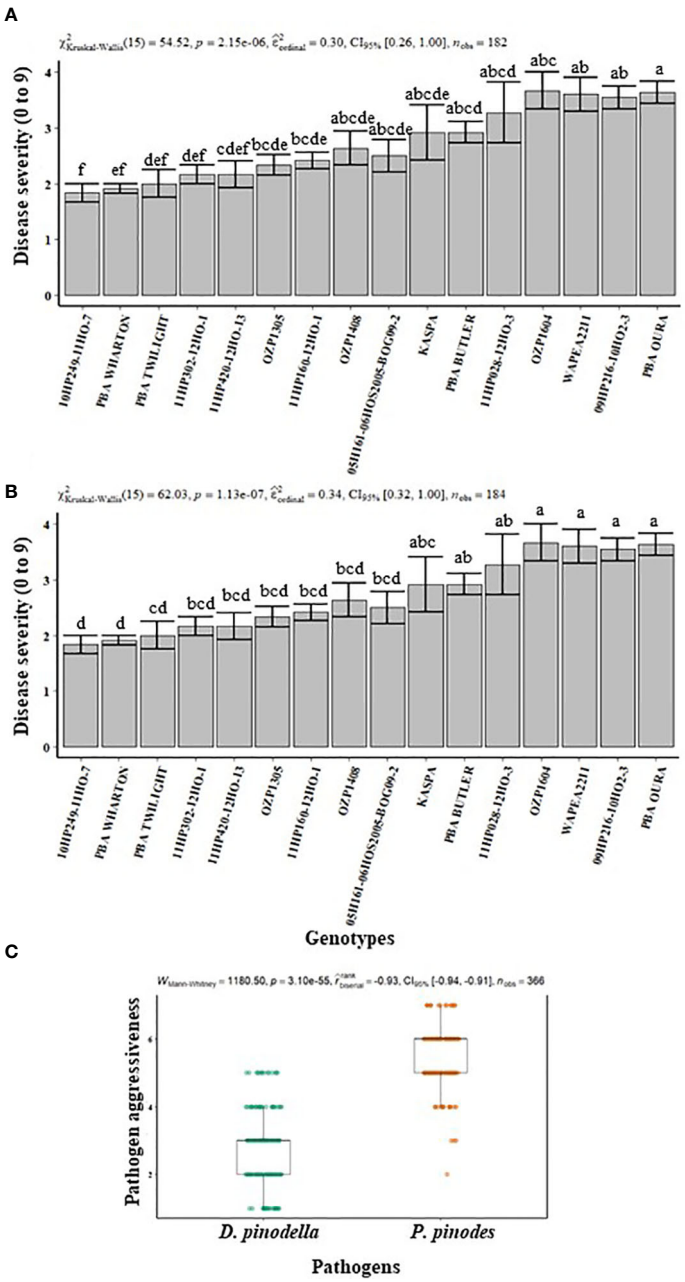

FIGURE 1
Disease severity **(A, B)** and pathogen aggressiveness **(C)** observed in field pea plants inoculated with *Peyronellaea pinodes* and *Didymella pinodella* at controlled environment facility in Horsham. The data are presented as mean ± SEM. Different letters on each bar signifies statistical significance among genotypes at $P < 0.05$ level for *P. pinodes* and *D. pinodella*.

aggressiveness of pathogen. The results of DAB staining corroborate with the scores obtained by the disease assay phenotyping.

The damaged leaf area (%) was quantified through image analysis. The extent of leaf damage recorded in the susceptible genotypes was in the range of 6.7% to 23.8% of total leaf area in the leaves inoculated with *P. pinodes*, and 1.5% to 4.2% in leaves inoculated with *D. pinodella* (Figures 3A, B). In partially resistant genotypes, the infection was at a minimal level and in the range of 1.6 to 2% in the leaves inoculated with *P. pinodes* and 0.1 to 1.1% in case of *D. pinodella* inoculated leaves. There was a close association between the leaf damage digital area of experiment 2 and disease severity of experiment 1 with a correlation co-efficient of r = 0.89 when inoculated with *P. pinodes* and r = 0.75 when inoculated with *D. pinodella* (Figures 4A, B).

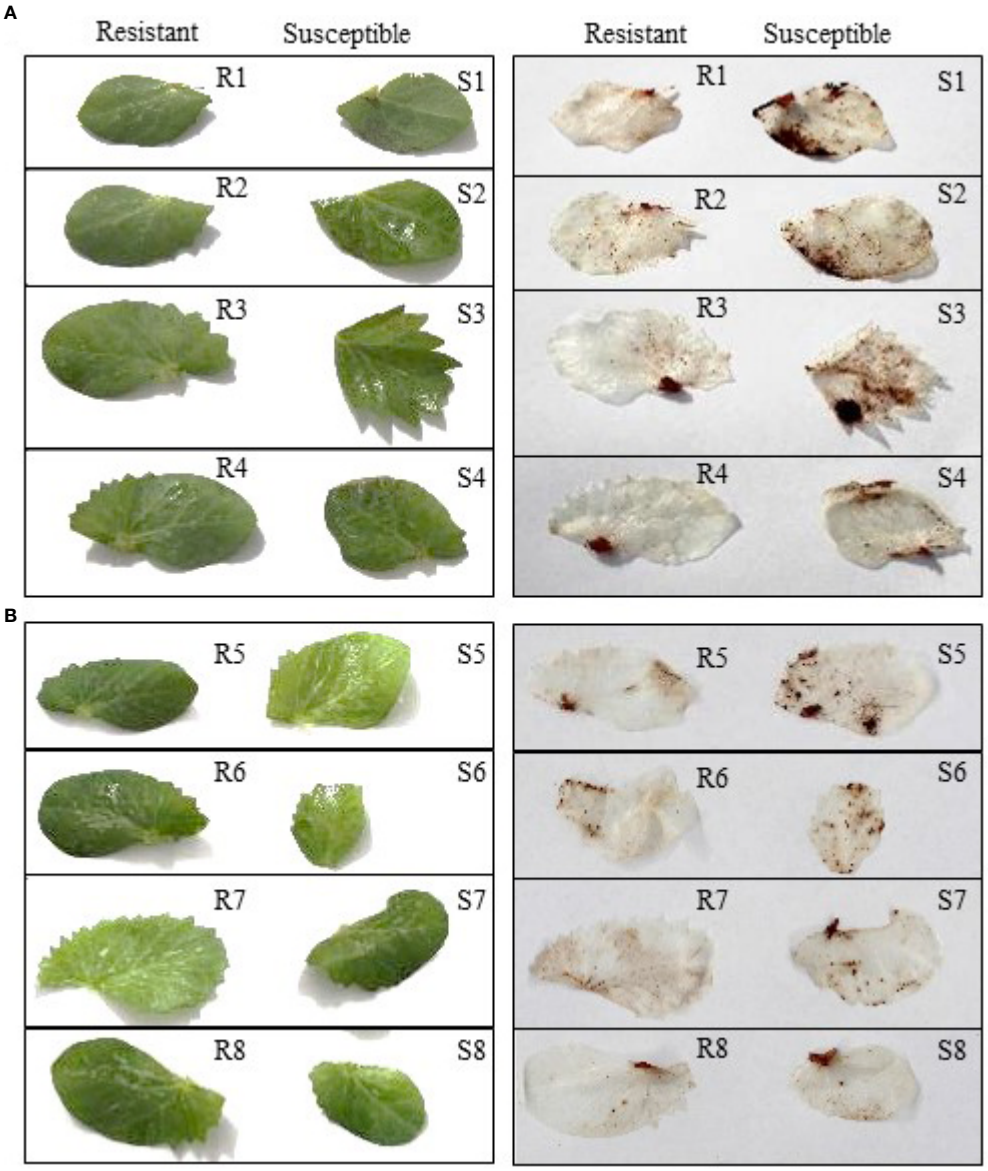

FIGURE 2
Detection of hydrogen peroxide through di-amino benzidine (DAB) staining in field pea leaves, visualized at 96 hours post inoculation with *Peyronellaea pinodes* **(A)** and *Didymella pinodella* **(B)**. R1, PBA Wharton; R2, 11HP302-12HO-1; R3, 05H161-06HOS2005-BOG09-2; R4 09HP216-10HO2-3; S1, OZP1604; S2, 11HP160-12HO-1; S3, PBA TWILIGHT; S4, 10HP249-11HO-7; R1-4 partially resistant and S1-4, susceptible to *P. pinodes*. R5, 10HP249-11HO-7; R6, PBA WHARTON; R7, PBA TWILIGHT; R8, 11HP420-12HO-13; S5, WAPEA2211; S6, OZP1604; S7, PBA OURA; S8, 09HP216-10HO2-3; R5-8 partially resistant and S5-8 susceptible to *D. pinodella*.

Induction of defense related gene expression in the leaves

The timing and degree of expression of defense related genes in leaves were investigated in field pea genotypes that had varying levels of resistance. The slopes derived from the standard curves of all the defense related and reference genes were found to range from 89.9% (*PsCHS3*) to 105.6% (*PsOPR1*) (Supplementary Figure S2). The Histone 3 (*PsHistone3)* gene was used as an internal control to normalize the expression of defense related genes. This gene had stable expression throughout the time course of the experiment. Among five defense related genes, peroxidase (*PsOX11*), showed an earlier upregulation while both 6a-hydroxymaachiain methyltransferase (*Pshmm6*) and chalcone synthase (*PsCHS3*) were upregulated and peaked at later time point, in the partially resistant genotypes. upon inoculation with

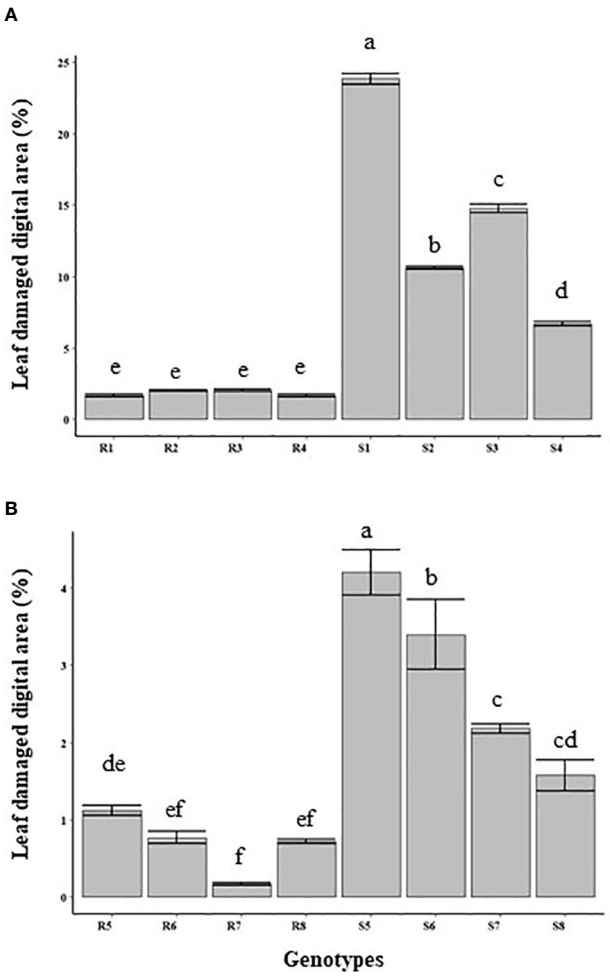

FIGURE 3
Quantification of the leaf area affected at 96 hours post inoculation with *Peyronellaea pinodes* **(A)** and *Didymella pinodella* **(B)**. The data are presented as mean ± SEM. Different letters on each bar signifies statistical significance among genotypes at $P < 0.05$ level for *P. pinodes* and *D. pinodella*. R1, PBA Wharton; R2, 11HP302-12HO-1; R3, 05H161-06HOS2005-BOG09-2; R4 09HP216-10HO2-3; S1, OZP1604; S2, 11HP160-12HO-1; S3, PBA TWILIGHT; S4, 10HP249-11HO-7; R1-4 partially resistant and S1-4, susceptible to *P. pinodes*. R5, 10HP249-11HO-7; R6, PBA WHARTON; R7, PBA TWILIGHT; R8, 11HP420-12HO-13; S5, WAPEA2211; S6, OZP1604; S7, PBA OURA; S8, 09HP216-10HO2-3; R5-8 partially resistant and S5-8 susceptible to *D. pinodella*.

P. pinodes (Figure 5A). Although the susceptible genotype OZP1604 showed an induction of *PsOX11* and *PsCHS3*, it was significantly lower than 11HP302-12HO-1 and PBA Wharton respectively. The gene *PsOX11* showed a gradual induction, reaching a peak at 72 HPI in the partially resistant genotypes 11HP302-12HO-1 (280 fold) and PBA Wharton (175 fold), followed by a reduced induction level at 96 HPI. In the partially resistant genotypes 11HP302-12HO-1 and PBA Wharton, the relative mRNA levels of 262 and 345 fold changes were observed for the gene *Pshmm6*, while an incremental fold change was observed from 24 to 72 HPI and peaking expression of 128 and 319 at 96 HPI was observed for the gene *PsCHS3*. A distinguishable level of induction in the expression of *PsAPX1* was observed in the susceptible genotypes PBA Twilight and OZP1604. Interestingly there was a rapid induction of *PsOPR1* that peaked at 24 HPI and was observed in both partially resistant and susceptible genotypes.

Upon inoculation with *D. pinodella*, the genes *PsOX11*, *PsAPX1, PsCHS3*, and *PsOPR1* showed clear induction in the partially resistant genotype PBA Wharton (Figure 5B). Interestingly there was very little induction of all the studied defense related genes except *Pshmm6* in the other partially resistant genotype 10HP249-11HO-7. In the genotype PBA Wharton, a gradual induction of *PsOX11* was observed at 72 HPI (52 fold) and peaked at 96 HPI (114 fold). *PsCHS3* (70 fold) and *PsOPR1* (172 fold) also showed a peak relative mRNA level in PBA Wharton at 96 HPI, while *PsAPX1* showed rapid significant induction at 24 HPI (83 fold) and 96 HPI (57 fold)

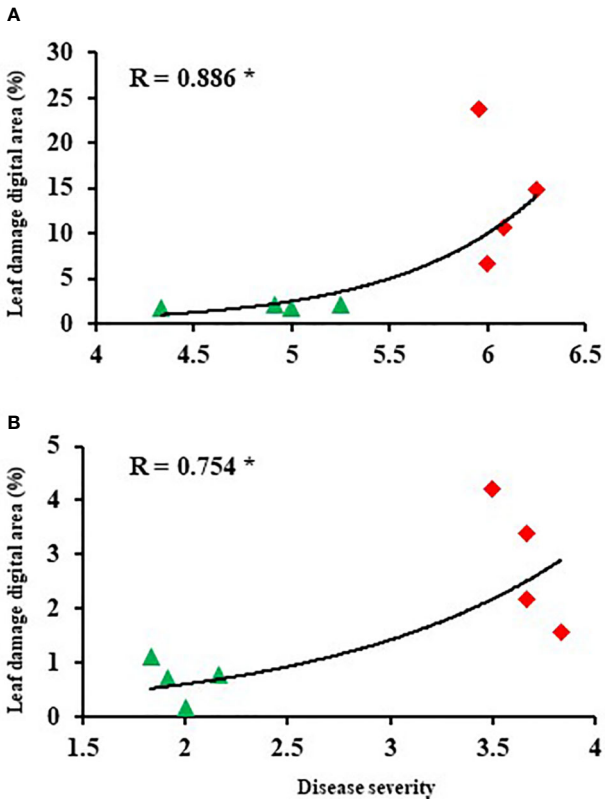

FIGURE 4
Correlations between disease score and leaf damage digital area at 96 hours post inoculation with *Peyronellaea pinodes* **(A)** and *Didymella pinodella* **(B)**. The asterisks show the significance level (* $p < 0.05$).

compared to the expression in susceptible genotypes OZP1604 and WAPEA2211. There was no difference in the expression of *Pshmm6* gene either in partially resistant or susceptible genotypes upon inoculation with *D. pinodella*.

Discussion

The ascochyta blight disease complex poses a continuous threat to the production of field pea worldwide. Understanding the resistance mechanisms initiated in field peas upon encountering the ascochyta blight pathogens will provide improved strategies to breed new genotypes that can effectively minimize yield loss. Despite previous efforts of characterizing of resistance responses of field pea against the pathogens *P. pinodes* (Fondevilla et al., 2011; Carrillo et al., 2013) and *A. koolunga* (Tran et al., 2018), these processes are still not completely understood. The polygenic nature of resistance in field peas against ascochyta blight indicates that there are complexities in understanding the associated mechanisms. The present study aimed at dissecting the resistance reactions in field pea genotypes inoculated separately with *P. pinodes* and *D. pinodella* using phenotypic, histochemical and molecular approaches.

The expression of five defense related genes, namely, *PsOXII*, *PsAPX1*, *Pshmm6*, *PsCHS3*, and *PsOPR1*, were studied to understand their role in providing resistance against necrotrophic pathogens that cause ascochyta blight in field pea. The genes selected for this study were from different classes such as the peroxidase superfamily (*PsOXII* and *PsAPX1*), the flavonoid and pisatin biosynthesis pathway (*PsCHS3* and *Pshmm6*), and the JA biosynthesis pathway (*PsOPR1*). In previous findings efforts were made to study the expression of these defense related genes against *P. pinodes* in leaf (Fondevilla et al., 2011; Tran et al., 2018) and against *A. koolunga* in the leaves and stems (Tran et al., 2018). In our study, an attempt was made to study the expression of these defense related genes and decipher their role in providing resistance against *P. pinodes* and *D. pinodella* in control and infected, resistant and susceptible lines.

Overall, changes in gene expression were much stronger for *PsOXII, Pshmm6* and *PsCHS3* upon inoculation with the more aggressive *P. pinodes* compared to the less aggressive *D.*

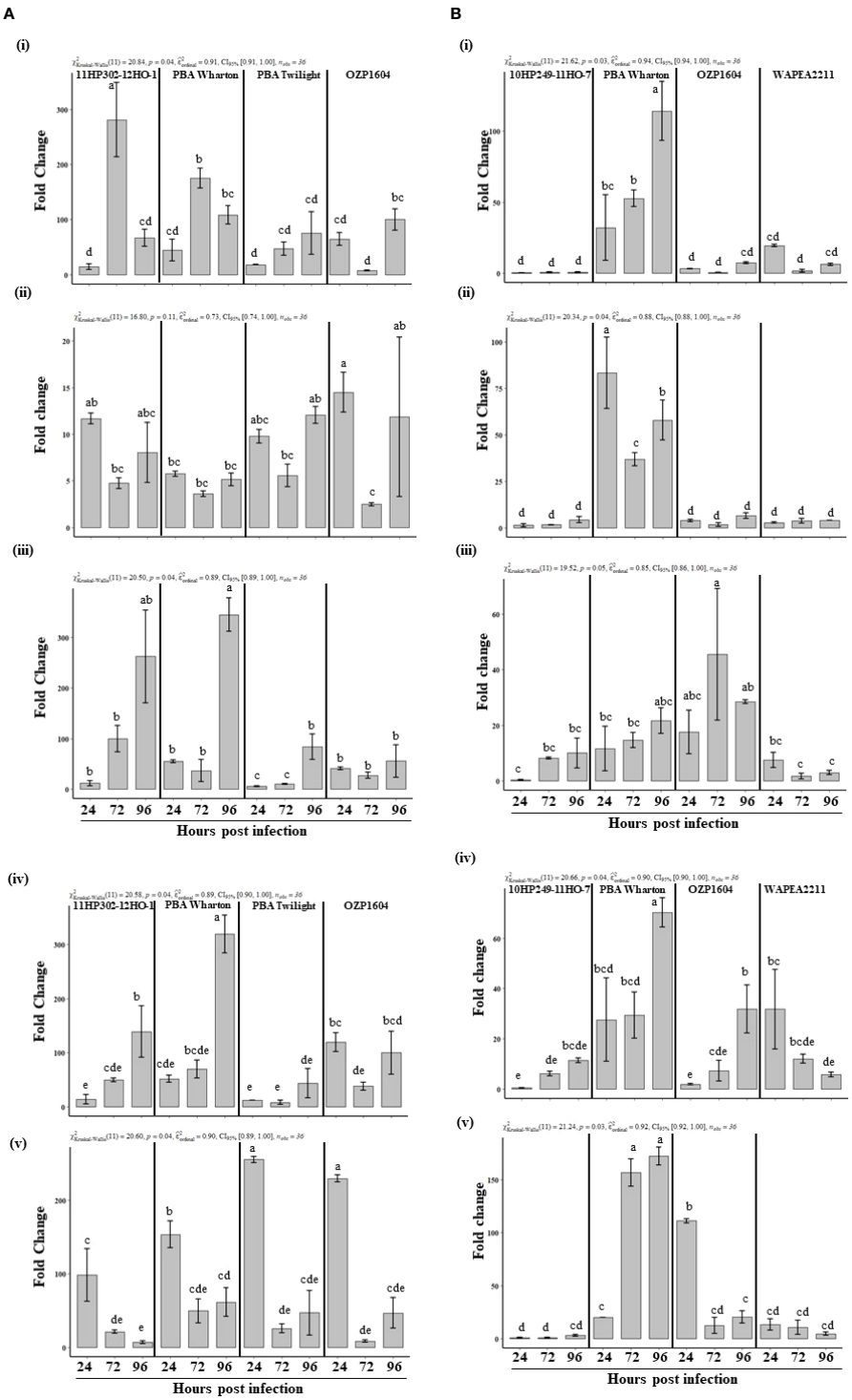

FIGURE 5
(A) Relative expression of defense related genes, *PsOXII* **(i)**, *PsAPX1* **(ii)**, *Pshmm6* **(iii)**, *PsCHS3* **(iv)**, *PsOPR1* **(v)** at 24, 72 and 96 hours post inoculation with *Peyronellaea pinodes*. The mRNA levels of defense related genes were normalized against reference gene *PsHistone3*. The data are presented as mean ± SEM. Different letters on each bar signifies statistical significance among genotypes at $P < 0.05$ level for *P. pinodes*. **(B)** Relative expression of defense related genes, *PsOXII* **(i)**, *PsAPX1* **(ii)**, *Pshmm6* **(iii)**, *PsCHS3* **(iv)**, *PsOPR1* **(v)** at 24, 72 and 96 hours post inoculation with *Didymella pinodella*. The mRNA levels of defense related genes were normalized against reference gene *PsHistone3*. The data are presented as mean ± SEM. Different letters on each bar signifies statistical significance among genotypes at $P < 0.05$ level for *D. pinodella*.

pinodella. These expression trends were in parallel to phenotypic assessments that showed that *P. pinodes* was 200% more aggressive in terms of disease severity and spread compared to *D. pinodella.* Similarly, (Hanssen et al., 2011) demonstrated a similar finding when the aggressive isolates of a pepino mosaic virus isolate against tomato seedlings elicited a stronger defense response than milder forms of the pathogen.

PsOXII codes for an extracellular enzyme while *PsAPX1* codes for an intracellular enzyme and these belong to class III and class I of the plant peroxidase superfamily, respectively. They play critical roles in plant defense by contributing to the formation of defense barriers (Jiang et al., 2019). Apart from plant defense, class III peroxidases are involved in physiological processes such as formation of lignin (Warinowski et al., 2016), auxin metabolism (Zhang et al., 2014), seed germination (Singh et al., 2015) and aging (Chen et al., 2020). Intrigued by the differential expression of *PsOXII* and *PsAPX1* in field pea against *A. koolunga* (Tran et al., 2018) the same two genes were evaluated against *P. pinodes* and *D. pinodella*. *PsOXII* showed an elevated expression of 170-200 fold in partially resistant genotypes upon inoculation with *P. pinodes*, whereas an inoculation with *D. pinodella* resulted in a 30-85 fold increase in expression in PBA Wharton. These results show that this gene is expressed more in an interaction with the aggressive pathogen *P. pinodes* both in partially resistant and susceptible genotypes although sooner in partially resistant genotypes. This gene expression was noticeably upregulated only in PBA Wharton when inoculated with the less aggressive pathogen *D. pinodella.*

PsAPX1 had similar patterns of induction in both resistant and susceptible genotypes when inoculated with *P. pinodes*, although induction was slightly higher in susceptible genotypes. Conversely, expression in the *D. pinodella* interaction was very low in three genotypes, but much higher in PBA Wharton. This suggests that on the one hand, APX1 plays role in a susceptible interaction with *P. pinodes*, but not with the more benign *D. pinodella.* This gene also appears to have a different role in PBA Wharton when challenged with this benign pathogen, and this is different than the other resistant line.

Similar elevated expression levels of five peroxidase genes have been previously demonstrated against treatment of *P. pinodes* elicitor (Kawahara et al., 2006) confirming the role of *PsOXII* in the pea and *P. pinodes* interaction. Similar differential response was demonstrated for *PsOXII* and *PsAPX1* where both the genes showed an elevated expression in resistant genotype against an inoculation with *A. koolunga* although the expression was more than 10 times higher in *PsAPX1* compared to *PsOXII*. (Tran et al., 2018). Although it indicates from this study and the previous studies that different resistance mechanisms exists against *P. pinodes, D. pinodella and A. koolunga*, further research with more genes would help confirm these results.

Peroxidases (POD) are a class of proteins that are induced in various biotic stresses (Sasaki et al., 2004). They play an important role in scavenging the excess H_2O_2 to maintain the ROS homeostasis in the cell (Ozyigit et al., 2016) and may have played a crucial role in providing partial resistance to the genotypes against the two pathogens under investigation. The leaves of partially resistant genotypes showed significantly fewer intensely stained lesions that was due to decreased cell death and lower generation of H_2O_2 compared to the leaves of susceptible plants. This was similar to the work in tomato that showed increased necrotic lesions, more intensely stained leaves, and lower activities of peroxidase enzymes in more susceptible mutants compared to the wild-type plants (Hong et al., 2019). The lack of an efficient scavenging mechanism may result in excessive generation of H_2O_2 and can cause oxidative stress resulting in chloroplast and peroxisome autophagy and triggering cell death (Smirnoff and Arnaud, 2019). The presence of antioxidant systems in plants help to eliminate excess H_2O_2 generated and thus maintains H_2O_2 levels in a normal dynamic balance (Quan et al., 2008). This could result in a lower detection of H_2O_2 in the partially resistant genotypes. Apart from the role of cellular signaling, ROS directly kills the pathogen and plays a key defensive strategy during pathogen attack (Paiva and Bozza, 2014). The results obtained in histochemical staining of the leaf samples and rapid induction in the expression of *PsOXII* and *PsAPX1* genes post inoculation with *P. pinodes* and *D. pinodella* confirmed that the association of elevated gene expression and low cell death in partially resistant genotypes compared to the susceptible counterparts. The lower accumulation of H_2O_2 in partially resistant genotypes may be due to the efficient scavenging mechanism by these peroxidase genes in comparison to susceptible genotypes. The result obtained in this study are in line with the findings that showed the removal of excessive H_2O_2 and limiting the damage caused during an interaction of wheat with *Pyricularia oryzae* ultimately provided greater resistance to the blast disease (Debona et al., 2012). More specifically, during the *D. pinodella* infection resulted an elevated the expression of *PsAPX1* gene which has played a key role to restrict the spread of the pathogen and this gene also played an important role in scavenging the excessive H_2O_2 and formed a part of defense reaction. In plants and algae *APX* enzyme catalyze the reduction of H_2O_2 and prevents the $H_2O_2^-$ mediated damage to cells and organs (Ozyigit et al., 2016).

Pshmm6 and *PsCHS3* encode for enzymes in the field pea isoflavonoid phytoalexin pisatin biosynthesis pathway (Liu et al., 2006). This phytoalexin has played a critical role in initiating defense responses upon inoculation with *P. pinodes* (Fondevilla et al., 2011) and *A. koolunga* (Tran et al., 2018) and the reduced ability to produced pisatin resulted in lower resistance to fungal infection (Wu and VanEtten, 2004). In this study the *Pshmm6* gene had the highest expression levels in partially resistant genotypes when challenged against the aggressive pathogen *P. pinodes* particularly at the later stages of infection (~ 260 – 345 fold) compared to susceptible genotypes (~ 55 – 83 fold). It is

clear that the induction levels were relatively low and at equal levels in partially resistant and susceptible field pea genotypes after inoculation with less virulent pathogen *D. pinodella* (~ 21 - 45 fold). This could be due to the presence of two highly conserved *hmm* genes which share 95.8% amino acid identity in field pea (Wu et al., 1997) where the other *hmm* gene may have played a role in initiating a defense response and providing resistance against *D. pinodella*. Further research is needed to decipher the role of these two *hmm* genes in providing resistance against the pathogens.

PsCHS3 showed greater induction up to ~ 319 fold late in the infection process in the partially resistant genotype PBA Wharton and *P. pinodes* interaction. Similarly low expression levels were observed in other partially resistant genotype (128 fold) and susceptible genotype OZP1604 (~ 120 fold) when inoculated with *P. pinodes*. In PBA Wharton and *D. pinodella* interaction the partially resistant genotype showed high expression level albeit late in infection. The lower disease severity in the partially resistant genotype PBA Wharton provides evidence that strong induction of *Pshmm6* and *PsCHS3* contributed to restrict the growth and spread of both the pathogens especially at later stage of infection. In a similar study in cotton, the knockdown of *GhCH3* gene resulted in the increased susceptibility to the *Verticillium dahliae* infection (Lei et al., 2018), which makes it clear that *GhCH3* gene plays a critical role in providing resistance against *V. dahliae*.

The hormone jasmonate has been shown to be involved in plant resistance against necrotrophic pathogens (Veronese et al., 2004). *AtOPR1* encodes for a 12-Oxophytodienoate reductase enzyme in the JA biosynthesis pathway in Arabidopsis (Biesgen and Weiler, 1999). In our studies *PsOPR1* showed a 153 – 229 fold induction at early stage of infection in both partially resistant and susceptible plants when inoculated with *P. pinodes* although the expression was significantly higher in the susceptible plants. These results were in agreement with that obtained by Fondevilla et al. (2011) where *OPR1* was shown to have high induction in susceptible genotype Messire and no induction in the resistant genotype P665 upon inoculation with *P. pinodes*. Interestingly there was clear high and gradual induction in partially resistant genotype PBA Warton up to 156 – 172 fold albeit late in the infection compared to its susceptible counterparts, when inoculated with *D. pinodella*. High induction of *PsOPR1* may not be enough to counter the aggressive pathogen like *P. pinodes* while a similar level of induction was sufficient enough to provide resistance against *D. pinodella*.

The visual quantification of damaged leaf area due to pathogen infection can be a challenging task due to its subjective nature. In recent times sensor based approaches have been widely used to assess the leaf damage post pathogen infection. Application of high throughput image processing techniques has enabled us to quantify the spread of the infection by *P. pinodes* and *D. pinodella*. The image processing techniques helped in the estimation of damaged leaf area due to infection and showed a 4.9 times higher detection of generated H_2O_2 in susceptible plants inoculated with *P. pinodes* than *D. pinodella*. Similar image processing techniques have been deployed to detect bacterial and fungal diseases in bean leaf (Singh and Misra, 2017) and estimation of the disease spread (Bock et al., 2010). Image analysis not only helps in detecting disease symptoms but also provides an enhanced ability to differentiate the genotypes with varying disease severity. In this regard our findings confirm those of another study investigating bacterial blight in bean (Xie et al., 2012). The results obtained by digital image analysis play a pivotal role in accurately phenotyping disease severity for detailed genetic analysis. This technique has been used as a tool in identifying quantitative trait loci for powdery mildew resistance in lettuce (Simko et al., 2014). The strong correlation between leaf damage digital area and disease severity shows that the value of using digital image analysis as a surrogate method in assessing disease severity.

The disease severity scores of experiment 1 and leaf damage digital area of experiment 2 inoculated against *P. pinodes* and *D. pinodella* showed high positive correlations, validating the disease assay and highlighting the value of the imaging technology. Furthermore, the reliability of the assay allows the selection of genotypes across different experiments and provides confidence in selecting improved lines for disease resistance breeding.

Conclusion

A range of field pea genotypes were evaluated to characterization the resistance against the two pathogens *P. pinodes* and *D. Pinodella* through phenotypic, histochemical and molecular approaches. Among the two pathogens *P. pinodes* was more aggressive compared to *D. pinodella*, exhibited a clear differential disease severity between genotypes against the two pathogen. The breeding lines 11HP-302-12HO-1 and 10HP249-11HO-7 showed lower disease severity and less accumulation of H_2O_2 against individual pathogens. The partially resistant genotype 11HP-302-12HO-1 showed an elevated early expression of *PsOXII*, late induction of *Pshmm6* to *P. pinodes*. Along with the breeding line PBA Wharton showed late expression of *PsCHS3* gene against *P. pinodes* and demonstrated high expression of *PsPOXII, PsAPX1, PsCHS3*, and *PsOPR1* against milder pathogen *D. pinodella* indicating that the resistance is multifaceted. The variation in responses exhibited against different pathogens of ascochyta blight can be harnessed through a recurrent selection breeding programs by combining different sources of partial resistance as identified in this work. The high correlation between data from two independent experiments show the stability of genotypes and these partially resistant breeding lines can be effectively used in disease resistance

breeding to develop varieties that produce sustainable yield by overcoming this disease complex.

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

SJ and GR conceived the experiment. SJ designed, conducted, performed statistical analysis and wrote the draft. GR supported the study. BP contributed scientific inputs. All authors edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

This study received funding from Grain Research Development Corporation. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.976375/full#supplementary-material

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Genetic mapping of the Andean anthracnose resistance gene present in the common bean cultivar BRSMG Realce

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The rajado seeded Andean bean (*Phaseolus vulgaris* L.) cultivar BRSMG Realce (striped seed coat) developed by Embrapa expressed a high level of anthracnose resistance, caused by *Colletotrichum lindemuthianum*, in field and greenhouse screenings. The main goal of this study was to evaluate the inheritance of anthracnose resistance in BRSMG Realce, map the resistance locus or major gene cluster previously named as *Co-Realce*, identify resistance-related positional genes, and analyze potential markers linked to the resistance allele. F_2 plants derived from the cross BRSMG Realce × BRS FC104 (Mesoamerican) and from the cross BRSMG Realce × BRS Notável (Mesoamerican) were inoculated with the *C. lindemuthianum* races 475 and 81, respectively. The BRSMG Realce × BRS FC104 F_2 population was also genotyped using the DArTseq technology. Crosses between BRSMG Realce and BAT 93 (Mesoamerican) were also conducted and resulting F_2 plants were inoculated with the *C. lindemuthianum* races 65 and 1609, individually. The results shown that anthracnose resistance in BRSMG Realce is controlled by a single locus with complete dominance. A genetic map including 1,118 SNP markers was built and shown 78% of the markers mapped at a distances less than 5.0 cM, with a total genetic length of 4,473.4 cM. A major locus (*Co-Realce*) explaining 54.6% of the phenotypic variation of symptoms caused by the race 475 was identified in Pv04, flanked by the markers snp1327 and snp12782 and 4.48 cM apart each other. These SNPs are useful for marker-assisted selection, due to an estimated selection efficiency of 99.2%. The identified resistance allele segregates independently of the resistance allele *Co-3*^{3} (Pv04) present in BAT 93. The mapped genomic region with 704,867 bp comprising 63 putative genes, 44 of which were related to the pathogen-host interaction. Based on all these results and evidence, anthracnose resistance in

BRSMG Realce should be considered as monogenic, useful for breeding purpose. It is proposed that locus *Co-Realce* is unique and be provisionally designated as *CoPv04*^{R} until be officially nominated in accordance with the rules established by the Bean Improvement Cooperative Genetics Committee.

KEYWORDS

***Phaseolus vulgaris* L., *Colletotrichum lindemuthianum*, molecular breeding, genetic resistance, allelism test, inheritance study**

Introduction

The common bean (*Phaseolus vulgaris* L.) is grown in more than 120 countries under different temperatures, light intensities, relative humidity, rainfall distributions and technological levels, aspects that contribute to the unstable global production (Pereira et al., 2018; FAO, 2022). Brazil is one of the main producer countries, harvesting 2,366,527 ton in 2020, 85% of which were the *carioca* and black seeded cultivars (Embrapa Rice and Beans, 2022).

The soil and climate conditions in regions with tropical and subtropical climates favor the occurrence of fungal diseases such as anthracnose, caused by *Colletotrichum lindemuthianum* (Basavaraja et al., 2020). This disease, which displays wide geographic distribution and pathogenic variability (Nabi et al., 2022), is more prevalent in areas with temperatures between 15 and 22°C, associated with high relative humidity (RU ≥ 95%) and frequent rainfall (Padder et al., 2017). Depending on the susceptibility level of cultivars, favorable environmental conditions and the presence of the initial inoculum, the disease can cause losses of up to 100% (Singh and Schwartz, 2010). In Brazil, where anthracnose races from the Mesoamerican gene *pool* is predominant, the introgression of resistance alleles from Andean gene *pool* is an important strategy to develop cultivars with durable and broad resistance spectrum (Miklas et al., 2006; Paulino et al., 2022). This strategy is supported by the high level of anthracnose resistance in the Andean cultivars developed by Embrapa in Brazil, particularly in BRSMG Realce, which is resistant to races 65, 73 and 81 (Melo et al., 2014; Aguiar et al., 2021). These races are the most prevalent in the main Brazilian common bean growing areas for the past 30 years (Paulino et al., 2022). The anthracnose resistance of BRSMG Realce has also shown to be stable over time, becoming one of the resistant controls in the final field trials – experiments of Value for Cultivation and Use (VCU) – conducted by the Embrapa breeding program (Aguiar et al., 2021). Thus, identifying resistance sources from the Andean gene *pool* and mapping the resistance alleles present in these genotypes is an indispensable target of common bean pre-breeding programs worldwide, enabling their effective use in the development of cultivars with durable and broad-spectrum resistance.

Disease integrated management and the use of resistant cultivars are considered the most promising, environmentally sustainable and economically profitable methods, in addition to being easily applied by growers (Miklas et al., 2006; Souza et al., 2013). Anthracnose resistance in common bean is largely conditioned by dominant alleles of major quantitative trait loci (QTLs), except for *co-8* (Paulino et al., 2022). Currently, 14 effective resistance loci have been identified; *Co-1* to *Co-17*, excluding *Co-7*, *Co-9* and *Co-10* which have been renamed as alleles from other loci. They were mapped in eight common bean chromosomes (Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, Pv09 and Pv11). Five of these loci have been identified in resistance sources from the Andean gene *pool*, namely as *Co-1*, *Co-12*, *Co-13*, *Co-14* and *Co-15* (BIC, List of Genes – *Phaseolus vulgaris* L.: http://www.bic.uprm.edu/wp-content/uploads/2019/10/Bean-Genes-List-2018-v2-1.pdf). *Co-1* is from the Michigan Dark Red Kidney resistance source and it was mapped in Pv01 (Zuiderveen et al., 2016). In this same genomic region, four alleles were identified: *Co-1*^{2} (Melotto and Kelly, 2000), *Co-1*^{3} (Melotto and Kelly, 2000), *Co-1*^{4} (Gonçalves-Vidigal et al., 2011), and *Co-1*^{5} (Gonçalves-Vidigal and Kelly, 2006). *Co-12* is a non-mapped resistance allele identified in the cultivar Jalo Vermelho (Gonçalves-Vidigal et al., 2008). *Co-13* was mapped on Pv03 in the Brazilian *landrace* Jalo Listas Pretas (Gonçalves-Vidigal et al., 2009; Lacanallo and Gonçalves-Vidigal, 2015). *Co-14* was mapped on Pv01, in the Pitanga resistance source (Gonçalves-Vidigal et al., 2012), while *Co-15* was mapped on Pv04 in the Brazilian *landrace* Corinthiano (Sousa et al., 2015).

Recent studies report new genomic regions associated with race-specific resistance to *C. lindemuthianum* in the common bean germplasm from Andean gene *pool*, such as the *Co-BF* (Marcon et al., 2021; Xavier et al., 2022), *Co-AC* (Gilio et al., 2020), *CoPv01*^{CDRK} (Gonçalves-Vidigal et al., 2020) and *Co-Pa* alleles (Lima-Castro et al., 2017), which have not been officially named in accordance with the rules established by the Bean Improvement Cooperative Genetics Committee (BIC, Genetics Committee: http://arsftfbean.uprm.edu/bic/wp-content/uploads/2018/04/Gene_Committee_Rules.pdf).

The main goal of this study was to evaluate the inheritance of anthracnose resistance in BRSMG Realce, map the resistance locus previously named as *Co-Realce*, identify resistance-related

positional genes, and analyze potential markers linked to the resistance allele. In addition, allelism tests have also been done to check if *Co-Realce* segregates independently of the resistance allele *Co-3*^{3} present in BAT 93, already used by the Embrapa common bean breeding program.

Materials and methods

Genetic material and crosses

BRSMG Realce is a *rajado* (striped seed coat) seeded cultivar from the Andean gene *pool* developed by Embrapa and partners in Brazil (Supplementary Figure 1). This cultivar presents a type I determinate growth habit, high yield potential and it is well suited to mechanized harvesting. In addition to anthracnose resistance, it is also resistant to powdery mildew (*Erysiphe polygoni*) and bacterial wilt (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*) (Melo et al., 2014). BRS FC104 is a Mesoamerican *carioca* seeded cultivar also developed by Embrapa, showing a super-early maturity and high yield potential (Melo et al., 2019). BRS Notável is also a Mesoamerican cultivar from *carioca* market class, but with a medium-early maturing cycle. It is resistant to anthracnose, *fusarium* wilt (*Fusarium oxysporum* f. sp. *phaseoli*), common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*) and bacterial wilt (Pereira et al., 2012). BAT 93 harbors the anthracnose resistance allele *Co-3*^{3}. It is a Mesoamerican breeding line developed by *Centro International de Agricultura Tropical* (CIAT, Cali, Colombia) from a double cross involving the parents Veranic 2, PI 207262, Jamapa, and Great Northern Tara (Geffroy et al., 2008).

For the inheritance studies, crosses between BRSMG Realce (female parent) and BRS FC104 (male parent) and between BRSMG Realce and BRS Notável (male parent) were carried out at Embrapa Rice and Beans (Santo Antônio de Goiás, Goiás, Brazil), under controlled conditions (greenhouse). The resulting F_1 plants were checked as true hybrids using 24 microsatellite markers, as described by Morais et al. (2016). F_1 checked plants were then advanced and F_2 seeds were obtained. For the allelism tests, using the same strategy, BRSMG Realce (female parent) was crossed with BAT 93 (male parent) and resulting F_2 seeds were obtained.

Phenotyping of F_2 populations

An inoculation test of the parents and control lines (BRSMG Realce, BRS FC104, BRS Notável, BAT 93, SEL 1308 and IPA 7419) was carried out under controlled conditions using the races 65, 73, 81, 91, 113, 475 and 1609 of *C. lindemuthianum*. The segregating F_2 populations were inoculated using the races that resulted in a better phenotypic contrast between their parents (Supplementary Table 2).

For the inheritance studies, 161 F_2 seedlings from the cross BRSMG Realce × BRS FC104 and 128 F_2 seedlings derived from the cross BRSMG Realce × BRS Notável were grown in expanded polystyrene trays filled with commercial substrate (Plantmax®). Each tray also contained 12 plants of the parents and the control lines (SEL 1308, resistant control; IPA 7419, susceptible control) (Sartorato et al., 2004). Before inoculation, plant tissue samples of each F_2 (BRSMG Realce × BRS FC104) plant and of their parents were collected and stored in a freezer at -20°C for genomic DNA extraction. For the allelism studies aiming to test the independence between the anthracnose resistance locus present in BRSMG Realce (*Co-Realce*) and *Co-3*^{3} present in BAT 93 (chromosome Pv04), which is already used by the Embrapa common bean breeding program, F_2 (BRSMG Realce × BAT 93) plants were independently inoculated with *C. lindemuthianum* races 65 (132 F_2 plants) and 1609 (183 F_2 plants).

Plants were inoculated seven days after sowing, in the V2 stage (fully expanded primary leaves) (Pastor-Corrales, 1992). The spore solution (1.2×10^6 spores/mL) was applied to the abaxial and adaxial leaves, using a manual atomizer (De Vilbiss, No. 15). After inoculation, the plants were incubated in a humidity chamber for 48 h, with temperature adjusted to 20 ± 2°C, 95% relative humidity controlled by nebulization and a 12-hour light/dark photoperiod. Later, nebulization was discontinued, and the inoculated plants were kept in a controlled environment under the same temperature and photoperiod conditions described above, where they remained until disease symptoms were screened.

Symptoms were evaluated seven days after inoculation, based on a 1-to-9 scale, where 1 = absence of symptoms; 2 to 3 = very small lesions, mostly on primary leaves; and 4 to 8 = numerous enlarged lesions or sunken cancers on the lower sides of leaves or hypocotyls; 9 = dead plants due to symptoms caused by the disease (Pastor-Corrales and Tu, 1989). Biologically, scores 1 to 3 represent incompatibility reactions between *C. lindemuthianum* and *P. vulgaris* and, therefore, are typical resistance reactions. On the other hand, the scores 4 to 9 indicate compatibility reactions and are characteristic susceptibility reactions (Pastor-Corrales, 1992). Thus, plants with scores between 1 and 3 are considered resistant (R) and the others susceptible (S). This threshold for R/S disease reactions is widely accepted and used by the bean research community (BIC, Research Techniques – Anthracnose: http://arsftfbean.uprm.edu/bic/wp-content/uploads/2018/04/Anthracnose.pdf).

Genotyping with SNP and SilicoDArT markers

Genomic DNA extraction from parental lines and F_2 plants (BRSMG Realce × BRS FC104) was performed according to the protocol described by Ferreira and Grattapaglia (1998). DNA concentration was estimated by fluorescence, using a Qubit® 2.0 Fluorometer (Invitrogen by Life Technology), and DNA integrity was checked *via* 1.0% agarose gel electrophoresis. The genotyping protocol was accomplished based on DArTseq technology, developed by DArT Pty Ltd (Kilian et al., 2012), from which SNP and SilicoDArT markers were extracted, as described by Valdisser et al. (2020).

Genetic mapping with SNP markers

The polymorphic SNP markers between parental lines were tested for Mendelian segregation at an expected ratio of 1:2:1 using the chi-squared test (χ^2; P-value < 0.05), followed by FDR (False Discovery Rate, P-value < 0.05) correction proposed by Benjamini and Hochberg (1995). The linkage groups were established using a LOD-score (logarithm of the odds) of 5 and maximum recombination fraction of 0.1. The order of markers was estimated using the RCD (Rapid Chain Delineation) method with a LOD-score of 3.0. In addition, the most likely position of each marker on the map was obtained using the safe function and later, the ripple function (5-marker windows and LOD-score of 3). Genetic distances were estimated using the Kosambi function (Kosambi, 1944). The coefficient of Spearman's correlation was estimated for the genetic marker positions and the physical marker positions on the reference genomes. The linkage map was constructed in the R software (R Core Team, 2022), using the OneMap package (Margarido et al., 2007).

QTL analysis and physical mapping

QTL (Quantitative Trait Loci) analysis was carried out using composite interval mapping (CIM) (Zeng, 1993), with a walkspeed of 0.5 cM and window size of 1.0 cM. The coefficient of determination (R^2) was calculated separately for each interval to determine the percentage of phenotypic variation explained by a single locus. The likelihood ratio values were converted into LOD values using the equation LOD = 0.2171*LTR (Churchill and Doerge, 1994). The minimum LOD value to declare the existence of a QTL was estimated using the criterion proposed by Churchill and Doerge (1994), with 1,000 permutations. Analyses were conducted using QTL-Cartographer software (Wang et al., 2012). The *Co-Realce* genomic region on the Pv04 was graphically represented using the software MapChart (Voorrips, 2002). The physical map was obtained using the positions of each marker linked with target alleles provided in base pairs (bp), according to the reference genome (Schmutz et al., 2014) and using the software MapChart (Voorrips, 2002).

Gene annotation

The genes annotated in the current version of the bean genome (Schmutz et al., 2014) were extracted from the sequences included in the locus interval identified in this study, using the Phytozome platform (*Phaseolus vulgaris* v2.1, DOE-JGI and USDA-NIFA, http://phytozome.jgi.doe.gov/).

Selection efficiency

Selection efficiency (%SE) of the SNP markers identified in the resistance locus interval was estimated according to the methodology described by Liu (1998), using the following estimator: SE (%) = ($1 - 4rf^2$), where "rf" is the recombination frequency between marker pairs.

Results

Reaction of parents to selected *C. lindemuthianum* races

Out of the seven *C. lindemuthianum* races used to screen the parents and controls (65, 73, 81, 91, 113, 475 and 1609), BRSMG Realce was resistant to six races, with mean score of 1.0, being susceptible only to race 113 (mean score of 5.2). BRS Notável was susceptible only to race 81 (mean score of 9.0). As expected, the resistant control SEL 1308 was resistant to all seven races, with mean score of 1.0, and the susceptible control IPA 7419 was susceptible, with mean score of 9.0. BRS FC104 was screened with five races (73, 81, 91, 475 and 1609), showing susceptibility to the races 81, 91, 475 and 1609. For the inheritance studies and allelism tests, the *C. lindemuthianum* races causing strongest contrasts for disease symptoms among parents were those selected and used to inoculate the segregating populations (Supplementary Table 2).

Inheritance studies and allelism tests

The screening of 161 F_2 (BRSMG Realce × BRS FC104) plants with the race 475 shown 127 resistant (scores 1-to-3) and

34 susceptible plants (scores 4-to-9), resulting in a segregation ratio of 3R:1S (χ^2 = 1.29; P-value = 26%). A total of 128 F_2 (BRSMG Realce × BRS Notável) plants were inoculated with the *C. lindemuthianum* race 81. The segregation ratio observed was also 3R:1S (χ^2 = 1.04 and P-value = 31%) (Table 1; Supplementary Table 1).

A total of 132 and 183 F_2 (BRSMG Realce × BAT 93) plants were inoculated with the *C. lindemuthianum* races 65 and 1609, respectively. In both cases, the segregation ratio observed was 15R:1S (χ^2 = 0.98 and P-value = 32%, and χ^2 = 0.55; P-value = 46%). The joint analysis using data from all 315 F_2 (BRSMG Realce × BAT 93) also shown a segregation ratio of 15R:1S (χ^2 = 0.005 and P-value = 94%) (Table 1).

These results strongly suggest that anthracnose resistance in BRSMG Realce is controlled by a single locus with complete dominance. In addition, that the resistance allele present in BRSMG Realce segregates independently of the resistance allele *Co-3*^{3} present in BAT 93 and mapped in Pv04.

Genetic map

The genotyping approach based on DArTseq technology resulted in 13,083 SNP and 16,186 DArT markers (Supplementary Table 3), with call rates ranging from 0.68 to 1.00 and from 0.56 to 1.00, respectively (Supplementary Table 4). A total of 6,304 (48.2%) SNP markers were polymorphic in the F_2 (BRSMG Realce × BRS FC104) population. The segregation test identified 4,175 (31.9%) of these markers as undistorted SNPs, once they fit to the segregation ratio of 1:2:1 (FDR ≥ 5%) and therefore were used for genetic mapping. Out of these markers, 4,129 (31.6%)

TABLE 1 Inheritance of anthracnose resistance in the Andean common bean cultivar BRSMG Realce from the rajado (striped seed coat) market class, and allelism test between BRSMG Realce *(Co-Realce)* and BAT93 *(Co-3*^{3}*)*.

Race[a]	Genotype	Hypothesis[d] R:S	Observed		Expected		χ^2	P-value
			R	S	R	S		
81	BRSMG Realce (*Co-Realce*)	1:0	12	0	12	0	–	–
	BRS Notável	0:1	0	12	0	12	–	–
	IPA 7419[b]	0:1	0	12	0	12	–	–
	F_2 (BRSMG Realce × BRS Notável)	3:1	101	27	96	32	1.0	0.31
475	BRSMG Realce (*Co-Realce*)	1:0	12	0	12	0	–	–
	BRS FC104	0:1	0	12	0	12	–	–
	IPA 7419	0:1	0	12	0	12	–	–
	F_2 (BRSMG Realce × BRS FC104)	3:1	127	34	121	40	1.3	0.26
65	BRSMG Realce (*Co-Realce*)	1:0	12	0	12	0	–	–
	BAT93	1:0	12	0	12	0	–	–
	IPA 7419	0:1	0	12	0	12	–	–
	F_2 (BRSMG Realce × BAT93)	3:1	121	11	99	33	19.6	$9.77e^{-06}$
		9:7	121	11	74	58	67.3	$2.36e^{-16}$
		13:3	121	11	107	25	9.4	0.002
		15:1	121	11	124	8	0.98	0.32
1609	BRSMG Realce	1:0	12	0	12	0	–	–
	BAT93 (*Co-3*^{3})	1:0	12	0	12	0	–	–
	IPA7419	0:1	0	12	0	12	–	–
	F_2 (BRSMG Realce × BAT93)	3:1	174	9	137	46	39.4	$3.52e^{-10}$
		9:7	174	9	103	80	112.1	$< 2.2e^{-16}$
		13:3	174	9	149	34	22.9	$1.64e^{-06}$
		15:1	174	9	172	11	0.55	0.46
F_2 (BRSMG Realce × BAT93) – Joint analysis[c]		3:1	295	20	236	79	58.4	$2.10e^{-14}$
		9:7	295	20	177	138	179.1	$< 2.2e^{-16}$
		13:3	295	20	256	59	31.8	$1.71e^{-08}$
		15:1	295	20	295	20	0.01	0.94

[a]Race of *Colletotrichum lindemuthianum*.
[b]Susceptible control.
[c]Joint allelism test performed using all resistant (121 + 174) and susceptible (11 + 9) F_2 (BRSMG Realce × BAT93) plants, considering the reaction to races 65 and 1609.
[d]R – Number of resistant plants, and S – Number of susceptible plants.

performed well for linkage analysis. Among them, 395 and 60 markers were positioned in *contigs* and *scaffolds*, respectively (Supplementary Figure 2; Supplementary Table 4).

A linkage map was built including 4,074 SNP markers covering the entire common bean genome. The linkage groups with the largest and smallest number of markers were Pv02 and Pv04, with 505 and 152 SNP markers, respectively. The average number of markers per linkage group was 370 (Supplementary Table 5). The SNPs mapped on *contigs* and *scaffolds* were allocated to the 11 chromosomes (Supplementary Table 6). When keeping only the markers with high statistical support (SAFE map), a total of 1,315 markers were mapped and well distributed in the common bean genome (Supplementary Figure 2), with an average of 120 markers per linkage group. The total genetic linkage distance of the SAFE map was 4,473.44 cM, with an average of 406.68 cM. Pv01 was the largest linkage group, with 561.32 cM, and the smallest one was Pv04, with 196.82 cM. In average, 78.1% of the markers were mapped at distances less than or equal to 5.0 cM, with an average distance of 4.07 cM between markers along the 11 chromosomes (Supplementary Table 5). Pv02 shown highest density (Supplementary Figure 2), with an average distance of 2.91 cM between markers and 89.7% of the markers were mapped at ≤ 5.0 cM (Supplementary Table 5). Markers ordered with a LOD-score < 3.0 were represented as accessory markers in their most likely position (Supplementary Table 6). The Spearman's correlation coefficients (ρ) between the positions of the markers on linkage map and physical map were positive (0.996-to-0.999) and highly significant (p-value < $2.2e^{-16}$), with an average of 0.999 (Supplementary Table 5).

Major locus associated with anthracnose resistance

The QTL analysis identified a major locus associated with anthracnose resistance in the Andean common bean cultivar BRS Realce on Pv04 (*Co-Realce*), with a LOD-score of 15.3 and explaining 54.60% of the phenotypic variation considering the symptoms incited by the *C. lindemuthianum* race 475. The size of this QTL was 4.48 cM flanked by the SNP markers snp1327 (position 477,285 bp) and snp12782 (1,182,123 bp) (Table 2; Figure 1). Simple linear regression analysis shown that markers snp1327 and snp12782 explain, respectively, 29% and 33% of the phenotypic variation (Table 3). The homozygous plants for the snp1327 reference allele (TT) associated with disease resistance shown a mean severity score of 1.62, while the mean score of homozygous plants for the respective susceptibility allele (CC) was 4.54 (Figure 2). Considering the locus snp12782, the homozygous plants for the resistance allele (CC) shown a mean severity score of 1.59, while the mean score of homozygous plants for the respective susceptibility allele (TT) was 4.86 (Figure 2). The joint selection of homozygous and heterozygous plants for *Co-Realce* using the markers snp1327 and snp12782 resulted in a set of plants showing a mean severity score of 1.54. The size of *Co-Realce* genomic region was 704,867 bp long (Pv04: 477,217 bp…1,182,084 bp) (Table 2) and a total of 63 genes were observed to be located in this interval, of which 44 are involved in signaling pathways of response to pathogen attack (Supplementary Table 7).

Increasing of mapping resolution in *Co-Realce* genomic region

In order to increase the mapping resolution in the genomic region containing the major locus *Co-Realce*, an additional set of 246 markers, including 135 SNPs and 111 SilicoDArTs previously known as located on Pv04 and with call rate of 0.58-to-1.0, were included in the genetic linkage analysis. The recombination fraction was estimated and 229 markers were mapped (Supplementary Table 8). By increasing markers density in the *Co-Realce* genomic region, its interval reduced from 704,867 bp to 20,405 bp (LOD of 16.3) and the phenotypic variation explained was 54% (Table 2). After this new approach, the closest and significantly markers identified as associated with *Co-Realce* were dart9817 (position 485,246 bp) and snp3308 markers (position 505,696 bp) spanning 2.9 cM (Table 2; Figure 1). A total two putative candidate genes associated with cell membrane processes were identified in the *Co-Realce* region. The Phvul.004G006800 transcript encodes proteins from the nuclear pore complex involved in the membrane transport system (Nuclear Pore Complex NPC - Nup210 GP210), and the transcript Phvul.004G006900 that encodes a protein from the glycosylphosphatidylinositol transamidase complex

TABLE 2 SNP and DArT markers flanking the major locus *(Co-Realce)* controlling anthracnose resistance in the Andean common bean cultivar BRSMG Realce, recombination frequency between the pair of markers flanking *Co-Realce*, interval size of the *Co-Realce* region, LOD-score and percentage of phenotypic variation explained by major locus *Co-Realce*.

Interval[a]	Pair of markers[b]	rf	Interval size	LOD-score	R^2 (%)
Pv04: 477,217…1,182,084	snp1327 and snp12782	4.48 cM	704,867 pb	15.3	54.60
Pv04: 485,246…505,651	dart9817 and snp3308	2.91 cM	20,405 pb	16.3	54.02

[a]Chromosome Pv04 (Chr04).
[b]Markers flanking the major locus *Co-Realce*.
rf – recombination frequency between the markers flanking *Co-Realce*.

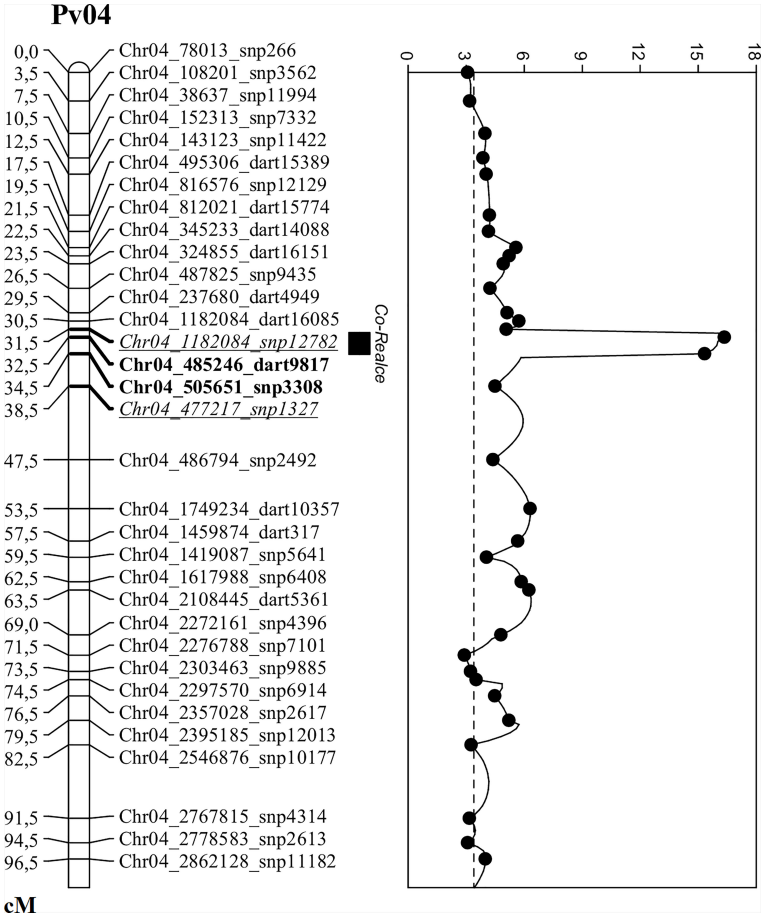

FIGURE 1
Genetic map of the *Co-Realce* genomic region on the common bean chromosome Pv04. QTL analysis was used to increase the mapping resolution in *Co-Realce* genomic region, performed using the F_2 population derived from the cross BRSMG Realce × BRS FC104 phenotyped with the *Colletotrichum lindemuthianum* race 475 and genotyped with SNP and SilicoDArT markers. The two underlined and italicized markers delimit the *Co-Realce* genomic region. The two bold markers delimit the *Co-Realce* genomic region after increasing the mapping resolution. The highest peak on Pv4 represents the major locus in the *Co-Realce* genomic region and the horizontal dashed line is the LOD-score threshold estimated after 1,000 permutations.

(Glycosylphosphatidylinositol transamidase-GAA1; Phvul.004G006900-GAA1; Phvul.004G006900), which generally act as membrane anchors for many cell surface proteins (Supplementary Table 7).

Discussion

Based on inheritance and allelism studies, and considering additional information from genetic and physical mapping, this study identified a major anthracnose resistance locus in the Andean common bean cultivar BRSMG Realce developed by Embrapa and partners in Brazil. This cultivar shows several important agronomic traits (Melo et al., 2014), including a high level, wide and durable resistance to anthracnose disease caused by the fungus *C. lindemuthianum*. It has being used as parent in crosses and as a resistant control in final field trials conducted by the Embrapa breeding program at least for the last decade (Aguiar et al., 2021), and its resistance has shown to be stable and durable over time. The use of genetic resistance is the most effective and sustainable tool to manage plant pathogens (Assefa et al., 2019). The potential to exploit resistance increases when the genetic control of the trait is well known, as well as its effects (Vollmann and Buerstmayr, 2016). For these reasons, and considering that the majority of anthracnose resistance genes described and mapped in common bean are from Mesoamerican gene *pool*, the efforts of the present work on characterization and mapping a new resistance allele in the Andean cultivar BRSMG Realce should be of great interest to the bean research community worldwide.

TABLE 3 Simple linear regression analysis between molecular markers (snp1327, snp12782, snp3308 and dart9817) flanking the genomic region of the major locus *Co-Realce* and the phenotype of F_2 (BRSMG Realce × BRS FC104) plants inoculated with the *C. lindemuthianum* race 475.

Source of variation	Df	SS	MS	F-value	p-value	R^2	Inclination[c]
			snp1327[a]				
Genotype	2	300.4	150.2	32.7	1.48E-12	0.29	–
TT vs CC[b]	1	203.5	203.5	44.33	4.68E-10	–	-0.13
CT vs CC	1	96.9	96.9	21.11	9.02E-06	–	-2.12
Residual	153	702.4	4.59	–	–	–	–
Total	155	1002.8	154.79	–	–	–	–
			snp12782				
Source of variation	Df	SS	MS	F-value	p-value	R^2	Inclination
Genotype	2	338.6	169.2	39.7	1.10E-14	0.33	–
CC vs TT	1	225.9	225.9	52.91	1.53E-11	–	-3.32
TC vs TT	1	112.8	112.8	26.42	8.02E-07	–	-2.24
Residual	158	674.5	4.27	–	–	–	–
Total	160	1013.1	173.47				
			snp3308				
Source of variation	Df	SS	MS	F-value	p-value	R^2	Inclination
Genotype	2	381.5	190.8	47.1	< 2e-16	0.37	–
CC vs TT	1	250.8	250.8	61.97	5.64E-13	–	-3.53
TC vs TT	1	130.7	130.7	32.29	6.39E-08	–	-2.43
Residual	155	627.3	4.05	–	–	–	–
Total	157	1008.8	194.81				
			dart9817				
Source of variation	Df	SS	MS	F-value	p-value	R^2	Inclination
1 vs 0	1	356.7	356.7	81.56	1.06E-15	0.36	-1.72
Residual	143	625.4	4.4	–	–	–	–
Total	144	982.1	361.1	–	–	–	–

[a]Df – degree of freedom, SS – sum of squares, MS – mean squares; the underline alleles are linked to disease resistance.
[b]Contrast considered in the regression analysis between marker alleles and the disease severity of *C. lindemuthianum* race 475.
[c]Angular coefficient of the linear regression equation; the negative sign on the inclination score indicates that the allele is associated with disease resistance.

The recent advances of genotyping by sequencing (GBS) methods resulted in the consequent development of high-density genetic maps using SNP markers. This approach allowed the identification of a large number of associations between genetic markers and genomic regions (major genes or QTLs), broadening the perspectives for marker-assisted selection (MAS) (Cobb et al., 2019). Berry et al. (2020) developed a linkage map for common bean containing 1,951 SNPs, with an average density of one marker every 0.52 cM and a total size of 1,011.7 cM, from a total of 48,244 SNPs and n =146 RILs. Almeida et al. (2021) used a population of 91 BC_2F_3 individuals and an initial set of 791,361 SNPs to develop a *P. vulgaris* genetic map with 1,091 markers and a total size of 1,923.16 cM, with an average distance between markers of 1.90 cM. In the present study, 13,083 SNPs were identified and a linkage map with 1,118 SNPs (n =161 F_2) was developed, with a total size of 4,473.4 cM and an average distance of 4.07 cM (Supplementary Table 5). However, it is important to highlight that in the present study only high quality not-distorted markers were used and that the markers' orders correlated well with their physical map positions (Spearman's coefficient > 99%) (Supplementary Table 5).

The resolution of a genetic map depends directly on the number of recombination events between the marker loci and potential target loci, what can be limited by the population size (Liu, 1998). In the perspective of value and usefulness for plant breeding, a low genetic distance could be redressed by the identification of markers flanking the target locus and explaining a significant part of the phenotypic variation (Ferreira et al., 2006). In this study, the initial genetic map built by linkage analysis shown a limitation of the population size to identify recombinant individuals, once the inclusion of *Co-Realce* locus inflated the genetic distances in its genomic region on Pv04. In addition, regarding the phenotypic data from the F_2 mapping population, the categorization of nine symptom-scores into only two phenotypic classes (1-to-3, resistance; and 4-to-9, susceptibility) may also explain the lack of precision in positioning the *Co-Realce* locus in the initial linkage map. For these reasons, and considering that the *Co-Realce* locus segregates as a major gene (Table 1) and that is has shown a

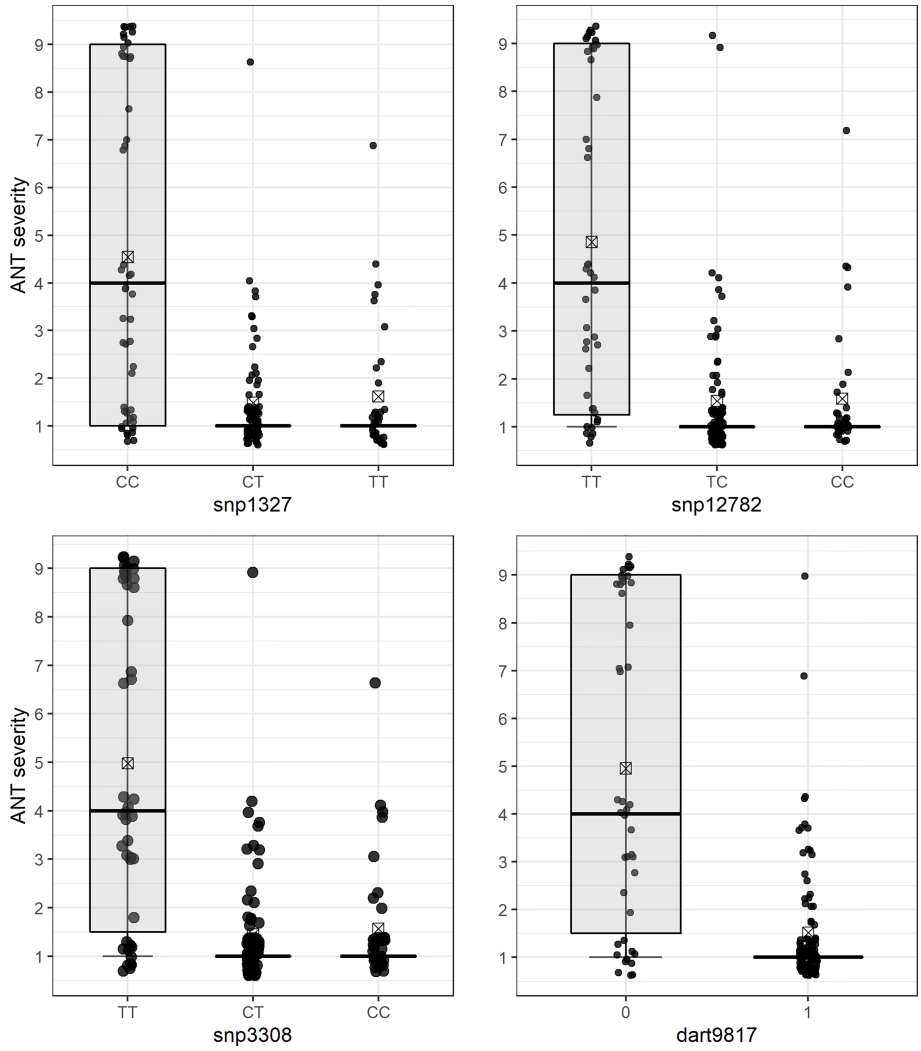

FIGURE 2
Differential reaction of F_2 (BRSMG Realce × BRS FC104) plants to *Colletotrichum lindemuthianum* race 475 for each molecular genotype class of SNP markers flanking the *Co-Realce* genomic region: snp1327 (CC, CT and TT), snp12782 (TT, TC and CC), snp3308 (TT, CT and CC) and dart9817 (0 and 1). The mean phenotypic scores are represented by a rectangle inside each box plot.

real value for the common bean breeding programs in Brazil, the QTL analysis was the approach used to map the major locus in the genomic region associated to anthracnose resistance and to identify useful SNP markers for MAS.

Using a panel of 189 common bean genotypes inoculated with the isolates Lv134 and Lv238 of the *C. lindemuthianum* race 65, Costa et al. (2021) identified by association study two genomic regions on Pv04 related with the resistance to Lv134 and Lv238. The SNP marker ss715649771 (96,165 bp) associated with the resistance to Lv134 and explaining 64.4% of the phenotypic variation and ss715646893 (1,165,722 bp) associated with the resistance to Lv238 and explaining 72.2% of the phenotypic variation. Mungalu et al. (2020) also report a major QTL (*ANT02.1*^{UC,SA}) for anthracnose resistance on Pv02, which explained 79.0 and 76.8% of the phenotypic variation. In both cases, major loci for resistance to anthracnose were identified by mapping using quantitative approaches.

The major anthracnose resistance locus (*Co-Realce*) identified in BRSMG Realce on over an interval of 704,867 bp (477,217-to-1,182,084 bp) of the *P. vulgaris* chromosome Pv04 explained 54.6% of the total phenotypic variation (Table 2). For this reason, anthracnose resistance in BRSMG Realce should be considered as a major gene or complex gene locus for breeding. It was also verified that *Co-Realce* segregates independently from *Co-3* (Table 1), the physically closest anthracnose resistance locus on Pv04 that has already been used by the Embrapa common bean breeding program to develop elite germplasm (Vieira et al., 2018). Still considering physical map evidences, the

positions of *Co-3* (1,286,490 bp) (Murube et al., 2019), *Co-15* (9,432,376 bp) (Sousa et al., 2015) and *Co-16* (1,537,169 bp) (Coimbra-Gonçalves et al., 2016) on Pv04 shown that those anthracnose resistance loci are distant from *Co-Realce* by 780,839 bp, 8,926,725 bp and 1,031,518 bp, respectively (Figure 3). The locus *Co-3* is the physically closest to *Co-Realce* but allelism tests demonstrated that they are distinct and independent from each other (Table 1). This evidence also indicates that the physically more distant loci *Co-15* and *Co-16* are also distinct and independent of *Co-Realce* (Figure 3). These results corroborate the hypothesis that BRSMG Realce harbors a new anthracnose resistance locus on Pv04. As already reported by Souza et al. (2016) and Nay et al. (2019b), physical position analysis using information from molecular markers linked to known resistance genes and the reference genome sequence of *P. vulgaris* has been used as an additional criterion to support the characterization of new disease resistance loci in common bean, as for angular leaf spot caused by *Pseudocercospora griseola*. However, to fully verify that *Co-Realce* does not coincide with any of the other two resistance loci previously mapped on Pv04, allelism tests between BRSMG Realce and Corinthiano (*Co-15*) and between BRSMG Realce and Crioulo 159 (*Co-16*) are also being carried out at Embrapa Rice and Beans. Other disease resistance genes have been mapped on Pv04, such as *Pse-6* for resistance to *Pseudomonas syringae*, *Ur-5* for resistance to *Uromyces appendiculatus*, *Phg-3* for resistance to *P. griseola*, and *Pm-2* for resistance to *Erysiphe difusa* (Pérez-Vega et al., 2013; Gonçalves-Vidigal et al., 2013; Cabrera, 2020). Some of these genes were mapped close to the genomic position of *Co-Realce* on Pv04, showing that this region is an important gene cluster for the coevolution between *P. vulgaris* and some of its relevant pathogen species.

Forty-four candidate genes related to pathogen-host interaction were annotated on *Co-Realce* genomic region (Supplementary Table 7). Among these genes, it is important to highlight those associated with response mechanisms to pathogen attack, including immunological receptors (Bent and Mackey, 2007), cellular communication between cytoplasm and nucleus (Zuiderveen et al., 2016; Vidigal Filho et al., 2020), association with kinase receptors (Zhou, 2019), elicitor molecule recognition and degradation (Craig et al., 2009), post-transitional processing (Manna, 2015), phosphate transport (Dong et al., 2019), transcription regulation and translation (Grafi et al., 2007; Woloshen et al., 2011), and extracellular pH modulation (Elmore and Coaker, 2011). There were also candidate genes that encode LRR proteins in different common bean chromosomes and that are associated with defense against fungi (Nay et al., 2019a; Mungalu et al., 2020; Nabi et al., 2022), bacteria (Wu et al., 2017) and virus (Seo et al., 2006). Furthermore, the upper portion of Pv04 contains a large cluster of resistance genes (Meziadi et al., 2016), over an interval of ~650 kb (from 345,784-to-993,499 bp) and including 28 genes related to resistance mechanisms in beans (Phytozome v11.0; *Phaseolus vulgaris* v2.1).

Three SNP markers linked to *Co-Realce* were identified by the QTL analysis (Figure 2). The snp12782 (position 1,182,123 bp) is positioned at around 5,164 bp from the Phvul.004G009500 gene (LRR), and the presence of the reference allele C (C/T) in homozygosis resulted in the selection of F_2 plants with an average score three times lower than that of plants without this allele ($p < 0.05$) (Figure 2). In addition, we assigned the markers snp1327 (position 477,285 bp) and dart9817 (position 485,246 bp) close to the Phvul.004G006800 gene region. This gene encodes the glycoprotein (NUP210) of the nuclear pore complex (NPC) and it has already been reported as associated with *P. vulgaris* resistance to anthracnose (Vidigal Filho et al., 2020; Shafi et al., 2022). It plays an important role in plant defense mechanisms, since they depend on the communication between the cytoplasm and the cell nucleus to be activated (Fang and Gu, 2021). NPC glycoproteins are necessary to make the nuclear envelope permeable to signaling macromolecules (Tamura and Hara-Nishimura, 2013). The snp3308 (position 505,696 bp) was mapped in the region of the Phvul.004G006900 (GAA1), which encodes the protein glycosylphosphatidylinositol transferase and helps recognize extracellular signals by associating with receptor-like kinases (Zhou, 2019). There are other candidate genes positioned in the *Co-Realce* genomic region, such as the Phvul.004G007600 and Phvul.004G009401 protein-encoding genes (RBP-RNA binding proteins) (Supplementary Table 7), essential to activate the defense response to pathogen attack in plants (Albà and Pagès, 1998; Woloshen et al., 2011). The main activities performed by RBP occur in the post-transcriptional processing of pre-RNA, and act to control splicing, polyadenylation of 3'extremity of RNA in the cap (modified guanine) added to the 5' extremity (Albà and Pagès, 1998; Woloshen et al., 2011). The Phvul.004G007600 gene is associated with *P. vulgaris* resistance to race 6 of *Pseudomonas syringae* pv. *phaseolicola* (Tock et al., 2017). Recently, Vidigal Filho et al. (2020) identified the gene Phvul.004G020900, which encodes RBP associated with *P. vulgaris* resistance to anthracnose race 65 (R^2 = 15%), corroborating the results of the present study.

The markers snp1327 (position 477,285 bp) and dart9817 explained 29 and 36% of phenotypic variation, respectively (Table 3). Selecting efficiency of the marker pairs snp1327/snp12782, snp1327/snp3308 and snp12782/snp3308 flanking the *Co-Realce* genomic region was 98.9%, 99.1% and 99.6%, respectively. This result support the high potential of these for MAS of *Co-Realce* during its introgression in elite lines and cultivars (Table 4; Supplementary Table 9). They are already being used by the Embrapa common bean breeding program in an allele pyramiding approach aiming to stack *Co-Realce* and the Mesoamerican resistance allele *Co-4*^{2}, present in the SEL 1308

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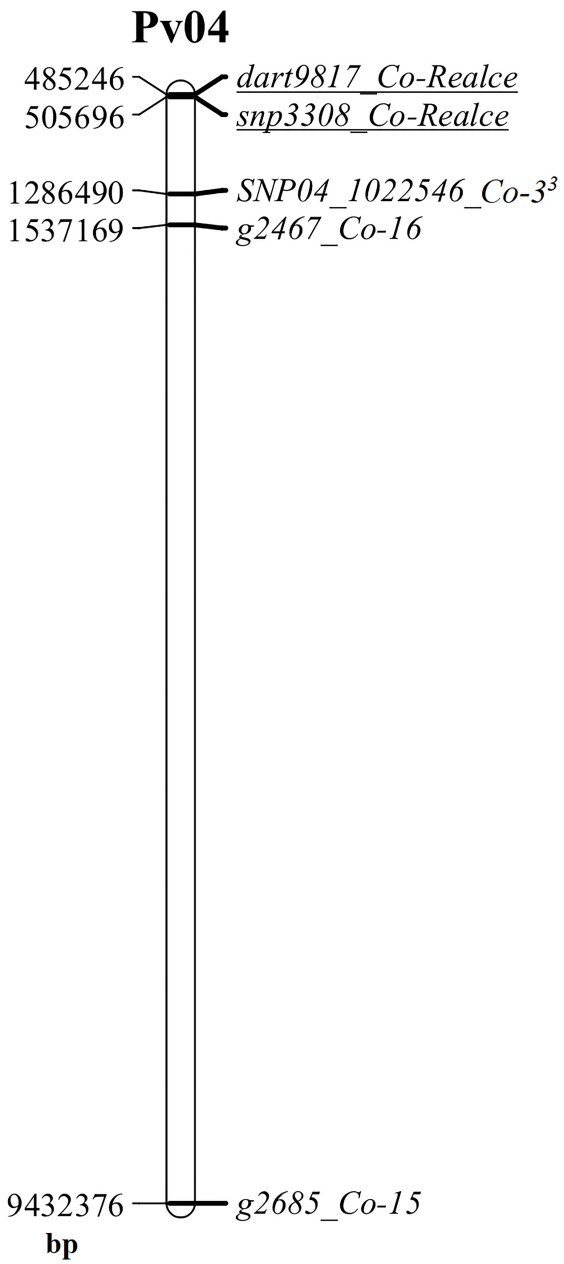

FIGURE 3
Physical map of the common bean chromosome Pv04 highlighting the location of the anthracnose resistance loci *Co-3, Co-15, Co-16* and *Co-Realce*, and their respective linked markers SNP04_1022546 (*Co-3*^{3}), g2685 (*Co-15*), g2467 (*Co-16*), dart9817 and snp3308 (*Co-Realce*). This physical map was built using the physical position of markers at the reference genome of *Phaseolus vulgaris* v2.1, available at www.phytozome.net (Paulino et al., 2022), using the software MapChart (Voorrips, 2002).

(Supplementary Table 2), in *carioca* seeded advanced lines. This breeding strategy aims to broadening the genetic resistance to anthracnose in the Brazilian common bean elite germplasm.

TABLE 4 Selection efficiency and recombination frequency of SNP markers positioned in the genomic interval of the major locus *Co-Realce*.

	snp1327	snp3308	snp12782	
snp1327	–	99.1	98.9	ES (%)[a]
snp3308	0.047	–	99.6	
snp12782	0.053	0.032	–	
		rf (cM)[a]		

[a]rf – Recombination frequency; ES – Selection efficiency.

Conclusions

Results obtained by the present work from inheritance studies, allelism tests, genetic and physical mapping shown that anthracnose resistance in the Andean common bean cultivar BRSMG Realce is controlled by a major locus (or complex gene locus) on Pv04, which has been previously named as *Co-Realce*. SNP markers useful for marker-assisted selection have been identified as linked to the dominant allele of this locus, showing a selection efficiency higher than 99.0%. Allelism tests and physical mapping of *Co-Realce* genomic region on Pv04 support that *Co-Realce* is different from other major loci already mapped on this same chromosome. The mapped genomic region included candidate genes related to pathogen-host interaction. Based on all these results and evidences, anthracnose resistance in BRSMG Realce should be considered as monogenic (major gene or complex gene locus) for breeding purpose. It is proposed that locus *Co-Realce* is unique and be provisionally designated as *CoPv04*^{R} until be officially nominated in accordance with the rules established by the Bean Improvement Cooperative Genetics Committee.

The cultivar BRSMG Realce is being already used by the Embrapa common bean breeding program as an anthracnose resistant donor parent from the Andean gene *pool*. This is because its resistance has shown to be stable and durable over time, even in final field trials conducted by the Embrapa in Brazil at least for the last 10 years. After the characterization of the anthracnose resistance in BRSMG Realce by the present work, this cultivar can now be used as a relevant donor source of an Andean resistance allele by common bean breeding programs worldwide, once it is already been successfully used for this propose in Brazil.

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

LMG-M, RPV, HSP, LCM, and TLPOS contributed to the conception and design of the study. LMG-M and LAR were in charge of laboratory analysis on DNA extraction and samples preparation and shipment. LMG-M, GRM, and TLPOS carried out crosses, plant material development, and the phenotyping assays. LMG-M, ASGC, and TLPOS performed the statistical analysis and elaborated graphs and figures. RPV, HSP, LCM, and TLPOS contributed with research grant funding application and management. LMG-M and RPV wrote the first draft of the manuscript. LMG-M and TLPOS wrote the final version of the manuscript. All authors reviewed and contributed to the article, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1033687/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Seeds of BRSMG Realce, an Andean common bean rajado (striped seed coat) seeded cultivar developed by Embrapa and partners in Brazil (Melo et al., 2014).

SUPPLEMENTARY FIGURE 2
Genetic map of the F_2 (BRSMG Realce × BRS FC104) population containing 1,118 SNP markers distributed across all 11 common bean chromosomes (Pv01-to-Pv11).

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Identification of a QTL region for ashy stem blight resistance using genome-wide association and linage analysis in common bean recombinant inbred lines derived from BAT 477 and NY6020-4

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Ashy stem blight (ASB), caused by the fungus *Macrophomina phaseolina* (Tassi) Goidanich is an important disease of the common bean (*Phaseolus vulgaris* L.). It is important to identify quantitative trait loci (QTL) for ASB resistance and introgress into susceptible cultivars of the common bean. The objective of this research was to identify QTL and single nucleotide polymorphism (SNP) markers associated with ASB resistance in recombinant inbred lines (RIL) derived from a cross between BAT 477 and NY6020-4 common bean. One hundred and twenty-six $F_{6:7}$ RIL were phenotyped for ASB in the greenhouse. Disease severity was scored on a scale of 1–9. Genotyping was performed using whole genome resequencing with 2x common bean genome size coverage, and over six million SNPs were obtained. After being filtered, 72,017 SNPs distributed on 11 chromosomes were used to conduct the genome-wide association study (GWAS) and QTL mapping. A novel QTL region of ~4.28 Mbp from 35,546,329 bp to 39,826,434 bp on chromosome Pv03 was identified for ASB resistance. The two SNPs, Chr03_39824257 and Chr03_39824268 located at 39,824,257 bp and 39,824,268 bp on Pv03, respectively, were identified as the strongest markers associated with ASB resistance. The gene Phvul.003G175900 (drought sensitive, WD repeat-containing protein 76) located at 39,822,021 – 39,824,655 bp on Pv03 was

recognized as one candidate for ASB resistance in the RIL, and the gene contained the two SNP markers. QTL and SNP markers may be used to select plants and lines for ASB resistance through marker-assisted selection (MAS) in common bean breeding.

KEYWORDS

ashy stem blight, common bean, *Macrophomina phaseolina*, *Phaseolus vulgaris*, quantitative trait loci, genome-wide association study

Introduction

Ashy stem blight (ASB) is a common disease in the common bean (*Phaseolus vulgaris* L.) in tropical and subtropical regions in the Americas and worldwide (Kaur et al., 2012; Ambachew et al., 2021). The disease is caused by the seed-transmitted fungus *Macrophomina phaseolina* (Tassi) Goidanich, and the pathogen can infect the roots and all aerial plant parts during the entire cropping season (Islam et al., 2012; Kaur et al., 2012; Viteri and Linares, 2022a). Damping off, leaf burning, plant wilting, premature defoliation, and stem blight are the most common symptoms observed in infected plants (Kaur et al., 2012). Microsclerotia, which is the major fungal structure for the primary infection, can survive in the soil for more than 10 years (Short et al., 1980; Kaur et al., 2012), and different levels of aggressiveness between isolates have been reported (Miklas et al., 1998a; Mayek-Pérez et al., 2001a; Viteri and Linares, 2017). Yield losses up to 80% were reported in susceptible common bean cultivars (Mayek et al., 2003; Kaur et al., 2012; Viteri and Linares, 2022a).

Genetic resistance is a better strategy than crop rotation to combat ASB, and the use of fungicides is not adequate to control this disease efficiently (Singh and Schwartz, 2010). Low to high levels of resistance have been reported in common bean and tepary bean (*Phaseolus acutifolius* A. Gray). For instance, the common bean genotypes of BAT 477, IPA 1, 'Negro Tacaná', 'Negro Perla', 'San Cristobal 83', TARS-MST1, and XAN 176 (Pastor-Corrales and Abawi, 1988; Mayek-Pérez et al., 2001b) and tepary bean accessions of Mex-114, PI 440806, and PI 321637 (Miklas et al., 1998a) were reported with higher levels of ASB resistance in field evaluations. Conversely, Andean common bean genotypes A 195, 'Badillo', 'PC 50', and PRA154 were reported in previous studies as having partial resistance in greenhouse evaluations (Viteri and Linares, 2017; Viteri et al., 2019). However, some breeding lines (e.g., BAT 477, NY6020-4, XAN 176) can have susceptible scores at later reproductive stages by the cut-stem method and two inoculations of *M. phaseolina* (Viteri et al., 2019). Furthermore, avoidance mechanisms (e.g., plants with upright growth habits) can help to reduce disease severity in the field and prevent a susceptible response of some genotypes (Mayek-Pérez et al., 2001a; Mayek-Pérez et al., 2001b; Viteri and Linares, 2022a).

ASB resistance can be inherited qualitatively or quantitatively depending on the resistant host genetic background and is affected by the screening method and environment used. For example, two complementary dominant genes (*Mp-1* and *Mp-2*) were identified to confer resistance in BAT 477/A 70 F_2 population screened in growth chambers (Olaya et al., 1996). Likewise, Mayek-Pérez et al. (2009) reported that two dominant genes with double recessive epistasis and nine quantitative trait loci (QTL) derived from BAT 477 were involved in field resistance to *M. phaseolina*. In addition, nine QTL on chromosomes Pv03, Pv05, Pv06, Pv08, Pv09, and Pv10 were reported to confer ASB resistance in the field and controlled environments in recombinant inbred line (RIL) population derived from BAT 477 and UI-114 (Méndez-Aguilar et al., 2017). Furthermore, Miklas et al. (1998b) reported that five QTL on Pv04, Pv06, Pv07, and Pv08 provided field resistance to ASB in the Dorado/XAN 176 RIL population, and they were derived from the black common bean XAN 176. More recently, Viteri and Linares (2019) identified two recessive genes and one recessive gene conferring resistance to *M. phaseolina* in PC 50/'Othello' and 'Badillo'/PR1144-5, respectively, under greenhouse conditions. These genes were derived from Andean genotypes PC 50 and Badillo. In the same study, one dominant gene was involved in resistance in the A 195/PC 50 population. To the best of our knowledge, the molecular identification of resistant QTL to ASB involving crosses between Andean and American genotypes has not been reported. This would be useful in marker assisted selection to increase the levels of resistance in common bean cultivars. The objective of this research was to identify QTL and SNP markers associated with ASB resistance in common bean RIL derived from a cross between BAT 477 and NY6020-4 genotypes. This would be useful in studying ASB resistance in different common bean genetic backgrounds, and the associated SNP markers could be used to select ASB resistant plants and lines in common bean molecular breeding through marker-assisted selection (MAS).

Materials and methods

Plant material and RIL development

A cross between BAT 477 and NY6020-4 common bean lines was made at the Isabela Research Substation at the University of Puerto Rico in January 2017. One hundred and twenty-six $F_{6:7}$ RIL from BAT 477/NY6020-4 was developed by single-seed-descent method from the F_2. NY6020-4 is an Andean snap bean with a determinate growth habit (Viteri et al., 2015) and low to partial levels of resistance to ASB (Viteri and Linares, 2017). BAT 477 is a common breeding line with indeterminate prostrate growth habit type III (Singh, 1982). This genotype was reported to be tolerant to drought stress (Arruda et al., 2018), and it has been widely used as a source of resistance to ASB. However, low to high levels of resistance were reported in previous studies in the greenhouse and field (Mayek-Pérez et al., 2009; Viteri and Linares, 2017; Viteri and Linares, 2022a; Viteri et al., 2019). BAT 477 was selected in this study because of the importance of identifying resistant QTL to the direct exposure of the pathogen, and to avoid a confounded effect of QTL expressed in field evaluations that could be associated with drought and heat stresses and/or disease avoidance mechanisms. NY6020-4 was selected because white beans are the most important market class in Puerto Rico (Beaver et al., 2020).

Macrophomina phaseolina isolates

PRI19 and PRL19 *M. phaseolina* isolates were collected from an infected stem tissue of common bean at R5 stage in the field of the Research Substations in Isabela (February, 2019) and Lajas (May, 2019), respectively. The fungi were isolated from infected stem tissue at reproductive stages (R5) with the characterized stem blight symptom. In addition, PRI21 was isolated from an infected seedling planted in the greenhouse in Isabela in January 2021. These three isolates were used in this study.

Phenotyping of ashy stem blight resistance

The 126 RIL and their parent strains were screened for resistance to PRI19 *M. phaseolina* isolate in Isabela and PRL19 in Lajas, respectively, in September 2020; they were screened for resistance to PRI21 isolate in Isabela in February 2021. A randomized complete block design (RCBD) with three replications were used, and four plants of each RIL line per replication were planted in each experiment in greenhouse trials.

One inoculation per plant of each of the aforementioned *M. phaseolina* isolates was conducted at the fourth internode (V5 growth stage). A 200 µL Eppendorf tip stacked with four mycelial plugs from a 48-hour-old *M. phaseolina* culture growth at 28°C on potato dextrose agar was used for each inoculation. Inoculated plants were exposed to high mean day temperatures > 27°C, and moisture ranged from 50–70%, which promoted an adequate ASB infection (Pastor-Corrales and Abawi, 1988; Mayek-Pérez et al., 2002; Viteri and Linares, 2022a). The disease severity was evaluated at 42 d after inoculation. A 1–9 scale was used, where 1 signified no sign of pathogen infection, 3 signified that the fungus did not pass the first node above/below the point of the inoculation, 6 signified that *M. phaseolina* reached the second node above/below the point of the inoculation, and 9 signified that the pathogen passed the third node below the point of inoculation with or without plant death (Singh et al., 2014; Viteri and Linares, 2017). Plants with scores of 1–3 were considered resistant, 4–6 intermediate, and 7–9 susceptible (Viteri and Linares, 2017).

Phenotypic data analysis

Disease scores of ASB phenotypic data were analyzed by analysis of variance (ANOVA) using the general linear model procedure of JMP Genomics 9 (SAS Institute, 2012 Cary, NC). The descriptive statistics were generated using 'Tabulate'; the distribution of the data was drawn using 'Distribution'; and Pearson's correlation coefficients (r) were calculated using "Multivariate Methods" of JMP Genomics 9 (SAS Institute, 2012 Cary, NC). The least squares mean of each isolate resistance for each RIL line was used as the phenotypic data for GWAS and QTL mapping using the ANOVA method.

Broad-sense heritability (H) was estimated using the following formula (Holland, 2003)

$$H = \sigma^2_G / \left[\sigma^2_G + (\sigma^2_{GE}/e) + (\sigma^2_E/re)\right]$$

where σ^2_G is the total genetic variance; σ^2_{GE} is variance between genetic and block interaction; σ^2_E is the residual variance; e is the number of environments; and r is the number of replications. The estimates for σ^2_G, σ^2_{GE} and σ^2_E are $\sigma^2_E = MS_E$; $\sigma^2_{GE} = (MS_{GE} - MS_E)/r$; and $\sigma^2_G = (MS_G - MS_{GE})/re$.

Phenotypic data of each of the three *M. phaseolina* isolates, PRI19, PRL19, and PRI21, were analyzed, separately. Because PRI19 and PRI21 were collected from the same location of Isabela, Puerto Rico, we merged the ASB phenotypic data as PRI. We also merged the ASB phenotypic data of the three isolates as PRI.L. Therefore, five ABS data sets performed GWAS and QTL mapping for ABS resistance in this study.

DNA extraction, sequencing, and SNP genotyping

Genomic DNA was extracted using the DNeasy® plant mini kit (Qiagen, Germantown, MD). The DNA was extracted from a

bulk sample of emerging trifoliate leaves collected from three plants of each parent and the 126 RIL. The DNA concentration was adjusted to 10 ug/mL using a Nanophotometer® P-class (Implen, Westlake Village, CA). Whole-genome resequencing (WGR) with 2x common bean genome size coverage took place on the 128 samples (126 RIL plus two parents) in Texas A&M Genomics and Bioinformatics Center. Libraries were prepared with PerkinElmer NEXTFLEX Rapid XP kit protocol, and common bean samples were sequenced on Illumina NovaSeq S4 XP using the 2x150 bp recipe. FASTQ files were processed with the Illumina Dynamic Read Analysis for Genomics (DRAGEN) Bio-IT processor. The DRAGEN pipeline (v3.8.4) was used to obtain SNP data for each individual sample based on the genome reference of *P. vulgaris* v2.1 common bean genome and annotation (https://phytozome-next.jgi.doe.gov/info/Pvulgaris_v2_1).

A total of 6,463,014 SNPs were identified in the 126 RIL and their parents, distributed on the 11 chromosomes. In the RIL population, the relevant SNP should contain two homozygous alleles in a 1:1 ratio with each other. A chi-square test was performed for each of the 6,463,014 SNPs found in DNA sequencing. We retained SNPs that had two homozygous alleles in a 1:1 ratio, those with a chi-square test P-value > 0.01, and the two parents which had different alleles and homogeneity. Meanwhile, we also filtered each SNP and kept the SNPs with missing alleles < 5%, heterogeneous rate < 5%, and minor allele frequency (MAF) > 35%. After filtering, the retained 72,017 SNPs distributed on 11 chromosomes were used in this study (Supplementary Figure S1). The 72,017 SNPs across the 126 RIL and their two parents (BAT 477 and NY6020-4) have been published at https://doi.org/10.6084/m9.figshare.19919221.v1.

Association analysis

GWAS was performed using the 72,017 SNPs across the 126 RIL by SMR (single marker regression), GLM (general linear model), and MLM (mixed linear model) methods in TASSEL 5 (Bradbury et al., 2007), and by GLM, FarmCPU (fixed and random model circulating probability unification), and BLINK (Bayesian-information and linkage-disequilibrium iteratively nested keyway) models in GAPIT 3 (Genomic Association and Prediction Integrated Tool version 3) (Wang and Zhang, 2021; https://zzlab.net/GAPIT/index.html; https://github.com/jiabowang/GAPIT3) by setting PCA = 2. In addition, a *t*-test was conducted for all 72,017 SNPs by using visual basic codes in Microsoft Excel 2016.

Multiple TASSEL and GAPIT models were used to find reliable and stable ASB resistance-associated SNP markers and candidate genes and QTL regions in the RIL. The significant threshold of associations was calculated using Bonferroni correction of P-value with an $\alpha = 0.05$ (0.05/SNP number) as the significance threshold (López-Hernández and Cortés, 2019), and LOD value of 6.16 was used as significance threshold based on the 72,017 SNPs in this study. In addition, a *t*-test was conducted for all 72,017 SNPs by using Visual Basic codes in Microsoft Excel 2016.

Genetic mapping and QTL analysis

Linkage maps were constructed for the RIL population using JoinMap 4 (Van Ooijen, 2006) and MSTmap (Wu et al., 2008; http://mstmap.org/). Single marker regression (SMR), single-trait multiple interval mapping (SMIM), and single-trait CIM MLE (SMLE, single-trait composite interval mapping maximum likelihood estimation) analyses were conducted for QTL mapping using QGene (Joehanes and Nelson, 2008).

Candidate gene identification/detection

Genes were searched within the QTL region from the *P. vulgaris* genome reference version v2.1 (https://phytozome-next.jgi.doe.gov/info/Pvulgaris_v2_1). Our objective was to find analogs of disease resistant genes near the significantly associated SNP markers in the QTL region for ASB resistance.

Results

Ashy blight resistance in the RIL

The scale (1–9) of ashy blight resistance in the 126 RIL derived from BAT 477 and NY6020-4 showed a near normal distribution in all five pathogen combinations (Figure 1). The mean disease rate ranged from 3.0–8.3, 2.9–7.9, 3.2–8.8, 3.2–8.6, and 3.2–8.4; averaged 5.0, 4.4, 5.7, 5.4, and 5.1 with a standard deviation of 1.09, 1.05, 1.21, 0.99, and 0.93, and the coefficient of variation (CV) was 21.6%, 23.6%, 21.1%, 18.4%, and 18.0%, for PRI19, PRL19, PRI21, PRI, and PRI.L, respectively (Supplementary Table S1). The data showed an extensive range and variation of the ASB disease scale in the 126 RIL, confirming the suitability of the RIL for GWAS and QTL analyses.

Broad-sense heritability was 46.3%, 63.5%, 53.2%, 71.2%, and 68.7% for PRI19, PRL19, PRI21, PRI, and PRI.L, respectively (Table S2), indicating the ASB resistance was mediate highly inheritable.

There were strong correlations (r = 0.36–0.98), where 5 of the 10 r values were greater than 0.80, and 8 out of 10 were greater than 0.60 of ASB resistance scores among the five pathogen combinations in the 126 RIL (Table S3), suggesting that the combinations had similar genetic resistance.

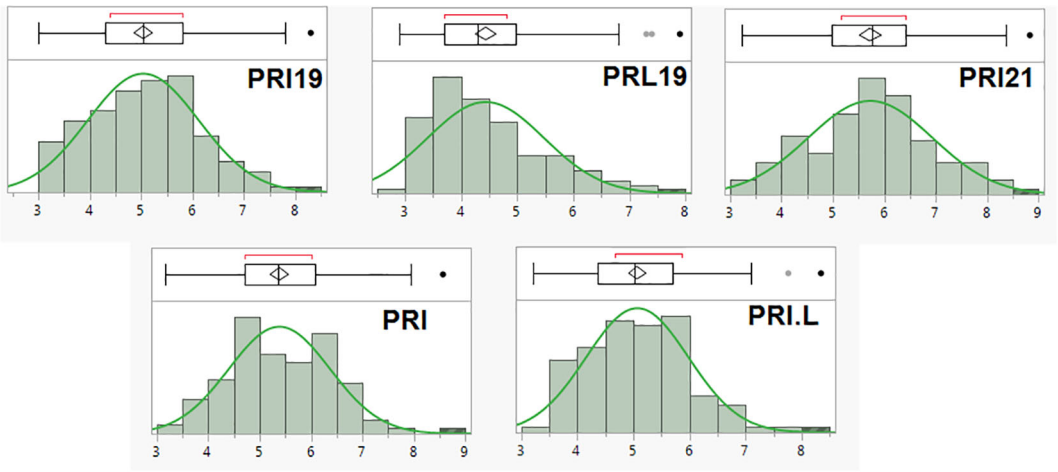

FIGURE 1
The distribution of ashy stem blight (ASB) score (1-9 scale) in 126 common bean RIL of BAT 477 and NY6020-4 for resistance to five *Macrophomina phaseolina* isolates or combinates, where the x axis represents ASB score (1-9 scale) and the y axis represents number of RIL.

Association study

Three models, GLM, MLM, and Blink in GAPIT 3, and three models, SMR, GLM, and MLM in TASSEL 5 when PCA = 2 performed association analysis for ASB resistance in this study. The observed vs expected LOD [-$\log_{10}$(p)] distributions in QQ-plots showed a large divergence from the expected distribution based on multiple QQ plots based on three models (GLM, MLM, and Blink) in PRI19, PRL19, PRI21, PRI, and PRI.L (Figure S2B on right side), indicating there were SNPs associated with ASB resistance in the association panel. The multiple Manhattan plots on three models (GLM, MLM, and Blink) in PRI19, PRL19, PRI21, PRI, and PRI.L (Figure S2A on left side) showed that a dozen SNPs with LOD value greater than 6.16 (significant threshold) were associated with ASB resistance. The multiple Manhattan and QQ plots based on the three models for ASB PRI19 resistance are also shown in Figure 2. The QQ-plots and Manhattan plots of three models in Tassel 5 (Figure S3 listed ASB PRI resistance) showed similar trends to GAPIT3 for ASB PRI resistance, indicating that there were significant SNP markers on Pv03 associated with ASB resistance. The Manhattan and QQ plots based on either Blink or GLM showed that there were SNPs on Pv03 associated with the ASB resistance for PRI19 (Figure 3), for PRL19 (Figure S4), for PRI21 (Figure S5), for PRI (Figure S6), and for PRI.L (Figure S7), further validated by QTL on Pv03 for ASB resistance.

Based on the three models in GAPIT 3 and the three models in TASSEL 5 when PCA = 2, 45 SNPs, located in the region of ~4.28 Mbp from 35,546,329 bp to 39,826,434 bp on chr 3, were associated with the ASB resistance with an LOD [-$\log_{10}$(p)] > 6.16 in one or more of the six models for one or more pathogen combination (Table S4; Figure S2). *t*-test showed all SNPs had an LOD > 2.0 accept Chr03_3572932 for PRI21 resistance (Table S4), validating 45 SNPs associated with ASB resistance at P=0.01 level. The averaged LOD ranged from 2.74 to 4.78 based on the six GWAS models and 3.52 to 6.12 based on *t*-test, and the R-square was 11.2 – 17.7% averaged from the six models (Table S4), indicating that there is a QTL on Pv03 for ASB resistance.

After combined analysis of the six GWAS models, four SNPs, Chr03_37381665, Chr03_37616128, Chr03_39824257, and Chr03_39824268 were associated with PRI19 resistance; three SNPs, Chr03_38912965, Chr03_38926573, and Chr03_39009342 with PRI21 resistance; four SNPs, Chr03_35546329, Chr03_35847673, Chr03_36036641, and Chr03_36036679 with PRL19; four SNPs, Chr03_38912965, Chr03_39009342, Chr03_39824257, and Chr03_39824268 with PRI; and five SNPs, Chr03_37616128, Chr03_38912965, Chr03_39009342, Chr03_39824257, and Chr03_39824268 with PRI.L resistance (Table 1). Among these SNPs, Chr03_37616128 was associated with both PRI19 and PRI.L resistance; Chr03_38912965 with both PRI21and PRI resistance; Chr03_39009342 with both PRI21 and PRI resistance; and Chr03_39824257 and Chr03_39824268 with PRI19, PRI, and PRI.L resistance (Table 1), indicating that these SNP markers had stable resistance. These SNP markers had an LOD > 4.5 in the *t*-test for associated ASB resistance.

The closest gene for Chr03_37381665 was Phvul.003G157500 with < 1 kb distance; for both Chr03_39824257 and Chr03_39824268 the gene Phvul.003G175900; Chr03_39009342 close to Phvul.003G168800 with a < 2 kb distance; and Chr03_35847673 to Phvul.003G148000 with <1 kb (Table S4), indicating that these genes may be associated with ASB resistance.

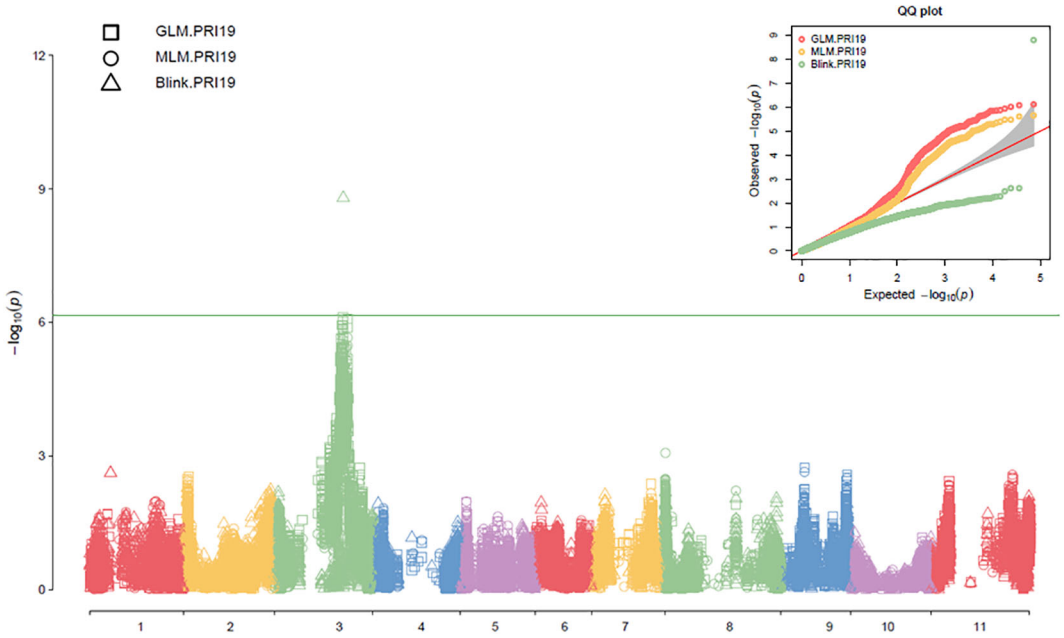

FIGURE 2
The multiple Manhattan and QQ plots of GLM, MLM, and BLINK models for ashy stem blight PRI 19 pathogen resistance in 126 common bean RIL of BAT 477 and NY6020-4.

Genetic mapping and QTL analysis

Eleven genetic maps consisting of of 35,787 SNPs from Pv01 to Pv11 were built by MSTmap (http://mstmap.org/) and JoinMap 4. There were 3,952 SNPs on Pv01; 3,841 SNPs on Pv02; 7,746 SNPs on Pv03; 2,366 SNPs on Pv04; 4,514 SNPs on Pv05; 2,358 SNPs on Pv06; 1,225 SNPs on Pv07; 3,712 SNPs on Pv08; 1,512 SNPs on Pv09; 2,815 SNPs on Pv10; and 1,746 SNPs on Pv11. The order of SNPs on each genetic map on Pv01, Pv03, Pv04, Pv07, Pv08, and Pv09 match well with their physical maps;

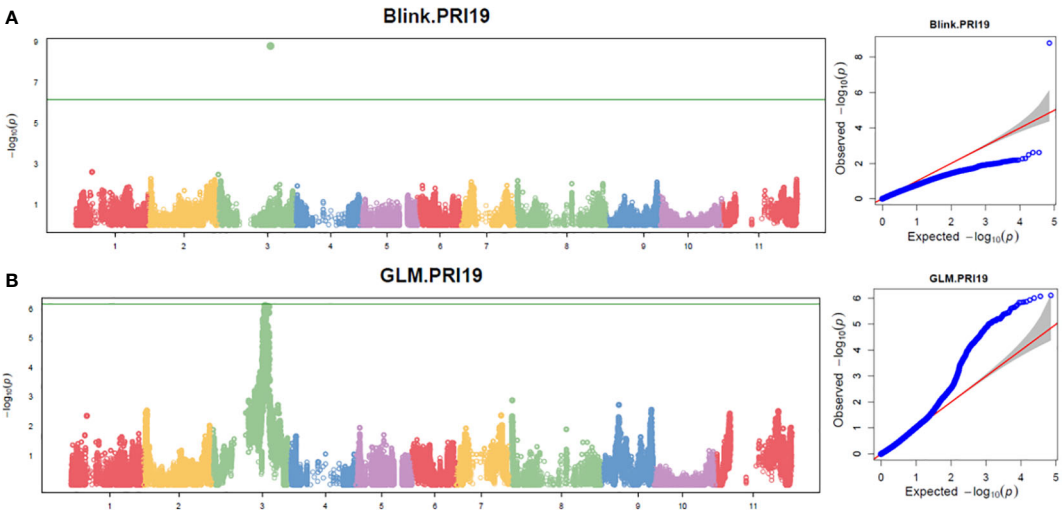

FIGURE 3
The Manhattan and QQ plots of Blink **(A)** and GLM **(B)** models for ashy stem blight PRI 19 pathogen resistance in 126 common bean RIL of BAT 477 and NY6020-4.

TABLE 1 SNP markers associated with five ashy stem blight pathogen combinations based on six models, listing the closest genes within 2 kb distance.

SNP	Chr	Position (bp)	-Log (P-value) in Tassel			-Log (P-value) using GAPIT 3			Average LOD	Associated pathogen	Gene	Distance between SNP andgene	*t*-test	Beneficial allele related to resistance/BAT477	Unbeneficial allele associated with susceptibility/NY6020-4
			SMR	GLM	MLM	Blink	GLM	MLM					-Log (P-value)		
Chr03_37381665	3	37381665	4.59	5.90	4.19	8.80	6.12	5.61	5.87	PRI19	Phvul.003G157500	<1 kb	6.19	C	T
Chr03_37616128	3	37616128	4.91	5.80	4.19	1.06	5.70	5.28	4.49				6.07	T	C
Chr03_39824257	3	39824257	6.21	7.23	4.64	1.23	6.08	5.65	5.17		Phvul.003G175900	on gene	6.43	A	G
Chr03_39824268	3	39824268	5.85	6.66	4.36	1.14	5.88	5.48	4.89				6.33	A	G
Chr03_38912965	3	38912965	4.28	4.61	2.83	3.70	4.23	3.39	3.84	PRI21			5.07	G	A
Chr03_38926573	3	38926573	3.13	3.54	2.50	3.43	3.96	3.30	3.31				4.54	T	C
Chr03_39009342	3	39009342	4.41	5.07	2.60	4.19	4.71	3.65	4.10		Phvul.003G168800	< 2 kb	5.25	G	A
Chr03_35546329	3	35546329	3.78	4.83	2.61	3.87	5.12	3.64	3.97	PRL19			5.29	G	C
Chr03_35847673	3	35847673	4.86	5.65	3.11	3.49	4.70	3.51	4.22		Phvul.003G148000	< 1 kb	5.63	G	A
Chr03_36036641	3	36036641	4.53	5.49	2.95	3.71	4.95	3.67	4.21				5.71	G	A
Chr03_36036679	3	36036679	4.51	5.32	2.85	3.68	4.91	3.66	4.15				5.72	C	A
Chr03_38912965	3	38912965	6.49	6.98	3.86	0.42	5.70	4.34	4.63	PRI			6.93	G	A
Chr03_39009342	3	39009342	5.85	7.05	3.51	9.29	6.41	4.83	6.16		Phvul.003G168800	< 2 kb	6.90	G	A
Chr03_39824257	3	39824257	5.38	6.62	3.82	0.65	6.14	5.10	4.62		Phvul.003G175900	on gene	6.63	A	G
Chr03_39824268	3	39824268	5.42	6.44	4.03	0.64	6.12	5.19	4.64				6.67	A	G
Chr03_37616128	3	37616128	5.41	6.76	4.01	0.82	6.40	4.91	4.72	PRI.L			6.84	T	C
Chr03_38912965	3	38912965	6.53	7.17	3.66	0.34	5.82	4.08	4.60				6.83	G	A
Chr03_39009342	3	39009342	6.03	7.46	3.59	9.73	6.70	4.79	6.38		Phvul.003G168800	< 2 kb	7.05	G	A
Chr03_39824257	3	39824257	5.80	7.27	3.88	0.64	6.36	4.96	4.82		Phvul.003G175900	on gene	6.61	A	G
Chr03_39824268	3	39824268	5.72	6.96	3.83	0.55	6.27	4.94	4.71				6.59	A	G

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Pv02 matches but not for the region from 12 Mbp to 26 Mbp; Pv05 and Pv10 had many SNPs located at the centromere and did not match well; Pv11 had a gap near the centromere; and Pv06 did not match well except from 25 Mbp up (Figure S8). This indicates that we can do QTL mapping for ASB resistance on Pv01, Pv03, Pv04, Pv07, Pv08, and Pv09; and it may be possible on Pv11 and partial regions of other chromosomes based on the 126 RIL derived from BAT 477 and NY6020-4.

QTL mapping by QGene showed that ASB resistance was observed only on chromosome Pv03. The 7,746 SNPs of Pv03 were too dense to do QTL mapping with a small RIL population with 126 individuals, and so we selected 179 SNPs on Pv03 to create a new linkage map to do QTL analysis for ASB resistance. The genetic and physical positions of the two linkage maps consisted of either 7,746 SNPs and 179 SNPs, listed in Table S5, where both combined maps between physical distance (Mbp) and genetic position (cM) were also included. The genetic map of Pv03 matches well to its physical map based on 179 SNPs (Figure 4).

QTL mapping by single-trait multiple interval mapping (SMIM) in Qgene showed a peak on chromosome Pv03 for each of PRI19, PRL19, PRI21, PRI, and PRI.L resistance (Figure 5) and the detailed QTL mapping in Pv3 for each ASB resistance was showed in the Supplementary Figure S9 with viewable and readable SNP marker names. The detailed QTL regions are shown in Supplementary Figure 10 in order to see the linked SNP markers, and an example of QTL mapping for PRI19 resistance included in the test can be found in Figure 6.

Twenty SNPs located at 446.5 - 555.9 cM on Pv03 were linked to ASB resistance in one of five combinations, either

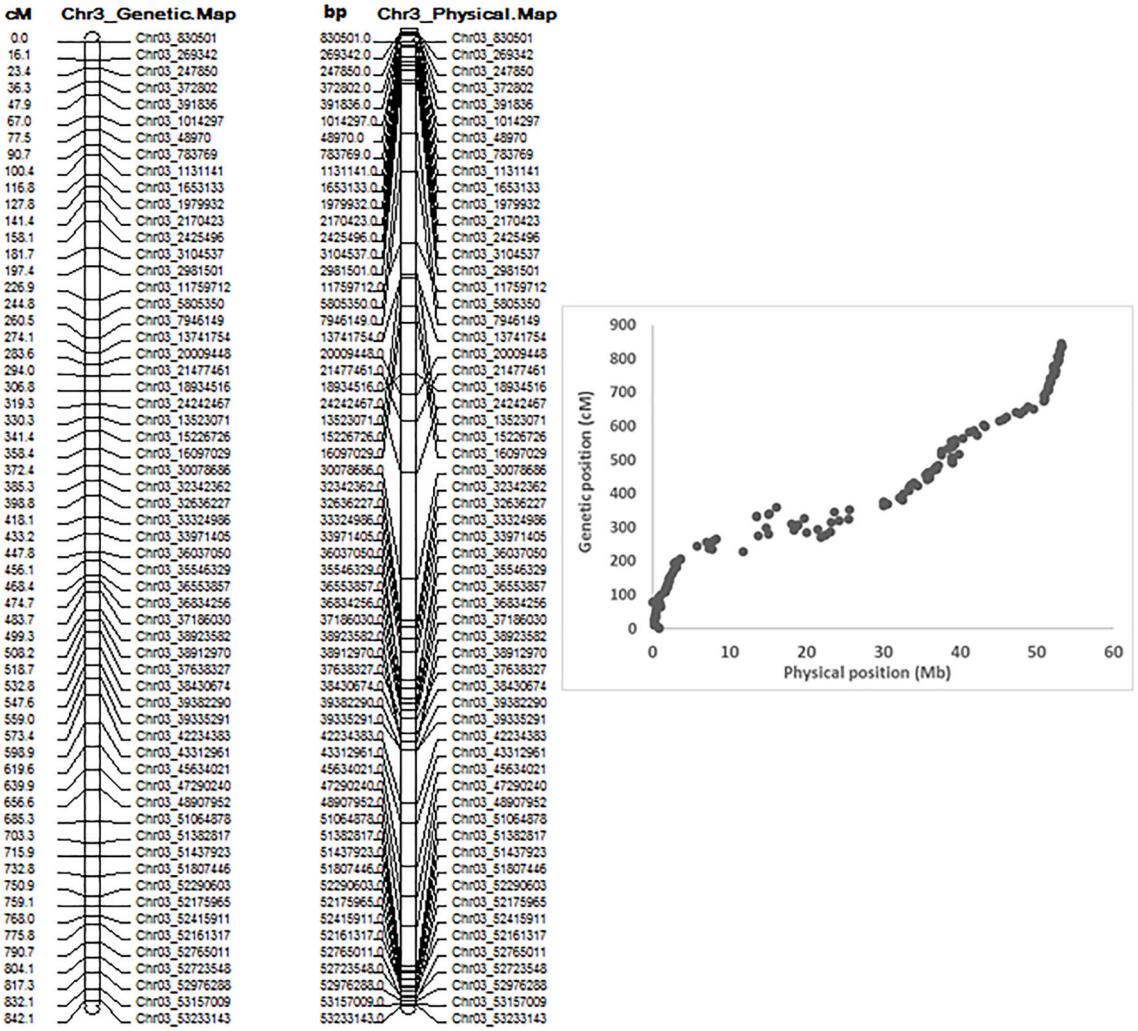

FIGURE 4
Genetic map (left), physical map, and combined map of physical and genetic map (right) consisting of 179 SNPs on chromosome 3 from 126 common bean RIL of BAT 477 and NY6020-4.

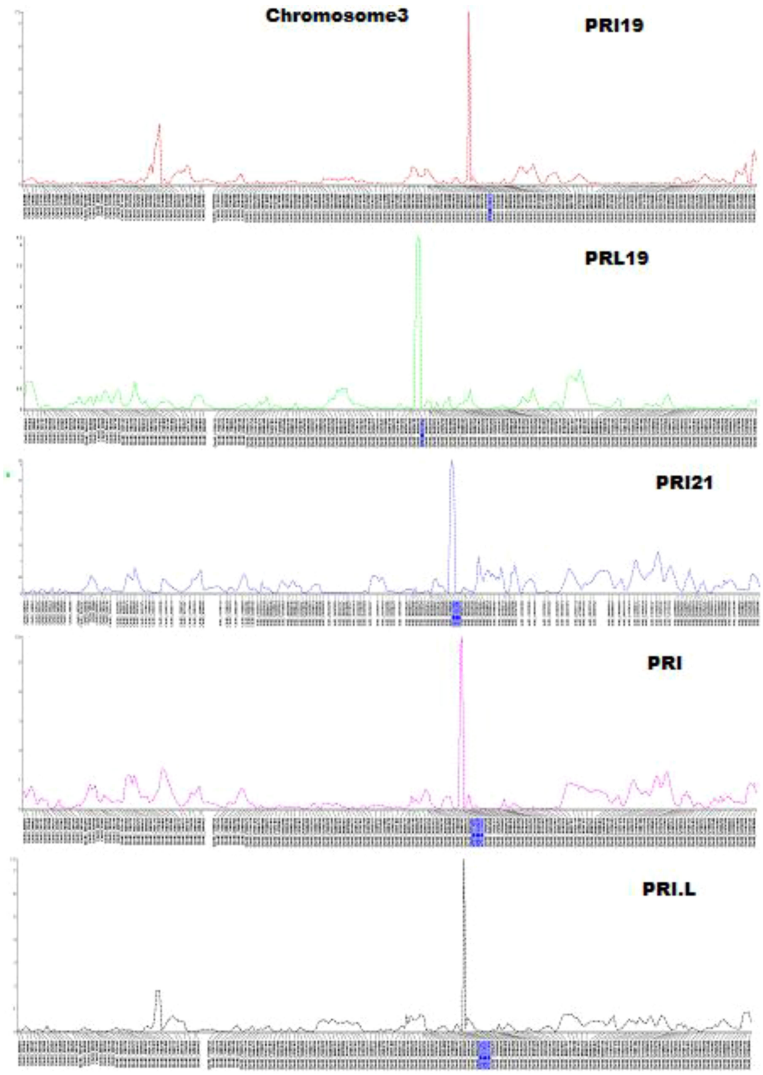

FIGURE 5
Five genetic maps at the QTL region on chromosome 3 created by single-trait multiple interval mapping (SMIM), where x-axis presents genetic map with SNP markers and y-axis presents LOD value (The detail information including the marker names was shown in Supplementary Figure S9).

PRI19, PRL19, PRI21, PRI, or PRI.L, based on SMR model in QGene (Table S6). QTL was identified at 452 – 514 cM on Pv03 based on SMIM model and at 448 – 554 cM on Pv03 based on SMLE (single-trait CIM MLE, single-trait composite interval mapping maximum likelihood estimation) for the five ASB combinations (Table S6), indicating that there is a QTL in the region for ASB resistance.

For PRI19 resistance, the QTL peak is at 514 cM of Pv03 based on SMR, SMIM, and SMLE analysis, confirmed by the two SNPs, Chr03_37616128 and Chr03_39824268 (Tables 2 and S6), and closer to Chr03_39824268 based on the peak of SMIM mapping (Figures 5, 6, S9 and S10), and the SNP is on the gene Phvul.003G175900.

For PRL19 resistance, the QTL peak is at 451 - 456 cM of Pv03 based on SMR, SMIM, and SMLE analysis, confirmed by the two SNPs, Chr03_36036679 and Chr03_35546329 (Tables 1 and S6; Figures 5, 6, S9 and S10), and a dozen genes are located at the region.

For PRI21 resistance, the QTL peak is at 490 - 494 cM of Pv03 based on SMR, SMIM, and SMLE analysis, confirmed by the two SNPs, Chr03_39009342 and Chr03_38926573 (Tables 2 and S6; Figures 5, 6, S9, S10), and three genes, Phvul.003G168500, Phvul.003G168700, and Phvul.003G168800 are located at this region.

For PRI resistance, the QTL peak is at 504 - 514 cM of Pv03 based on SMR, SMIM, and SMLE analysis, confirmed by the two

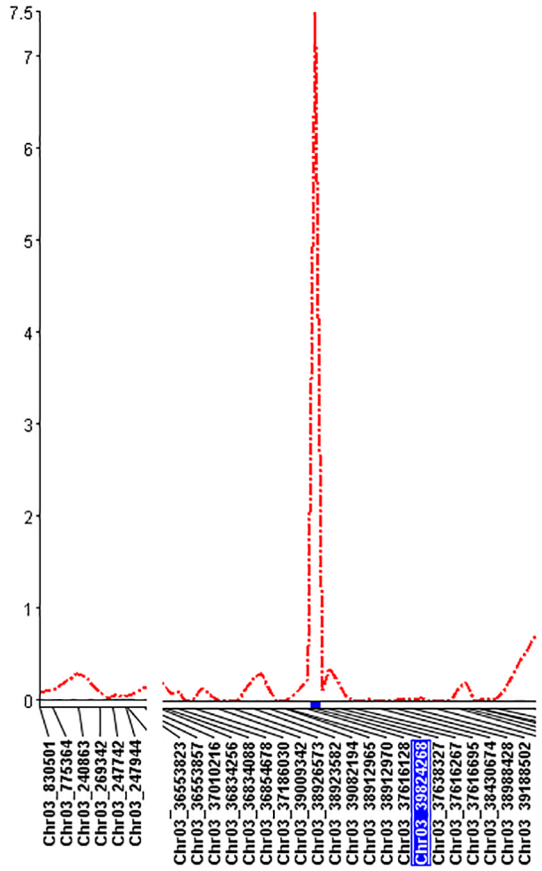

FIGURE 6
The genetic map at the QTL region with the viewable SNPs on chromosome 3 created by single-trait multiple interval mapping (SMIM) for PRI19 *M. phaseolina* isolate resistance, where the x axis indicates genetic map with SNP markers and the y axis indicates LOD value.

SNPs, Chr03_39082194 and Chr03_39824268 (Tables 2 and S6), and closer to Chr03_38912965 based on the peak of SMIM mapping (Figures 5, 6, S9, S10), and dozens genes are located at this region.

For PRI.L resistance, the QTL peak is at 514 cM of Pv03 based on SMR, SMIM, and SMLE analysis, confirmed by the two SNPs, Chr03_37616128 and Chr03_39824268 (Tables 2, S6), and closer to Chr03_39824268 based on the peak of SMIM mapping (Figures 5, 6, S9, S10), and the SNP is on the gene Phvul.003G175900, which showed similar PRI19 resistance.

Candidate gene identification/detection

There are 305 genes in the QTL region from 36.17 Mbp to 9.83 Mbp on chromosome Pv03 for ASB to PRI19, PRI21, PRL19, PRI, and PRI.L, based on six GWAS models in GAPIT 3 and three QTL models in QGene (Table S7). Among the 305 genes, there are 11 disease gene analogues (Table 3), where three genes, Phvul.003G152900, Phvul.003G156366, and Phvul.003G168000, link to one or more SNP markers identified by GWAS and listed in Table S4. Four genes, Phvul.003G148000, Phvul.003G157500, Phvul.003G168800, and Phvul.003G175900, are located at an associated SNP marker for ASB with < 2kb distance (Table 3) based on the GWAS and QTL analyses in Tables 1 and 2.

Discussion

Ashy stem blight resistance in the RIL

In this study, BAT 477 showed intermediate to high ABS resistance, and NY6020-4 was intermediately susceptible to ABS based on PRI19 and PRL19 *M. phaseolina* isolates collected from common bean fields planted in Isabela and Lajas, Puerto Rico, respectively, in October 2019; PRI21 isolate collected from an infected seedling planted in the greenhouse in Isabela in January

10.3389/fpls.2022.1019263

TABLE 2 QTL and linked SNP markers for ashy stem blight resistance of five combinations based on three models in Qgene.

Mapping model	SNP	Position /region (cM)	Add effect	LOD	%R^2
Single marker regression (SMR)	Chr03_37616128	513.5	-0.456	4.718	15.8
	Chr03_39824268	515.7	-0.472	5.032	16.8
Single-trait multiple interval mapping (SMIM)	Chr03_37616128 - Chr03_39824268	514	-0.565	7.488	23.9
Single-trait CIM MLE (SMLE)		514	-0.474	5.141	17.1
Single marker regression (SMR)	Chr03_36036679	451.5	-0.421	4.503	15.2
	Chr03_35546329	456.1	-0.399	4.002	13.6
Single-trait multiple interval mapping (SMIM)	Chr03_36036679 - Chr03_35546329	452	-0.402	3.967	13.5
		454	-0.419	4.191	14.2
		456	-0.401	4.115	14
Single-trait CIM MLE (SMLE)		452	-0.402	3.967	13.5
		454	-0.419	4.191	14.2
		456	-0.401	4.115	14
Single marker regression (SMR)	Chr03_39009342	489.9	-0.477	3.903	13.3
	Chr03_38926573	494	-0.447	3.574	12.2
Single-trait multiple interval mapping (SM IM)	Chr03_39009342- Chr03_38926573	490	-0.459	3.752	12.8
		492	-0.495	4.11	13.9
Single-trait CIM MLE (SMLE)		490	-0.459	3.752	12.8
		492	-0.495	4.11	13.9
Single marker regression (SMR)	Chr03_39082194	503.4	-0.412	4.622	15.5
	Chr03_38912965	507.9	-0.452	5.726	18.9
	Chr03_38912970	508.1	-0.448	5.585	18.5
	Chr03_37616128	513.5	-0.432	5.127	17.1
	Chr03_39824268	515.7	-0.457	5.766	19
Single-trait multiple interval mapping (SMIM)	Chr03_39082194- Chr03_39824268	504	-0.453	5.403	17.9
		506	-0.469	5.857	19.3
		514	-0.202	0.483	1.7
Single-trait CIM MLE (SMLE)		504	-0.453	5.403	17.9
		506	-0.469	5.857	19.3
		508	-0.436	5.308	17.6
		510	-0.46	5.528	18.3
		512	-0.455	5.418	18
		514	-0.446	5.595	18.5
Single marker regression (SMR)	Chr03_37616128	513.5	-0.418	5.506	18.2
	Chr03_39824268	515.7	-0.43	5.823	19.2
Single-trait multiple interval mapping (SMIM)	Chr03_37616128- Chr03_39824268	514	-0.491	7.481	23.9
Single-trait CIM MLE (SMLE)		514	-0.428	5.921	19.5

2021; PRI (combined PRI19 and PRI21); and PRI.L (combined PRI19, PRL19, and PRI21) (Table S8). Although the ASB rate difference between the two parents was not large, the 126 RIL showed large variation, with an extensive range for PRI19, PRL19, PRI21, PRI, and PRI.L between the two parents (Figure 1, Table S1), confirming the suitability of the RIL for GWAS and QTL analyses. High broad-sense heritability (46.3% - 71.2%) was also observed (Table S2), indicating that ASB resistance in BAT 477 can be transferred to other common bean cultivars and lines.

Variability of *Macrophomina phaseolina*

In this study, three *M. phaseolina* pathogen sources, PRI19, PRI21, and PRL19, were used to evaluate ASB resistance in the RIL. Although we were unsure whether they belonged to the same race, similar results were observed with variability (Figure 1; Tables S1, S2), and strong correlations (r = 0.36 - 0.98) also observed with majority (80%) r > 0.60 (Table S3). QTL and association mapping of ASB resistance showed the

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TABLE 3 Eleven disease gene analogues located at the QTL region between 35.8 Mbp and 39.9 Mbp on chromosome Pv03, and four genes located within 2 Kb distance from one or more SNP associated with ashy stem blight resistance.

Gene	Chr	Gene_Start_pos	Gene_End_pos	Gene-defined	Close SNP	From gene start	From gene end		Comment
Phvul.003G152900	3	36779067	36784453	Leucine-rich repeat protein kinase family protein	Chr03_36834088	55021	49635	< 50 kb	SNP markers listed within 50 Kb distance
					Chr03_36834256	55189	49803	< 50 kb	
Phvul.003G154000	3	36948002	36951212	Leucine-rich receptor-like protein kinase family protein					
Phvul.003G156366	3	37209311	37212260	Leucine-rich repeat protein kinase family protein	Chr03_37185993	-23318	-26267	< 25 Kb	
					Chr03_37186030	-23281	-26230	< 25 Kb	
					Chr03_37186035	-23276	-26225	< 25 Kb	
Phvul.003G158700	3	37486636	37489949	Cysteine-rich RLK (receptor-like protein kinase)					
Phvul.003G159700	3	37734035	37737603	Leucine-rich repeat protein kinase family protein					
Phvul.003G161500	3	38073321	38075049	Protein kinase superfamily protein					
Phvul.003G163700	3	38264295	38267316	P-loop containing nucleoside triphosphate hydrolases superfamily protein					
Phvul.003G165700	3	38535457	38548714	Protein kinase superfamily protein					
Phvul.003G168000	3	38867946	38872171	Protein kinase superfamily protein	Chr03_38912965	45019	40794	< 45 kb	
					Chr03_38912970	45024	40799	< 45 kb	
Phvul.003G170900	3	39293276	39299144	Avirulence induced gene (AIG1) family protein					
Phvul.003G172400	3	39452306	39462226	Leucine-rich repeat family protein					
Phvul.003G148000	3	35848190	35865660	FGGY family of carbohydrate kinase	Chr03_35847673	-517	-17987	< 1kb	< 2 Kb
Phvul.003G157500	3	37378080	37381316	Tetratricopeptide repeat (TPR)-like superfamily protein	Chr03_37381665	3585	349	< 1kb	
Phvul.003G168800	3	39004746	39007688	Raffinose synthase family protein	Chr03_39009342	4596	1654	< 2 kb	
Phvul.003G175900	3	39822021	39824655	Drought sensitive, WD repeat-containing protein 76	Chr03_39824257	2236	-398	on gene	
					Chr03_39824268	2247	-387	one gene	

same QTL region on chromosome 3 for ASB resistance for PRI19, PRL19, PRI21, PRI, and PRI.L, but different significant SNP markers for each pathogen source were identified (Tables 1, 2; Tables S4, S6; Figures 6, S9, S10 and S11), indicating that there was variability of the *M. phaseolina* pathogen used in this study. The variability of the *M. phaseolina* pathogen was reported by Reyes-Franco et al. (2006); Mahdizadeh et al. (2012), and Yesil and Bastas (2016), who also studied the genetic diversity of *M. phaseolina* collected from Iran, Mexico, Turkey, and other countries.

QTL identification of ashy stem blight resistance

QTL mapping is based on phenotypic data and genotypic data (molecular markers) to map QTL to chromosome(s) or linkage group(s) (LGs) in segregating population(s) such as F_2, $F_{2:3}$, or RIL using a statistic model, and it has been widely used in tagging major or minor genes/alleles in crops. Except for single marker analysis such as single marker regression and *t*-test, QTL mapping requires an LG or chromosome with ordered markers, known as genetic maps. Different genetic maps will result in different results for QTL mapping. The marker number, marker density, and marker order in each chromosome or LG affect the results in QTL mapping, as do the mapping populations. Even using same marker number, the marker order in each chromosome or LG will be different depending on the mapping populations (parents, generation, size, etc.) and mapping tools such as MSTmap and JoinMap.

In this study, we used JoinMap 4 (Van Ooijen, 2006) and MSTmap (Wu et al., 2008; http://mstmap.org/) to create the genetic linkage maps in an RIL population of 126 $F_{6:7}$ for RIL derived from a cross between BAT 477 and NY6020-4. We found it was easy to create genetic maps but hard to create stable and uniform genetic maps of the 11 chromosomes. The order of the SNPs in each chromosome was different depending on the SNP number, but the physical position of the SNPs did match well on the chromosomes. Although a total of 6,463,014 SNPs were identified in the 126 RIL and their parents, distributed on the 11 chromosomes, and 35,787 SNPs mapped to create the genetic maps (Figure S8), the genetic and physical distances and the order of SNPs in each chromosome still did not match well. However, the chromosome Pv03 did have good matched genetic and physical maps, using either 7,746 SNPs or 179 SNPs (Table S5, Figure 4), on which we identified the QTL for ASB resistance. The orders of the genetic and physical maps in the QTL region were not exactly the same (Table S5), such as for the three ABS SNP markers, Chr03_39009342, Chr03_37616128, and Chr03_39824268, where the physical order was Chr03_37616128-Chr03_39009342-Chr03_39824268 with position 39,009,342 bp, 37,616,128 bp, and 39,824,268 bp, respectively, on Pv03, but their genetic map order was Chr03_39009342-Chr03_37616128-Chr03_39824268 with genetic position 489.999 cM, 513.509 cM, and 515.756 cM, respectively, on Pv03, based on 179 SNPs on this chromosome; however, the genetic order was Chr03_39009342-Chr03_39824268- Chr03_37616128 based on 7,746 SNPs on chromosome Pv03 (Tables 2, S5), which may be caused by the map population size with 126 RIL.

In order to overcome the disadvantage of QTL mapping caused by the genetic order error, we also performed GWAS for ASB resistance in this RIL using three models – GLM, MLM, and Blink – in GAPIT 3 and three models – SMR, GLM, and MLM – in TASSEL 5 when PCA = 2, and combined QTL mapping using SMR, SMIM, and SMLE in QGene. A QTL was identified to be located at 35,546,329 - 39,826,434 bp on Pv03, and two SNPs, Chr03_39824257 and Chr03_39824268, located at 39,824,257 bp and 39,824,268 bp on Pv03, respectively, were identified as being the strongest markers associated with ASB resistance in this study.

Resistant QTL to ASB derived from the BAT 477 breeding line have been reported in previous studies (Mayek-Pérez et al, 2009; Méndez-Aguilar et al., 2017) with different results. Mayek-Pérez et al. (2009) reported that BAT 477 had two and nine genes for *M. phaseolina* resistance in field conditions. Hernández-Delgado et al. (2009) detected one QTL associated to charcoal rot resistance in BAT 477 using a F2 population and the markers BPC40M127 and BPC54M150 associated with charcoal rot (=ASB) resistance (Méndez-Aguilar et al., 2017). Méndez-Aguilar et al. (2017) identified QTL for ABS resistance in a 94 $F_{2:9}$ RIL population derived from a cross between BAT 477 and cv. Pinto UI-114 using 476 AFLP polymorphic markers, and mapped the QTL on Pv03, Pv05, Pv06, Pv08, Pv09, and Pv10 LG based on 68 AFLP markers distributed in 10 linkage groups (LG) with coverage of 718.1 cM and two QTL in Pv03 by use of only six AFLP markers on Pv03. The ASB resistant QTL on Pv03 was identified using 7,746 SNPs on chromosome 3 by QTL and associated mapping with several models.

However, these reported QTL were identified under natural infestations of *M. phaseolina* in the field, where avoidance mechanisms (i.e., plants with upright growth habits, open canopy, and/or resistance to lodging) may be associated with lower severity to this pathogen (Mayek-Pérez et al., 2001b; Viteri and Linares, 2022a). However, our novel QTL on Pv03 chromosome was identified in the greenhouse, which is the appropriate environment used to detect physiological resistance to necrotrophic fungus such as *M. phaseolina* (Viteri and Linares, 2017; Viteri et al., 2019) and *Sclerotinia sclerotiorum* L. de Bary (Soule et al., 2011; Schwartz and Singh, 2013; Viteri et al., 2015).

Candidate gene for ashy stem blight resistance

There are 305 genes in the QTL region from 36.17 Mbp to 9.83 Mbp on chromosome Pv03 for ASB resistance to PRI19, PRI21, PRL19, PRI, and PRI.L based on six GWAS models in GAPIT 3 and three QTL models in QGene (Table S7). Among the 305 genes, there are 11 disease gene analogues (Table 3), which may be associated with the ASB resistance. From this study, the QTL for ASB resistance in the RIL of BAT 477/NY6020-4 was located at 35,546,329 - 39,826,434 bp on Pv03. Two SNPs, Chr03_39824257 and Chr03_39824268 located at 39,824,257 bp and 39,824,268 bp on Pv03, respectively, were identified as the strongest markers associated with ASB resistance, and they were on the gene Phvul.003G175900 (drought sensitive, WD repeat-containing protein 76), thus Phvul.003G175900 located at 39,822,021 – 39,824,655 bp on Pv03 was recognized as the candidate for ASB resistance in the

RIL. The two SNP markers and the gene can provide information for selecting ASB resistance in common bean breeding through MAS.

Utilization of the RILs for ashy stem blight resistance

Among 126 RIL, 10 lines showed high resistance to ASB pathogens, with 4 or lower as an average score across two years in two locations (Supplementary Table S8), where either PRI.L or RPI score was <= 4; PRI19 or PRI19 <= 3.8 (except 20373Vit_92 with score = 4.1); and PRI21 <=4.1 (except 20373Vit_85 with score = 4.8 and 20373Vit_128 = 4.2), indicating that the 10 RIL were more ASB resistant in this RIL population, suggesting they can be used as parents in common bean breeding.

The 126 RIL can be divided into two clusters (groups) (Figure S11) based on each of the two parents, BAT 477 and NY 6020-4. The top 10 ASB resistant RIL were also distributed into two groups analyzed by MEGA 7 using Maximum Likelihood (ML) method either among 128 lines (126 RILs plus 2 parents) or 12 lines (10 R-line plus two parents) (Figure S11), indicating that the ASB resistant QTL 'ASB-qtl-3' on chromosome Pv03 can be transferred from the BAT 477 breeding line to an NY 6020-4 genetic background and utilized in common bean breeding programs to develop new ASB resistant lines. New common bean germplasms UPR-Mp-42 and UPR-Mp-48 have been developed with BAT 477, Andean PRA154, and NY6020-4 as parents in their lineage, with enhanced levels of resistance to ASB (Viteri and Linares, 2022b). However, it has been necessary to pyramid higher levels of resistance derived from the Andean gene pool (i.e., A 195, 'PC 50', and PRA154) (Viteri and Linares, 2017; Viteri et al., 2019). It has been reported that BAT 477 and NY6020-4 can reach susceptible scores under a severe screening method (i.e., two inoculations per plant) (Viteri and Linares, 2022b).

Conclusion

In this study, a QTL region for ASB resistance was identified in an RIL population derived from BAT 477 and NY6020-4. The QTL was located at 35,546,329 - 39,826,434 bp on chromosome Pv03. Two SNPs, Chr03_39824257 and Chr03_39824268 located at 39,824,257 bp and 39,824,268 bp on Pv03, respectively, were identified as the strongest markers associated with ASB resistance, and they were on the gene Phvul.003G175900 (drought sensitive, WD repeat-containing protein 76), thus Phvul.003G175900 located at 39,822,021 – 39,824,655 bp on Pv03 was recognized as the candidate for ASB resistance in the RIL. The two SNP markers and the gene can provide information for selecting ASB resistance in common bean breeding through MAS.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories in FigShare https://doi.org/10.6084/m9.figshare.19919221.v1 and accession number(s) can be found in the article/Supplementary Material.

Author contributions

DV was the principal investigator (PI) for the project. DV, AL, and ZM were involved in phenotyping and performing ashy stem blight resistant evaluation. AS performed genomic and statistical analysis. AS and DV wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1019263/full#supplementary-material

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Conventional and new-breeding technologies for improving disease resistance in lentil (*Lens culinaris* Medik)

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Lentil, an important cool season food legume, is a rich source of easily digestible protein, folic acid, bio-available iron, and zinc nutrients. Lentil grows mainly as a sole crop in the winter after harvesting rice in South Asia. However, the annual productivity is low due to its slow growth during the early phase, competitive weed infestation, and disease outbreaks during the crop growth period. Disease resistance breeding has been practiced for a long time to enhance resistance to various diseases. Often the sources of resistance are available in wild crop relatives. Thus, wide hybridization and the ovule rescue technique have helped to introgress the resistance trait into cultivated lentils. Besides hybridization, induced mutagenesis contributed immensely in creating variability for disease tolerance, and several disease-resistant mutant lines have been developed. However, to overcome the limitations of traditional breeding approaches, advancement in molecular marker technologies, and genomics has helped to develop disease-resistant and climate-resilient lentil varieties with more precision and efficiency. This review describes types of diseases, disease screening methods, the role of conventional and new breeding technologies in alleviating disease-incurred damage and progress toward making lentil varieties more resilient to disease outbreaks under the shadow of climate change.

KEYWORDS

lentil, disease outbreaks, conventional breeding, new breeding technologies, biotic stress, yield

1 Introduction

Lentil (*Lens culinaris* Medik 2n=2x=14), belonging to the Fabaceae family, is one of the oldest domesticated cool season food legumes (Zohary 1999). At present the accepted name for lentil is *Vicia lens* (L.) Coss. & Germ. Lentil grains are a rich source of protein, vitamins, fiber, and micronutrients such as iron, zinc, magnesium, and folate, consumed in various raw, cooked, and processed forms (Mitchell et al., 2009; Sen Gupta et al., 2013; Joshi et al., 2017; Raina et al., 2022a). Besides, lentil enriches soil nitrogen through biological nitrogen fixation and condition soil health in long-term cereal-legume cropping sequences. The crop is cultivated over sub-tropical to temperate areas worldwide and is one of South Asia's famous and highly consumed pulse crops (Alghamdi et al., 2014). Lentil occupies the 5th position in total production among pulses worldwide and supports nutrition in low- and middle-income countries (Joshi et al., 2017; Warne et al., 2019). Worldwide lentil production has increased by 49% over the last 10 years and surpassed 6.5 million tons in the year 2020 (Yang et al., 2021; FAOSTAT, 2022) (Figure 1). Due to early domestication, lentil is grown as a sole pulse crop in rice fallow or *paira* crop in South Asia. It is usually grown in lower elevated land during winter, at higher altitudes during spring, and as green lentils during summer in some parts of the World beyond South Asia.

Several biotic stresses cause a huge yield loss and are emerging as threats to be addressed quickly for yield stability (Erskine et al., 1994). Early interventions of disease resistance breeding involving intra-specific hybridization has increased the average yield of lentils from 560 kg/ha to 950 kg/ha within a few decades (Singh et al., 2014). However, climate change exposes lentils to extreme weather events (drought and terminal heat), leading to increased disease outbreaks and eventually hampering yield stability (Raza et al., 2019). Among various diseases, fungal pathogens are the most threatening that reduce plant population drastically at every growth period from seedling to the pod-bearing stage. For instance, *Ascochyta* blight infection caused 30-70% yield reductions in Canada, the United States of America, Australia, and northern parts of India (Morrall and Pedersen, 1991; Singh et al., 2013a). In comparison, *Colletotrichum truncatum* caused 60% yield reductions in Canada (Morrall. 1997; Buchwaldt et al., 2013). Stemphylium blight incurs nearly 95% yield loss in India (Sinha and Singh, 1993). Further, North East India, Nepal and Bangladesh reported considerable yield loss in lentil due to Stemphyllium blight (Bakr and Ahmed, 1992). Besides yield reductions, fungal blight disease induces leaf drop, wilting, pod and seed lesions, and complete plant mortality (Taylor et al., 2007). The best way to mitigate the dreadful consequences of fungal diseases is to develop disease-resistant varieties.

Disease screening among available germplasm has not yielded desired results in identifying extremely resistant lines for Stemphyllium blight except for a few moderate resistant sources in Eastern India (Mondal et al., 2017). Uncertainty in rainfall and rise in atmospheric temperature facilitate disease outbreaks and turns some minor diseases into prominent dreadful diseases. For instance, anthracnose caused by *Colletotrichum truncatum*, is becoming a major disease in Canada (Buchwaldt et al., 2018). Thus, it is worth putting efforts into guarding the lentil crop against durable, multiple minor, and major diseases in climate-changing scenarios (Cowling, 1996). In addition, lentils possess a narrow genetic base due to their limited domestication involving very few traits that leads to very sporadic resistance to disease in cultivated gene pools (Ladizinsky, 1987; Zohary, 1989). Moreover, the quick co-evolution of pathogens causes more yield losses and demands increased genetic diversity using CWRs and new breeding lines with improved resistance (Dodds and Thrall, 2009; Tullu et al., 2006a; Singh et al., 2014). Therefore, efforts are needed to minimize the quick pace of pathogen co-evolution (Negussie et al., 2005). Still there is a opportunity for screening available

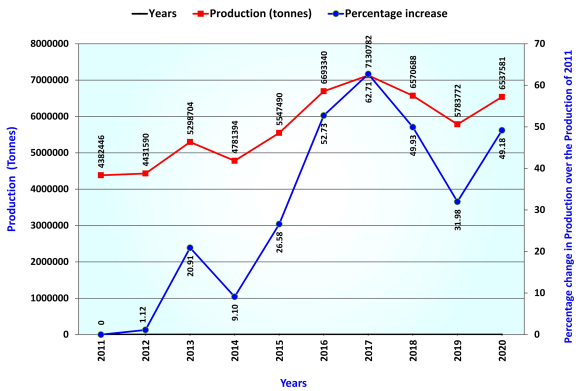

FIGURE 1
Trend of worldwide production volume of lentil over the last ten years.

germplasm for disease resistance at various environments which will provide a source materials for disease inheritance, QTL identification and gene isolation study. Therefore, artificial screening protocols are required to confirm the resistance source vis-à-vis newly identified QTLs. New plant breeding technologies like, genomics assisted breeding (GAB), genomic selection (GS) and gene editing should be painstakingly carried out for developing lentil cultivars with improved tolerance against all dreadful diseases. Recent advances in genomics, including identifying specific QTLs associated with disease tolerance and a few differentially expressed genes from the QTL region, have broadened the understanding of lentil disease resistance (Saha et al., 2010a; Cao et al., 2019). This review describes important aspects of disease resistance and the role of breeding strategies in developing disease-resistant lentil varieties.

2 Major diseases of lentil

Despite the high demand for lentil in South-east Asia, a declining trend in farmers' adoption of lentil is being observed due to several biotic stresses, which limit the yielding potential. Several biotic constraints, such as fungi, bacteria, viruses, insects, nematodes, phytoplasmas, and weeds, cause a substantial reduction in average annual yield (Chen et al., 2009; Darai et al., 2017). For instance, Fusarium wilt can cause a 50 to 100% reduction in yield (Tiwari et al., 2018). However, fungal pathogens are the most dreadful and infect almost all parts, such as stems, roots, leaves, pods, and seeds, thus reducing their marketability (Bayaa et al., 1994; Bhadauria et al., 2017a). Most foliar fungal pathogen affects photosynthetic apparatus after successful colonization and sporulation, produce toxins, and cause blight (Chen et al., 2009; Chen et al., 2013). In the case of a wilt pathogen, xylem vessels get blocked and eventually restrict the upward movement of water (Erskine et al., 1994; Darai et al., 2017). Disease cycle of a foliar fungal pathogen, *Ascochyta lentis* (Figure 2), and a wilt pathogen, *Fusarium oxysporum* f.sp. *lentis* (Figure 3) represented contrasting features of these two major pathogens of lentil. The new infection of *Ascochyta* can occure through infected seed (from pycnidium) or through the resting spores (from pseudothecium) from crop debris of previous season (Figure 2). In case of wilt pathogen, the new infection arises from soil borne chlamydospore or micro/macro-conidia (Figure 3). In addition to fungal diseases, lentil production is substantially reduced by bacterial diseases such as bacterial leaf spot, bacterial root rot, bacterial blight, etc. In general, bacteria overwinter in infected seed and crop debris and sequentially infect cotyledons, leaves, and vascular system, multiply rapidly in the xylem and cause systemic infection producing stem and leaf lesions. Internally, bacteria move between cells, up or down in the vessels and ooze out through splits in the tissue and re-enter stems or leaves through stomata or wounds (Glazebrook et al., 2005). A detailed list of lentil diseases is furnished in Table 1.

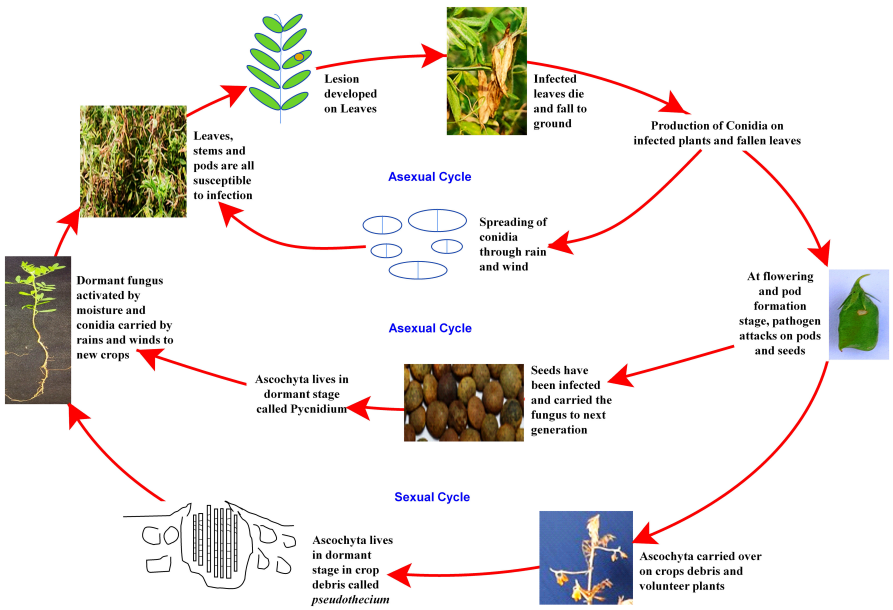

FIGURE 2
Disease cycle of Ascochyta blight disease of lentil.

It is important to note that some diseases are common in almost every lentil-growing region of the world, such as Fusarium wilt and Ascochyta blight. In contrast, many are limited to areas such as Alternaria blight (restricted in India, Ethiopia, and Egypt) (Taylor et al., 2007). However, the economic importance of a disease is not necessarily characterized only by its geographical distribution. A disease with limited occurrence may still cause significant economic losses and lead to devastating effects in conducive conditions (Chen et al., 2009). The extent of yield loss in lentil as a result of different pathological diseases has been reported by several researchers (Table 1).

In addition to fungi and bacteria, viruses are also capable of affecting lentil productivity across the globe (Beniwal et al., 1993). About 30 virus species belonging to 16 genera, representing 9 families, with single-stranded RNA or DNA, affect lentil productivity (Chen et al., 2009). Viruses hijack the plant cell machinery, use its nucleic acids and proteins for their multiplication, and can traverse through plasmodesmata from one cell to another. At least ten viruses infect lentil in field conditions (Bos et al., 1988; Makkouk et al., 1992). Of these viruses, pea seed-borne mosaic virus (PSbMV) is more common and dreadful, decreasing the seed yield by up to 72% (Aftab et al., 1992; Kumari et al., 2009). The important viral diseases, their causal organism, and their genomic features are furnished in Table 2.

Besides viruses, phytoplasma from 16SrII-C group also causes a significant loss in lentil productivity and produces symptoms like floral malformation, little leaf, chlorosis, and excessive growth of branches (Akhtar et al., 2016). However, literature is scanty on the extent of damage caused by phytoplasmas. While discussing the diseases, it is imperative to mention the role of weeds in reducing the overall productivity of lentils. By virtue of the short height and slow growth rate of lentils in the early seasons of their development, weeds outperform the crop for nutrients, light, space, and water and result in huge yield losses ranging from 20-84% (Basler, 1981; Yenish et al., 2009). Some of the crucial weeds critically reducing lentil productivity include *Avena fatua*, *Loliu multiflorum*, *Phalaris minor, Poa annua*, *Setaria viridis*, *Convolvulus arvensis*, *Cirsium arvense*, *Cuscuta campestris, C. chinensis Cynodon dactylon*, *Cyperus rotundus* and parasitic flowering plants (*Orobanche crenata*, *O. aegyptiaca*, *Phelipanche aegyptiaca*) (Rubiales et al., 2009). *Orobanche* infestations in Turkey resulted in 59% yield losses (Yolcu et al., 2020). Similarly, *Cuscuta chinensis* are dreadful weeds and can reduce the lentil productivity by 87%. Moreover, weed-borne insects, pests, and pathogens compounded adverse effects in lentils (Moorthy et al., 2003). Irrespective of the nature of devastation and causative agents, disease resistance can be improved using different genetic resources.

3 Genetic resources for disease resistance

Successful plant breeding depends on accessible genetic variability in the germplasm and its sustainable exploitation

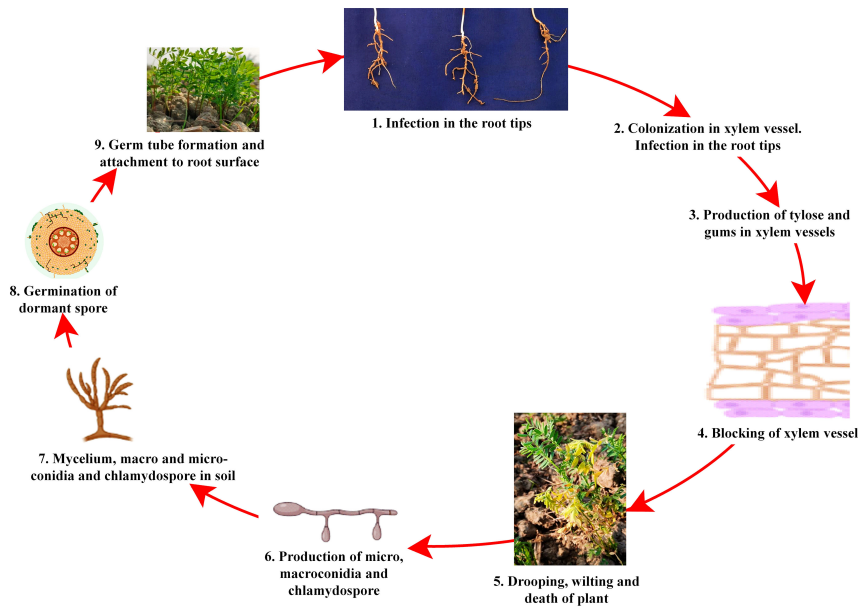

FIGURE 3
Disease cycle of Fusarium wilt disease of lentil.

TABLE 1 Economically important fungal and bacterial diseases and their extent of yield loss in lentil.

Name of the disease	Causative agent	Extent of yield loss
Anthracnose	*Colletotrichum truncatum* (Schw.) Andrus & Moore.	60% (Morrall. 1997; Buchwaldt et al., 2013)
Aphanomyces root rot	*Aphanomyces euteiches* C. Drechsler	80% (Gaulin et al., 2007)
Ascochyta blight	*Ascochyta lentis* Bond. &Vassil.	30 – 70% (Gossen and Morrall, 1983; Singh et al., 2013a)
Botrytis grey mould	*Botrytis cinerea* Pers. Ex Fr. and *Botrytis fabae* Sard.	50 – 100% (Haware and McDonand, 1992; Bayaa and Erskine, 1998; Davidson et al., 2004)
Collar rot	*Sclerotium rolfsii* Sacc.	Up to 50% (Asghar et al., 2018)
Fusarium wilt	*Fusarium oxysporum* f.sp. *lentis*	67 – 100% (Garkoti et al., 2013; Tiwari et al., 2018)
Lentil rust	*Uromyces viciae-fabae* (Pers.) Schroet.	60 – 69% (Sepulveda, 1985; Chen et al., 2009)
Powdery mildew	*Erysiphe trifolli*, *E. diffusa*, *E. pisi* and *Leveillula taurica* (Lév.) Arnaud.	5.5 – 15.5% (Singh et al., 2013b)
Sclerotinia rot	*Sclerotinia sclerotiorum* (Lib.) de Bary.	80% (Ahmed and Akhond, 2015)
Stemphylium blight	*Stemphylium botryosum* Wallr.	95% (Sinha and Singh, 1993)
Bacterial leaf spot	*Xanthomonas* sp.	94% (Richardson and Hollaway, 2011)
Bacterial blight	*Pseudomonas syringae* pv. *Syringae*	5% (Adhikari et al., 2018)

(Upadhyaya et al., 2011). Therefore, it is imperative to have extensive knowledge of lentil genotypes that are potential sources of disease resistance (Table 3). *Lens culinaris* ssp *culinaris* categorised as cultivated lentil in the genus consisting of a primary gene pool (including *Lens orientalis*) and a secondary gene pool (including *Lens nigricans*, *Lens ervoides* and *Lens odomensis*) (Muehlbauer et al., 1995). However, Fratini and Ruiz (2006) categorized *Lens ervoides* and *Lens nigricans* in the tertiary gene pool. Recently, *Lens culinaris*, *Lens tomentosus*, and *Lens orientalis* have been categorized in the primary gene pool; *Lens odemensis* and *Lens lamottei* were placed in the secondary gene pool; *Lens ervoides* was kept in the tertiary gene pool, and *Lens nigricans* were kept in the quaternary gene pool (Wong et al., 2015). Wild relatives of lentil such as *Lens culinaris* ssp *orientalis*, *Lens ervoides*, *Lens odemensis*, and *Lens nigricans* are potential and promising donors of foliar disease resistance (Bayaa et al., 1994; Ye et al., 2000). *Lens ervoides* possess genetic loci that confer partial disease resistance against Stemphylium blight (Podder et al., 2013), Fusarium wilt (Singh et al., 2017), Ascochyta blight (Tullu et al., 2010). Vail et al. (2012) also reported partial resistance to antrhacnose in cultivated lentil (*Lens culinaris*) genotypes. While, *L. ervoides* and interspecific-hybridization-

TABLE 2 Economically important viral diseases in lentil, causative agents, their taxonomy, genetic constitution and extent of damage.

Name of the virus	Genus	Family	Genome	Extent of damage
Alfalfa mosaic virus or AMV	*Alfamovirus*	*Bromoviridae*	(+) ssRNA	–
Bean leaf roll virus or BLRV	*Luteovirus*	*Luteoviridae*	(+) ssRNA	50 - 91%
Bean yellow mosaic virus or BYMV	*Potyvirus*	*Potyviridae*	(+) ssRNA	34 - 96%
Beet western yellows virus or BWYV	*Polerovirus*	*Luteoviridae*	(+) ssRNA	–
Broad bean stain virus or BBSV	*Comovirus*	*Comoviridae*	(+) ssRNA	14 - 61%
Cucumber mosaic virus or CMV	*Cucumovirus*	*Bromoviridae*	(+) ssRNA	75 - 84%
Faba bean necrotic yellows virus or FBNYV	*Nanovirus*	*Nanoviridae*	ssDNA	80 - 90%
Pea enation mosaic virus-1 or PEMV-1	*Enamovirus*	*Luteoviridae*	(+) ssRNA	16 - 50%
Pea seed borne mosaic virus or PSbMV	*Potyvirus*	*Potyviridae*	(+) ssRNA	23 – 73%
Pea streak virus or PeSV	*Carlavirus*	*Flexiviridae*	(+) ssRNA	–
Subterranean clover red leaf virus or SCRLV	*Luteovirus*	*Luteoviridae*	(+) ssRNA	–

derived lines showed significantly more resistance than the cultivated lentil genotypes (Vail et al., 2012). Similarly, *Lens lamottei*, *Lens ervoides* and *Lens nigricans* showed the highest resistance against anthracnose disease (Tullu et al., 2006a). However, complete resistance to Stemphylium blight has been reported in *Lens tomentosus* (Guerra-García et al., 2021). The wild species, *Lens ervoides*, *Lens nigricans* and *Lens odomensis* harbor resistance against rust, fusarium wilt and powdery mildew. In addition, *Lens culinaris* ssp *orientalis* and *Lens culinaris* ssp *tomentosus* revealed complete resistance against fusarium wilt and powdery mildew (Gupta and Sharma, 2006). A total of 58,045 accessions of lentil are maintained worldwide (FAO, 2010). National Bureau of Plant Genetic Resource (NBPGR), New Delhi, India, maintains 7712 lentil accessions, including exotic and indigenous *Lens culinaris* ssp *culinaris*. In comparison, International Centre for Agricultural Research in Dry Areas (ICARDA) maintains 14597 accessions in its gene bank (Guerra-García et al., 2021). Among the countries, maximum lentil collection is available in Syria, Australia, Iran, USA, Russia, India, Chile, Canada, and Turkey where genotypes are conserved as *ex-situ* germplasm (Malhotra et al., 2019). Despite World-wide collection, few genotypes have been used extensively in lentil breeding to improve disease resistance. For instance, foliar disease-resistant accession ILL 5588 was exploited in Australia for disease resistance breeding against Ascochyta blight (Ford et al., 1999). Later, a novel resistance source ILL 7537 was identified (Nguyen et al., 2001). Future studies should be directed to screen large number of available lentil germplasm for various disease resistance in both field based screening and artificial screening in phytotron or high throughput phenotyping facility. In developing disease-resistant cultivars, proper germplasm screening is an important step towards developing disease-resistant cultivars.

4 Screening methodologies for disease resistance breeding

Establishing suitable and effective screening techniques is the major component of the breeding programs for disease resistance. A complete understanding of resistance type, pathogenicity, virulence pattern, and the effective breeding strategy is required to obtain desirable results. A sufficient amount of research work has been carried out in the last decade to delineate the nature and durability of resistance, and effective methods of screening for resistance to several pathogens have been devised (Tullu et al., 2003; Negussie et al., 2005; Stoilova and Chavdarov, 2006; Podder et al., 2013). The following paragraphs briefly explain some of the commonly used screening methods for various diseases.

TABLE 3 Genetic resource of lentil for resistance to diseases.

Lentil Disease	Resistant Sources	Reference
Ascochyta Blight	VL Masoor 3, CDC Robin, 964a-46, ILL 7537, ILL 5588, ILL 358, ILL5684, Laird, Rajah, Masoor-93, ILL 4605, ILL 857, ILL5590, ILL 5593, ILL 5244, ILL 5725, ILL 179, ILL 195, ILL 201, ILL 5698, ILL 5700, ILL 5883, ILL 6212, ILL 2439, ILL 5562, Indianhead, 96, 507, 712, 859, 112082, 123452, 123514 and 123801	Nguyen et al., 2001; Ye et al., 2002; Tivoli et al., 2006; Sari et al., 2018; Bedasa, 2021
Lentil rust	IPL81, PL639, L4147, L4149, DPL 15, LL147, L4076, Pant Lentil 4, LH82-6, NDL92-1, Gudo (a resistant cultivar), R 186	Negussie et al., 2005; Dikshit et al., 2016; Singh et al., 2020
Wilt	JL 3, NDL92-1, P177-12, PL-639, Jawahar Lentil-1, VL Masoor 4, Pant Lentil 4, DPL-15, ILL 5883', 'ILL 5588', 'ILL 4400' and 'ILL 590' Pant L 406, Pant L 4, Priya, Seri, VL507, IPL 306, IPA 98, Idleb 2, Idleb 3, Idleb 4, Ebla 1, ILL 6256, Firat 87, Syran 96, Talya 2, Rachayya, Hala, RL-13, RL-21, ILL 6468, ILL 9996,\ILL 6024, ILL 6811, ILL 7164, Arun, Maheswar bharti, L 7920 and DPL 58, PL 101, L 4076 (cultivar)	Pandya et al. (1980); Singh et al. (1994); Erskine et al. (1994); Sarker and Erskine (2002); Joshi and Maharjan (2003); El-Ashkar et al. (2004); Rahman et al. (2009); Parihar et al., 2017; Yadav et al., 2017; Singh et al., 2020; Taranam et al., 2021
Alternaria Blight	EC866132, IC267 67, IC201778	Roy et al., 2021
Anthracnose	*L. ervoides* accession: PI72847, IG72815 PI572330, PI572334, PI57233, BGE001814, PI298644, PI283604, PI477921, PI431809, PI432005, PI432033, PI432071, PI297287, PI572327. W627758 (*L. culinaris* spp. *culinaris*) PI320937, PI320952 (cv. Indianhead), PI345629, PI468901	Buchwaldt et al., 2004; Buchwaldt et al., 2018; Barilli et al. (2020)
Resistant to Blight, rust and Viral disease	66013-6	Hussain et al., 2008
Resistant to Stemphylium blight	LL 1370, VL 151, LL 1375, RLG 195, L 4727, L 4769, LL 1397, DL 14-2, VL 526, VL 126, RKL 14-20, IPL 334, L 4710, PL 210, PRECOZ (RC), RL-13, RL-21, ILL 6468, ILL 9996, \ILL 6024, ILL 6811, ILL 7164, Arun, Maheswar bharti	Mondal et al., 2017; Yadav et al., 2017

4.1 Screening in a natural field condition

Screening genotypes in the field condition under natural disease epidemics for selecting resistant genotypes requires an extensive knowledge of the disease epidemics and 'hot-spots'. Diseases have different hotspots based on their congenial growth conditions. The test genotypes are grown in the hotspot regions and screened for the target disease. While screening the genotypes, some known resistant and susceptible cultivars are also planted as checks under the same environmental conditions (Ye et al., 2002). However, this approach suffers a major drawback due to its dependence upon the epidemic year for the screening and thus reduces the breeding progress. Besides, it is also dependent upon the severity of disease infestation (Porta-Puglia et al., 1994). Therefore, screening and selection of resistant genotypes may be performed under artificial conditions to achieve reliable outcomes (Ye et al., 2002). Using the field screening teachnique, Buchwaldt et al. (2004) identified 16 lentil germplam which were resistant to the antracnose disease caused by isolate Ct1. But no accessions were found resistant to isolate Ct0. Bedasa (2021) conducted field screening for Aschochyta blight in hot spot condidtion of Alemtena and Minjar (Ethiopia) and identified eight resistant lentil genotypes which showed resistant to moderate resistant reactions in both seasons.

4.2 Screening in the field through artificial infections

Screening of disease-resistant genotypes under artificial conditions could overcome major limitations that are frequently encountered in the natural screening method. Artificial epidemics for a particular disease can be generated in the field by using the following three methods viz., preparation of inoculums in the laboratory and application in the individual plant, scattering the diseased plant debris throughout the experimental field, and inter-planting of susceptible genotypes (spreader rows) after every 6-8 rows of test genotypes to increase pathogen populations over the field (Ye et al., 2002). Regular irrigation through flooding or sprinkler may be provided to generate the optimum relative humidity in the field (Ahmed and Morrall, 1996). Inoculums may be applied in plants by spraying the suspension (for foliar pathogen), mixing the pure culture of pathogens into the planting soil (soil-borne pathogen) or by leaf clipping method (Stoilova and Chavdarov, 2006). Methods of preparation of inoculums for artificial infection may vary significantly according to the nature of pathogen. After the inoculation, proper conditions will be required to facilitate the pathogen growth, multiplication, and disease progression. The chances of disease severity will be considerably less in the absence of optimum conditions. Furthermore, inter-planting susceptible genotypes with test genotypes may be a viable and most feasible option for screening disease resistance. To date, most of the resistant genotypes released were identified through artificial infection at field method. The effectiveness of this method may be affected by the interaction between genotype and environment, physiological age of the tested plant and tissue-specific expression of disease resistance (Ahmed and Morrall, 1996). Dikshit et al. (2016) screened a RIL population of lentil to screen for rust resistance by following a spreader row technique in field and successfully phenotyped the population towards identification linked molecular markers for rust resistance. Almost one-third of screened F2 population (119 plants) was found resistant to rust disease.

4.3 Screening in the glasshouse/ greenhouse through artificial infections

Screening of genotypes for any disease resistance may be performed in a glasshouse under controlled environmental conditions. Optimum photoperiod, relative humidity, and temperature may be easily adjusted in the controlled glasshouse/ greenhouse according to the requirements for disease progression. These parameters may differ as per the nature of pathogens. Using this method, test genotypes are planted under a glasshouse along with the susceptible genotypes, followed by artificial infections. Artificial infection-based screening at glasshouse could overcome major limitations of field screening. Following are the major advantages in this method, i) disease screening may be performed in off-season and at any developmental phenophase, ii) manipulation of environmental conditions can be accustomed easily for proper disease development, iii) interference from other biological agents can be avoided by creating clean environments, iv) the inoculums can be more evenly distributed and consequently reduce the chance of escapes (Ye et al., 2002; Porta-Puglia et al., 1994). Looking at the merits of this method, it may be best suited for screening disease-resistant genotypes and understanding the genetic mechanism of disease resistance. This method is also suitable for screening genotypes with novel resistance genes for new virulent strains using a range of pathotypes or isolates. However, this method is quite costly and may not be useful for screening large size of segregating population. For soil borne disease like Fusarium wilt, sick plot techniques in field used for the identification of resistant germplasm (Bayaa and Erskine, 1990; Bayaa et al., 1995; Bayaa et al., 1997; Eujayl et al., 1998). The resistance against vascular wilt caused by *Fusarium oxysporum* f.sp. *lentis* Vasud. & Srin, was screened in a sick plot technique in a polyhouse using artificial inoculation of a Syrian isolate of this fungus at the seedling stage (Bayaa et al., 1995). Three accessions each of *Lens culinaris ssp. orientalis* and *L. nigricans* ssp. *nigricans* and two of *L. nigricans ssp. ervoides* were found to possess resistance at the reproductive growth stage. Further, three accessions (ILWL 79 & ILWL 113 of *L. culinaris ssp. orientalis* and ILWL 138 of *L. nigricans ssp. ervoides*) were tolerant. Negussie et al. (2005) identified Gudo and R-186 as sources of rust resistance based on glasshouse

screening. While, Fiala et al. (2009) conducted experiment to screen a segregating population for anthracnose resistance under controlled conditions in a Conviron growth chamber (Model GR178; Winnipeg, MB) maintained at 21°C day and 18°C night temperatures with an 18-h photoperiod under fluorescent and incandescent lighting with artificial inoculation Ct0 and Ct1 isolates. They found 103 $F_{5:6}$ RILS were resistant to Ct0 isolates, while only 19 were resistant to Ct1 isolate.

4.4 Screening of disease resistance in laboratory

When creating disease epiphytotics is difficult in field and greenhouse conditions, then some laboratory based screening methods like detached leaf test (Hanounik and Maliha, 1986); culture filtrates or purified phytotoxins based selection method (Buiatti and Ingram, 1991) and cut-twig method (Sharma et al., 1995) may be performed to assess host reactions (Porta-Puglia et al., 1994). Hanounik and Robertson (1988) employed a detached leaf test to evaluate disease resistance against chocolate spots in faba bean and concluded that this method could easily be followed for foliar disease resistance screening in a laboratory environment. In detached leaf test, fully expanded leaflets of a similar age were detached from the fifth node position of test plants and susceptible check plants. These leaflets were laid flat on a 2 cm thick moist sponge lining the bottoms of 90 X 40 X 5 cm galvanized metal pans, then inoculated separately with fungal spores (around 0.1 ml suspension containing 600,000 spores). One droplet was placed on each half of the upper lamina surface of each leaflet, then the pans were covered immediately and incubated at room temperature for disease development. Sharma et al. (1995) used the 'cut-twig' technique to screen resistance against *Ascochyta rabiei* in chickpea. This method includes inoculation of spores in single cut branches with spores. Culture filtrates or purified phytotoxins based selection method was used first by Carlson in 1973 using haploid cell lines of *Nicotiana tabacum* (Carlson, 1973). It was suggested that some purified phytotoxins positively correlate with plant tolerance and resistance behavior to pathogens. Therefore, this method has been used to screen genotypes for various disease resistance. However, the results sometimes seem to be contradictory, and there were systems where such a correlation seems to be proven only for some cultivars and not in other cultivars (Buiatti and Ingram, 1991; Buiatti and Scala, 1984; Buiatti et al., 1985; Kono, 1989). Therefore this method was not used regularly by the breeders for screening purposes. Laboratory testing is instrumental in selecting a resistant plant in earlier generations when the number of seeds per line is limited. Since disease reaction can be confirmed using twigs/leaf (with petiole)/branch, the entire plant is kept aside for seed production and further multiplication. However, there is a lack of research regarding the possible use of such methods in food legumes like lentil.

4.5 Screening of disease resistance genes using molecular markers

With the advent of molecular markers and next-generation sequencing approaches, it became easy to identify the genes/QTLs associated with specific disease resistance. The linkage between genes and molecular markers may be accurately calculated with the help of recent genomic approaches, *viz.* bi-parental QTL mapping (Collard et al., 2005), association mapping (Chakraborty and Weiss, 1988; Kruglyak, 1999; Yu et al., 2006), QTLSeq (Takagi et al., 2013). Development of molecular markers include generation of mapping population, screening of polymorphic markers, phenotyping of the mapping population, genotyping of population with polymorphic markers, generation of linkage map and QTL analysis and validation of linked markers. A molecular marker tightly linked with the gene of interest/QTL may be used to screen a large number of segregating populations with the minimum phenotyping in the field. This is relatively effortless, feasible, and much more reliable than other methods. However, disease-resistant genes' penetrance and expressivity may vary in genotypes and environmental conditions. Furthermore, the results may be confirmed by field screening due to the occurrence of recombination event between resistance gene and marker loci. Tar'an et al. (2003) screened a recombinant inbred line (RIL) population using markers linked to *ral1* (for ascochyta blight), *AbR1* (for ascochyta blight) and to the major gene for resistance to anthracnose using molecular markers UBC2271290, RB18680 and OPO61250, respectively and confirmed pyramiding of resistance genes for both Ascochyta blight and Anthracnose disease in 11 RILs. There are many more examples of such available markers for different diseases of lentil (Table 4). All these markers have potential to screen the segregating populations towards resistant genotypes identification. Moreover, these markers will help to pyramid multiple resistance genes in a agronomically superior lentil variety.

5 Resistance breeding in lentil

Reducing the pathogen entry at the initial phase of infection is the basic strategy for inhibiting disease progress (Nene et al., 1998). It has been stated that open canopy architecture is less sensitive to foliar diseases than the closed canopy. Hence, breeding for the canopy architecture in lentils will indirectly provide resistance to biotic stresses (Pedersen and Morrall, 1994). Similarly, leafless branches in pea tolerated lodging and were less prone to foliar diseases (Heath and Hebbelthwaite, 1985). A similar strategy, improving the harvest index, can be followed in selecting disease-resistant lentil genotypes. Epidemiology of disease is very important to decide the breeding strategies to be followed in field. For instance, *Aschochyta* blight heavily infests lentil during cool and wet weather conditions and infection frequency reaches a maximum at 10-15°C (Pedersen and Morrall, 1994; Nene et al., 1998). Artificial infection in the field, glasshouse and laboratory are initial and important steps

in screening true resistance for breeding programme and developing standard off-season disease-specific screening protocols (Ye et al., 2002). ICARDA led multilocational disease screening throughout the centres around the globe has facilitated the registration of disease-resistant cultivars in many countries (Russell, 1994; Singh et al., 1994; Erskine et al., 1996). A small seeded *Lens culinaris* variety 'Pant Lentil 4' was developed through pedigree selection in a 3-way cross (UPL175 × (Pant L 184 × P288)) in the North-Western plains of India. This variety has higher seed yield and resistance to rust, wilt and Ascochyta blight (Singh et al., 1994). In the last 15 years, about 38 disease resistant/tolerant lentil varieties were developed through recombination breeding technique in India that were either released by central varietal release committee or state variety release committee (Project Coordinator's Report, Annual Group Meet on MULLaRP, AICRP, ICAR, IIPR, Kanpur 2017-18; https://www.seednet.gov.in). The pedigree method developed a multiple disease-resistant variety 'Debine' in Ethiopia recently. This variety had comparable resistance/tolerant levels to major lentil diseases such as Aschocyta blight, rust, and root rot (Tekalign et al., 2022). The bulk method is ideal for applying natural selection for disease resistance in segregating populations. While early generation selection using disease nursery or the creation of

TABLE 4 Details of QTLs and genes identified and mapped for disease resistance in lentil.

S. No.	Trait	Type of marker	Marker name/QTLs/Genes	Mapping Populations	Phenotypic variationExplained by the QTL (%)	References
1.	Resistance to *Ascochyta* blight	RAPD	*RV01–RB18*	ILL5588 × ILL6002	90	Ford et al. (1999)
		RAPD, SCAR	$UBC227_{1290}$ and $OPD\text{-}10_{870}$ for *ral 2* gene	Eston x Indian head	–	Chowdhury et al. (2001)
		RAPD, ISSR	$OPB18_{680}$ $OPV1_{800}$	ILL5588 x L692-16-1	29 – 36	Tar'an et al. (2002)
		RAPD	$UBC227_{1290}$ for *ral1* gene, $RB18_{680}$ for *AbR1* gene.	CDC Robin x 964a-46	–	Tar'an et al. (2003)
		RAPD, AFLP, and ISSR	Five QTLs on LG1, LG2, LG4 LG5.	ILL5588 × ILL7537,	7 – 69	Rubeena et al. (2006)
		RAPD, AFLP, and ISSR	Four QTLs on LGI and LG II.	ILL7537 × ILL6002	6 – 34	Rubeena et al. (2006)
		AFLP and RAPD	ctcaccB *and LCt2*	Eston × PI320937	41	Tullu et al. (2006b)
		EST-SSR/ SSR	DK 225–UBC825c	North Weld (ILL5588) × Digger (ILL5722)	61	Gupta et al. (2012)
		SNP, SSR	Two major QTLs on LG1 and LG 2. LcC12416p463 and LcC03040p469 are the SNP markers for respective QTL	CDC Robin × 964a-46	–	Sari (2014)
		SNP and SSR	Three QTLs: AB_IH1, AB_IH2.1 & AB_NF1. Markers: SNP20005010, SNP20002370, SNP20001370, and SNP20001765	Indianhead × Northfield	7 – 47	Sudheesh et al. (2016)
		SNP and SSR	Two QTLs: AB_IH1 & AB_IH2.2 Marker: SNP20005010	Indianhead × Digger	22 - 30	Sudheesh et al. (2016)
		SNPs and short InDels	AS-Q1, AS-Q2, and AS-Q3	*L. culinaris* (Alpo) x *L. odemensis* (ILWL235)	28.46	Polanco et al. (2019)
2.	Resistance to Stemphylium blight	SSR, SRAP, RAPD	$QLG4_{80}$, $QLG2_{49}$, $QLG3_{3}$, $QLG4_{81}$ Markers: ME4XR16c, MR5XR10, and UBC34	ILL6002 × ILL5888	25 - 46	Saha et al. (2010a)

(Continued)

TABLE 4 Continued

S. No.	Trait	Type of marker	Marker name/QTLs/Genes	Mapping Populations	Phenotypic variationExplained by the QTL (%)	References
		SNP	qSB-2.1, qSB-2.2, qSB3 Markers: Contig271180p29128, Contig313227p47568, Contig406212p17766	L01-827A x IG 72815	9.9 -18.30	Bhadauria et al. (2017a)
3.	Resistance to Lentil Rust	SSR and SRAP	GLLC527 (SSR)	PL8 x L4149	–	Dikshit et al. (2016)
		SSR	GLLC106	FLIP-2004-7L x L-9-12	–	Fikru et al. (2016)
		SRAP	F7XEM4a	ILL-4605 x ILL-5888.		Saha et al. (2010b)
4.	Resistance to Anthracnose	RAPD, AFLP	*LCt-2 locus* Markers: OPEO6(1250), UBC-704(700), EMCTTACA(350), EMCTTAGG(375), EMCTAAAG(175)	Eston lentil x PI 320937	–	Tullu et al. (2003); Tullu et al. (2006a)
		RAPD	$OPO6_{1250}$	CDC Robin x 964a-46	–	Tar'an et al. (2003)
		SNP	qANTH0-3, qANTH0-5.1 and qANTH0-5.2 for race *Ct0* qANTH1-3.2, qANTH1-5.1 and qANTH1-5.2 for race *Ct1*	L01-827A x IG 72815	47.58 for Ct0 and 54.82 for Ct1	Bhadauria et al. (2017a)
		RNA Seq.	-	LR-66-528 x LR-66-524	–	Bawa (2020)
5.	Resistance to Fusarium wilt	RAPD	*Fw locus* Markers: $OPK\text{-}15_{900}$, $OP\text{-}BH_{800}$ and $OP\text{-}DI5_{500}$, $OP\text{-}C04_{650}$	ILL5588 x L692–16-l(s)	–	Eujayl et al. (1998)
		AFLP	p17m30710	ILL5588 x L 692-16-1(s)	–	Hamwieh et al. (2005)
		SSR	SSR59-2B	ILL5588 x L 692-16-1(s)	–	Hamwieh et al. (2005)
6.	Resistance to Aphanomyces Root Rot	SNP (GBS)	Q.RRI-Lc2.1, Q.BLU-Lc2.1, Q.SAT-Lc2.1, Q.CAN-Lc2.1, Q.AGI-Lc2.1, Q.RRI-Lc5.1 and Q.AGI-Lc5.1	K192-1 x K191-2	5.2 – 12.1	Ma et al. (2020)
		SNP (GBS) Association mapping	G.RRI-Lc1.1 and G.BLU-Lc1.1, G.RDL-Lc4.1 and G.RPL-Lc4.2, G.RRI-Lc5.1 and G.SAT-Lc5.1	326 accessions (AM)	1.4- 21.4	Ma et al. (2020)

artificial disease epidemic will be good for pedigree breeding methods. The combined bulk and pedigree method has been used for a long time in lentil resistance breeding (Singh, 1993; Muehlbauer et al., 1995). Hybrid plants (*Lens culinaris* x *Lens ervoides*) with improved disease resistance have been developed using embryo culture techniques (Ladizinsky et al., 1985). Further, the rearrangement of resistant alleles through chromosome translocation and recombination has developed novel resistance against a fungal disease that originated from *Lens ervoides* (Bhadauria et al., 2017a). Recently, successful gene introgression from wild lentil *L. ervoides* was evident in an advanced backcross population that showed significant variation in anthracnose and Stemphylium blight disease resistance and held a promise to provide valuable disease-resistant genetic stocks in a future breeding program (Gela et al., 2021). Simple crossing involving multiple resistance sources can lead to the study of the complex inheritance of a particular disease. The RIL population among contrasting parents can deliver information regarding the distribution of disease reactions in a defined population. Gene pyramiding can be used to accumulate such multiple disease resistance in a single genotype through marker-assisted selection. Barilli et al. (2020) proposed using moderate to highly resistant germplasm of diverse origin as donors for Aschochyta blight resistance to broaden the genetic diversity of the evolved resistant

genotypes. Interspecific hybridization among newly identified resistant lentil species is very important to develop pre-breeding materials for disease resistance breeding. To maintain the genetic base of the crop and reduce its genetic erosion, the classical breeding approach must be continued to develop the genotypes with new gene combinations. Till now, interspecific and intraspecific hybridization in lentil has eveloved many resistant recombinants. These resistant recombinants/breeding lines will be of great use in transferring resistance into well adapted varieties. Efficient selection in such process requires linked molecular markers that will help in pyramiding diverse resistant alleles in a superior variety.

6 Marker-assisted breeding for disease resistance

Identifying and mapping genes/QTLs controlling the desired phenotype is the basic and important step in marker-assisted breeding for crop improvement. Among various genomic resources, molecular markers have played a significant role in speeding up crop improvement and understanding the genetic basis of economically important traits (Varshney and Tuberosa, 2007). The availability of polymorphic markers and genetic linkage maps makes it easier to identify and map the QTLs for a trait of interest through family-based linkage mapping or germplasm-based association mapping approaches (Mackay and Powell 2007). Linkage-based QTL identification and mapping require a properly developed experimental population with a suitable size, developed from two contrasting parents (Bohra et al., 2014; Mitchell-Olds 2010). However, association mapping or linkage disequilibrium mapping requires a set of genetically diverse genotypes, landraces, or natural populations (Mackay and Powell 2007). Linkage and association-based QTL mapping follows the principles of the forward genetic approach and hence depend on phenotypic expressions or variations available in the experimental population for the trait of interest.

Most of the researchers followed the identification and mapping of QTL through a linkage-based approach for economically important traits. Several QTLs have been identified and mapped for agronomic traits (days to flowering, plant height, seed size, pod dehiscence, winter hardiness, growth habit, seed yield), disease resistance (ascochyta blight, stemphylium blight, rust, anthracnose, fusarium wilt and aphanomyces root rot) and abiotic stress tolerance (boron tolerance) by utilizing both inter- and intraspecific maps (Ford et al., 1999; Rubeena et al., 2006; Tullu et al., 2008; Saha et al., 2010a; Bohra et al., 2014; Dikshit et al., 2016; Sudheesh et al., 2016; Bhadauria et al., 2017a; Polanco et al., 2019). A comprehensive list of identified QTLs for resistance to Ascochyta blight, Stemphylium blight, rust, anthracnose, Fusarium wilt, and Aphanomyces root rot in lentil is presented in (Table 4). Saha et al. (2010a) employed SSR, SRAP, and RAPD markers to identify QTL (*QLG480–81*) for Stemphylium blight resistance. In contrast, Bhadauria et al. (2017a) identified two QTLs (*qSB-2.1 and qSB-2.2)* for resistance to Stemphylium blight using SNP markers. Three QTLs viz., *F7XEM4a*, *GLLC527* and *GLLC106* conferring resistance to lentil rust were identified by Saha et al. (2010b); Dikshit et al. (2016), and Fikru et al. (2016), respectively using SSR markers (Table 4). Substantial research in resistance to anthracnose is also evident in the number of identified QTLs viz., *OPO61250* (Tar'an et al., 2003), *LCt-2* (Tullu et al., 2003), *qANTH1.2-1, ANTH1.2-2* and *qANTH1.3-2* (Bhadauria et al., 2017b) in lentil. Besides anthracnose, Eujayl et al. (1998) identified one QTL (*fw1*) using the RAPD marker, while Hamwieh et al. (2005) identified two QTLs viz., *p17m30710* using AFLP and *SSR59-2B* using SSR markers for resistance to Fusarium wilt.

However, very few reports are available for identifying QTLs for disease resistance through association mapping. Identification of QTLs through linkage-based mapping is not as much robust as association mapping due to some limitations *viz.*, lack of high resolution, inefficiency, and requiring a long time to develop a bi-parental population (Parisseaux and Bernardo, 2004). Moreover, bi-parental mapping approach also had some inherent genetic constraints like, moderate to high segregation distortion and non-universality of linked marker reaction to other inter-specific/intra-specific populations. Alternatively, association mapping can potentially address these limitations of bi-parental linkage mapping. Association analysis may identify QTLs further through high-resolution mapping using historical recombination available in diverse genotypes or natural populations (Mackay and Powell 2007; Ma et al., 2020). A well-designed set of association panels represented by a global mini-core collection of lentil with a high amount of genetic variation may save time and cost while performing marker-assisted breeding in this crop. Genome-wide association study (GWAS) was first demonstrated in lentil to reveal marker-trait association for Aphanomyces root rot resistance (Ma et al., 2020). Later, GWAS was used for identification of marker for improtant agronomic traits (Rajendran et al., 2021), prebiotic carbohydrates (Johnson et al., 2021) and salt tolerance (Dissanayake et al., 2021).

Molecular markers linked to desirable genes/QTL affecting a phenotype are being used now to introgress that QTL in the genetic background of improved genotypes using marker-assisted breeding (Collard et al., 2005). Several tightly linked markers (<5 cM) with high phenotypic effect are now available in lentil that may be used in marker-assisted breeding (MAB), marker-assisted backcrossing (MABC), marker-assisted gene pyramiding, marker-assisted recurrent selection (MARS), and genome-wide selection (GWS) (ana et al., 2019). Pyramiding of multiple QTLs/genes may be conducted through the multiple parent crossing, backcrossing, and recurrent selection. Pyramiding three or four genes can be achieved through three-way, four-way, or double-crossing. Ta'ran et al. (2003) identified

two QTLs *viz.*, *ral1* (UBC2271290) and *AbR1* (RB18680) for Ascochyta blight resistance and one QTL (OPO61250) for resistance to anthracnose using RAPD marker. While integrating these QTLs into a single genotype through marker-assisted breeding, they found 11 RILs with all three genes. These pyramided genes explained about 55% contribution to resistance to Ascochyta blight and anthracnose. With this work's help, they could develop a durable variety of lentils. Availibility of genomic resources in lentil will help breeders to fine map each disease resistance locus and develop candidate gene-based markers in lentil for efficient selection in future.

7 Role of mutation breeding in lentil improvement

Even though genomics-assisted breeding is popular in other legumes, its pace is slow in lentil due to its large genome size, narrow genetic base, low-density genetic linkage map, and difficulty in identifying beneficial alleles (Kumar et al., 2015). However, these limitations or genetic bottlenecks can be overcome by mutation breeding in popular lentil cultivars (Erskine et al., 1998). Molecular tools have infrequently been used to realize the genetic basis of a few traits related to biotic (ascochyta blight, anthracnose, rust, fusarium wilt, Stemphylium blight) and abiotic (drought, frost, cold, boron, salinity) stresses (Kumar et al., 2014). Further use of hybridization for crop improvement in lentils is limited due to its tiny flower, flower drop, low seed set in interspecific hybridization, and unease in tissue culture-based embryo rescue technique. In such inherent constraints, the narrow genetic base could be broadened using induced mutation breeding, a coherent tool for increasing genetic variability (Laskar et al., 2015; Rana and Solanki, 2015; Khursheed et al., 2018; Shahwar et al., 2019; Raina et al., 2022b; Raina et al., 2022c).

7.1 Types of mutants and mutant varieties of lentil for disease resistance

Lentil is responsive to both chemical and physical mutagens indicating the scope of improvement using mutation breeding (Sharma and Sharma, 1979; Gaikwad and Kothekar, 2004; Solanki and Phogat, 2005; Solanki et al., 2007). Mutation breeding was considered in lentils to improve several agronomical traits (Tyagi and Gupta, 1991; Ali and Shaikh, 2007; Ali et al., 2010; Tabti et al., 2018a), herbicide tolerance (Rizwan et al., 2017; McMurray et al., 2019), fascinating fertile mutants (Tyagi and Gupta, 1991), early maturing and dwarf mutants (Sinha, 1988; Sinha, 1989; Solanki, 2005; Solanki and Phogat, 2005), disease resistance (Bravo, 1983; http://mvgs.iaea.org , MVD, 2020) and yield (Ali and Shaikh, 2007; Ali et al., 2010). Laskar et al., 2017 and Laskar et al., 2018a; Laskar et al., 2018b) developed lentil mutant lines with improved yield and nutrient density using gamma rays and hydrazine hydrates. Mutagenic lentil populations developed at ICARDA through the treatment of ethyl methane sulfonate has shown the promise in isolation of pod shattering, herbicide tolerance and *Orobanche* tolerance (Kumar et al., 2015). Chemical mutagens mostly react with nucleotide base and modified it. This modified base impairs in base pairing and thus causes base substitution. Physical mutagen like gamma rays also causes base substitutions due to the base damage by free radicals along with its direct action in single and/or double strand break in DNA that leads to deletions, insertions, inversions, and translocations. Punia et al. (2014) reported hypervariable spontaneous generation of mutation for earliness, seed coat colour and seed size in a commercial population of lentil cultivar DPL-62. However, the frequency of spontaneous mutations is not adequate to meet the needs for genetic improvement and necessitates the use of induced mutations. Occasionally such behaviour is explained due to the activity of transposable elements (Gowda et al., 1996). It is reported that genomic shock/stress (ionizing radiations, base-damaging chemicals) induces the transposition of mobile genetic elements and causes an indirect mutation in plants (Koturbash, 2017). Success story towards induction of disease-resistant lentil variety 'NIAB MASOOR 2002' through gamma rays mutagenesis is well documented in mutant variety database (https://mvd.iaea.org/#!Variety/3379). Another successful example of induction of a high-yielding variety with multiple disease resistance (Ascochyta blight, rust, and Botrytis grey mould) is 'NIAB MASOOR 2006' obtained from 200 Gy gamma rays treatment of ILL 2580 in Pakistan (Sadiq et al., 2008). Mutation breeding in lentils is tilted more towards enhancing tolerance to biotic stress rather than direct yield improvement. Till now, a total of 18 mutant varieties have been developed in lentil crop (http://mvgs.iaea.org) (Table 5). Of which, nine lentil mutants were resistant to various diseases. For instance, mutant lines viz., Binamasur-1, Binamasur-2, Binamasur-3 and NIAB Masoor-2006 are resistant to rust and ascochyta blight. Zomista and Mutant 17 MM are resistant to anthracnose and viral diseases. Another mutant, Djudje, is resistant to Fusarium wilt and Botrytis grey mould diseases (http://mvgs.iaea.org , MVD, 2020). In addition to appropriate plant material and an optimum mutagen dose, a large M_2 population is also important for achieving success in the mutation breeding program. The success of mutation breeding in developing mutant varieties with improved yield, grain quality, and tolerance to biotic and abiotic stress is determined by factors like the genetic background of parents, the dose of mutagen, mutagenized plant population, selection criteria, successive handling of advanced mutant generation.

7.2 Selection of genotypes and dose determination

In mutation breeding programs, selecting appropriate genotypes usually well-adapted farmer's preferred variety, is important for the genetic improvement of existing lentil cultivars (Laskar et al., 2018a). Besides, a traditional landrace suitable for cultivation in a particular agroclimatic condition is also preferred to improve yield and quality traits. Moreover, an interspecific derivative line that may still have linkage drag can also be used as source material for further improvement. The tight linkage between the disease resistance and undesirable traits in interspecific-cross derived lines can easily be broken down using mutagens such as gamma rays, electron beams, charged particles, and fast neutrons (Joshi et al., 2020). After the selection of source material, it is recommended to study the dose-response of the particular genotypes for the evaluation of GR30 and GR50 values following probit analysis. It is always recommended to use an optimum dose that lies between GR30 and GR50 values to achieve the highest frequency of mutation and less biological damage. Combinations of physical and chemical mutagens have also been employed in the genetic improvement of lentil cultivars (Laskar and Khan, 2017). A study revealed that 0.4% of hydrazine hydrates and 400 Gy of gamma rays were maximum non-lethal strength of respective mutagens for mutation induction in lentils (Laskar et al., 2017). In contrast, lower concentrations of ethyl methanesulfonate (0.1 and 0.2%), hydrazine hydrate (0.02 and 0.03%), and sodium azide (0.01 and 0.02%) were used to develop a large mutagenized population of lentil for screening tolerant mutant for herbicide (Rizwan et al., 2017). A lower dose of gamma rays 100 Gy on cv. Idlib-3 (ILL6994) effectively generated significant variability for most lentil quantitative traits (Tabti et al., 2018b). For most of the seed propagating crops, pure seed (nucleus seed) was used as a source material for treatment with mutagens. Various factors are responsible for optimum dose determination of seeds. For gamma rays, initial moisture content and oxygenated environment are very crucial to get optimum DNA damage in seeds. For chemical mutagens, pre-treatment, types of buffer, time of treatment, cell cycle stages, and temperature are the major determinants for determining the concentration of chemicals used for mutation breeding experiments.

7.3 Mutant population development

Mutations are random events induced at a very low frequency and further reduced by plant recovery mechanisms. To effectively screen a desired mutant, a large-sized mutagenized population developed by using an optimum mutagen dose is required (Raina et al., 2020). Moreover, the cytotoxic effect of higher mutagen dose leads to the mortality of M_1 plants and ultimately results in lower M_2 population size (Goyal et al., 2021). On the contrary, a lower mutagen dose is not enough to induce a mutation and results in the progression of wild-type progeny. Thus, prior to the mutation breeding experiment, optimization of mutagen dose must be carried out using above-mentioned methods. Further a large-sized mutagenized population is recommended to screen the desired lentil mutants effectively. In case of rice, a small mutagenized population (with 10000 plants) can saturate the genome with mutations (Viana et al., 2019). Lentil possesses nearly ten times bigger genome size than rice (4063 Mbp); therefore, it requires a large mutagenized population (with at least 50000 plants) to screen desired mutants. Few successful examples demonstrated the advantage of a large size population in literature. A total of 83083 M_2 plants were screened for isolating herbicide-resistant (against sulfonylurea herbicide) mutants in lentils (Rizwan et al., 2017). Recently, McMurray et al. (2019) selected two mutant lines (M043 and M009) from 9,500,000 M_2 population developed from 'PBA Flash' variety through ethyl methyl sulphonate (EMS) based mutation breeding. Interestingly, both the mutant lines were tolerant to metribuzin herbicide (a broad-spectrum herbicide affecting photosystem II). Therefore, it is quite evident that induction and effective screening of desired mutants requires an adequate size of a mutagenized population.

7.4 Screening methodology for identification of mutants

Mutations are recessive in nature and hence are not visible in M_1 generation, therefore the screening for mutants with improved agronomical traits including disease resistance in the M_2 generation is recommended (Mondal et al., 2011; Raina et al., 2017). Single plant harvest of all M_1 plants may be grown in single row by following the plant to row method with standard spacing. Based on availability of facilities, the M_2 population may be artificially infested by the pathogens of the targeted disease (Ali and Shaikh, 2007; Ali et al., 2010; Rizwan et al., 2017; Ayala-Doñas et al., 2022). Thereafter, the disease resistant plants may be selected based on the visual performance of the plants in the field. In the earlier generations, breeders often select only high yielding plants with good agronomic features and mutants are artificially screened for targeted disease in advanced generations. Plants selected in M_2 generation may be grown to raise M_3 generation followed by screening for a targeted disease to evaluate their true to type behaviour and resistance to disease or targeted traits (Punia et al., 2014; McMurray et al., 2019). While growing the M_3 population, best susceptible check variety must be grown after every 10^{th} row to create a natural epiphytotic environment (Nene et al., 1981). Based on the availability of pathogens, individual plants may be artificially

TABLE 5 List of disease resistant lentil mutants developed and registered under the Joint FAO/IAEA Database of Mutant Variety and Genetic Stock (http://mvgs.iaea.org).

	Variety Name	Parent name	Mutagen	Dose	Local/ National Registration Year	Character Improvement Details	Institute
1	Binamasur-1	L-5 (local genotype)	Extract of Dhatura seeds	NA	2001	High yield, tolerant to rust and blight, black seed coat	Bangladesh Institute of Nuclear Agriculture (BINA) & Bangladesh Agriculture University (BAU), Bangladesh
2	Binamasur-2	Utfala (local genotype)	Gamma rays	200 Gy	2005	High yield, early maturity, tolerant to rust and blight	BINA, Bangladesh
3	Binamasur-3	L-5 (local genotype)	EMS	0.50%	2005	High yield, early maturity, rust and blight tolerance	BINA, Bangladesh
4	Djudje	Tadjikskaya 95	Gamma rays	30 Gy	2000	High yield, dwarf bushy habit, suitable for mechanized harvesting, non-shattering, resistance to fusarium and botrytis, high protein content (27.9%), good culinary and organoleptic quality	Dobrudzha Agricultural Institute (DAI), General Toshevo, Bulgaria
5	Elitsa	Tadjikskaya 95	Gamma rays	40 Gy	2001	High yield (34.4%) and resistance to the major disease	DAI, General Toshevo, Bulgaria
6	Mutant 17 MM	NA	NA	NA	1999	Vigorous growth habit, large leaflet, pods and seeds, resistance to anthracnose, stemohylium and viruses, high yield, drought tolerance and improved cooking quality	DAI, General Toshevo, Bulgaria
7	NIAB MASOOR 2002	NA	Gamma rays	NA	2002	Erect growth habit, early maturity (120 days), black seed coat color, high grain yield, diseases recsistance and synchronous pod maturity	Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan
8	NIAB MASOOR-2006	ILL-2580	Gamma rays	200 Gy		Higher number of pods, resistance to lodging and resistance to blight and rust	NIAB, Faisalabad, Pakistan
9	Zornitsa	Tadjikskaya 95	EMS	0.10%	2000	High yield, high protein content (28.7%), good culinary and organoleptic quality, resistance to anthracnose, viruses and ascochyta blight	DAI, General Toshevo, Bulgaria

treated with a critical load of inoculums of the targeted disease (Bravo, 1983; Solanki and Phogat, 2005). Mutants showing resistance to the disease with good agronomic features may be selected for further advancement. If the isolated mutants showed consistent and stable performance in the M_4 generation, the seeds may be bulked and stored to raise the M_5 generation and evaluated in replicated yield trials (Laskar and Khan, 2017; Rizwan et al., 2017). Based on their performance, they may be evaluated in multi-location and national trials in M_7 and M_8 generations by following appropriate experimental design along with recommended agronomic practices. Multi-location testing may be repeated for 2-3 years to confirm the adaptability and stability of the mutant lines. Based on the performance of mutants in multi-location trials and national trials, the mutant genotype may be recommended for release to a particular location by the state variety release committee or for the whole country by the central variety release committee (Toker et al., 2007). Upon release of the mutant by the technical committee, they may be submitted for notification from the government authority for entering into a quality seed production channel.

7.5 Role of mutation breeding for induction of disease resistance in lentil

7.5.1 Possibility for loss of function mutation to behave as disease resistant/tolerant

Pathogen exploit disease susceptibility gene products to gain access into the plant cell and take over replication machinery (Eckardt, 2002). Mutations in these susceptibility genes may disrupt their functions and thus impede the pathogen entry and multiplication inside plant cells and eventually affects pathogenesis. Such types of resistance behave as recessive genes and impart durable broad-spectrum resistance to crop plants (Bravo, 1983; Solanki and Sharma, 2001; Liu et al., 2021; Koseoglou et al., 2022). These resistances are well documented

against the virus (mutant *eIF4E* gene in pepper against potato virus Y; Ruffel et al., 2002), fungus (*mlo* in barley for resistance against *Blumeria graminis* f. sp. *hordei*; Brown, 2015), bacteria (*xa13/OsSWEET11* in case of rice for *Xanthomonas oryae* pv. *oryzae* race 6; Yang et al., 2006).

7.5.2 Possibility of gain of function mutations to behave as disease resistant/tolerant

Most of the resistance (R) genes are either non-functional or may play a role in association with other R genes in providing disease resistance. The binding of the pathogen's avirulence (AVR) gene product on the leucine-rich repeat (LRR) domain of R-protein induces a conformational change that helps binding of ATP in nucleotide-binding site (NBS) domain. Hydrolysis of ATP induces another conformational change in the protein that led to aggregation of R-protein to form either resistosome complex (Wang et al., 2019) or three-dimensional conformation changes in Toll Interleukin-1 Receptor (TIR) domain which hydrolyze NAD+/NADP+ (Horsefield et al., 2019). All these above protein-protein interactions mediate through domain-specific non-covalent interactions between specific amino acids. Thus, changes in any amino acids through point mutations involving non-synonymous mutations in the interacting helix/loops may lead to gain of function.

7.6 Present thrust and requirement in mutation breeding for disease resistance

7.6.1 Strategy against stemphyllium blight disease

Stemphyllium blight, caused by *Stemphyllium botryosum* is an important fungal disease that is predominant in all major lentil growing regions. In a recent coordinated effort, FAO-IAEA joint division has formulated a project to induce resistance against Stemphyllium blight in lentil through induced mutagenesis. Cao et al., 2019 recently undertook a leaf transcriptome analysis to detect the differentially expressed genes (DEGs) in resistant and susceptible bulk of a recombinant inbred line population derived from wild lentil species, *Lens ervoides*. This analysis reported several DEGs in resistant plants and an upregulated transcript in susceptible plant/bulk. It was hypothesized that this upregulated gene (codes for uncharacterized protein Lc07593) in susceptible genotypes is a candidate for 'genes for susceptibility' in lentil. Mutations can be created in this gene through random mutagenesis/Targeting Induced Local Lesions IN Genomes (TILLING) or targeted mutagenesis approach like clustered regularly interspaced short palindromic repeats and CRISPR associated protein 9 (CRISPR-Cas9) in these genes. These mutants can be bio-assayed in the field or controlled laboratory conditions to detect resistance against *Stemphyllium botrysum*. The same approach could also be followed to induce resistance against rust and Ascochyta blight and anthracnose disease in lentil.

7.6.2 Strategy against pea seed-borne mosaic virus

PSbMV is more common viral diseases and infestation at earlier stage causes a substantial reduction in the seed yield (up to 72%) (Aftab et al., 1992). Viral disease including PSbMV are often transmitted in the field by means of aphids. Eukaryotic translation initiation factor 4E (eIF4E) is exploited by the PSbMV virus to translate its RNA into other viral proteins for multiplication and cell to cell movement. Gao et al. (2004) while working in pea reported that the *sbm1* mediated resistance to two pathotypes P1 and P4 of PSbMV is a consequence of mutations in an *eIF4E* homolog. In contrast, Kang et al. (2005) showed that transient expression of susceptible-*eIF4E* in a resistant background complemented PSbMV infection. The above genetic basis for resistance against PsbMV will pave the way to find mutations or allelic variation in a homologue of *eIF4E* of lentil toward a generation of field resistance through conventional mutation breeding and TILLING approach.

7.6.3. Strategy against fusarium wilt disease (is there any genome editing target)?

Host oxylipin pathways are important for pathogenesis, successful colonization, reproductive development, and biosynthesis of mycotoxins by certain fungal pathogens including *Fusarium* sp. *Fusarium* exploits the jasmonate pathway in plants to create an initial infection. The enzyme lipoxygenase (lox) catalyzes the conversion of α-linolenic acid to its 13-hydroperoxide derivative leading to jasmonate production (Wasternack and Strnad, 2018). Gao et al. (2007) showed that disruption of maize 9-lipoxygenase (*lox 9*) resulted in increased resistance to *Fusarium verticillioides* and reduced levels of fumonisin (a mycotoxin) production. Direct evidence of *in vitro* mutagenesis using ethyl methane sulphonate for wilt resistance also exists in the development of five Fusarium wilt-resistant lines of banana (*Musa* spp., AAA) (Chen et al., 2013). Subsequently, Ghag et al. (2014) identified a down-regulated lipooxygenase (LOX) gene responsible for providing resistance against *Fusarium oxysporum* f. sp. *cubense* in a somaclonal mutant of banana. Lanubile et al. (2021) confirmed the strategic role of *ZmLOX4* in controlling defense against *F. verticillioide* through induction of Mutator-insertion mutagenesis. The above example in the disruption of an isoform of *lox* genes reiterates the practice of mutation breeding for induction of mutations in such equivalent genes in lentil to enhance the resistance against Fusarium wilt without compromising plant vigour and seed yield.

8 Role of new breeding technologies in disease resistance breeding

New breeding technologies including genomics assisted breeding (GAB), speed breeding and gene editing, and next-generation breeding targets developing climate-resilient varieties using all sorts of strongly associated marker identification, phenotyping based on machine learning and artificial intelligence (Razzaq et al., 2021). Genomic data along with added information from pan genomes, modification in CRISPR technology, innovation in genome editing and advanced form of base editing were considered for food security in this era of new breeding technologies (Fasoula et al., 2020).

8.1 Genomics assisted breeding in lentil

Significant progress in gene-based SSR and SNP markers, availability of draft genome sequence of lentil and cost-effective sequencing of functional regions of lentil genome has made the journey smooth for efficient MAB by virtue of the development of tightly linked markers for disease resistance (Sari, 2014; Sudheesh et al., 2016; Bhadauria et al., 2017a; Bhadauria et al., 2017b; Polanco et al., 2019; Bawa, 2020; Ma et al., 2020). A breeder-friendly marker should have tight linkage having a distance of <1.0 cM from the genes/QTL controlling a trait of interest and explain high phenotypic variation (Collard et al., 2005). Later employment of next-generation sequencing techniques like 'genotyping by sequencing (GBS)' has helped in the identification of three nested QTLs on linkage group 5 (9.5-11.5% PVE) and a QTL on linakge group 2 (9.6% PVE) for Ascochyta blight resistance and identification of putative causal genes (Dadu et al., 2021). Ma et al. (2020) used GBS strategy to genotype a RIL population and identified 19 QTLs for Aphanomyces root rot resistance in lentil. In parallel, genome wide association studies (GWAS) were also practiced in lentil for identification of marker-trait association for Aphanomyces root rot resistance (Ma et al., 2020), agronomic traits (Rajendran et al., 2021), prebiotic carbohydrates (Johnson et al., 2021) and salt tolerance (Dissanayake et al., 2021). All these above examples of detecting QTLs/associated SNPs for a targeted trait in lentil have shown promise to apply genomic selection to select genotypes with multiple disease resistance. The concept of GAB evolved to deal with complex traits like yield through involvement of genome-wide markers for selection. The well-characterized training population help to identify such markers for GAB and then applied in a test population after validating them in a subset of training population. In context to disease resistance breeding, GAB will be more helpful to pyramid all the resistance genes in selected plants. Towards this, the Multiparental Advanced Generation Intercross (MAGIC) population involving hybridization among different sources of resistance and elite lines will be helpful to get a genotype with multiple disease resistance through GAB. Whatever products will generate through these above new breeding teachniques (GAB, MAB and GWAS) should be stabilized before testing in a multilocation yield trial. Speed breeding can help to stabilize the selected plants rapidly in a breeding scheme. Normal greenhouse can produce 2-3 generations, whereas rapid generation cycle in speed breeding facilities 4-6 generations in several crops such as wheat, barley, durum wheat, pea and canola (Watson et al., 2018). Manipulating light sources with a very low red:far red ratio was standardized to cause the earliest flowering in lentils (Mobini et al., 2014; 2016). The materials developed in such a speed breeding facility can also be screened for multiple disease resistance and shared with partners for varietal evaluation.

8.2 Possible application of gene editing technology for disease resistance breeding

The era of gene/genome editing offers targeted alternations of a particular gene or portion of genome without no alternations in other parts of the genome. Thus the derived product will have same agronomic potential except the targeted change. It offers to rectify some drawbacks of a megavariety within a short span of time. Targeted knockout of negative regulators of disease resistance gene and/or susceptibility genes *via* genome editing tools is a rapid and powerful approach for disease resistance plant breeding (Ahmad et al., 2020). But, before implementing such new techniques in genotype improvement, scientists must take care about possible off-targets through the careful design of guide-RNA. Xu et al. (2019) had demonstrated the successful induction of broad-spectrum bacterial blight resistance by using CRISPR/Cas9 mediated gene editing of two *OsSWEET* genes (S genes) in rice. Further, targeted mutation of *Oryza sativa* ethylene responsive factor 922 (a negative regulator of disease resistance gene) yielded enhanced disease resistance against rice blast (Wang et al., 2016). Such an example in model crop plants shows promise of using gene editing technologies to induce disease resistance in lentils. A working gegetic transformation protocol is a prerequisite to demonstrating these gene editing tools in lentil. Several genetic transformation methods including *Agrobacterium*-mediated genetic transformation have been attempted in lentil (Gulati et al., 2002; Sarker et al., 2012). There are few reports on successful lentil transformation, but transformation efficiency is less than 1.0% (Atkins and Smith, 1997; Sarker et al., 2019). *In vitro* plant regeneration of explants from different lentil tissues, including shoot apices, epicotyls, nodal segments, embryo axes, cotyledonary nodes, and roots, has been attempted for genetic transformation (Mahmoudian et al., 2002; Sarker et al., 2003; Akcay et al., 2009). Cotyledon-attached decapitated embryos appeared to provide the best response toward *in vitro* regeneration following genetic transformation.

8.3 Putative candidate disease resistance genes in lentil

WRKY genes are important in disease resistance due to their involvement in several secondary metabolite production and senescence pathways (Yoda et al., 2002). Among several putative candidate genes for disease resistance β-1,3-glucanase, a Bet v I (a pathogenesis-related protein 10), disease resistance response protein homologue of pea, disease resistance response protein G49-C, pathogenesis related protein-4 and antimicrobial protein SNAKIN-2 are fully sequenced lentil disease resistance genes (Kumar et al., 2015). NBS family resistance gene analogue have also been identified in *Lens* species (Yaish et al., 2004). Expression study of defense responsive genes, including pathogenesis-related protein, chitinase etc., have explained their role in plant immunity and can be utilized in genomics lead breeding (Tarafdar et al., 2018). Genomics breeding in lentil was started using orthologous gene information and taking help from a synteny crop like *Medicago tranculata* and *Lotus japonicas* (Weller et al., 2012). EST search-based effector identification revealed CtNUDIX and CtToxB effector are involved in *Collectotrichum lentis* infection (Bhadauria et al., 2013a). In a transcriptome study using wild *Lens ervoides* for Stemphylium blight resistance, various genes of oxidation-reduction process, asparagine metabolism were differentially expressed. Of which, a specifically calcium transporting ATPase and glutamate receptor 3.2 showed differential expression between resistant and susceptible bulk (Cao et al., 2019). CC-NBS-LRR R gene has been identified in the lentil, showing differential expression upon *Colletotrichum lentis* infection (Bhadauria et al., 2013b). Transcriptomic analysis of host-pathogen interaction revealed complex molecular interplay between 26 resistance genes in lentil and 22 effector genes in *Colletotrichum lentis*. Both positive and negative regulators of plant immunity such as suppressor of npr1-1 constitutive 1 (SNC 1) and dirigent as well as markers of antagonistic defense signaling pathways such as PR 1, PR 5 (for salisylic acid mediated pathway) and PR 4 (for jasmonic acid mediated pathway) were found upregulated during the compatible lentil - *Colletotrichum lentis* interaction (Bhadauria et al., 2017b). The challenge remains in identifying the susceptibility genes from these above disease resistance genes in lentil. The future breeding strategy will involve exploiting such S genes in site-directed mutagenesis through gene editing technology.

9 Conclusion and future perspectives

Intensive selection pressure for certain agronomic traits on segregating populations derived from hybridization between closely related and common breeding lines has narrowed down the genetic variability of lentil. Crop vulnerability due to the limited genetic variability heightened the risk for biotic and abiotic stresses. Such infestations are turning into disastrous looks due to climatic changes in some pockets of the World. Genetic diversity plays a decisive role in the development of novel plant varieties. Genetic improvement of lentil requires introducing new alleles that extend beyond the existing adapted germplasm pool. New genes and alleles must be identified or generated either through introgression from wild relatives or through induced mutagenesis in lentil genetic resources to attain further breakthroughs in biotic stress resistance with high stability. Induced mutagenesis and site-directed mutagenesis offers a solution for creating new variations and genes. Deployment of CRISPR-Cas9 technology will hasten the process of creating new alleles. Such new breeding technology demands the design of sequence-specific sgRNA cassettes. Availability of reference genome of lentil (Redberry) (https://knowpulse.usask.ca/lentilgenome) will offer a strong foundation for designing such specific sgRNAs towards trait improvement. Gene editing can provide an easier, cheaper, and more precise way of disrupting genes for lentil improvement. Before implementing the new breeding technology for lentil improvement, generating trait variation through induced mutagenesis is essential. Induced mutagenesis offers to understand the nature of mutations and apply the knowledge to rapidly improve the trait through targeted genome editing using CRISPR/Cas9 technology in lentil.

Further, deploying precise gene-editing technology in lentil requires good regeneration and efficient transformation protocols. Optimization of the protocol with an appropriate combination of mineral media and hormones is required in near future. Whatever means are there to improve the plant traits, the selections must be stabilized from early generation to near cent percent homozygosity. Rapid generation advancement through speed breeding technique offers a solution to stabilize the generated mutants in lentil in a short span of time. It is possible to stabilize the lentil plant to complete homozygosity within two years through the use of speed breeding technique. Still, there is a scope to improvise this speed breeding protocol in terms of various optimized parameters like type of light-emitting diodes, quality of light, spectral composition and red/far-red light ratio. Resistance breeding in lentil has sufficiently shown a path to exploit the crop wild relatives (CWRs) to better this crop. A schematic depicting the future road map for disease resistance breeding in lentil is presented here (Figure 4). Future works must continue in this direction to untap available genetic resources along with CWRs. Such usage can be accelerated by deploying a high throughput phenotyping facility for disease screening in pre-breeding materials. Utilization of elite lines in recombination breeding with pre-breeding materials can be made to generate mapping populations, including MAGIC and Nested Association Mapping (NAM). This will offer to tag the resistance genes and develop more dense flanking markers for disease resistance QTL. Rapid advancement in high-density and low-cost genotyping assay will further help to accelerate the process of marker development and offers great promise in precise genomic selection and/or marker-assisted selection. Integrating advanced mutagenesis tools and speed breeding techniques will further identify new genes/alleles for disease resistance and rapidly develop the varieties. All these new research

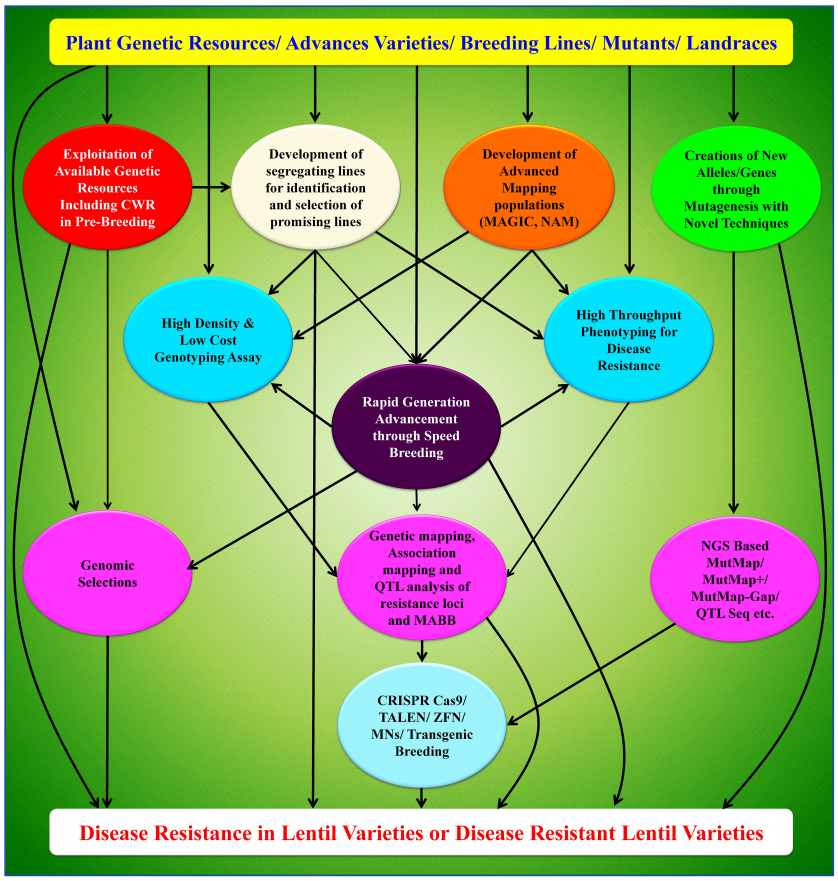

FIGURE 4
A schematic depicting the future road map for disease resistance breeding in lentil.

initiatives lead to developing disease resistance genotypes/varieties that can be deployed to the farmers' field through productive linkages between research institutes and private institutes/ enterprises towards quality seed production.

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

Abstract: SM. Background/Introduction (Importance of lentil, Recent trends in production, Concepts of disease resistance in general): AnR, and AaR. Biotic production constraints of lentil: AnR, AaR & SM. Available genetic resources to tackle these biotic constraints: AnR, CD. Screening methodologies available for disease resistance breeding: PS. Resistance breeding of lentil (Different breeding approaches, Examples): AnR & PS. Role of mutation breeding in lentil improvement: SM and PS. Role of new breeding technologies in disease resistance breeding: AnR & SM. Conclusion and future perspectives: SM. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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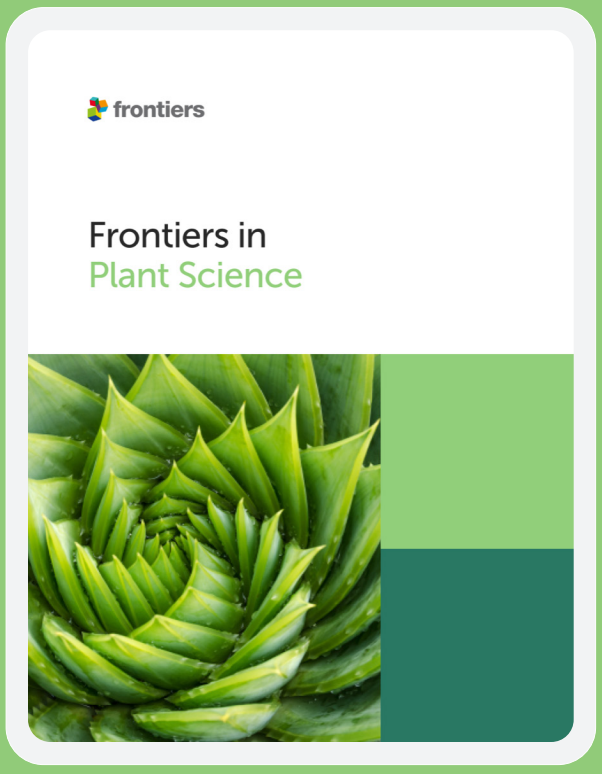

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