# ADVANCES IN ASCOCHYTA RESEARCH

EDITED BY: Diego Rubiales, Sara Fondevilla, Weidong Chen and Jennifer Davidson

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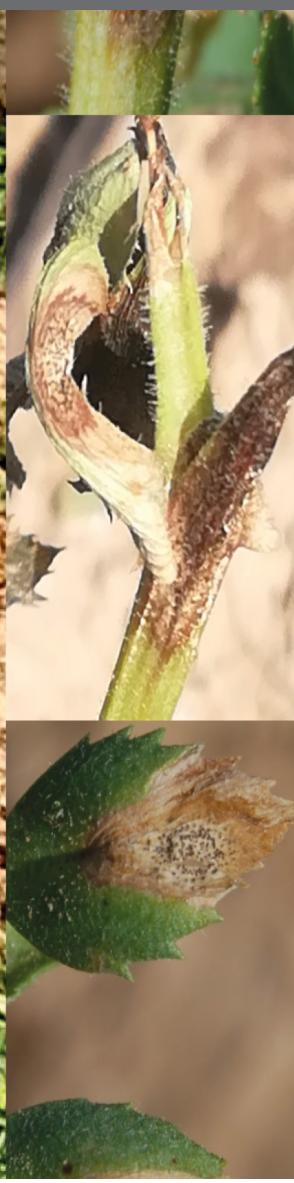

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# ADVANCES IN ASCOCHYTA RESEARCH

Topic Editors:
**Diego Rubiales,** Institute for Sustainable Agriculture, CSIC, Spain
**Sara Fondevilla,** Institute for Sustainable Agriculture, CSIC, Spain
**Weidong Chen,** USDA-ARS, Washington State University, United States
**Jennifer Davidson,** SARDI, Australia

Chickpea cvs. resistant (back) vs susceptible (front) to ascochyta blight, with details of symptoms.
Image: Diego Rubiales and R. Kimber

Legume crops provide an excellent source of high quality plant protein and have a key role in arable crop rotations reducing the need for fertilizer application and acting as break-crops. However, these crops are affected by a number of foliar and root diseases, being ascochyta blights the most important group of diseases worldwide. Ascochyta blights are incited by different pathogens in the various legumes. A number of control strategies have been developed including resistance breeding, cultural practices and chemical control. However, only marginal successes have been achieved in most instances, most control methods being uneconomical, hard to achieve or resulting in incomplete protection. This eBook covers recent advances in co-operative research on these diseases, from agronomy to breeding, covering traditional and modern genomic methodologies.

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EDITORIAL
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# Editorial: Advances in Ascochyta Research

*Diego Rubiales*[1]*, *Sara Fondevilla*[1], *Weidong Chen*[2] and *Jennifer Davidson*[3]

[1] Institute for Sustainable Agriculture, CSIC, Córdoba, Spain, [2] USDA-ARS, Washington State University, Pullman, WA, United States, [3] SARDI, Adelaide, SA, Australia

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## Editorial on the Research Topic

## Advances in Ascochyta Research

Legume crops provide an excellent source of high quality plant protein and have a key role in arable crop rotations reducing the need for fertilizer application and acting as break-crops facilitating management of pests, diseases and weeds. However, these crops are themselves affected by a number of foliar and root diseases, with ascochyta blights being one of the most important groups of diseases worldwide. Ascochyta blights are incited by different pathogens in the various legume crops.

A number of control strategies have been developed including cultural practices and chemical control. However, only partial successes have been achieved since control methods can be uneconomical, hard to implement or result in incomplete protection. Nevertheless, the control methods available today represent major progress when compared to what was available one to two decades ago. Crops can be protected by cultural methods or by resistance, by selective fungicides, and by biocontrol agents, that did not exist before.

Infection of seed is one of the major survival mechanisms of *Ascochyta* spp. and an important means of transmission into previously uninfected areas. For some species this can also represent a major source of inoculum for the developing crop. Kumar and Banniza assessed the effect of seed infection with *A. pisi* on field pea in Canada. Although infected seeds may be an important way for the pathogen to survive in nature, their study concluded it cannot be regarded as a source of inoculum in the epidemiology of *A. pisi* under western Canadian growing conditions.

The use of resistant cultivars is widely acknowledged as the most economic and environmentally friendly control method. Breeding for ascochyta blight resistance has been a priority for breeding programs across the globe and consequently, a number of resistance sources have been identified and extensively exploited. However, ascochyta resistance breeding is not an easy task. The combination of genomic resources, effective molecular genetic tools and high resolution phenotyping tools will improve the efficiency of selection for ascochyta blight resistance and accelerate varietal development. Rodda et al. reviews current progress and future directions of molecular breeding for ascochyta blight resistance in lentil.

A detailed understanding of the genetic basis of ascochyta blight resistance is hence highly desirable, in order to obtain insight into the number and influence of resistance genes. Sudheesh et al. developed single nucleotide polymorphism (SNP)-based linkage maps from three recombinant inbred line (RIL) populations identifying totals of two and three quantitative trait loci (QTLs) explaining 52 and 69% of phenotypic variation for resistance in lentil. Evaluation of markers associated with ascochyta blight resistance across a diverse lentil germplasm panel revealed that the

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***Edited by:***
*Juan Moral,*
*University of California, Davis, United States*

***Reviewed by:***
*Kevin E. McPhee,*
*Montana State University, United States*
*Omer Frenkel,*
*Agricultural Research Organization (Israel), Israel*

***Correspondence:**
*Diego Rubiales*
*diego.rubiales@ias.csic.es*

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identity of alleles associated with one of the QTLs predicted the phenotypic responses with high levels of accuracy (∼86%), and therefore have the potential to be widely adopted in lentil breeding programs.

Resistance breeding in legume crops has been slow due to the complex nature of resistance and the relatively low investment in genetics, genomics and biotechnology of legume crops, but also, mainly because of limited knowledge of the biology of the causal agents and pathogen variation. Davidson et al. investigated field reactions of lentil cultivars against *A. lentis* and the pathogenic variability of the *A. lentis* population in southern Australia on commonly grown cultivars, confirming the change in reaction on the foliage of the previously resistant cultivars. The impact of dominant cultivars in cropping systems and loss of effective disease resistance is discussed. Future studies are needed to determine if levels of aggressiveness among *A. lentis* isolates are increasing against a range of elite cultivars.

The recently reported changes in aggressiveness of *A. lentis* have led to decreased resistance within cultivars, reinforcing the utility of wild relatives as new sources of resistances. Dadu et al. reported novel resistance in wild lentil species *Lens orientalis*. This was consistently resistant against highly aggressive isolates recovered from diverse geographical lentil growing regions and host genotypes, suggesting stability and potential for future use of this resistance in lentil breeding.

A few major ascochyta blight R-genes have been characterized in different lentil genotypes. Sari et al. compared cellular and molecular defense responses to *A. lentis*. Histological examinations indicated that cell death triggered by the pathogen might be operative in some accessions, whereas limited colonization of epidermal cells might operate in others. Resistant accession differed also in timing and magnitude of SA and JA signaling pathway activation, corroborating the existence of diverse resistance mechanisms in lentil.

Large temporal and spatial variations have been detected within *Ascochyta* populations, and this can vary with the species and the region. Mehmood et al. showed that the Australian *A. rabiei* population has low genotypic diversity with only one mating type detected to date, potentially precluding substantial evolution through recombination. However, a large diversity in aggressiveness exists. In an effort to better understand the risk from selective adaptation to currently used resistance sources and chemical control strategies, the population was examined in detail concluding that the most common haplotype, ARH01, represents a significant risk to the Australian chickpea industry, being not only widely adapted to the diverse agro-geographical environments of the Australian chickpea growing regions, but also containing a disproportionately large number of aggressive isolates, indicating fitness to survive and ability to replicate on the best resistance sources in the Australian germplasm.

Temperature stresses might affect the resistance as well as pathogen aggressiveness. Kemal et al. showed that chilling temperature predisposed chickpea to *D. rabiei* infection. There were significant interactions of genotypes and isolates with temperature but this did not cause changes in the rank orders of the resistance of chickpea genotypes and aggressiveness of pathogen isolates.

Quinone outside inhibitor (QoI) fungicides (pyraclostrobin and azoxystrobin) have been the choice of farmers for managing ascochyta blight in pulses. However, Owati et al. detected and characterized resistance to these fungicides in *D. rabiei*. This indicates that where resistant isolates are located, fungicide failures may be observed in the field. *D. rabiei*-specific polymerase chain reaction primer sets and hydrolysis probes were developed to efficiently discriminate QoI-resistant from QoI-sensitive isolates.

The genetic resistance to ascochyta blight in chickpea is complex and governed by multiple QTLs. The molecular mechanism of quantitative disease resistance to ascochyta blight and the genes underlying these QTLs are still unknown. Most often disease resistance is determined by resistance R-genes, the most predominant of which contain nucleotide binding site and leucine rich repeat (NBS-LRR) domains. Sagi et al. performed a genetic analysis of NBS-LRR gene family in chickpea and their expression profiles in response to ascochyta blight infection. Thirty of the NBS-LRR genes co-localized with nine of the previously reported ascochyta blight QTLs. Of these, 27 showed differential expression in response to ascochyta blight infection.

Li et al. sequenced a collection of resistant chickpea genotypes, and identified more than 800,000 SNPs. Population structure analysis revealed relatively narrow genetic diversity amongst recently released Australian varieties and two groups of varieties separated by the level of ascochyta blight resistance. A 100 kb region (AB4.1) on chromosome 4 was significantly associated with ascochyta blight resistance collocating to a large QTL. This region was validated by GWAS in an additional collection of 132 advanced breeding lines. This study demonstrates the power of combining whole genome re-sequencing data with relatively simple traits to rapidly develop "functional makers" for marker-assisted selection and genomic selection.

Verma et al. performed a genome-wide identification and analysis of transcription factors (TFs) in *A. rabiei*, taking advantage of *A. rabiei* genome sequence. The *A. rabiei* secretome was predicted to be mainly regulated by Myb TFs. Expression profile of TFs varied with pathotype of *A. rabiei* and the cultivar of chickpea. The analyses would provide the basis for further studies to dissect the molecular mechanisms of *A. rabiei* pathogenesis.

The species *Didymella pinodes* is the principal causal agent of ascochyta blight, one of the most important fungal diseases of field pea worldwide. Understanding its host specificity has crucial implications in epidemiology and management. Barilli et al. delineated the host range of *D. pinodes* among legume crops and wild relatives, and compared it with that of other close species. *D. pinodes* was highly virulent on field pea accessions, although differences in virulence were observed among isolates. *D. pinodes* host range is larger than that of *D. fabae*, *D. lentil*, and *D. rabiei*. This has relevant implications in epidemiology and control as these species might act as alternative hosts for *D. pinodes*.

Suzuki et al. examined the histology and ultrastructure of early infection events and fungal development in the pathosystem *Medicago truncatula*/*D. pinodes*. Successful penetration and subsequent growth of infection hyphae were considerably restricted in the resistant ecotype. The oxidative burst reaction

leading to the generation of reactive oxygen species is associated with a local host defense response in the resistant ecotype, since no clear $H_2O_2$ accumulation was detectable in the susceptible ecotype.

QTL mapping studies in several field pea crosses have resulted in identification of genomic regions associated with ascochyta blight resistance. However, these QTLs cover large regions which may not be effective for marker-assisted selection. Jha et al. fine mapped two of these QTLs using a high density SNP-based genetic linkage map and identified markers in heterogeneous inbred family populations. Resistance to lodging was also associated with these two QTLs. The identified SNP markers will be useful in marker assisted selection for development of field pea cultivars with improved ascochyta blight resistance.

## AUTHOR CONTRIBUTIONS

DR, SF, WC, and JD were guest editors of the RT. The four of them contributed to this editorial.

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**Conflict of Interest Statement:** 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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# Assessment of the Effect of Seed Infection with *Ascochyta pisi* on Pea in Western Canada

*Nimllash T. Sivachandra Kumar and Sabine Banniza**

*Crop Development Centre, Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK, Canada*

The role of seed infection with *Ascochyta pisi* using naturally infected seeds with an incidence from 0.5 to 14.5% was studied in field pea experiments in western Canada at locations with historically low inoculum pressure. A significant effect of *A. pisi* seed infection on the emergence of seedlings was observed in one experiment and when all data were pooled, but emergence was only reduced minimally, and symptoms of *A. pisi* on the aerial parts of the seedlings were rarely observed. The level of seed infection at planting had no impact on *A. pisi* disease severity on mature plants, on seed yield and size, or on the incidence of *A. pisi* infection of harvested seeds although *A. pisi* was the dominant species recovered from seeds. Results suggest that the disease did not progress significantly from seeds to seedlings, hence did not contribute to infection of aerial parts of the plants, and therefore infected seeds cannot be regarded as a source of inoculum in the epidemiology of this pathogen under western Canadian growing conditions. Assessing seed components of seeds with varying levels of *A. pisi* infection and seed staining revealed that the pathogen was present in all components of the seed, regardless of the severity of seed staining. This indicates that infected seeds may be an important way for the pathogen to survive in nature.

**Keywords:** ***Peyronellaea pinodes*, *Mycosphaerella pinodes*, ascochyta blight, seed components, seed-to-seedling transmission**

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***Edited by:***
*Jennifer Davidson,*
*South Australian Research and Development Institute, Australia*

***Reviewed by:***
*Omer Frenkel,*
*Agricultural Research Organization, Israel*
*Christophe Le May,*
*Agrocampus Ouest, France*

****Correspondence:***
*Sabine Banniza*
*sabine.banniza@usask.ca*

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## INTRODUCTION

Ascochyta blight, also referred to as the ascochyta blight complex, is one of the major diseases affecting field pea production and can be caused by several pathogens with anamorphs in the genus *Ascochyta* (Tivoli and Banniza, 2007). Worldwide, *Peyronellaea pinodes* (syn. *Mycosphaerella pinodes*), *Ascochyta pisi*, and *Phoma pinodella* have been associated with this disease. In Australia other species of *Phoma* including *Phoma koolunga* (Davidson et al., 2009), *Phoma herbarum* (Li et al., 2011), and *Phoma glomerata* (Tran et al., 2014) were also shown to be pathogenic on pea and have been associated with ascochyta blight. Among the causal agents of ascochyta blight, *P. pinodes* is considered most damaging with yield losses of 28–88% depending on environmental conditions (Bretag et al., 1995a; Tivoli et al., 1996; Xue et al., 1997; Garry et al., 1998). Symptoms of *P. pinodes* and *Phoma pinodella* are very similar with brown to purplish lesions of irregular shape and without a distinct margin (Jones, 1927). *A. pisi*, in contrast has light brown lesions with a distinct darker

brown margin. Pycnidia are easily visible in mature lesions of *A. pisi*, but not in those of the other two species.

Infection of pea seed is one of the major survival mechanisms of *Ascochyta* spp. and an important way of transmission into previously uninfected areas, but for some species can also represent a major source of inoculum for the developing crop (Tivoli and Banniza, 2007). Infection reduces seed germination, and seedlings that do develop from infected seeds may be diseased resulting in poor plant development and stands (Jones, 1927; Maude, 1966; Moussart et al., 1998). Higher severity of seed staining could be correlated with deeper penetration of *P. pinodes* into the seed, which in turn reduced emergence rates (Moussart et al., 1998). Under controlled conditions, seed-to-seedling transmission was up to 100% for *P. pinodes* (Xue, 2000) and 40% for *A. pisi* (Maude, 1966).

The impact of seed-borne inoculum is influenced by factors including rainfall and temperature, and areas with low rainfall often produce disease-free seeds in the field (Bathgate et al., 1989; Bretag et al., 1995b). Surface sterilization of pea seeds results in a reduction of seed infection with *P. pinodes* by 60%, indicating that the pathogen may be mostly carried on the seed coat (Bathgate et al., 1989). Seed infection levels with *P. pinodes* higher than 10% can cause severe economic damage to the crop under conducive environmental conditions (Xue, 2000). Seed-borne infection of other species of the ascochyta blight complex such as *Phoma* spp. has not been identified as very important in initiating epidemics of ascochyta blight in the field. *Ascochyta* spp. can survive on pea seed coats for several years (Bretag et al., 1995b), and for *A. pisi* specifically, it was estimated that the fungus will be eliminated from seed after 5 to 7 years of seed storage in cool and dry conditions (Wallen, 1955).

Until 1961, *A. pisi* was the dominant pathogen recovered from pea seeds in Canada (Wallen et al., 1967a). Incidences of 85% seed infection with *A. pisi*, 27.5% with *P. pinodes* and 10% with *Phoma pinodella* were reported from Canada in the mid-1950s (Skolko et al., 1954). In 1961, the pea variety Century (originally released as Creamette [Gfeller and Wallen, 1961]) was introduced and quickly gained in acreage due to its high level of resistance to *A. pisi*. Simultaneously, *P. pinodes* became the dominant foliar pea pathogen in Canada (Wallen et al., 1967a). In the early 2000s, a resurgence of *A. pisi* was noted in western Canada based on increasing levels of this pathogen on harvested seeds (Morrall et al., 2011). In response to this, experiments were conducted to reassess the impact of seed infection in the epidemiology of *A. pisi*, to evaluate the importance of seed-to-seedling transmission under field conditions, and to determine the nature of seed-borne infection by *A. pisi*. It was hypothesized that pea plants developing from seeds infected with *A. pisi* would be infected with the pathogen and that seed infection would thus promote the development of *A. pisi* infection in the developing crop canopy. It was also hypothesized that low levels of seed coat staining would be indicative of no or a low incidence of embryo infection with *A. pisi* whereas high seed coat staining would be correlated with a high incidence of embryo infection.

# MATERIALS AND METHODS

## Field Experiments

Seeds of CDC Patrick, a green cotyledon field pea cultivar, were used for this experiment. Two commercial seed lots with an incidence of natural *A. pisi* seed infection of 0.5 and 14.5%, and 0 and 4% *P. pinodes* infection, respectively, confirmed by a commercial seed testing lab, were obtained from a seed grower. Samples were combined to obtain *A. pisi* incidence levels of 0.5, 5, 10, and 14.5%, which were confirmed through seed testing by plating four replicates of 100 seeds per incidence level onto potato dextrose agar (PDA) after 2.5 min surface sterilization in 0.6% NaOCl. Field experiments were established in the Canadian province of Saskatchewan in May at Outlook, Saskatoon, and Milden where levels of *A. pisi* infection had been low in previous years, and experiments were harvested in August. Detailed dates and general agronomic practices are presented in Supplementary Table S1. Experiments were designed as randomized complete block designs with four replicates. Plot size was 1.2 m × 3.7 m with 26 seeds $m^{-1}$ row, or 86 seeds $m^{-2}$ at a row spacing of 30 cm.

During the growing season, plant emergence was assessed by counting the number of seedlings per one meter plant row in four arbitrarily selected rows or row segments of each plot. The severity of symptoms caused by *A. pisi* and *P. pinodes* was assessed at the seedling stage, during flowering, at the podding stage and at maturity using the 0–10 rating scale based on 10% incremental increases in the percentage of disease severity together on leaves, stems and eventually pods. Five arbitrarily selected plants were rated in each plot and data were transformed to percentage disease severity using the class mid points. The averages per plot were calculated for further data analyses.

At harvest, seed yields were determined for each plot, seeds were assessed for thousand seed weight (TSW) and the incidence of seed infection with pathogens.

For seed testing, 100 seeds per plot were surface-sterilized by soaking in 0.6% NaOCl for 3 min with constant agitation, rinsing with sterile distilled water for 2 min, and drying on a sterile distilled paper towel before plating on PDA plates at 10 seeds per 9 cm Petri dish. Seeds were incubated at 20°C for 7 days under continuous fluorescent light on the bench top. Each seed was assessed for infection by *A. pisi*, *P. pinodes*, and other pathogens, and the percentage incidence of infection was recorded per plot for each pathogen.

## Seed Component Study

The same source of CDC Patrick seeds as above with an incidence of *A. pisi* infection of 14.5% was used for the seed component study. Based on the relatively low level of 4% *P. pinodes* infection in this sample, it was assumed that seed coat staining was primarily caused by *A. pisi* infection. The seeds were visually categorized into five categories based on the amount of seed coat staining of individual seeds: 0% (clean seeds without any staining), 1 to 25%; 26 to 50%; 51 to 75%; 76 to 100% of the seed coated stained. The latter also included a small number of underdeveloped and shriveled seeds assumed to be caused by

*A. pisi* (**Supplementary Figure S1**). For each category, seven replicates of 50 seeds were soaked in sterile distilled water for 2 h to soften the seed coat. Seeds were dissected into seed coat, cotyledon, and embryo. Seed components were surface-sterilized by soaking in 0.6% NaOCl for 3 min with constant agitation, rinsing with sterile distilled water for 2 min, and drying on a sterile distilled paper towel before being placed on PDA in Petri dishes. Seeds were incubated at 20°C for 7 days under continuous fluorescent light in a bench top incubator. Each Petri dish was assessed for infection and fungal growth was morphologically identified to the species level for *A. pisi* and *P. pinodes*, and to the genus level for other common fungi.

## Data Analysis

All data were analyzed using in SAS (Version 9.4, SAS Institute Inc.). All data were tested for normality and heterogeneity of variances of residuals. Data of emergence, yield, TSW, disease severity and the incidence of *A. pisi* infection were analyzed with the regression procedure where the seed infection level was the regressor. Incidence data for *A. pisi* and *P. pinodes* from the seed component study were analyzed with the mixed model procedure where seed staining categories and seed components were considered fixed effects, whereas replications were considered random effects. Initially, other pathogens detected in seed samples were used as covariates. Final modeling of *A. pisi* data was done with the significant covariate(s) and means were separated by Fisher's least significant difference test.

# RESULTS

## Field Experiments

Seedling emergence ranged from 10 to 24 seedlings per meter row in plots, with an overall average of 16 seedlings per meter row. Emergence was lowest at Milden in 2013 and highest at the same location in 2014, which was most likely associated with soil moisture conditions during emergence. Infection of CDC Patrick seeds with *A. pisi* only reduced emergence at Outlook in 2012 ($P = 0.0306$) and when data from all years and locations were pooled ($P = 0.0031$; **Figure 1**). However, in both cases, seed infection only explained a small proportion of the variability in emergence (29% for Outlook 2012, 9% for pooled data), and based on pooled data emergence was reduced by 1 plant $m^{-1}$ row for every 7% increases in the incidence of seed infection.

The average severity of *A. pisi* symptoms on seedlings after emergence was 1% in 2012 and 2013, and 5% in 2014, and many seedlings were disease-free. Overall, disease development on peas was higher at Saskatoon and Outlook in 2012 than in other years and locations because of higher precipitation (359 and 343 mm, respectively, compared to 143 to 234 mm in other years and locations) during the growing season (May to August). Temperatures were similar with maximum deviations among average daily temperatures for each month of 3°C. Seed infection with *A. pisi* had no effect on *A. pisi* development of pea seedlings (data not shown) or plants close to maturity when average *A. pisi* symptom severity ranged from 17 (Milden 2014) to 55% (Outlook in 2012). The severity of *P. pinodes* ranged from 18 (Saskatoon 2014) to 62% (Saskatoon 2012), and was always higher than *A. pisi* severity, with the exception of Saskatoon in 2014, when the severity of *A. pisi* reached 22%, whereas it was 18% for *P. pinodes* when averaged across all treatments. There were no significant differences in *P. pinodes* severity among treatments in any of the experiments.

Seed infection with *A. pisi* had no effect on seed yields, TSW or the incidence of *A. pisi* infection of harvested pea seeds (**Figure 1**). *A. pisi* infection of harvested seed was close to 0 at Outlook in 2012, but reached an average of 7% at Saskatoon in 2012. The incidence of *P. pinodes* infection ranged from 0.4% at Saskatoon in 2013 to 9% at Milden in 2014, and similar to *A. pisi*, there were no treatment effects. Except for Outlook 2012 and Milden 2014, harvested seeds had more *A. pisi* than *P. pinodes* infection.

## Seed Component Study

Seed components without staining of the seed coat were not infected with *A. pisi*. Seed coats, cotyledons, and embryos of all other four seed staining categories were infected with *A. pisi*. In addition to *A. pisi*, other organisms, such as *Colletotrichum* spp., *Fusarium* spp., *Alternaria* spp., *Epicoccum* spp., unidentified green molds and bacteria were also identified on the stained seed components (**Table 1**). Only incidence data of *Epicoccum* spp. had a significant effect on the model as a co-variate ($P = 0.0212$) and were included in the model. Seed staining category, seed components, and their interaction had significant effects on the incidence of *A. pisi* infection ($P < 0.0001$). Seed staining categories 51–75% and 76–100% had a higher incidence of seed coat infection compared to that in staining category of 26–50%. Seeds staining categories 1–25% and 76–100% had a higher incidence in cotyledon infection compared to staining category 51–75%, whereas there was no difference in the incidence of embryo infection among the seed staining categories (**Figure 2**).

# DISCUSSION

Pea seedling emergence was slightly, but statistically significantly affected by the incidence of *A. pisi* infection of seeds. Based on the regression model here, an increase in the incidence by 7% *A. pisi* infection in seeds is predicted to reduce seedling emergence by 1 plant $m^{-1}$ representing 4% in our experiment with 26 plants $m^{-1}$. This indicates that even an incidence of 14.5% of seed infection, the highest infection level assessed here, will only have a minor impact on plant stands. A much more significant impact of *A. pisi* seed infection on emergence was reported previously by Jones (1927) who found 69 and 76% seedling emergence under field, and 75% under greenhouse conditions from a seed sample with an incidence of *A. pisi* infection of 8%, when compared to emergence of seeds from the same sample treated with organic mercuric dust. In contrast, assessments of seed samples from several years and locations with *A. pisi* infection rates of 10% resulted in seedling emergence of 85% (Wallen, 1955). In that study, samples with 44 and 46% *A. pisi* infections were assessed as well and had emergence rates of 87 and 67%, respectively, supporting observations here that *A. pisi* infection does not have

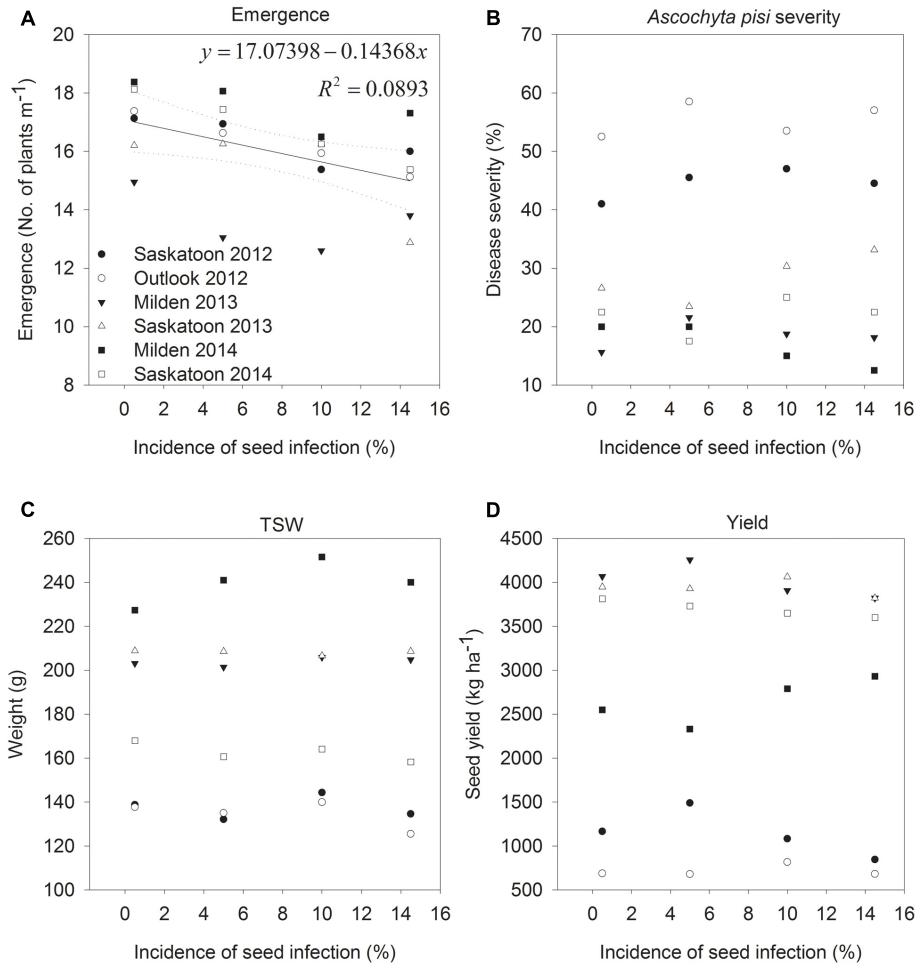


**FIGURE 1 |** Seedling emergence **(A)**, *Ascochyta pisi* severity on mature plants **(B)**, seed yields **(C)**, and thousand-seed weight (TSW; **D**) of pea cv. CDC Patrick grown from seeds with incidence levels of *A. pisi* infection of 0.5, 5, 10, and 14.5% in field experiments conducted at two locations in 2012 to 2014.

a major impact on emergence, although the confounding impact of organisms other than *A. pisi*, observed in all of these studies, has not been quantified. When comparing these numbers it is important to keep in mind that the earlier reports used pea varieties that are now 60 to more than 100 years old, and were most likely more susceptible to *A. pisi* than modern CDC Patrick.

**TABLE 1 |** Incidence levels (%) of *Ascochyta pisi* and other fungi (mean of 3 seed components) on naturally infected seeds of pea cv. CDC Patrick seeds that were separated into four seed coat staining categories.

| | Staining category | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Pathogens** | **1–25%** | | **26–50%** | | **51–75%** | | **76–100%** | |
| *Ascochyta pisi* | 45.36 | (1.24) | 43.71 | (2.46) | 43.50 | (2.46) | 52.00 | (2.78) |
| *Peyronellaea pinodes* | 0.14 | (0.14) | 0.14 | (0.14) | 2.14 | (0.70) | 6.29 | (1.34) |
| *Alternaria* spp. | 3.71 | (0.97) | 7.57 | (1.34) | 10.00 | (1.46) | 25.14 | (2.51) |
| *Colletotrichum* spp. | 0.71 | (0.42) | 1.14 | (0.86) | 3.57 | (1.13) | 7.14 | (1.20) |
| *Stemphylium* spp. | 0.57 | (0.20) | 3.86 | (0.94) | 3.43 | (1.02) | 3.71 | (1.02) |
| *Epicoccum* spp. | 0.14 | (0.14) | 0.86 | (0.34) | 0.29 | (0.18) | 1.00 | (0.44) |
| Green mold | 2.86 | (0.80) | 2.43 | (0.53) | 9.86 | (1.74) | 14.29 | (1.71) |
| Bacteria | 0.57 | (0.57) | 0.14 | (0.14) | 1.57 | (0.53) | 1.71 | (0.81) |

*Numbers in brackets represent standard errors of the mean.*

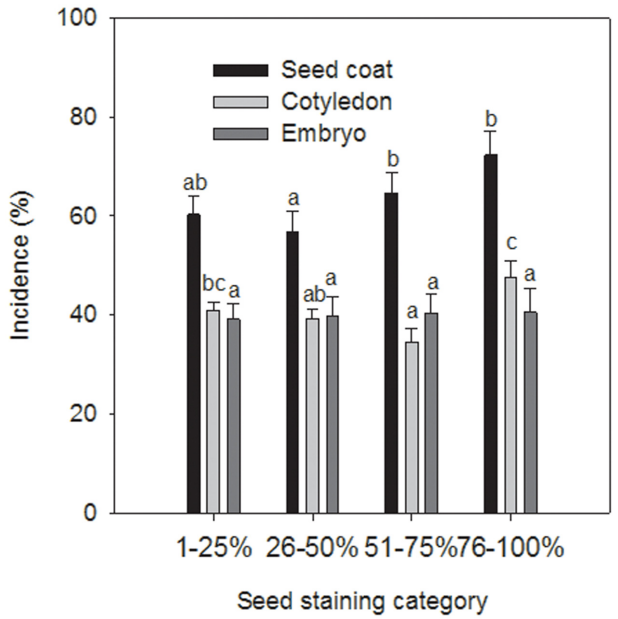


**FIGURE 2 |** Incidence (%) of *Ascochyta pisi* infection of seed coats, cotyledons, and embryos of commercial pea seeds of cv. CDC Patrick that were separated into four seed coat staining categories. Bars indicate standard errors of the mean. Letters above columns indicate significant differences: columns of each series with a letter in common are not significantly different.

Even though the first highly *A. pisi* resistant pea variety was only released in 1961 (Gfeller and Wallen, 1961), it can be speculated that pea varieties studied by Wallen (1955) may have already had improved resistance compared to those used by Jones (1927) 28 years earlier, as resistance to *A. pisi* will have been a primary breeding objective. A negligible impact of *A. pisi* on pea seedling emergence observed here is in stark contrast to *P. pinodes* where seed infection levels of 24 to 46% resulted in germination rates of 19 to 23% (Xue, 2000), and seeds with more than 50% seed coat staining had a seed-to-seedling transmission of 100% (Moussart et al., 1998).

Precipitation during the growing seasons of 2012 to 2014 at experimental locations was average or above average, so conditions generally were conducive for the initiation of epidemics. Very low levels of seedling infections and no effect of *A. pisi* seed infection on disease severity on the developing plants here indicated that infection of seeds with *A. pisi* used for seeding does not pose a risk for initiating epidemics in the field under western Canadian growing conditions. There was also no effect on seed yield, seed size or the infection levels with this pathogen of harvested seeds. In general, *A. pisi* is considered to be less aggressive than other pathogens, with reported yield losses of 11% compared to 45 and 25% due to *P. pinodes* and *Phoma m.* var *pinodella*, respectively (Wallen, 1965).

In four of the six field experiments seed infection of harvested seeds with *A. pisi* was higher than with *P. pinodes* despite the fact that for three of those four experiments, *P. pinodes* severity on pea plants was higher than *A. pisi* severity. Wallen et al. (1967b) pointed out a natural antagonism between *A. pisi* and *P. pinodes*, and also found that seed-borne infection tends to be higher with *A. pisi* compared to *P. pinodes* (Wallen, 1965). A higher incidence of *A. pisi* infection had been observed for certain seed lots in commercial seed testing labs in recent history as well (Morrall et al., 2011), which had triggered a re-assessment of the importance of *A. pisi* here. When assessing seed components for infection, the embryo of all seeds were infected with *A. pisi* irrespective of the amount of seed staining as long as there was some seed coat staining. This is distinctly different from seed infection by *P. pinodes* where the amount of seed coat staining is positively correlated with the depth of infection into the seed and the frequency of embryo infection (Moussart et al., 1998). For this pathogen, no necrosis on seed components other than on the seed coat was observed for seeds with less than 25% seed staining. Seeds with higher seed coat staining always showed evidence of necrosis caused by *P. pinodes* on the outward facing side of cotyledons, and a gradual increase in the incidence of necrosis on the inward-facing side of cotyledons from 12 to 100% as outer seed coat staining increased from 25 to 100%. Similarly, the incidence of necrosis on embryos increased from 10 to 100% once seed coat staining exceeded 25% and increased to 75 to 100%. This positive correlation between increasing outer seed coat staining and infection of inner seed components suggests that *P. pinodes* infects the more or less immature pod and penetrates from there into the seeds. The relatively high incidence of *A. pisi* in embryos and cotyledons irrespective of the amount of seed coat staining may indicate that *A. pisi* infection already occurs during flowering. The lack of correlation between foliar infection, from which water-splashed conidia could infect flowers, and the incidence or depth of seed infection indicates that airborne ascospores of *A. pisi* rather than water-splashed conidia may infect flowers and seeds, considering that windborne ascospores can be blown in from remote inoculum sources, and ascospore concentration will likely be more equal across a field. Little is known about the life cycle of *A. pisi* whereas that of *P. pinodes* has been well studied. The latter is homothallic and readily produces sexual fruiting structures (pseudothecia) which are thought to overwinter on pea stubble generating airborne ascospores that represent the initial inoculum for the new pea crop in the following season (reviewed in Roger and Tivoli, 1996). Studies in France showed that ascospores of *P. pinodes* are released throughout the growing season, but peak toward its end when large numbers of pseudothecia develop almost exclusively on senescent plant tissue, and mostly on stems of the maturing, increasingly diseased and senescent pea plants.

The teleomorph of the heterothallic species *A. pisi, Didymella pisi*, was only described relatively recently and it was shown that pseudothecia matured within 2 months at a constant temperature of 10°C, but their development ceased at 23°C (Chilvers et al., 2009). Historically, the daily maximum temperature in many parts of the Canadian Prairies exceeds 23°C during the growing season, but the daily average temperature often does not due to cool nights, so depending on the effect of fluctuating temperatures on perithecial development in *A. pisi* the climate may be conducive for ascospore production. To date, no studies have been conducted to determine whether pseudothecia

develop under field conditions, nor have there been attempts to trap ascospores of this species. Indeed, such research would be complicated by the fact that *P. pinodes* tends to also be present. Although pseudothecia of *A. pisi* are slightly larger than those of *P. pinodes,* ascospores overlap in size (Punithalingam and Holliday, 1972, Chilvers et al., 2009), hence differentiating sexual structures of both species by microscopying or spore trapping would be highly complicated. Molecular probes readily differentiate between them, but do not allow to determine whether fruiting structures and spores are of sexual or asexual origin. In future, it may be possible to conduct studies of this nature through a combination of sophisticated imaging technology and molecular techniques.

Jones (1927) suggested that *A. pisi* overwinters as mycelium on pea straw after inoculating pea stems with this pathogen and incubating them under natural winter conditions in Wisconsin, United States. However, based on Wallen et al. (1967b) isolation of *A. pisi* from agricultural soil of eastern Canadian fields or from sterilized soil inoculated with spore suspensions of the ascochyta blight pathogens was unsuccessful whereas *P. pinodes* and *Phoma pinodella* were isolated on a regular basis. Incubation studies in sterilized soil each inoculated with one of the three ascochyta blight pathogens and incubated at temperatures ranging from −20 to +30°C revealed that *A. pisi* only survived in the soil for a period of 12 months at +5 and −20°C (Wallen and Jeun, 1968). At −20°C, *P. pinodes* and *Phoma pinodella* survived for that period as well, but with lower recovery rates than *A. pisi*. Both, *P. pinodes* and *Phoma pinodella* also survived up to 12 months in soil incubated at 5 to 25°C and were recovered at high rates, indicating clear temperature optima for *A. pisi*, and *P. pinodes* and *Phoma pinodella*. When sterilized soil was co-inoculated with the ascochyta blight pathogens in all possible pairwise combinations, *P. pinodes* was always recovered at the highest rate. In the presence of *P. pinodes*, *Phoma pinodella* survived for at least 9 months, whereas *A. pisi* was least competitive in the presence of either partner.

Jones (1927) also noted that seedlings developing from infected seed had lesions on the first leaves, so may represent a second source of initial inoculum. Testing commercial seed samples, Maude (1966) only found 40% of seed-to-seedling transmission for *A. pisi* compared to close to 100% for *P. pinodes*, and research here with a modern cultivar of pea revealed rare seed-to-seedling transmission under western Canadian field conditions. Considering that the pathogen is not readily isolated from soil (Wallen et al., 1967b), competes poorly with *Phoma pinodella* and *P. pinodes* in soil and does not, or rarely, produces chlamydospores (Wallen and Jeun, 1968), it can be speculated that infected seeds may play a much more important role for the survival of *A. pisi* than is the case for the other two common ascochyta blight pathogens. This would explain why the incidence of seed infection with *A. pisi* historically, and in some years in recent times, has been higher compared to *P. pinodes*.

## CONCLUSION

The effect of *A. pisi* infection in seed on emergence was minimal under western Canadian growing conditions, *A. pisi* symptoms on seedlings were rare, and incidence levels of *A. pisi* infection of seed up to 14.5% did not increase the amount of disease on mature plants or harvested seeds. Infection with *A. pisi* of harvested seeds was common across all seed infection categories used for seeding, and staining was significant, so while seed infection up to the incidence level tested here may not impact pea production when the seed is used for seeding, the staining caused by *A. pisi* infection of seeds can result in lower quality of seeds to be sold as food or feed. The common infection of embryos and cotyledons of seeds of all staining categories may be indicative for a more dominant role of the seeds in the survival of *A. pisi* compared to *P. pinodes* that survives well in soil. Whether seed infection is initiated by ascospores during flowering, as speculated here, will only be revealed when more is known about the life cycle of this pathogen.

## AUTHOR CONTRIBUTIONS

NSK conducted the research experiments as part of his MSc thesis, he contributed to the data analyses and to drafting the manuscript. SB was the principal investigator of this research project, supervised NSK, contributed to data analyses and the drafting of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00933/full#supplementary-material

**FIGURE S1** | Seed staining categories based on the percentage of seed coat staining of commercial CDC Patrick field pea with 14.5% incidence of *Ascochyta pisi* infection due to natural infection. Top right: The category with 76 to 100% seed coat staining also included a small number of shriveled and undeveloped seeds.

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REVIEW
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# Molecular Breeding for Ascochyta Blight Resistance in Lentil: Current Progress and Future Directions

*Matthew S. Rodda[1], Jennifer Davidson[2], Muhammad Javid[1], Shimna Sudheesh[3], Sara Blake[2], John W. Forster[3,4] and Sukhjiwan Kaur[3]**

[1] Agriculture Victoria, Grains Innovation Park, Horsham, VIC, Australia, [2] Pulse and Oilseed Pathology, Plant Health and Biosecurity, Sustainable Systems, South Australian Research and Development Institute, Urrbrae, Adelaide, SA, Australia, [3] Agriculture Victoria, AgriBio, Centre for AgriBioscience, La Trobe University, Bundoora, VIC, Australia, [4] School of Applied Systems Biology, La Trobe University, Bundoora, VIC, Australia

Lentil (*Lens culinaris* Medik.) is a diploid (2n = 2x = 14), self-pollinating, cool-season, grain legume that is cultivated worldwide and is highly valuable due to its high protein content. However, lentil production is constrained by many factors including biotic stresses, majority of which are fungal diseases such as ascochyta blight (AB), fusarium wilt, rust, stemphylium blight, anthracnose, and botrytis gray mold. Among various diseases, AB is a major -problem in many lentil-producing countries and can significantly reduce crop production. Breeding for AB resistance has been a priority for breeding programs across the globe and consequently, a number of resistance sources have been identified and extensively exploited. In order to increase the efficiency of combining genes from different genetic backgrounds, molecular genetic tools can be integrated with conventional breeding methods. A range of genetic linkage maps have been generated based on DNA-based markers, and quantitative trait loci (QTLs) for AB resistance have been identified. Molecular markers linked to these QTLs may potentially be used for efficient pyramiding of the AB disease resistance genes. Significant genomic resources have been established to identify and characterize resistance genes, including an integrated genetic map, expressed sequence tag libraries, gene based markers, and draft genome sequences. These resources are already being utilized for lentil crop improvement, to more effectively select for disease resistance, as a case study of the Australian breeding program will show. The combination of genomic resources, effective molecular genetic tools and high resolution phenotyping tools will improve the efficiency of selection for ascochyta blight resistance and accelerate varietal development of global lentil breeding programs.

**Keywords: legume, pulse, mapping, molecular markers, fungal disease resistance**

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****Correspondence:***
*Sukhjiwan Kaur*
*sukhjiwan.kaur@ecodev.vic.gov.au*

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## INTRODUCTION

Lentil is a self-pollinating diploid (2n = 2x = 14) grain legume crop with a genome size of c. 4 Gbp (Arumuganathan and Earle, 1991). Lentil is cultivated globally and is highly valued as an efficient source of dietary protein. The global cool-season grain legume production is largely represented by chickpea (*Cicer arietinum* L.), pea (*Pisum sativum* L.), and cultivated lentil

(*Lens culinaris* Medikus ssp. *culinaris*) (Khazaei et al., 2016). Lentil was one of the oldest domesticated grain legumes, derived from a center of origin in the Near East (Zohary, 1999), and the highest levels of contemporary diversity are still located in this region, particularly Turkey, Syria, and Iraq. Lentil cultivation subsequently spread to the Nile valley, Central Asia and the Mediterranean Basin, followed by Pakistan, India, and South America (Cubero, 1981; Khazaei et al., 2016). The crop is currently grown widely throughout the Indian sub-continent, the Middle East, northern Africa, southern Europe, North and South America, Australia, and western Asia (Fikiru et al., 2007; Kaur et al., 2014a). The total (global) lentil production is estimated at 4.4 million metric tons from an estimated 4.2 million hectares, with an average yield of 1,068 kg/ha (FAO, 2015; Kumar et al., 2015). Lentil cultivation in rotation with cereals provides benefits to the cropping systems through biological nitrogen fixation, breaking of disease cycles and effective control of weeds, and significant support for the livelihood of small-scale farmers practicing agriculture in the dryland agricultural ecosystems of South Asia, Sub-Saharan Africa, West Asia, and North Africa (Kumar et al., 2013).

Lentil production is limited by many factors including abiotic stresses such as terminal drought, heat stress, low soil fertility, and various biotic stresses including infection by the pathogens causing ascochyta blight (*Ascochyta lentis* Vassilievsky), fusarium wilt (*Fusarium oxysporum* f.sp. *lentis*), anthracnose (*Colletotrichum truncatum*), stemphylium blight (*Stemphylium botryosum*), rust (*Uromyces viciae-fabae*), botrytis gray mold (*Botrytis cinerea* and *B. fabae*), and white mold (*Sclerotinia sclerotiorum*) (Sharpe et al., 2013; Kumar et al., 2015). Among these diseases, ascochyta blight (AB) is one of the most widespread, being of economic concern in the majority of lentil-producing regions, especially under the mild, wet winter conditions of Mediterranean and maritime climates (Erskine et al., 1994; Ye et al., 2002; Ford et al., 2011). *A. lentis* (teleomorph *Didymella lentis*) is the causal agent of AB of lentil (Kaiser et al., 1997). Symptoms include lesions on stems, leaves, petioles and pods. Plant death is common following seedling infection, while infection of mature plants can lead to significant reduction in yield and seed quality (Morrall and Sheppard, 1981). The foliar infection can cause yield losses of up to 40%, but the loss of economic value due to seed staining and mold may be more than 70%, as it can result in a failure to meet export quality standards (Gossen and Morrall, 1983, 1984; Brouwer et al., 1995). AB can be managed through the application of fungicides, however the most economically viable and environmentally sustainable method of control is the development of disease resistant varieties (Ford et al., 2011).

As a decade may typically be required for release of a commercial variety, development and implementation of new molecular genetics tools will support a transition from conventional to genomics-assisted breeding approaches in order to accelerate the release of improved lentil cultivars. Molecular tools, including marker-assisted selection, have the potential to accelerate and improve the effectiveness of breeding for disease resistance in lentil. For this reason, during the last two decades substantial efforts have been made to understand the genetics and genomics of lentil, including a focus on understanding the genetic basis of resistance to *A. lentis*. Genetic linkage maps of lentil have been constructed based on a range of molecular genetic marker types such as randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), sequence characterized amplified regions (SCARs), resistance gene analogs (RGAs), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), and single nucleotide polymorphism (SNPs) (Eujayl et al., 1998; Rubeena et al., 2003; Tullu et al., 2006; Sharpe et al., 2013; Kaur et al., 2014a; Verma et al., 2015). Through the use of these maps, a number of genomic regions controlling AB resistance have been identified (Ford et al., 1999; Rubeena et al., 2006; Sudheesh et al., 2016).

In addition to lentil, AB is one of the most important diseases of the other cool season food legumes such as field pea, chickpea, and faba bean, although the causal pathogens of AB differ for each crop host. The status of AB as a disease of significant economic concern in each of these crops has led to a large number of QTL studies aimed at identifying the genomic regions associated with AB resistance; in field pea (Timmerman-Vaughan et al., 2002, 2004; Tar'an et al., 2003a; Prioul et al., 2004; Fondevilla et al., 2008, 2011; Jha et al., 2016, 2017), chickpea (Udupa and Baum, 2003; Lichtenzveig et al., 2006; Tar'an et al., 2007; Sabbavarapu et al., 2013), and faba bean (Román et al., 2003; Avila et al., 2004; Kaur et al., 2014b; Atienza et al., 2016). Within Australia breeding germplasm, lentil is the most advanced of these four crop species in the implementation of MAS for AB resistance (pers. comm. Rodda, Agriculture Victoria). Breeding for resistance to AB in field pea is complicated by the co-occurrence of three to four species in the disease complex also known as blackspot (Bretag and Ramsey, 2001; Davidson et al., 2009). In addition, there are limited sources of major gene resistance available in field pea (Kraft et al., 1998; Zhang et al., 2006). AB is one of the most important diseases of faba bean and chickpea and resistance has been a major focus of molecular marker development for these crops in Australia. Unfortunately, in both these species, there have recently been shifts in the pathogen population, overcoming key resistance genes (Kimber et al., 2016; Moore et al., 2016) which have rendered their available markers unusable for the most aggressive forms of the ascochyta blight pathogens.

In this review paper, the progress of and prospects for breeding for ascochyta blight resistance in lentil is discussed, along with potential impact of genomic technologies on future crop improvement.

## THE PATHOGEN

*A. lentis* can infect cultivated and wild species of lentil including *L. culinaris* subsp. *orientalis*, *L. culinaris* subsp. *odemensis*, *L. ervoides*, *L. lamottei*, *L. nigricans,* and *L. tomentosa* (Bayaa et al., 1994; Hernandez-Bello et al., 2006; Tullu et al., 2010). However, the pathogen appears to be host-specific to the *Lens* genus, being unable to cause disease symptoms on other legume crops including chickpea (*C. arietinum*), faba bean (*Vicia faba*), field pea (*P. sativum*), or hairy vetch (*V. villosa*) (Hernandez-Bello et al., 2006; Peever et al., 2007).

A comparison of the related *Ascochyta* pathogens from wild and cultivated legume hosts, including *A. lentis*, *A. fabae*, *A. rabiei, A. pinodes, A. pinodella,* and *A. pisi*, has revealed near-identical ribosomal DNA internal transcribed spacer (ITS) regions. In contrast, analyses of protein-coding genes of fungal isolates obtained from the same host species demonstrated clustering even when collections had been made from diverse regions. A co-evolutionary history between the pathogens and their respective hosts is likely to have resulted in the observed host specificity of *Ascochyta* fungi (Peever et al., 2007).

Early morphological studies revealed that *A. lentis* could not be separated from *A. fabae* (the causal pathogen of ascochyta blight on faba bean) on the basis of cultural or morphological characteristics, and so the two pathogens were proposed to be synonymized as two special forms i.e., *A. fabae* f. sp. *lentis* and *A. fabae* f. sp. *fabae*, respectively (Gossen et al., 1986). *A. lentis* was later confirmed as a species distinct from *A. fabae* on the basis of pathology tests, RAPD markers and the results of controlled crosses between complementary mating types of *A. fabae* and *A. lentis* (Kaiser et al., 1997). Notably, the crosses showed the inability of the progeny to produce fertile pseudothecia that induce disease on either host parent (Kaiser et al., 1997). In contrast, progeny from successful matings between *A. lentis* and an Italian isolate from ascochyta-type lesions on grasspea (*Lathyrus sativus* L.) produced a normal culture morphology, demonstrating that these isolates could not be placed into separate taxa. The variant, which is able to infect grasspea but not lentil, has recently been described as *A. lentis* var. *lathyri*, and shows 99–100% sequence identity to the *A. lentis* genome, despite significant morphological differences between conidia of the two variants. The differences in conidial dimensions and host specificity suggest that these variants have arisen from a speciation process (Infantino et al., 2016).

As a heterothallic fungus, *A. lentis* requires two mating types (MAT1-1 and MAT1-2) for sexual reproduction to occur in order to produce the *Didymella* teleomorph (Kaiser et al., 1997; Galloway et al., 2004; Hernandez-Bello et al., 2006). The two mating types are encoded by alternate alleles at a single (*MAT*) locus. PCR amplicons of sizes 450 and 700 bp have been amplified from MAT1-1 and MAT1-2 isolates, respectively (Cherif et al., 2006) although a MAT1-2-specific amplicon at 750 bp has also been consistently amplified (pers. comm. Herdina, SARDI, March 2017). Both mating types have been identified in isolates from Algeria, Canada, Hungary, India, Russia, Spain, USA (Ahmed et al., 1996a), and Australia (Galloway et al., 2004). MAT1-1 is reported to occur more frequently than MAT1-2 in Australia in the *A. lentis* population by a ratio of 2:1 (Nasir, 1998 cited in Skiba and Pang, 2003) and 5:1 in Canada (Ahmed et al., 1996a).

Sexual reproduction between the mating types results in the development and maturation of pseudothecia on infested lentil straw under cool moist conditions (Kaiser, 1997; Galloway et al., 2004). These structures, which have only been observed on straw (Skiba and Pang, 2003), develop within 17 days at 10°C in controlled conditions (Ahmed et al., 1996a). The dome-shaped pseudothecia contain many bitunicate asci each with 8 hyaline, two-celled ascospores (Skiba and Pang, 2003; Galloway et al., 2004). Asexual flask-shaped pycnidia also develop on infested straw and produce conidia (Skiba and Pang, 2003). The maturation of pseudothecia and discharge of ascospores from infested lentil straw overlap with the vegetative stage of the crop (unpublished data, Davidson, SARDI, June 2016), indicating that ascospores may serve as primary inoculum for the disease, similar to the case of *Didymella fabae* (Rubiales and Trapero-Casas, 2002). Mature ascospores of *D. lentis* are wind-dispersed to a distance of 50 m from the infested straw (Galloway and MacLeod, 2002). Epidemics can also be initiated by infested seed (Kaiser and Hannan, 1986) and by asexual conidia which are splash-dispersed from infested straw onto lentil plants during rainfall (Morrall and Sheppard, 1981; Kaiser and Hannan, 1986; Nasir and Bretag, 1997b). Spores can germinate within 6 h of inoculation, and germ tubes grow to form an appressorium within 10 h (Roundhill et al., 1995). Under optimal conditions of temperature (15–20°C) and leaf wetness, the period from inoculation to expression of disease symptoms for *A. lentis* is 6–7 days (Pederson and Morrall, 1994), but may take up to 10–14 days (Roundhill et al., 1995). Necrotic lesions, initially pale green and then turning light brown, develop on all above-ground parts, leading to leaf drop, stem breakage, reduction in pod size, and shriveled and/or stained seed. Pycnidia and conidia develop within the lesions on diseased plants during the growing season, and the epidemic spreads to adjacent plants through successive cycles of rain-splashed conidia (Pederson et al., 1994; Ford et al., 2011).

## HOST-PATHOGEN INTERACTIONS

*A. lentis* populations are highly variable in terms of aggressiveness on different lentil cultivars and wild accessions (Bayaa et al., 1994; Ahmed et al., 1996b; Nasir and Bretag, 1997a; Ahmed and Morrall, 1999; Tullu et al., 2010; Davidson et al., 2016). There was also a greater degree of variability identified in populations of *Ascochyta* spp. isolated from wild host species, suggesting that the collections from cultivated hosts constitute sub-sets of the variation present in wild populations (Peever et al., 2007). Studies using different host sets of *L. culinaris* each identified five or six pathotypes of *A. lentis* in Australia (Nasir and Bretag, 1997a, 1998; Sambasivam et al., 2017) and Pakistan (Iqbal et al., 2006). Pathogenic groups were also separated by differences in pre-penetration events (spore germination, germ tube length, and appressoria development), and early differences in defense responses (Sambasivam et al., 2017).

There is no evidence to suggest that mating type influences the aggressiveness or virulence of pathogen isolates (Ahmed et al., 1996a). However, the presence of both mating types of *A. lentis* leads to a high potential for adaptation through sexual reproduction, since heterothallism ensures a diverse population (Ford et al., 2000; Cherif et al., 2006). In addition, the movement of infected seed between regions, as well as the introduction of isolates via international germplasm (Kaiser, 1997), increases the potential for pathogenic variability and generation of isolates with increased aggressiveness. RAPD analysis revealed greater variability among isolates from Western Australia than those

from a larger geographical area in eastern Australia, presumably due to multiple introductions from international sources into Western Australia (Ford et al., 2000).

Intensive cropping of single cultivars can lead to loss of resistance by selection for aggressive isolates that are already present in the naturally variable population (Davidson et al., 2016). Recent changes in the foliar response of the previously resistant lentil cvs. Northfield (ILL5588) and Nipper were identified and experimentally confirmed in Australia (Davidson et al., 2016), the latter being a progeny of the resistant cvs. Northfield x Indianhead. ILL5588 was used extensively as a source of resistance to AB in the Canadian and Australian lentil breeding programs (Tullu et al., 2010; Davidson et al., 2016), and these changes may have a wide impact on resistant sources. There was also loss of resistance to AB in Canada on lentil cv. Laird, leading to 50% yield reduction (Morrall, 1997). Rapid loss of resistance to AB indicates resistance conferred by major genes, but the general continuum of aggressiveness that is also present among *A. lentis* isolates is indicative of polygenic resistance, leading to the conclusion that both major and minor genes are involved (Ye et al., 2002; Banniza and Vandenberg, 2006; Gupta et al., 2012; Davidson et al., 2016).

Inheritance of pathogen virulence on cv Northfield (ILL5588) was reported to be controlled by two independently segregating genes, operating in mutual epistasis, based on a 3:1 segregation ratio in the $F_1$ progeny (ascospores) (Skiba and Pang, 2003). Because *A. lentis* is a haploid organism, the $F_1$ progeny between virulent and avirulent isolates should segregate, while two virulent isolates should only produce virulent progeny. Ahmed and Morrall (1999) identified avirulent progeny from crosses between parents of intermediate virulence and also crosses between two highly virulent parents. Some progeny of each cross showed intermediate reactions as compared to the parents. These results may indicate the involvement of multiple genes with additive effects, and/or gene interaction. In addition, some progeny displayed higher virulence than either parent, showing that sexual recombination can generate novel isolates capable of attacking AB resistant cultivars.

In order to begin to understand the complexity of genes involved in resistance to *A. lentis*, a micro-array experiment with 762 probes was used to investigate gene expression changes in the susceptible lentil line ILL6002 and the resistant line ILL7537 (Mustafa et al., 2009). Several differentially expressed genes encoding pathogenesis-related (PR) proteins were identified in the early stages of infection, including a PR4 protein, three PR10 proteins and a β-1,3-glucanase, all up-regulated in the resistant ILL7537 line but not the susceptible ILL6002 line. β-1,3-glucanases cause lysis of the fungal cell wall, while PR4 disrupts cell growth through chitinase activity. Both mechanisms may work in tandem: the first opening the cell wall, so allowing the second to enter the cell and disrupt function. The pathogenesis-related PR4-encoding gene *LcPR4a* (Vaghefi et al., 2013), which was induced in lentil plants following infection by *A. lentis*, was detected at 12 h post-infection in both compatible and incompatible interactions of plant and pathogen. However, the magnitude of *LcPR4a* expression continued to increase in the resistant line to 114-fold by 48 h post-infection. Recombinant *LcPR4a* protein significantly reduced fungal biomass in an *in vitro* antifungal assay, further suggesting a role in the defense response to *A. lentis* (Vaghefi et al., 2013).

## GENETICS OF AB RESISTANCE

Several studies have been performed to explore the genetics of both seed/pod and foliar AB resistance in lentil, with resistant germplasm identified in both the cultivated and wild species (Bayaa et al., 1994; Tullu et al., 2010). An early study identified foliar AB resistance in wild lentil species, controlled by two dominant genes in both *L. ervoides* (Brign.) Grande and *L. odemensis* Ladz., and by a single dominant gene in *L. culinaris* ssp. *orientalis* (Ahmad et al., 1997). Several studies have described the roles of both dominant and the recessive genes in conferring AB resistance in cultivated lentil. For example, two foliar AB resistance genes, designated *Ral2* (dominant) and *ral2* (recessive), were identified as being present in the cultivars Northfield and Indianhead, respectively (Andrahennadi, 1994; Chowdhury et al., 2001). In addition, a third distinct dominant gene for foliar resistance ($AbR_1$) has been reported from Northfield (Tay and Slinkard, 1989). Two dominant complementary genes have been found to be associated with inheritance of foliar AB resistance in lentil accession ILL7537 (Nguyen et al., 2001). Previous reports indicated that screening of this accession with molecular genetic markers linked to, and flanking, the resistance gene $AbR_1$ failed to identify the resistance marker alleles, indicating that the AB resistance in ILL7537 may potentially be unique (Nguyen et al., 2001; Rubeena et al., 2006).

Both dominant and recessive genes were reported to control the seed-based AB resistance in lentil. For example, in one study, a three-gene model for seed-based AB resistance was proposed, including the effects of two dominant genes and a single recessive gene (Tay, 1989). In contrast, another study reported only one dominant and one recessive gene for seed-based AB disease resistance (Sakr, 1994), and a third study reported control by a single dominant gene (Vakulabharanam et al., 1997).

The studies conducted to date on both foliar and seed-based AB resistance have provided a detailed understanding of the role of dominant and recessive genes. The variable number and nature of genes observed in such studies was often due to the different sources of genetic resistance used, with their independent genetic control of plant resistance. In addition, there may be due to differences in AB screening assays, environmental conditions, *A. lentis* isolates and the variable size of populations being evaluated (Ford et al., 1999).

Wild species have the potential to be an important source of resistance to biotic stresses in lentil, compensating for the comparatively low intraspecific variability that is characteristic of domesticated lentil species (Abo-elwafa et al., 1995; Tullu et al., 2010). Interspecific crosses and populations are already being exploited by lentil breeders to introgress diverse resistance genes for a number of other biotic stresses (pers. comm. Vandenberg, University of Saskatchewan).

## MARKER-ASSISTED BREEDING FOR ASCOCHYTA BLIGHT RESISTANCE

Marker-assisted selection (MAS) allows the selection of a desirable trait with a marker, or suite of markers, based on associated sequence variation, in the absence of direct phenotypic assessment. This approach is dependent on establishment of a close linkage between the molecular genetic marker and the chromosomal location of the gene(s) that control the trait that is to be selected in a particular environment. For example, disease resistance can be evaluated using MAS in the absence of infection, and in the early stages of plant development.

In the major crop species, a large number of genetic markers for key traits relevant to plant breeding are available, providing a critical tool to increase selection efficiency (Dwivedi et al., 2007; Xu and Crouch, 2008). Although application of MAS to lentil has been limited until recently (Kumar et al., 2015), the advent of next-generation sequencing (NGS) technologies provided opportunities for the development of DNA sequence-based markers, which are being implemented in the modern lentil breeding programs of Australia and Canada (pers. comm. Vandenberg, University of Saskatchewan; pers. comm. Rodda, Agriculture Victoria).

A broad range of genetic and genomic resources have recently been generated for lentil through delivery of large numbers of expressed sequence tag (EST)-derived (and hence gene-associated) SSR and SNP markers (Kaur et al., 2011; Sharpe et al., 2013; Kaur et al., 2014a; Sudheesh et al., 2016). They have been extensively used to construct densely populated intraspecific genetic linkage maps, and to identify QTLs (Sharpe et al., 2013; Kaur et al., 2014a; Sudheesh et al., 2016). The information from multiple population-specific genetic maps can be integrated to produce high-density consensus structures utilizing the sequence-linked genetic markers which enables the identification of bridging loci between maps (Sudheesh et al., 2015a,b).

In lentil, molecular markers have been developed for traits with both simple (qualitative) and complex (quantitative) control. In the case of traits controlled by major genes, relatively simple phenotyping methods have been developed, allowing the accurate mapping of the gene. Traits such as boron toxicity tolerance are predominantly controlled by single genes, permitting deployment of a small set of flanking markers (Kaur et al., 2014a). However, to establish reliable marker-trait associations for more complex traits, rapid and reliable screening methods, together with marker saturated target regions and validated QTLs in multiple environments and genetic backgrounds are essential.

A number of independent studies (summarized in **Table 1**) have used molecular genetic marker technology to determine the basis for AB resistance, based on the construction of genetic maps for segregating populations derived from crossing of parental genotypes with divergent phenotypes. Several generations of marker technology have been used, from low-fidelity and non-locus-specific systems such as RAPDs, AFLPs, and ISSRs (Ford et al., 1999; Chowdhury et al., 2001; Tar'an et al., 2003b; Rubeena et al., 2006; Tullu et al., 2006) to high-fidelity, locus-specific and frequently gene-associated systems such as RFLPs, SSRs, and SNPs (Gupta et al., 2012; Sudheesh et al., 2016). Cultivar Northfield (ILL5588) has been a common parent in the majority of the published studies. Evaluation of resistance has generally been performed at the seedling stage, 11–28 days after infection, although Gupta et al. (2012) co-assessed resistance in both the seedling and mature pod-bearing plant. Most studies revealed multiple QTLs for AB resistance, with magnitude varying from 3 to 89% of the phenotypic variance ($V_p$). Due to differing nomenclature systems for linkage groups (LGs) and a dearth of common marker loci between genetic maps, common QTL locations between studies are difficult to establish. Nonetheless, AB_NF1 on LG6 in the study of Sudheesh et al. (2016) is comparable in position to QTL5 on LG1 of Rubeena et al. (2006) and QTL1 on LG1 of Gupta et al. (2012), based on a common SSR locus location. QTLs have also been correlated with known resistance determinants such the dominant *Ral2* and $AbR_1$ and recessive *ral2* genes (Ford et al., 1999; Chowdhury et al., 2001; Tar'an et al., 2003b).

Although the full genetic basis of AB resistance is not known, screening of a range of lentil genotypes against differential *A. lentis* isolates has identified putative groupings of genotypes based on resistance profiles (Davidson et al., 2016). Molecular genetic marker studies have identified three trait-linked markers relevant to Australian breeding germplasm (Sudheesh et al., 2016). One of these markers, AB_IH1 (see **Table 1**), is linked to a key resistance gene, which predicted field AB resistance in more than 85% of diverse validation panel, composed both Australian and international germplasm. The currently described AB resistance-associated markers permit selection of two major resistance genes of importance, one from Indianhead and one from ILL5588. However, other lentil germplasm displays resistance to AB that is not explained by these resistance genes, implying that there are additional important resistance genes still to be located in the genome.

## PROGRESS IN BREEDING FOR AB RESISTANCE: AN AUSTRALIAN CASE STUDY

The Australian lentil cropping zone is located predominantly in regions of mild, wet winters, in which conditions conducive to fungal disease occur in most years. For this reason, resistance to AB has been considered a priority since the crop was first introduced to Australia, with a significant amount of research and breeding effort put into accessing and introgressing sources of resistance to *A. lentis*.

Given the similarities of climate, the Australian lentil breeding program was based on germplasm developed at ICARDA in Syria, which has been found to be the most readily adapted to Australian conditions. Indeed, many of the early lentil varieties were direct introductions from the ICARDA breeding program, namely cvs. Northfield (ILL5588), Nugget (ILL7180), Digger (ILL5722), Aldinga (ILL5750), and Cumra (ILL0590). Traits for appropriate phenology, yield potential, red lentil seed quality, as well as one source of aschochyta resistance (ILL5588) have

**TABLE 1 |** Details of the genetic linkage maps and linked markers for ascochyta blight resistance in lentil.

| Name of population | Assessment tissue–period (DAI*) | QTL name | Chromosome/ linkage group | Marker type | Markers associated with QTL | Phenotypic variation explained (%) | References |
|---|---|---|---|---|---|---|---|
| ILL5588 (cv. Northfield) x ILL6002 | Seedling-11 | – | – | RAPD | RV01–RB18 | 89 | Ford et al., 1999 |
| ILL5588 (cv. Northfield) x L692-16-1 | Seedling-14 | QTL 1 | LG 2 | RAPD, ISSR, RFLP, AFLP | $OPB18_{680}$ | 36 | Tar'anr et al., 2002 |
| | | QTL 2 | LG 4 | | $OPV1_{800}$ | 29 | |
| ILL5588 (cv. Northfield) x ILL7537 | Seedling-14 | QTL-1 | LG2 | RAPD, ISSR, AFLP | $W03_{1050}$–$S01_{750}$ | 11 | Rubeena et al., 2006 |
| | Seedling-21 | QTL-2 | LG2 | | $G04_{530}$–$AC02_{480}$ | 7 | |
| | | QTL-3 | LG4 | | $T16_{500}$–$C04_{580}$ | 7 | |
| | | QTL-4 | LG5 | | $U14_{560}$–$B08_{520}$ | 69 | |
| | | QTL-5 | LG1 | | $B18_{1100}$–$W08_{800}$ | 55 | |
| | Seedling-28 | QTL-2 | LG2 | | $P08_{1200}$–$G04_{530}$ | 9 | |
| | | QTL-4 | LG5 | | $U14_{560}$–$B08_{520}$ | 52 | |
| | | QTL-5 | LG1 | | $B18_{1100}$–$W08_{800}$ | | |
| ILL7537 x ILL6002 | Seedling-14 | QTL-6 | LGI | RAPD, ISSR, AFLP | C-CTA/M-$ACC_{190}$–C-TTA/M-$AC_{285}$ | 8 | Rubeena et al., 2006 |
| | | QTL-7 | LGI | | C-TTA/M-$AC_{285}$–C-TTA/M-$AC_{165}$ | 27 | |
| | | QTL-8 | LGII | | $M20_{700}$–C-GTA/M-$GC_{191}$ | 6 | |
| | Seedling-21 | QTL-6 | LGI | | C-CTA/M-$ACC_{190}$–C-TTA/M-$AC_{285}$ | 11 | |
| | | QTL-7 | LGI | | C-TTA/M-$AC_{285}$–C-TTA/M-$AC_{165}$ | 34 | |
| | | QTL-8 | LGII | | $M20_{700}$–C-GTA/M-$GC_{191}$ | 9 | |
| | Seedling-28 | QTL-6 | LGI | | C-CTA/M-$ACC_{190}$–C-TTA/M-$AC_{285}$ | 16 | |
| | | QTL-7 | LGI | | C-TTA/M-$AC_{285}$–C-TTA/M-$AC_{165}$ | 31 | |
| | | QTL-8 | LGII | | $M20_{700}$–C-GTA/M-$GC_{191}$ | 10 | |
| Eston x PI 320937 | Seedling-10 | QTL | LG-6 | RAPD, AFLP, SSR | cagaggE | 41 | Tullu et al., 2006 |
| ILL5588 (cv. Northfield) x ILL5722 (cv. Digger) | Seedling-14 | QTL 1 | LG1 | EST-SSR/SSR, ISSR, RAPD, ITAP | DK 225–UBC825c | 6 | Gupta et al., 2012 |
| | | QTL 3 | LG9 | | UBC890–ARG10 | 3 | |
| | Seedling-21 | QTL 2 | LG1 | | AC097a–V20a | 8 | |
| | | QTL 3 | LG9 | | UBC890–ARG10 | 6 | |
| | Seedling-28 | QTL 2 | LG1 | | AC097a–V20a | 7 | |
| | | QTL 3 | LG9 | | UBC890–ARG10 | 4 | |
| | Pod/maturity-14 | QTL 4 | LG1 | | ILMs25–UBC857b | 7 | |
| | | QTL 5 | LG4 | | UBC855a–UBC830b | 7 | |
| | | QTL 6 | LG5 | | UBC807a–Lup91 | 7 | |
| | Pod/maturity-21 | QTL 4 | LG1 | | ILMs25–UBC857b | 8 | |
| | | QTL 5 | LG4 | | UBC855a–UBC830b | 7 | |
| | | QTL 6 | LG5 | | UBC807a–Lup91 | 6 | |
| | Pod/maturity-28 | QTL 4 | LG1 | | ILMs25–UBC857b | 6 | |
| | | QTL 5 | LG4 | | UBC855a–UBC830b | 7 | |
| | | QTL 6 | LG5 | | UBC807a–Lup91 | 6 | |

*(Continued)*

**TABLE 1** | Continued

| Name of population | Assessment tissue–period (DAI*) | QTL name | Chromosome/ linkage group | Marker type | Markers associated with QTL | Phenotypic variation explained (%) | References |
|---|---|---|---|---|---|---|---|
| Indianhead x Northfield | Seedling-14 | AB_IH1 | LG2 | Genomic DNA-derived SSR, –EST-SSR, SNP | PBA_LC_0629–SNP_20005010 | 47 | Sudheesh et al., 2016 |
| | | AB_IH1.2 | LG3 | | SNP_20002370–SNP_20002371 | 15 | |
| | | AB_NF1 | LG6 | | SNP_20001370–SNP_20001765 | 7 | |
| Indianhead x Digger | Seedling-14 | AB_IH1 | LG2 | Genomic DNA-derived SSR, –EST-SSR, SNP | SNP_20005010–SNP_20004695 | 30 | Sudheesh et al., 2016 |
| | | AB_IH1.3 | LG3 | | SNP_20000505–SNP_20000553 | 22 | |

*DAI, Days after inoculation.*

been derived from germplasm originating in the Near East. Traits for early vigor and improved biomass were introduced from green lentil germplasm, via North American germplasm, such as cv. Palouse. In terms of disease resistance, the Australian lentil breeding program has benefitted directly from research on AB conducted at the University of Saskatchewan, having utilized AB resistance genes obtained from cv. Indianhead and one of its progeny, cv. CDC Matador.

In the 25 years of lentil breeding in Australia, the program has successfully combined multiple sources, both major and minor, of resistance to AB. These have included the unique resistance sources of ILL5588, Indianhead and potentially another unidentified resistance source (represented by cv. PBA Jumbo2), as well partial (minor) resistance genes, such as those from cv. Digger. To achieve this outcome, the program has relied heavily on field selection for resistance within breeding germplasm, using simple selection methods such as spreading of naturally infected crop residues at sites with reliably cool, wet winters, such as at Horsham, Victoria. Phenotypic selection for resistance has been routinely performed on a whole plot basis, so selecting within families to maintain key resistance genes in breeding germplasm.

The result of this breeding effort has been to obtain a high incidence of resistance to current AB pathogen populations in Australian lentil germplasm. This resistance is also robust, and there are multiple lentil cultivars, such as PBA Ace and PBA Jumbo2, which are effectively immune to the dominant AB isolates when tested in the field or under highly controlled conditions.

Under the disease pressure conditions of the southern Australian cropping environment, intensive lentil production has more recently led to the selection of aggressive pathogen isolates that are able to overcome the major resistance genes which are found in dominant cultivars. As previously described, this has resulted in the loss of effectiveness of the important resistance gene derived from ILL5588 (Davidson et al., 2016). Subsequently, in 2016, pathologists found evidence of isolates able to overcome the key resistance gene linked to AB_IH1 (unpublished data, Blake and Davidson, SARDI, March 2017). This gene, derived from cv. Indianhead, has been widely incorporated in both the Australian and Canadian breeding programs, and the finding identifies a significant threat to the AB resistance of current lentil germplasm. A significant change in the pathogen population could render a substantial proportion of existing Australian breeding material more susceptible. For this reason, beginning in 2016, a pre-emptive breeding strategy based on controlled environment selection was initiated to address the challenge. The other ramification of a potential change is that unless new genetic markers are identified, the Australian breeding program may soon be without a means to effectively predict field-based AB resistance.

For several years, the Australia lentil breeding program has been investing in crosses to incorporate additional unique resistance sources, including ILL7537, which has not been extensively exploited within program. However, field-based selection for new resistance genes is not possible while the current genes are effective. New molecular genetic markers would address this problem, and current research is underway to address the issue. In the meantime, as research effort has applied to study of pathogen diversity in Australia (Davidson et al., 2016), differential sets of isolates offer a good opportunity for phenotypic selection.

## FUTURE PROSPECTS FOR RESISTANCE BREEDING IN THE GENOMIC ERA

Recent significant advancements in genomic technologies have opened up new opportunities and enabled new strategies in crop breeding. The genome sequences from model and non-model legumes such as *Medicago truncatula* Gaertn., *Lotus japonicas* L., soybean and chickpea are available in the public domain, and

so may be used for comparative genomics analysis. These model plant species permit better understanding of plant development, responses to biotic stresses, and evolution. Conserved synteny between the genomes of legume species has been investigated over the last 20 years, as revealed by comparisons of both genetic maps and fully sequenced genomes (Gujaria-Verma et al., 2014). An international sequencing effort is currently underway with the goal to deliver a reference lentil genome, leading to the recent release of an initial draft genome assembly from the cultivar CDC Redberry (Bett et al., 2016). However, this draft genome sequencing information has limited usage with minimal gene annotation and restricted access (http://knowpulse.usask.ca/portal/). Availability of an improved and well annotated lentil genome assembly in future will allow the identification of diagnostic markers for ascochyta blight resistance and assist breeders to track the trait more effectively. This will eventually improve the rate of selection for ascochyta blight resistance and accelerate the rate of varietal development.

Trait dissection for AB resistance has been exclusively based on the use of biparental genetic mapping populations. However, this approach is a laborious and resource-intensive way to identify marker-trait associations from multiple germplasm sources, including ecotypes and land-races. The availability of large numbers of genome-wide distributed SNP markers, especially following completion of the current effort to determine the lentil genome sequence, will permit implementation of genome-wide association mapping studies (GWASs) (Huang and Han, 2014), based on analysis of customized germplasm collections. The resolution of such studies is typically higher than for linkage mapping, permitting discovery of more closely associated genetic markers. The identification of such sequence polymorphism to physical locations within the genome, either through comparative genomics with model legume species such as *M. truncatula*, or on the draft lentil genome sequence directly, will support prediction of candidate genes for AB resistance. Such genes may include resistance (R) genes involved in pathogen race-specific interactions, such as the nucleotide binding site—leucine-rich repeat (NBS-LRR) class, or more generic defense response genes such as chitinases and glucanases. Given identification of such candidate genes, direct modification through the use of genetic transformation or gene editing may be used to verify identity and potentially transfer specific resistance genes into recipient varieties, in order to accelerate the breeding process. Such approaches, however, will require highly efficient plant transformation and regeneration processes for lentil (Akcay et al., 2009). Densely distributed genome-wide markers will also support the use of genomic selection strategies (Meuwissen et al., 2001; Newell and Jannink, 2014), in which the genetic merits of individual genotypes within a breeding program are predicted on the basis of a genomic estimated breeding value (GEBV) derived from the summation of contributory gene effects across the genome.

## CONCLUSIONS

AB, caused by *A. lentis*, is an important disease of lentil throughout the world, causing serious yield losses of up to 70% in extreme cases (Gossen and Morrall, 1983). The most efficient means to control this disease is to breed for host resistance without the need for additional inputs. Extensive searches for AB resistance in lentil have been conducted through screening of germplasm, including cultivated varieties, landraces, and closely related species. To accelerate the process of introgressing AB resistance genes into elite backgrounds, molecular genetic tools can be combined with conventional breeding approaches. Molecular markers associated with AB resistance QTLs have been positioned on linkage maps, and these markers can be used for efficient pyramiding of the disease resistance genes.

Significant achievements have been made in lentil genomics to detect important genes that are involved in AB resistance. Valuable resources, such as an integrated genetic linkage map, EST libraries, gene based markers, and draft genome sequences have been generated. The comparative genomics approaches enabled the identification of candidate genes, however, they have not yet been used directly to improve lentil cultivars in the field, but it is highly likely that these approaches will be more commonly used in near future. The availability of large numbers of molecular genetic markers in lentil will also allow the implementation of GWAS and genomic selection approaches. This will further assist in the identification of more closely linked markers for AB resistance in lentil that can be effectively used in breeding. Genomic selection methods will be useful to calculate prediction values for AB resistance in different sets of germplasm and help to trace inheritance of the trait in future generations.

## AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript text. SK prepared the sections on introduction, molecular markers, and genomics. MR wrote the section on breeding aspects. JD and SB drafted the pathology section. SS contributed to the molecular markers and marker assisted selection. MJ drafted the section on genetics basis for resistance and JF prepared the section on future prospects as well as edited the article. All authors read and approved the manuscript.

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# SNP-Based Linkage Mapping for Validation of QTLs for Resistance to Ascochyta Blight in Lentil

*Shimna Sudheesh[1], Matthew S. Rodda[2], Jenny Davidson[3], Muhammad Javid[2], Amber Stephens[1], Anthony T. Slater[1], Noel O. I. Cogan[1,4], John W. Forster[1,4] and Sukhjiwan Kaur[1]**

[1] Biosciences Research, Agriculture Victoria, AgriBio, La Trobe University, Bundoora, VIC, Australia, [2] Biosciences Research, Agriculture Victoria, Horsham, VIC, Australia, [3] South Australia Research and Development Institute, Plant Research Centre, Urrbrae, SA, Australia, [4] School of Applied Systems Biology, La Trobe University, Bundoora, VIC, Australia

Lentil (*Lens culinaris* Medik.) is a self-pollinating, diploid, annual, cool-season, food legume crop that is cultivated throughout the world. Ascochyta blight (AB), caused by *Ascochyta lentis* Vassilievsky, is an economically important and widespread disease of lentil. Development of cultivars with high levels of durable resistance provides an environmentally acceptable and economically feasible method for AB control. A detailed understanding of the genetic basis of AB resistance is hence highly desirable, in order to obtain insight into the number and influence of resistance genes. Genetic linkage maps based on single nucleotide polymorphisms (SNP) and simple sequence repeat (SSR) markers have been developed from three recombinant inbred line (RIL) populations. The IH × NF map contained 460 loci across 1461.6 cM, while the IH × DIG map contained 329 loci across 1302.5 cM and the third map, NF × DIG contained 330 loci across 1914.1 cM. Data from these maps were combined with a map from a previously published study through use of bridging markers to generate a consensus linkage map containing 689 loci distributed across seven linkage groups (LGs), with a cumulative length of 2429.61 cM at an average density of one marker per 3.5 cM. Trait dissection of AB resistance was performed for the RIL populations, identifying totals of two and three quantitative trait loci (QTLs) explaining 52 and 69% of phenotypic variation for resistance to infection in the IH × DIG and IH × NF populations, respectively. Presence of common markers in the vicinity of the AB_IH1- and AB_IH2.1/AB_IH2.2-containing regions on both maps supports the inference that a common genomic region is responsible for conferring resistance and is associated with the resistant parent, Indianhead. The third QTL was derived from Northfield. Evaluation of markers associated with AB resistance across a diverse lentil germplasm panel revealed that the identity of alleles associated with AB_IH1 predicted the phenotypic responses with high levels of accuracy (~86%), and therefore have the potential to be widely adopted in lentil breeding programs. The availability of RIL-based maps, a consensus map, and validated markers linked to AB resistance provide important resources for lentil improvement.

**Keywords: legume, pulse, single nucleotide polymorphism, fungal disease resistance, molecular breeding**

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*Liezhao Liu, Southwest University, China*

***Correspondence:**
*Sukhjiwan Kaur*
*sukhjiwan.kaur@ecodev.vic.gov.au*

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## INTRODUCTION

Lentil (*Lens culinaris* Medik.) is a self-pollinating, cool-season, grain legume crop that is produced throughout the world and is valued due to its high protein content. However, lentil production is limited by a number of abiotic and biotic stress factors (Erskine et al., 1994), and fungal diseases of particular significance are ascochyta blight (AB), fusarium wilt, rust, stemphylium blight, anthracnose, and botrytis gray mold (Taylor et al., 2007).

AB is the major disease problem in many lentil-producing countries, including Australia, Canada, Argentina, Ethiopia, India, New Zealand, and Pakistan (Erskine et al., 1994; Ye et al., 2002). AB in lentil is caused by the ascomycete species *Ascochyta lentis* Vassilievsky. The disease causes lesions on stems, leaves, petioles, and pods. Plant death is common following seedling infection, while infection of mature plants leads to the reduction in vigor, with subsequent decrease in the yield and quality of the seed (Morrall and Sheppard, 1981). Yield losses of up to 40% due to foliar infection have been reported, but the loss of economic value due to seed damage may be more than 70%, as seed can quickly become unsaleable (Gossen and Morrall, 1983, 1984; Brouwer et al., 1985). AB may be controlled through the use of fungicides (Bretag, 1989; Ahmed and Beniwal, 1991), but the most effective, economic, and environmentally sustainable method of control is the development of disease resistant cultivars (Ye et al., 2002).

A number of sources of genetic resistance to AB have been identified (Ahmad et al., 1997; Ford et al., 1999; Nguyen et al., 2001; Ye et al., 2003), including in cultivars such as Indianhead and Northfield (syn. ILL5588) which have been extensively exploited by lentil breeding programs, especially in Australia and Canada (Tar'an et al., 2003). Two independent AB resistance genes, *Ral2* (dominant) and *ral2* (recessive) were identified from Northfield and Indianhead, respectively (Andrahennadi, 1994; Chowdhury et al., 2001). A third (dominant) resistance gene $AbR_1$, that is active in foliar tissue was also identified in Northfield on the basis of genetic segregation (Tay and Slinkard, 1989) and genetic mapping studies (Ford et al., 1999). Ye et al. (2003) also identified two dominant resistance genes in Northfield, controlling major and moderate resistance, respectively, as well as two additive recessive genes in Indianhead. However, a limited number of disease resistance genes have been placed on lentil genetic linkage maps (Ford et al., 1999; Tar'an et al., 2003). Molecular genetic marker loci such as those generated by random amplified polymorphic DNA (RAPDs) and sequence characterized amplified region (SCARs) systems, were associated with all the known AB resistance genes from Indianhead and Northfield (Ford et al., 1999; Chowdhury et al., 2001; Tar'an et al., 2003).

An additional novel source of resistance, accession ILL7537, exhibits resistance to a number of Australian pathogen groups at a higher level of resistance than either Indianhead or Northfield (Nguyen et al., 2001). The resistance phenotype is thought to be due to at least two dominant resistance genes, distinct to those of Northfield, which were characterized by crosses with susceptible genotypes and subsequent quantitative trait loci (QTL) identification (Rubeena et al., 2006).

Some conflicting results have been obtained from studies of the genetic basis of resistance to infection by *A. lentis*, possibly due to the effects of multiple phenotypic screening methods, variable environmental conditions and variation in the size of evaluated populations (Ford et al., 1999). Pathogen diversity also contributes to the variable assessments of resistance status. Isolates capable of overcoming the dominant resistance gene derived from Northfield are now well-characterized, and have been found in Australia and Canada (Tar'an et al., 2003; Davidson et al., 2016). Given the historical importance in Australia of the formerly resistant cultivar Nipper, of which Northfield and Indianhead are parents, the newer aggressive isolates have been termed "Nipper-virulent" (Davidson et al., 2016). The breakdown in resistance in Nipper also coincided with a reduction in the resistance of a number of Australian cultivars for which resistance was derived from Northfield. However, it has been determined that Nipper does not contain a major resistance gene from Indianhead, unlike a number of other resistant cultivars, suggesting that Indianhead-derived genes are still capable of conferring full resistance against a large proportion of a pathogen population in the field (Davidson et al., 2016). This is also the case in Canada (Albert Vandenberg, pers. comm.), in which AB is an important fungal diseases.

Molecular genetic markers in close linkage with AB resistance genes would permit accelerated development of elite lentil genotypes with resistance to this disease. However, the technologies that have previously been used (Ford et al., 1999; Chowdhury et al., 2001; Tar'an et al., 2003; Rubeena et al., 2006) are not optimal for diagnostic screening in a breeding programme. In addition, previous molecular genetic marker-based maps of lentil have typically been low-density, which limit the capacity to identify marker loci in sufficiently close linkage. However, a number of transcriptome sequencing studies for lentil have generated expressed sequence tag (EST) databases, delivering large numbers of EST-derived (and hence gene-associated) simple sequence repeat (SSR) and single nucleotide polymorphisms (SNP) markers (Kaur et al., 2011, 2014; Sharpe et al., 2013). These marker systems have been used to construct dense genetic linkage maps, and to identify QTLs (Sharpe et al., 2013; Kaur et al., 2014). Sequence-linked genetic markers also facilitate the identification of bridging loci between population-specific genetic maps, and subsequent integration to produce high-density consensus structures (Sudheesh et al., 2015a,b).

The present study describes the construction of genetic maps for three populations derived from pair-wise combinations of the lentil varieties Indianhead, Northfield, and Digger. Although partial breakdown of the Northfield-type AB resistance has occurred (Davidson et al., 2016), QTLs for the effective Indianhead-type resistance were identified. The predictive capacity of markers linked to AB resistance genes was also tested using a diverse germplasm collection, or "validation

**Abbreviations:** AB, Ascochyta blight; EST, Expressed sequence tag LG, Linkage group; MAS, Marker-assisted selection; QTL, Quantitative trait locus; RAPD, Random amplified polymorphic DNA; RIL, Recombinant inbred line; SCAR, Sequence characterized amplified region; SNP, Single nucleotide polymorphism; SSR, Simple sequence repeat; R, Resistance.

panel." The population-specific maps were integrated to form a consensus structure suitable for application in lentil molecular breeding.

# MATERIALS AND METHODS

## Plant Materials

Two segregating genetic mapping populations were developed from crosses between single genotypes of Indianhead (resistant to AB) with Northfield (previously resistant to AB) and Digger (moderately resistant to AB), respectively. The third genetic mapping population was developed by crossing single genotypes from Northfield and Digger. All three populations were initiated at DEDJTR-Horsham in 2002, based on single seed descent from $F_2$ progeny for four generations in the glasshouse to generate the following $F_6$ recombinant inbred line (RILs): Indianhead × Northfield [IH × NF] – 117 RILs; Indianhead × Digger [IH × DIG] – 112 RILs; and Northfield × Digger [NF × DIG] – 114 RILs.

A germplasm panel composed of a set of 79 diverse lentil genotypes was used for validation of AB resistance-linked markers. The panel included Australian lentil cultivars, varieties, and breeding germplasm, along with international lentil germplasm from the International Center for Agricultural Research in the Dry Areas (ICARDA) and North American breeding programs (see **Supplementary Table 1** for list).

Plants were grown in glasshouse at 20 ± 2°C under a 16/8 h light/dark photoperiod regime. Genomic DNA was extracted from young leaves using the DNeasy® 96 Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Approximately 6–8 leaflets per sample were used for each extraction, and were ground using a Mixer Mill 300 (Retsch®, Haan, Germany). DNA was resuspended in milliQ water to a concentration of 50 ng/μl and stored at −20°C until further use.

## SSR and SNP Genotyping and Genetic Linkage Mapping

Genomic DNA-derived (Hamwieh et al., 2005) and EST-derived SSRs (Kaur et al., 2011) were screened on the mapping parents for polymorphism detection, and the resulting polymorphic markers were screened on the RILs as described previously (Schuelke, 2000; Kaur et al., 2014). For SNP genotyping, a previously described set of 768-plex SNPs (Kaur et al., 2014), was selected and genotyped using the GoldenGate™ oligonucleotide pooled assay (OPA; Illumina Inc., San Diego, USA). Genetic linkage mapping and visualization of the linkage groups (LGs) of RILs were performed as described previously (Kaur et al., 2014). All sequences underlying mapped SNP markers from the present study were analyzed with BLASTN against the equivalent sequences of Sharpe et al. (2013) and the *Medicago truncatula* genome (Mt4.0) at a threshold E-value of $10^{-10}$. This information was used to assign identity and orientation to the lentil LGs. Visual comparisons between genetic linkage maps were performed using the Strudel software package (Bayer et al., 2011).

## Consensus Linkage Map Construction

The genetic maps from the present study were combined with the Cassab × ILL2024 map of Kaur et al. (2014), which shared a high proportion of common markers, to generate a consensus map using MergeMap (Wu et al., 2011). Each LG from the consensus linkage map was visualized using MapChart (Voorrips, 2002). The visual comparison of the consensus map with individual RIL-based maps was performed using the Strudel software package (Bayer et al., 2011).

A comparative analysis of this consensus map was made to the sequence-based map of Sharpe et al. (2013) as well as the pseudomolecules of *M. truncatula*, using BLASTN analysis of sequences underlying mapped SNP markers. Dot-plots of the comparison of the consensus linkage map and *M. truncatula* pseudomolecules were generated using the R software package with the xyplot function from the Lattice CRAN library (Sarkar, 2014).

## Phenotypic Assessment of AB Resistance

Resistance to AB was assessed for the three RIL mapping populations in four separate experiments (single experiment for each of IH × NF and IH × DIG, two experiments for NF × DIG). A total of 3–6 seeds from each RIL and respective parents were sown into individual pots (8 × 18 × 6.5 cm), filled with Van Schaik's Bio Gro (Bio Gro Pty. Ltd., Victoria, Australia) pine bark potting mix. The potting mix consisted of 1000 L of composted pine bark (Bio Gro), 1 kg Floranid® N32 (Compo, Münster, Germany), 1 kg 8–9 month Osmocote® (Scotts, NSW, Australia), 1 kg 3–4 month Osmocote® (Scotts), 225 g micronutrients MicroMax® Complete (Scotts), 225 g SP Quality® FeEDDHA Chelate (6% Fe; Libfer-BASF, Victoria, Australia), 30 kg agricultural lime (Sibelco, Victoria, Australia), and 2 kg Saturaid® (Debco, NSW, Australia). After sowing, the pots were placed in a controlled environment room (CER) at 15°C, under a 12/12 h light/dark cycle regime in four plastic tents (160 × 80 × 80 cm) in a randomized complete block design, with one replicate per tent. Pots were watered by hand as required. Seedlings were inoculated 2 weeks after sowing, as described below.

Three different isolates of *A. lentis* (**Supplementary Table 2**) were used, the Australian reference isolate AL4 being applied to NF × DIG, isolate FT13038 to IH × NF, and isolate FT12013 to IH × DIG. Parents of the mapping populations exhibit different level of resistance to *A. lentis* isolates, hence isolates which could most effectively distinguish between the parents were selected for application on mapping populations. AB resistance-specific screening of the germplasm panel was conducted using FT12013, which has been isolated recently and is known to have overcome the resistance of at least one of the resistance (R) genes present in cultivars Northfield and Nipper (Davidson et al., 2016). A sub-set of germplasm panel lines were further evaluated using multiple pathogen isolates (**Supplementary Table 2**).

To initiate fungal cultures, mycelial plugs of *A. lentis* isolates were transferred from storage vials to potato dextrose agar (PDA) in 9 cm diameter culture dishes and incubated for 14 days under fluorescent light and near ultraviolet light under a 12/12 h light/dark cycle regime at room temperature. A 1

L conidial suspension of the isolate was prepared by flooding the plates with sterile reverse osmosis (RO) water and gently rubbing the culture surface with a sterile glass rod to suspend the conidia. The concentration was determined with the aid of a hemocytometer, adjusted to $1 \times 10^6$ conidia $mL^{-1}$ and the surfactant Tween 20 [0.02% (v/v)] was added. The conidial suspension was sprayed onto the seedlings until run-off occurred. Each tent had two ultrasonic humidifiers, one at either end, using RO water to maintain leaf wetness. The two ultrasonic humidifiers within each tent were switched on immediately after inoculation for 1 h and every day thereafter for 1 h to promote lesion development which continued until disease assessment could be performed. Disease incidence was assessed for each seedling 14 days after inoculation as percentage area of plant diseased (% APD), incorporating leaf and stem lesions (Davidson et al., 2016).

Data was analyzed to estimate genotype-specific adjusted means for any spatial effects using residual maximum likelihood (REML) in Genstat v14.1 (Lane et al., 2011). Means of % APD data from each trial were used to construct frequency distribution histograms.

## QTL Analysis and Identification of Sequences Associated with Flanking Genetic Markers

QTL detection was performed using marker regression, simple interval mapping (SIM) and composite interval mapping (CIM) in QTL Cartographer v 2.5 (Wang et al., 2012). For SIM, an arbitrary LOD threshold of 2.5 was used to determine significance, while for CIM, significance levels for LOD thresholds were determined using 1000 permutations. SIM and CIM analysis of the NF × DIG mapping population for both experimental treatments failed to identify any QTL associated with AB resistance. Data for this population was consequently not considered further for trait-dissection purposes, but was used for consensus linkage map construction.

## Genotyping of the Diverse Germplasm Panel

Genetic markers flanking AB resistance QTL-containing intervals from the IH × NF and IH × DIG mapping populations were used for genotypic analysis. SSR primer synthesis and PCR amplifications were performed as described above. SNP genotyping was performed using KASP™ genotyping chemistry (LGC, Middlesex, UK) as described in Javid et al. (2015).

# RESULTS

## Polymorphic Markers for Map Construction

A total of 546 publicly available SSR markers (30 genomic DNA-derived SSRs and 516 EST-SSRs) were screened for polymorphism detection in the mapping populations. Of the former, up to 87% detected polymorphisms, while relatively smaller proportions of the EST-SSR markers were polymorphic (**Table 1**). After the $\chi^2$ analysis ($P < 0.05$), final sets containing a maximum of 61 (IH × NF) and a minimum of 31 (IH × DIG) segregating SSR markers were used for linkage mapping.

A commonly used set of 768 SNPs was screened on the mapping populations, of which 328 (NF × DIG) to 435 (IH × NF) detected polymorphism (**Table 1**). A small number (24) of polymorphic loci were shared between all three mapping populations, but up to 490 loci were common between any two populations. After the $\chi^2$ analysis ($P < 0.05$), SNP markers that did not segregate in accordance with the expected Mendelian inheritance ratio were excluded, which resulted in a final set of up to 422 SNP markers (IH × NF; **Table 1**).

## Genetic Linkage Mapping

A total of 483 loci (IH × NF), 346 loci (IH × DIG), and 367 loci (NF × DIG) were used for linkage mapping (**Table 1**). Details of the number of assigned LGs, markers, and the cumulative length of maps are provided in **Table 2** and **Supplementary Table 3**. The proportion of loci assigned to LGs was 95.3, 95.1, and 89.9% for the IH × NF, IH × DIG, and NF × DIG maps, respectively, while the remaining markers were unlinked. The IH × NF map contained a higher number of markers with lower average marker density than the other two maps. The distribution of markers was not uniform across the LGs, as some regions of high and low marker density were observed. Significant commonality of marker order was observed between the three maps, although distances were not always in similar proportion (**Supplementary Figures 1A–C**). Some markers (52 in total) were assigned to different LGs on the various maps. One such major anomaly was observed for the IH × DIG and NF × DIG maps, in which 49 markers (45 SNPs and 4 SSRs) were located in a segment on LG4, while for the IH × NF map, the corresponding positions of those markers were on LG6 (**Supplementary Figure 1D**). Sequence similarity searches against the *M. truncatula* genome of DNA sequences underlying those SNP loci revealed matches to MtChr7 (**Supplementary Table 4**), which displays macrosynteny with lentil LG6.

BLASTN analysis of the DNA sequences corresponding to 163 map-assigned SNPs detected significant similarity matches to 102 sequences assigned to the genetic map of Sharpe et al. (2013) (**Supplementary Table 4**). This analysis supported establishment of bridging loci between six LGs (LG1, 2, 3, 4, 5, and 7), although no common markers could be identified for LG6 (**Table 3**). Marker order was generally co-linear between the two studies, although minor discrepancies were observed for some markers.

## Consensus Linkage Map Construction

Data from the mapping populations described in the present study and a previously published mapping population (Cassab × ILL2024, containing 318 markers) was used to construct the consensus linkage map of lentil. The common markers on homologous LGs from the RIL-based maps served as bridges for integration into a consensus structure. A total of 149 markers were unique to single populations (62 – IH × NF; 15 – IH × DIG; 34 – NF × DIG; 38 – Cassab × ILL2024), the remainder acting as bridging loci between two or more maps. As the SNP marker sets were selected to obtain a large number of

**TABLE 1 | Total number of markers analyzed, tested for polymorphism, and assigned to genetic linkage map locations.**

| Marker type | Total number of markers screened | Polymorphic markers | | | Markers used for linkage mapping | | | Mapped markers | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IH × NF | IH × DIG | NF × DIG | IH × NF | IH × DIG | NF × DIG | IH × NF | IH × DIG | NF × DIG |
| Genomic DNA-derived SSR | 30 | 26 | 19 | 22 | 17 | 9 | 22 | 11 | 7 | 16 |
| EST SSR | 516 | 45 | 42 | 35 | 44 | 22 | 35 | 31 | 22 | 28 |
| SNP | 768 | 435 | 329 | 328 | 422 | 315 | 310 | 418 | 300 | 286 |
| Total markers | 1314 | 577 | 451 | 442 | 483 | 346 | 367 | 460 | 329 | 330 |

**TABLE 2 | Marker distribution over the LGs of IH × NF, IH × DIG, and NF × DIG genetic linkage maps.**

| Linkage group | Map length (cM) | | | Number of mapped marker | | | Average marker density | | |
|---|---|---|---|---|---|---|---|---|---|
| | IH × NF | IH × DIG | NF × DIG | IH × NF | IH × DIG | NF × DIG | IH × NF | IH × DIG | NF × DIG |
| LG1 | 199.1 | 143.4 | 74.1 | 44 | 32 | 8 | 4.5 | 4.5 | 9.3 |
| LG1.2 | | 18.8 | 182.3 | | 7 | 29 | | 2.7 | 6.3 |
| LG2 | 195.7 | 87.9 | 126.3 | 84 | 29 | 21 | 2.3 | 3.0 | 6.0 |
| LG2.2 | 13.5 | 211.5 | 155.3 | 3 | 35 | 30 | 4.5 | 6.0 | 5.2 |
| LG3 | 151.7 | 245.5 | 163.8 | 53 | 65 | 26 | 2.9 | 3.8 | 6.3 |
| LG3.2 | 31.9 | 14 | 29 | 12 | 3 | 7 | 2.7 | 4.7 | 4.1 |
| LG3.3 | | | 73.9 | | | 7 | | | 10.6 |
| LG3.4 | | | 11.3 | | | 5 | | | 2.3 |
| LG4 | 310.2 | 205.7 | 431.6 | 80 | 78 | 88 | 3.9 | 2.6 | 4.9 |
| LG5 | 187.3 | 197.2 | 310 | 58 | 38 | 46 | 3.2 | 5.2 | 6.7 |
| LG6 | 190.4 | 22.8 | 38.8 | 69 | 4 | 6 | 2.8 | 5.7 | 6.5 |
| LG7 | 176.3 | 13.2 | 280 | 54 | 6 | 52 | 3.3 | 2.2 | 5.4 |
| LG7.2 | 5.5 | 40.5 | 37.7 | 3 | 8 | 5 | 1.8 | 5.1 | 7.5 |
| LG7.3 | | 102 | | | 24 | | | 4.3 | |
| | 1461.6 | 1302.5 | 1914.1 | 460 | 329 | 330 | 3.2 | 4.1 | 6.2 |

**TABLE 3 | Marker distribution over the LGs of the consensus linkage map.**

| Linkage group | Predicted Mt chromosome | LG from Sharpe et al. (2013) | Number of mapped markers | Map length (cM) | Average marker density |
|---|---|---|---|---|---|
| LG1 | 1/5 | 1 | 79 | 332.9 | 4.2 |
| LG2 | 2/6 | 2 | 131 | 429.7 | 3.3 |
| LG3 | 3 | 3 | 110 | 353.0 | 3.2 |
| LG4 | 4/7/8 | 4 | 117 | 398.3 | 3.4 |
| LG5 | 5/1 | 5 | 94 | 403.1 | 4.3 |
| LG6 | 7 | | 72 | 192.7 | 2.7 |
| LG7 | 4/8 | 7 | 86 | 319.9 | 3.7 |
| Total | | | 689 | 2429.6 | 3.5 |

polymorphic markers for all populations under study, only a small number of markers (18) were common across all four RIL-based maps. The largest number of common markers (113) was between the IH × NF – IH × DIG maps, followed by the IH × NF – NF × DIG (92) and NF × DIG – Cassab × ILL2024 (17) comparisons. The 52 markers that did not display consistent LG assignment were excluded. IH × NF linkage map was used as the skeleton map as that map contained the highest number of markers, with lower average marker density than other three maps. Also, the IH × NF linkage map revealed a high degree of colinearity of marker order when compared to previously published lentil maps (Sharpe et al., 2013; Kaur et al., 2014), as well as a superior level of conserved synteny with the genome of *M. truncatula*. Finally, 689 marker loci (94 SSRs and 595 SNPs) were assembled into seven LGs (**Figure 1**, **Table 3**, **Supplementary Table 5**), with a total length of 2429.6 cM, lengths of LGs varying from 192.7 cM (LG6) to 429.7 cM (LG2), with an average density of one marker per 3.5 cM. The marker order of consensus map was largely colinear between the individual RIL-based maps, although several inversions and local rearrangements were observed (**Supplementary Figures 2A–C**).

Of the 689 markers assigned to the seven LGs of the lentil consensus map, 522 (76%) identified orthologous sequences on the eight *M. truncatula* chromosomes, with a minimum of 62% (LG1) and a maximum of 88% (LG6). The relative correspondences and orientations of consensus map LGs and *M. truncatula* pseudomolecules were determined by examining dot-plots, which showed large segments of conserved macrosynteny, as anticipated (**Figure 2**). LGs 3 and 6 were relatively colinear along their entire length with pseudomolecules

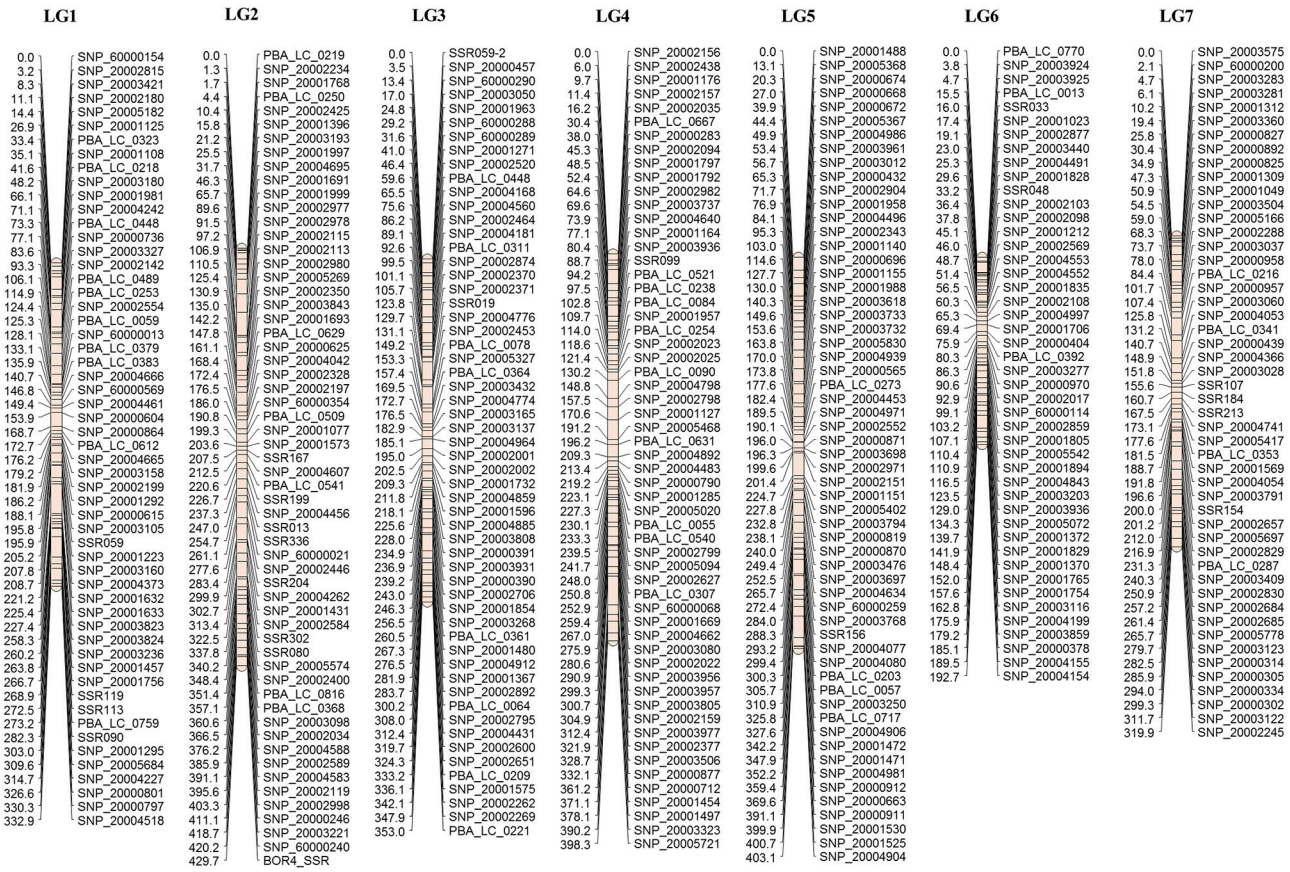


**FIGURE 1 | Consensus map of lentil, with marker loci shown on the right of LGs, and distances between markers indicated in cM on the left.** For presentation purposes, only one of a set of co-located genetic markers is shown on the map.

3 and 7. Comparative analysis also indicated that some genome rearrangements have occurred in lentil. For example, LG2 exhibited macrosynteny with pseudomolecules 2 and 6 (**Figure 2**), and major evolutionary translocations were observed for pseudomolecules 1 and 5 relative to LG1 and 5 of lentil. LG7 showed significant matches to positions on pseudomolecules 4 and 8, while LG4 showed similarity to genomic regions on pseudomolecules 4, 7, and 8.

## Phenotypic Analysis of RIL Populations and QTL Detection

Significant differences in plant symptom scores (%) for parents and RILs of each mapping population were observed following infection with *A. lentis* isolates. Severity of AB infection varied significantly for the IH × NF mapping population, scores ranging from 0 to 30%. The Indianhead and Northfield parents showed 0 and 12% infection, respectively, while a small proportion of RILs displayed transgressive segregation toward scores characteristic of higher susceptibility than Northfield. Similar effects were observed for the IH × DIG mapping population (Indianhead, 0%; Digger, 5%; RILs, 0–30%; **Supplementary Figure 3**).

For IH × NF, CIM analysis detected three QTLs (AB_IH1, AB_IH2.1, and AB_NF1) associated with AB resistance, on LG2, LG3, and LG6, explaining c. 47, 15, and 7% of the phenotypic variance ($V_p$), respectively (**Figure 3**, **Table 4**). For AB_NF1, the resistance determinant was derived from Northfield, while the other two QTL regions were associated with the resistant parent, Indianhead. For IH × DIG, CIM detected two QTLs from IH (AB_IH1 and AB_IH2.2), which were at the same locations (LG2 and LG3) as those identified in the IH × NF mapping population, accounting for c. 30 and 22% of $V_p$, respectively (**Figure 3**, **Table 4**). The LOD peak of AB_IH1 coincided with the markers PBA_LC_0629 and SNP_20005010 for both mapping populations, while the markers flanking AB_IH2.2 (SNP_20000505 and SNP_20000553) were not variant on the IH × NF map. However, the presence of common markers between these two maps in the vicinity of the QTL-containing region supports the inference that a common genomic region on LG3 is responsible for conferring resistance.

## Phenotypic and Genotypic Analysis of Diverse Germplasm Panel

Responses of the germplasm panel members to inoculation treatments with *A. lentis* isolate FT12013 were consistent. Severity of AB infection varied significantly, with scores from 0 (no symptoms) to a highest score of 25% APD.

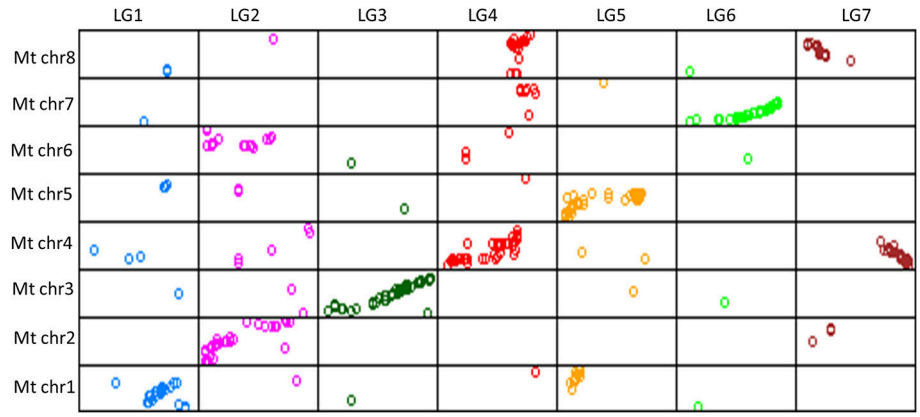


**FIGURE 2 | Dot-plot representing correspondence between lentil consensus map linkage groups 1 through 7 (top) and *M. truncatula* chromosomes 1 through 8 (left side).**

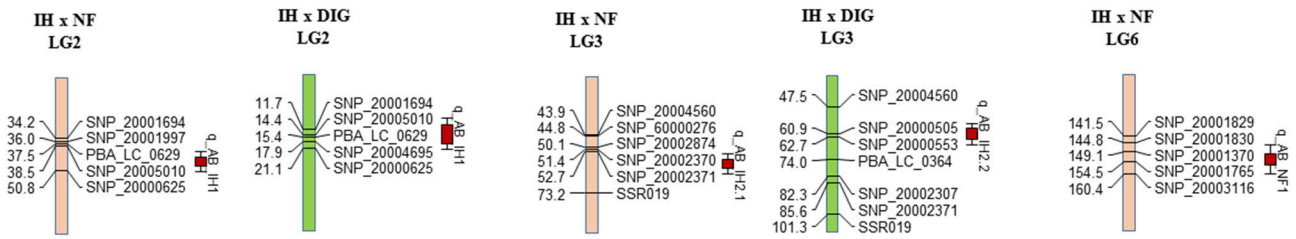


**FIGURE 3 | Localization of QTLs associated with resistance to *A. lentis* on the IH × NF-derived genetic map and IH × DIG-derived genetic map.** The name is provided at the top of each LG. Distances of the loci (cM) are shown to the left and names of loci are shown to the right side of LGs. For presentation purposes, only selected markers are shown on the map.

**TABLE 4 | Identification of QTLs for AB resistance on IH × NF and IH × DIG genetic maps based on CIM.**

| Mapping population | QTL name | Linkage group | Flanking markers | Position (cM) | LOD threshold | Maximum LOD score | % Phenotypic variance | Additive effect |
|---|---|---|---|---|---|---|---|---|
| IH × NF | AB_IH1 | LG2 | PBA_LC_0629<br>SNP_20005010 | 37.5–38.5 | 3.1 | 16.8 | 47 | 3.9174 |
| | AB_IH2.1 | LG3 | SNP_20002370<br>SNP_20002371 | 51.4–52.7 | 3.1 | 7 | 15 | 2.2097 |
| | AB_NF1 | LG6 | SNP_20001370<br>SNP_20001765 | 149.1–154.5 | 3.1 | 3.5 | 7 | −1.4697 |
| IH × DIG | AB_IH1 | LG2 | SNP_20005010<br>PBA_LC_0629<br>SNP_20004695 | 14.4–17.9 | 3.1 | 13.1 | 30 | 3.7020 |
| | AB_IH2.2 | LG3 | SNP_20000505<br>SNP_20000553 | 60.9–62.7 | 3.1 | 9.7 | 22 | 3.2251 |

Approximately half of the panel lines, including those with a known high level of resistance (cultivars Indianhead, CDC Matador and PBA Ace) showed no foliar infection symptoms, while the majority of the remaining lines displayed intermediate scores (5–18%). A total of four lines, including cultivars Cumra and PBA Flash, showed a susceptible reaction to AB, with foliar infection levels of 20–25% (**Supplementary Figure 4**).

The allelic identity for genetic markers linked to AB resistance QTLs was highly correlated with the phenotypic assessment data, and clearly distinguished between resistant, moderately resistant, and susceptible genotypes. As the largest proportion

of phenotypic variance (47%) was explained by AB_IH1, precedence was given to allelic identity data for markers linked to that QTL. Of the 79 genotypes tested, marker allele predictions were accurate for 68 (86%). For those lines where the phenotype and genotype data were discordant, six contained the resistance allele but were susceptible (false-positive), while five were resistant but only contained the moderate resistance-associated allele from Digger (false-negative; **Supplementary Table 6**). The 11 anomalous genotypes were further examined using pedigree information, revealing that all false-positive genotypes lacked the RIL parental lines in their pedigrees.

A detailed analysis of phenotype-genotype data correlations for the sub-set of panel lines that were infected with multiple Australian *A. lentis* isolates is provided in **Table 5**. The SNP marker SNP20005010 was found to most reliably predict the presence of the resistance allele for AB_IH1. For AB_NF1, SNP 20001370, and SNP20001765 were found to be associated with a minor gene that appeared to confer partial resistance to a FT14125 isolate (2014 isolate from Horsham, Victoria). For AB_IH2.1, the marker identified in the IH × NF population, SNP20002370, provided a prediction of partial resistance to isolates such as FT14125. Markers associated with AB_IH2.2 were apparently not correlated with resistance to *A. lentis* isolates.

# DISCUSSION

## Genetic Linkage Mapping

A substantially lower proportion of EST-SSR markers detected polymorphism (10%) as compared to genomic derived-SSRs (87%), as previously reported for the same marker set (Kaur et al., 2014). SNP genotyping revealed a total of 583 markers (75%) as polymorphic, but only a small number were found to be common between the three RIL populations. The 768-plex SNP assays used in this study were developed from a range of cultivated genotypes (including the parental genotypes of the mapping populations) and further selected to maximize the number of population-specific SNPs (Kaur et al., 2014), accounting for the observed variable proportions of polymorphic loci and limited commonality between populations.

A number of genetic linkage maps have recently been developed for lentil, through the use of SSR and SNP marker technologies (Sharpe et al., 2013; Gujaria-Verma et al., 2014; Kaur et al., 2014). The cumulative lengths of maps from this study were marginally higher than those from previous studies (Sharpe et al., 2013; Kaur et al., 2014), possibly due to the effects of a higher number of map-assigned markers, or the genetic constitution of mapping populations (potentially influencing rates of recombination). The distribution and order of markers across LGs in each genetic linkage map were highly comparable, except for those markers anomalously assigned to LG4 in individual RIL-based maps, but confirmed to be located on LG6 on the basis of known macrosynteny with MtChr7. This discrepancy could be due to chromosomal rearrangement events in specific genotypes, but may also be attributable to paralogous sequence effects. As the EST-derived markers may have been derived from individual members of a gene family, an assay designed to detect a polymorphism between two contrasted genotypes in one gene copy (but with no variation in a second gene copy) may inadvertently detect the reverse situation between a second pair of contrasted genotypes, thus generating the appearance of a re-located marker locus (Schwarz-Sommer et al., 2003; Somers et al., 2004). Identification of multiple loci of this nature may reflect the presence of ancestral segmental duplication events, which are known to have been common during the evolution of the Fabaceae.

Merger of the four RIL-based maps through use of common genetic markers generated a consensus map containing a total of 689 markers, higher than any previously constructed population-specific map for lentil (Sharpe et al., 2013; Kaur et al., 2014). Comparative analysis also supported identification of bridging loci between six LGs of the consensus map with that of Sharpe et al. (2013), but the two maps could not be integrated into a single structure due to insufficient common markers, especially for LG6 which is devoid of such markers.

Legumes display extensive conservation of gene order, even between species which differ dramatically in terms of genome size (Choi et al., 2004; Phan et al., 2006). In the present study, comparative analysis was performed between the *M. truncatula* genome and the gene sequences associated with markers assigned on the consensus map. As previously reported (Sharpe et al., 2013; Gujaria-Verma et al., 2014; Kaur et al., 2014), direct and simple correspondences are observed between *M. truncatula* pseudomolecules and lentil LGs, although some evolutionary translocations and non-colinear relationships were also detected.

Although draft or complete genome sequences for many plant species have been made available [e.g., *M. truncatula* (http://www.medicago.org), chickpea (*Cicer arietinum*); Varshney et al., 2013), soybean (*Glycine max*; Schmutz et al., 2010), and pigeon pea (*Cajanus cajan*; Varshney et al., 2011), crop improvement programs based on recombinational assortment of favorable gene variants require the construction of genetic maps. Moreover, whole genome assemblies require high-density linkage maps to assist assembly and assess the quality of sequenced genomes. An international effort to deliver a reference lentil genome sequence is currently underway, leading to the recent release of an initial draft assembly from the cultivar CDC Redberry. However, this assembly is still in a preliminary form, with minimal gene annotation and limited access (Bett et al., 2016). The consensus map generated from the current study could potentially help to further improve the current draft lentil genome.

## Identification of QTLs and Validation of Linked Genetic Markers

Multiple studies have been conducted in order to identify superior sources of resistance to AB in lentil, corresponding to genes of major effect (Ahmad et al., 1997; Ford et al., 1999; Nguyen et al., 2001). The results of the present study are consistent with previous studies (Rubeena et al., 2006; Gupta et al., 2012) that demonstrated the presence of multiple genes for AB resistance with different modes of action in different lentil genotypes. The identification of common QTLs (AB_IH1, AB_IH2.1/AB_IH2.2) between mapping populations

**TABLE 5 | Phenotypic scores of AB infection on lentil germplasm panel inoculated with *A. lentis* isolates alongside genotyped markers for three QTL regions.**

| Lentil line | Pedigree | AB field rating | Phenotypic scores of AB infection on lentils inoculated with five separate *A. lentis* isolates in controlled environment | | | | | AB_IH1 | AB_IH2.1 | AB_NF1 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Historical isolate, Kewell | FT14125 | FT10002 | FT12013 | FT13013 | SNP_20005010 | SNP_20002370 | SNP_20001370 |
| ILL7537 | | R | 0 | 0 | | 0.6 | 0 | + | + | − |
| Indianhead | | R | 3.7 | 0 | | 0 | 0 | + | + | − |
| CIPAL1504 | PBAACE/PBABOLT | MR | 10.4 | 1.1 | | 0 | 0.1 | + | + | + |
| CIPAL1522 | PBAACE/04-299L-05HG1001-05HSHI2006 | R | 4.5 | 0.2 | | 0 | 0 | + | + | + |
| PBA Ace | CIPAL0501/96-047L*99R099 | R | 8.7 | 1.9 | 0 | 0 | 0 | + | + | + |
| PBA Herald XT | 96-047L*99R060-EMS02 | R | 2.5 | 0.9 | 0 | 0 | 0 | + | + | + |
| PBA Jumbo2 | CIPAL0205/BOOMER//CIPAL0401 | R | 0 | 0 | 0 | 0.6 | 0 | + | − | − |
| CIPAL1501 | PBABOLT*06G1001/CIPAL0804 | R | 0 | 0.4 | | | 0.1 | + | − | + |
| CIPAL1301 | PBABOLT/02-325*03HS001 | R | 0.1 | 0.6 | 0 | 0 | 0.6 | + | − | + |
| CIPAL1502 | PBABOLT*06G1002/CIPAL0804 | R | 0.3 | 0.1 | | | 0 | + | − | + |
| CIPAL1523 | PBABLITZ/04-190L-05HG1001-05HSHI2001 | MR | 5.7 | 5.1 | | 0 | 0 | + | − | + |
| PBA Bolt | ILL7685/96-047L*99R060 | MR | 5.9 | 4.6 | 0 | 0 | 0 | + | − | + |
| CIPAL1521 | PBABOLT/04-299L-05HG1001-05HSHI2006 | R | 7.3 | 2.4 | | 1.3 | 0 | + | − | + |
| PBA Hurricane XT | PBAFLASH/96-047L*99R060M3 | MR | 12.8 | 4.7 | 0.1 | 0 | 1.1 | + | − | − |
| CIPAL1422 | PBAHERALD/PBABOLT | R | 16.0 | 3.4 | 0 | 0 | 0.7 | + | − | + |
| Boomer | DIGGER/PALOUSE | MR | 0.9 | 0.1 | 0.1 | 0.6 | 3.0 | − | + | − |
| Nipper | INDIANHEAD/NORTHFIELD//NORTHFIELD | MR/MS | 0.3 | 0 | 0.1 | 9.4 | 10.6 | − | + | − |
| PBA Giant | PBAFLASH/BOOMER | MR/MS | 3.4 | 1.3 | 1.3 | 2.6 | 4.7 | − | + | + |
| Nugget | NORTHFIELD/ILL5714 | MR/MS | 3.6 | 3.0 | 4.7 | 7.5 | 4.3 | − | + | + |
| Northfield | | MR/MS | 0 | 0.1 | 3.7 | 8.1 | 5.6 | − | − | + |
| PBA Flash | ILL7685/NUGGET | MS | 3.1 | 2.0 | 4.6 | 16.9 | 5.8 | − | − | + |
| PBA Blitz | CUMRA/INDIANHEAD//CASSAB | MR/MS | 1.8 | 0.2 | 3.6 | 5.6 | 3.9 | − | − | − |
| PBA Greenfield | CIPAL0205/BOOMER//PBAFLASH | MR/MS | 0 | 0 | 3.6 | 14.4 | 6.1 | − | − | − |
| PBA Jumbo | ALDINGA/CDCMATADOR | MR/MS | 1.0 | 0 | 3.3 | 15.6 | 9.4 | − | − | − |
| Cumra | | S | 22.9 | 3.1 | | 14.4 | 8.0 | − | − | − |

*+, Preferred (resistance) allele present; −, Non-preferred (susceptible) allele present.*

with a shared parent (Indianhead) provides confidence in the process of QTL identification. Differences observed between the $V_p$ proportions accounted for the common QTLs could be due to variability between conditions of the two screening experiments, or the influence of partial resistance genes contributed by Northfield and Digger.

In the context of a fungal pathogen population that is able to overcome plant resistance genes, the properties of the three QTLs identified in this study largely explain the observed genetic resistance to two alternative AB pathotypes recently isolated from field-grown crops. The QTL of largest effect (AB_IH1, identified in both IH × NF and IH × DIG mapping populations) accounted for the majority of AB resistance when using the current, most aggressive, field-derived isolates (Davidson et al., 2016). The Indianhead-derived QTL allele conferred resistance to these isolates, of which FT12013 was a representative. Recombination in the vicinity of this QTL appears to have occurred in Australian germplasm such as cultivar Nipper, which contains the allele of the flanking SSR marker PBA_LC_0629 characteristic of Indianhead (which was a parent of Nipper), but not the corresponding allele at the coincident SNP marker (SNP20005010). Nipper, and others with the same genotype (such as PBA Greenfield) do not have resistance to isolate FT12013, probably indicating that the candidate R gene is closer to the SNP than the SSR marker. Allelic identity at AB_IH1 was found to be predictive of resistance to the aggressive "Nipper-virulent" isolate (FT12013) in the majority of Australian lentil germplasm testing in the panel. However, this relationship was not conserved for all diverse germplasm, such as ICARDA lines ILL2024 and ILL6788 (two parental lines that have used in the Australian lentil breeding program), which were susceptible to FT12013. As a consequence, resistance status was not predictable for cultivar PBA Bounty, which was derived from selected progeny of a cross with ILL6788.

In 2014, a field isolate (FT14125) with a different pattern of pathogenicity on lentil genotypes was identified from a population of *A. lentis* at Horsham, Victoria. This isolate is hence thought to belong to a pathotype grouping differing from the currently dominant field isolates that has overcome the resistance derived from Northfield (which is also found in cultivars Digger and Nugget). The Indianhead-derived allele at locus SNP20005010 was not found to be necessary for resistance to FT14125, and cultivars Nipper, PBA Blitz, PBA Jumbo, and PBA Greenfield (which are susceptible to Nipper-virulent isolates) exhibited complete resistance to this isolate in a controlled environment trial having only the Digger-derived allele at SNP20005010 and the Indianhead-derived allele at AB_NF1 in common. The Indianhead-derived allele at AB_IH2.1 (SNP_20002370) also appeared to confer partial resistance to this isolate in the absence of the previous two alleles (e.g., for PBA Ace, PBA Herald XT, and CIPAL1522).

There is also evidence for AB resistance genes apart from the three identified QTLs. Northfield and Boomer demonstrate greater resistance to AB than expected on the basis of QTL-associated genotype. Northfield has the same genotype as the susceptible cultivar PBA Flash (lacking Indianhead-derived alleles), and a similar susceptibility to "Nipper-virulent" isolates (such as FT12013), but is at present significantly more resistant than PBA Flash, in the field environment and to isolate FT14125. Similarly, Boomer lacks the Indianhead-derived allele at SNP20005010, but displays moderate resistance to field isolates of AB, significantly higher than for cultivars such as Nipper and Nugget (which have a similar genotype at the three identified QTLs).

A direct comparison of QTL-flanking loci identified in the current study with those from previous studies (Rubeena et al., 2006; Gupta et al., 2012) could not be performed, due to the lack of common markers. Furthermore, previous LG nomenclature differed from that used in more recent studies (Sharpe et al., 2013). However, AB_NF1 on LG6 is comparable in location to a previously described QTL (QTL5 on LG1—Rubeena et al., 2006, QTL1 on LG1—Gupta et al., 2012), based on a common SSR locus location. Moreover, the various mapping populations in these studies were related through the common parent Northfield, which conferred seedling-based AB resistance. A previous study (Chowdhury et al., 2001) reported the development of two SCAR markers from RAPD markers linked to the *ral2* ($UBC227_{1290}$ and $OPD\text{-}10_{870}$) gene. However, that study revealed that SCAR marker developed from $UBC227_{1290}$ was monomorphic, and the other SCAR marker developed from $OPD\text{-}10_{870}$ was not efficient in discriminating different phenotypes among $F_2$ progeny (Chowdhury et al., 2001), and so was not screened in the present study.

The markers identified in the present study will be highly useful for deployment of desirable R genes into a lentil breeding program, allowing pyramiding with other effective genes to confer durable resistance. The current data suggests that AB_IH1 confers the highest level of field resistance, but may be enhanced by the presence of AB_IH2.1, while the value of AB_NF1 from the Northfield background has been mostly non-conclusive. Different R alleles from these QTLs have been noted to respond differently to various *A. lentis* isolates, and so further in-depth knowledge of the population structure of pathogen may be required to understand the effects of AB_NF1 on AB resistance. An immediate use of the identified markers will therefore be to select for QTL combinations capable of matching the resistance profile of Indianhead.

As has been recently demonstrated (Davidson et al., 2016), the *A. lentis* pathogen is capable of adaptation to overcome R genes deployed in lentil germplasm. For this reason, continuous surveillance of resistance status is necessary, including analysis of other structured genetic populations in order to locate for AB resistance coming in germplasm such as Boomer and ILL7537, as well as the partial resistance genes present in Northfield and Digger.

In conclusion, the present study has developed valuable genetic resources including RIL-based maps and a consensus linkage map, which will collectively assist other trait-dissection studies for future lentil breeding activities. Evaluation of AB resistance under controlled conditions permitted identification of three and two QTLs in the IH × NF and IH × DIG mapping populations, respectively. Common genomic regions (AB_IH1 and AB_IH2.1/AB_IH2.2) were identified as responsible for AB resistance in both mapping populations, and were associated with the resistant parent, Indianhead while the third genomic region was associated with Northfield parent. Validation of flanking

markers across a diverse germplasm demonstrated that these markers predicted the phenotypic responses with high levels of accuracy. The tightly linked molecular markers for AB resistance will enable marker-assisted selection (MAS) of AB resistant cultivars, based on introgression of QTL-containing genomic regions from donor to recipient germplasm.

## AUTHOR CONTRIBUTIONS

SS performed map construction, QTL analysis, marker validation, and contributed to drafting the manuscript. MR contributed to data interpretation and assisted in drafting the manuscript. JD performed the phenotyping of the mapping populations and validation panel and contributed to data interpretation. MJ assisted in performing phenotyping of validation panel. AS performed the genotyping of the mapping populations. JF, ATS, and NC co-conceptualized the project and assisted in drafting the manuscript. SK co-conceptualized and coordinated the project and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01604/full#supplementary-material

**Supplementary Figure 1 | Comparison between linkage groups of IH × NF, IH × DIG, and NF × DIG maps.** This file shows visual representation of the comparison of **(A)** all LGs in three different maps and the common marker loci between them, **(B)** LG5 from three maps and the common marker loci between them, **(C)** LG7 from three maps and the common marker loci between them, **(D)** LG6 from three maps and the common marker loci between them. White lines represent the corresponding positions of common markers.

**Supplementary Figure 2 | Comparison between linkage groups of IH × NF, IH × DIG maps with consensus linkage map.** This file shows visual representation of the comparison of **(A)** all LGs in IH × NF and IH × DIG maps with consensus map and the common marker loci between them, **(B)** LG5 from three maps and the common marker loci between them, **(C)** LG6 from three maps and the common marker loci between them. White lines represent the corresponding positions of common markers.

**Supplementary Figure 3 | Frequency distribution histogram.** This file contains frequency histograms generated from AB resistance scores in IH × NF and IH × DIG mapping populations.

**Supplementary Figure 4 | Frequency distribution histogram.** This file contains frequency histograms generated from AB resistance scores from lentil germplasm lines.

**Supplementary Table 1 | List of validation panel lines.** The table provides the list of lentil validation panel lines used for marker validation with their respective germplasm category.

**Supplementary Table 2 | List of *A. lentis* isolate used for phenotyping.** This file contain details of different *A. lentis* isolates used for phenotyping lentil mapping populations and the validation panel.

**Supplementary Table 3 | Linkage map statistics from IH × NF, IH × DIG, and NF × DIG maps.** This file contains details of different markers (SSRs and SNPs) and their corresponding positions on different LGs.

**Supplementary Table 4 | BLASTN results of the markers.** The data represents the BLASTN-based sequence analysis of DNA sequences underlying SNP markers assigned to three maps against *M. truncatula* genome (Mt4.0) and DNA sequences underlying SNP markers assigned on the Sharpe et al. (2013) map.

**Supplementary Table 5 | Consensus genetic linakge map statistics.** This file contains details of different markers (SSRs and SNPs) and their corresponding positions on different LGs.

**Supplementary Table 6 | Phenotypic scores and genotypic data of the lentil germplasm set for the validation of AB resistance markers.** This file contains the phenotypic scores of AB infection on lentil germplasm panel inoculated with *A. lentis* isolate FT12013 alongside genotyped markers for three QTL regions.

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# Changes in Aggressiveness of the *Ascochyta lentis* Population in Southern Australia

*Jennifer Davidson[1]*, Gabriel Smetham[2], Michelle H. Russ[1], Larn McMurray[3], Matthew Rodda[4], Marzena Krysinska-Kaczmarek[1] and Rebecca Ford[5]*

*[1] Pulse and Oilseed Pathology, Plant Health and Biosecurity, Sustainable Systems, South Australian Research and Development Institute, Adelaide, SA, Australia, [2] Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, VIC, Australia, [3] New Variety Agronomy, Sustainable Systems, South Australian Research and Development Institute, Clare, SA, Australia, [4] Biosciences Research, Department of Economic Development, Jobs, Transport and Resources, Horsham, VIC, Australia, [5] School of Natural Sciences, Environmental Futures Research Institute, Griffith University, Nathan, QLD, Australia*

Anecdotal evidence identified a change in the reaction of the resistant lentil cv Nipper to ascochyta blight in South Australia in 2010 and subsequent seasons, leading to infection. This study investigated field reactions of lentil cultivars against *Ascochyta lentis* and the pathogenic variability of the *A. lentis* population in southern Australia on commonly grown cultivars and on parental germplasm used in the Australian lentil breeding program. Disease data recorded in agronomic and plant breeder field trials from 2005 to 2014 in southern Australia confirmed the change in reaction on the foliage of the previously resistant cvs Nipper and Northfield. Cultivar responses to seed staining from *A. lentis* did not change. The change in foliar response was confirmed in a series of controlled environment experiments using single, conidium-derived, isolates of *A. lentis* collected over different years and inoculated onto differential host sets. Specific isolate/cultivar interactions produced a significant range of disease reactions from high to low aggressiveness with a greater percentage of isolates more aggressive on cvs Nipper, Northfield and PBA Flash than previously detected. Specific isolates were tested against Australian lentil cultivars and breeding lines in controlled conditions, again verifying the aggressiveness on cv Nipper. A small percentage of isolates collected prior to the commercial release of cv Nipper were also able to infect this cultivar indicating a natural variability of the *A. lentis* population which subsequently may have been selected in response to high cropping intensity of cv Nipper. Spore release studies from naturally infested lentil stubbles collected from commercial crops also resulted in a high percentage of infection on the previously resistant cvs Nipper and Northfield. Less than 10% of the lesions developed on the resistant differentials ILL7537 and cv Indianhead. Pathogenic variation within the seasonal populations was not affected by the cultivar from which the stubble was sourced, further indicating a natural variability in aggressiveness. The impact of dominant cultivars in cropping systems and loss of effective disease resistance is discussed. Future studies are needed to determine if levels of aggressiveness among *A. lentis* isolates are increasing against a range of elite cultivars.

**Keywords: ascochyta blight, *Didymella lentis*, lentil, *Lens culinaris*, aggressiveness**

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****Correspondence:***
*Jennifer Davidson*
*jenny.davidson@sa.gov.au*

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## INTRODUCTION

*Ascochyta lentis* (teleomorph *Didymella lentis*) is the causal agent of ascochyta blight of lentil (*Lens culinaris*) (Kaiser et al., 1997), a disease of global importance and considered the major biotic constraint to lentil production in Australia (Salam et al., 2011). Australia is currently the second largest exporter of lentil behind Canada (FAOSTAT, 2014), producing 348,000 tons in 2014. Production is almost entirely in winter cropping areas of Victoria and South Australia, with less than 1% production in the states of New South Wales and Western Australia (Pulse Australia, 2014). On average 3% of arable land is cropped to lentils across South Australia each year and less than 2% of arable land across Victoria (Pulse Australia, 2014). Seasons characterized by frequent and prolonged winter rainfall events as can occur in these regions favor *A. lentis* infection and development of ascochyta blight leading to yield losses and reduced marketability of resultant stained and distorted seeds (Hawthorne et al., 2012).

*A. lentis* is specific to cultivated and wild species of lentil (Tullu et al., 2010). It is morphologically indistinct from *A. fabae* but the latter is unable to infect lentil species (Kaiser et al., 1997). Movement of the host germplasm has disseminated the pathogen worldwide (Kaiser, 1997) where it is primarily introduced to new sites through infected seed (Morrall and Sheppard, 1981; Kaiser and Hannan, 1986; Nasir and Bretag, 1997b). Wind dispersal of ascospores from infected lentil stubble into neighboring fields is considered the primary source of inoculum in Australia (Hawthorne et al., 2012) while splash dispersal of asexual pycnidiospores in prolonged damp conditions leads to epidemics. Sexual ascospores are produced on lentil stubble from the previous crop when both fungal mating types, MAT1-1 and MAT1-2, are present (Kaiser and Hellier, 1993), leading to increased genetic diversity and adaptive potential (Martin et al., 2013). Both mating types are present in Australia (Skiba and Pang, 2003) and the teleomorph has been identified in the field in both Victoria and Western Australia (Galloway et al., 2004). In Australia the ascospores are produced during the growing season in late autumn and winter (May to July) and are wind dispersed to a distance of 50 m from infected stubble (Galloway and MacLeod, 2002).

Control of the disease currently consists of the integrated selection of the most resistant varieties and best cultural practices, plus applications of fungicides on seed and foliage (Hawthorne et al., 2012). Fungicide applications are a considerable cost, both financially and environmentally, and can be difficult to apply in a timely fashion due to adverse weather and soil conditions therefore the development of highly resistant lentil varieties continues to be a primary breeding goal. Traditional breeding techniques have been used to date since the sources of genetic resistance to *A. lentis* are still largely uncharacterised (Ahmad et al., 1997; Ford et al., 1999; Gupta et al., 2012).

The cv Northfield, a selection from the ICARDA breeding line ILL5588 (originally from Jordan) was identified as resistant to ascochyta blight (Ali, 1995), and registered in 1995 to become one of the first cultivars to be grown in Australia, particularly in South Australia (Muehlbauer et al., 2009). Subsequently, it was replaced by the cv Nugget with moderate resistance to ascochyta blight (Hawthorne et al., 2011) and by cv Nipper with high resistance (McMurray et al., 2011). The cv Nipper, the progeny of two resistant cultivars *viz.* Indianhead and Northfield (Pulse Australia, 2011), was released to industry in 2006 (Taylor et al., 2007). Like Northfield, the parental line Indianhead also has a high level of resistance to ascochyta blight (Ye et al., 2001) and has been used extensively in the Australian lentil breeding program, along with the resistant breeding line ILL7537.

Resistant hosts, however, may instigate the selection of more aggressive individuals (Pariaud et al., 2009), where aggressiveness is "the quantitative variation of pathogenicity on susceptible hosts." An early RAPD study on the *A. lentis* population in Australia (Ford et al., 2000) found the diversity of this fungal population was similar to that of isolates originating from outside of Australia. The authors concluded that the diversity came about through multiple introductions from different international sources and warned that this diversity and the presence of mating types provided a high potential for adaptation via sexual reproduction. Glasshouse studies of aggressiveness of *A. lentis* isolates in Australia in the late 1990's (Nasir and Bretag, 1997a, 1998), described 39 isolates as five or six pathotypes. Similarly, the other published Australian study to date (Sambasivam, 2011) also classified 17 isolates into six pathotypes although on a different host set, making comparisons difficult. This range of reactions from mostly resistant to highly susceptible is consistent with international studies (Bayaa et al., 1994; Ahmed and Morrall, 1996; Ahmed et al., 1996). However, a Canadian study of a larger number of isolates (84) against 10 lentil differentials indicated there was a continuum of aggressiveness without cultivar specificity (Ahmed et al., 1996).

Anecdotal evidence identified of a change in reaction to ascochyta blight on the cv Nipper in South Australia in 2010 and subsequent seasons, leading to infection on this cultivar. This study investigates the pathogenic variability of the *A. lentis* population in southern Australia on commonly grown cultivars and on parental germplasm used in the Australian lentil breeding program. A suite of lentil field trials are conducted each season across southern Australia for agronomic and breeding purposes and these trials were used, along with commercial crops, as a resource for determining host reaction to natural pathogen infection and for pathogen collection. Therefore, the aims of this study were to determine (1) The field reactions of lentil hosts against *A. lentis* over a number of seasons (2) The overall range of aggressiveness among recent Australian isolates of *A. lentis* against lentil differentials under a controlled environment, (3) If isolates with higher aggressiveness than identified from previous studies are present, and (4) If distinct isolate per host interactions exist in the Australian population.

## MATERIALS AND METHODS

### Field Trials

Ascochyta blight naturally infected a number of lentil field trials, including Pulse Breeding Australia (PBA) selection trials, National Variety Trials (NVT; http://www.nvtonline.com.au/) and agronomic research trials in South Australia in 2005,

2010, 2013 and Victoria in 2014. These trials were assessed for disease as described below to provide data to breeders and agronomists on cultivar reactions and efficacy of disease management practices. In these seasons, rainfall was up to 189% above the 50 year long term average (105 mm compared to 90 mm long term average) (Bureau of Meteorology, 2016) in August and September when crops are starting to flower and conidial splash of *A. lentis* spreads the pathogen. Very limited ascochyta blight was evident in the intervening years due to dry seasonal conditions (18–84 mm during August and September) which prevented the development and spread of disease. Trials (**Table 1**) were randomized blocks, sown in 6.75 or 13.5 $m^2$ plots, with 3 replicates, and trial management represented local grower practice in the region with respect to sowing date, seeding rate, fertilizer, herbicides, and pesticides. All seed was treated with P-Pickle T® (a.i. 360g$L^{-1}$ thiram plus 200 g$L^{-1}$ thiabendazole) fungicide seed dressing at 200 ml per 100 kg of seed prior to sowing.

In 2005 and 2010 ascochyta blight symptoms were scored on the foliage in each plot during flowering and podding growth stages (August to September), using the 1–9 categorical scale; 1 = no disease, 3 = individual leaf lesions, 5 = leaf and stem lesions, 7 = leaf, stem, and pod lesions, 9 = plant death. Data were analyzed with Friedman's non-parametric analysis of variance. In 2013 and 2014, the disease on foliage was assessed as % Leaf Area Diseased (%LAD) of total foliage in each plot during flowering or early podding and these data were analyzed using Analysis of Variance for randomized blocks in Genstat® version 16.

All trials were harvested at maturity and 100 seeds per plot were sampled at random from trials in 2005 and 2013. Ascochyta blight seed staining was scored on 2005 grain samples using a categorical scale of 0–3; 0 = no staining; 1 = ascochyta blight lesions $\leq$ 1 mm diameter; 2 = ascochyta blight lesions $>$ 1 mm diameter and $<$25% seed coverage; 3 = $\geq$ 25% seed coverage. Seed from the 2013 trial at Mallala was scored using a 0–5 categorical scale; 0 = no staining; 1 = ascochyta blight lesions $\leq$ 1 mm diameter; 2 = ascochyta blight lesions $>$ 1 mm and $<$ 10% seed coverage; 3 = ascochyta blight lesions $>$ 2 mm and $<$ 10% seed coverage; 4 = ascochyta blight lesions $>$ 3 mm and between 10–25% seed coverage; 5 = $\geq$ 25% seed coverage. The number of seeds in each category was summed and a disease index (DI) was calculated for each plot as follows:

DI = $[\sum_{n}^{i=0} (d_i * s_i / N] * 100/C$ where; $s_i$ refers to the number of seeds in each disease category, $d_i$ is the value of the disease category, N is the total number of assessed seeds per plot and C is the number of disease categories. Data from 2005 were square root transformed to normalize residuals and analysis of variance was performed on the transformed DI. Data from PBA Mallala 2013 did not require square root transformation for analysis. All data were analyzed using Genstat® version 16 and significant differences were based on 95% confidence intervals.

## Isolate Collection from Field Trials and Commercial Crops

Lentil plants with typical ascochyta blight leaf or stem lesions as well as seeds with ascochyta blight lesions were collected from the above-mentioned trials and from commercial crops in South Australia from 2010 to 2014, including the years with limited disease incidence, and from plant material in field trials in Victoria in 2012. Diseased plants were collected in August and September each year during the growing seasons and seeds were collected after harvest. The host cultivar and location was recorded for each collection. Diseased plant material was surface sterilized by dipping in 70% ethanol, followed by 30 s in 1% hypochlorite then rinsed in sterile water. Seeds were soaked in 2% hypochlorite for 2 min then drained through muslin cloth and dried on Whatman® sterile filter paper in a laminar flow. Seed or plant material were placed onto potato dextrose agar (PDA) (Oxoid®) amended with 0.01% streptomycin and plates were incubated 10–14 days under fluorescent lights (two Phillips TLD 36W/840 daylight tubes and one NEC black fluorescent light) for 12 h day/night at 22°C. The resulting isolates were identified as *A. lentis* based on the morphological characteristics of the conidia and cultures (Morrall and Sheppard, 1981). Single conidium-derived isolates were prepared and stored in sterile water at 4°C. An additional 17 single conidium-derived isolates of *A. lentis* had been collected from within South Australia and stored as described above at the SARDI Pulse and Oilseed Pathology Laboratory between 1989 and 2006, prior to the commercial release of cv Nipper.

### Isolate Collection from Infested Lentil Stubble

After harvest in December 2012, lentil stubble naturally infested with *A. lentis* was collected from three commercial crops including two crops of cv PBA Flash (moderately susceptible to ascochyta blight) (Hawthorne et al., 2012) and one crop of cv Nipper. All crops were located within the Yorke Peninsula region of South Australia, which has a comparatively high intensity of lentil cropping (13% of arable land compared to the state average of 3% arable land) (Pulse Australia, 2014). The three stubbles were placed, separately, into large (150 × 75 cm) nylon mesh bags with 1 kg stubble approximately 10 cm deep in each bag. These were placed on benches, one bag per bench, in an external environment in a shadehouse at SARDI exposed to ambient conditions from 21st January 2013 to encourage release of spores.

Seed of eight lentil lines were sown, 25 pots per cultivar, four seedlings per pot (90 × 90 × 180 mm) filled with Van Schaik's Biogro (Biogro Pty. Ltd.) pine bark potting mix plus half a teaspoon of super fine agricultural lime (Biogro Pty. Ltd.) to raise pH to 7.0. These lentil lines were the ascochyta blight resistant sources in the PBA breeding program *viz.* cvs Northfield, Indianhead, breeding line ILL7537, and selected commercial cultivars grown in South Australia *viz.* cvs Nipper, PBA Flash, Nugget, PBA Herald XT (the latter resistant to ascochyta blight) (Hawthorne et al., 2012) as well as the susceptible cv Cumra (Siddique, 2000). On 25th June five pots of each cultivar containing 4 week old seedlings were placed around each bag of stubble; all pots were at equidistance and immediately adjacent to the stubble. Seedlings were watered as required. Following initial *A. lentis* symptom observation the infected leaves were detached each week to count and collect the lesions until 28th August 2013. Single conidium-derived isolates were produced from lesions, as described above.

**TABLE 1 | Ascochyta blight scores on foliage of lentil lines in field trials in South Australia and Victoria from 2005 to 2014.**

| Site | | Melton | Paskeville Trial 1 | Paskeville Trial 2 | Melton Trial 1 | Melton Trial 2 | Maitland | Maitland | Minlaton | Willamulka | Mallala | Horsham |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | | **2005** | **2010** | **2010** | **2010** | **2010** | **2010** | **2013** | **2013** | **2013** | **2013** | **2014** |
| **Disease scores** | | **1–9[a]** | **1–9** | **1–9** | **1–9** | **1–9** | **1–9** | **%LAD[b]** | **%LAD** | **%LAD** | **%LAD** | **Sqrt %LAD** |
| **Lentil cultivar** | **Foliar resistance rating** | | | | | | | | | | | |
| Aldinga | MR/MS[c] | | | | 3.0 | 3.2 | | | | | 15.0 | |
| Boomer | MR | 5.6 | 2.5 | 1.7 | 1.0 | 1.3 | 1.0 | | | | 0.0 | 1.1 (1.2)[d] |
| Cumra | S | | | | 5.3 | | | | | | | |
| Nipper | R-MR/MS[e] | 1.0 | 3.8 | 5.1 | 3.0 | 2.7 | 4.3 | 6.7 | 5.0 | 3.7 | 13.3 | 2.2 (4.9) |
| Northfield | R-MR/MS | 1.0 | 2.5 | | 3.7 | 1.8 | | | | | 3.3 | 1.9 (3.5) |
| Nugget | MR/MS | 6.0 | 4.3 | 6.1 | 4.0 | 4.2 | 4.3 | 6.7 | 8.3 | 4.0 | 8.3 | 2.2 (5.2) |
| PBA Ace | R | | | | | | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 (0.1) |
| PBA Blitz | MR | | | | | | 2.3 | 1.3 | 0.7 | 0.0 | 3.3 | 1.2 (1.4) |
| PBA Bounty | MR/MS | | 3.5 | 4.5 | 3.3 | 2.8 | 3.7 | | | | 10.0 | 2.1 (4.3) |
| PBA Flash | MS | | 6.3 | 5.4 | 4.0 | 3.5 | 6.7 | 8.3 | 11.7 | 9.3 | 28.3 | 3.5 (12.3) |
| PBA Herald XT | R | | | | 1.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 (1.0) |
| PBA Hurricane XT | MR | | | | | | | 0.2 | 1.7 | 0.0 | 0.0 | 0.8 (0.4) |
| PBA Jumbo | MR/MS | | | | | | 1.7 | 4.0 | 5.0 | 0.3 | 8.3 | 2.3 (5.0) |
| Friedman's statistic (*P*-value) | | 25.74 (0.001) | 25.0 (0.002) | 45.2 (0.001) | 32.1 (0.001) | 36.3 (0.001) | 44.0 (0.001) | | | | | |
| LSD ($P \leq 0.05$) | | | | | | | | 2.4 | 2.2 | 1.3 | 5.7 | 0.3 |

[a] *Foliar score 1–9; 1, no disease; 3, individual leaf lesions; 5, leaf and stem lesions; 7, leaf, stem and pod lesions; 9, plant death.*

[b] *%Leaf Area Diseased (%LAD).*

[c] *Cultivar foliar resistance rating designated by Pulse Breeding Australia; R, resistant; MR, moderately resistant; MR/MS, moderately resistant/moderately susceptible; MS, moderately susceptible; S, susceptible.*

[d] *Raw data in parentheses.*

[e] *Cultivars had a different field reaction over several seasons.*

This experiment was repeated the following year with lentil stubble naturally infested with ascochyta blight, collected after harvest in December 2013 from three commercial crops, *viz*. one crop of cv PBA Flash and two crops of cv Nipper, and stubble from a lentil trial consisting of a mixture of cvs Nipper and Northfield. One of the cv Nipper crops was in the lower north region of South Australia, where the density of lentil cropping is 4.5% of the arable land (PIRSA, 2014) while the other stubble lots were sourced from the Yorke Peninsula region. Stubble was incubated as described above from 10th December 2013 and pots of the lentil lines with 4 week old seedlings were sown on 6th May 2014 and placed adjacent to the stubble in ambient conditions as described above. Lesions of ascochyta blight were first observed on plants on 4th June, and infected leaves were detached each week to count and collect the lesions until 30th July 2014. Single conidium-derived isolates were collected from these lesions and stored as described above.

The cumulative number of lesions per pot were tested for homogeneity using Bartlett's variance homogeneity test and pooled data were analyzed using a generalized linear mixed model in Genstat® version 16. Where the homogeneity test was significant, data sets were analyzed separately. Significant differences were based on 95% confidence interval.

## Phenotyping Isolates Under Controlled Environment Conditions

Single conidium-derived isolates were tested on differential sets of lentil lines in a series of four experiments at SARDI Pulse and Oilseed laboratory and three experiments at The University of Melbourne, Faculty of Veterinary and Agricultural Science. A fifth experiment was also conducted at SARDI comprising NVT lentil entries from 2014.

### SARDI Isolate Phenotyping Experiments

Seventeen single conidium-derived isolates collected between 1989 and 2006 (designated 1989–2006 collection), 63 isolates collected between 2010 and 2013 (designated 2010–2013 collection) and 22 isolates collected in 2014 (designated 2014 collection) were tested on a differential set of five lentil lines in a series of four experiments in controlled conditions. Two reference isolates from Victoria, AL4 from 1998 and Kewell from 2001 (Nasir and Bretag, 1997a; Nguyen et al., 2001), were also included in each experiment to enable the ranking of isolate aggressiveness across trials. The differential set comprised Indianhead, ILL7537, cv Northfield, cv Nipper and the susceptible cv Cumra. Disease ratings of these lines had been determined by field assessments in previous seasons and in growth room experimental conditions (Sambasivam, 2011).

Seed of the five lentil lines were sown separately into pots as described above. In three experiments, each comprising 21 different isolates collected from 2010 to 2013 as described above plus the two control isolates, each lentil line was sown into 96 pots, four seeds per pot, which were thinned to three seedlings per pot after emergence. A fourth experiment, comprising the 17 isolates from 1989 to 2006 plus the two control isolates, consisted of 80 pots per line while a fifth experiment, comprising the 22 isolates from 2014 and control isolates, consisted of 100 pots per line. The experiments were of a split plot design, with isolates as the main plots and lentil lines randomly allocated to subplots. After sowing, the pots were placed in a controlled environment room (CER) at 15°C, 12 h/ 12 h light/ dark cycle in 4 plastic tents (160 × 80 × 80 cm), one replicate per tent. Pots were watered by hand as required. Seedlings were inoculated after 2 weeks as described below.

Cultures of the isolates were grown for 14 d on PDA as previously described. A conidial suspension of each isolate was prepared by flooding the plates with sterile distilled water and gently rubbing the culture surface with a sterile glass rod to suspend the conidia. The spore concentration was determined by haemocytometer and adjusted to between $9 \times 10^5$ to $1 \times 10^6$ conidia per mL. Conidial suspensions of 75 mL per isolate were produced and surfactant Tween 20 (0.01%) (Merck Pty. Ltd.) was added. Each conidial suspension was sprayed separately until runoff onto four replicate pots of each lentil line. Control seedlings (four pots per lentil line) were sprayed with sterile distilled water plus Tween 20 (0.01%) until runoff.

After inoculation, an ultrasonic humidifier using reverse osmosis water was turned on in each tent for 2 h and then for 1 h each day until disease assessment to maintain leaf wetness. Disease was assessed on each seedling 10–14 days after inoculation as % area of plant diseased (%APD), incorporating leaf and stem lesions of the 4 nodes and internodes that were spray inoculated. Data were square root transformed to normalize residuals where necessary and each experiment was analyzed separately using split plot analysis of variance with isolate as the main plot in GenStat® version 16. Cultivar x isolate reactions were placed into category Resistant (0–4.2%APD), Moderately Resistant (> 4.2–8.5%APD), Moderately Susceptible (> 8.5–13.0%APD) or Susceptible (>13.0%APD) based on the least significant difference between mean disease scores. Chi-square analysis in GenStat® version 16 was used to compare the number of isolates within each resistant category per host for the isolates in the different collection periods and for cultivar of origin for the isolates. A comparison of disease severity scores was made between isolates collected before 2006 and the isolates collected after 2006. The data were first averaged within pots to reduce variability and to satisfy assumptions of normality. Data were logarithm transformed to stabilize variance except for among the hosts and a generalized mixed linear model in GenStat® version 16 was used to analyse the data for each host independently. Significant differences between the two collection periods were based on 95% confidence interval.

### University of Melbourne Isolate Phenotyping Experiments

A total of 29 Australian *A. lentis* isolates were assessed (**Table 2**) in this experiment. These were predominately isolated in South Australia from field plants or seed stocks in 2010, 2011, and 2012. The other isolates were from field plants from Victoria isolated in 2012 with the exception of the two reference isolates Kewell and AL4. Most isolates were from lentil cvs PBA Flash and Nipper, three were from cv Nugget, two from cv Northfield and one from cv Aldinga. Inoculum was prepared as described above and the concentration adjusted to $10^6$ spores per ml before

**TABLE 2 | Mean disease score at 28 days after inoculation for *Ascochyta lentis* isolate/cultivar interactions.**

| Cultivar | ILL7537 | Indianhead | Nipper | Northfield | Flash | ILL6002 |
|---|---|---|---|---|---|---|
| **Foliar resistance rating[a]** | **R** | **R** | **R-MR/MS[b]** | **R-MR/MS** | **MS** | **S** |
| **ISOLATE** | | | | | | |
| ALP2 | 3.40 | 2.90 | 4.90 | 5.74 | 6.74 | 7.24 |
| P3040 | 3.16 | 3.83 | 5.50 | 5.00 | 5.83 | 7.00 |
| FT12022 | 3.74 | 3.07 | 5.07 | 5.24 | 6.07 | 7.07 |
| AL4 | 3.02 | 4.52 | 4.97 | 4.47 | 5.47 | 7.36 |
| P3046 | 2.73 | 3.73 | 5.06 | 4.39 | 5.73 | 7.73 |
| 61/10 | 3.12 | 3.95 | 4.62 | 5.29 | 5.12 | 7.12 |
| P3012 | 2.40 | 4.40 | 4.57 | 4.90 | 5.57 | 7.40 |
| P3026 | 3.25 | 3.25 | 4.58 | 5.41 | 5.25 | 7.41 |
| ALK1 | 3.29 | 3.46 | 4.13 | 5.29 | 5.63 | 6.79 |
| P3044 | 2.64 | 3.80 | 4.80 | 4.64 | 5.30 | 6.80 |
| FT12023 | 3.21 | 3.05 | 5.21 | 4.71 | 5.05 | 6.71 |
| FT12013 | 3.37 | 3.04 | 5.04 | 5.54 | 4.87 | 6.04 |
| FT10001 | 2.97 | 3.80 | 4.14 | 4.64 | 5.14 | 7.14 |
| MEL1 | 2.57 | 3.57 | 4.23 | 4.23 | 5.73 | 7.40 |
| ALM8 | 3.18 | 3.02 | 3.35 | 5.68 | 5.35 | 7.02 |
| P3047 | 2.81 | 3.15 | 3.65 | 4.81 | 5.48 | 7.31 |
| MEL2 | 2.63 | 3.79 | 4.63 | 3.79 | 5.63 | 6.63 |
| 68/10 | 2.91 | 3.07 | 4.24 | 4.74 | 5.41 | 6.74 |
| P3065 | 2.66 | 3.16 | 4.16 | 4.83 | 5.83 | 6.33 |
| FT12025 | 3.16 | 3.49 | 4.16 | 4.16 | 4.99 | 6.66 |
| FT12029 | 3.05 | 2.71 | 3.21 | 5.55 | 5.88 | 5.88 |
| FT10017 | 2.92 | 3.26 | 4.09 | 3.59 | 5.42 | 6.76 |
| 58/10 | 2.41 | 3.24 | 4.24 | 3.74 | 5.74 | 6.58 |
| ALR1 | 3.35 | 2.52 | 3.68 | 4.85 | 4.68 | 6.18 |
| FT10012 | 2.63 | 2.96 | 3.96 | 3.46 | 4.96 | 7.13 |
| FT10007 | 2.76 | 2.93 | 5.26 | 3.93 | 3.59 | 6.26 |
| 48/10 | 2.65 | 2.98 | 4.15 | 3.15 | 4.32 | 6.98 |
| FT10016 | 2.68 | 2.52 | 3.35 | 3.52 | 4.35 | 6.35 |
| Kewell | 2.64 | 3.09 | 3.31 | 3.25 | 3.75 | 6.48 |
| Mean | 2.94 | 3.32 | 4.35 | 4.57 | 5.27 | 6.84 |
| Standard deviation | 0.33 | 0.49 | 0.62 | 0.75 | 0.66 | 0.45 |

*Lentil cultivars are presented in descending overall resistance from left to right and isolates are listed in descending order of overall aggressiveness. Scores: 0 = no disease to 9 = severe disease/plant death. LSD 5% = 0.54–1.09.*

[a]*Cultivar foliar resistance rating designated by Pulse Breeding Australia; R, resistant; MR, moderately resistant; MR/MS, moderately resistant/moderately susceptible; MS, moderately susceptible; S, susceptible.*

[b]*Cultivars had a different field reaction over several seasons.*

adding a drop of Tween 80 (Merck Pty. Ltd.). Control seedlings were sprayed with sterile water plus Tween 80. Plants were inoculated until runoff using a 500 ml hand sprayer producing a fine mist, and the pots rotated during the procedure to achieve an even spread of inoculum. The host differential set consisted of ILL7537, cvs Northfield, Indianhead, Nipper, PBA Flash and the susceptible check ILL6002. Each accession was sown as five seeds per 5 cm forestry tube filled with a 1:1 pine bark/sand mix, ameliorated with dolomite to achieve pH 7.0, and grown in a growth room at 20°C with a 12 h photoperiod. After 2 weeks, these were thinned to three seedlings per pot immediately prior to inoculation. Seedlings were watered to field capacity twice a week, and fertilized weekly from 2 weeks old with Nitrosol (Amgrow) as per the manufacturer's instructions.

The 29 isolates were tested using three separate randomized, nested, complete block design trials. Each trial assessed three plants/accession/treatment and trials were repeated three times (total of four replicates). There were 12 treatments per trial consisting of nine unknown isolates, two positive control isolates (AL4 and Kewell) and one uninoculated control. The following method was adapted from those previously used (Nasir and Bretag, 1997a; Ford et al., 1999; Sambasivam, 2011) to promote infection and maintain conditions for disease development. The six pots, each containing a different accession, were placed randomly in a solid 2 L plastic container, assigned a treatment, inoculated as described above then placed randomly in one of two 200 L plastic crates to minimize air flow present in the growth room. The crate also contained water (2–4 cm depth) to maintain humidity. The crate was covered tightly with a lid and wrapped in black plastic for 48 h post inoculation to provide dark conditions with 100% humidity to promote infection. After removal of the coverings, the crates were misted three times a day and covered with damp hessian for 48 h each week to provide conditions conducive to disease. The growth room conditions were the same as those described above for seedling production.

Final disease assessment was made on whole plants 28 days after inoculation (dai) when discrimination of disease reaction between susceptible and resistant plants was distinct (Ford et al., 1999). One observation was made from each seedling. The subjective 1–9 disease index used by previous researchers (Nasir and Bretag, 1997a; Ford et al., 1999; Sambasivam, 2011) was modified by specifying a size limit of small lesions and percentage leaf drop. The scores were: 1 = no visible disease symptoms; 3 = leaf lesions only, chlorosis of affected leaves, < 10% leaf drop; 5 = leaf lesions, up to 25% leaf drop, stem flecks or lesions < 2 mm; 7 = leaf lesions, up to 50% leaf drop, stem lesions > 2 mm; 9 = leaf lesions, potential defoliation, stem girdling, potential plant death.

Statistical analysis was performed using GenStat® version 16. Data from all three trials were then pooled and analyzed using Linear Mixed Model analysis. The use of the same two controls in each trial provided a means of ranking isolates across trials. Data from control seedlings was excluded from all analyses to prevent bias since the scores were consistently 1. Means of disease score were calculated for isolates, cvs and the isolate /cv interaction using Least Square Difference (LSD) 5%. Interaction plots for each of the three trials were performed using Minitab 16 Statistical Software to provide a means of observing deviations from common patterns of interaction. Mean with 95% confidence limit was used to compare aggressiveness of isolates originally isolated from cv PBA Flash or cv Nipper. Mean scores were used to place isolate reactions on cultivars into categories of Resistant (score 1), Moderately Resistant (score 1.1–4.9), Moderately Susceptible (score ≥ 5–6.0) or Susceptible (score > 6.0) (Nasir and Bretag, 1998).

### NVT Lentil Lines Tested against *A. lentis* Isolates in Controlled Conditions at SARDI

Twenty seven lentil lines from 2013 NVT trials (**Table 3**) were tested in controlled conditions against isolate F13013 (collected in 2013) and isolate F10002 (collected in 2010) which were aggressive and non-aggressive, respectively, to cv Nipper based on the controlled screening described above. Isolate Kewell (collected in 2001) was also included as a control. This four replicate split plot experiment was conducted and analyzed as described above for SARDI experiments. Mean disease scores for each line from the controlled screening were regressed against mean disease field scores from Mallala 2013 and Horsham 2014 using linear regression in GenStat® version 16. The disease scores against the three isolates and the field disease scores were used to place each lentil line into one of six response groups.

## RESULTS

## Ascochyta Blight in Field Trials

In 2005 at the Melton field site, significant differences ($P = 0.001$) in ascochyta blight occurred such that no ascochyta blight was recorded on foliage of the resistant cv Nipper or on the resistant cv Northfield while the moderately resistant cvs Nugget and Boomer recorded disease scores ranging from 3.4 to 6.0 at the same site (**Table 1**). Five years later in 2010, cv Nipper recorded similar disease scores to cv Nugget (up to 5.1 and 6.1, respectively) and in 2013 cv Nipper again had a similar disease score to cv Nugget at the Mallala trial (13.3%LAD and 8.3%LAD, respectively, Least Significant Difference [LSD] = 5.7, $P < 0.05$). The resistant cvs PBA Ace, PBA Bolt, PBA Herald XT, and PBA Hurricane XT recorded a maximum disease score of 1.8 in 2010 and between 0 to 3.3%LAD in the 2013 trials. At Horsham in 2014, cv Nipper had a similar disease score to cv Nugget (4.9 and 5.2%LAD respectively, LSD = 0.3, $P < 0.05$) while the resistant cultivars listed above recorded significantly less disease than cv Nipper (0.1–1.0%LAD) (**Table 1**).

The highest seed staining DI (**Table 4**) was on cvs Boomer (18.0) and Nugget (14.0) in the Melton 2005 trial (LSD = 0.5, $P < 0.05$). The DI from the Sandilands 2005 trial were generally lower than from Melton but again cvs Boomer and Nugget had significantly higher DI than cv Nipper or cv Northfield. Very low DI was recorded on cvs Nipper (0.70) and Northfield (0.41) in both trials. In the Mallala 2014 trial, the highest seed DI was on cvs PBA Jumbo (7.9) and PBA Flash (4.9) (LSD = 1.6, $P < 0.05$). All other cultivars, including Nipper and Northfield, had a DI not significantly greater than zero.

### Isolate Collection from Infested Lentil Stubble

Bartlett's variance homogeneity test was significant between years for the stubbles incubated in 2013 and 2014 (Chi-square 30.1 on 6 df, $P < 0.001$) but was not significant within each year, hence the 2013 and 2014 data sets were analyzed separately. In 2013 the origin of the stubble had no significant influence on lesion production but significant differences ($P < 0.001$) were observed in cultivar reactions such that the majority of the lesions developed on the susceptible cv Cumra and moderately susceptible cv PBA Flash, and least number of lesions developed on the remaining cultivars which ranged from an intermediate resistance (moderately resistant/moderately susceptible) to resistant (**Figure 1A**). In 2014 there was a significant interaction between stubble source and lesion host ($P < 0.001$). However responses mirrored those of 2013 in that for each stubble source the majority of lesions developed on either cv Cumra or cv PBA Flash, followed by either the cvs Nipper or Northfield and then cv Nugget. Least or no lesions developed on the three resistant cvs Indianhead, PBA Herald XT and ILL7537 (**Figure 1B**).

## Phenotyping Isolates Under Controlled Environment Conditions

The interaction between cultivar and isolate was significantly different for disease scores in the SARDI and University of Melbourne tests. Disease scores in SARDI tests ranged from 0 to 33.2%APD with $LSD_{interaction}$ ($P < 0.05$) ranging from 2.9 to 6.7 for individual experiments. The disease scores in the University of Melbourne tests ranged from 2.4 to 7.73 (1–9 scale) with $LSD_{interaction}$ ($P < 0.05$) ranging from 0.54 to 1.09. The analyzed results were used to place the isolate reactions on the differential hosts into resistance and susceptible categories (**Table 5**). The majority of isolates in the SARDI collection (73–100%) caused susceptible or moderately susceptible reactions on cv Cumra, and all isolates screened at The University of Melbourne tests caused a susceptible reaction on ILL6002 (the most susceptible of the lines tested) while ILL7537 was the most resistant line in both series of tests (Sambasivam, 2011). The cv Indianhead was resistant or moderately resistant to all isolates in both SARDI and University of Melbourne tests, although a small amount of disease (<4.2%APD) was recorded on this line. The cv PBA Flash showed moderate disease reaction overall in line with its field rating (Hawthorne et al., 2012).

In SARDI tests, the previously resistant cv Nipper was susceptible to a greater number of isolates in the more recent collection compared to earlier collections (**Table 5**). Only 23% of the isolates collected between 1989 and 2006 produced a susceptible or moderately susceptible reaction on this cultivar but this significantly increased ($P = 0.006$) to 68% in the 2014 isolate collection. The resistant cv Northfield was susceptible or moderately susceptible to 91% of isolates from the 2014 collection. This was an increase from 23% of isolates collected during 1989–2006 ($P < 0.001$). The percentage of isolates with a resistant reaction on the susceptible cv Cumra was significantly higher ($P = 0.02$) in the 2010–2013 collection compared to the 1989–2006 collection and the 2014 collection (**Table 5**). The severity of disease on cv Nipper assessed in the controlled conditions, averaged for isolates from each collection period, significantly increased (Wald statistic 15.4, $P < 0.001$) over time (**Table 6**); i.e., 4.0%APD by isolates collected from 1989 to 2006 compared to 6.5%APD for isolates collected after 2006. Similar results were obtained at The University of Melbourne whereby the cvs Northfield and Nipper appeared less resistant than in the previous study conducted by Sambasivam (2011). The disease severity on ILL7537 did not vary with the two collection periods

**TABLE 3 | Ascochyta blight disease scores on National Variety Trial lentil entries for 2014 inoculated with three separate isolates of *Ascochyta lentis* in a controlled environment room, and in Mallala Pulse Breeding Australia (PBA) trial in 2013 and Horsham PBA trial 2014.**

| Lentil line | Pedigree | Controlled environment study % area of plant diseased, averaged for 4 replicates × 4 plants | | | Field trials % leaf area diseased (%LAD), averaged for 3 replicates | | Response group |
|---|---|---|---|---|---|---|---|
| | | Control isolate | Non-aggressive isolate on cv Nipper | Aggressive isolate on cv Nipper | Mallala PBA trial 2013 | Horsham PBA trial 2014 | |
| | | Kewell | FT10002 | FT13013 | %LAD | Sqrt %LAD | |
| Flash | ILL7685/Nugget | 3.7 | 4.6 | 22.9 | 28.3 | 3.51 (12.3)[a] | 1: Most susceptible line in these tests, lacking major resistance genes and little partial resistance |
| [b]CIPAL1421 | CIPAL0105-EMS03/CIPAL611//PBA Blitz | 8.1 | 5.8 | 15.0 | 4.3 | 2.19 (4.8) | 2: Lacking major resistance genes but displays partial resistance in field |
| PBA Jumbo | Aldinga/CDC Matador | 1.4 | 3.3 | 14.2 | 8.3 | 2.25 (5.0) | 3a: Susceptible to the new isolate (FT13013)and some susceptibility to older isolate (FT10002) |
| Nugget | Northfield/ILL5714 | 1.8 | 5.5 | 12.5 | 8.3 | 2.80 (5.2) | |
| CIPAL1405 | PBA Blitz/CIPAL804//CIPAL611 | 1.4 | 10.1 | 16.7 | 2.1 | 1.82 (3.3) | |
| Northfield | | 0.8 | 3.6 | 17.9 | 3.3 | 1.88 (3.5) | |
| PBA Greenfield | CIPAL205/Boomer//PBA Flash | 0.0 | 3.6 | 5.9 | 1.7 | 2.37 (5.6) | 3b: Susceptible to the new isolate (FT13013) and resistant to older isolates |
| Nipper | Indianhead/Northfield//Northfield | 0.0 | 0.1 | 8.3 | 13.3 | 2.20 (4.9) | |
| PBA Bounty | ILL6788/Nugget | 0.1 | 1.6 | 9.6 | 10.0 | 2.08 (4.3) | |
| CIPAL1403 | CIPAL405/CIPAL503//PBA Flash | 0.0 | 0.3 | 6.7 | 2.3 | 2.09 (4.4) | |
| CIPAL1404 | Nipper/CIPAL401//PBA Flash | 0.1 | 1.3 | 6.8 | 3.1 | 1.68 (2.8) | |
| PBA Blitz | Cumra/Indianhead//Cassab | 1.0 | 5.3 | 3.1 | 3.3 | 1.18 (1.4) | 4a: Moderate resistance to new isolate (FT13013) but some susceptibility to older isolate (FT10002) |
| PBA Giant | PBA Flash/Boomer | 1.1 | 1.3 | 1.6 | 3.3 | 2.00 (4.0) | 4b: Moderate resistance to tested isolates but some susceptibility in field |
| CIPAL1302 | 96-047L-99R099/CIPAL204//CIPAL401 | 13.8 | 0.0 | 0.1 | 0.0 | 0.53 (0.3) | 5a: Resistant to recent isolates F13013 and FT10002 but susceptible to the control isolate Kewell; all except CIPAL901 have CDC Matador in pedigree (96-047L being a Nugget/ CDC Matador cross) |
| CIPAL1303 | 96-047L-99R099/CIPAL204//CIPAL401 | 7.1 | 0.0 | 0.9 | 0.0 | 0.41 (0.2) | |
| CIPAL1422 | PBA Herald XT/PBA Bolt | 8.4 | 0.0 | 0.1 | 0.0 | 0.94 (0.9) | |
| CIPAL901 | CIPAL501/CIPAL205//PBA Flash | 6.3 | 0.0 | 0.0 | 0.0 | 0.55 (0.3) | |
| CIPAL1204 | 96-047L-99R099/CIPAL0204// CIPAL0401 | 2.7 | 0.0 | 0.4 | 0.0 | 0.54 (0.3) | |
| CIPAL1402 | 99-068L-2-02H042/ 02-325*03HS001 | 3.4 | 0.1 | 0.0 | 0.0 | 0.46 (0.2) | |
| PBA Hurricane XT | PBA Flash/96-047L*99R060M3 | 8.12 | 0.1 | 0.1 | 0.0 | 0.67 (0.4) | |

*(Continued)*

**TABLE 3 | Continued**

| Lentil line | Pedigree | Controlled environment study % area of plant diseased, averaged for 4 replicates × 4 plants | | | Field trials % leaf area diseased (%LAD), averaged for 3 replicates | | Response group |
|---|---|---|---|---|---|---|---|
| | | Control isolate | Non-aggressive isolate on cv Nipper | Aggressive isolate on cv Nipper | Mallala PBA trial 2013 | Horsham PBA trial 2014 | |
| | | Kewell | FT10002 | FT13013 | %LAD | Sqrt %LAD | |
| Boomer | Digger/Palouse | 0.0 | 0.1 | 1.4 | 0.0 | 1.10 (1.2) | 5b: Resistant to the recent isolates (FT13013 and FT10002) and resistant to the control isolate Kewell; all except PBA Jumbo2 and Boomer have CDC Matador in pedigree |
| CIPAL1401 | PBA Bolt/02-325*03HS001 | 1.8 | 0.0 | 0.1 | 0.0 | 0.75 (0.6) | |
| PBA Jumbo2 | CIPAL205/Boomer//CIPAL401 | 0.8 | 0.0 | 0.0 | 0.0 | 0.03 (0.0) | |
| PBA Bolt | ILL7685/96-047L*99R060 | 0.7 | 0.0 | 0.0 | 1.7 | 0.53 (0.3) | |
| PBA Herald XT | 96-047L*99R060-EMS02 | 0.1 | 0.0 | 0.0 | 0.0 | 1.00 (1.0) | |
| CIPAL1301 | PBA Bolt/02-325*03HS001 | 0.0 | 0.0 | 0.1 | 0.0 | 0.36 (0.1) | |
| PBA Ace | CIPAL501/96-047L*99R099 | 0.0 | 0.0 | 0.0 | 0.0 | 0.36 (0.1) | |
| Least significant difference ($P < 0.05$) | | | 3.9 | | 1.9 | 0.34 | |

[a] *Raw data in parentheses.*

[b] *CIPAL refers to lentil crosses made by the Coordinated Improvement Program of Australian Lentils multiplied for potential release.*

**TABLE 4 | Ascochyta blight seed staining scores, Disease Index (DI), on seed harvested from cultivars in three field trials in South Australia at Melton (2005), Sandilands (2005) and Mallala (2014).**

| Site | Melton | Sandilands | Mallala |
|---|---|---|---|
| Year | 2005 | 2005 | 2013 |
| Disease rating | Sqrt DI[a] | Sqrt DI[a] | DI[b] |
| **LENTIL CULTIVAR** | | | |
| Boomer | 4.17 (18.0)[c] | 1.91 (3.9) | 0.7 |
| Nipper | 0.71 (0.65) | 0.69 (0.5) | 0.4 |
| Northfield | 0.72 (0.60) | 0.52 (0.3) | 0.07 |
| Nugget | 3.72 (14.0) | 1.00 (1.05) | 1.4 |
| PBA Ace | | | 0.0 |
| PBA Blitz | | | 1.3 |
| PBA Bounty | | | 0.6 |
| PBA Flash | | | 4.9 |
| PBA Herald XT | | | 0.07 |
| PBA Hurricane XT | | | 0.3 |
| PBA Jumbo | | | 0.1 |
| LSD ($P \leq 0.05$) | 0.5 | 0.3 | 1.6 |

[a] *Disease Index (DI) assessed using a categorical scale of 0–3 whereby 0, no staining; 1, ascochyta blight lesions ≤ 1 mm diameter; 2, ascochyta blight lesions > 1 mm diameter and <25% seed coverage; 3, ≥25% seed coverage.* $DI = [\sum_{i=0}^{n} (d_i * s_i / N] * 100/C$ *where* $s_i$ *refers to the number of seeds in each disease category,* $d_i$ *is the value of the disease category, N is the total number of assessed seeds per plot and C is the number of disease categories.*

[b] *Disease Index assessed using a 0–5 categorical scale; 0, no staining; 1, ascochyta blight lesions ≤ 1 mm diameter; 2, ascochyta blight lesions > 1 mm and <10% seed coverage; 3, ascochyta blight lesions > 2 mm and < 10% seed coverage; 4, ascochyta blight lesions > 3 mm and between 10 and 25% seed coverage; 5, ≥25% seed coverage.*

[c] *Raw data in parentheses.*

in the SARDI tests while cv Indianhead had significantly less (Wald statistic 43.3, $P < 0.001$) severe reaction to the later isolates although disease scores were low ($\leq$ 1%APD) on this host (**Table 6**).

Chi-square analysis of the effect of cultivar of origin of the isolates on resistant and susceptible reactions was not significant in this study. Isolates that caused a susceptible or moderately susceptible reaction in SARDI tests on cv Nipper originated from a range of host cultivars *viz*: cvs Nipper, Cumra, PBA Flash, PBA Blitz, PBA Herald XT. Five isolates aggressive to cv Northfield were originally isolated from cvs Nipper, Northfield, Cumra and PBA Flash. Three other isolates that originated from cv Northfield caused small lesions (resistant or moderately resistant) on cvs Nipper and Northfield. As mentioned above a small amount of disease was occasionally recorded on cv Indianhead and ILL7537, including one isolate collected from cv Indianhead and three from ILL7537 in the stubble experiments. These isolates only developed a small amount of disease on the other hosts including cv Cumra. In the University of Melbourne tests the isolates derived from cv PBA Flash had a similar mean aggressive score to isolates from cv Nipper (means ± 95%CI: PBA Flash 4.62 ± 0.16; Nipper 4.45 ± 0.24). The two isolates from cv Northfield were ranked 8th and 26th out of 29 for aggressiveness while the single isolate from cv Aldinga was in the top 10 for aggressiveness (ranked 7th).

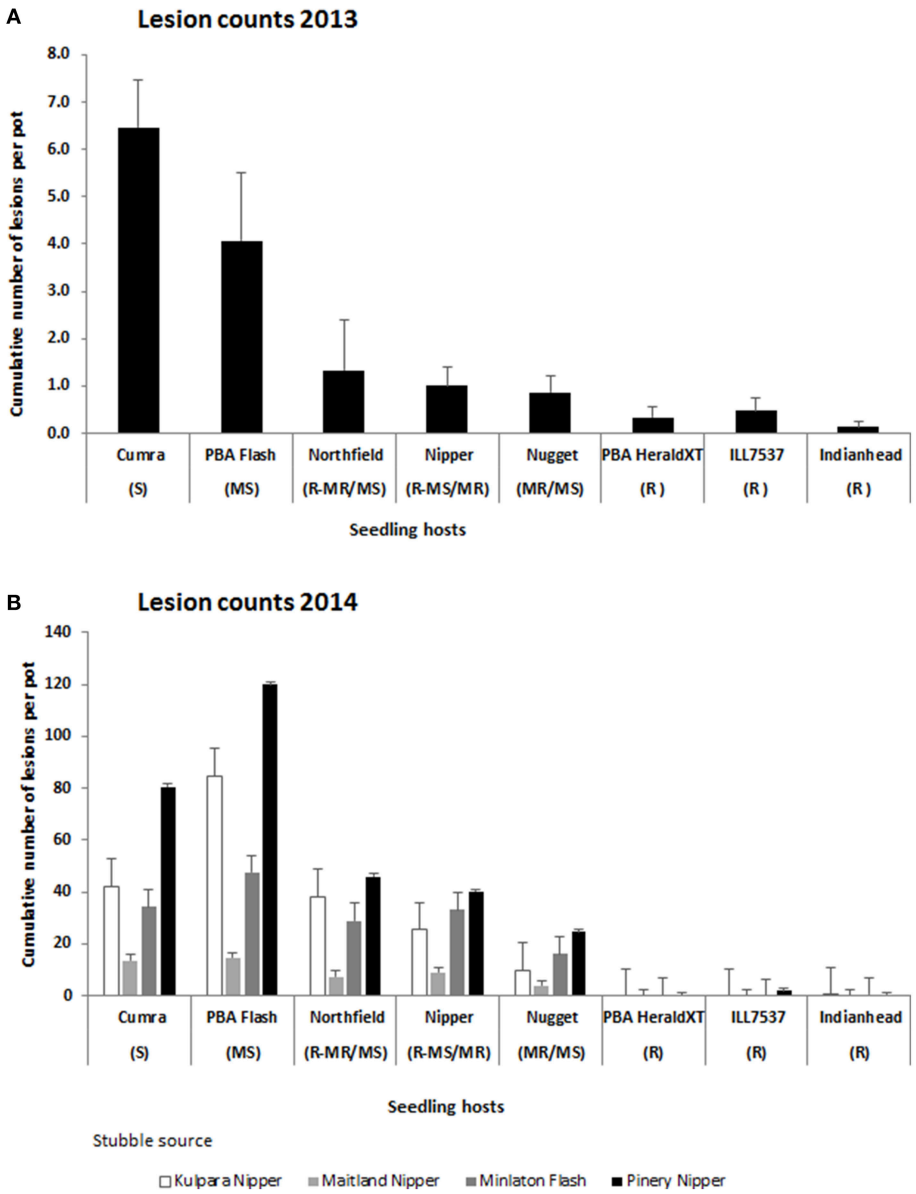


**FIGURE 1 | Cumulative number of ascochyta blight lesions assessed on 5 lentil seedlings per pot adjacent to naturally infested lentil stubble from commercial crops or field trials incubated in (A) 2013, average of three stubble sets, LSD 5% = 2.1 and; (B) 2014, LSD 5% (interaction stubble set x seedling host) = 13.8.** Vertical bars represent standard errors of the means. R, resistant; MR, moderately resistant; MR/MS, moderately resistant/moderately susceptible; MS, moderately susceptible; S, susceptible.

The disease scores of the 29 isolates screened on the six differentials at The University of Melbourne (**Figure 2**) overlapped, indicating a range of aggressiveness without distinction. Similar observations were made in the SARDI experiments (data not shown). Isolate/cultivar interaction scores showed that while ILL7537 was broadly resistant and ILL6002 susceptible to all isolates tested, significant differences in disease severity were produced by specific isolates on specific cultivars (**Table 2**). Similarly, eight isolates in the 2010–2013 SARDI collection caused a susceptible reaction on cvs Nipper and Northfield but the converse did not hold with an additional five isolates causing a susceptible reaction on cv Northfield. Only two isolates in the SARDI 2014 collection did not show the same reaction on cvs Nipper and Northfield, with one causing a moderately resistant reaction on cv Nipper but moderately susceptible on cv Northfield and vice versa for the other isolate. In The University of Melbourne tests, the cv PBA Flash produced the largest range of disease response (disease score range 3.75–6.74), followed in descending order by cvs Northfield, Nipper and Indianhead, thus providing data on specific isolate/cultivar combinations producing high, medium or low disease responses. Again similar observations were made

**TABLE 5 | Number of isolates from different collection periods that cause resistant or susceptible reactions when tested against differential hosts in controlled conditions at (A) SARDI and (B) University of Melbourne.**

| Differential hosts | Isolate × Host resistance category | *A. lentis* isolates | | | Chi-square between Collection 1 and 2 ($df = 1$) | Chi-square between Collection 1 and 3 ($df = 1$) |
|---|---|---|---|---|---|---|
| | | Collection 1 1989-2006 | 2010–2013 Collection 2 | Collection 3 2014 | | |
| **(A) SARDI** | | | | | | |
| Cumra[a] | Resistant[b] | 0 (0%) | 17 (27%) | 1 (5%) | 5.8 ($P = 0.02$) | Not significant |
| | Susceptible[c] | 17 (100%) | 46 (73%) | 21 (95%) | | |
| Northfield | Resistant | 13 (77%) | 50 (79%) | 2 (9%) | 0.07 ($P = 0.08$) | 18.4 ($P < 0.001$) |
| | Susceptible | 4 (23%) | 13 (21%) | 20 (91%) | | |
| Nipper | Resistant | 13 (77%) | 55 (87%) | 7 (32%) | Not significant | 7.7 ($P = 0.006$) |
| | Susceptible | 4 (23%) | 8 (13%) | 15 (68%) | | |
| ILL7537 | Resistant | 17 (100%) | 63 (100%) | 22 (100%) | –[d] | – |
| | Susceptible | 0 (0%) | 0 (0%) | 0 (0%) | | |
| Indianhead | Resistant | 17 (100%) | 63 (100%) | 22 (100%) | – | – |
| | Susceptible | 0 (0%) | 0 (0%) | 0 (0%) | | |
| | Total | 17 | 63 | 22 | | |

[a] *Susceptible check.*
[b] *Resistant (combined R and MR categories) = ≤ 8.5% area of plant diseased.*
[c] *Susceptible (combined S and MS categories) = > 8.5% area of plant diseased.*
[d] *Data could not be analyzed due to zeros in the Susceptible category.*

| Differential hosts | Isolate × Host resistance category | Collection 4 2010–2013 | Chi-square between Collections 2 and 4 ($df = 1$) |
|---|---|---|---|
| **(B) UNIVERSITY OF MELBOURNE TESTS** | | | |
| ILL6002[a] | Resistant[b] | 0 (0%) | |
| | Susceptible[c] | 27 (100%) | |
| PBA Flash | Resistant | 7 (26%) | |
| | Susceptible | 20 (74%) | |
| Northfield | Resistant | 18 (67%) | Not significant |
| | Susceptible | 9 (33%) | |
| Nipper | Resistant | 21 (78%) | Not significant |
| | Susceptible | 6 (22%) | |
| ILL7537 | Resistant | 27 (100%) | –[d] |
| | Susceptible | 0 (0%) | – |
| Indianhead | Resistant | 27 (100%) | |
| | Susceptible | 0 (0%) | |
| | Total | 27 | |

[a] *Susceptible check.*
[b] *Resistant (combined R and MR categories) = 1–4.9 (1–9 scale).*
[c] *Susceptible (combined S and MS categories) = ≥ 5.0 (1–9 scale).*
[d] *Data could not be analyzed due to zeros in the S-MS category.*

in SARDI tests whereby the range of disease scores were highest in cv Northfield (0.1–26.2%APD), followed by Nipper (0.1–16.3%APD) and Indianhead (0–4.0%APD).

## Comparison of NVT Lentil Lines Tested against *A. lentis* Isolates in Controlled Conditions and in Field Trials

The field disease scores from Mallala 2013 and Horsham 2014 were more correlated with the disease scores resulting from the isolate aggressive on cv Nipper (isolate FT13013) than with the non-aggressive isolate FT10002 (**Table 3**). The correlation coefficient for comparison of isolate FT13013 with Mallala field scores = 0.82 ($P < 0.001$) and with Horsham = 0.87 ($P < 0.001$); the correlation coefficient for comparison of isolate FT10002 with Mallala = 0.53 ($P < 0.02$) and with Horsham (raw data) = 0.68 ($P < 0.001$). Results were comparable between the two field sites i.e., correlation coefficient = 0.92 ($P < 0.001$).

**TABLE 6 | Comparison of mean disease scores for *Ascochyta lentis* isolates collected prior to 2006 compared to isolates collected after 2006 tested on five lentil hosts in controlled conditions at SARDI.**

| Host (Foliar disease rating) | Mean of log (disease +0.05) (raw data in parentheses) | | Standard error of difference | Wald statistic | *P*-value |
|---|---|---|---|---|---|
| | 1989–2006 collection | Post 2006 collection | | | |
| Cumra (S)[a] | 2.561 (14.1%) | 2.312 (12.2%) | 0.132 | 3.5 | 0.061 |
| Northfield (R-MS/MR)[b] | 1.52 (6.9%) | 1.553 (7.3%) | 0.185 | 0.0 | 1.0 |
| Nipper (R-MS/MR) | 0.815 (4.0%) | 1.503 (6.5%) | 0.176 | 15.4 | 8.75E-05 |
| ILL7537 (R) | −0.571 (0.2%) | −0.5827 (0.1%) | 0.048 | 0.1 | 0.752 |
| Indianhead (R) | −0.155 (1.0%) | −0.6698 (0.01%) | 0.0781 | 43.3 | 4.7E-11 |

[a]*Cultivar foliar disease rating designated by Pulse Breeding Australia; R, resistant, MR, moderately resistant; MR/MS, moderately resistant/moderately susceptible; MS, moderately susceptible; S, susceptible.*
[b]*Cultivars had a different field reaction over several seasons.*

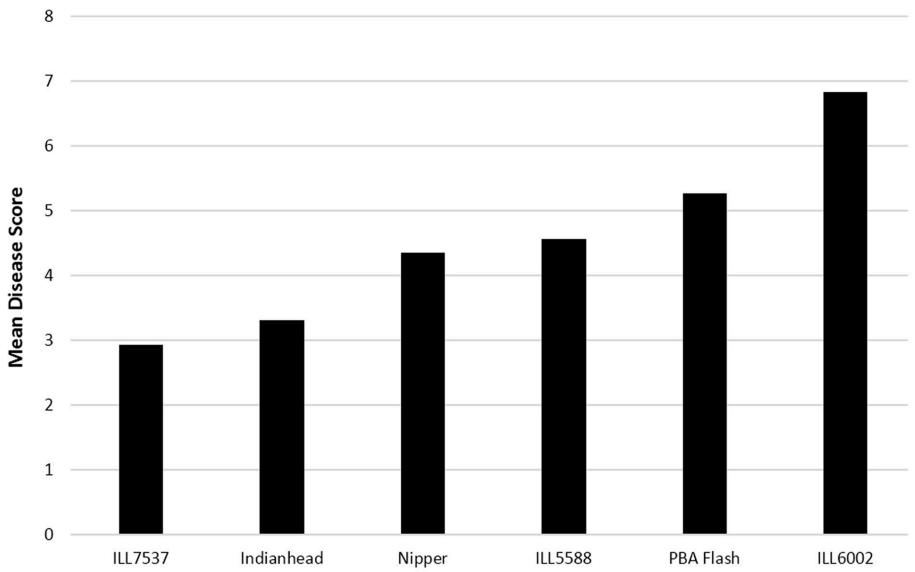


**FIGURE 2 | Mean disease score for lentil accession at 28 dai with 29 *Ascochyta lentis* isolates.** Disease scored from 0 = no disease to 9 = severe disease/plant death. LSD 5% = 0.17. R, resistant; MR, moderately resistant; MR/MS, moderately resistant/moderately susceptible; MS, moderately susceptible; S, susceptible.

Five disease response groups were identified in this set of 27 lentil lines (**Table 3**). Group 1 only contained cv PBA Flash which was the most susceptible of the lines tested, and lacked field resistance. The breeding line CIPAL1421 was the only entry in Group 2 and was also susceptible to the isolates in the controlled testing but appeared to have some field resistance. Group 3 consisted of nine entries including cvs Nipper and Northfield that were susceptible to the isolate aggressive on cv Nipper (FT13013) but resistant to the control isolate Kewell. However two subgroups were identified that were susceptible or resistant to the recent isolate FT10002. Group 4 consisted of cvs PBA Blitz and PBA Giant which were moderately resistant to FT13013 but showed differential responses to isolate FT10002. Group 5 includes accessions that were resistant to the recent isolates (FT13013 and FT10002) but separated into two subgroups based on susceptibility or resistance to the control isolate Kewell.

## DISCUSSION

The field experiments in this study were either designed as selection trials for plant breeders or were agronomic management trials and the disease assessment scales differed for these purposes. These trials were placed in fields in which lentil is part of the normal cropping rotation and management of the trials reflected local practices. Epidemics of ascochyta blight developed naturally and were similar to epidemics in surrounding commercial crops. Using isolates from breeding trials has the potential to bias results since there is a wide diversity of resistance genes in the trials, however additional isolates were collected from commercial fields. Irrespective of these issues the disease data presented here is sufficiently robust to confirm the change in field response of cv Nipper after 2010 which had also been observed in commercial crops. The phenotyping of *A. lentis* isolates on a differential host set was initiated independently in

the two institutes and data were compared after the experiments were completed. While there were minor differences in the details of experimental procedures, the methodologies were very similar and the results from the two laboratories were in agreement. Although the results of growth room studies may not be directly translatable to the field, they do enable the effects of different environmental factors on disease occurrence and progress to be assessed and compared among host genotypes and growth stages. They also provide the necessary environmental controls and repeatability required for experiments to dissect the mechanisms of resistance in lentil specifically deployed against *A. lentis*, as well as the opportunity to select isolates and lentil cultivars with identified differential disease interactions. Despite the range of sources from which the isolates of *A. lentis* were collected, the phenotyping gave similar results between each source, demonstrating that a large percentage of isolates in the most recent collections were able to infect the previously resistant cv Nipper.

This study identified a natural diversity in aggressiveness of the *A. lentis* population leading to the loss of effective resistance in the widely used cv Nipper in lentil growing regions of southern Australia just 4 years after its commercialisation. A similar increase in aggressiveness of *A. lentis* isolates over a similar time period was detected in Canada, with possible breakdown of resistance in cv Laird (Ahmed et al., 1996; Banniza and Vandenberg, 2006). The correlation of isolate screening in controlled conditions with field observations indicates that isolates aggressive to cv Nipper have become more frequent and widespread in the *A. lentis* population, possibly as a selective response to the widespread presence of this cultivar in the farming system. This cultivar reached maximum cropping in 2012, covering 20% of the total lentil area, but was grown most frequently on the Yorke Peninsula in South Australia, comprising 30% of the lentil area (S. Crane, Seednet, personal communication). The aggressive isolates detected in southern Australia did not infect other resistant cultivars and breeding lines i.e. Indianhead, ILL7537 and PBA Herald XT.

Lentils are an important cash crop, especially on Yorke Peninsula where, anecdotally, the loss of resistance in cv Nipper was first observed. PBA cultivars with adapted traits are rapidly adopted in this region and cv Nipper was widely grown due to a premium price for its small round seed, its ability to withstand lodging and its resistance to both botrytis gray mold and ascochyta blight (Pulse Australia, 2011). Subsequently the area planted to cv Nipper has fallen and largely replaced by cv PBA Hurricane XT, made popular by its improved tolerance to Group B herbicides and resistance to ascochyta blight. This cultivar has now been planted over a greater area than cv Nipper. Commercialized in 2013 (Pulse Australia, 2013), in the following season 94% of South Australian PBA Hurricane XT seed sales went to Yorke Peninsula, while other regions had a wider spread of cultivar seed sales. It is predicted that this cultivar may occupy around 50% of the Australian lentil cropping area in the future (J. Sounness, PBSeeds Pty. Ltd., personal communication).

PBA Hurricane XT and a number of other cultivars, including PBA Ace, PBA Bolt and PBA Herald XT, share the parent line CDC Matador which in turn has Indianhead parentage. These cultivars maintain their resistant status in field conditions and are resistant to the isolates identified as aggressive on cv Nipper in the controlled screening. This indicates they contain different resistance gene(s) to cv Nipper although further research is required to confirm this. While no isolates in this study were able to completely overcome the resistances in cv Indianhead or ILL7537 some caused a moderately resistant reaction confirming an earlier study (Nguyen et al., 2001) in which isolates from Victoria were virulent on cv Indianhead. These results suggest a natural variability in the *A. lentis* population. Widespread planting of lentil cultivars with Indianhead/Matador heritage could lead to the selection of aggressive isolates against this resistance with a similar outcome to that observed on cv Nipper.

While the cvs Northfield and Nipper were field resistant to ascochyta blight in South Australia prior to 2006, it is apparent from this study that isolates able to infect them were already present in the *A. lentis* population, further evidence of a natural variability in the population. As well as the increased number of isolates that showed a susceptible reaction on cv Nipper, a significant number of the isolates from all collections were also able to infect cv Northfield mirroring results of Nasir and Bretag (1997a) in Victoria. This variability in the *A. lentis* population does not appear to be affected by the host cultivar although more studies are required to confirm this. A study specifically addressing host susceptibility and related isolate aggressiveness in wheat following epidemics of *Mycosphaerella graminicola* found that isolates recovered at the end of the season from moderately resistant cultivars were more aggressive than those from susceptible ones (Cowger and Mundt, 2002), and similar selective pressure on the *A. lentis* population may also be happening. Certainly in Canada, isolates collected in 1992 were found to be more aggressive than those collected in 1978 and 1985 (Ahmed et al., 1996). The cultivation in Australia of at least moderately resistant lentil cultivars indicates that continual monitoring of aggressiveness in the local *A. lentis* population is needed.

The cultivar of the naturally infested lentil stubbles had no influence on the number of lesions observed on adjacent lentil cultivars. However the proportion of aggressive isolates may increase with the introduction of resistant cultivars, as demonstrated by the controlled screening experiments whereby a greater number of isolates collected after 2010 were aggressive on cv Nipper. The isolates collected from stubble in 2013 and 2014 showed similar characteristics to isolates collected from the field in the same year, in that at least 50% were aggressive on cvs Nipper and Northfield. However there is no data for isolates produced from stubble prior to 2013, and so no information on how variability of isolates from stubble may have changed over the years of lentil cultivation in southern Australia. This study identified very low infection of *A. lentis* on cv PBA Hurricane XT in field trials and controlled experiments but the presence of low infection combined with the high selection pressure brought about by high cropping intensity could result in the selection of aggressive forms of the pathogen as seen with cv Nipper.

Ascochyta blight infection on lentil seed and pods can affect grain yield and quality through seed abortion and seed staining (Hawthorne et al., 2012). While infection on the foliage

influences severity of seed and pod infection via rain-splash of conidia, cultivar responses to ascochyta blight on seed and foliage appear to differ (Hawthorne et al., 2012). In this study seed infection was low for cv Nipper in the field trials that were assessed, suggesting that seed resistance has remained effective. The genetics of resistance in cv Nipper are not understood however two recessive genes for foliar resistance have been identified in the parent line Indianhead (Ye et al., 2001) and a single recessive gene identified for seed resistance (Chowdhury et al., 2001). Two dominant genes have been identified in the other parent, cv Northfield, that confer resistance to foliar infection (Ford et al., 1999; Ye et al., 2001) but it is not known if these are the same genes that confer seed resistance (Tay and Slinkard, 1989; Chowdhury et al., 2001). Further research is required to understand the resistance in cv Nipper and whether shared or separate genes confer resistance to foliage and seed.

McDonald and Linde (2002) identified five evolutionary forces that contribute to the loss of effective resistance genes. Four of these five forces potentially demonstrate a high evolutionary risk for *A. lentis viz*. (1) large overseasoning populations survive on stubble maintaining virulent alleles, (2) asexual conidia are dispersed by air and the pathogen may transfer long distance on seed, (3) the reproduction system involves both annual sexual outcrossing and asexual propagules, and finally (4) the resistance genes are deployed in high cropping intensities. The fifth force is mutation rate but there is insufficient information in *A. lentis* to comment. McDonald and Linde (2002) hypothesized that pathogens like *A. lentis* that have mixed reproduction systems pose the highest risk of evolution since many new genotype combinations are created through recombination and these are "tested" in different environments, leading to the most fit types increasing in frequency through asexual reproduction. The rate of increase can be slowed by deploying genes in mixtures or in rotations through space and time which either reduces the efficiency or disrupts selection. They also state that these pathogens require most effort to achieve durable resistance and so breeding effort should concentrate on quantitative resistance which is renewed regularly to stay ahead of the pathogen. Consequently an ongoing study aimed at assessing temporal changes in aggressiveness of the *A. lentis* isolates on a range of elite Australian lentil cultivars is required to determine if potential selective evolution is occurring in relation to host resistances (Cowger and Mundt, 2002; Pariaud et al., 2009).

The identification of highly significant differences in disease reactions between specific isolates against specific cultivars in the phenotyping experiments provides opportunity for further study into the genetic differences involved. In particular, the broad range of disease severity from high to low among isolates on cvs PBA Flash, Northfield and Nipper will enable fine dissection of the interactions. The rapid loss of resistance in cv Nipper indicates there may be one or more major genes for resistance that have been rendered ineffective by changes in the pathogen population. However in addition to changes on specific hosts there is an apparent continuum of aggressiveness among the *A. lentis* isolates when assessing the mean reaction across the entire host set. This supports similar findings in the Canadian study (Ahmed et al., 1996), and is in broad agreement with the theory that the resistance that plants deploy against necrotrophs is polygenic, and can be quantitative as well as qualitative, rather than only the discreet responses seen against biotrophs (Thrall et al., 2005). While the genetic mechanisms of resistance that lentil uses against *A. lentis* are still poorly understood, reviews of this pathosystem report that both major and minor genes are inferred in the interaction, either singly or in complement, although the allelic nature of the genes is yet to be identified (Ye et al., 2001; Gupta et al., 2012). Races of necrotrophs have been identified in other pathosystems, for instance the *Phytophthora nicotianae*-tobacco interaction (Van Jaarsveld et al., 2002), based on the pathogen's ability to infect different cultivars expressing different resistance genes. However, the lack of cultivar specificity observed in the earlier Canadian study (Ahmed et al., 1996) indicates that the resistance mechanisms in lentil may be more complex. Recent evidence suggests that many plants respond to necrotrophs not only with quantitative responses but also with those activated depending on the pathogen species involved (Lai and Mengiste, 2013). Future planned sequencing of the transcriptome of lentil cultivars when challenged by *A. lentis* isolates with known aggressiveness will aid in uncovering these.

The strategy of the PBA lentil breeding program has been to develop lines with different sources of resistance to ascochyta blight from a range of parents, as demonstrated in **Table 3**. Many entries have the resistant cv CDC Matador in their pedigree while the resistance in cv PBA Jumbo2 has most likely come from parent CIPAL205, a line used extensively for ascochyta blight resistance in the Australian lentil breeding program. Relatively minor resistances have also been pyramided and one of the resulting cultivars (Boomer) shows effective resistance in the field and also in controlled conditions although the origin of this resistance is unclear since neither of the parent lines, cvs Digger and Palouse, are resistant to *A. lentis* (Ford et al., 1999; Sambasivam, 2011). However the agronomic success of individual lines such as cv Nipper and now cv PBA Hurricane XT has led to the rapid and dominant uptake of single cultivars. This intensity threatens the durability of ascochyta blight resistance in PBA Hurricane XT and related lines, and if resistance is rendered ineffective this will reduce the number of resistant sources that can be used in the Australian lentil breeding program. Better genetic understanding and molecular tools for rapid inclusion of major and minor genes is paramount to maintaining resistance to ascochyta blight in the Australian lentil industry. While additional sources of resistance must be sought, it is also important to encourage cultural practices that maintain disease resistance.

In conclusion, a broad range of aggressiveness and natural variability exists among recent Australian isolates of *A. lentis*. Also significant differences in disease severity exist among specific isolates, enabling researchers' choice of highly aggressive isolates for targeted resistance breeding efforts and individual isolate/cultivar combinations with high, medium and low levels of disease severity for future investigation of the potentially differential defense responses. Detailed understanding of the genetics of resistance to *A. lentis* is essential for the successful

future deployment of ascochyta blight resistance in lentil cultivars.

## AUTHOR CONTRIBUTIONS

LM and MR conducted field experiments; JD, LM, and MR assessed and analyzed data from field trials; GS and RF conducted and analyzed controlled experiments at Melbourne University; MHR, MK, and JD conducted controlled experiments and stubble experiments at SARDI; JD analyzed data from SARDI experiments; JD, GS, and RF wrote the manuscript; all authors reviewed the manuscript before submission.

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# A Novel *Lens orientalis* Resistance Source to the Recently Evolved Highly Aggressive Australian *Ascochyta lentis* Isolates

*Rama H. R. Dadu[1]*, Rebecca Ford[2], Prabhakaran Sambasivam[2] and Dorin Gupta[1]**

*[1] Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Dookie, VIC, Australia, [2] Environmental Futures Research Institute, School of Natural Sciences, Griffith University, Nathan, QLD, Australia*

Substantial yield losses and poor seed quality are frequently associated with Ascochyta blight infection of lentil caused by *Ascochyta lentis*. Recently reported changes in aggressiveness of *A. lentis* have led to decreased resistance within cultivars, such as Northfield and Nipper in Australia. Furthermore, the narrow genetic base of the current breeding program remains a risk for further selective pathogen evolution to overcome other currently used resistances. Therefore, incorporation of potentially novel and diverse resistance genes into the advanced lines will aid to improve cultivar stability. To identify these, 30 genotypes sourced from five wild species (*Lens orientalis, L. odomensis, L. ervoides, L. nigricans* and *L. lamottei*), including eight previously reported resistance sources, were screened for disease reaction to two recently isolated and highly aggressive isolates. Subsequently, two *L. orientalis* accessions were found highly resistant and a further six *L. nigricans,* one *L. odomensis*, one *L. ervoides*, one *L. lamottei*, and one *L. orientalis* accessions were moderately resistant. Several of these were more resistant than the currently deployed resistance source, ILL 7537. Furthermore, *L. orientalis* accession ILWL 180 was consistently resistant against other highly aggressive isolates recovered from diverse geographical lentil growing regions and host genotypes, suggesting stability and potential for future use of this accession in the Australian lentil breeding program.

**Keywords: ascochyta blight, *Ascochyta lentis,* lentil, wild lentils, screening**

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*Sabine Banniza,*
*University of Saskatchewan, Canada*

***Correspondence:**
*Rama H. R. Dadu*
*rdadu@student.unimelb.edu.au*
*Dorin Gupta*
*dorin.gupta@unimelb.edu.au*

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## INTRODUCTION

Lentil (*Lens culinaris* Medikus ssp. *culinaris*) (2n = 14), a cool season high protein (26%) food legume cultivated around the world, is ranked fifth in size of global production among legumes at 4.88 million tons (mt) (FAOSTAT, 2014). However, a significant reduction in lentil productivity (30%) was reported during 2013–2014 in Australia (FAOSTAT, 2014), largely due to the disease ascochyta blight, caused by necrotrophic fungus *Ascochyta lentis (A. lentis)*. This disease is of global concern (Kaiser and Hannan, 1986; Erskine et al., 1994; Nasir and Bretag, 1997a; Muehlbauer and Chen, 2007), reducing yields and seed quality (Morrall and Sheppard, 1981; Gossen and Morrall, 1983). It causes an estimated $15.3 million AUD in losses to the Australian lentil industry alone due to reduced production and disease management costs (Murray and Brennan, 2012).

To date, integrated disease management approaches combining best cultivation practices, application of fungicides and cultivars with moderately resistant or resistant ratings have sustained the industry in the presence of *A. lentis* (Hawthorne et al., 2012). However, continuous cultivation of relatively few resistant cultivars with narrow genetic base has likely led to episodes of resistance breakdown through selection of adapted and aggressive isolates (Nasir and Bretag, 1997b; Davidson et al., 2016; Sambasivam et al., 2017). This has also occurred for several Canadian cultivars including Laird (Ahmed and Morrall, 1996) and breeding line ILL 5588 (Tullu et al., 2010). ILL 5588 was also introduced into Australia after its success in Canada and Northfield, a selection from ILL 5588 (Ali, 1995) along with Indianhead were employed either individually or in combination to breed resistant cultivars. However, an increased susceptibility of Northfield to the Australian *A. lentis* population was detected within six seasons after its commercialization (Nasir and Bretag, 1997b). Consequently, this most likely led to the demise of the new Australian cultivar Nipper after just four seasons though carrying an Indianhead pedigree, which is still resistant to major Australian isolates (Davidson et al., 2016). Meanwhile, other Australian cultivars, such as PBA Ace, PBA Blitz, PBA Bolt, PBA Jumbo, PBA Jumbo2, PBA Herald XT, and PBA Hurricane XT, were developed containing a CDC Matador pedigree with *A. lentis* resistance from Indianhead (Pulse Australia, 2016). Several of these were found susceptible or moderately susceptible to recently detected highly aggressive Australian isolates, with predicted increasing industry reliance on those that remained somewhat resistant, such as PBA Jumbo2 and PBA Harricane XT (Davidson et al., 2016). This will again likely lead to increased selection pressure on the highly variable pathogen population (Nasir and Bretag, 1997b, 1998; Davidson et al., 2016; Sambasivam et al., 2017), to evolve and overcome the relatively few resistance sources upon which the industry is currently reliant.

Therefore, a major goal for the Australian lentil breeding program remains to introgress novel resistance genes/alleles or combinations thereof to improve the stability and further enhance durability of resistance to *A. lentis* within elite cultivated backgrounds. Several previous investigations have uncovered sources for novel *A. lentis* resistance in all five wild relative *Lens* taxa (*L. orientalis, L. odomensis, L. ervoides, L. nigricans*, and *L. lamottei*) (Bayaa et al., 1994; Ahmad et al., 1997; Tullu et al., 2010). Although crossing incompatibility exists among such broad germplasm (Ladizinsky, 1979; Ladizinsky et al., 1984), inter-specific fertile hybrids were produced through conventional techniques between accessions of *L. culinaris* and *L. orientalis* within the primary gene pool (Ladizinsky, 1999; Fratini et al., 2004; Gupta and Sharma, 2007). Success was achieved with the aid of GA3 application and embryo rescue techniques for the more incompatible crosses (Cohen et al., 1984; Ahmad et al., 1995; Tullu et al., 2013). Subsequently, segregating populations for ascochyta resistance were successfully produced from *L. culinaris* × *L. orientalis* and *L. culinaris* × *L. ervoides* crosses, within which resistance was simply inherited (Ahmad et al., 1997). More recently, Fiala et al. (2009) successfully transferred anthracnose resistance from *L. ervoides* to *L. culinaris* and developed RIL population which was later evaluated by Vail (2010). This cross was also used to generate backcrosses which were reported to be stable and without any phenotypic linkage drag with yield. Selected breeding lines evaluated under field conditions were reported to be highly resistant to anthracnose under high disease pressure.

The hypothesis is that the wild species of lentil possess novel and diverse resistance alleles/genes to ascochyta blight and the resistance conferred is potentially durable. Therefore, the aims of the current study were to 1) uncover potentially novel wide germplasm sources of resistance to the most aggressive isolates of *A. lentis* recently detected in Australia and 2) determine potential stability of the resistance(s) through screening against a diverse collection of isolates from the current population.

# MATERIALS AND METHODS

## Plant and Fungal Materials

Thirty wild lentil accessions were provided by the Australian Grain Gene bank (AGG), Horsham, Victoria (**Table 3**). Two cultivars routinely used to discriminate the reaction of *A. lentis* (Davidson et al., 2016; Sambasivam et al., 2017), ILL 6002 (susceptible) and ILL 7537 (resistant) were included as controls. Three seeds per genotype were sown in 10 cm pots filled with pine bark potting mix, fertilized with Nitrosol, Amsgrow® (4.5 mL/L) on a weekly basis and watered on every alternative day. Three replications (three inoculated and three non-inoculated/control pots) were included per each treatment combination (genotype × isolate) After sowing, pots were placed in a glass house at the Dookie Campus, University of Melbourne, Victoria maintained at 20 ± 5°C under 16/8 h day/night photoperiod until inoculation. Considering germ inhibition in wilds, 21 day old seedlings were used for bioassay, such that the leaf number and number of nodes were a minimum of 8–10 and a minimum of 4, respectively, in both wilds and 14 day old cultivars. Post inoculation, pots were moved into a Conviron growth cabinet replicating glass house conditions.

Single spore cultures of four highly aggressive isolates (FT13037, FT13038, FT13050, and FT13027) and one low aggressive isolate (F13082) of *A. lentis* were obtained from the South Australian Research and Development Institute (SARDI) (**Table 1**). These were sub cultured on the 8th day from wild lentil genotypes sowing on potato dextrose agar media (PDA) plates and incubated for 14 days at 22°C, 12/12 h dark/light cycle under florescent (OSRAM TLD/18W) and near Ultra Violet (UV) lights (PHILIPS BLB/18W).

## Experimental Design

Preliminary bioassays were conducted to reconfirm the aggressiveness of the two isolates (FT13037 and FT13038) by screening them against three host differentials with known resistance levels comprising ILL 7537 (resistant), Nipper (moderately resistant-moderately susceptible) and ILL 6002 (susceptible) (Davidson et al., 2016; Sambasivam et al., 2017).

Later, experiments were carried out in two stages. Initially, all 30 genotypes were screened against isolates FT13037 and FT13038 to determine disease responses and identify those

**TABLE 1 |** Details of *A. lentis* isolates used in the study.

| Isolate | Nature of isolate | Lentil–cultivar | Location in Australia | Date collected |
|---|---|---|---|---|
| FT 13027 | Aggressive | Blitz | Maitland, South Australia (SA) | 2/08/2013 |
| FT 13037 | Aggressive | Flash | Urania, SA | 29/07/2013 |
| FT 13038 | Aggressive | Cumra | Urania, SA | 29/07/2013 |
| FT 13050 | Aggressive | Nipper | Mallala, SA | 30/08/2013 |
| F13082 | Non-aggressive | Nipper | Pinery, SA | 24/09/2013 |

with lowest disease severity. Subsequently, the highly resistant genotype (ILWL 180) identified was assessed for its reaction to all five isolates. All the experiments were set out in a completely randomized design under controlled conditions with 3 replications. Initial screening included 120 treatment combinations (30 genotypes × 2 isolates × 2 inoculation treatments (inoculated or non-inoculated)), whereas stability experiments included 10 treatment combinations (1 genotype × 5 isolates × 2 inoculation treatments (inoculated or non-inoculated)).

## Preparation of Inoculum and Bioassay

Preparation of spore suspension and subsequent inoculation of pots was followed as described in previous studies (Ford et al., 1999; Davidson et al., 2016). Fourteen-day-old fungal plate cultures were flooded with sterile water and pycnidiospores were harvested by gently disturbing the surface with a sterile glass rod. Spore suspensions were filtered through a 250 mm sieve to separate the spores from mycelia and the resultant concentration adjusted to $1 \times 10^6$ spores/mL using a haemocytometer. Two to three drops of Tween 20 (0.02% v/v) per 100 mL of spore suspension was added as a surfactant. Subsequently, 3-week-old seedlings of each wild *Lens* genotype and 2 week old seedlings of both controls were uniformly inoculated using an air pressurized hand sprayer until run off. Control/non-inoculated pots were sprayed with water mixed with Tween 20 (0.02% v/v).

Meanwhile, bioassay conditions were adapted from Chen and Muehlbauer (2003) and Davidson et al. (2016) to stimulate the development of blight symptoms on plants. Post inoculation, all pots were covered with long inverted solid paper cups and placed in plastic crates filled with 2–4 cm of water to facilitate 24 h of leaf wetness and darkness. After 48 h, the cups were removed and the plants were covered with wet hessian bags to maintain high humidity until first appearance of disease symptoms. Further, plants were also misted thrice daily to improve the spore germination of the fungus.

### Disease Assessment

Each of the three seedlings per pot was scored for symptoms of *A. lentis* infection at 14 and 21 days post inoculation (dpi) (Ford et al., 1999; Sambasivam et al., 2017) using a non-destructive 1–9 scoring scale specifying a size limit on leaf and stem lesions and percentage leaf drop (Ford et al., 1999; Davidson et al., 2016; Sambasivam et al., 2017). The scores were 1 = no disease symptoms; 3 = leaf lesions only, chlorosis of affected leaves, < 10% leaf drop; 5 = leaf lesions, up to 25% leaf drop, stem flecks, or lesions <2 mm; 7 = leaf lesions, up to 50% leaf drop, stem flecks or lesions >2 mm; 9 = leaf lesions, potential defoliation, stem girdling and potential plant death (adapted from Davidson et al., 2016).

### Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics software. Data from all the control (non-inoculated) replicates were excluded from analysis since plants were symptom free with a consistent score of 1. Modes of disease scores of each pot were calculated to study each (genotype × isolate) interaction and Friedman's non-parametric analysis of variance was used to assess the modal variances among them. Most frequently observed scores pooled from three inoculated replicates were used to calculate modal disease score at 14 and 21 dpi. Modal disease scores were used to categorize the genotypes into resistant (1–3), moderately resistant (5) and susceptible (7–9) (Ford et al., 1999; Nguyen et al., 2001; Rubeena et al., 2006). Area under disease progress curve (AUDPC) was used to summarize the disease intensity over time and was estimated as described by Campbell and Madden (1990).

$$\text{AUDPC} = \sum_{i=1}^{n} \left( \frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i) \quad (1)$$

Where; n = total number of observations, $y_i$ = modal disease score at the *i*th observation and $t$ = time at the *i*th observation.

# RESULTS

## Phenotyping of Wild Genotype Resistance to Two Most Aggressive Isolates of *A. lentis*

From preliminary screening on ILL 7537, Nipper and ILL 6002, both isolates FT13038 and FT13037 were deemed aggressive, producing a susceptible reaction on ILL 6002 and Nipper with extensive leaf lesions, stem girdling and subsequent plant death at 21 dpi. Further, both isolates produced leaf lesions on ILL7537, with isolate FT13037 (Modal disease score of 7) more aggressive than isolate FT13038 (Modal disease score of 3) ($P = 0.001$) (**Table 2**).

Following inoculation of the 30 wild *Lens* genotypes with these isolates, first visual symptoms (leaf lesions) occurred from 7 dpi and stem lesions coalesced leading to stem girdling and plant death by 21 dpi on the most susceptible genotypes. Disease symptoms did not appear

**TABLE 2 |** Modal disease score of three host differentials at 14 and 21 dpi for *A. lentis* isolates FT13038 and FT13037.

| S. No | Isolate/Genotype | FT13037 | | FT13038 | |
|---|---|---|---|---|---|
| | | 14 DPI | 21 DPI | 14 DPI | 21 DPI |
| 1 | ILL 6002 | 7 | 9 | 7 | 9 |
| 2 | ILL 7537 | 5 | 7 | 3 | 3 |
| 3 | Nipper | 7 | 9 | 7 | 9 |

until 11 dpi on other genotypes and several were observed to overcome the infection. This demonstrated a range of disease reactions, from susceptible to resistant based on Friedman's test ($P = 0.002$). Two genotypes of *L. orientalis,* ILWL 180 and ILWL 7, were resistant to both isolates at 21 dpi with modal disease scores of 1 and 3, respectively, whereas five genotypes of *L. nigricans,* ILWL 37, PI 572348, PI 572351, PI 572359, and PI 615677, were resistant to just isolate FT13038 (**Table 3**).

Unsurprisingly, disease severity increased significantly between 14 and 21 dpi for most of the genotypes assessed and when inoculated with either isolate. However, disease severity on ILWL 221, ILWL 235, ILWL 261, ILWL 325, PI 572334, PI 572345, PI 572347, PI 572348, and PI 572360, to isolate FT13038 did not progress after 14 dpi, potentially indicating stability of the resistance response(s) to this isolate. These accessions did however become susceptible at 21 dpi following inoculation with isolate FT13037. Similarly, ILL 7537 was resistant to isolate FT13038 but susceptible to isolate FT13037 at 21 dpi (**Table 3**).

Significant differences were observed among the AUDPC of genotypes following inoculation with either of the highly aggressive isolates. Isolate FT13037 was able to cause significantly more disease on 25 of the genotypes, and on the two controls, compared to isolate FT13038. The remaining five genotypes, ILWL 70, ILWL 160, PI 572347, ILWL 7, and ILWL 180, had equal or significantly higher disease over time when inoculated with isolate FT13038 compared to isolate FT13037. The highest and lowest disease severity and AUDPC was observed on genotypes ILWL 206 (90.41) and ILWL 180 (19.46), respectively when inoculated with isolate FT13038. Meanwhile, the highest and lowest disease severity and AUDPC was observed on genotypes PI 572362 (123.66) and ILWL 180 (18.69), respectively when inoculated with isolate FT13037.

Five genotypes, PI 572348, PI 572359, PI 615677 PI 572351, and ILWL 180, were more resistant than ILL7537 to isolate FT13038, and 11 genotypes, ILWLW 146, ILWL 160, PI 572333, PI 572348, PI 572347, PI 572359, ILWL 37, PI 615677, PI 572351, ILWL 7, and ILWL 180, were more resistant than ILL7537 to isolate FT13037. Likewise, two genotypes, ILWL 206 and PI 572342, were more susceptible than ILL6002 to isolate FT13038, and six genotypes, PI 572362, ILWL 172, PI 572330, PI 572317, ILWL 116, and ILWL 206, were more susceptible than ILL6002 to isolate FT13037 (**Table 3**).

## *Lens orientalis* ILWL 180 as a Potential Novel Resistance Source

The genotype ILWL 180 remained resistant at 21 dpi following repeated screening with the initial two isolates as well as three further isolates FT13027, FT13050, and FT13082 ($P = 0.534$). This remained so even against what appeared to be the most aggressive isolate FT13037, which was able to overcome ILL7537 ($P = 0.001$) (**Figure 1**; **Table 4**).

# DISCUSSION

Evolution of the pathogen population toward more highly aggressive isolates has likely contributed to failure and reclassification of the resistance status of widely grown cultivars, such as Laird and breeding line ILL 5588 in Canada (Morrall, 1997; Morrall et al., 2004), and Northfield and Nipper in Australia (Nasir and Bretag, 1997b; Davidson et al., 2016), although this requires further spatial and temporal population assessment for validation. Furthermore, the broad diversity within the *A. lentis* population will likely maintain pressure on the few remaining resistance sources within the Australian cultivars (Davidson et al., 2016). Hence, introduction of potentially novel resistance sources from diverse germplasm, such as wild relatives is pivotal for maintaining production stability within the lentil industry.

The recent inclusion of ILL7537 as a resistance source within the Australian breeding program was largely consistent with the findings of this study, whereby this accession was resistant against the majority of isolates assessed. Although none of the existing varieties have ILL7537 as one of the parent in their pedigree, isolate FT13037, which was isolated in 2013 from Urania, the Yorke Peninsula of South Australia, was able to cause severe disease on ILL7537 under the bioassay conditions. Therefore, caution should be taken when relying upon this source of resistance for future resistance breeding strategies. The resistance status of this source and Indianhead was previously questioned following controlled bioassays (Nguyen et al., 2001; Davidson et al., 2016).

The quantitative summary of disease severity and progression in this study identified resistant genotypes from *L. orientalis* (2) and *L. nigricans* (5) but not from *L. odomensis, L. ervoides* or *L. lamottei*. This agreed with the findings of Tullu et al. (2010), who reported ILWL 206 (*L. ervoides*) as susceptible and ILWL 146 (*L. orientalis*) as moderately resistant against Canadian isolates. However, this was in contrast to the previous findings of Bayaa et al. (1994), who reported that ILWL 69, ILWL 116, ILWL 172, ILWL 206, and ILWL 261 were resistant to Syrian isolates, potentially indicating a higher aggressiveness of isolates within the current Australian population. The two *L. orientalis* genotypes identified in this study as resistant (ILWL 180 and ILWL 7) and moderately resistant (ILWL 146) were previously also reported to be resistant to Syrian isolates (Bayaa et al., 1994), potentially highlighting the stability of these resistance sources. Similarly, Tullu et al. (2010) identified *L. ervoides, L. nigricans,* and *L. orientalis* genotypes resistant to both Canadian and Syrian

**TABLE 3 |** Details of the genotypes used in the study along with corresponding modal disease scores at 14 and 21 DPI and AUDPC.

| S. No | Isolate/Genotype | Species | Country | FT13038 | | | | FT13037 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 14 dpi | 21 dpi | AUDPC | Category | 14 dpi | 21 dpi | AUDPC | Category |
| 1 | ILL 6002[a] | *culinaris* | A pure line selection from Argentinian variety, Precoz | 5 | 9 | 79.94 | S | 7 | 9 | 100.38 | S |
| 2 | ILL 7537[b] | *culinaris* | Jordan | 3 | 3 | 46.66 | R | 5 | 7 | 71.75 | S |
| 3 | PI 572330 | *ervoides* | Israel | 5 | 7 | 74.69 | S | 7 | 9 | 107.1 | S |
| 4 | PI 572317 | *ervoides* | Italy | 5 | 7 | 73.89 | S | 7 | 9 | 106.54 | S |
| 5 | PI 572362 | *odomensis* | Unknown | 5 | 5 | 76.23 | MR | 9 | 9 | 123.66 | S |
| 6 | PI 572336 | *ervoides* | Turkey | 5 | 7 | 79.35 | S | 5 | 9 | 84 | S |
| 7 | ILWL 172 | *odomensis* | Syria | 3 | 7 | 57.54 | S | 9 | 9 | 120.54 | S |
| 8 | ILWL 206 | *ervoides* | Bosnia and Herzegovina | 7 | 7 | 90.41 | S | 7 | 9 | 105.14 | S |
| 9 | ILWL 116 | *odomensis* | Syria | 5 | 5 | 72.35 | MR | 7 | 9 | 105.77 | S |
| 10 | PI 572342 | *nigricans* | France | 5 | 9 | 82.81 | S | 7 | 9 | 100.28 | S |
| 12 | ILWL 221 | *odomensis* | Turkey | 5 | 5 | 75.46 | MR | 5 | 9 | 77.77 | S |
| 11 | ILWL 235 | *odomensis* | Syria | 5 | 5 | 73.89 | MR | 5 | 7 | 80.12 | S |
| 14 | PI 572345 | *nigricans* | Italy | 5 | 5 | 72.35 | MR | 5 | 7 | 77.77 | S |
| 16 | PI 572334 | *ervoides* | Turkey | 5 | 5 | 68.46 | MR | 7 | 7 | 87.89 | S |
| 17 | ILWL 261 | *ervoides* | Turkey | 5 | 5 | 70.81 | MR | 5 | 7 | 75.43 | S |
| 19 | ILWL 325 | *orientalis* | Jordan | 5 | 5 | 70 | MR | 5 | 7 | 75.81 | S |
| 21 | ILWL 69 | *orientalis* | Former Soviet Union | 3 | 5 | 52.12 | MR | 5 | 7 | 75.08 | S |
| 25 | ILWL 70 | *orientalis* | Iran | 5 | 7 | 77 | S | 5 | 7 | 77 | S |
| 13 | ILWL 146 | *orientalis* | Syria | 3 | 5 | 49 | MR | 5 | 5 | 70 | MR |
| 15 | PI 572360 | *odomensis* | Israel | 5 | 5 | 66.12 | MR | 5 | 5 | 74.66 | MR |
| 18 | ILWL 437 | *lamottei* | Turkey | 5 | 5 | 71.96 | MR | 5 | 5 | 74.66 | MR |
| 20 | PI 572399 | *orientalis* | Bosnia and Herzegovina | 3 | 7 | 59.12 | S | 5 | 5 | 73.89 | MR |
| 22 | ILWL 160 | *odomensis* | Syria | 5 | 7 | 76.97 | S | 5 | 5 | 69.97 | MR |
| 23 | PI 572348 | *nigricans* | Yugoslavia | 3 | 3 | 45.12 | R | 3 | 5 | 53.69 | MR |
| 24 | PI 572347 | *nigricans* | Italy | 5 | 5 | 66.89 | MR | 3 | 5 | 52.92 | MR |
| 26 | PI 572359 | *nigricans* | Turkey | 3 | 3 | 44.31 | R | 3 | 5 | 52.12 | MR |
| 27 | PI 572333 | *ervoides* | Turkey | 3 | 5 | 49.39 | MR | 5 | 5 | 66.12 | MR |
| 28 | PI 615677 | *nigricans* | Yugoslavia | 3 | 3 | 45.12 | R | 3 | 5 | 49.77 | MR |
| 29 | ILWL 37 | *nigricans* | Turkey | 3 | 3 | 48.23 | R | 3 | 5 | 50.58 | MR |
| 30 | PI 572351 | *nigricans* | Bosnia and Herzegovina | 3 | 3 | 41.23 | R | 3 | 5 | 48.62 | MR |
| 31 | ILWL 7 | *orientalis* | Turkey | 3 | 3 | 50.58 | R | 3 | 3 | 44.31 | R |
| 32 | ILWL 180 | *orientalis* | Syria | 1 | 1 | 19.46 | R | 1 | 1 | 18.69 | R |

*Genotypes are arranged in descending order of overall resistance for FT13037 isolate. Scores 0 = no disease to 9 = plant death.*

[a] *ILL 6002–susceptible control.*

[b] *ILL 7537–resistant control.*

isolates, also highlighting that the wild species may possess broad resistances.

Interestingly, the resistant genotypes ILWL 180 and ILWL 146 originated from a common geographical region of Syria and other moderately resistant genotypes originated from Turkey (Bayaa et al., 1994). Associations between geographical origin and the *A. lentis* resistance trait have previously been reported in larger germplasm collections representative of different geographical regions (Bayaa et al., 1994), indicating potential co-evolution of resistance mechanisms with selection from regional populations. Given that these accessions are also resistant to the most aggressive Australian isolates, shared environmental-trait (resistance) based relationships would be useful to consider when seeking further resistance sources within germplasm collections. For this, researchers at the International Centre for Agricultural Research in Dry Areas (ICARDA) have developed a Focused Identification of Germplasm Strategy (FIGS) (Mackay, 1990, 1995, 2011; Street et al., 2008).

After identification in a wild relative species (subspecies), the next hurdle is to bring the desirable genes/alleles across to an elite cultivated background. For *Lens*, inter-species crossing has been encumbered with pre- and post-fertilization barriers, such as reduced pollen fertility, chromosomal aberrations and embryo abortion (Abbo and Ladizinsky, 1991, 1994; Gupta and Sharma, 2007). To date, no successful deployment of wild relative-derived resistance for improved *A. lentis* resistance has been reported. Nevertheless, fertile and phenotypically normal hybrids have been created between primary gene pool species, such as


**FIGURE 1 |** Response of wild *Lens* ILWL 180 to five isolates. **(a,h)** Response of resistant and susceptible controls ILL 7537 and ILL 6002, respectively to a representative isolate FT13038 21 days post inoculation. **(b)** control pot of wild *Lens* ILWL 180. **(c–g)** Response of wild *Lens* ILWL 180 to isolates FT13050, FT13027, F13082, FT13037 and FT13038 21 days post inoculation

**TABLE 4 |** Modal disease scores of ILWL 180 and controls at 14 and 21 dpi against five *A. lentis* isolates

| S. No | Isolates | ILL 6002 | | ILWL 180 | | ILL 7537 | |
|---|---|---|---|---|---|---|---|
| | | 14 dpi | 21 dpi | 14 dpi | 21DP1 | 14 dpi | 21 dpi |
| 1 | FT13037[a] | 7 | 7 | 3 | 3 | 5 | 7 |
| 2 | FT13038[a] | 5 | 7 | 1 | 3 | 3 | 3 |
| 3 | FT13050[a] | 7 | 7 | 1 | 3 | 3 | 3 |
| 4 | FT13027[a] | 7 | 7 | 3 | 3 | 3 | 3 |
| 5 | FT13082[b] | 5 | 5 | 1 | 1 | 1 | 3 |

[a]*aggressive.*
[b]*non-aggressive.*

*L. culinaris* and *L. orientalis* through conventional techniques (Wong et al., 2015). Thus, exploiting the resistance detected in *L. orientalis* would be a practical choice rather than pursuing that detected in secondary, tertiary or quaternary gene pools, which would be time consuming and laborious.

In conclusion, substantial variation for resistance to *A. lentis* is present in wild relative genepools and the *L. orientalis* accession ILWL 180 was most resistant to the most highly aggressive isolates detected in the recent Australian population. Further investigation into this resistance source is required to validate its stability against the breadth of the pathogen population and to identify resistance loci for selective breeding purposes.

## AUTHOR CONTRIBUTIONS

RD conducted bioassays, wrote the manuscript and analyzed the data. DG, RF, and PS conceived the study, participated in its design and assisted to draft the manuscript. All authors read and approved the final manuscript.

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# Genotype-Dependent Interaction of Lentil Lines with *Ascochyta lentis*

*Ehsan Sari†, Vijai Bhadauria†, Albert Vandenberg and Sabine Banniza**

*Department of Plant Sciences/Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada*

Ascochyta blight of lentil is a prevalent disease in many lentil producing regions and can cause major yield and grain quality losses. The most environmentally acceptable and economically profitable method of control is to develop varieties with high levels of durable resistance. Genetic studies to date suggest that ascochyta blight resistance genes (R-gene) in lentil lines CDC Robin, ILL 7537, 964a-46, and ILL 1704 are non-allelic. To understand how different R-genes manifest resistance in these genotypes and an accession of *Lens ervoides*, L-01-827A, with high level of resistance to ascochyta blight, cellular and molecular defense responses were compared after inoculation with the causal pathogen *Ascochyta lentis*. Pathogenicity testing of the resistant lines to *A. lentis* inoculation revealed significantly lower disease severity on CDC Robin and ILL 7537 compared to ILL 1704 and 964a-46, and no symptoms of disease were observed on L-01-827A. Histological examinations indicated that cell death triggered by the pathogen might be disrupted as a mechanism of resistance in CDC Robin. In contrast, limiting colonization of epidermal cells by *A. lentis* is a suggested mechanism of resistance in 964a-46. A time-series comparison of the expressions of hallmark genes in salicylic acid (SA) and jasmonic acid (JA) signal transduction pathways between CDC Robin and 964a-46 was conducted. These partially resistant genotypes differed in the timing and the magnitude of SA and JA signaling pathway activation. The SA signaling pathway was only triggered in 964a-46, whereas the JA pathway was triggered in both partially resistant genotypes CDC Robin and 964a-46. The expression of JA-associated genes was lower in 964a-46 than CDC Robin. These observations corroborate the existence of diverse ascochyta blight resistance mechanisms in lentil genotypes carrying different R-genes.

**Keywords: histology, cell death, salicylic acid, jasmonic acid, PR-genes**

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*Carlos Polanco, Universidad de León, Spain*

****Correspondence:***
*Sabine Banniza*
*sabine.banniza@usask.ca*

***†Present address:***
*Ehsan Sari, Aquatic and Crop Resource Development Portfolio, National Research Council, Saskatoon, SK, Canada;*
*Vijai Bhadauria, Swift Current Research and Development Centre, Agriculture and Agri-Food Canada, Swift Current, SK, Canada*

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## INTRODUCTION

Ascochyta blight of lentil (*Lens culinaris* Medik.) caused by *Ascochyta lentis* Vassilievsky (teleomorph: *Didymella lentis* W.J. Kaiser, B.C. Wang, and J.D. Rogers) is prevalent throughout many temperate lentil production regions of the world and has been reported to cause yield losses of up to 70% in Canada, 30–50% in the USA, and 50% in Australia (Gossen and Morrall, 1983; Kaiser, 1992; Brouwer et al., 1995). The most environmentally acceptable and economically

**Abbreviations:** AOC, allene oxidase cyclase; CLSM, confocal laser scanning microscopy; $C_t$, cycle of threshold; hpi, hours post-inoculation; JA, jasmonic acid; PR, pathogenesis-related proteins; qRT-PCR, quantitative real time PCR; R-gene, resistance gene; SA, salicylic acid.

profitable method of control is to develop varieties with high levels of durable resistance. A few major ascochyta blight R-genes have been characterized in different lentil genotypes (Tay and Slinkard, 1989; Andrahennadi, 1994, 1997; Ahmad et al., 1997; Ford et al., 1999; Ye et al., 2000; Nguyen et al., 2001), and varieties partially resistant to ascochyta blight have been released (Ali, 1995; Vandenberg et al., 2001, 2002).

Due to continuous exposure to insects and pathogens, plants are armed with a sophisticated immune system that recognizes various types of stimuli and responds accordingly by activating intricate and effective defense pathways (Jones and Dangl, 2006; Howe and Jander, 2008). Conclusive evidence points to the involvement of the phytohormones SA, JA, ethylene (ET), and abscisic acid (ABA) as primary signals in fine-tuning the plant immune system (Pieterse et al., 2009; Verhage et al., 2010). The accumulation of individual or blends of phytohormones upon pathogen challenge can generally be linked to the infection strategy of pathogens. The SA-dependent pathway induces resistance against biotrophic pathogens, but is also activated upon invasion by hemi-biotrophs. The JA/ET induces resistance against necrotrophs and hemibiotrophs (Kunkel and Brooks, 2002). The defense responses induced by the ABA signaling pathway are more complicated, and both, augmented resistance and susceptibility to pathogens have been reported in ABA defective mutants (Ton et al., 2009). By balancing the biosynthesis of these signaling compounds through an intricate network of cross-talk, plants are able to spatially and temporarily adjust their defense responses (Pieterse et al., 2009). However, compatible pathogens can harness these pathways to their own benefit by secreting effectors that directly or indirectly antagonize the host immune responses (Pieterse and Dicke, 2007; Grant and Jones, 2009). Recent evidence suggests that some necrotrophs even hijack resistance mechanisms that are effective against biotrophs to induce cell death and promote host cell colonization (Hammond-Kosack and Rudd, 2008; Kazan and Lyons, 2014).

As separate groups of pathogenesis-related (PR) proteins are induced when SA and JA/ET pathways are triggered, pathway-specific PR proteins have frequently been used to indirectly monitor the activation of SA and JA/ET signaling in various plant-pathogen interaction studies (e.g., Penninckx et al., 1996; Lorenzo et al., 2003). Previous studies revealed the requirement of SA signaling for induction of *PR-1*, *PR-2*, and *PR-5*, and JA signaling for *plant defensin like protein 1.2* (*PDF1.2)*, *hevein-like protein* (*HEL*), *basic chitinase* (*CHI-B*), *PR-3*, and *PR-4* (Thomma et al., 1998). *PR-1* has been widely accepted as a hallmark of SA signaling in *Arabidopsis thaliana* (L.) Heynh. (Rogers and Ausubel, 1997) and some crop species such as tomato (*Lycopersicon esculentum* Mill., Niderman et al., 1995; Tornero et al., 1997). PR-1 proteins also appear to possess anti-microbial activity (Alexander et al., 1993). Proteins of the PR-5 family are homologous to thaumatin- and osmotin-like proteins and show destructive effects on the permeability of fungal plasma membranes (Abad et al., 1996). The only PR proteins studied in lentil to date are those of the PR-4 family. Transcriptome analysis of lentil genotypes partially resistant to ascochyta blight revealed up-regulation of *PR-4* upon pathogen challenge in the partially resistant but not in the susceptible genotype (Mustafa et al., 2009). The antifungal activity of PR-4 proteins has been shown in other plant-pathogen systems (Caruso et al., 2001). Vaghefi et al. (2013) demonstrated *in vitro* the antifungal activity of a recombinant lentil PR-4 protein (LcPR4a) on *A. lentis*.

Allene oxidase cyclase is a key enzyme in the JA pathway, involved in JA biosynthesis from α-linolenic acid (Vick and Zimmerman, 1983). The *AOC* gene has been cloned from plants such as *Arabidopsis* (Stenzel et al., 2003), *Lycopersicon* (Ziegler et al., 2000), and *Medicago truncatula* Gaertn and is of primary importance in JA signaling for legume mycorrhization (Isayenkov et al., 2005). Moreover, *AOC* has potential utility as a marker for monitoring the JA signaling pathway (Leon-Reyes et al., 2010).

Microscopic examination of cellular reactions to a plant pathogen have been widely used in the study of plant-fungal interactions (Hood and Shew, 1996). Success in microscopic studies depends on the application of staining techniques that allow differentiation of plant and pathogen tissues, enabling detection of cascades of cytological events after infection. Understanding the developmental stages of a pathogen in time and space in the host plant is a prerequisite for determining the sampling intervals required for gene expression analysis of plant-pathogen interactions, and allows gene expression profiles to be correlated with cellular events. The initial infection process of *A. lentis* was studied by Roundhill et al. (1995), who showed that colonization of epidermal cells by *A. lentis* occurred after the disruption of cytoplasm indicating that *A. lentis* is either a necrotroph or hemi-biotroph with a short biotrophic phase. Recently, Sambasivam et al. (2017) compared the cellular reaction of lentil genotypes to two isolates of *A. lentis* with distinctly different levels of virulence. They found that the resistant genotypes reacted faster to pathogen infection, resulting in delayed and reduced formation of the fungal infection structures. The rapid generation of $H_2O_2$ and triggering of the hypersensitive reaction was reported as a common early response to fungal penetration in the resistant genotypes.

Research on fusarium headblight resistant wheat (Foroud et al., 2012) and powdery mildew resistant tomato lines (Bai et al., 2005) has shown that different R-genes can confer resistance through different mechanisms. The non-allelic nature of several ascochyta blight R-genes widely used in lentil breeding programs for the development of partially resistant varieties was recently confirmed (Sari, 2014). Based on the hypothesis that these non-allelic R-genes trigger different resistance mechanisms, the present study was designed to determine whether lentil genotypes carrying non-allelic R-genes differ in their cellular reactions to *A. lentis* infection. For two genotypes, the differential activation of SA and JA signal transduction pathways was also assessed.

# MATERIALS AND METHODS

## Plant Materials

Four lentil genotypes that have been widely used for improving resistance to ascochyta blight in lentil breeding programs were

used in this study: CDC Robin, 964a-46, ILL 1704, and ILL 7537. Also included were *L. ervoides* (Brign.) Grande accession L-01-827A (a single plant selection from the ICARDA accession IG 72847, Fiala et al., 2009) and lentil cv. Eston (susceptible control). CDC Robin has a recessive ascochyta blight resistance R-gene (*ral2*) derived from cultivar Indianhead and is also partially resistant to race 1 of *Colletotrichum lentis* Damm causing anthracnose (Andrahennadi, 1994; Vail et al., 2012). Breeding line 964a-46 has a dominant R-gene (*AbR1*) derived from ILL 5588, which is also the source of resistance for cv. Northfield (Ali, 1995). CDC Robin, 964a-46, and Eston were developed at the Crop Development Centre (CDC), University of Saskatchewan, Canada (Slinkard, 1981; Vandenberg et al., 2002). ILL 7537 and ILL 1704 are landraces from Jordan and Ethiopia, respectively, with resistance to ascochyta blight as reported in previous studies (Rubeena et al., 2006; Tullu et al., 2010). Nguyen et al. (2001) showed that ILL 7537 carries a R-gene different from that in ILL 5588. Using recombinant inbred lines developed from crosses among four partially resistance lines, Sari (2014) determined that non-allelic R-genes condition resistance to ascochyta blight in ILL 7537, CDC Robin, 964a-46, and ILL 1704. Analysis of a population derived from Eston and *L. ervoides* L-01-827A indicated the presence of two complementary recessive ascochyta blight R-genes in L-01-827A (Sari, 2014).

## Inoculation Procedure and Ascochyta Blight Disease Severity Rating

A conidial suspension was prepared from a monoconidial culture of *A. lentis* isolate AL57, an aggressive isolate from Landis, Saskatchewan, Canada (Banniza and Vandenberg, 2006). AL57 was stored in a cryopreservation solution containing 10% skim milk and 20% glycerol at −80°C. Spores were revitalized on 50% oatmeal agar plates (30 g oatmeal [Quick Oats, Quaker Oats Co., Chicago, IL, USA], 8.8 g agar [Difco, BD®, Sparks Glencoe, MD, USA], 1 L $H_2O$), and incubated for 7 days at room temperature. The spore suspension was prepared following the protocol described by Vail and Banniza (2008). The concentration of the spore suspension was adjusted to $5 \times 10^5$ conidia $mL^{-1}$ using a hemocytometer.

Four seeds of each lentil genotype were sown in 10 cm square pots containing a mixture of Sunshine Mix No. 4 (Sun Grow Horticulture® Ltd., Vancouver, BC, Canada) and Perlite™ (3/1 V/V). Pots were maintained for 21 days in a greenhouse with average daily temperature of 23.5°C, relative humidity of 66% and a 18 h/6 h day/night light regime supplied from the integration of natural an artificial lighting. Seedlings with 10–15 expanded leaves were inoculated with the spore suspension at a rate of 2 mL per seedling using an airbrush, and were incubated in a humidity chamber for 48 h. Since L-01-827A grew slower than the other lentil genotypes, inoculation was repeated 2 weeks after the first one for this genotype to rule out any confounding effect of growth stage with host response in this genotype. Plants were then incubated under the same greenhouse conditions as before, but on a mist bench where they were misted with water for 30 s every 90 min during the day for the remainder of the test. The experiment was conducted as a randomized complete block design with four replicates and was conducted twice. Disease severity data were collected for each of the four plants grown in each pot, 3 weeks after inoculation, using a scale of 1–10 based on 10% incremental increases in the percentage of symptomatic area on leaflets and stems. Data were converted to percentage disease severity using the class midpoints and averaged across the four plants for each replicate for data analysis.

## Microscopy of Cellular Reaction of Lentil Genotypes to *A. lentis* Infection

### Quantitative Assessment of Fungal Infection Structures

Quantitative microscopy was used to investigate how defense mechanisms counteracted the growth and development of *A. lentis* on each lentil genotype. The experiment was conducted as a randomized complete block design with three replicates and was conducted twice. Plants were grown and inoculated as described for pathogenicity testing. All inoculated leaflets of four lentil seedlings grown in one pot were pooled, representing one biological replicate, after collection at 10, 12, 24, 30, and 48 hpi). Fungal structures were stained with Uvitex-2b (Polyscience Inc., Warrington, PA, USA) following the protocol of Moldenhauer et al. (2006) with minor modification. Leaflet tissue was immersed in ethanol-chloroform (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid immediately after collection and cleared for at least 18 h followed by washing in 50% ethanol. Leaflets were then soaked in 0.1 M Tris-HCL buffer (pH = 5.8) for 30 min and stained in 0.1% (w/v) Uvitex-2b in 0.1 M Tris-HCL buffer (pH = 5.8) for 5 min. Samples were de-stained by washing four times for 10 min in water. Specimens were mounted in 50% glycerol for slide preparation.

Three leaflets were arbitrarily selected from the pool of leaflets for each biological replicate of each treatment and subjected to quantitative measurements. Percentage of conidial germination was determined for samples collected at 10 and 12 hpi by examining 100 conidia per three fields of vision. Conidia were considered germinated when they produced germ tubes equal to or longer than the conidial diameter. To determine the length of infectious hyphae, leaflets collected at 24, 30, and 48 hpi were examined in 10 fields of vision (each containing a minimum of 10 germinated conidia) and images were recorded for each field using an AxioCamICc1 digital camera installed on a Zeiss Axioplan fluorescent microscope (Carl Zeiss, Göttingen, Germany). The length of infectious hyphae was determined using the curve spline tool of Axiovision 4.7 digital image processing software. All quantitative data were collected with BP excitation/emission cubes (546/FT580/LP590).

### Description of Epidermal Cell Response to *A. lentis* Infection Using Confocal Laser Scanning Microscopy

For CDC Robin, 964a-46 and Eston descriptive microscopy was used to determine the underlying cellular mechanisms of defense and differences among genotypes in cellular reactions to *A. lentis* infection. Ten infected leaflets were arbitrarily selected from the pool of leaflets collected from single plants of each

genotype at 60 and 90 hpi, and were discolored and stained with Uvitex-2b following the protocol described above. The reaction of epidermal cells to pathogen penetration was studied using a two photon Carl Zeiss confocal laser scanning microscope as described by Moldenhauer et al. (2006). The specimens were excited with UV-laser beams at 351 and 364 nm, then scanned with filter settings at 400–500 nm for Uvitex 2b-stained fungal structures, and with argon-laser beams at 514 and 543 nm, and then scanned with filter settings at 560–680 nm for epidermal cells responses. Observations of pathogen and plant cells located at different tissue depth were conducted by collecting images in a number of Z stacks at 0.5 μm intervals. The Z stacks were then compiled to a single micrograph using the Z projection tool in Image J 1.7 p (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA[1], 1997–2012).

### Test of Cell Viability by Light Microscopy

The viability of epidermal cells of CDC Robin, 964a-46, and Eston was investigated following the method of O'Connell et al. (1991) with the following modifications. Samples of 10 infected leaflets, arbitrarily selected from the pool of leaflets collected from single plants of each genotype at 60 and 72 hpi, were subjected to viability staining as follows: Leaflets were cut in half and then vacuum-infiltrated in 0.85 M $KNO_3$ containing 0.01% Neutral Red (Sigma-Aldrich, St. Louis, MO, USA) for 5 h. The specimens were then mounted in the infiltration solution and fungal structures stained with a drop of 0.1% Aniline Blue (BDH Prolabo, UK) in lactic acid solution. The specimens were examined under a Zeiss light microscope (Carl Zeiss, Göttingen, Germany) and images were recorded using an AxioCamICc1 digital camera.

## Analysis of the SA and JA Signal Transduction Pathways by qRT-PCR

The temporal pattern of SA and JA signaling after *A. lentis* infection was indirectly assessed by expression analysis of *PR-1* and *PR-5* as hallmarks of the SA pathway, and *PR-4* and *AOC* as hallmarks of the JA pathway. Lentil genotypes CDC Robin and 964a-46 were selected for this test, with the addition of Eston as the susceptible control. Plants were inoculated as described for pathogenicity testing, except that a higher concentration of conidia ($10^6$ conidia $mL^{-1}$) was used. The experiment was arranged in a randomized complete block design with three replicates.

All inoculated leaflets of seedlings were collected at 6, 12, 18, 24, 36, 48, and 60 hpi and flash frozen in liquid nitrogen. Leaflets were also collected from non-inoculated control plants sprayed only with water. Leaflet samples were stored at −80°C. Leaflets pooled for each biological replicate were ground in an RNAse-free mortar, pre-cooled with liquid nitrogen. Two subsamples from the pool of ground tissue collected for each biological replicate were subjected to RNA extraction. RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was then treated with DNAse I (Invitrogen, Carlsbad, CA, USA) to remove any trace of genomic DNA contamination according to the manufacturer's recommendation. The purity and quantity of RNA were determined using a NanoDrop ND8000 (Thermo Scientific, Wilmington, DE, USA). Samples with an A260/280 ratio less than 2.0 were discarded. The integrity of RNA was determined by denaturing agarose gel electrophoresis (Barill and Nates, 2012).

Total RNA (1 μg) was used for reverse transcriptase-dependent first strand cDNA synthesis, primed by $Oligo(dt)_{12-18}$ primer (Invitrogen, Carlsbad, CA, USA) according to Klickstein et al. (2001). Residual genomic DNA contamination of total RNA samples was detected by running a PCR using ubiquitous actin primer pairs designed for an exon-exon junction and first strand cDNA following the protocol of Vaghefi et al. (2013). PCR was conducted in a 20 μL reaction mix containing 4 μL of 1:10 diluted cDNA, 1X taq reaction buffer, 0.13 μM of each primer, 0.25 mM dNTPs, 3 mM $MgCl_2$, and 1 U Taq polymerase (GenScript, Piscataway, NJ, USA). The PCR cycles were 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C, followed by a final extension of 72°C for 7 min. PCR products were visualized by staining with 1:1000 dilution of GelRed® (Invitrogen, Carlsbad, CA, USA) added to the loading dye, after separation on a 1.4% agarose gel. Samples with genomic DNA contamination were discarded and cDNA synthesis was repeated after total RNA treatment with a doubled concentration of DNAse I.

Primer sequences of Vaghefi et al. (2013) were used for *PR-4*, whereas primer pairs for *PR-1* and *PR-5* (LcPR-1 and LcPR-5) were designed using the mRNA sequences available in the Expressed Sequence Tag (EST) library of lentil infected with the *C. lentis* (Bhadauria et al., 2013). The mRNA sequence of β-*actin* and *AOC* of *M. truncatula* were used to retrieve their orthologs in lentil cv. Redberry transcriptome using the BLASTn tool available at http://knowpulse.usask.ca/portal/blast/nucleotide/nucleotide (**Table 1**). LcActin-257 and LcAOC-69 were selected from a group of primers designed for lentil β-*actin* and *AOC*, respectively, based on their high fidelity

**TABLE 1 | Names, sequences, and gene bank accession number of source sequences of gene-specific primer pairs used for quantitative real-time PCR.**

| Primer name | Sequence 5′→3′ | GenBank accession |
|---|---|---|
| LcPR-1 | F: AGATCCGAGGTTGGTGTTTC | JG294109 |
| | R: CCCACAATTTCACAGCATCT | |
| LcPR-5 | F: CACTGTATGGCCAGGAACAC | JG293995 |
| | R: TACCAAAGTTGCTGGTGGAA | |
| LcAOC-69 | F: AGAGTAGGCATAACTGCAGGCT | AJ866733* |
| | R: TGGTACGTCAGATAAGCTCCCTGT | |
| LcActin-257 | F: CACTGTACTTCCTCTCCGGC | EU664318* |
| | R: TATGTTCCCCGGGATTGCTG | |

**The mRNA sequence of β-actin and Allen Oxidase Cyclase of Medicago truncatula were used to identify their orthologs in lentil cv. Redberry transcriptome using the BLASTn tool available at http://knowpulse.usask.ca/portal/blast/nucleotide/nucleotide.*

[1]http://imagej.nih.gov/ij/

and amplification efficiency. Primers were designed using the primer BLAST search tool provided by the National Centre for Biotechnology Information (NCBI[2]).

The qPCR reaction included 10 μL of Power SYBR® Green master mix (Applied Biosystems, Warrington, UK), 0.2 μM of each primer, and 5 μL of 1:10 diluted cDNA. The cycling program was executed in an ABI StepOnePlus™ Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA) and comprised 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s followed by a melting curve from 60 to 95°C with 0.3°C intervals. PCR was conducted in duplicate. The expression level was reported relative to the non-inoculated control by calculating fold changes following the method of Livak and Schmittgen (2001).

Amplification efficiency was calculated for each primer pair using cDNA samples serially diluted 1:4 (v/v) five times (total six dilutions). Dilutions were used as a template for qPCR following the protocol described above. A linear equation was fitted to the cycle threshold ($C_t$) values obtained for various cDNA dilutions. Percentile of amplification efficiency ($E$) was calculated from the slope of the regression line using the equation $E = 10^{(-1/slope)} - 1$.

## Statistical Analysis

All data analyses were performed using Statistical Analysis System (SAS) version 9.3 (SAS Institute Inc., Cary, NC, USA). Homogeneity of variances was tested using the Levene's test and, in the case of heterogeneity, the variances were modeled using the SAS mixed model procedure.

Percentage of disease severity data were subjected to mixed model analysis with genotypes assigned as fixed, block nested in repeat and repeat as random effects. Quantitative microscopy data were subjected to mixed model analysis with genotypes and sampling time points assigned as fixed, block nested in repeat and repeat as random and sampling time points as repeated measure effects. Means of conidial germination and length of infectious hyphae on the genotypes were compared at each individual time point based on least significant differences with the Tukey adjustment ($\alpha = 0.05$).

For statistical analysis of qRT-PCR data, the mean $C_t$ of two technical qPCR replicates was normalized ($\Delta C_t$) and converted to $2^{-\Delta Ct}$. Data were subjected to generalized linear mixed model analysis using the SAS generalized linear mixed model procedure. Genotypes and sampling time points were considered fixed effects, replicates were random effects and sampling time points were identified as repeated measurements. A log-normal distribution with an identity link function was specified to account for the non-normal distribution, and a first-order ante-dependence covariance structure was used to accommodate unequally spaced sampling time points and heterogeneous variances. Differences among genotypes and sampling time points were assessed based on least significant differences with the Tukey adjustment ($\alpha = 0.05$) in the generalized linear mixed model procedure. To confirm the validity of the reference gene (β-*actin*) for normalization, the $C_t$ values generated for LcActin-257 primer of non-inoculated leaflets were compared with those of inoculated samples using the Kruskal–Wallis test (Schmittgen and Zakrajsek, 2000).

[2]https://www.ncbi.nlm.nih.gov/tools/primer-blast

# RESULTS

## Reaction of *Lens* Genotypes to *A. lentis* Inoculation

Genotypes had a significant effect on disease severity ($P = 0.0263$), and all the partially resistant genotypes had lower disease severity than the susceptible control Eston (**Figure 1**). No symptoms were observed on *L. ervoides* L-01-827A despite a second inoculation 2 weeks after the first one, indicating that a high level of resistance to ascochyta blight is age-independent. Disease severity was not different between CDC Robin and ILL 7537, but both had significantly lower disease severity than the partially resistant genotypes ILL 1704 and 964a-46. There was no significant difference in ascochyta blight severity between ILL 1704 and 964a-46.

## Quantitative Assessment of Fungal Infection Structures

Percent conidial germination was determined for lentil genotypes to investigate the potential association between germination inhibition and resistance to ascochyta blight. Conidial germination was a host genotype-independent trait as differences among genotypes were not significant ($P = 0.47$). Incubation time ($P = 0.0012$) and the interaction ($P = 0.04$) had significant effects on germination, and germination significantly increased from 10 to 12 hpi for Eston and L-01-827A, but not for the other genotypes. Percent conidia germination at 12 hpi ranged from 73.4 to 93% and *L. ervoides* accession L-01-827A and CDC Robin had the highest and lowest germination rate, respectively (**Figure 2A**).

As a second step to understanding the point at which *A. lentis* growth was inhibited in the resistant genotypes, the length of infectious hyphae was measured at three time points to cover

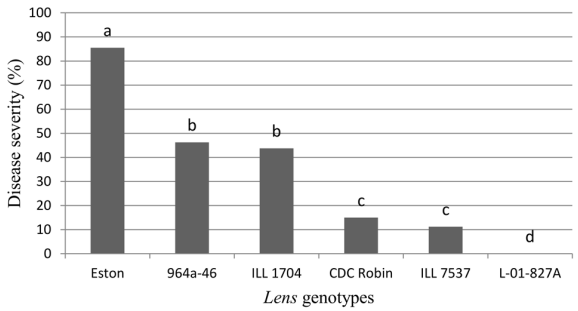


**FIGURE 1 | Percentage of ascochyta blight severity of *Lens* genotypes.** All genotypes are accession of *L. culinaris* except L-01-827A (*Lens ervoides*). Estimates of the means were generated from four biological replicates using a mixed model analysis. Disease severity was rated using a 1–10 scale with 10% incremental increase in disease severity and converted to percentage disease severity using the class midpoint. Means with one letter in common are not significantly different.

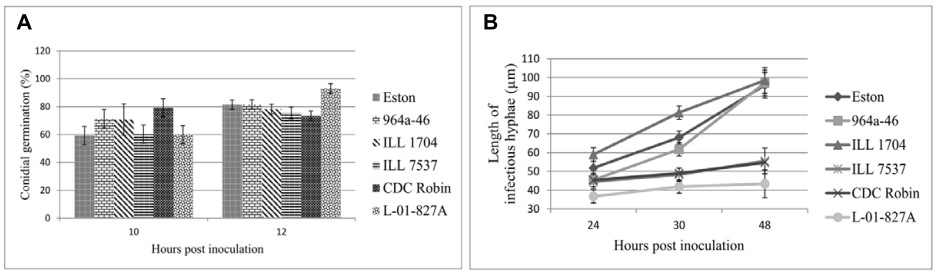


**FIGURE 2 | Mean germination (%) of conidia (A)**, and mean length of infectious hyphae (µm; **B**) extending from germinated *Ascochyta lentis* conidia on the leaflet surface of *Lens* genotypes at 10 to 48 hpi). All genotypes are *L. culinaris* except L-01-827A (*L. ervoides*). Estimates of means were generated from three biological replicates using a mixed model analysis. For each biological replicate 300 conidia were assessed for conidial germination, and a minimum of 100 for infectious hyphae length.

post-penetration stages of infection. Analysis of variance showed significant effects of genotypes ($P < 0.001$), sampling time points ($P < 0.001$), and their interaction ($P = 0.0019$), suggesting that length of infectious hyphae was a genotype-dependent trait and the effect of genotypes on the length of infectious hyphae changed over time. For all genotypes, the length of infectious hyphae increased over time (**Figure 2B**). At 24 hpi, conidia on ILL 1704 had developed longer infectious hyphae than on all other genotypes except for Eston. Increasing incubation time to 30 and 48 h resulted in consistently shorter infectious hyphae in CDC Robin, ILL 7537, and L-01-827A compared to ILL 1704, 964a-46 and Eston. No significant differences in the length of infectious hyphae were observed between 964a-46 and Eston at any time point. Based on the length of infectious hyphae, lentil genotypes could be separated into two groups, one with restricted infectious hyphae growth (CDC Robin, ILL 7537 and L-01-827A), and one with long infectious hyphae (ILL 1704, 964a-46 and Eston).

## Cellular Reaction of Lentil Genotypes to Infection by *A. lentis*

Partially resistant genotypes CDC Robin and 964a-46 were selected for descriptive microscopy as representatives of the two groups with short and long infectious hyphae, respectively, and cellular reactions were compared with the susceptible genotype Eston.

In Eston, the fluorescent signals emitted from the entire cell protoplast in response to infection. Cell wall reinforcement and papillae at the site of penetration attempts was observed in Eston at 60 hpi (indicated by arrow in **Figures 3A,a**). Cellular events induced following *A. lentis* infection in 964a-46 were similar to those of Eston at 60 hpi (**Figures 3C,c**), although 964a-46 developed thinner papillae compared to Eston. Destruction of papillae by infection vesicles was detected in both 964a-46 and Eston at 60 hpi. In CDC Robin, a concentrated autofluorescent signal was detected at the site of penetration attempts at 60 hpi, and the destruction of papillae was not observed in this genotype at this time point (**Figures 3B,b**).

The reactions of CDC Robin epidermal cells to *A. lentis* did not change from 60 to 90 hpi (**Figures 3B,b,E,e**), unlike those of Eston and 964a-46 where massive colonization of epidermal cells was observed at 90 hpi (**Figures 3D,d,F,f**). The colonization by fungal mycelium was denser in 964a-46 compared to Eston and pycnidia were often developed by 90 hpi in 964a-46.

Results of cell viability test showed that cell death occurred in Eston and 964a-46 at 72 hpi, but not in CDC Robin (**Figure 4**). In Eston, most cells attacked by the pathogen had lost their viability. Cell death was detected in a few non-infected cells neighboring the infection site in 964a-46 at 72 hpi. Penetration into epidermal cells was observed in Eston and 964a-46 but not in CDC Robin at this time point.

## Quantitative Measurement of Gene Expression of *PR-1*, *PR-5*, *PR-4*, and *AOC*

All gene-specific primers had amplification efficiencies close to 100% (data not presented). No significant differences were observed between the $C_t$ values generated for the LcActin-257 primer of non-inoculated leaflets and those of inoculated samples (Eston: $P = 0.4414$; CDC Robin: $P = 0.4159$; 964a-46: $P = 0.1037$). Variance analyses showed that genotype ($P < 0.0001$) and incubation time ($P < 0.0001$) and their interaction had very highly significant effects on the expression of *PR-1*, *PR-4*, *PR-5,* and *AOC*.

CDC Robin and Eston were similar in relative *PR-1* expression over the tested time points except for 12 hpi, when *PR-1* expression in Eston was significantly higher than in CDC Robin (**Figure 5A**). *PR-1* expression in 964a-46 increased exponentially at 18 hpi and peaked at 24 hpi, when expression was estimated to be 7084 times higher than in non-inoculated samples. The expression then declined and all three genotypes had similar levels of expression at 36 hpi. Subsequently, the expression increased again in 964a-46, but less rapidly and to a lower peak than the first fold increase starting at 18 hpi.

*PR-5* expression was not significantly different between the susceptible check Eston and CDC Robin at all sampling time points except for 48 hpi (**Figure 5B**). At 48 hpi, Eston had significantly higher *PR-5* expression than CDC Robin, but lower than 964a-46. In 964a-46, *PR-5* expression was not different from the others at 6 and 12 hpi. However, its expression exponentially

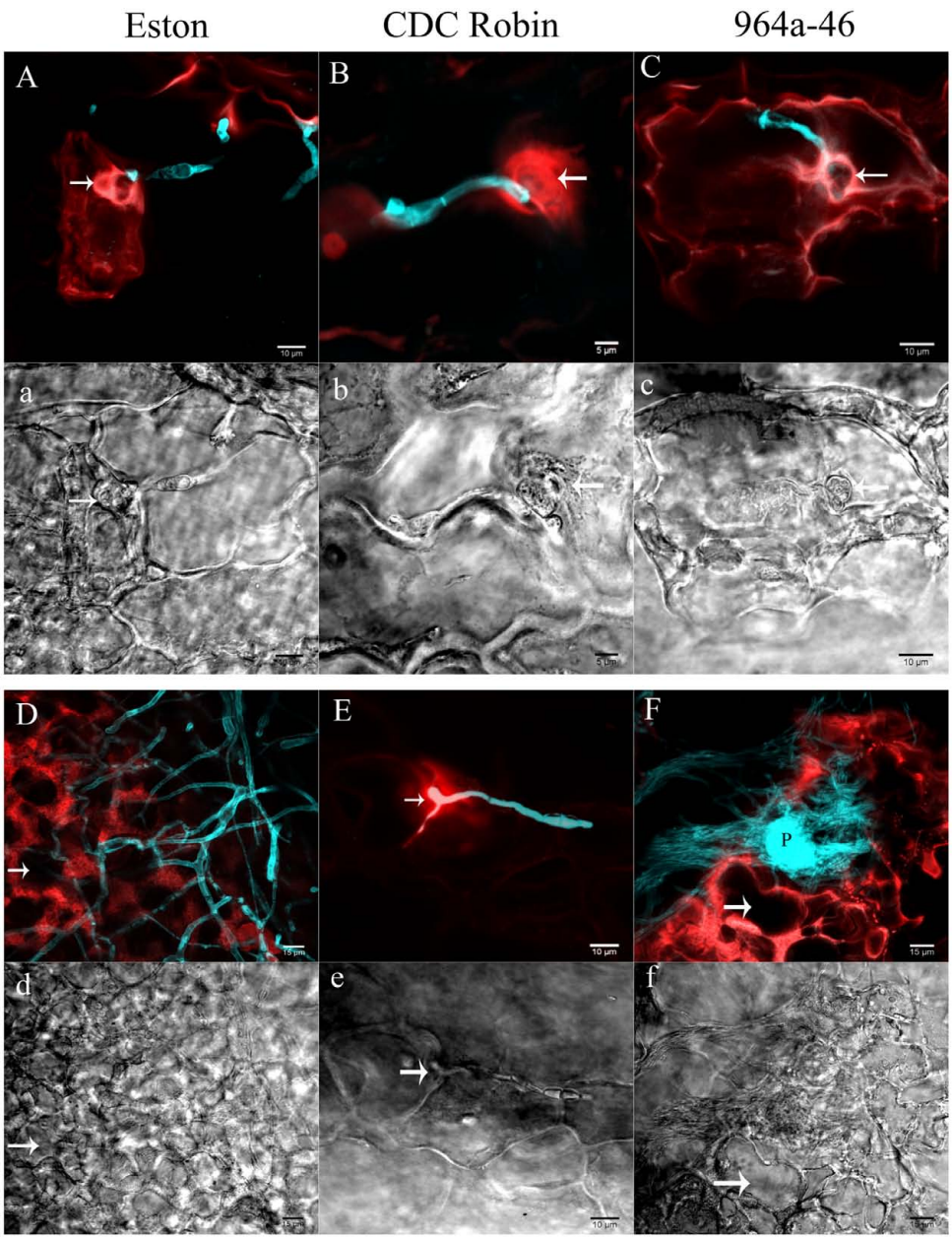


**FIGURE 3 | Cellular reactions of partially resistant lentil genotypes to *A. lentis* infection captured by two photon CLSM at 60 (A,a,B,b,C,c)** and 90 hpi **(D,d,E,e,F,f)**. Microscopic fields of vision were simultaneously scanned using fluorescence (capital letters) and differential interference contrast (DIC) filters (small letters). Fungal structures (cyan) were stained with Uvitex-2b. Autofluorescent signals developed in the host epidermal cells in response to pathogen infection are in red. Arrows in images show the penetration site except for **(d)** and **(f)**, where arrows indicate the cavity developed as a result of the destruction of cell contents. P in **(F)** shows a newly developed pycnidium formed on the mass of mycelium. Scale bars are indicated at the bottom right of each image.

increased after 12 hpi and reached a peak that was 4910 times that of the non-inoculated plants at 24 hpi. The peak detected in 964a-46 at 24 hpi was negligible in Eston and absent in CDC Robin. Although expression in 964a-46 had declined at 36 hpi, levels were still higher than for CDC Robin and Eston. *PR-5* expression increased again at 48 hpi in 964a-46 to a level similar to that at 24 hpi, then declined at 60 hpi to a level similar to that at 6 and 12 hpi. Eston and CDC Robin experienced an increase in *PR-5* expression at 48 hpi similar to that noted for, but of lower magnitude than in 964a-46. As in 964a-46, *PR-5* expression then declined in CDC Robin and Eston to levels initially observed at 6 and 12 hpi.

*PR-4* expression was the same in all genotypes at 6 and 12 hpi (**Figure 5C**). In Eston, it remained low until 24 hpi, and then increased to a peak at 36 hpi before gradually declining. For CDC Robin, *PR-4* expression increased at 18 hpi, reached a peak

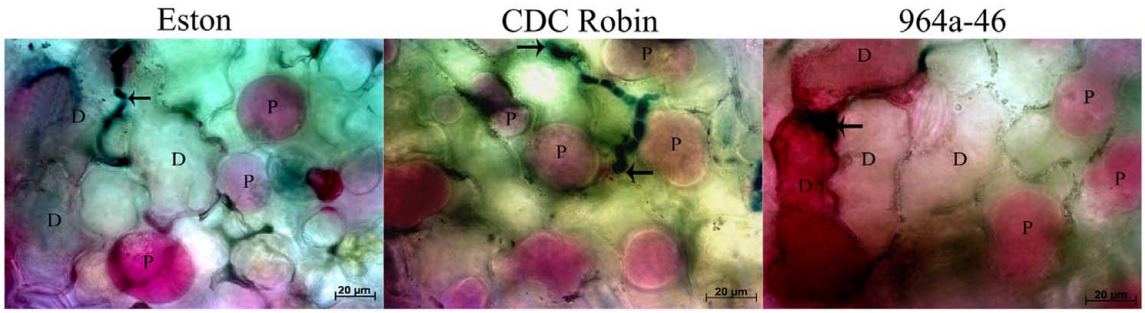


**FIGURE 4 | Changes in viability of epidermal lentil cells 72 h after inoculation with *A. lentis*.** Fungal tissues were stained with Aniline Blue-lactic acid solution (dark blue). Arrows indicate the penetration site. Viability was postulated when the host protoplasm (P) contracted and absorbed red pigments after vacuum infiltration of leaflet tissues in 0.85 M $KNO_3$/0.01% Neutral Red solution. Dead cells (D) absorbed the red pigments but did not show contracted protoplasm. Scale bars are indicated at the bottom right of each image.

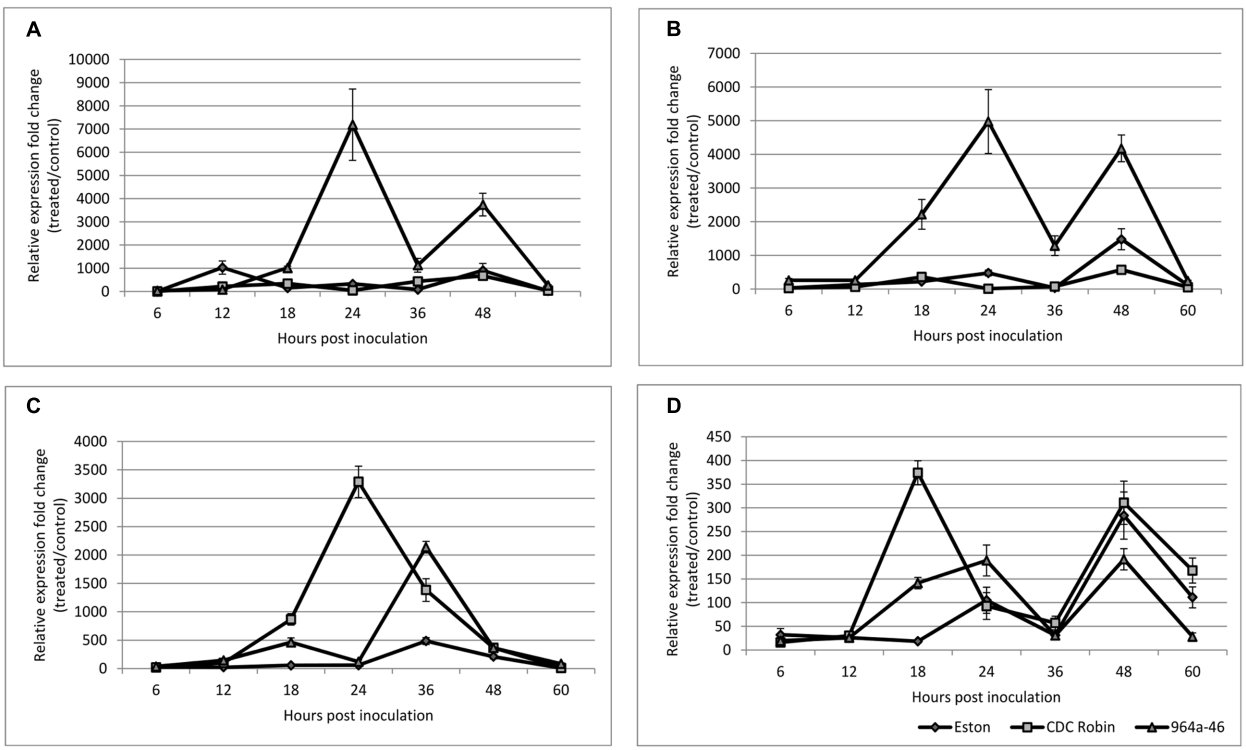


**FIGURE 5 | Quantitative assessment of *PR-1* (A)**, *PR-5* **(B)**, *PR-4* **(C)**, and *AOC* **(D)** expression in susceptible lentil genotype Eston and two partially resistant CDC Robin and 964a-46 by quantitative real-time PCR after inoculation with *A. lentis*. Data are mean of three replicates. Error bars indicate standard errors of the mean. Gene expression was reported relative to non-inoculated samples collected just prior to inoculation. Data were normalized using β-*actin* gene expression as a reference gene.

level at 24 hpi (3281 times that of non-inoculated plants), and then declined. There were significant differences between the expression levels of *PR-4* in CDC Robin and Eston at 18–36 hpi. *PR-4* expression in 964a-46 increased starting at 18 hpi but began to decline at 24 hpi. At 24 hpi, Eston and 964a-46 had almost identical levels of *PR-4* expression. The expression of *PR-4* increased again for 964a-46 at 36 hpi to levels significantly higher than that observed for CDC Robin and Eston, but then returned to similarly low levels.

Allene oxidase cyclase expression in Eston remained at the baseline until 18 hpi, but increased at this time point for both CDC Robin and 964a-46 (**Figure 5D**). This increase was more than double in CDC Robin compared to 964a-46. *AOC* expression then declined at 24 hpi in CDC Robin, but the decline was not observed for 964a-46, which had significantly higher *AOC* expression at 24 hpi compared to 18 hpi. All genotypes showed a decline in *AOC* expression at 36 hpi, and no significant differences were observed among them. All genotypes

experienced a fold-change increase at 48 hpi and a decline in expression at 60 hpi.

## DISCUSSION

This study investigated for the first time the differences in cellular reactions and the activation of SA and JA signaling pathways among lentil genotypes with partial resistance to ascochyta blight in response to *A. lentis* infection. Microscopic examination of infected leaflets of these genotypes indicates that the process of cell death is of relative importance in resistance of lentil to ascochyta blight. Genotypes showed different patterns in the expression of genes connected with the SA and JA signal transduction pathways. The involvement of both the SA and JA pathways in the reaction of lentil to ascochyta blight is implicated. There were differences among genotypes in deployment of the the signaling pathways during the course of infection with *A. lentis*.

Conidial germination was confirmed to be genotype-independent, similar to the results of previous microscopic studies of the related fungal species *A. rabiei* when infecting chickpea (Höhl et al., 1990). Sambasivam et al. (2017) found higher conidial germination for a highly virulent *A. lentis* isolate on three lentil genotypes with different levels of resistance than for a less virulent isolate at 2 hpi, but this difference only persisted on the most resistant genotype ILL7537, indicating that there may be an interaction between isolates and lentil genotypes in terms of germination. After germination, *A. lentis* conidia develop germ tubes that penetrated into the epidermal cells by differentiating to appressoria and penetration pegs. Minor differences in the length of infectious hyphae were apparent at 24 hpi, but starting at 30 hpi, the differences became more obvious. Infectious hyphae observed on partially resistant genotypes CDC Robin, ILL 7537 and L-01-827A were consistently shorter than those on the susceptible genotype Eston. Although not analyzed in detail, Sambasivam et al. (2017) also appear to have observed shorter germ tubes on the resistant than the more susceptible genotypes, lending more supports to an inhibition of host colonization as a common mechanism of resistance to *A. lentis* in lentil. At less than 30% disease severity, CDC Robin, ILL 7537 and L-01-827A had high levels of partial resistance to *A. lentis* isolate AL57 infection. In contrast, on ILL 1704 and 964a-46, the length of infectious hyphae was similar to that of conidia on the susceptible control Eston. In the pathogenicity tests, ILL 1704 and 964a-46 were significantly more resistant than Eston, but had higher disease severity than CDC Robin, ILL 7537, and L-01-827A. These results were the first indications for the involvement of different defense mechanisms, or components thereof, in these lentil genotypes for which resistance is conferred by non-allelic R-genes. Differences during the infection phase on lentil genotypes were previously reported from the hemibiotrophic pathogen *C. lentis* (Armstrong-Cho et al., 2012). Similarly, Kema et al. (1996) found differences in the number of epidermal cells colonized among wheat genotypes that varied in their level of resistance to *Mycosphaerella graminicola* (Fückel) Schroeter.

Assessment of cellular responses by CLSM revealed an accumulation of autofluorescent compounds at the sites of penetration attempts in both susceptible and partially resistant genotypes starting at 60 hpi, but the emission of fluorescent signals from cell protoplasts was only observed in Eston and 964a-46. The viability tests indicated that emission of fluorescent signals from cell protoplasts was due to disruptions of cell protoplasm and cell death. This was also implied by a previous report of microscopic studies of *A. lentis* on two lentil genotypes with different levels of resistance to ascochyta blight (Roundhill et al., 1995). They reported differences among susceptible and resistant genotypes at the penetration stage. While susceptible cells became necrotic, followed by growth of the penetration peg into the cell lumen in that study, the penetration peg on the resistant genotypes was surrounded by electron-dense materials and the cells remained viable. These and the present findings suggest that cell death might facilitate the colonization of epidermal cells by the fungus. Necrotrophic and hemi-biotrophic plant pathogens are dependent on cell death for their pathogenesis and cell death promotes colonization of plants by necrotrophs such as *Botrytis cinerea* Pers.: Fr (Govrin and Levine, 2000). Enhanced cell death *Arabidopsis* mutants show comparatively higher susceptibility to necrotrophs (Veronese et al., 2004).

Additional support for the role of cell death in the pathogenicity of *A. lentis* is that cell death was rarely detected in CDC Robin at 60 and 90 hpi. Rare cases of cell death observed in CDC Robin at 90 hpi correspond with the low levels of ascochyta blight symptoms observed on this genotype. Ascochyta blight resistance identified to date is partial, so the inconsistent inhibition of cell death in CDC Robin may contribute to the partial nature of resistance. Induction of cell death was suggested as a defense response in the resistant genotype ILL7537 (Sambasivam et al., 2017). Allelism tests suggested that ascochyta blight R-genes in CDC Robin and ILL 7537 are different (Sari, 2014), lending support to the presence of different resistance mechanisms between these genotypes.

Similar cascades of cellular events were observed in 964a-46 and Eston, with the only differences being that relatively higher numbers of cells surrounding the infection site lost viability, and relatively denser fungal colonies and thinner papillae formed in 964a-46. The engagement of non-infected cells in 964a-46 may be due to a systemic signal transduced to the neighboring cells. The occurrence of systemic signaling was previously suggested as the main difference between two genotypes of wheat with resistance to fusarium head blight (Foroud et al., 2012). Transduction of systemic signals to non-infected cells around the infection site could have primed defense responses and decreased the aggressiveness of the pathogen, thereby limiting the area of colonization in 964a-46. The formation of thicker papillae in Eston than 964a-46 suggest that papillae are not involved in the *A. lentis* resistance of 964a-46. Further research on the frequency and variation in size of papillae would be required to fully resolve their role in resistance. Overall, microscopic studies could not provide conclusive evidence for phenotypic separation of the colonization process of 964a-46 and the susceptible control

Eston. Improvement of microscopy techniques may lead to better phenotypic differentiation in future studies.

Analysis of quantitative expression of *PR-1*, *PR-5*, *PR-4*, and *AOC* suggested that genotypes differed with respect to their activation of the SA and JA signaling pathways. The rapid increase in *PR-1* and *PR-5* expression in 964a-46 at 24 hpi suggests the involvement of the SA pathway in the interaction of this genotype with *A. lentis*. The SA-mediated signaling pathway activates defense responses that are only effective against biotrophic and hemi-biotrophic fungi (Kunkel and Brooks, 2002), but is also part of the host response to hemibiotrophic infection (Liu et al., 2007). Roundhill et al. (1995) previously suggested that *A. lentis* is either a necrotrophic or a hemi-biotrophic fungal pathogen, but activation of both pathways as shown here support a hemi-biotrophic lifestyle of this pathogen. In the case of the hemibiotrophic fungal pathogen *Fusarium graminearum* Schwabe of wheat, the SA pathway was triggered very early at 6 hpi and the levels of expression were higher in resistant than in susceptible lines (Ding et al., 2011).

The increased levels of *PR-1* and *PR-5* expression were transient and declined at 36 hpi in 964a-46. It is likely that *A. lentis* AL57 suppresses SA-mediated plant defense in 964a-46 by deploying effectors interfering with the SA signaling pathway. Effectors interfering with the SA pathway have previously been identified in various types of plant pathogens. Recently, Liu et al. (2014) reported the secretion of isochorismatases by *Phytophthora sojae* Kaufm. & Gerd. and *Verticillium dahlia* Kleb. that degrades the SA precursor isochorismate and suppress the defense responses induced by the SA signaling pathway. The decline in SA-related genes at 36 hpi could also be attributed to physiological factor affecting photosynthesis. Seyfferth and Tsuda (2014) indicated that "pathogen-induced SA is mainly synthesized via the isochorismate pathway in chloroplasts." Plants were incubated in humidity chambers for 48 h after inoculation, where they were exposed to a dark period at 36 hpi. Prevention of photosynthesis at 36 hpi might explain the lower production of SA and thus the decline in the expression of SA-depended genes. Similarly, Mouradov et al. (1994) also found a decline followed by a peek in the accumulation of *PR-1* mRNA in barley leaves challenged with *Erysiphe graminis f.sp. hordei* Marchal. The fact that the transient activation of SA signaling was not observed in Eston and CD Robin suggests that these genotypes may not perceive the pathogen in a similar way as 964a-46, either as a result of the successful manipulation of the defense apparatus by the pathogen or due to lack of the receptor genes, respectively.

In a study of the *A. lentis* transcriptome, the involvement of a complex toxin model was proposed in which the quantitative nature of resistance in lentil is attributed to the interactions of numerous toxins produced by the pathogen with their hypothetical corresponding susceptibility factors in the plant (Lichtenzveig et al., 2012). Notably, reactions to ascochyta blight in 964a-46 varied from highly to moderately resistant when challenged with two different isolates of *A. lentis* (Tar'an et al., 2003). The reactivation of the SA pathway in 964a-46 after 36 hpi may be due to the recognition of specific pathogen toxins by receptor genes in this genotype, resulting in cell death and successful infection through effector triggered susceptibility (ETS). Previous results suggest that ETS-facilitated infection by some host-specific necrotrophs occurs following a gene-for-gene interaction between a host specific toxin and a host receptor protein (reviewed in Oliver and Solomon, 2010). In wheat it has been speculated that the receptor Tsn1 for the host-specific ToxA released by some races of *Pyrenophora tritici-repentis* may be associated with a gene that could be involved in the SA-dependent pathway (Manning et al., 2004). Induction of cell death in the epidermal cells neighboring the infection site (observed in the viability test) along with higher levels of *PR-4* accumulation might have fortified the plant basal defense and increased the level of resistance in 964a-46 compared to Eston.

The putative role of *PR-4* in lentil resistance to *A. lentis* was described previously (Mustafa et al., 2009; Vaghefi et al., 2013). Its antifungal activity was also demonstrated *in vitro* using a recombinant protein (Vaghefi et al., 2013). The suggested involvement of *PR-4* in JA-triggered defense (Thomma et al., 1998) was the reason it was selected for analyzing the role of JA in the present study. *PR-4* expression could explain differences in resistance levels among genotypes. The expression of *PR-4* was not induced in Eston until 24 hpi, yet peaked in CDC Robin at this time. A similar expression peak occurred in 964a-46, but 12 h later and at significantly lower expression levels. Previous studies suggest that the SA signaling pathway is ineffective during the necrotrophic phase of infection and instead JA plays a crucial role (Glazebrook, 2005). The lower susceptibility of 964a-46 to *A. lentis* than that of Eston could be attributed to the potential of 964a-46 to induce higher levels of *PR-4* expression.

Expression of *PR-4* in 964a-46 peaked at 36 hpi, concurrent with the decline in the expression of *PR-1* and *PR-5,* that was expected considering the reciprocal antagonistic effects of SA on JA pathways (Schenk et al., 2000; Kunkel and Brooks, 2002; Glazebrook et al., 2003). Expression of *PR-4* in CDC Robin peaked at 24 hpi, concurrent with low expression of *PR-1* and *PR-5* in this genotype. The antagonistic effects of JA on SA might be the reason for the peak in *PR-1* and *PR-5* expression being absent in CDC Robin at 24 hpi. Suppression of SA-mediated cell death along with the higher levels of *PR-4* expression could be the cause of lower frequency of cell death in CDC Robin and its higher levels of partial resistance than in 964a-46.

The *AOC* expression from 12 to 24 hpi was concomitant with the ranking of disease severity rating of the genotypes, corroborating the putative role of JA signaling in resistance to *A. lentis* as well. Similar to the pattern of *PR-4* expression, *AOC* expression increased at a slower rate in 964a-46 compared to CDC Robin. *AOC* expression peaked about 12 h earlier than *PR-4* (at 24 hpi). As explained previously, *AOC* is a component of the JA biosynthesis pathway (Vick and Zimmerman, 1983). Usually, PR proteins are expressed downstream of the SA and JA signaling cascades, with a time interval between defense activation and expression. This might explain the 12 h delay in the induction of *PR-4* compared to *AOC*.

The current study analyzed the expression of signature genes involved in SA and JA signaling pathways using attached leaf assays. We adopted this method since previous studies on

*A. thaliana* suggested distinct reactions in attached and detached leaf assays to hemibiotroph *Colletotrichum* spp. (Liu et al., 2007). The drawback of using attached leaf assays is that it is nearly impossible to completely synchronize the development of fungal individuals on the leaves. We tried to minimize this type of variation by pooling leaflets and including two RNA-extraction batches per biological replicate in the qRT-PCR tests, but independently inoculated time-course experiments are required to confirm observations here.

## CONCLUSION

Results indicated that genotypes partially resistant to ascochyta blight differ in the timing and magnitude of gene induction associated with the SA and JA signaling pathways. Infection by *A. lentis* caused intensive activation of SA-related genes in 964a-46, suggesting large differences between this and the other resistant genotype CDC Robin and the susceptible check Eston. Expression levels of genes associated with the JA pathway were associated with differences among genotype in the levels of resistance. Microscopy studies suggested that lower disease severity is associated with a lower cell death frequency in CDC Robin. This could not, however, explain the differences between the reaction of 964a-46 and Eston to *A. lentis*. Application of more advanced microscopy with modified staining protocols may enable capture of these differences. The combined results suggested that lentil genotypes carrying different R-genes possess divergent cellular and molecular mechanisms of resistance. Complete understanding of signal transduction pathways activated upon *A. lentis* infection requires further analyses of additional components of SA and JA signaling in relation to the other signals and their downstream pathways.

## AUTHOR CONTRIBUTIONS

ES conducted experiments under primary supervision of SB and VB. The data were analyzed by SB. Authors equally contributed to the experimental designs. ES drafted the manuscripts with contributions by VB and SB, who also critically reviewed the manuscript. AV was principal investigator and involved in overall design of the project, provided some supervision to ES and critically reviewed the manuscript. All authors have read the manuscript and agreed to its publication.

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# Evidence and Consequence of a Highly Adapted Clonal Haplotype within the Australian *Ascochyta rabiei* Population

*Yasir Mehmood[1]*, Prabhakaran Sambasivam[1], Sukhjiwan Kaur[2], Jenny Davidson[3], Audrey E. Leo[4], Kristy Hobson[5], Celeste C. Linde[6], Kevin Moore[5], Jeremy Brownlie[1] and Rebecca Ford[1]**

[1] Environmental Futures Research Institute, School of Natural Sciences, Griffith University, Nathan, QLD, Australia, [2] Agriculture Victoria, AgriBio, The Centre for AgriBioscience, Bundoora, VIC, Australia, [3] South Australian Research and Development Institute, Urrbrae, SA, Australia, [4] New South Wales Department of Primary Industries, Wagga Wagga Agricultural Institute, Wagga Wagga, NSW, Australia, [5] Department of Primary Industries, Tamworth Agricultural Institute, Tamworth, NSW, Australia, [6] Research School of Biology, Australian National University, Canberra, ACT, Australia

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*Petr Smýkal,*
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*Teresa Millan,*
*Universidad de Cordoba, Spain*

***Correspondence:**
*Rebecca Ford*
*rebecca.ford@griffith.edu.au*
*Yasir Mehmood*
*yasir.mehmood@griffithuni.edu.au*

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The Australian *Ascochyta rabiei* (Pass.) Labr. (syn. *Phoma rabiei*) population has low genotypic diversity with only one mating type detected to date, potentially precluding substantial evolution through recombination. However, a large diversity in aggressiveness exists. In an effort to better understand the risk from selective adaptation to currently used resistance sources and chemical control strategies, the population was examined in detail. For this, a total of 598 isolates were quasi-hierarchically sampled between 2013 and 2015 across all major Australian chickpea growing regions and commonly grown host genotypes. Although a large number of haplotypes were identified (66) through short sequence repeat (SSR) genotyping, overall low gene diversity ($H_{exp} = 0.066$) and genotypic diversity ($D = 0.57$) was detected. Almost 70% of the isolates assessed were of a single dominant haplotype (ARH01). Disease screening on a differential host set, including three commonly deployed resistance sources, revealed distinct aggressiveness among the isolates, with 17% of all isolates identified as highly aggressive. Almost 75% of these were of the ARH01 haplotype. A similar pattern was observed at the host level, with 46% of all isolates collected from the commonly grown host genotype Genesis090 (classified as "resistant" during the term of collection) identified as highly aggressive. Of these, 63% belonged to the ARH01 haplotype. In conclusion, the ARH01 haplotype represents a significant risk to the Australian chickpea industry, being not only widely adapted to the diverse agro-geographical environments of the Australian chickpea growing regions, but also containing a disproportionately large number of aggressive isolates, indicating fitness to survive and replicate on the best resistance sources in the Australian germplasm.

**Keywords: chickpea, *Ascochyta rabiei*, resistance sources, SSR genotype, haplotype and highly aggressive**

## INTRODUCTION

Chickpea (*Cicer arietinum* L.), is the most widely cultivated legume, grown in over 50 countries across the Indian subcontinent, North Africa, the Middle East, southern Europe, the Americas and Australia. The global production in 2014 was 14 million tons with yields of[1] 982 kg/ha. The crop is grown in rotation, largely for its high cash return and ability to fix atmospheric nitrogen (Gan et al., 2006). However, significant yield instability remains, largely due to Ascochyta blight caused by the necrotrophic fungal pathogen *Ascochyta rabiei* (Nene, 1982). The disease causes extensive crop losses globally (Pande et al., 2005), and remains the major biotic constraint to the winter-grown crop in Australia, with all growing regions affected (Bretag et al., 2008). Subsequent inoculum release following increased precipitation over the 2013 to 2016 growing seasons has led to non-manageable epidemics on "resistant" host genotypes (Moore et al., 2016). The recent severity of the disease is likely due to the dispersal of isolates that are highly aggressive, widely adapted and able to survive between the growing seasons in the harsh Australian summer climate.

*Ascochyta rabiei* is a bipolar heterothallic fungus with one mating type locus and two mating types (Wilson and Kaiser, 1995). Large temporal and spatial variations have been detected within populations from other global regions where both mating types exist (Udupa et al., 1998; Jamil et al., 2000; Peever et al., 2004; Ali et al., 2012). On a global scale, the total gene diversity detected with 19 sequence tag microsatellite primers was estimated to be 0.29 among *A. rabiei* populations worldwide. Maximum gene diversity was detected among intra-country populations in Canada (0.38), followed by the United States (0.36) and Syria (0.32) (Phan et al., 2003). In other studies, based on different sets of short sequence repeat (SSR) loci, the diversity of the population was estimated to be even higher; 0.55 in Tunisia (Rhaïem et al., 2006), and 0.79 in Iran (Nourollahi et al., 2011). This is in stark contrast to the population diversity observed in Australia, where despite trapping of the putative ascospore in the field (Galloway and MacLeod, 2003), only one mating type has been detected (Barve et al., 2003; Leo et al., 2015). Accordingly, multiple studies have shown a very low gene diversity within the population (ranging from 0.02 to 0.094), consistent with an organism that is reproducing asexually (Phan et al., 2003; Rhaïem et al., 2006; Leo et al., 2015).

The variation in aggressiveness detected within sexually recombinant *A. rabiei* populations worldwide has led to the erosion of resistant host genotypes (McDonald and Linde, 2002; Peever et al., 2012; Mahiout et al., 2015; Vafaei et al., 2015; Tekin et al., 2017). Although not directly comparable due to a number of factorial differences such as host genotype, isolate and bioassay conditions, several in country studies have identified diversity of aggressiveness within *A. rabiei* populations. Sets of isolates have been identified that react similarly or differently to a group of host genotypes (Grewal, 1984; Udupa et al., 1998; Jayakumar et al., 2005; Pande et al., 2005; Imtiaz et al., 2015; Vafaei et al., 2015; Baite et al., 2016). Jan and Wiese (1991) reported the presence of 11 "virulent forms" among 39 isolates assessed from the Palouse region of the United States. Navas-Cortés et al. (1998) identified 11 "pathotypes" in India, Pakistan, Spain, and the United States. Next, Jamil et al. (2000) classified 102 isolates from Pakistan into eight virulence forms and 14 "pathotype groups" were identified among 40 Canadian isolates assessed for disease reaction on eight chickpea differential lines (Chongo et al., 2004). Pouralibaba et al. (2008) reported three "pathotype groups" present in north-western Iran, whereas Ghiai et al. (2012) reported 10 "virulent forms" and 16 "pathogenic groups," respectively, from Iran. Most recently, a new highly virulent "pathotype IV" was reported in Syria and the existence of the four previously identified Syrian pathotypes (Atik et al., 2013) were confirmed (Imtiaz et al., 2015).

In Australia, although it appears that the population is largely clonal based on neutral genetic markers, a broad range of aggressiveness exists (Elliott et al., 2013). Hence the Australian chickpea industry is at risk from selective propagation and dispersal of the fittest and best adapted *A. rabiei* clones. Indeed, since host resistance is multigenic and partial (Cho et al., 2004), there is a heightened risk of resistance erosion caused by selection and increasing frequency of individual clones, with the ability to overcome singular or multiple defense genes/strategies as well as maintain peak fitness (Andrivon et al., 2007).

Two types of adaptation are recognized in fungal species, generalized adaptation and localized adaptation, both resulting in the production of unique haplotypes with high aggressiveness levels and frequencies, due directly to high survival rates (Leonard, 1977). Elliott et al. (2013) first detected clones with differing levels of aggressiveness within the Australian *A. rabiei* population and proposed that despite its clonal nature, the population contained a large potential to evolve and adapt to overcome chemical and host resistance management strategies. This proposal was based on a small number of isolates. To better understand and manage this risk a much larger study, encompassing a greater number of isolates from multiple growing regions and host genotypes collected over several growing seasons was required. This rationale is supported by observations over recent seasons of severe disease symptomology on host genotypes widely adopted throughout the Australian growing regions and that, until very recently, were considered "resistant" (in the case of Genesis 090 in the southern Victoria and South Australia regions) or "moderately resistant" (in the case of PBA HatTrick in the northern New South Wales and southern Queensland regions).

In order to assess risk to currently employed host resistance and chemical control strategies, as well as to better select for resistance longevity, an in depth understanding of the genetic and pathogenic structure of the Australian *A. rabiei* population is required. Therefore the aims of this study were to (1) assess the genetic structure of the *A. rabiei* population and any changes in the structure within and between the major chickpea growing regions of Australia and host genotypes sown, and (2) assess the spread and frequency of the most frequently occurring haplotypes containing the most aggressive isolates, to identify those isolates of highest risk to the Australian chickpea industry. Used together, this new knowledge of diversity, haplotype frequency and aggressiveness will enable strategic choice of

[1] http://faostat3.fao.org

isolates for application to resistance breeding programs and to assess for sustainability of resistance in newly deployed and soon to be widely adopted host genotypes.

# MATERIALS AND METHODS

## Population Structure: SSRs

### Isolate Collection and Culturing

To determine the structure of the Australian *A. rabiei* population, isolates were collected from commercial chickpea crops and National Variety Trial (NVT) sites during 2013–2015. This was done in a quasi-hierarchical manner, in that wherever possible, infected material was collected from the four corners and one central location within each field. At NVT sites, infected material was collected from as many host genotypes as possible, one sample from each genotype row at each location. For the overall Australian *A. rabiei* population study, a total of 598 isolates were collected from across the six agro-geographical chickpea growing regions in eastern and southern Australia (**Figure 1**). The full list of isolates and their available passport data (place of collection, year of collection, and host genotype) is provided in the additional material (Online source 1).

To assess for selective adaptation on widely grown Australian host genotypes, isolates were intensively collected from Genesis 090 and PBA HatTrick. At the time of study, these were rated as "resistant" and "moderately resistant," respectively (Pulse Australia, 2009).

In order to assess for effect of location and track any shift in population structure associated with a single host genotype over time, isolates were collected repeatedly over three consecutive growing seasons (2013, 2014, and 2015) from PBA HatTrick grown in the same two locations (locations A and B), each with a 50 km radius and 247 km apart (**Figure 2**).

Individual isolates were recovered from pycnidia of only one lesion per infected plant to minimize the likelihood of sampling clones due to short distance dispersal of conidia through rain splash. A single pycnidium per lesion was picked with a sterile needle from an infected chickpea leaf, stem or pod and inoculated into 2 mL of sterile distilled water before streaking onto V8 juice growth agar. Leaf lesions with no visible pycnidia were surface sterilized and placed on V8 juice growth agar. All agar plates were incubated for 14 days at 22 ± 2°C with a 12/12 h near-UV light irradiation (350–400 nm)/dark photoperiod, and resulting cultures were single spored on V8 juice agar media (Elliott et al., 2013).

### DNA Extraction and SSR Genotyping

Five hundred and ninety-eight single spored isolates were inoculated separately into 25 mL falcon tubes containing Czapek Dox broth (Difco, Australia) and incubated for 2 weeks at 22 ± 2°C in the dark. Mycelia were then harvested and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, United States) according to the manufacturer's instructions.

Seven previously characterized and informative SSR loci were used for determining the genetic structure of the population (Leo et al., 2011). Genotyping was performed using the Multiplex-Ready PCR technique (Hayden et al., 2008), products were separated on a 96 capillary ABI 3730 DNA electrophoresis analyser and allele sizes were analyzed using GeneMapper v4.0 software (Applied Bio-systems) at the Australian Genome Research Facility (AGRF). Allele data was incorporated in population analysis of sizes only relevant to the previously characterized loci repeat polymorphisms.

### Molecular Data and Population Structure Analysis

Number of alleles (Na), number of effective alleles (Ne) and Nei's unbiased gene diversity ($H_{exp}$) (Nei, 1978) was used to calculate the genetic diversity was calculated in GenAlex 6.5 (Peakall and Smouse, 2012). The number of multilocus genotypes (MLG), the number of expected MLGs at the smallest sample size based on rarefaction (*eMLG*), the corrected genotypic diversity index (*D*), MLG and genotypic evenness (*E.5*) were calculated using the Poppr package (Kamvar et al., 2014) in R (R Core Team, 2013). Analysis of molecular variance (AMOVA) was performed to examine the variation within and among the above mentioned sub-populations and multilocus analysis was performed to group isolates into haplotypes (online source 1) using GenAlex 6.5. (Peakall and Smouse, 2012).

To visualize the relationships among MLGs in the six sub-populations, SSR data were used to construct a minimum spanning network based on Bruvo's distance (Bruvo et al., 2004) using the R package Poppr on non-clone-corrected data. The network was visualized using the package igraph (Csardi and Nepusz, 2006). Subsequently, the frequencies of most common haplotype were evaluated separately.

## Pathogenic Population Structure

### Plant Material

Four chickpea genotypes with known disease reactions were used as a differential host set to assess isolate aggressiveness (**Table 1**). ICC3996 is used widely as a resistance source in the Australian chickpea breeding program, and Genesis090 and PBA HatTrick are the most widely grown "resistant" host genotypes in southern and northern regions, respectively. Meanwhile, Kyabra remains a widely grown host genotype in the harsher regions of New South Wales and southern and central regions of Queensland due to high yield and quality but is considered "susceptible" and used as a disease check in NVT sites. Seedlings were grown in 15 cm diameter pots containing commercial grade potting mix. Two replicates were sown for each of the genotype × isolate combinations assessed, with five plants grown per pot/rep. All plants were grown and maintained in the glasshouse facility at 22 ± 5°C under 16 h/8 h day/night photoperiod at The University of Melbourne, Parkville campus, Victoria, Australia.

### Fungal Materials, Inoculation and Disease Assessment

Two hundred and sixty single spored *A. rabiei* isolates were selected for phenotyping, representative of the years, regions and host genotype origins within the 2013–2015 collection. This included sub-sets of isolates from targeted regions and genotypes as mentioned in section "Population Structure: SSRs". Single spored isolates were cultured in V8

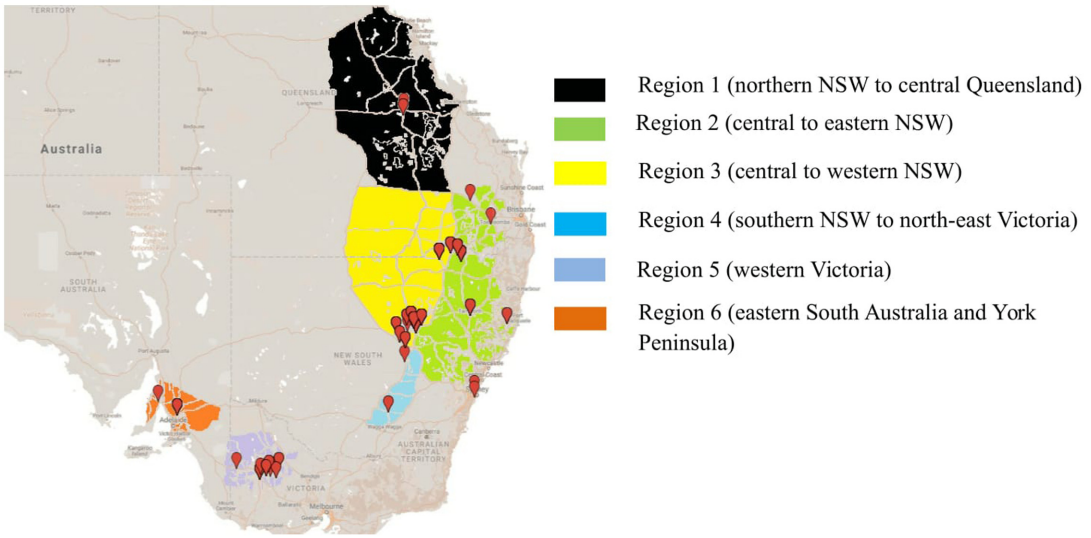


**FIGURE 1 |** Isolate collection of *Ascochyta rabiei* from six different agro-geographically classified chickpea growing regions during three consecutive growing seasons (2013–2015).

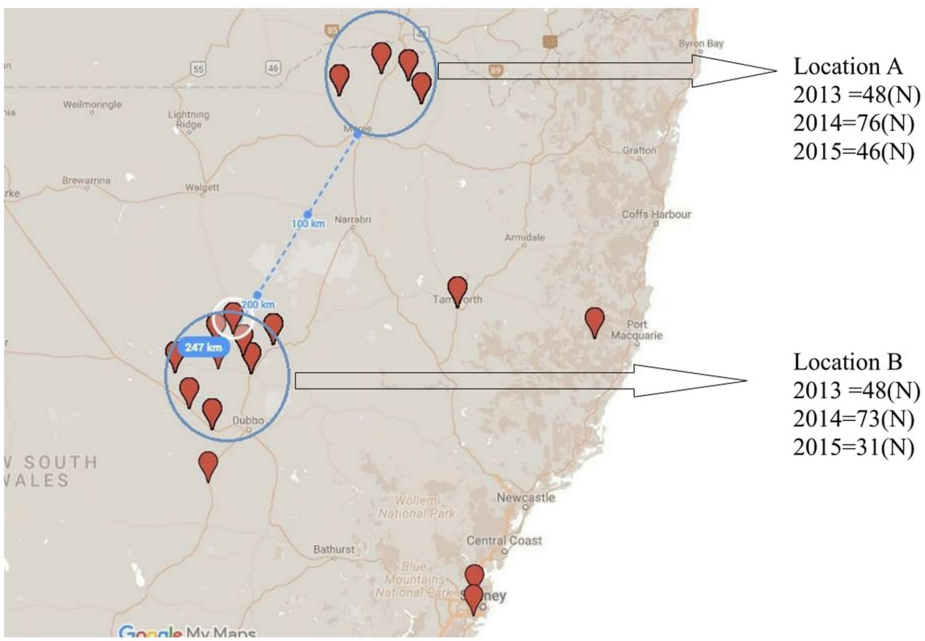


**FIGURE 2 |** Collection locations and isolate numbers recovered (N) from PBA HatTrick over three consecutive years (2013–2015) for potential association of spatial and temporal effects.

juice agar and maintained in the incubator for 14 days at 22 ± 2°C with a 12/12 h near-UV light irradiation (350–400 nm)/dark photoperiod prior to being used in the inoculation bioassay.

Inoculum was prepared as described in Sambasivam et al. (2017) and the mini-dome technique of Chen et al. (2005) was used to initiate disease. The disease severity of each isolate on each of the host genotypes was assessed using the qualitative 1–9 scale of Singh et al. (1981) at 21 days after inoculation (dai) where; scores of 1 or 3 represented a low disease severity; 5 represented a moderate disease severity without significant stem infection, and 7 or 9 represented a high disease severity with stem lesions that would lead to major difficulties in transpiration, photosynthesis and/or breakage.

### Highly Aggressive Isolates and Pathogenicity Grouping

Isolates identified as highly aggressive produced a cumulative leaf score of at least 7 on >80% and a stem score of at least 7 on >10% of all of the host plants assessed. Subsequently, this sub-set of isolates were placed into pathogenicity groups based on their ability to cause low, moderate or high disease severity independently on ICC3996, Genesis090 and PBA HatTrick (**Table 2**).

### Highest Risk Isolates to the Australian Chickpea Industry

Isolates of highest risk were identified on the basis of genotype and phenotype data. Accordingly, these belonged to the most frequently detected haplotype and were the most aggressive on the best resistance sources used within the advanced breeding program. Highest risk isolates were also aggressive on the currently deployed "resistant" host genotypes (pathogenicity group 4).

# RESULTS

## Population Structure: SSRs

Between two and eight alleles were identified for each of the SSR loci across the collection of 598 isolates. The maximum gene diversity ($H_{exp}$) at each ranged from 0.020 to 0.183 with an average of 0.066 ($H_{exp}$). Locus ArA03T ($H_{exp} = 0.183$) was the most informative, followed by ArH05T ($H_{exp} = 0.132$) (**Table 3**).

In total, 66 haplotypes were detected, of which 34 were detected just once ($n = 1$) (Supplementary Table 1). The detection frequency of each haplotype and the genetic relationships among them revealed by the seven SSR loci are presented in **Figure 3**. The most frequently detected haplotype, ARH01 accounted

**TABLE 1 |** Differential host genotypes and their known disease response ratings to *A. rabiei* in Australia.

| Genotype | Resistance level | Reference |
|---|---|---|
| Genesis 090 (kabuli) | R | Pulse Australia, 2017 |
| ICC3996 (desi) | R | Nasir et al., 2000 |
| PBA HatTrick (desi) | MR | Pulse Breeding Australia, 2017 |
| Kyabra (desi) | S | Moore et al., 2015 |

**TABLE 2 |** Criteria used for pathogenicity grouping of the highly aggressive isolates.

| Pathogenicity group | Description |
|---|---|
| 1 | High disease on PBA HatTrick and low disease on Genesis090 and ICC3996 |
| 2 | High disease incidence on PBA HatTrick, moderate disease on Genesis090 and low disease on ICC3996 |
| 3 | High disease on PBA HatTrick, moderate disease on Genesis090 and moderate disease on ICC3996 |
| 4 | High disease on PBA HatTrick, high disease on Genesis090 and moderate disease on ICC3996 |

**TABLE 3 |** The informative microsatellite loci used for genotyping the Australian *A. rabiei* population.

| Locus | Allele size | Number of allele | Number of Allele in regions | | | | | | Size of allele | Diversity ($H_{exp}$) ± SE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Region 1 (*N* = 18) | Region 2 (*N* = 233) | Region 3 (*N* = 130) | Region 4 (*N* = 92) | Region 5 (*N* = 56) | Region 6 (*N* = 69) | | |
| ArA03T | 409–439 | 8 | 5 | 3 | 4 | 2 | 3 | 4 | 409, 412, 415, 421, 424, 427, 430, 433, 439 | 0.183 ± 0.057 |
| ArH05T | 221–257 | 6 | 2 | 4 | 2 | 3 | 2 | 3 | 221, 233, 239, 242, 248, 254 | 0.132 ± 0.035 |
| ArR12D | 185–191 | 3 | 2 | 1 | 2 | 2 | 2 | 2 | 185, 187, 189, 191 | 0.038 ± 0.017 |
| ME14-1-56 | 379–383 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 379, 383 | 0.039 ± 0.039 |
| ME14-1-63 | 313–319 | 3 | 1 | 2 | 1 | 3 | 2 | 1 | 313, 316, 319 | 0.033 ± 0.021 |
| ME14-1-83 | 283–285 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 283, 285 | 0.020 ± 0.019 |
| ME14-1-91 | 339-342 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 333, 339, 342 | 0.021 ± 0.018 |
| | | | | | | | | | Mean ($H_{exp}$) = 0.066 ± 0.015 | |

*N = Number of individuals observed. $H_{exp}$ = Nei's unbiased gene diversity (Nei, 1978). SE = Standard error.*

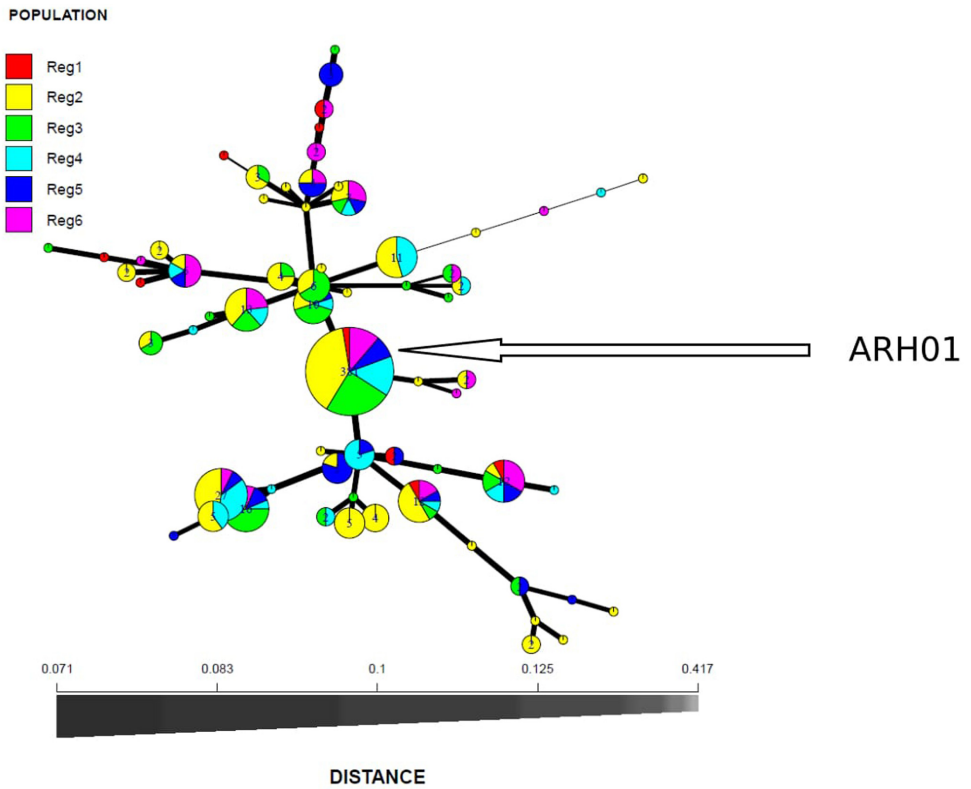


**FIGURE 3 |** Minimum spanning network based on Bruvo genetic distances representing 66 MLGs observed in six *A. rabiei* populations from Australia. Node colors represent population membership proportional to the pie size. Node sizes are relatively scaled to logl.75n, where *n* is the number of samples in the nodes to reduce node overlap. Edge thickness (lines) represent minimum genetic distance between haplotype.

for 55.35 to 72.30% of the six regional population across all growing seasons (**Table 4**). In accordance with the overall gene diversity detected, the highest gene diversity was observed in Region 1 ($H_{exp}$ = 0.161), which contained the most unique and effective alleles (2.28 and 1.20, respectively). Gene diversity in Region 1 was significantly higher than in all other regions ($P \leq 0.001$). The *eMLG* (9.00), *E.5* values (0.49), Ne (1.20), and Na (2.28) values were also highest for Region 1, indicating a more diverse population in this region compared to the other analyzed regions. However, the corrected Simpson's genotypic diversity index (D) did not differ greatly among regions The mean low genotypic diversity ($D = 0.57$) indicated the consistent, low diversity detected within the entire Australian *A. rabiei* population (**Table 4**).

A total of 430 isolates were collected from the two widely adopted and "resistant" or "moderately resistant" host genotypes, Genesis090 and PBA HatTrick. Within these sub-populations, a total of 17 ($N = 55$) and 47 ($N = 373$) MLGs were observed on Genesis090 and PBA HatTrick, respectively (**Table 5**). Regardless of the host genotypes, the most frequently detected haplotype

**TABLE 4 |** The genetic structure of the population detected within each of the six growing regions.

| Regions | N | MLG | *eMLG* ± SE | Na ± SE | Ne ± SE | $H_{exp}$ ± S.E | *E.5* | *D* | % of ARH01 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 18 | 9 | 9 ± 0.00 | 2.28 ± 0.47 | 1.20 ± 0.08 | 0.161 ± 0.05 | 0.498 | 0.555 | 55.55 |
| 2 | 233 | 38 | 6.8 ± 1.73 | 1.85 ± 0.45 | 1.05 ± 0.03 | 0.049 ± 0.03 | 0.269 | 0.555 | 63.09 |
| 3 | 130 | 21 | 5.38 ± 1.52 | 1.85 ± 0.40 | 1.04 ± 0.04 | 0.039 ± 0.03 | 0.312 | 0.592 | 72.30 |
| 4 | 92 | 18 | 6.37 ± 1.49 | 1.85 ± 0.34 | 1.07 ± 0.04 | 0.061 ± 0.03 | 0.382 | 0.592 | 60.86 |
| 5 | 56 | 16 | 7.78 ± 1.49 | 1.71 ± 0.28 | 1.06 ± 0.03 | 0.055 ± 0.02 | 0.393 | 0.554 | 55.35 |
| 6 | 69 | 16 | 6.82 ± 1.50 | 1.85 ± 0.45 | 1.03 ± 0.02 | 0.034 ± 0.01 | 0.362 | 0.652 | 62.31 |

*Mean D = 0.57. N = Number of individuals observed. MLG = Number of multilocus genotypes (MLG) observed. eMLG = The number of expected MLG at the smallest sample size based on rarefaction. SE = Standard error. Na = Number of different alleles (Kalinowski, 2005). Ne = Number of effective alleles (Kalinowski, 2005). $H_{exp}$ = Nei's unbiased gene diversity (Nei, 1978). E.5 = Genotypic evenness (Pielou, 1975; Ludwig and Reynolds, 1988; Grünwald et al., 2003). D = Corrected Simpson's Index (Simpson, 1949).*

was ARH01, which accounted for 54 and 63% of the isolates detected on Genesis090 and PBA HatTrick, respectively (**Table 5**). Although more than five times the number of isolates were collected from PBA HatTrick than Genesis090, no significant difference in gene and genotypic diversity measures ($P \leq 0.45$) was detected among the isolate groups. Furthermore, genetic diversity were all low (**Table 5**).

### No Evidence of Temporal or Spacial Population Shift

Although some differences in gene diversity ($H_{exp}$) were observed between the 1st and 3rd years of sampling, increasing from 0.024 to 0.053 at Location A, and from 0.024 to 0.059 at Location B, these changes were not significantly different to those detected in the 2nd year of sampling. Also, the frequency of the ARH01 haplotype remained almost static at each independently sampled geographical location, A and B, and across the 3 years of sampling (ranging from 58.62 to 66.67%; **Figure 2**). Thus, no evidence of either a temporal or spacial population shift in SSR diversity was observed over the period of the study, at the epidemic locations sampled (**Table 6**).

### Pathogenic Population Structure and Highest Risk Isolates

Among the 260 isolates assessed, 54 (21%) were highly aggressive and categorized into highly aggressive pathogenicity groups. Of these, 62% belonged to pathogenicity group 1, 2% belonged to pathogenicity group 2, 13% belonged to pathogenicity group 3 and 23% belonged to pathogenicity group 4 (Supplementary Table 2).

Among the 54 highly aggressive isolates identified, 75% belonged to haplotype ARH01, far more than detected in any other haplotype group, and recovered across all of the growing regions and hosts assessed. Far lower frequencies of the highly aggressive isolates were detected within other haplotype groups (**Figure 4**; Supplementary Table 3).

## DISCUSSION

Together with the strategic application of fungicides, chickpea production is reliant on the host containing the optimal combination of *A. rabiei* resistance alleles for timely recognition and defense initiation. Meanwhile, the pathogen population is under constant selective pressure to alter, to reproduce and spread new versions of itself that are able to survive in new environments, evade detection by the host and potentially overcome early host defenses (Pandey et al., 2016). In a clonal population, this occurs through opportunistic mutation and spread of the fittest and most widely adaptable isolates (Messer et al., 2016). Given the clonal nature of *A. rabiei* in Australia (Leo et al., 2015), it is unsurprising that we have detected a limited number of haplotypes that occur frequently throughout the chickpea growing regions, independent of host genotype.

### Population Structure: SSRs

The overall low genetic diversity found within the comprehensive assessment of the Australian *A. rabiei* population in this study is a common finding with Leo et al. (2015). Even if compatible mating types were present in the population, it seems likely that the expansion of this population to date has occurred through clonal means, perhaps due to forces suppressing recombination such as an initial imbalance in the mating type ratio of the founder isolates. The life cycles of many fungal species alternate between asexual and sexual multiplication (Hawker, 2016; Laloi et al., 2016) and asexual reproduction is often the major reproductive mechanism during epidemics to quickly increase the frequency of fit individuals (Laloi et al., 2016).

**TABLE 5 |** The genetic structure of the *A. rabiei* population detected on two widely adopted host genotypes.

| Hosts | *N* | MLG | eMLG ± SE | Na ± SE | Ne ± SE | $H_{exp}$ ± SE | *E.5* | D | % of ARH01 |
|---|---|---|---|---|---|---|---|---|---|
| Genesis090 | 57 | 17 | 4.91 ± 1.32 | 1.85 ± 0.45 | 1.06 ± 0.03 | 0.052 ± 0.02 | 0.37 | 0.662 | 57.89 |
| PBA HatTrick | 373 | 47 | 4.28 ± 1.37 | 2.28 ± 0.42 | 1.05 ± 0.03 | 0.048 ± 0.02 | 0.26 | 0.575 | 64.61 |

*N = Number of individuals observed. MLG = Number of multilocus genotypes (MLG) observed. eMLG = The number of expected MLG at the smallest sample size based on rarefaction. SE = Standard error. Na = Number of different alleles (Kalinowski, 2005). Ne = Number of effective alleles (Kalinowski, 2005).* $H_{exp}$ *= Nei's unbiased gene diversity (Nei, 1978). E.5 = Genotypic evenness (Pielou, 1975; Ludwig and Reynolds, 1988; Grünwald et al., 2003). D = Corrected Simpson's Index (Simpson, 1949).*

**TABLE 6 |** The genetic structure of the population detected within each of the six growing regions.

| Year | Population locations (*N*) | MLG | eMLG ± SE | Na ± SE | Ne ± SE | $H_{exp}$ ± S.E | *E.5* | *D* | % of ARH01 |
|---|---|---|---|---|---|---|---|---|---|
| 2013 | A (48) | 12 | 8.86 ± 1.27 | 1.42 ± 0.29 | 1.02 ± 0.02 | 0.024 ± 0.018 | 0.382 | 0.544 | 66.67 |
| | B (48) | 12 | 8.86 ± 1.27 | 1.43 ± 0.29 | 1.02 ± 0.02 | 0.024 ± 0.018 | 0.382 | 0.540 | 66.66 |
| 2014 | A (72) | 21 | 10.51 ± 1.81 | 1.42 ± 0.29 | 1.03 ± 0.03 | 0.031 ± 0.021 | 0.308 | 0.584 | 63.88 |
| | B (72) | 21 | 10.51 ± 1.81 | 1.43 ± 0.29 | 1.03 ± 0.02 | 0.031 ± 0.021 | 0.308 | 0.630 | 63.88 |
| 2015 | A (42) | 10 | 8.15 ± 1.05 | 1.71 ± 0.47 | 1.06 ± 0.02 | 0.053 ± 0.029 | 0.418 | 0.630 | 66.66 |
| | B (29) | 9 | 8.867 ± 9.0 | 1.43 ± 0.20 | 1.06 ± 0.03 | 0.059 ± 0.028 | 0.491 | 0.576 | 58.62 |

*N = Number of individuals observed. MLG = Number of multilocus genotypes (MLG) observed. eMLG = The number of expected MLG at the smallest sample size based on rarefaction. SE = Standard error. Na = Number of different alleles (Kalinowski, 2005). Ne = Number of effective alleles (Kalinowski, 2005).* $H_{exp}$ *= Nei's unbiased gene diversity (Nei, 1978). E.5 = Genotypic evenness (Pielou, 1975; Ludwig and Reynolds, 1988; Grünwald et al., 2003). D = Corrected Simpson's Index (Simpson, 1949).*

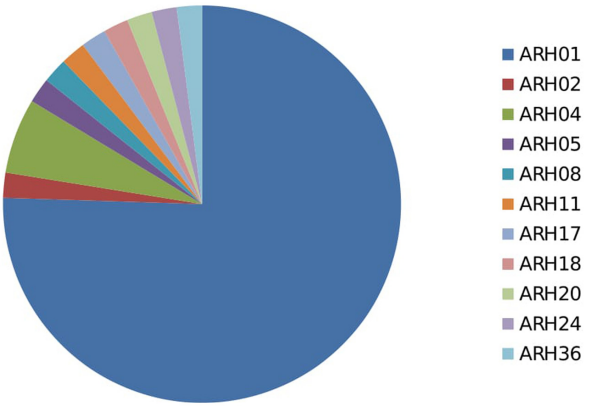


**FIGURE 4 |** Proportion of highly aggressive isolates from different haplotype group.

Within the Australian *A. rabiei* population, the large haplotype group (ARH01) occurs at extremely high frequencies of up to 63% of the entire population within a region. This haplotype likely established during an initial founder effect (Rivas et al., 2004) due to specific fitness characteristics that enabled survival at the point of introduction. The isolates then likely spread to other growing regions through infected seed distribution (Galloway and MacLeod, 2003). Subsequently, isolates that were highly adapted to survive in the range of agro-geographical regions and able to overcome host resistance proliferated through clonal propagation, causing severe disease epidemics when optimal climatic conditions prevailed. The resultant genetic and genotypic diversities observed in the Australian *A. rabiei* population reflect this founder effect, whereby the establishment and success of the pathogen has occurred through an available niche, provided by the abundance of susceptible host and an optimal environment. The number of founder events that have occurred for *A. rabiei* in Australia is unknown, but potentially the increasing frequencies of several haplotypes other than ARH01 in the population is an indication of slow evolution of other groups of highly adapted isolates, which should be monitored for increases in highly aggressive isolate frequency.

The genotypic diversity detected among isolates recovered from Genesis090 was not significantly different to that detected among those recovered from PBA HatTrick. Whilst it is likely that the host genotype would contribute to shaping the structure of the pathogen population (Jones and Dangl, 2006; Ley et al., 2006), the number of isolates assessed over the time period in the current study may not have been sufficient to visualize this phenomenon. Given the relatively short period for potential adaptation (<40 years), the clonal Australian population may still be experiencing the original founder effects. More in-depth investigation is required to determine if host factors are contributing to population adaptation. This might be through tracking of specific isolates over time and observations of the host defense responses that are instigated within each of the hosts under investigation. A similar, but smaller scale study was previously conducted by Leo et al. (2016), who found some host-specific differences in defense-related gene expressions. Expression of differential host defense responses to specific isolate populations might also identify factors that impact on survival and reproduction of specific populations and hence inform management strategies within growing regions where particular hosts are grown (Hollomon and Brent, 2009; Bertolini et al., 2012; Ali et al., 2016).

The non-significant differences detected in genotypic diversity over time at both of the locations assessed in New South Wales was unsurprising given the clonal nature of the pathogen and the short period since introduction to Australia. In a similar study, no significant changes were observed over 3 years within a *Mycosphaerella graminicola* population, DNA fingerprints were used to identify colones produced through asexual reproduction, suggesting and genetic stability of fungus was proposed (Chen et al., 1994).

Although overall seemingly stable and despite being clonal, we cannot ignore the potential for the existing population to change and evolve rapidly in response to an external factor (Messer et al., 2016). Rapid evolution of the wheat pathogen *Zymoseptoria tritici* was determined to be due to clustering of transposable elements leading to generation of extensive rearrangements and multiple independent gene losses (Hartmann et al., 2017). Rapid population changes may also occur through selective sweeps, potentially linked to host genotypes and/or chemical controls, resulting in "adaptive walk" and genetic shift among a limited number of frequently occurring haplotypes (Orr, 1998; Messer et al., 2016). Evidence of this may become more apparent as the industry adopts new resistant host genotypes such as PBA Seamer, which will need careful monitoring for pathogen population shift (Pulse Australia, 2017).

## The Pathogenic Population Structure

Evaluating *A. rabiei* populations on a set of differentials with different levels of resistance is useful for monitoring aggressiveness changes and to identify the most aggressive isolates. These isolates are required for selective breeding and, potentially, disease management strategies, particularly if differential factors underpinning aggressiveness are able to be dissected. Despite the SSR clonal composition, a similar wide diversity in aggressiveness was detected within the Australian population as previously detected elsewhere (Iqbal et al., 2004; Benzohra et al., 2011; Atik et al., 2013; Mahiout et al., 2015). Highly aggressive isolates were able to cause differential disease severities across a host set including two of the most widely adopted cultivars that underpin the Australian chickpea industry, Genesis090 and PBA HAtTrick. Genesis090 was introduced from ICARDA, Syria, where it was tested as FLIP94-090C, while PBA HatTrick is a cross of *cv.* Jimbour and the resistant Iranian landrace ICC14903 (Pulse Australia, 2017). However, these host genotypes have in recent years experienced an increase in susceptibility to *A. rabiei* (Moore et al., 2016), in keeping with our observation of increased frequencies of highly aggressive isolates within the population.

Increased aggressiveness within the Australian *A. rabiei* population was highlighted by the recent first observation of pycnidia formation on ICC3996, one of the resistance pillars

of the chickpea breeding program, in field trials (Moore et al., 2016). Consequently, erosion of resistance in host genotypes that contain alleles from this source is highly likely as the adapted highly aggressive isolates spread and most likely become more frequent. Other sources of resistance will be necessary in the immediate future to underpin the chickpea breeding programs in Australia. Such material should be selected based on ability to resist the diversity of the pathogen population since different genetic mechanisms are likely to be controlling aggressiveness between the different pathogenicity groups (Hamid and Strange, 2000). Although the most destructive isolates detected in this study were of Pathogenicity Group 4, we must remember that this classification was based on disease severity on ICC3996. It is highly likely that differential reactions would occur on other resistance sources and that these should be more fully characterized for their own disease reactions to the representative pathogen population before being used within the breeding program.

## CONCLUSION

Within the adapted clonal groups detected in this study, we can surmise that isolates were selected by their ability to overcome resistance within the widely adopted "resistant" host genotypes such as Genesis090 and PBA HatTrick. This would help to explain the occurrence of a greater frequency of highly aggressive isolates within the ARH01 haplotype group, creating "super isolates" of the very highest pathogenicity ranking able to survive in many locations and on a wide range of host genotypes. These isolates represent the very highest risk to the Australian chickpea industry. However, several factors must be considered when selecting accessions that appear "resistant" to these: (1) This study suggests rapid changes in aggressiveness of the pathogen population, (2) only one mating type of *A. rabiei* has been detected in Australia but, if both mating types were present, the sexual reproductive cycle may quickly become active to create a recombinant pool population, and (3) isolate sampling and testing is limited by time and resources leading to the possibility of missing some important aggressive isolates. To extend stability of resistance, our growers must maintain their best practice in farming systems including growing clean seed, a minimum of 3-year rotations, effective distances between chickpea crops and fungicide spray regimes. Meanwhile, further studies are required to better understand the genetics of resistance in order to develop host genotypes with different resistance gene combinations, to potentially reduce selective adaptation of the pathogen.

## AUTHOR CONTRIBUTIONS

YM assessed the molecular structure of the *A. rabiei* population and constructed the manuscript. PS assessed the pathogenic structure of the *A. rabiei* population and drafted the manuscript. JD, KH, KM, CL, SK, JB, and AL contributed to analyses and manuscript production. RF supervised the research and edited the manuscript.

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# Effects of Temperature Stresses on the Resistance of Chickpea Genotypes and Aggressiveness of *Didymella rabiei* Isolates

*Seid Ahmed Kemal[1]*, Sanae Krimi Bencheqroun[2], Aladdin Hamwieh[3] and Muhammad Imtiaz[4]*

*[1] Biodiversity and Integrated Gene Management Program, International Center for Agricultural Research in the Dry Areas, Rabat, Morocco, [2] Centre Régional de la Recherche Agronomique de Settat, Institut National de la Recherche Agronomique, Settat, Morocco, [3] Biodiversity and Integrated Gene Management Program, International Center for Agricultural Research in the Dry Areas, Giza, Egypt, [4] International Maize and Wheat Improvement Center, Islamabad, Pakistan*

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***Correspondence:**
*Seid Ahmed Kemal*
*s.a.kemal@cgiar.org*

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Chickpea (*Cicer arietinum* L.) is an important food and rotation crop in many parts of the world. Cold (freezing and chilling temperatures) and Ascochyta blight (*Didymella rabiei*) are the major constraints in chickpea production. The effects of temperature stresses on chickpea susceptibility and pathogen aggressiveness are not well documented in the Cicer-Didymella pathosystem. Two experiments were conducted under controlled conditions using chickpea genotypes and pathogen isolates in 2011 and 2012. In Experiment 1, four isolates of *D. rabiei* (AR-01, AR-02, AR-03 and AR-04), six chickpea genotypes (Ghab-1, Ghab-2, Ghab-3, Ghab-4, Ghab-5 and ICC-12004) and four temperature regimes (10, 15, 20, and 25°C) were studied using 10 day-old seedlings. In Experiment 2, three chickpea genotypes (Ghab-1, Ghab-2, and ICC-12004) were exposed to 5 and 10 days of chilling temperature exposure at 5°C and non-exposed seedlings were used as controls. Seedlings of the three chickpea genotypes were inoculated with the four pathogen isolates used in Experiment 1. Three disease parameters (incubation period, latent period and disease severity) were measured to evaluate treatment effects. In Experiment 1, highly significant interactions between genotypes and isolates; genotypes and temperature; and isolate and temperature were observed for incubation and latent periods. Genotype x isolate and temperature x isolate interactions also significantly affected disease severity. The resistant genotype ICC-12004 showed long incubation and latent periods and low disease severity at all temperatures. The highly aggressive isolate AR-04 caused symptoms, produced pycnidia in short duration as well as high disease severity across temperature regimes, which indicated it is adapted to a wide range of temperatures. Short incubation and latent periods and high disease severity were observed on genotypes exposed to chilling temperature. Our findings showed that the significant interactions of genotypes and isolates with temperature did not cause changes in the rank orders of the resistance

of chickpea genotypes and aggressiveness of pathogen isolates. Moreover, chilling temperature predisposed chickpea genotypes to *D. rabiei* infection; developing multiple stress resistance is thus a pre-requisite for the expansion of winter-sown chickpea in West Asia and North Africa.

**Keywords: aggressiveness, chickpea, cold, *Didymella rabiei*, pre-disposition, resistance**

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important cool-season food legume crop grown in many parts of the world. The crop is produced for local consumption, to generate export earnings and act as a break crop to improve soil fertility and health. Chickpea is mainly grown in spring in many parts of the world where drought, heat, wilt/root rots, Ascochyta blight and insect pests limit crop productivity and production (Singh et al., 1989; Jha et al., 2014; Li et al., 2015). Chickpea yield can be substantially increased by adopting early winter sowing at low to medium altitudes in the West Asia and North African (WANA) region (Hawtin and Singh, 1984; Singh et al., 1989; Mazid et al., 2013). However, sowing chickpea in winter can increase the risk of exposing the crop to subzero temperatures as low as −10°C for up to 60 days and to chilling temperature and Ascochyta blight epidemics during the cropping season (Malhotra and Singh, 1991; Singh et al., 1993; Croser et al., 2003; Nezami et al., 2012).

To tap the potential of winter chickpea sowing in low to medium altitude areas, the International Center for Agricultural Research in the Dry Areas (ICARDA) has initiated a chickpea breeding program targeting winter sowing since the 1974/75 cropping season. The two key traits for the success of winter sown chickpea are cold (freezing) tolerance at the seedling and vegetative stages and resistance to Ascochyta blight (*Didymella rabiei*). ICARDA develops cold tolerant chickpea germplasm in key cold testing sites in Syria (Tel Hadya Research Station), Turkey (Eskishehr Research Station), Lebanon (Terbol Research Station) and Iran (Maraghahe Research Station). Breeding lines and germplasm accessions (cultivated and wild relatives) are exposed to freezing temperatures ranging from −5°C in Lebanon and Syria to −20°C in Turkey and Iran. The first cultivars released for winter sowing with cold tolerance and Ascochyta blight resistance were ILC-482 and ILC-3279 (Malhotra and Singh, 1991; Singh et al., 1992a,b). The cultivar ILC-482 has moderate levels of Ascochyta blight resistance and tolerance to freezing temperatures as low as −10°C and can yield up to 4 t/ha (Hawtin and Singh, 1984). Besides high productivity, winter-sown chickpea cultivars are taller than spring sown chickpea and allows mechanical harvesting that solves labor shortages during harvesting and threshing (Singh et al., 1997). Moreover, wilt/root rots and leaf miner are less problematic in winter-sown than in spring-sown chickpea crops.

Ascochyta blight is a major biotic factor contributing to high yield gaps in chickpea in many countries (Pande et al., 2005; Singh et al., 2007). The pathogen is heterothallic and requires the pairing of two compatible mating types (MAT1-1 and MAT1-2) for sexual reproduction. In the presence of the two mating types, fertile pseudothecia can develop on overwintering chickpea straw; ascospores act as primary inoculum sources to initiate disease foci that can lead to epidemics under favorable environmental conditions. Many races and pathotypes of *D. rabiei* have been reported in the WANA region (Udupa et al., 1998; Nourollahi et al., 2011; Atik et al., 2013). Different approaches are available to manage Ascochyta blight in chickpea crops with varying levels of effectiveness. These include foliar fungicide applications, seed treatment, agronomic practices, growing resistant cultivars and integration of two or more control options (Gan et al., 2006; Chang et al., 2007a; Dusunceli et al., 2007; Lobna et al., 2010). Although different Ascochyta blight management options are available for growers, breeding for host plant resistance is given the highest priority by national and international chickpea breeding programs (Singh and Reddy, 1996; Muehlbauer and Chen, 2007; Rubiales and Fondevilla, 2012; Sharma and Ghosh, 2016). The resistance of cultivars released in many countries is controlled by both major and minor genes (Lichtenzveig et al., 2002; Muehlbauer and Chen, 2007; Rubiales and Fondevilla, 2012; Labdi et al., 2013; Sharma and Ghosh, 2016). For example, the first two cultivars (genotypes ILC-482 and ILC-3279) released for winter sowing in many countries in WANA region have rate reducing resistance to the *D. rabiei* population existing in the early 1980s in Syria (Reddy and Singh, 1993) but this resistance was eroded through the appearance of more aggressive pathogen populations (Imtiaz et al., 2011; Atik et al., 2013; Hamwieh et al., 2013).

Interactions between biotic and abiotic stresses have been reported in many host-pathogen pathosystems where plants exposed to abiotic stresses showed either increased or decreased resistance/tolerance to subsequent infections by pathogens (Atkinson and Urwin, 2012; Bostock et al., 2014; Suzuki et al., 2014; Moyer et al., 2015). Temperature has a significant effect on host plant resistance genes and pathogen virulence and aggressiveness in many pathosystems. For example, in lupin, resistant cv. Wonga became susceptible to anthracnose when the temperature increased from 12–18° to 26°C (Thomas et al., 2008). When infected with different *Fusarium oxysporum* f. sp. *ciceris* races, chickpea cv. Ayala was moderately resistant at 24/21°C but susceptible at 27/25°C (Landa et al., 2006). In wheat, some isolates of *Puccinia striiformis* f. sp. *tritici* showed increased aggressiveness at high temperature (Milus et al., 2009). In sunflower, resistance to *Orobanche aegyptiaca* was found to be temperature dependent (Eizenberg et al., 2003). Studies on biotic-abiotic interactions in *Ascochyta*-legume pathosystem have mainly focused on the role temperature and wetness period play in inoculum production and disease development (Trapero-Casas and Kaiser, 1992, 2007; Pedersen and Morrall, 1994; Roger et al., 1999; Tivoli and Banniza, 2007; Golani et al., 2016), but studies on the role temperature stress plays

in host resistance, pathogen aggressiveness and predisposing chickpea to pathogen infection are lacking. Therefore, this study was designed to assess: (1) the effect of different temperature ranges on host resistance and pathogen aggressiveness in *Cicer-Didymella* pathosystem; and (2) how chilling temperature predisposes chickpea genotypes to *D. rabiei* infections.

## MATERIALS AND METHODS

Two independent experiments were conducted in 2011 and 2012 at ICARDA Tel Hadya Research Station, Syria. Experiment 1 was conducted in two controlled environment growth cabinets (Conviron Model E15, Winnipeg, Canada), adjusted to a 16-h photoperiod of approximately 450 mmol $m^{-2}$ $s^{-1}$ light intensity provided by fluorescent light (Jhorar et al., 1998; Udupa et al., 1998); Experiment 2 was conducted under plastic house conditions. In both experiments, four *D. rabiei* isolates, namely, AR-01 (Pathotype-1, weakly aggressive), AR-02 (Pathotype-2 moderately aggressive), AR-03 (Pathotype-3, aggressive) and AR-04 (Pathotype-4, highly aggressive), were used (Udupa et al., 1998; Imtiaz et al., 2011). The four isolates are routinely used to screen ICARDA chickpea breeding lines for Ascochyta blight resistance under field and controlled conditions (Hamwieh et al., 2013).

### Effects of Temperature on Host Resistance and Pathogen Aggressiveness (Experiment 1)

Four temperature regimes (10, 15, 20, and 25°C) and five released Kabuli chickpea genotypes for winter sowing, namely Ghab-1 (ILC-482), Ghab-2 (ILC-3279), Ghab-3 (FLIP-150-82), Ghab-4 (FLIP-85-122), Ghab-5 (FLIP-88-85), and one desi chickpea genotype (ICC-12004) were used in this experiment. All released cultivars in this study were resistant to pathogen populations existing during the year of their release. Seedlings (five seeds per 10-cm diameter pot) of each genotype planted in a sterilized soil-peat moss mixture in plastic house at 20 ± 2°C for 10 days were used for this experiment. The experiment was laid out in a split-split plot design (main plot: temperature; sub-plot: genotypes and sub-sub plots: isolates) with three replications and performed four times. The four *D. rabiei* isolates were grown on chickpea dextrose agar (4% chickpea flour; 2% dextrose and 2% agar in 1 l of distilled water) for 7–10 days inside a culture room at 21–23°C under 16/8 h light and darkness cycles. Spore suspensions were prepared the day of seedling inoculations by flooding the surface of the culture in Petri dishes with distilled water and then scraping the surface of the culture with a glass rod to release spores from pycnidia. Spore concentrations were determined using a Neubauer hemacytometer and adjusted to the desired concentration by diluting with distilled water. Ten-day old chickpea raised in the plastic house were inoculated with a spore suspension ($5 \times 10^5$ spores $m^{-l}$) of each isolate using hand sprayers until runoff for each temperature regime. Inoculated seedlings were covered with transparent polyethylene sheets for 72 h. After removing the plastic covers, seedlings were misted 3–4 times daily with distilled water to maintain favorable conditions (leaf wetness and 70% or more relative humidity in the growth cabinets) and favor disease development.

### Effect of Chilling Temperature in Predisposing Chickpea Genotypes to *D. rabiei* Infection (Experiment 2)

Three chickpea genotypes (Ghab-1, Ghab-3, and ICC-12004) were used in this experiment. Cultivars Ghab-1 and Ghab-3 are tolerant to cold but the reaction of genotype ICC-12004 is not documented. The isolates, number of seedlings per pot, experimental design, number of replications, spore concentrations and inoculation and incubation methods were similar to those described under **Experiment 1**. Seedlings (10 day-old) were raised in a plastic house at 20 ± 2°C and 16/8 h light/dark cycles and exposed to chilling temperature (5°C) in a cold chamber (16/8 h light/ dark and light cycles) for 5 and 10 days before inoculation. After chilling exposure, seedlings were returned to the plastic house for inoculation with pathogen isolates. Sets of seedlings of each genotype not exposed to chilling temperature and raised at 20 ± 2°C in a plastic house were used as controls. High humidity (>75%) was kept in the plastic house by misting water for 30 s at 2-3-h intervals. The chilling temperature (5°C) was selected because the cold chamber cannot be adjusted at a lower temperature; the temperature closer to the low temperatures prevailing during the seedling and vegetative stages of winter-sown chickpea at ICARDA, Tel Hadya Research Station, Syria (**Figure 1**) and based on published works on cold tolerance research on chickpea (Nayyara et al., 2005; Bakht et al., 2006). The Tel Hadya Research Station experiences 17–56 days of freezing temperature (Malhotra and Singh, 1991; Singh et al., 1993).

### Disease Parameters and Data Analyses

Three disease parameters, incubation period (IP) as the interval between inoculation and the first appearance of symptoms; latent period (LP) as the interval between inoculation and the first appearance of pycnidia on infected seedlings; and disease severity were recorded for seedlings in each pot in both experiments. Disease severity was recorded using a 1–9 rating scale where 1 = healthy plant, no disease; 2 = lesions present, but small and inconspicuous; 3 = lesions easily seen, but plants are mostly green; 4 = severe lesions clearly visible, stem infection is clear; 5 = lesions girdle stems, most leaves show lesions; 6 = plants collapsing, tips die back; 7 = plants dying, but at least three green leaves present; 8 = nearly all plants dead but still have a green stem; and 9 = dead plants (Chen et al., 2004). Final disease severity (the average of the scores in 5 plants per pot) was scored approximately 20 days after inoculation in Experiment-1 and 15 days after inoculation in Experiment-2 after one disease cycle was completed. All data for the three disease parameters were analyzed using the residual (restricted) maximum likelihood (REML) method in a generalized linear mixed model (Garrett et al., 2004; Onofri et al., 2010) using Genstat Software (16th edition). In the model, temperature, duration of chilling temperature exposure, genotypes and isolates were assigned as fixed effects and number

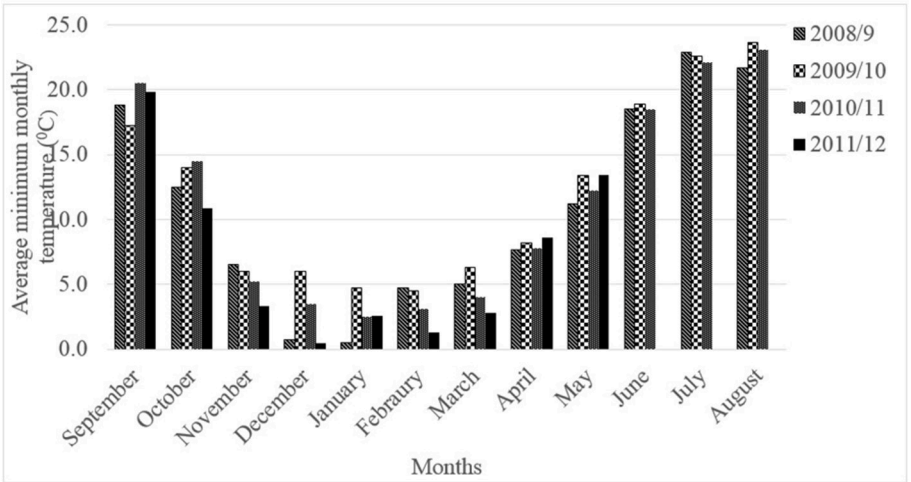


**FIGURE 1 |** Average monthly minimum temperature (°C) at ICARDA Tel Hadya Research Station, 2008–2012 cropping seasons.

**TABLE 1 |** Generalized linear mixed model analysis of fixed factors for three disease parameters on chickpea genotypes inoculated with *Didymella rabiei* isolates and incubated at different temperature ranges.

| Fixed factors | Degree of freedom | Incubation period | Latent period | Disease severity |
|---|---|---|---|---|
| Genotypes (G) | 5 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| Isolates (I) | 3 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| Temperature (T) | 3 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| GXI | 15 | $P < 0.01$ | $P < 0.005$ | $P < 0.003$ |
| GXT | 15 | $P < 0.001$ | $P < 0.001$ | $P < 0.061$ |
| IXT | 9 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| GXPXT | 45 | $P < 0.006$ | $P < 0.001$ | $P < 0.003$ |

**TABLE 2 |** Mean incubation and latent periods (days) of chickpea genotypes inoculated with four isolates of *Didymella rabiei*.

| Parameters | Genotypes | Isolates | | | | |
|---|---|---|---|---|---|---|
| | | AR-01 | AR-02 | AR-03 | AR-04 | Mean |
| Incubation period | Ghab-1 | 8.4 | 7.8 | 7.3 | 6.0 | 7.4 |
| | Ghab-2 | 8.7 | 7.6 | 7.6 | 7.2 | 7.8 |
| | Ghab-3 | 9.6 | 8.3 | 8.0 | 6.5 | 8.1 |
| | Ghab-4 | 9.2 | 9.1 | 7.5 | 6.4 | 8.1 |
| | Ghab-5 | 8.9 | 9.2 | 8.0 | 6.6 | 8.2 |
| | ICC-12004 | 9.1 | 9.7 | 8.9 | 7.5 | 8.8 |
| | Mean | 9.0 | 8.6 | 7.9 | 6.7 | 8.0 |
| LSD (0.01) for genotype by isolate interaction = 1.05 | | | | | | |
| Latent period | Ghab-1 | 11.2 | 10.5 | 10.3 | 9.4 | 10.3 |
| | Ghab-2 | 13.1 | 11.6 | 11.0 | 10.0 | 11.4 |
| | Ghab-3 | 12.6 | 11.5 | 11.5 | 9.5 | 11.3 |
| | Ghab-4 | 12.6 | 11.5 | 10.6 | 9.6 | 11.1 |
| | Ghab-5 | 12.9 | 11.9 | 11.7 | 9.5 | 11.5 |
| | ICC-12004 | 12.3 | 13.0 | 13.1 | 10.4 | 12.2 |
| | Mean | 12.4 | 11.6 | 11.3 | 9.7 | 11.3 |
| LSD (0.01) for genotype by isolate interaction = 0.62 | | | | | | |

of times the experiments were repeated and replications as random effects. Least square differences were calculated from the standard errors of the differences for mean comparisons. Correlation analyses were made between disease parameters.

# RESULTS

## Effects of Temperature on Host Resistance and Pathogen Aggressiveness

The variance component analyses for fixed effects of the three disease parameters are presented in **Table 1**. Interactions between genotypes and isolates; genotypes and temperature, and isolates and temperature were highly significant for IP and LP. Highly significant interactions were observed for genotype and isolate and isolate and temperature interactions for disease severity. No significant differences were observed between genotypes and temperature for disease severity.

### Incubation and Latent Periods

The IP among chickpea genotypes ranged from 7 to 9 days and LP ranged from 10 to 12 days (**Table 2**). Across all chickpea genotypes, IP and LP were the shortest when infected by isolate AR-04, and the longest when infected by AR-01. In the genotype × temperature interactions ($P < 0.001$), IP and LP were significantly longer at 10°C than the other temperatures across all chickpea genotypes (**Figures 2A,B**). Both IP and LP showed a decreasing trend from 15 to 20°C and increased when the temperature increased to 25°C. The shortest IP was observed in Ghab-1 and longest in ICC-12004 at 20°C. A similar trend was observed for LP where genotypes showed long LP at 10°C and then decreased during incubation at 15

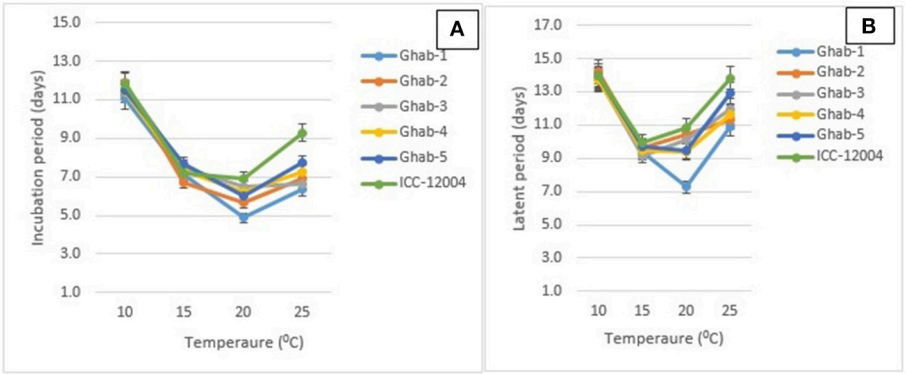


**FIGURE 2 |** Effects of temperature on mean incubation **(A)** and latent periods **(B)** of chickpea genotypes inoculated with four isolates of *Didymella rabiei*. Vertical bars indicate standard errors of the means.

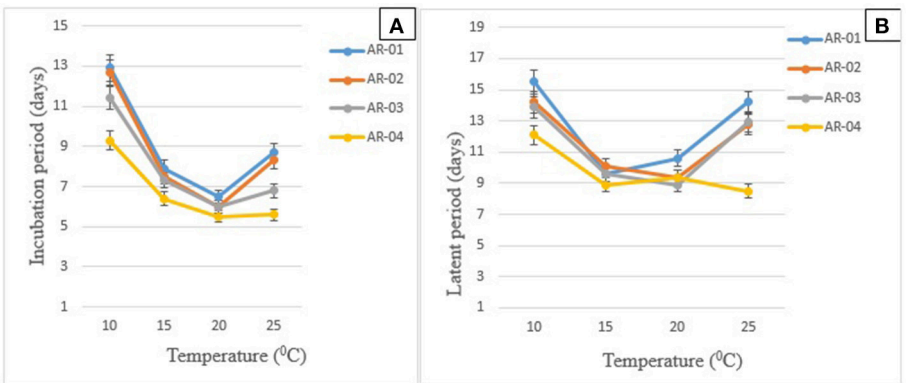


**FIGURE 3 |** Effects of temperature on the mean duration of the development of symptoms **(A)** and pycnidia **(B)** on chickpea genotypes inoculated with four isolates of *Didymella rabiei*. Vertical bars indicate standard errors of the means.

and 20°C. The shortest LP was observed at 20°C on Ghab-1.

In the isolate × temperature interaction ($P < 0.001$), all isolates took a long time to cause symptoms and produce pycnidia on chickpea genotypes incubated at 10°C (**Figures 3A,B**). Short IP was observed at 15–25°C for all isolates. However, except AR-04, the other isolates showed long IP at 25°C incubation temperature. Isolate AR-04 caused symptoms and produced pycnidia within a short period of time under all temperature regimes.

### Disease Severity

Overall there were differences among isolates in their aggressiveness that caused disease severity ranging from 3 to 7, while the susceptibility of genotypes was narrower, and across all isolates ranged from 5 to 6. In genotype × isolate interactions, isolates AR-04 and AR-03 caused high disease severity across all chickpea genotypes (**Table 3**). Isolate AR-01 showed low levels of disease severity (<4) on all genotypes. Isolate AR-03 caused high disease severity on Ghab-1 compared to its aggressiveness on the other genotypes. In isolate by temperature interactions, isolate AR-04 caused high disease severity at 10–15°C and the lowest severity at 25°C (**Table 4**). Isolate AR-01 caused high disease severity at 10–15°C than at the other two temperature regimes. The highly aggressive isolate AR-04 caused high disease severity at 25°C compared to the other isolates.

## Effect of Chilling Temperature in Predisposing Chickpea Genotypes to *Didymella rabiei* Infection

Non-significant interactions were observed between chilling duration × genotypes for LP and disease severity. Moreover, genotype × isolate interactions were not significant for IP and LP (**Table 5**). Highly significant interactions were observed between chilling duration and genotypes for IP; between chilling duration and isolate for the three disease parameters; and between genotype and isolates for disease severity.

### Incubation and Latent Periods

The mean IP for chickpea genotypes ranged from 7 to 9 days, while for LP it ranged from 10 to 12 days. Shorter IP and LP were observed on chickpea genotypes infected with the

four isolates and exposed to chilling temperature than on the controls. The 5-day chilling period shortened the duration of pycnidia formation on the chickpea genotypes infected with across all isolates. In chilling duration x isolate interactions, all pathogen isolates caused symptoms within a shorter period on all chickpea genotypes exposed to chilling temperature than on the controls (**Figures 4A,B**). Isolates AR-01 and AR-02 caused disease symptoms within a significantly shorter period on chickpea genotypes exposed to chilling temperature than on the controls. The pycnidia formation period was shorter under chilling temperature for isolates AR-01 and AR-03, while the highly aggressive isolate AR-04 produced symptoms and pycnidia within a short period under all treatments (**Table 6**).

### Disease Severity

The genotype × chilling exposure interaction did not show significant differences but genotypes Ghab-1 and ICC-12004 showed increased disease severity under chilling temperature (data not shown). In the genotype x isolate interaction ($P < 0.003$), isolate AR-04 caused high disease severity on all chickpea genotypes, while genotype ICC-12004 showed low levels of susceptibility to all pathogen isolates (**Figure 5**). The genotype did not show changes in rank order due to infection by *D. rabiei* isolates with varying levels of aggressiveness as affected by chilling temperature.

In the chilling duration × isolate interactions, isolate AR-04 caused high disease severity on chickpea genotypes in all treatments (**Figure 6**). The weakly aggressive isolate AR-01 caused more disease on chickpea exposed to chilling temperature than on the controls. Isolate AR-02 caused more disease on seedlings exposed to chilling temperature. However, isolate AR-3 caused more disease on seedling exposed to 5 days chilling temperature that 10 days exposure.

## Correlations among Disease Parameters

All correlations between two disease parameters were highly significant ($P < 0.001$) in both experiments. In Experiment 1, the correlation between IP and LP was positive and high ($r = 0.71$), while the correlation between LP and disease severity was intermediate and negative ($r = -0.55$). The correlation between IP and disease severity was low and negative ($r = -0.30$). In Experiment 2, the correlation between IP and LP was positive ($r = 0.73$), while the correlations between IP and disease severity ($r = -0.63$) and LP and disease severity ($r = -0.73$) were high and negative.

**TABLE 3 |** Mean disease severity on chickpea genotypes inoculated with four isolates of *Didymella rabiei*.

| Genotypes | Isolates | | | | |
|---|---|---|---|---|---|
| | AR-01 | AR-02 | AR-03 | AR-04 | Mean |
| Ghab-1 | 3.5 | 6.1 | 6.5 | 7.5 | 5.9 |
| Ghab-2 | 3.0 | 4.9 | 5.8 | 7.1 | 5.2 |
| Ghab-3 | 3.0 | 4.6 | 5.4 | 7.6 | 5.2 |
| Ghab-4 | 2.7 | 4.5 | 5.2 | 7.4 | 4.9 |
| Ghab-5 | 2.7 | 4.8 | 5.3 | 7.2 | 5.0 |
| ICC-12004 | 2.9 | 4.7 | 5.0 | 6.6 | 4.8 |
| Mean | 3.0 | 4.9 | 5.5 | 7.3 | 5.2 |

LSD (0.01) for genotype by isolate interaction = 0.56

**TABLE 4 |** Combined reaction of six chickpea genotypes to four isolates of *Didymella rabiei* on mean disease severity.

| Genotypes | Temperature regimes | | | | |
|---|---|---|---|---|---|
| | 10 | 15 | 20 | 25 | Mean |
| AR-01 | 4.0 | 3.5 | 2.2 | 2.2 | 3.0 |
| AR-02 | 5.0 | 6.5 | 5.6 | 2.7 | 4.9 |
| AR-03 | 5.0 | 6.7 | 5.8 | 4.6 | 5.5 |
| AR-04 | 7.9 | 8.0 | 7.2 | 5.9 | 7.3 |
| Mean | 5.5 | 6.2 | 5.2 | 3.9 | 5.2 |

LSD (0.01) for isolate by temperature interaction = 0.56

**TABLE 5 |** Generalized linear mixed model analysis of fixed factors for three disease parameters on chickpea genotypes exposed to chilling temperature.

| Fixed factors | Degree of freedom | Incubation period (days) | Latent period (days) | Disease severity |
|---|---|---|---|---|
| Duration (D) | 2 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| Genotypes (G) | 2 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| Isolates (I) | 3 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| DXG | 4 | $P < 0.03$ | $P < 0.551$ | $P < 0.706$ |
| DXI | 6 | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| GXI | 6 | $P < 0.104$ | $P < 0.159$ | $P < 0.001$ |
| DXGXI | 12 | $P < 0.002$ | $P < 0.360$ | $P < 0.856$ |

# DISCUSSION

Ascochyta blight and low temperature (cold and frost) will remain key production constraints to both winter and spring sown chickpea crops in many countries in the world. Ascochyta blight epidemics depend on weather conditions (moisture and temperature), level of variety resistance and aggressiveness of the pathogen population. The effect of biotic-abiotic interactions on pre-disposing hosts and the effectiveness of resistance genes is becoming an important issue since it affects disease management practices and breeding strategies (Bostock et al., 2014). Hence, knowledge on abiotic and biotic interactions on winter-sown chickpea is important in developing appropriate disease management practices to reduce the impact of Ascochyta blight and expand winter chickpea technology in WANA.

In Experiment 1, significant genotype x isolate interactions did not show changes in resistance of chickpea genotypes to the four isolates measured based on the three disease parameters indicating that reactions of the chickpea genotypes were temperature independent. Although resistance reactions of the chickpea genotypes are based on disease severity rating, they showed differences in IP and LP that could be used as selection criteria for partial resistance in chickpea resistance breeding.

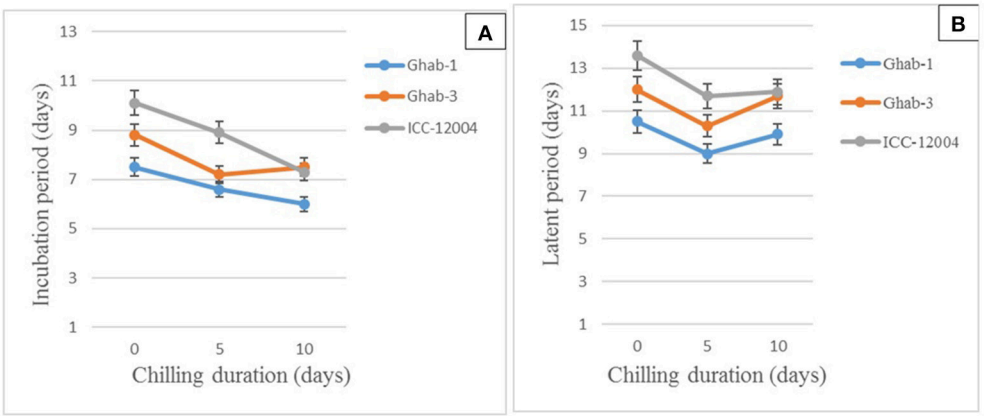


**FIGURE 4 |** Effects of chilling duration on mean incubation **(A)** and latent periods **(B)** on chickpea genotypes inoculated with four isolates of *Didymella rabiei.* Vertical bars indicate standard errors of the means.

**TABLE 6 |** Effects of chilling temperature on incubation and latent period of four isolates of *Didymella rabiei* inoculated on three chickpea genotypes.

| Parameters | Chilling duration (days) | Isolates | | | | |
|---|---|---|---|---|---|---|
| | | AR-01 | AR-02 | AR-03 | AR-04 | Mean |
| Incubation period | 0 | 13.2 | 8.8 | 7.2 | 6.0 | 8.8 |
| | 5 | 10.1 | 7.1 | 7.3 | 7.6 | 7.6 |
| | 10 | 6.9 | 6.7 | 7.6 | 6.6 | 7.0 |
| | Mean | 10.1 | 7.5 | 7.4 | 6.1 | 7.8 |
| LSD (0.01) for chilling duration x isolate interaction = 1.44 | | | | | | |
| Latent period | 0 | 16.7 | 12.3 | 10.7 | 8.5 | 12.1 |
| | 5 | 13.2 | 9.8 | 10.0 | 8.2 | 10.3 |
| | 10 | 9.8 | 10.0 | 12.9 | 9.6 | 10.6 |
| | Mean | 13.2 | 10.7 | 11.2 | 8.8 | 11.0 |
| LSD (0.01) for chilling duration x isolate interaction = 1.83 | | | | | | |

Temperature also did not affect the level of aggressiveness of the isolates where the highly aggressive isolate AR-04 showed short IP, LP and high disease severity across all temperature ranges. Isolate AR-04 is highly aggressive to all available chickpea genotypes developed at ICARDA, indicating more attention in developing resistant genotypes from cultivated and wild relatives to manage highly aggressive pathogen population like isolate AR-04 in the future since many isolates with similar aggressiveness with AR-04 are reported in Syria (Atik et al., 2013). High temperature prevails during podding stage of chickpea and if supplementary irrigation is given, it provides long wetness period and isolates adapted to high temperature range can cause heavy pod and seed infections that affect seed quality and increased the chance of pathogen spread to new areas through germplasm exchanges. Under controlled conditions, Frenkel et al. (2010) found that *D. rabiei* isolates collected from cultivated chickpea showed high temperature adaptation than those isolates from wild chickpea.

In Experiment 2, chickpea genotypes showed short IP and LP and high disease severity when exposed to chilling temperature. Cold exposure can pre-dispose resistant chickpea genotypes to both weakly and highly aggressive pathogen populations and can cause disease epidemics under field conditions if conditions are favorable for disease development. The roles of abiotic stress in affecting resistance genes, increasing pathogen virulence and pre-disposing crops to subsequent pathogen attacks have been reported by many researchers (Landa et al., 2006; Thomas et al., 2008; Bostock et al., 2014). The effects of cold in pre-disposing crops to pathogen infections are reported in rice to blast (Koga et al., 2004) and cotton to Alternaria blight (Zhao et al., 2012). Our findings showed that chilling temperature can pre-dispose chickpea genotypes to *D. rabiei* infection and that could lead to blight epidemics in winter-sown crops in WANA.

The negative high and significant correlation between LP and disease severity in both experiments is an indication that isolates that produce pycnidia within a short period can cause many disease cycles compared with isolates with a long pycnidia formation period. Moreover, LP could be an important fitness component in dominating the population of *D. rabiei* during epidemics. This study showed no significant tradeoff between IP, LP and disease severity under controlled conditions. Incubation period was found to differentiate field pea genotypes to *Mycosphaerella pinodes* and correlated with final disease severity, area under the disease progress curve and rate of disease development under controlled condition (Prioul et al., 2003).

The chilling duration did not show a clear trend on the aggressiveness of isolates inoculated on chickpea genotypes. Some isolates showed increased aggressiveness over others and this could lead different isolates to dominate the pathogen population during the epidemic period. In wheat- *Zymoseptoria tritici* pathosystem, variations in aggressiveness were observed between initial pathogen isolates in winter and final population in spring (Suffert et al., 2015).

The expansion of winter-sown chickpea in WANA requires germplasm that tolerates freezing and chilling and has high levels of Ascochyta blight resistance. At ICARDA, separate

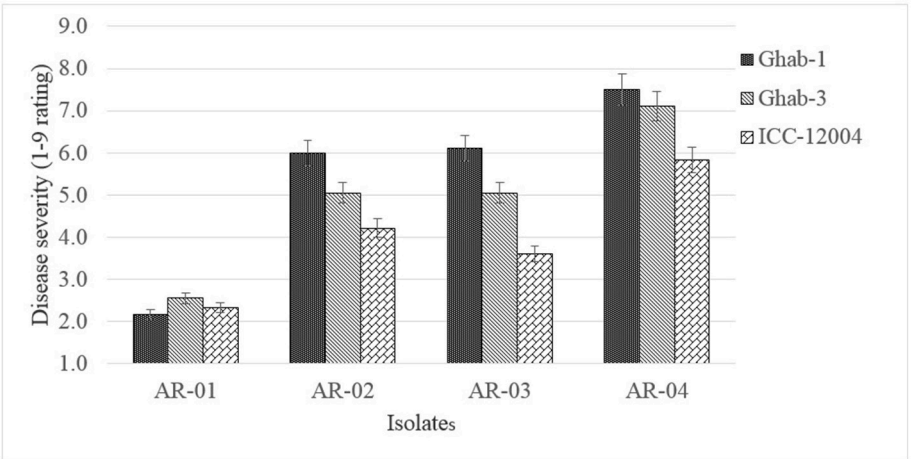


**FIGURE 5 |** Mean disease severity in chickpea genotypes inoculated with four isolates of *Didymella rabiei*. Vertical bars are standard errors of the means.

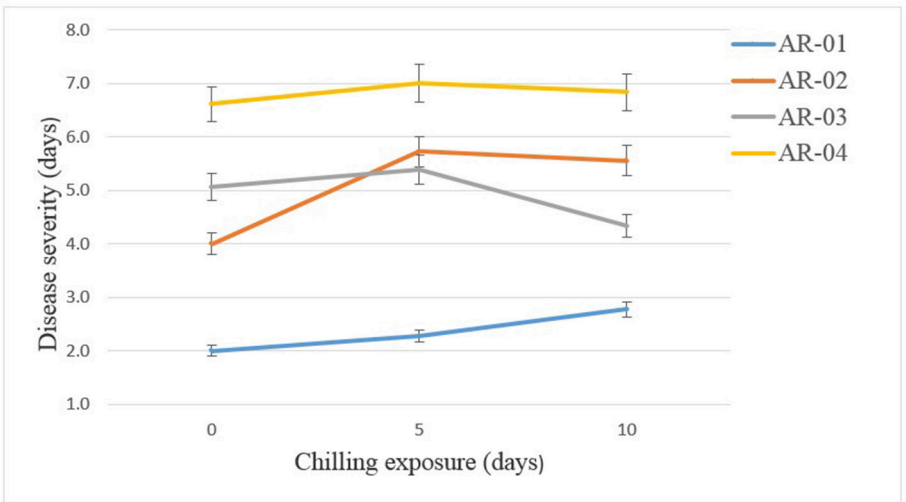


**FIGURE 6 |** Effects of chilling exposure on the aggressiveness of *Didymella rabiei* isolates inoculated on chickpea genotypes. Vertical bars are standard errors of the means.

screening germplasm and breeding lines are developed for freezing tolerance and Ascochyta blight resistance. However, Ascochyta blight screening is done by planting chickpea in December, which exposes the crop to chilling temperatures that could predispose the crop to *D. rabiei* infections when temperature increases in February-April at Tel Hadya and Terbol Research Stations. During some growing seasons, chickpea breeding lines planted for Ascochyta blight screening are hit by freezing temperature; some genotypes regenerate from cold damage but show high susceptibility to Ascochyta blight (Ahmed, personal observation). Regeneration ability could be a trait of interest for cold tolerance; thus there is a need to look for genotypes that have the ability to regenerate as well as good levels of Ascochyta blight resistance.

Pre-disposing effects of chilling temperature on partially resistant winter-sown chickpea cultivars could lead to severe Ascochyta blight epidemics that may require more fungicide spraying to manage the disease. Repeated fungicide applications will increase farmers' production costs and could create *D. rabiei* populations that are insensitive to the available fungicides. In addition to insensitivity to the fungicides most widely used in WANA, there are reports of fungicide tolerance in *D. rabiei* populations from chickpea fields in USA and Canada (Chang et al., 2007b; Wise et al., 2008). In order to mitigate climate change and variability, conservation cropping is being adopted in WANA where chickpea is a key rotation crop in cereal cropping system where infected chickpea straws are left in the farm. Hence, chilling temperature favors sexual reproduction (Navas-Corte's et al., 1998) where the two mating types of *D. rabiei* are existing.

The discharge of ascospores usually coincides with the onset of the vegetative growth of the chickpea crop and infection and subsequent disease epidemics will be high on chickpea crops pre-disposed by chilling temperature during this crop stage of the crop. To improve selection for cold tolerance combined with Ascochyta blight resistance, conventional screening methods should be supported by marker-assisted selection.

This study was limited to a few temperature regimes and pathogen isolates to study the impact of temperature on host plant resistance and pathogen aggressiveness. Moreover, chilling temperature, duration of chilling exposure and crop growth stages were very limited due to shortage of space. Changing the physiological and other tolerance mechanisms to chilling temperature of the studied chickpea genotypes was not attempted. Studies showed that the natural plant growth regulators Abscisic acid (ABA) plays a role in cold tolerance in chickpea (Bakht et al., 2006; Kumar et al., 2008). However, ABA produced during chilling temperature is reported to expose crops to pathogen infections (Mauch-Mani and Mauch, 2005) but the role of ABA in pre-disposing chickpea to *D. rabiei* infection in winter planted chickpea is not known and future investigation is required.

In conclusion, our study showed that Ascochyta blight resistance in the studied chickpea genotypes and the aggressiveness of pathogen isolates are not temperature dependent. Hence, resistance screening and genetic studies can be done in temperature ranging from 15 to 20°C since resistance and aggressiveness are temperature independent. Chilling temperature pre-disposes chickpea genotypes to *D. rabiei* infections and increased disease severity. As a result, emphasis should be given to developing germplasm with high levels of cold tolerance and Ascochyta blight resistance. Our study clearly showed the existence of D. rabiei isolates that can cause high disease severity under wide ranges of temperature.

## AUTHOR CONTRIBUTIONS

SAK, SK and MI planned the idea and did preliminary controlled experiments in 2011 at ICARDA Tel Hadya Research Station, Syria and later SAK modified the original experiments and implemented 2011 and 2012 at the same place. Data analyses and first draft and revisions of the manuscript were done by SAK, AH, MI, and SK and agreed before submissions.

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frontiers in Plant Science

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# The Detection and Characterization of QoI-Resistant *Didymella rabiei* Causing Ascochyta Blight of Chickpea in Montana

*Ayodeji S. Owati[1], Bright Agindotan[1], Julie S. Pasche[2] and Mary Burrows[1]**

*[1] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT, United States, [2] Department of Plant Pathology, North Dakota State University, Fargo, ND, United States*

Ascochyta blight (AB) of pulse crops (chickpea, field pea, and lentils) causes yield loss in Montana, where 1.2 million acres was planted to pulses in 2016. Pyraclostrobin and azoxystrobin, quinone outside inhibitor (QoI) fungicides, have been the choice of farmers for the management of AB in pulses. However, a G143A mutation in the *cytochrome b* gene has been reported to confer resistance to QoI fungicides. A total of 990 isolates of AB-causing fungi were isolated and screened for QoI resistance. Out of these, 10% were isolated from chickpea, 81% were isolated from field peas, and 9% isolated from lentil. These were from a survey of grower's fields and seed lots (chickpea = 17, field pea = 131, and lentil = 21) from 23 counties in Montana sent to the Regional Pulse Crop Diagnostic Laboratory, Bozeman, MT, United States for testing. Fungicide-resistant *Didymella rabiei* isolates were found in one chickpea seed lot each sent from Daniels, McCone and Valley Counties, MT, from seed produced in 2015 and 2016. Multiple alignment analysis of amino acid sequences showed a missense mutation that replaced the codon for amino acid 143 from GGT to GCT, introducing an amino acid change from glycine to alanine (G143A), which is reported to be associated with QoI resistance. Under greenhouse conditions, disease severity was significantly higher on pyraclostrobin-treated chickpea plants inoculated with QoI-resistant isolates of *D. rabiei* than sensitive isolates ($p$-value = 0.001). This indicates that where resistant isolates are located, fungicide failures may be observed in the field. *D. rabiei*-specific polymerase chain reaction primer sets and hydrolysis probes were developed to efficiently discriminate QoI- sensitive and - resistant isolates.

**Keywords: Ascochyta blight, pyraclostrobin, QoI-fungicide resistance, G143A mutation, hydrolysis probe assay**

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****Correspondence:***
*Mary Burrows*
*mburrows@montana.edu*

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## INTRODUCTION

The production of cool season pulse crops including chickpea (*Cicer arietinum* L.), field pea (*Pisium sativum* L.), and lentil (*Lens culinaris* Medik) in the Northern Great Plains of the United States is rapidly increasing. Montana is the leading producer of field peas and lentil in the United States, where 1.2 million acres were planted to pulses in 2016 (United States Department of Agriculture and National Agriculture Statistics Service, 2016). However, an increase in pulse production is accompanied by potentially yield-limiting diseases. Chief among these diseases is Ascochyta

blight (AB). This is a host-specific disease caused by fungal species including *Didymella rabiei* (Kovachevski) v. Arx (anamorph *Ascochyta rabiei* (Pass) Labr) on chickpea, a species complex consisting of *Didymella pisi* (Barilli et al., 2016), *Peyronellaea pinodes*, and *Peyronellaea pinodella* on field pea (Aveskam et al., 2010), and *Didymella lentis* Kaiser, Wang and Rogers (anamorph *A. lentis* Vassiljevsky) on lentil (Barilli et al., 2016). AB can infect crops at all developmental stages and cause over 40–50% yield reduction under conditions suitable for disease development (Mondal et al., 2005; Wise et al., 2011). In faba bean, 90% losses have been reported (Pande et al., 2005; Barilli et al., 2016). Symptoms of AB can develop on foliar and stem parts of the plant and also cause seed rot. AB is seed- and residue-borne. In the field, disease onset is normally post-flowering (growth stage R1) through plant maturity (growth stage R8). "Infected seeds from diseased pods may be small, shrunken or discolored" (Ye et al., 2000; Gossen et al., 2011). In addition to seed as a source of inoculum, *D. rabiei*, *D. pisi*, and P. *pinodes* also can subsist in the sexual and/or asexual forms (pseudothecia, pycnidia, and perithecia, respectively), producing ascospores and conidia that can provide a source of inoculum for disease epidemics (Tivoli and Banniza, 2007; Chilvers et al., 2009; Wise et al., 2011).

Management of AB requires an integrated approach including the use of certified disease-free seeds, deep seeding depth, crop rotations of at least 3 years, tillage to bury plant debris, fungicide seed treatment to reduce seed transmission, the use of resistant cultivars and foliar fungicides for prevention or treatment of disease symptoms (Gossen and Derksen, 2003; Wise et al., 2011). The use of resistant varieties and cultural practices can reduce AB, however, resistant varieties are not widely available in the Northern Great Plains. Breeding for resistance to AB is challenging in chickpea. This is because this trait is reported to be rare in the genetic resources available for chickpea (Sharma and Ghosh, 2016). In addition, negative genetic correlation has been reported between resistance to AB and other desirable traits. This was illustrated by Lichtenzveig et al. (2002), who pointed out the negative genetic correlation that existed when combining good resistance to AB and early flowering in chickpea. Current studies in the Middle East, North America and Australia are targeted at developing AB-resistant chickpea genotypes, especially resistance to pathotype IV *D. rabiei,* which is considered highly virulent (Bayaa et al., 2004; Imtiaz et al., 2011; Sharma and Ghosh, 2016). Integration of molecular tools and conventional breeding approaches are being used to accelerate introgression of AB-resistance genes in chickpea genotypes (Sharma and Ghosh, 2016). Fungicides are still widely used to achieve an acceptable level of disease control (Davidson and Kimber, 2007; Lonergan et al., 2015). Broad spectrum protectant fungicides (chlorothalonil) are typically applied pre-flowering and can delay the onset of AB; however, once symptoms appear it is imperative for the grower to apply fungicides that provide a high level of control and move beyond the site of application in plant tissues due to canopy closure (Gan et al., 2006; Davidson and Kimber, 2007; Wise et al., 2009; Lonergan et al., 2015). This concern is heightened in chickpea which is more susceptible to AB when compared with field peas and lentils. Thus, fungicides are frequently applied to chickpea fields and sparingly in field peas and lentils. Three registered fungicide classes that provide a premium level of control for the management of AB include succinate dehydrogenase inhibitors (SDHI; FRAC code 7), demethylation inhibitors (DMI; FRAC code 3), and quinone outside inhibitors (QoI; Fungicide Resistance Action Committee [FRAC] code 11) (Burrows, 2013; Lonergan et al., 2015). These classes of fungicides are considered to have high to medium risk of resistance development (Fungicide Resistance Action Committee, 2015, 2016). This concern is elevated by the site-specific mode of action (MOA) of the fungicides, the polycyclic nature of the disease, airborne spores of the AB pathogens, and of the option of sexual reproduction for most species, allowing rapid mutation and allow inheritance by offspring. The polycyclic nature of the disease predisposes growers to repeat fungicide applications as disease severity can increase rapidly when the weather is favorable, particularly with AB of chickpeas (Banniza et al., 2011; Lonergan et al., 2015; Fungicide Resistance Action Committee, 2016).

Currently, of the three classes of fungicide, QoI fungicides are the choice of most pulse growers for pre- and post-infection management of AB in the United States and Canada (Wise et al., 2008; Delgado et al., 2013; Lonergan et al., 2015; Bowness et al., 2016). Prior to 2007, it was the only available fungicide MOA on pulse crops and resistance developed rapidly in North Dakota and Canada (Gossen and Anderson, 2004; Wise et al., 2011; Bowness et al., 2016). In 2012, SDHIs were registered for use and these have largely been released as blends with other fungicide MOAs due to the high risk of resistance development. In addition, grower's preference of QoI- fungicides for disease control in pulse fields got a boost when the US Environmental Protection Agency (EPA) approved the use of pyraclostrobin (Headline®) to benefit plant health on federally issued labels (Agweb, 2009). This plant health benefit of the QoI-fungicide was reported by Dimmock and Gooding (2002) to prolong grain filling in wheat crops. Furthermore, QoI-fungicide was reported to lower transpiration rates and also reduce the rate of senescence in wheat plants (Petit et al., 2012; Mahoney et al., 2014).

This QoI class of fungicide inhibits mitochondrial respiration in the cytochrome bc1 complex (also known as respiratory chain complex III). The cytochrome bc1 complex facilitates electron transfer from ubiquinol to cytochrome c and links this transfer to proton translocation across the bc1 complex membrane via a mechanism called the proton-motive Q cycle (Brandt and Trumpower, 1994), resulting in ATP/energy production. The fungicide binds to the center of the quinone (Qo) site of the cytochrome bc1 complex (complex III) on the positive side of the inner mitochondrial membrane. This causes depletion of adenosine triphosphate (ATP) that ultimately halts spore germination due to energy inadequacy (Grasso et al., 2006a; Wise et al., 2009; Delgado et al., 2013).

Resistance to QoI fungicides has been reported in wheat pathogens such as *Microdochium nivale Blumeria graminis* f. sp. *tritici*, *Microdochium majus, Ourcosphaerella graminicola*, (Sierotzki et al., 2000; Amand et al., 2003; Walker et al., 2009; Patel et al., 2012), and several other fungal pathogens including

*Cercospora sojinia, Colletotrichum graminicola, Alternaria alternata, Botrytis cinerea, Pyricularia grisea, Podosphaera fusca, Pythium aphanidermatum, Pyrenophora teres*, and *Pseudoperonospora cubensis* (Ishii et al., 2001; Gisi et al., 2002; Avila-Adame et al., 2003; Kim et al., 2003; Ma et al., 2003; Sierotzki et al., 2007; Banno et al., 2009; Samuel et al., 2011; Zeng et al., 2015; ). In addition, QoI resistance has been reported in *D. rabiei* in North Dakota and Canada (Gossen and Anderson, 2004; Wise et al., 2009; Delgado et al., 2013). "The mechanism of resistance of *D. rabiei* has been attributed to single amino acid replacement in the cytochrome b protein of the *cytochrome bc1* complex" (Delgado et al., 2013). Currently, three amino acid substitutions are found in the cytochrome b protein of fungal plant pathogens that confer different degrees of resistance to QoI fungicides (Grasso et al., 2006a; Delgado et al., 2013). Low levels of resistance are bestowed by a substitution from phenylalanine to leucine at position 129 (F129L) and a substitution from glycine to arginine at position 137 (G137R) while a high level of resistance is conferred by the amino acid change from glycine to alanine at position 143 (G143A). Fungicide insensitivity have been categorized to two types: quantitative and qualitative. With quantitative insensitivity, the pathogen becomes less sensitive to the fungicide, although higher rates of the fungicide are still effective. Qualitative insensitivity predisposes the pathogen to become completely insensitive to the active ingredient and disease control is no longer achieved at recommended field application rates. Insensitivity to the QoI-fungicides has previously been reported to be qualitative (Delgado et al., 2013; Bowness et al., 2016). QoI-resistant *D. rabiei* isolates have been identified in North Dakota and Montana (Wise et al., 2008, 2009), where the mechanism of resistance was identified as the G143A mutation in the former (Delgado et al., 2013). However, in Montana, there has not been a statewide survey to monitor for resistance to QoI in pulse crops. There is an urgent need to develop a robust screening and monitoring strategy for QoI resistance to help prevent the spread of QoI-resistant AB pathogens in the rapidly increasing pulse acreage in Montana. Thus, the objectives of this study were to (1) determine the presence of resistance to QoI fungicides in AB pathogens from chickpea, field pea, and lentil in Montana; (2) determine the mechanism of resistance associated with QoI-resistant isolates; and (3) develop a robust multiplex real-time PCR diagnostic tool for screening and monitoring of QoI resistance.

## MATERIALS AND METHODS

### A Collection of *D. rabiei, D. pisi,* and *D. lentis* Isolates

Isolates of *D. rabiei, D. pisi*, and *D. lentis* were obtained from four general sources. Most isolates were obtained from chickpea, field pea and lentil seed lots submitted by growers in 23 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT for testing during the 2014, 2015, and 2016 growing seasons (**Table 1**). A second set of isolates were obtained from chickpea and field pea production fields in Montana where QoI fungicides had been applied. Fields containing chickpea and field pea plants with AB symptoms were sampled on a "W" pattern, with samples taken at a set of intervals of approximately 15 m. The third set of isolates were collected from chickpea and field pea plants with AB symptoms sampled by growers and submitted to the Schutter Diagnostic Laboratory, Bozeman, MT. Finally, some isolates were also obtained courtesy of Julie Pasche at North Dakota State University, Fargo, ND, United States.

**TABLE 1 |** Isolates of *Didymella rabiei, D. pisi,* and *D. lentis* were obtained from chickpea, field pea, and lentil seed lots sent by growers to the Regional Pulse Crop Diagnostics Laboratory (RPCDL) in Bozeman, MT, United States for planting during 2014, 2015, and 2016 growing season.

| Collection location by county | Number of seed lots sampled | Total number of isolates | Isolates with quinone outside inhibitor (QoI) resistance[a] |
|---|---|---|---|
| Cascade | 6 | 39 | 0 |
| Chouteau | 2 | 3 | 0 |
| Daniels | 28 | 235 | 5 |
| Dawson | 7 | 38 | 0 |
| Gallatin | 5 | 16 | 0 |
| Glacier | 2 | 5 | 0 |
| Hill | 7 | 45 | 0 |
| Liberty | 2 | 6 | 0 |
| McCone | 24 | 92 | 4 |
| Musselshell | 1 | 1 | 0 |
| Philips | 4 | 9 | 0 |
| Pondera | 5 | 6 | 0 |
| Richland | 3 | 16 | 0 |
| Roosevelt | 23 | 133 | 0 |
| Sheridan | 15 | 81 | 0 |
| Teton | 2 | 4 | 0 |
| Valley | 24 | 213 | 2 |
| Yellowstone | 2 | 9 | 0 |
| Toole | 1 | 2 | 0 |
| Broadwater | 1 | 2 | 0 |
| Flathead | 1 | 5 | 0 |
| Blaine | 2 | 15 | 0 |
| Garfield | 2 | 15 | 0 |
| Total | 169 | 990 | 11 |

[a]*An isolate was considered resistant if it has the G143A mutation in its cytochrome b gene.*

For a standard AB seed test, seed (chickpea $n = 600$, field pea $= 400$, and lentil $= 400$) were sterilized in a 1% free chlorine solution for 10 min (International Rules for Seed Testing [IRST], 2017). The solution was drained and seeds were air-dried in the biological cabinet for 30 minutes. Dried seeds ($n = 10$ per plate) were plated on potato dextrose agar (PDA) (Alpha Biosciences Inc., Baltimore, MD, United States). Mycelial growth was noticed from the plated seeds after 11 to 14 days incubation at 20°C ± 1°C in the presence of a routine cycle of cool white fluorescent light (12 h light followed by 12 h dark). The presence of AB pathogens was confirmed by viewing the conidia at 40× magnification.

From plants, isolates were also obtained from a single lesion on symptomatic leaves and stems by cutting the tissue into 3- to 4-cm sections. Stem or leaf sections were immersed in 1% NaOCl for 30 s and rinsed for 30 s in sterile distilled water. Sterilized

stem or leaf sections were air-dried in a biological cabinet, placed on PDA and incubated under the conditions described above. Confirmation of the pathogen was conducted as previously described. Conidium of individual isolates from infected seed lots ($n = 5$ to 10) and from symptomatic leaves or stems were incubated on PDA under the conditions previously described. To isolate single spores, three pycnidia from a 10 day old culture were dropped into 2 ml screw cap tube (MP Biomedicals) containing five ceramic beads (MP Biomedicals), 300 μl of sterile water and 0.05% (v/v) tween-20. The mixture was homogenized using a Beadbug homogenizer (Benchmark BeadBug Homogenizer, Benchmark Scientific, NJ, United States) for 60 s at 4000 rpm. The supernatant was removed into a clean 1.5 ml eppendorf tube and diluted 100-fold in sterile water. From the diluted suspension, 100 μl was inoculated on fresh PDA plates and incubated at 20°C±1°C under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark). Single spores germinated after 3–5 days. Isolates were stored long-term as conidia on sterile filter paper and as mycelia in 15% sterilized glycerol at −80°C (Skoglund et al., 2011).

## Screening of *Didymella* spp. Isolates for QoI Fungicide Resistance Using a Discriminatory Dose

A total of 990 AB causing isolates were screened for QoI resistance. Of these, 10% were from chickpea, 81% from field peas, and 9% from lentil from seed lots (chickpea = 17, field pea = 131, and lentil = 21) submitted to the RPCDL from 23 counties in Montana. Screening of the isolates was conducted using an *in vitro* agar plate assay according to published methods (Wise et al., 2008) with some modifications. Stock solutions of technical grade formulations of pyraclostrobin (99% active; BASF Corporation, Research Triangle Park, NC, United States) were prepared at a concentration of 5 μg/ml and diluted in acetone. Salicylhydroxamic acid (SHAM; Sigma–Aldrich) was dissolved in methanol and added to all fungicide-amended media at a concentration 100 μg/ml. SHAM minimizes the effect of the alternative oxidative pathway that some fungi use to evade QoI fungicide toxicity in *in vitro* fungicide sensitivity assays (Olaya and Köller, 1999; Bartlett et al., 2002; Wise et al., 2008, 2009; Lonergan et al., 2015). *D. rabiei* and other AB pathogens can utilize this alternative pathway in the presence of QoI fungicides. SHAM has been reported to have no side effects on conidial germination (Wise et al., 2008). The 0 μg/ml treatment served as a control and was amended with 100 μg/ml SHAM, 1 ml of acetone, and 1 ml of methanol per liter.

In addition to agar plate assay, isolates from all the three hosts were screened using a mismatch amplification mutation assay PCR (MAMA-PCR) (Delgado et al., 2013). This PCR-based assay was used to detect mutant isolates of *D. rabiei* bearing the A143 allele of the *cytochrome b* gene. Isolates that had a mycelial growth on fungicide amended media that was 70% of the control plate (without pyraclostrobin fungicide) and that also amplified with the MAMA-PCR were selected for total RNA extraction. Only 11 isolates of *Didymella rabiei* met the two criteria. No isolates of *D. pisi* or *D. lentis* met either of the criteria.

## Total RNA Extraction

Selected isolates were cultured on PDA at 22°C for 7 days at 12 h light. Total RNA was isolated from the fungal isolates using the RNeasy Plant Mini kit (QIAGEN) with alterations in the starting process. Fresh fungal mycelium of each isolate (100 mg) from a 7-day old culture was scraped into a 2 mL screw cap tube (MP Biomedicals) containing 450 μL RLC buffer. The mycelium was disrupted using the BeadBug Benchtop homogenizer (Benchmark Scientific, NJ, United States) set at 3500 rpm for 60 s, and centrifuged at 13, 000 *g* for 1 min. About 400 μL lysate was then transferred to a QIAshredder spin column placed in a 1.5 mL collection tube. From this stage onward, the protocol followed the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000c at 260 nm (Thermo Scientific, United States) and adjusted to a final concentration of 100 ng/μL.

## Synthesis of Complementary DNA, RT-PCR, and Sequencing

The first-strand complementary DNA (cDNA) was synthesized using a RevertAid-Reverse Transcriptase kit (Thermo scientific). The cDNA was used in a PCR assay to amplify the coding sequence for amino acid codons 127–276 of the *cytb* gene from *D. rabiei*. This region has been reported to have the G143A mutation and other mutations that confer resistance to QoI fungicides (Fraaije et al., 2002; Delgado et al., 2013).

Standard PCR was conducted in a T100 Biorad thermocycler (Bio-Rad Inc.) with Phusion High-Fidelity PCR master mix, 10 *p*mol each of primer (Delgado et al., 2013) and 50 ng of cDNA template in a final volume of 50 μL. The reaction conditions were: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min. PCR was terminated with an extension at 72°C for 5 min. PCR products were analyzed on ethidium bromide-stained 1.5% (w/v) agarose gels run in the 1x tris-acetate-EDTA buffer and exposed to UV light to visualize DNA fragments. Isolates with an expected product of 675 bp were purified directly from PCR products using alcohol precipitation. The purified PCR products were sequenced with primer pair used for the amplification (**Table 2**), in both directions (MCLAB DNA sequencing services).

## Effect of G143A Mutation on *D. rabiei* Fungicide Sensitivity on Disease Control

Greenhouse trials were conducted to determine the level of *in vivo* disease control attainable with QoI fungicides against isolates classified as susceptible or resistant to QoI fungicides based on sequencing results. Five QoI-sensitive *D. rabiei* isolates (AR-405, AR-407, AR-419, AR-439, and AR-430) (Wise et al., 2008) and five QoI-resistant isolates (AR-R001 to AR-R005) were included in the trial (**Table 3**). The five QoI-resistant isolates were isolated from a chickpea seed lot submitted to the RPCDL for seed testing, and five QoI-sensitive isolates were from a baseline population (Lonergan et al., 2015). The QoI sensitivity of these

**TABLE 2 |** Primers pairs used for amplification of the *cytochrome b* gene fragment of *Didymella rabiei* and for detecting the G143A mutation.

| Primers | Primer sequence (5′–3′) | Annealing temperature | Reference | Primer pair purpose |
|---|---|---|---|---|
| A99 | TATTATGAGAGATGTAAATAATGG | 46°C | Delgado et al., 2013 | Sequencing of *cytochrome b* gene |
| A100 | CCTAATAATTTATTAGGTATAGATCTTA | 46°C | Delgado et al., 2013 | Sequencing of *cytochrome b* gene |
| A243 | GCTTTCCTGGGTTACGTTCT | 64°C | This study | Multiplex TaqMan PCR |
| A244 | CCAACTCATGGTATAGCACTCAT | 64°C | This study | Multiplex TaqMan PCR |
| A245res | FAM-TGGGCAAATGTCACTATGAGCTGCTACAG-BHQ1 | 64°C | This study | QoI-resistant probe (A143 allele) |
| A246sens | Cy5-TGGGCAAATGTCACTATGAGGTGCTACAG-BBQ | 64°C | This study | QoI-sensitive probe (G143 allele) |

**TABLE 3 |** List of *D. rabiei* isolates used for the *in vivo* assay.

| Isolate ID | QoI status | Location by state |
|---|---|---|
| AR-R001 | Resistant | Montana |
| AR-R002 | Resistant | Montana |
| AR-R003 | Resistant | Montana |
| AR-R004 | Resistant | North Dakota |
| AR-R005 | Resistant | North Dakota |
| AR 405 | Sensitive | Idaho |
| AR 407 | Sensitive | Idaho |
| AR 439 | Sensitive | Washington State |
| AR 411 | Sensitive | Idaho |
| AR 430 | Sensitive | Idaho |

five isolates was determined using pyraclostrobin amended PDA, MAMA-PCR, and mutation analysis of their *cytb* gene.

The greenhouse experiments were performed following Pasche et al. (2004, 2005) and Wise et al. (2009). Briefly, chickpea seeds (cv. Troy) were obtained from Washington State Crop Improvement Association (WSCIA). Troy is a moderately resistant chickpea cultivar. The seeds were tested free of seed borne AB and were sown at one plant per pot in 80 ml plastic cones filled with a mixture of peat and Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA, United States) at ratio 1:1, and grown at 22 ± 2°C. Fourteen days after planting, chickpea plants were treated with commercial formulations of pyraclostrobin (Headline, 2.09 EC; BASF Corporation) at concentrations of 0, 0.1, 1.0, 10, and 100 μg a.i./ml of water. Fungicides were applied to runoff using a $CO_2$-powered Generation III Research Track sprayer (DeVries Manufacturing, United States). About 24 h after fungicide application, chickpea plants were inoculated with a conidial suspension obtained from 14 old culture of QoI-resistant and sensitive *D. rabiei* isolates. Within an hour made conidial suspensions were adjusted to a concentration of $3 \times 10^5$ conidia/ml and applied to chickpea plants. Inoculum from each isolate was applied to plants using hand-held spray bottles. Chickpea plants were placed in a mist chamber and held at >90% relative humidity for 36 h at a 14 h photoperiod under artificial lighting. After 11 days, disease severity was assessed visually based on the percent leaf area infected of the whole plant (Reddy and Singh, 1984). The experiment was laid out as a randomized completely block design (RCB). Nine replicates (one plant per replicate) were included in each experiment, and the disease severity was calculated for each observational unit. Percent disease control was calculated by: "[1 – (% diseased tissue/% disease on 0 μg/ml control)] × 100" (Wise et al., 2009). Homogeneity of the variances from the two greenhouse experiments was determined by the Levene's test (Levene, 1960). Data were converted to percent disease control to enhance direct comparisons between QoI-sensitive and resistant isolates at each fungicide concentration and analyzed using the generalized linear mixed-effects model in lm4/ nlme statistical package (R Core Team, 2013).

## Development of a Multiplex Hydrolysis Probe Assay for the Detection of QoI-Resistant (G143A) and QoI-Sensitive *D. rabiei* Isolates

For simultaneous detection and differentiation of the *D. rabiei* G143A mutants from the sensitive isolates, a primer pair (A243 and A244) and two hydrolysis probes (A245res and A246ses) were designed for a multiplex real-time PCR assay to amplify a 92 bp fragment of the *cytb* gene. The 5′ ends of the probes A245res and A246ses were labeled with 6-carboxy fluorescein (FAM) and cyanine 5 (Cy5), while their 3′ ends were labeled with Iowa black-FQ and Iowa black-RQ quencher, respectively. The primers were designed to flank the region of the G143A mutation, while the two fluorogenic dyes enable the multiplex differentiation between resistant and sensitive alleles. To enhance the efficiency of the probes both probes were designed to have $T_m$ values at least 7°C higher (69°C) than that of the primers (62°C), and their GC content was higher than 45%. The multiplex TaqMan assay was optimized in a final volume of 20 μL containing 10 μL of EconoTaq Plus 2x master mix according to manufacturer's recommendations (Lucigen Corporation, Middleton, WI, United States), 25 pM of each primer (A-243, A-244), 10 pM of each probe (A-245res, A-246sens) and 3 μL of DNA extract. Cycling parameters were 4 min at 94°C, followed by 35 cycles of 15 s at 94°C and 30 s at 64°C and a final extension at 72°C for 5 min completed the PCR. Primers and hydrolysis probes were synthesized by Integrated DNA Technology (IA, United States). The assay was


**FIGURE 1 |** The partial protein sequence of the *cytochrome b* gene of five isolates of *Didymella rabiei* with different QoI sensitivities. Star indicates the G143A amino acid substitution responsible for decreased sensitivity to QoI fungicides. Dark gray highlighted areas represent 100% identities.

developed, evaluated and analyzed on the Biorad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

In addition, evaluation of the assay efficiency was determined by plotting cycle thresholds for a six times tenfold dilution starting with 1000 ng of DNA obtained from resistant and sensitive isolates against DNA concentration to yield the standard curves. The result from experiments in the multiplex assay was compared to the uniplex assay using tenfold dilutions from 1000 ng to 1 pg DNA extracts from only isolates that contained the G143A mutation.

## RESULTS

From the screening, only 11 isolates of *D. rabiei* amplified with the MAMA-PCR and also had a mycelial growth on fungicide amended media that was 70% of the control plate (without pyraclostrobin fungicide). Multiple alignment analysis of amino acid sequences of the *cytb* gene of the detected QoI-resistant *D. rabiei* isolates showed a mutation that replaced the codon for amino acid 143 from GGT to GCT, resulting in an amino acid change from glycine to alanine (G143A) (**Figure 1**). Other known mutations such as (F129L) and (G137R) were not found in the protein sequences of our QoI-resistant isolates. However, none of the isolates of *D. pisi* and *D. lentis* amplified using MAMA-PCR.

### Effect of the G143A Mutation on *D. rabiei* Fungicide Sensitivity

Independent analysis of greenhouse disease control experiments showed that variances were homogeneous and the two experiments were combined for further analysis ($p$-value = 0.05). Disease severity was significantly higher on chickpea plants inoculated with G143A mutant isolates at all concentrations of pyraclostrobin. Percent disease control from the non-treated was calculated to directly compare the two isolate groups. Disease control of G143A mutant isolates was significantly reduced in the pyraclostrobin treatments when compared to wild type isolates at all fungicide concentrations ($p$-value <0.001) (**Figure 2**). About 75% disease control was observed at 10 and 100 μg/ml in wild-type isolates while <25% disease control was observed in G143A mutant isolates.

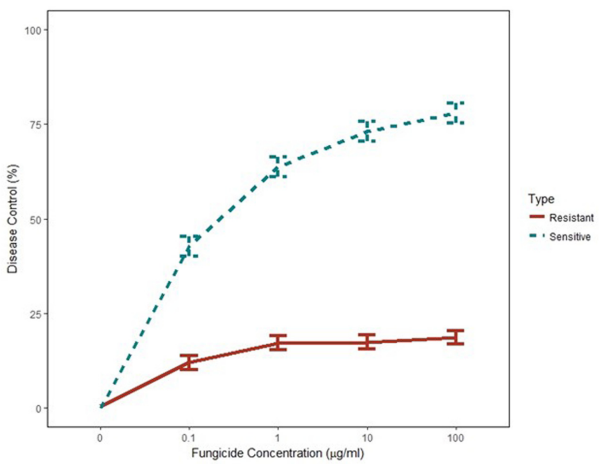


**FIGURE 2 |** Mean *in vivo* percent disease control for five QoI-sensitive and five QoI-resistant *Didymella rabiei* isolates to pyraclostrobin fungicide concentration (μg/ml). Values include standard errors of disease control measurements obtained from one plant across nine replications in two experiments.

### Detection of QoI-Resistant (G143A) and QoI-Sensitive *D. rabiei* Isolates Using a Multiplex Hydrolysis Probe Assay

By deploying the single nucleotide polymorphism (SNP) hydrolysis probe assay, it was possible to detect the G143A

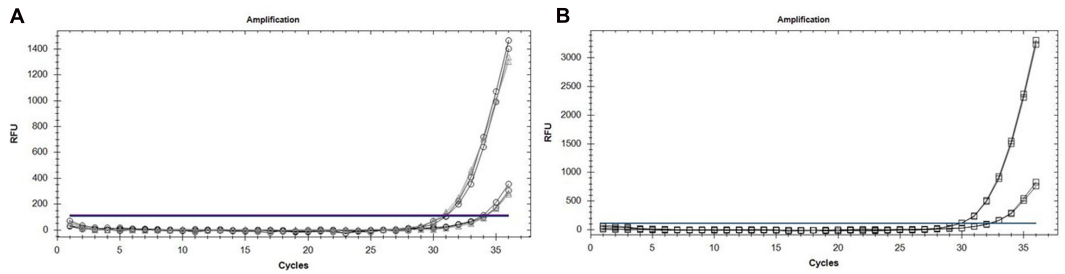


**FIGURE 3 | (A)** Amplification curves showing the detection efficiency of sensitive (○) and resistant (△) alleles using the mixtures of DNA from QoI-resistant and QoI-sensitive isolates as a template. **(B)** Amplification curves showing the detection efficiency of resistant (□) alleles in uniplex TaqMan real-time PCR using DNA from QoI-resistant isolates as a template.

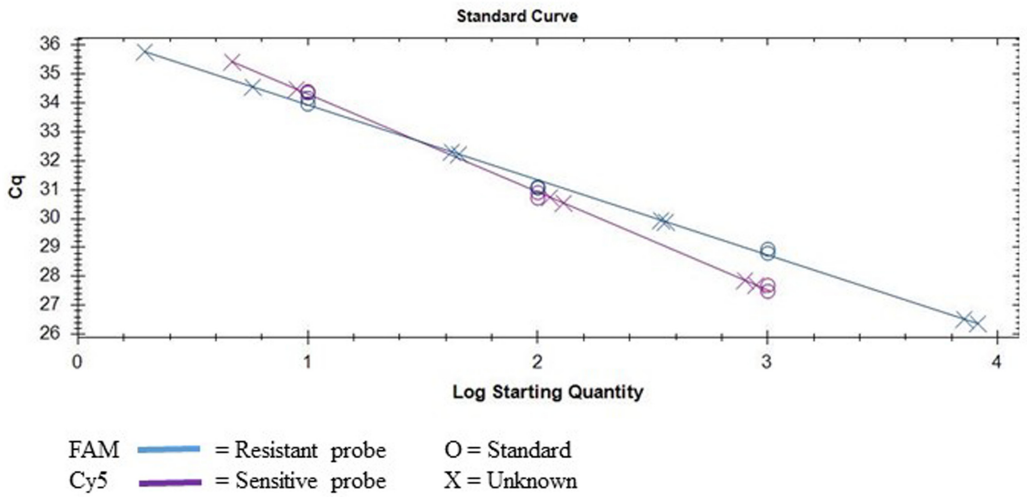


**FIGURE 4 |** Standard curve obtained by using the multiplex SNP TaqMan assay to detect the G143A mutation in *D. rabiei* isolates collected from Montana.

mutation and discriminate between QoI- resistant and QoI-sensitive isolates. The SNP could be distinguished using specific probes in which a nucleotide proximal to the 3′ is complementary to one allele but forms as a mismatch with the second allele. The annealing temperature was validated in a temperature gradient assay, the optimum annealing temperature was 64°C for both multiplex and uniplex (data not shown). The optimum concentration of primers and probes that gave the highest reporter fluorescence and the lowest threshold cycle was 20 and 10 μM, respectively, in both tests. To confirm the assay can simultaneously detect the two alleles, DNA from wild-type (QoI-sensitive) and G143A mutant (QoI-resistant) isolates were mixed in the same proportion. Satisfactory discrimination was achieved between the two alleles (**Figure 3**). This emphasized the accuracy of this assay, by its capacity to detect either of the alleles both in a multiplex or uniplex assays, respectively. Standard curves were constructed based on the tenfold dilution series of the wild-type and mutant isolates (**Figure 4**). There was linearity in the amplification across the DNA dilutions and correlation coefficients for the standard curve of the DNAs from wild types and G143A mutant isolates were 0.998 and 0.991, respectively (**Table 4**). The y-axis on the amplification plot measures the relative fluorescence units (RFU), a measure of the amplified DNA, while cycling threshold value (cq) on the x-axis, is inversely proportional to the initial concentration of nucleic acid template in each sample, which correlates to the number of copies in each sample.

## DISCUSSION

According to our findings, the G143A mutation is responsible for QoI fungicide resistance in *D. rabiei* isolates from Montana chickpea fields. The gene structure of the *cytochrome b* gene of *D. rabiei* appears to be favorable for the development of a SNP associated with QoI resistance at codon 143 (Delgado et al., 2013). In contrast, the G143A mutation has not been reported in fungal species that have an intron downstream of codon 143 (Grasso et al., 2006b; Sierotzki et al., 2007; Banno et al., 2009; Samuel et al., 2011; Delgado et al., 2013; Zeng et al., 2015). Thus, there is no reported case of QoI resistance in this type of *cytochrome b* gene structure. For example, fungal species such as

**TABLE 4 |** Slope, efficiencies, correlation coefficients ($R^2$), and *y*-intercepts from amplification of serial dilutions of DNA from *Didymella rabiei* isolates sensitive and resistant to quinone outside inhibitor fungicides using TaqMan single-nucleotide polymorphism assay to detect G143A mutation.

| Allele | Slope | Efficiency (%) | $R^2$ | *y*-intercepts |
|---|---|---|---|---|
| Wild-type-sensitive | 3.39 | 97.2 | 0.998 | 37.705 |
| Mutant-resistant | 2.59 | 142.7 | 0.991 | 36.531 |

**TABLE 5 |** Distribution of Ascochyta isolates collected per crop in 2014–2016 from Montana.

| Crop | Number of Counties sampled | Total Number of seed lots | Total number of isolates | Isolates with QoI resistance |
|---|---|---|---|---|
| Chickpea | 9 | 17 | 88 | 11 |
| Field pea | 17 | 131 | 810 | 0 |
| Lentil | 7 | 21 | 92 | 0 |
| Total | | 169 | 990 | 11 |

*A. solani* do not show a G143A mutation due to the lethal effect of proximal exonic flanking sequences on the 5′-splice (Lambowitz and Belfort, 1993; Pasche et al., 2005; Grasso et al., 2006b; Sierotzki et al., 2007; Banno et al., 2009; Delgado et al., 2013). However, G143A mutation has been reported to be responsible for QoI fungicide resistance in *C. sojina*, the causal organism of frog eye leaf spot of soybean (Zeng et al., 2015) and in *B. cinerea,* the causal organism of gray mold (Samuel et al., 2011). In these two organisms, the mutation does not occur in the 5′-splicing site. QoI fungicides do not control fungi bearing the G143A mutation, while those containing the F129L and G137R are controlled to some degree, but at a lower level than wildtype isolates. Similar results were obtained in a study in North Dakota (Wise et al., 2009; Delgado et al., 2013), where the mechanism of resistance of QoI-resistant *D. rabiei* isolates was reported to be a G143A mutation. Noticeably, all the three counties where QoI-resistant *D. rabiei* isolates were found are near North Dakota indicating the QoI-resistant isolates detected could have either spread from North Dakota via seed or were selected for in Montana chickpea fields.

Quinone outside inhibitor fungicide resistance was observed in isolates of *D. rabiei* collected in Montana during the 2012 and 2015 growing seasons (M. Burrows, Personal communication). Prior to this, QoI-resistant isolates were reported in 2009 from chickpea fields in North Dakota (Wise et al., 2009) and have been largely maintained in the population (Delgado et al., 2013). From both seed testing results and the current in-field survey of Montana pulse fields, the frequency of resistant isolates is thus far low (B. Agindotan, personal communication, March 2016). This is different from the report of high frequency of QoI-resistant isolates in North Dakota (Wise et al., 2009; Delgado et al., 2013). This may be due to the comparatively low relative humidity and reduced disease pressure in Montana vs. North Dakota. The relative humidity level in North Dakota is 12.1% higher than Montana in chickpea growing areas (ClimaTemps, 2015). Though this study was targeted at AB of chickpea, field pea, and lentil, only 11 isolates of *D. rabiei* from three seed lots from a larger study of 88 isolates from 17 seed lots were confirmed to be resistant to pyraclostrobin. All isolates contained the G143A mutation which confers resistance to all QoI fungicides (**Table 5**). The frequency of QoI resistance might be on the rise in *D. rabiei* because, from observations during the 2016 crop year, USDA-NASS statistics and grower testimonies, there is increasing chickpea acreage and multiple applications of fungicides to ward off potential fungal attacks. Many of these applications were QoI products solely and in combination with either chlorothalonil or SDHI fungicides such as fluxapyroxad. The proportion of QoI fungicide applied solely is higher than that applied in combination with other fungicides. However, continuation of the practice of applying multiple applications of fungicides including high-risk products such as QoI and SDHI fungicides will select for resistance development. These active ingredients are available as seed treatments and foliar products. Although education is underway, fungicide decisions are often driven by the price and efficacy of the product more than the MOA.

In contrast with chickpea, field pea and lentil fields are rarely treated with fungicides. This is due to the low foliar disease occurrence in Montana to date. Since seed testing was started in Montana in 2000, the percent of seed lots with at least one seed of 500 infected by AB has increased from 0% (2000–2002) to as high as 25% through 2009. Since the 2010 crop year, the level has increased to 60%. It was 80% in 2017. To date, we have very rarely observed QoI resistance in AB pathogens recovered from a field pea seed lots and never observed QoI resistance in AB recovered from lentil seed lots. This correlates with the low frequency of fungicide application in these crops.

Differences in disease control were observed when QoI-resistant and QoI-sensitive *D. rabiei* isolates were inoculated on pyraclostrobin-treated chickpea plants. Applications of pyraclostrobin at a concentration of 100 μg/ml provided less than 25% control of disease on chickpea plants infected with QoI-resistant isolates. This amount of control is unacceptable in field production. Disease severity of AB was higher on fungicide-treated chickpea plants inoculated with QoI-resistant *D. rabiei* isolates that on plants inoculated with QoI-sensitive isolates study. Several studies have not observed a fitness cost associated with G143A substitution in *cytb* in fungal pathogens (Chin et al., 2001; Avila-Adame et al., 2003; Karaoglanidis et al., 2011; Veloukas et al., 2014). However, once established, resistance is likely to be preserved in the population due to the selective advantage if QoI fungicides continue to be applied. This lack of disease control in QoI-resistant isolates was confirmed a report from North Dakota (Wise et al., 2009). In that study, <50% disease control was achieved with the applications of 100 μg a.i/ml pyraclostrobin to chickpea plants inoculated with QoI-resistant isolates. Considering this, monitoring of QoI resistance in AB pathogens infecting pulse crops is important to prevent the establishment of resistant populations. Due to the widespread occurrence of AB pathogens in seed lots in Montana, favorable environmental conditions will likely lead to a widespread epidemic of the pathogen. Frequent fungicide applications in those circumstances will lead to the establishment of resistant isolates and then additional management strategies

will need to be used more effectively to manage fungicide resistant AB pathogens. The risk of fungicide resistance development can be lowered by limiting the number of applications of single-site fungicides with the same MOA, rotation among fungicides with different biochemical modes of action or by using blends of fungicides with different modes of action. This approach was very effective in the control of fungicide-resistant strains of sclerotinia dollar spot and pythium blight in turfgrass (Couch, 2002, 2003).

The development of QoI resistant isolates in the epicenter of pulse production in Montana could cause significant problems for the industry, which in 2016 occupied 1.2 million acres and was valued at $322 million dollars (United States Department of Agriculture and National Agriculture Statistics Service, 2016). To contain the spread of QoI- resistance, monitoring is key. PCR-based tools like the one developed in this study are needed to monitor the shift in fungicide sensitivity of field population of fungal pathogens. Furthermore, integrated pest management practices including crop rotation, tillage to bury infested residue, and host resistance (where available) can help reduce the risk of fungicide resistance (Zeng et al., 2015). There should be caution in the application of foliar fungicides for reasons other than disease control including 'plant health benefit.' Other practices to prevent fungicide resistance development include restricting the number of QoI fungicide applications to two to four per season as specified on the label, using QoIs preventatively rather than after disease has developed, not allowing sequential applications of QoI products, as specified on the label, rotating fungicide modes of action when multiple applications are necessary, using pre-mixtures or tank mixtures different modes of action, and always applying the recommended labeled rate (Kitchen et al., 2016). The minimum recommended rates of each fungicide in the tank mix should be used (Damicone and Smith, 2009). Failure to practice these guidelines will apply additional selection pressure on *D. rabiei* and other fungal pathogens which will consequentially result in fungicide resistance development.

Various polymerase chain reaction- based methods such as PCR-RFLP and CAPS methods have been developed for the detection of G143A mutation. However, limited quantitative methods such as allele-specific real-time PCR have been developed for a few pathogens (Samuel et al., 2011; Zeng et al., 2015). The real-time PCR assay enhances high throughput and accuracy during QoI-sensitivity screening of isolates when compared to the conventional *in vitro* test using fungicide amended PDA plates. This test takes about 7 to 10 days to get a result, in addition to the logistics required to screen multiple isolates. The hydrolysis probes were designed to have $T_m$ values at least 7°C higher (69°C) than that of the primers (62°C), and their GC content was higher than 45%, improving the specificity of the assay in discriminating sensitive from resistant alleles. Furthermore, modification of the primers and probes avails this assay the potential to monitor G143A mutation in *D. pisi, D. lentis*, and *A. alternata*, thus it can serve as a tool to monitor QoI resistance in other fungal pathogens and also for routine screening of fungal isolates in diagnostic laboratories. In addition, this technique is suitable for future research targeted at determining modification in the frequency of G143 and A143 alleles and also to determine the fitness of QoI-resistant and sensitive isolates of fungal pathogens.

## CONCLUSION

This study was successful in detecting the presence of QoI-resistant *D. rabiei* isolates, characterizing the mechanism of resistance and developing a diagnostic tool for QoI resistance in *D. rabiei* that will allow high throughput and accurate screening of G143A mutants. Other researchers have been successful in developing a molecular technique to detect the G143A mutation (Delgado et al., 2013). This is a qualitative assay that cannot be used to monitor the frequency of G143 and A143 alleles both in individual isolates and in mixed field populations. The assay reported in this study streamlines the detection process. This process could be used for large-scale surveys, as well as rapid identification of insensitivity to QoI fungicides. Furthermore, this technique can be used in studies to determine changes in the frequency of G143 and A143 alleles in *D. rabiei*.

## AUTHOR CONTRIBUTIONS

AO and MB designed the greenhouse experiments. AO carried out the greenhouse experiments. Isolates of *D. rabiei* were donated by JP. AO and BA designed the hydrolysis probe assay experiment. AO carried out the assay development. AO conducted out most of the analysis and wrote the manuscript. The manuscript was reviewed by MB, BA, and JP.

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# Genetic Analysis of NBS-LRR Gene Family in Chickpea and Their Expression Profiles in Response to Ascochyta Blight Infection

*Mandeep S. Sagi, Amit A. Deokar and Bunyamin Tar'an**

*Department of Plant Sciences, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK, Canada*

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***Correspondence:**
*Bunyamin Tar'an*
*bunyamin.taran@usask.ca*

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Ascochyta blight is one of the major diseases of chickpea worldwide. The genetic resistance to ascochyta blight in chickpea is complex and governed by multiple QTLs. However, the molecular mechanism of quantitative disease resistance to ascochyta blight and the genes underlying these QTLs are still unknown. Most often disease resistance is determined by resistance (R) genes. The most predominant R-genes contain nucleotide binding site and leucine rich repeat (NBS-LRR) domains. A total of 121 NBS-LRR genes were identified in the chickpea genome. Ninety-eight of these genes contained all essential conserved domains while 23 genes were truncated. The NBS-LRR genes were grouped into eight distinct classes based on their domain architecture. Phylogenetic analysis grouped these genes into two major clusters based on their structural variation, the first cluster with toll or interleukin-1 like receptor (TIR) domain and the second cluster either with or without a coiled-coil domain. The NBS-LRR genes are distributed unevenly across the eight chickpea chromosomes and nearly 50% of the genes are present in clusters. Thirty of the NBS-LRR genes were co-localized with nine of the previously reported ascochyta blight QTLs and were tested as potential candidate genes for ascochyta blight resistance. Expression pattern of these genes was studied in two resistant (CDC Corinne and CDC Luna) and one susceptible (ICCV 96029) genotypes at different time points after ascochyta blight infection using real-time quantitative PCR. Twenty-seven NBS-LRR genes showed differential expression in response to ascochyta blight infection in at least one genotype at one time point. Among these 27 genes, the majority of the NBS-LRR genes showed differential expression after inoculation in both resistant and susceptible genotypes which indicates the involvement of these genes in response to ascochyta blight infection. Five NBS-LRR genes showed genotype specific expression. Our study provides a new insight of NBS-LRR gene family in chickpea and the potential involvement of NBS-LRR genes in response to ascochyta blight infection.

**Keywords: NBS-LRR genes, expression profiling, ascochyta blight, chickpea**

## INTRODUCTION

Plant pathogens are diverse in nature which include bacteria, virus, fungi, and nematodes. Each of them deploys various approaches to draw nutrition from plants such as biotrophy, necrotrophy, or hemi-biotrophy. The necrotrophic fungi are well known to have broad host ranges and can cause severe economic losses in agriculture (Pusztahelyi et al., 2015). Plants and pathogens have co-evolved together and each has adapted different survival strategies. Plant pathogens have evolved the ability to invade plants, suppress plant defense response and colonize plant tissue for their growth and reproduction. To cope with the wide array of pathogens, plants have developed a sophisticated immune system (Hammond-Kosack and Jones, 1997; Qi and Innes, 2013). Plant immune system differs from vertebrate animals, as plants lack adaptive immune system. Alternatively, plants solely rely on its bi-layered cell-autonomous immune system to perceive and respond to the invading pathogens (Jones and Dangl, 2006). Most pathogen infections are prevented by non-host resistance conferred by the first layer of plant basal defense response which is triggered by recognition of pathogen-associated molecular patterns (PAMPs) by the plant pattern recognition receptors (PRRs) localized on the plasma membrane. Some specifically adapted pathogens can overcome the first barrier by delivering effector proteins into plant cells to suppress the host basal defense. Such host-specific pathogens are countered by the second layer of defense termed as effector-triggered immunity (ETI) mediated by the intracellular receptors encoded by the plant disease resistance gene (R-genes) that recognize the presence of pathogen effector protein and activate downstream immune responses to inhibit pathogen infection.

Several classes of R-genes have been identified and classified based on their putative protein domain organization and their localization in the plant cell (van Ooijen et al., 2007). The most abundant class of R-genes is characterized by the encoding proteins that consist of central Nucleotide Binding Site (NBS) and carboxyl/C-terminal Leucine Rich Repeat (LRR) domain, hence called NBS-LRR genes (McHale et al., 2006). Plant NBS-LRR proteins are mainly intracellular receptors that can perceive the presence of pathogen effector directly by binding to the pathogen effector proteins or indirectly by recognition of any modification in the pathogen effector target proteins in host and activate multiple defense signal transductions which often result in hypersensitive response and other biochemical changes that limit pathogen growth (Meyers et al., 2003; DeYoung and Innes, 2006). The central NBS domain (also known as NB-ARC domain) is composed of strictly ordered conserved motifs which are required for ATP and GTP binding and hydrolysis. Plant NBS domain shows structural homology between Apaf-1 and CED4 domains that are involved in animal cell apoptosis, which suggests similarity in their modes of action (Dangl and Jones, 2001; DeYoung and Innes, 2006). The NBS domain is followed by several tandem LRRs which are known to provide recognition specificity toward pathogen effector molecules (Innes, 2004).

The plant NBS-LRR genes can be classified into two sub-classes based on the presence or absence of amino/N-terminal domain. The first sub-class comprises proteins that carry *Drosophila* Toll and INTERLEUKIN1 like receptor (TIR) domain at the N-terminal position and are termed as TIR-NBS-LRR (TNL). The other sub-class comprises proteins which often carry Coiled-coil (CC) domain and are known as CC-NBS-LRR (CNL; Meyers et al., 1999). Other domains such as Zinc Finger or RPW8 domain are also found in the N-terminal position instead of CC domain which is often classified under CNL class (Sukarta et al., 2016). Recent studies demonstrated the function of TIR and CC domain in pathogen recognition and downstream signaling (Maekawa et al., 2011; Williams et al., 2014). The distribution of TNL and CNL gene classes is species specific as dicots contain both classes while monocots lack the TNL class (Shao et al., 2016).

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid crop with genome size of 738 Mb and the world's second most cultivated food legume crop after dry bean (FAOSTAT, 2015). Chickpea production is vital for food security and for improving the nutritional quality of diets in many developing countries particularly in South Asia, where it serves as a staple for human protein. Global chickpea production has increased by 56% in the last decade (2004–2013; Gaur et al., 2016). Both biotic and abiotic stresses are the major challenges of chickpea production limiting the crop to express its maximum yield potential. Ascochyta blight caused by the necrotrophic fungus *Ascochyta rabiei* (Pass) Labrousse is one of the major diseases of chickpea worldwide which lowers both grain yield and grain quality.

*Ascochyta rabiei* infects all above ground parts of chickpea plants which can result in total crop loss if favorable environmental conditions prevail for its infection and further growth. Ascochyta blight is a major concern as most of the cultivated chickpea accessions are susceptible to the disease, which limits the efforts to breed for ascochyta blight resistant cultivars. Genetic resistance to ascochyta blight is a complex trait and is highly influenced by environmental conditions. To date, several quantitative trait loci (QTLs) for ascochyta blight resistance have been identified in diverse genetic backgrounds on linkage groups (LGs) 2, 3, 4, 6 and 8 (Santra et al., 2000; Flandez-Galvez et al., 2003; Cho et al., 2004; Iruela et al., 2006; Tar'an et al., 2007; Anbessa et al., 2009; Sabbavarapu et al., 2013; Stephens et al., 2014). Most of these QTLs were tagged with SSR markers. The genes underlying the resistance are still unknown. So far two candidate genes CaETR-1 (EIN-4 like) and *ethylene insensitive 3-like gene* (*Ein3*) from ethylene pathway were identified in ascochyta blight resistance $QTL_{AR1}$ on LG4 and $QTL_{AR3}$ on LG2, respectively (Madrid et al., 2012, 2014). These studies suggest the possible involvement of ethylene pathway in ascochyta blight resistance in chickpea. In a recent study, expression profiling of 15 defense-related genes in response to *Ascochyta rabiei* infection identified six differentially expressed genes among ten chickpea genotypes (Leo et al., 2016). Identification of candidate genes involved in resistance to ascochyta blight associated QTLs will help in understanding the resistance mechanism and further assists in the development of resistant cultivars using marker assisted selection.

Homologs of NBS-LRR genes have been identified in *Arabidopsis* (Meyers et al., 2003), rice (Monosi et al., 2004), *Medicago* (Ameline-Torregrosa et al., 2008), cassava (Lozano

et al., 2015), soybean (Kang et al., 2012), *Brassica* (Mun et al., 2009), potato (Lozano et al., 2012) and in many other plant species. Most studies identified variable numbers of NBS-LRR genes ranging from 50 in papaya (Porter et al., 2009) to 1,015 in apple (Arya et al., 2014). The NBS-LRR genes are unevenly distributed in plant genomes and are often found in clusters. The availability of the draft genome sequence of chickpeas (Jain et al., 2013; Varshney et al., 2013) provided an opportunity to explore genome-wide distribution of several gene families such as Aux/IAA gene family (Singh and Jain, 2015), F-box genes (Gupta et al., 2015), ERF genes (Deokar et al., 2015), CaNAC genes (Ha et al., 2014), UDP-glycosyltransferase genes (Sharma et al., 2014) and many others. Recently the genome assemblies of both desi and kabuli chickpeas have been significantly improved and updated (Edwards, 2016a,b). Considering the critical role of NBS-LRR genes in plant defense system against multiple pathogens, it is important to explore this gene family in chickpea and examine their response to ascochyta blight infection. In this study, we identified NBS-LRR gene homologs in the chickpea genome. We analyzed the structural diversity of the chickpea NBS-LRR genes and further classified these genes based on their protein domain architectures. Annotation of the functional domains, phylogenetic tree construction and analysis of genomic distribution of this gene family were completed. We identified co-localized NBS-LRR genes with the previously reported ascochyta blight QTLs (AB-QTLs) and further examined the expression profiles of these co-localized NBS-LRR genes using quantitative real-time PCR (qRT-PCR) at different time points after ascochyta blight infection. This study provides an insight of the NBS-LRR gene family in the chickpea genome and their potential involvement in response to ascochyta blight infection.

# MATERIALS AND METHODS

## Identification and Classification of Chickpea NBS-LRR Genes

To identify the NBS-LRR genes in the chickpea genome, the genome assembly of CDC Frontier including the predicted gene model annotation was downloaded from NCBI (National Centre of Biotechnology Information[1]). Predicted protein sequences of 28,269 genes in the chickpea genome were initially scanned for the Hidden Markov Model (HMM) profile of NBS/NB-ARC domain (pfam00931) in HMMER v3.1b2 using "hmmsearch" with an expected value (*e*-value) threshold of $<1e{-}04$. The presence of NBS domain was further confirmed using NCBI conserved domain database (CDD) tool using *e*-value of 0.01 (Marchler-Bauer et al., 2011). The CDD results also confirmed the presence or absence of additional domains such as TIR, CC, and RPW8 in the N-terminal position and a variable number of LRR domains in the C-terminal position. The chickpea NBS-LRR genes were classified based on their protein domain arrangements.

## Identification of Conserved Motifs

The central NBS domain contains several conserved motifs such as P-loop, Kinase-1, GLPL etc. Eight distinct motifs within the NBS domain have been reported in *Arabidopsis* (Meyers et al., 2003). We used a similar approach to identify homologous conserved motifs in NBS domains of chickpea. The protein sequence of NBS domain from each NBS-LRR gene was retrieved and subjected to MEME (Multiple Expectation Maximization for Motif Elicitation; Bailey et al., 2006) for prediction of the conserved motifs.

## Gene Structure, Sequence Alignment, and Phylogenetic Analyses

The exon/intron structure of the chickpea NBS-LRR genes was retrieved from the general feature format (GFF) file of the chickpea genome annotation from NCBI. Multiple sequence alignments were conducted on the full length of the 121 NBS-LRR protein sequences using the default parameters of the Clustal W program. Due to pairwise distance calculation problem, four protein sequences (LOC101489470, LOC105851382, LOC101488657, and LOC101498409) were removed from further analysis. Neighbor-Joining (NJ) phylogenetic tree of 117 protein was constructed with 1,000 bootstrap replications using MEGA7.0. Gene Structure Display Server (GSDS) was used to align the phylogenetic tree and the gene structure of the NBS-LRR genes along with the domain positions.

## Distribution and Cluster Analysis of NBS-LRR Genes

The NBS-LRR genes were distributed across the eight chickpea chromosomes based on the CDC Frontier genome assembly v1. The genes were also mapped on the advanced version CDC Frontier genome assembly v2 for comparison (Edwards, 2016b). To define the gene cluster, the following parameters were established: a cluster must contain at least two genes, the distance between two neighboring NBS-LRR genes should be <200 kb and no more than eight genes between the neighboring NBS-LRR genes.

## Co-localization of NBS-LRR Genes with Ascochyta Blight QTLs

The information of the chickpea AB-QTLs was retrieved from the cool season legume database[2]. The physical locations of the markers associated with AB-QTLs in the chickpea genome were obtained via sequence similarity analysis of both forward and reverse primer sequences of each marker using NCBI BlastN. Only hits with 100% coverage of both query and subject were selected. Based on the physical position of the markers, the physical positions of the corresponding AB-QTLs were inferred on both versions of the CDC Frontier genome assembly. The physical positions of the two candidate genes (CaETR1 and *EIN3*) tagged with $QTL_{AR1}$ and $QTL_{AR3}$ were also retrieved to confirm the physical location of the QTLs. The co-localizations between the NBS-LRR genes and AB-QTLs were analyzed using Microsoft

[1]http://www.ncbi.nlm.nih.gov/assembly/GCF_000331145.1

[2]https://www.coolseasonfoodlegume.org

Excel. For visualization, a physical map of the chickpea genome was constructed by combing the distribution of the NBS-LRR genes and the physical location of the AB-QTLs using Mapchart.

## Ascochyta Blight Screening

Three chickpea genotypes including CDC Corinne, CDC Luna (both moderately resistant to ascochyta blight) and ICCV 96029 (susceptible) were used in the greenhouse trial. The experiment was conducted in completely randomized design (CRD). A total of three independent biological replications of each chickpea genotype at each time point under control (non-inoculated) and inoculated were analyzed. Three-week old seedlings were inoculated with single spore derived conidial suspension culture of *Ascochyta rabiei* isolate *AR-170*. About 3 mL of conidial suspension with a concentration of $2 \times 10^{-5}$ conidia $mL^{-1}$ was sprayed on each plant using an air compressor. Control plants were mock-inoculated with water. Following inoculation, all plants were kept under humidity chambers equipped with two humidifiers which maintain relative humidity of 100% for 48 h. Later all plants were moved to greenhouse benches equipped with the overhead misting system and all sides of the bench were covered with plastic sheets to maintain high humidity. Leaf samples were collected at four time points at 12, 24, 48, and 72 hours post inoculation (hpi) from each of the three biological replicates of both control and inoculated plants. Collected tissue samples were immediately frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

## Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted and treated with DNase I using SV Total RNA Isolation kit following manufacturer's instruction (Promega, USA). Extracted RNA sample quantity was determined by an optical density reading at 260 nm and the OD260/OD280 absorption ratio using NanoDrop 800 UV-vis spectrophotometer (Thermo Fisher Scientific, Inc. USA) and RNA integrity was checked on 1.5% agarose gel electrophoresis. Total RNA (1 μg) was reverse transcribed to cDNA using SensiFAST cDNA synthesis kit (Bioline, Inc.). The cDNA used for qRT-PCR was diluted 5× with DNase/RNase free water. Specific primers were designed for each of the co-localized NBS-LRR genes in AB-QTLs and five reference genes (*18SrRNA*, Elongation factor [*Ef1α*], *GAPDH*, Initiation factor [*IF4a*] and *ACTIN*) using IDT Primer quest tool (Integrated DNA Technologies, Inc). All primer sequences are presented in Supplementary Table 1. The primer pairs were designed to span exon–exon junction with PCR product size between 55–180 bp, the length of primer sequence of 18–25 nucleotides, T*m* between 50–60°C and GC content of 50–60%. Each primer was tested on cDNA and gDNA samples to ensure amplification of the target region. Primer efficiencies of each target and reference gene were calculated by making the 10-fold serial dilution of cDNA using equation $(1 + E) = 10$ slope (Ramakers et al., 2003). SensiFAST SYBR No-ROX kit was used for the target gene expression using optical 384 well plate on BIO-RAD CFX384 real-time PCR detection system (Bio-Rad laboratories) in accordance with the manufacturer's protocols. For each gene, two technical replicates of each three biological replication per chickpea genotype at each time point were performed in a single plate along with controls [negative reverse transcription control (−RTC) and no template control (NTC)] for detection of DNA contamination or primer dimers. PCR product specificity of each gene was checked by melting curves analysis carried out by PCR machine after 40 amplification cycles. All experimental samples for each amplicon had a single sharp peak at the amplicon melting temperature.

## qRT-PCR Data Analysis

Among the tested five reference genes, *GAPDH* was selected and used to normalize the relative quantities of the target genes based on its consistency across the different time points and genotypes. The comparative $C_T$ method was used for the quantification of the expression of the co-localized NBS-LRR genes in AB-QTLs in which fold changes in expression were calculated by $2^{(-\Delta\Delta CT)}$ method (Schmittgen and Livak, 2008). A mean fold change expression level of 2.0 was used as a cut-off point. Differentially expressed genes were clustered using hierarchical cluster analysis. UPMG method was used to generate a dendrogram using *K*-means clustering with Cluster v3.0 program. The heatmap was constructed and displayed using Treeview v1.60. The complete procedure of the expression profiling experiment is summarized in **Figure 1**.

# RESULTS

## Identification and Classification of Chickpea NBS-LRR Genes

We identified a total of 121 NBS-LRR genes in the CDC Frontier genome assembly v1 using our search criteria as explained in the materials and methods. Based on the protein domain combinations, the NBS-LRR genes were grouped into eight classes (**Table 1**). Among the 121 genes, we identified 98 complete genes that carried both the NBS and LRR domains and 23 partial genes that carried the NBS domain but lacked the LRR domain. The majority of the genes belong to the TNL class (39) followed by the CNL class (34) and the NL class (21). We also identified five genes with the RPW8 domain in the N-terminal position other than TIR and CC domain and classified them as the RPW8-NBS-LRR (RNL [4]) and the RPW8-NBS (RN [1]). Sixteen genes that only carried the central NBS domain and lacked both the N-terminal domain and the C-terminal LRRs were classified as the NBS class.

## Identification of Conserved Motifs within NBS Domain

The MEME motif analysis within the chickpea NBS domain identified eight conserved motifs similar to the *Arabidopsis* NBS domain motif structure. The eight major motifs varied in their divergence within and between the TNL and CNL classes (**Table 2**). Six conserved motifs (P-loop, Kinase-2, RNBS-B, RNBS-C, GLPL and MHDV) were consistently detected in each class. Two motifs RNBS-A and RNBS-D were more diverse in sequence which distinguished the CNL and TNL class. All eight motifs were present in strict order from P-loop to MHDV.

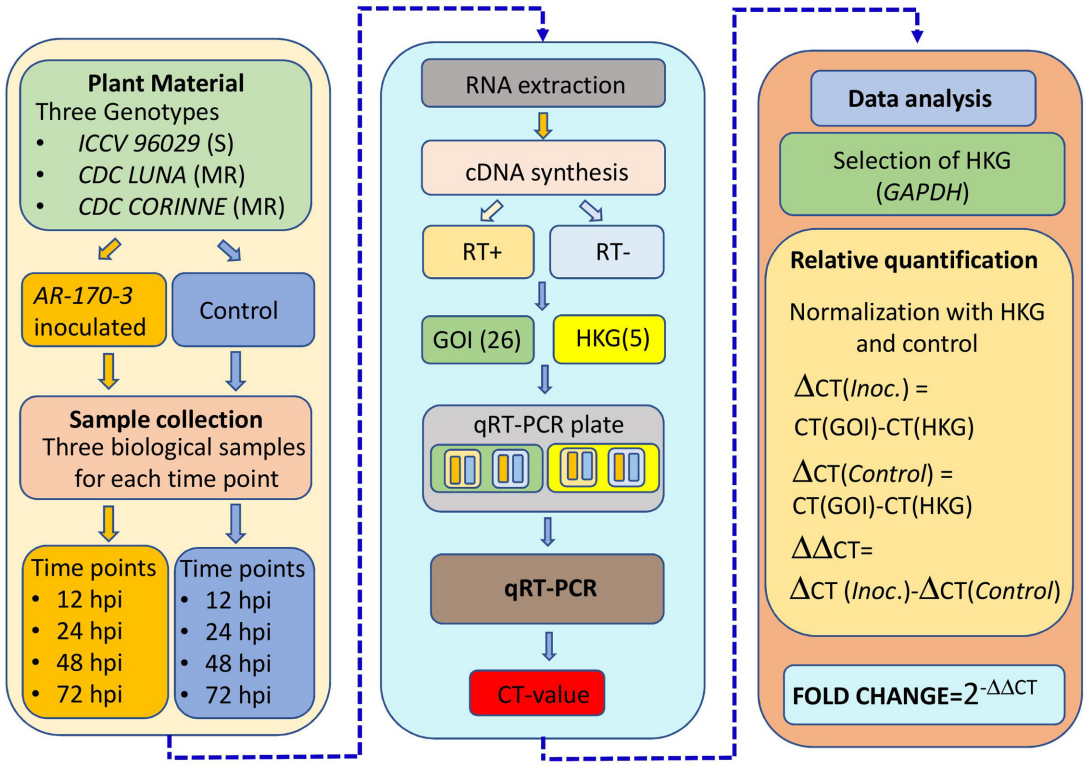


**FIGURE 1 | Overview of the experiment and data analysis for the expression profiling of the co-localized NBS-LRR genes with the known QTLs for ascochyta blight resistance in chickpea.**

## Sequence Alignment and Phylogenetic Analyses

A NJ phylogenetic tree of 117 complete NBS-LRR proteins was constructed to examine the evolutionary relationships among the NBS-LRR genes (**Figure 2**). Four truncated protein sequences were removed from the analysis after multiple-sequence protein alignment. The NJ tree displayed two clear clades which distinctly separated the TNL class from the non-TNL class. The TNL clade consists of three TNL sub-clades. The non-TNL clades were separated into CNL and NL sister clades. Furthermore, the CNL clade was clearly separated into two sub-clades consisting of CNL clade and RPW8 clade. Phylogenetic clustering of the genes with similar sequences from different chromosomes and the same chromosomes was observed. The alignment of phylogenetic tree with gene structure revealed that exon–intron structure tends to remain the same within the genes present in the same clade reflecting strong conservation of the gene structure during evolution.

## Distribution of the NBS-LRR Genes

The physical locations of the NBS-LRR genes were determined based on the chickpea gene annotation and the GFF3 file. Using CDC Frontier genome assembly v1, we were able to place 93 genes on the eight chickpea chromosomes and 28 genes on the unanchored scaffolds. While using the advanced genome assembly version v2, we were able to map 109 NBS-LRR genes on the chickpea chromosomes and 12 were located on the unplaced scaffolds. The chromosomal location of the NBS-LRR genes revealed the uneven distribution of the genes on the eight chickpea chromosomes and showed tandemly located gene clusters (**Figure 3**). Chromosome 5 has the highest number (29) of the NBS-LRR genes (27% of mapped genes), while chromosome 8 has the lowest number (5) of the NBS-LRR genes. At least one CNL gene was present on each chickpea chromosome while the TNL class was limited to seven chromosomes (absent on chromosome 4). Out of the 121 NBS-LRR genes, 58 genes were

**TABLE 1 | Classification of the NBS-LRR genes in the chickpea genome.**

| Set | Class | No. of genes |
|---|---|---|
| With LRR | (1) CC-NBS-LRR | 34 |
| | (2) TIR-NBS-LRR | 39 |
| | (3) RPW8-NBS-LRR | 4 |
| | (4) NBS-LRR | 21 |
| Without LRR | (5) CC-NBS | 3 |
| | (6) TIR-NBS | 3 |
| | (7) RPW8-NBS | 1 |
| | (8) NBS | 16 |
| Total | | 121 |

*NBS, Nucleotide Binding Site; LRR, Leucine Rich Repeat; CC, Coiled Coil; TIR, Toll/Interleukin Receptor; RPW8, Resistance to Powdery Mildew 8.*

**TABLE 2 | Consensus sequence of the major motifs identified in the chickpea NBS domain of the CNL and TNL proteins.**

| Motif | CNL | TNL |
|---|---|---|
| *P-loop* | VIPIVGMGGLGKTTLAQLVYND | LGIWGMGGIGKTTLAKAIYNKIXR |
| *RNBS-A* | DLKAWVCVSDDFDVLKVTKXI | FEGRCFLENVRENSE |
| *Kinase-2* | LQGKRFLLVLDDVWNEDY | IIKRRLCRKKVLLVLDDVDKLEQ |
| *RNBS-B* | PCGAKGSKILVTTRNQKVAS | WFGPGSRIIITTRDKHLLXGH |
| *RNBS-C* | HSLEXLSDEDCWSLFAKHAFR | YEVKELNEKESLELFSWHAFKQDX |
| *GLPL* | LEKIGKEIVKKCGGLPLAAVT | VVXYAGGLPLALEVLGSFLFGKDI |
| *RNBS-D* | DKKDLILLWMAEGFL | LDDTEKEIFLDIACF |
| *MHDV* | FVMHDLVHDLATLVSGEFYFR | MHDLLQDMGREIVREESPKEP |

present in 23 clusters each carrying two to four genes while 68 genes were present as singletons (**Table 3**). Among the 23 clusters, 18 were monophyletic clusters containing 45 genes and 5 were in mixed clusters containing 13 genes. The maximum of four genes per cluster was found in each mono-cluster and mixed-cluster on chromosome 5 and 7, respectively.

## Co-localization of the NBS-LRR Genes with Ascochyta Blight QTLs

Based on the physical position of the SSR markers on the chickpea chromosomes, 16 QTLs for ascochyta blight resistance previously reported were physically mapped on chromosomes 2, 3, 4, 5, 6 and 8 (Supplementary Table 2). Nine QTLs were co-localized with the NBS-LRR genes. Out of the nine QTLs, three QTLs (Cho et al., 2004; [QTL-AR2] Iruela et al., 2006; [AB-Q-SR-4-2] Sabbavarapu et al., 2013) were mapped on chromosome 4, three QTLs ([QTL4] Tar'an et al., 2007; [AB-Q-APR-6-1, AB-Q-APR-6-2] Sabbavarapu et al., 2013) were mapped on chromosome 6 and one QTL each on chromosome 2 ([QTL1] Anbessa et al., 2009), chromosome 3 ([QTL2] Tar'an et al., 2007) and chromosome 8 ([QTL5] Anbessa et al., 2009). The QTLs ([QTL-AR2] Iruela et al., 2006; [AB-Q-SR-4-2] Sabbavarapu et al., 2013) identified on chromosome 4 in different genetic populations were mapped on the same physical locus. Similarly, QTLs ([QTL4] Tar'an et al., 2007; [AB-Q-APR-6-2] Sabbavarapu et al., 2013) on chromosome 6 were over-lapping. We identified 30 NBS-LRR genes co-located within the flanking markers corresponding to these nine AB-QTLs (**Figure 4**). Among the co-localized NBS-LRR genes, 24 genes were complete genes, i.e., they carry all essential domains for their independent functions. Among these 24 genes, 13 belong to the TNL class, eight belong to the CNL class and three belong to the NL class. Six co-localized genes were incomplete that belong to the RN class (1), CN class (2) and NBS class (3). The majority of the genes (17) co-localized with the AB-QTLs were present in clusters of 2 to 3 genes. On chromosome 2, QTL1 (Anbessa et al., 2009) was co-localized with three mono-clusters; cluster 2, 3 and 4 consisting of two genes from the TNL class, two genes from the NBS class and three genes from the TNL class, respectively (**Table 3**). The QTL2 (Tar'an et al., 2007) on chromosome 3 overlaps with the mixed-cluster 20 consisting of three genes, each from the NL, CN and CNL class. On chromosome 4, the QTL reported by Cho et al. (2004) co-localized with the cluster 5 consisting of three CNL

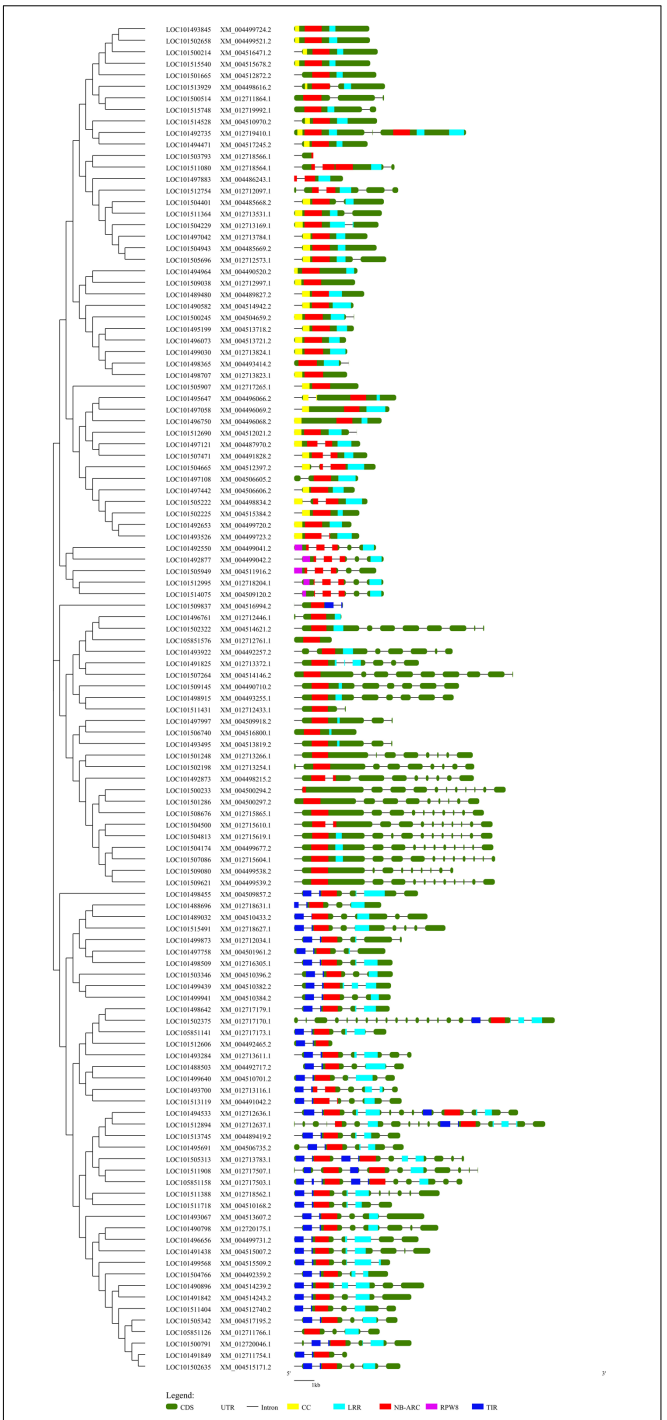


**FIGURE 2 | A Neighbor-Joining phylogenetic tree depicting the evolutionary relationship of the chickpea NBS-LRR genes aligned with the exon-intron structure of each gene along with the domain distribution.** This tree was constructed using 117 complete NBS-LRR protein sequences with 1000 bootstraps. Evolutionary distance was calculated using p-distance method. The gene structure was retrieved from the chickpea annotation and the General Feature File (GFF3). The position of the domains in each gene was obtained from the NCBI conserved domain database (CDD). The phylogenetic tree was aligned with the gene structure along with the domain position using GSDS. Different domains are indicated by different colors.

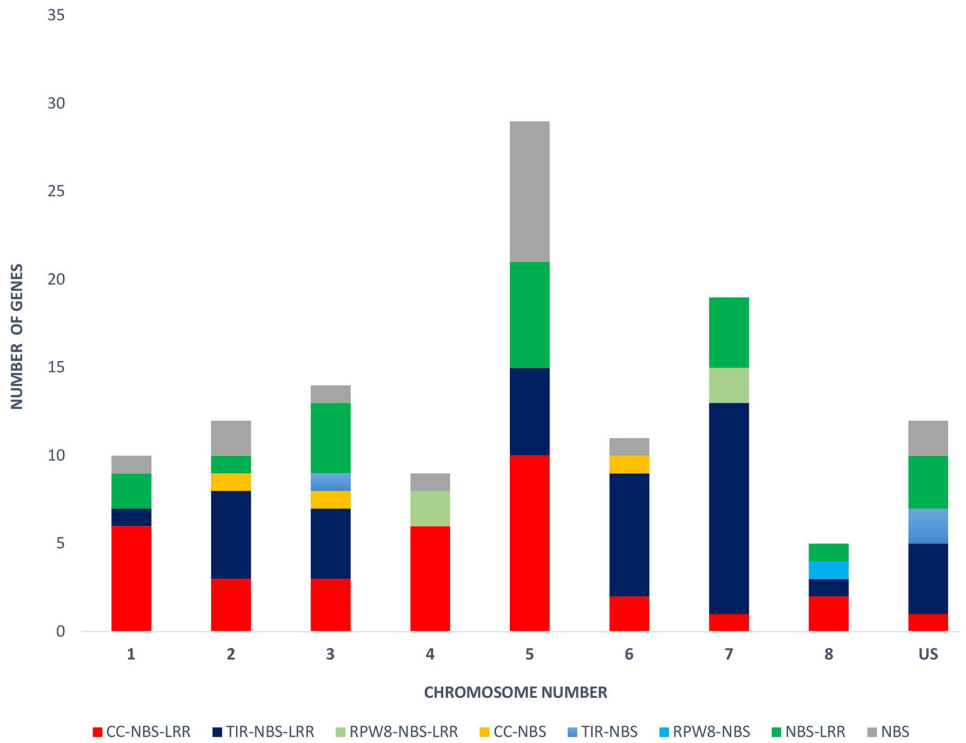


**FIGURE 3 | Distribution of the NBS-LRR genes on each chickpea chromosome (1–8) and unplaced scaffold (US).** Different colors reflect different gene classes.

class genes. Two mono-clusters, cluster 13 and cluster 14 each consisting of two genes from the TNL class were co-localized with the AB-Q-APR-6-2 (Sabbavarapu et al., 2013) on chromosome 6.

## Quantitative Real-Time Expression Profiling of the NBS-LRR Genes

Among the 30 co-localized NBS-LRR genes in nine AB-QTLs, 27 genes showed differential expression in response to ascochyta blight infection in at least one genotype at one time point compared to the control (**Figure 5**). The expression of the remaining three genes (LOC101493700, LOC101513119, and LOC101494533) was below the cut-off level of 2.0 mean fold change across all time-points in all genotypes. Five genes showed genotype-specific expression, two genes (LOC101509145, LOC101498915) showed up-regulation only in CDC Corinne at 48 and 72 hpi, and down-regulated or no regulation in ICCV 96029 and CDC Luna at all time points. In contrast, three genes (LOC101512894, LOC101513745, and LOC101497042) showed up-regulation in ICCV 96029 and CDC Luna but no regulation or down-regulated in CDC Corinne. One gene (LOC101505949) constantly expressed in all three genotypes at all time points, except at 12 hpi in CDC Corinne. In terms of the levels of expression, a range of 2–13 mean fold change expression was observed. The highest of 13 mean fold change expression was observed for two genes (LOC101498365 and LOC101511908) in CDC Corinne at 72 hpi when compared to control, followed by 12 mean fold change in three genes; two genes (LOC101505907 and LOC101511908) in CDC Luna and one gene (LOC101500245) in ICCV 96029. Expression profiling of the NBS-LRR genes allowed to differentiate the three genotypes, the susceptible cultivar was separated from the moderately resistant cultivars, and the moderately resistant cultivars were distinguished with respect to the up-regulation of these genes at different time points after inoculation. In ICCV 96029, the highest number of genes (20) were up-regulated at early hours of infection (12 hpi), while 12 NBS-LRR genes were up-regulated in CDC Luna and only 4 NBS-LRR genes were up-regulated in CDC Corinne at 12 hpi. In CDC Luna, the highest number of genes (21) were upregulated at 24 hpi, while 15 and 13 genes were up-regulated in ICCV 96029 and CDC Corinne, respectively. In CDC Corinne, the highest number of NBS-LRR genes (20) were upregulated at both 48 and 72 hpi, while 12 and 18 genes were up-regulated in ICCV 96029 and CDC Corinne, respectively, at 48 hpi, and 14 and 3 genes were up-regulated in ICCV 96029 and CDC Luna, respectively, at 72 hpi. On average, most genes showed up-regulation at 12 and 24 hpi in ICCV 96029, whereas in CDC Luna and CDC Corinne higher expression was observed at 24 and 48 hpi and 48 and 72 hpi, respectively (**Figure 5**).

## Patterns of Gene Expression within and among Genotypes

Cluster analysis of the 27 NBS-LRR genes revealed the underlying expression patterns within and among the genotypes at different time points (**Figure 5**). Two major clusters of expression

**TABLE 3 | Cluster analysis of the NBS-LRR genes in chickpea.**

| Cluster type | Cluster | Cluster size (KB) | No. of Genes | Chromosome | Gene ID |
|---|---|---|---|---|---|
| Mono-cluster | 1 | 16.4 | 3 | 1 | LOC101504943, LOC101504401, LOC101505696 |
| | 2 | 3.2 | 2 | 2 | LOC101513119, LOC101493700 |
| | 3 | 0.8 | 2 | 2 | LOC101501248, LOC101502198 |
| | 4 | 53.3 | 3 | 2 | LOC101494533, LOC101512894, LOC101513745 |
| | 5 | 18.9 | 3 | 4 | LOC101495647, LOC101496750, LOC101497058 |
| | 6 | 71.3 | 2 | 4 | LOC101495199, LOC101496073 |
| | 7 | 2.9 | 2 | 4 | LOC101492550, LOC101492877 |
| | 8 | 57.6 | 3 | 5 | LOC101508676, LOC101500233, LOC101501286 |
| | 9 | 4.4 | 2 | 5 | LOC101497758, LOC101498509 |
| | 10 | 21.9 | 2 | 5 | LOC101509621, LOC101509080 |
| | 11 | 144.1 | 4 | 5 | LOC101507086, LOC101504174, LOC101504500, LOC101504813 |
| | 12 | 45.2 | 3 | 5 | LOC101492653, LOC101493526, LOC101493845 |
| | 13 | 0.7 | 2 | 6 | LOC105851141, LOC101502375 |
| | 14 | 40.3 | 2 | 6 | LOC101511908, LOC105851158 |
| | 15 | 5.6 | 2 | 7 | LOC101512995, LOC101514075 |
| | 16 | 11.9 | 3 | 7 | LOC101488696, LOC101515491, LOC101489032 |
| | 17 | 199.2 | 3 | 7 | LOC101499439, LOC101499941, LOC101503346 |
| | 18 | 20.0 | 2 | 7 | LOC101490896, LOC101491842 |
| Mixed-cluster | 19 | 116.0 | 2 | 1 | LOC101511364, LOC101512754 |
| | 20 | 12.0 | 3 | 3 | LOC101498365, LOC101498707, LOC101499030 |
| | 21 | 18.5 | 2 | 5 | LOC101497108, LOC101497442 |
| | 22 | 145.1 | 2 | 5 | LOC101499568, LOC101500514 |
| | 23 | 47.0 | 4 | 7 | LOC101503793, LOC101511080, LOC101511388, LOC101511718 |
| Total clusters | 23 | | 58 | | |
| Non-clustered | | | 63 | | |
| Total genes | | | 121 | | |

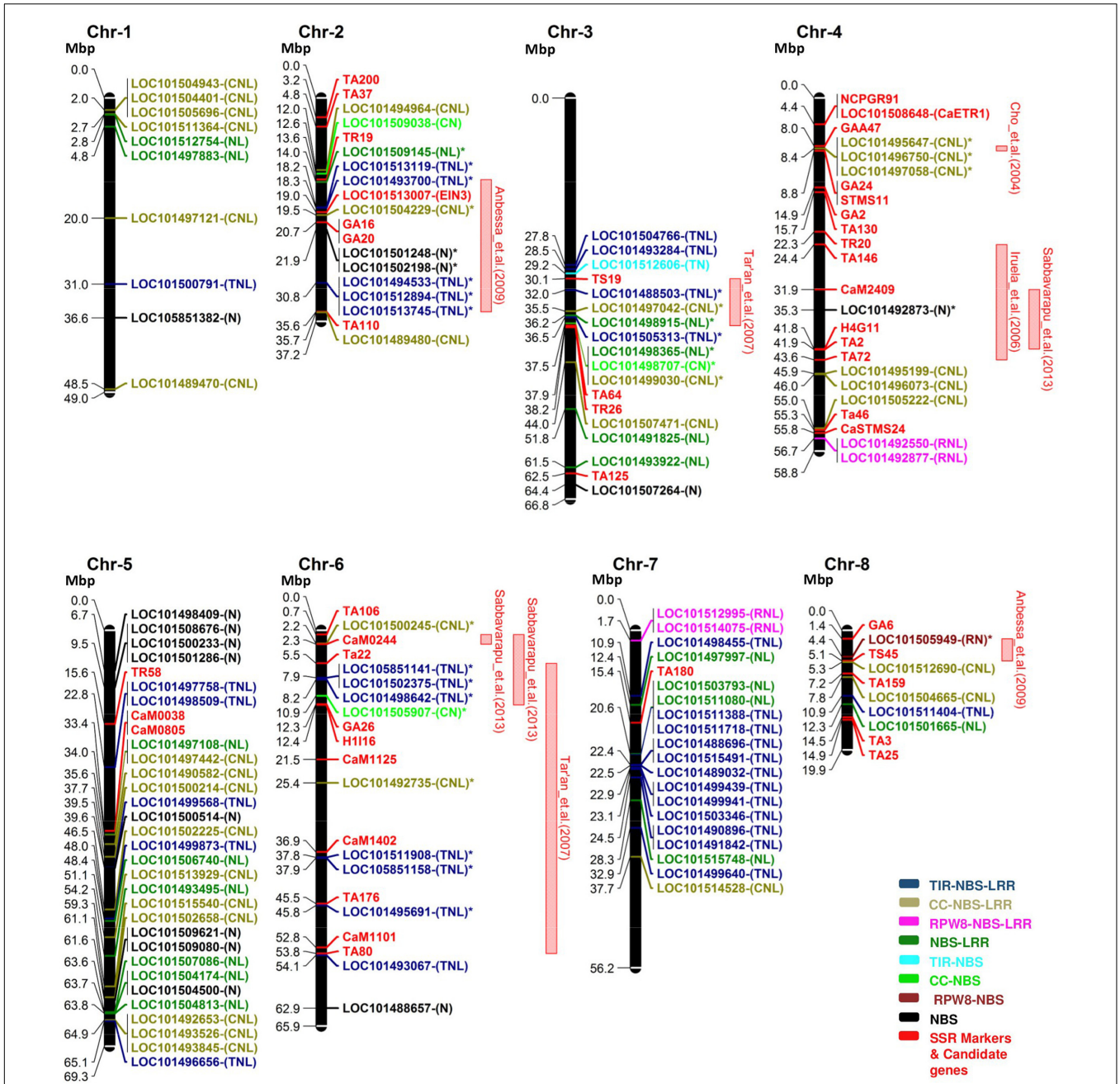


**FIGURE 4 | The distribution of NBS-LRR genes on the physical map of CDC Frontier v2 along with the position of the markers corresponding to the physical positions of the quantitative trait loci (QTLs) for ascochyta blight resistance.** Eight chromosomes (Chr) of chickpea were represented as black bars. Gene and marker names are shown on the right-handed side and their physical positions in megabase pair (Mbp) are shown on the left. Color codes are defined in the legend. QTLs with co-localized NBS-LRR genes were shown as red bars on the right side of the chromosomes. Co-localized NBS-LRR genes are shown with (*) along with their name. We identified 30 NBS-LRR genes co-localized within the nine QTLs for ascochyta blight resistance.

patterns were observed. Cluster 1 consisted of 3 genes and cluster 2 consisted of 24 genes. Three genes (LOC101512894, LOC101513745, and LOC101497042) in cluster 1 were only up-regulated in ICCV 96029 and CDC Luna, but their expression was below the cut-off limit in CDC Corinne. Cluster 2 consisted of two sub-clusters, cluster 2.1 and cluster 2.2. Eight genes were present in cluster 2.1. Among these, two genes (LOC101509145, LOC101498915) showed contrasting expression pattern to genes present in cluster 1 as they only showed higher expression in CDC Corinne. This suggests that these genes are specific to CDC Corinne in response to ascochyta blight infection. The other six genes in cluster 2.1 were up-regulated in ICCV 96029 at 12 and

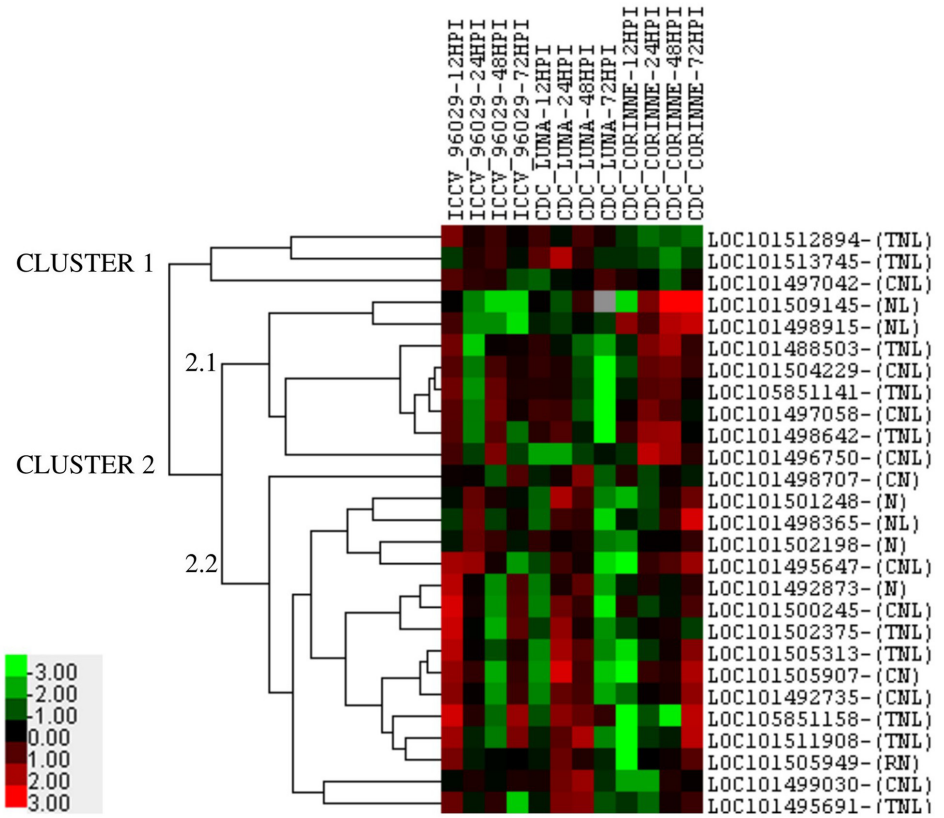


**FIGURE 5 | Heatmap of 27 NBS-LRR genes representing the mean fold change expression levels at four different time points in three chickpea cultivars (ICCV 96029, CDC Luna and CDC Corinne) after infection with *Ascochyta rabiei* isolate *AR-170-3*.** The mean fold change expression values were calculated after normalization with the reference gene (GAPDH) and the non-infected control samples. Red color represents up-regulation, black represents no change and green represents down-regulation as presented in color bar.

48 hpi, in CDC Luna at 12 and 24 hpi and in CDC Corinne at 24 and 48 hpi. The sub-cluster 2.2 consisted of 16 genes, with few exceptions, most genes were up-regulated at 12 and 72 hpi in ICCV 96029, 24 and 48 hpi in CDC Luna, and at 48 and 72 hpi in CDC Corinne.

## DISCUSSION

Ascochyta blight is one of the major yield limiting factor of chickpea production worldwide. However, disease severity is more significant in areas with cooler and wet growing season such as Western Canada (Tar'an et al., 2007). Limited success has been achieved in developing ascochyta blight resistant cultivars due to lack of complete resistance in the chickpea germplasm. To date, several QTLs associated with ascochyta blight resistance have been identified in diverse genetic backgrounds. Yet, the precise mechanism of resistance to ascochyta blight is still unknown.

Numerous plant disease resistance genes including NBS-LRR genes that play a major role in resistance against diverse array of pathogens have been identified and cloned in many plant species (Hammond-Kosack and Jones, 1997). The majority of the NBS-LRR genes were known to provide resistance against biotrophic pathogens following a "gene-for-gene" or "guard" model of host-pathogen interaction leading to the activation of salicylic acid (SA) defense response (Glazebrook, 2005). The knowledge of resistance mechanism against necrotrophic pathogens was limited to phytotoxin production and activation of jasmonic acid (JA) and ethylene pathway (Glazebrook, 2005). An association of the NBS-LRR genes with susceptibility against necrotrophic pathogen has been observed in different studies (Lorang et al., 2007; Nagy and Bennetzen, 2008; Faris et al., 2010). Recent studies also showed the involvement of NBS-LRR genes in resistance reaction against necrotrophic pathogens. For example, *Arabidopsis* TNL class RLM3 gene provides resistance against three necrotrophic fungi; *Botrytis cinerea*, *Alternaria brassicicola*, and *Alternaria brassicas*, and one hemibiotrophic fungus *Leptosephaeria maculans* (Staal et al., 2008). In wheat, over-expression of the *TaRCR1* gene, a member of CNL class increased resistance against the necrotrophic fungus *Rhizoctonia cerealis* (Zhu et al., 2016).

The involvement of NBS-LRR genes against ascochyta blight infection in chickpea has not been reported so far. Chickpea genome consists of 121 NBS-LRR genes, which is about 0.43% of the total 28,269 annotated genes. Chickpea has relatively low number of NBS-LRR genes compared to *Glycine max* (0.58%),

*Medicago truncatula* (0.66%), and *Arabidopsis thaliana* (0.75%; Meyers et al., 2003; Ameline-Torregrosa et al., 2008; Kang et al., 2012). The frequency of NBS-LRR genes is highly variable among plant species, as low as 0.21% in papaya (Porter et al., 2009) and as high as 1.6% in apple (Arya et al., 2014). The chickpea NBS-LRR gene frequency falls within this range. Several studies showed that there is no correlation between the NBS-LRR gene frequency and the genome size or the total annotated genes. One hypothesis for the explanation of the low copy number of NBS-LRR genes might be due to the fitness cost and lethal effect of NBS-LRR genes on plant cells which restrict the number of NBS-LRR genes in plant genome (Zhang et al., 2016). Despite the relatively low number of NBS-LRR genes, most of the chickpea NBS-LRR gene family possess the essential conserved domains as observed in other plant species. Out of the 121 NBS-LRR genes, 98 genes encode proteins consisting of both NBS and LRR domains and the remaining 23 genes were incomplete. The presence of all necessary structural motifs depicts their capacity to function as independent R-proteins. However, the truncated or incomplete genes have also been reported to have a function in co-operation with the complete genes. For example, two tandem NBS-LRR genes; *RPP2A* and *RPP2B* are required to provide resistance against *Peronospora parasitica* isolate Cala2 in *Arabidopsis*. *RPP2A* is incomplete TIR-NBS gene with truncated LRR domain and *RPP2B* is a complete gene, both genes complement each other by providing recognition specificity or signaling lacking by its partner and confer resistance against isolate Cala2 (Sinapidou et al., 2004). Our expression study also showed up-regulation of truncated NBS-LRR genes upon ascochyta blight infection.

Chickpea NBS-LRR gene family were grouped into eight major classes based on their domain architecture. In general, the TNL class is often lacking in monocot species. Chickpea being a dicot species contained TNL class and their numbers (39) were relatively higher than CNL class (34), a pattern similar to *Arabidopsis, Medicago*, soybean and other dicot species. This indicated that the evolution of NBS-LRR genes diversified significantly between monocots and dicots. The evolutionary divergence of TNL from non-TNL/CNL class had been observed in many studies (Meyers et al., 2003; Ameline-Torregrosa et al., 2008; Lozano et al., 2015). It was also observed that RPW8 formed separate sub-clade within CNL clade which supports the functional divergence of the RPW8 from common CNL genes (Collier et al., 2011). Our phylogenetic analysis indicated that chickpea NBS-LRR genes followed the similar patterns. Our analysis showed that among the non-TNL clades, the NL clade was separated from the CNL clade as different clades represent the structural difference among these classes. The phylogenetic analysis supports our criteria of classification into TNL, CNL, RNL and NL and similar classes that lacked the C-terminal LRR domain.

The NBS-LRR genes were distributed across all chickpea chromosomes. However, the distribution was not even across the chromosomes as chromosome 5 contains the highest number of NBS-LRR genes and chromosome 8 has the lowest. Frequently it has been observed that the NBS-LRR genes are present in clusters, which may contribute to the genetic variation and the rapid evolution (Hulbert et al., 2001). In chickpea, nearly half of the NBS-LRR genes (48%) were found in clusters. Among these clustered genes, mono-clusters (78%) were abundant than mixed clusters which reflect that these genes might have evolved through tandem duplications. Another significance of clustering of NBS-LRR genes is that tandem clustering of functionally related genes facilitates co-expression and forms functional heterodimers which might interact with pathogen effector molecules to govern resistance as observed in rice (Ashikawa et al., 2008) and *Arabidopsis* (Sinapidou et al., 2004). Our expression analysis showed similar expression pattern of the NBS-LRR genes present in the cluster. Two NBS-LRR genes (LOC101501248 and LOC101502198) of cluster 3 present in QTL1 (Anbessa et al., 2009) on chromosome 2 showed similar induction pattern in each genotype. Cluster 2 co-located with QTL2 (Tar'an et al., 2007) on chromosome 3, comprising of three NBS-LRR genes (LOC101498365, LOC101498707, and LOC101499030) also showed similar expression pattern in each genotype. Co-expression of these clustered genes reflects their potential involvement in common resistance mechanism.

Co-localized genes in QTL regions have been successfully used to identify potential candidate genes associated with different traits. In chickpea, CaETR1 and *Ein3* have been identified as candidate genes for ascochyta blight resistance based on their co-localization with $QTL_{AR1}$ and $QTL_{AR3}$, respectively, for ascochyta blight resistance (Madrid et al., 2012, 2014). In soybean, the strong positive correlation between the number of NBS-LRR genes and the disease resistance QTLs on each chromosome reflects the contribution of this gene family in soybean disease resistance (Kang et al., 2012). The association of NBS-LRR genes with the ascochyta blight response in chickpea has not been reported. Here, we reported 30 NBS-LRR genes that were co-localized with the physical position of the nine ascochyta blight resistance QTLs on chromosome 2, 3, 4, 6, and 8. Previously it has been reported that cluster of the NBS-LRR genes provides effective resistance in rice and *Arabidopsis* against *Magnaporthe grisea* and *P. parasitica*, respectively (Sinapidou et al., 2004; Ashikawa et al., 2008). Clusters of the NBS-LRR genes were also identified within the AB-QTLs on chickpea chromosome 2, 3, 4 and 6. A cluster of three CNL class genes co-localized with AB-QTL (Cho et al., 2004) on chromosome 4 showed high sequence similarity with the *Arabidopsis* RPP13 gene which provides resistance against five isolates of *P. parasitica* via novel signaling pathway that function independently of SA-mediated response (Bittner-Eddy and Beynon, 2001).

The *Ascochyta rabiei* isolate *AR-170-3* infects both the susceptible and the moderately resistant genotypes as evident by the production of necrotic lesions in all three genotypes. The moderately resistant genotypes (CDC Luna and CDC Corinne) showed delayed symptom development in comparison to the susceptible genotype (ICCV 96029). The majority of the co-localized NBS-LRR genes in AB-QTLs showed differential expression in at least one genotype at one time point compared to control. However, up-regulation of these genes were observed at early hours of infection in the susceptible genotype compared to the resistant genotypes, which correlates with the disease progression on these genotypes. Genotype-specific expression pattern of some of the NBS-LRR genes was also observed.

Two NBS-LRR genes (LOC101509145 and LOC101498915) were up-regulated only in the moderately resistant cultivar CDC Corinne. One gene (LOC101505949) co-localized with QTL5 (Anbessa et al., 2009) on chromosome 8 showed up-regulation in all ascochyta blight inoculated samples, except at 12 hpi in CDC Corinne. This gene had very high sequence similarity with the *Arabidopsis ARD1*-like genes (AT4G33300). The *Arabidopsis ARD1*-like gene is an RPW8-NBS-LRR class gene which accumulates SA and provides broad resistance against the biotrophic pathogen (Grant et al., 2003). The presence of biotrophic resistance gene in ascochyta blight resistance QTL suggests common defense mechanism might be involved in providing resistance against biotrophic and necrotrophic pathogens. As cross communication between SA and JA pathway has been previously suggested (Kunkel and Brooks, 2002; Derksen et al., 2013).

Besides the NBS-LRR genes, other disease resistance related genes might also be present within the AB-QTL interval, such as the *EIN3* gene was co-localized with the NBS-LRR genes in QTL2 and $QTL_{AR3}$ (Anbessa et al., 2009; Madrid et al., 2014). *EIN3* is a plant-specific transcription factor which plays an important role in mediating ethylene responses (Madrid et al., 2014). Plant defense response induces several signaling molecules including ethylene, SA and JA which are involved in downstream of the NBS-LRR proteins (McHale et al., 2006). Thus, it is likely that both the ethylene pathway and the NBS-LRR genes might be involved in providing resistance to ascochyta blight. Therefore, it would be interesting to further explore the interaction between the NBS-LRR and the defense responsive ethylene pathway.

In summary, our genetic analysis identified 121 NBS-LRR genes in the chickpea genome. The NBS-LRR genes were classified into eight distinct classes. We identified NBS-LRR genes that are potentially involved in response to ascochyta blight infection based on their co-localization with the known QTLs for ascochyta blight resistance and based on their expression profiles. Our study provides resources for further functional studies to validate the association of NBS-LRR genes with disease resistance in chickpea.

## AUTHOR CONTRIBUTIONS

MS conducted the experiments, analyzed the data and wrote the manuscript. AD and BT designed the experiment, assisted with data analysis, wrote and edited the manuscript. BT conceived and directed the project. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00838/full#supplementary-material

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# Genome Analysis Identified Novel Candidate Genes for Ascochyta Blight Resistance in Chickpea Using Whole Genome Re-sequencing Data

*Yongle Li[1], Pradeep Ruperao[2], Jacqueline Batley[3], David Edwards[3], Jenny Davidson[4], Kristy Hobson[5] and Tim Sutton[1,4]**

[1] School of Agriculture, Food and Wine, University of Adelaide, Adelaide, SA, Australia, [2] School of Agriculture and Food Sciences, University of Queensland, Brisbane, QLD, Australia, [3] School of Plant Biology and Institute of Agriculture, University of Western Australia, Crawley, WA, Australia, [4] South Australian Research and Development Institute, Urrbrae, SA, Australia, [5] New South Wales Department of Primary Industries, Tamworth, NSW, Australia

Ascochyta blight (AB) is a fungal disease that can significantly reduce chickpea production in Australia and other regions of the world. In this study, 69 chickpea genotypes were sequenced using whole genome re-sequencing (WGRS) methods. They included 48 Australian varieties differing in their resistance ranking to AB, 16 advanced breeding lines from the Australian chickpea breeding program, four landraces, and one accession representing the wild chickpea species *Cicer reticulatum*. More than 800,000 single nucleotide polymorphisms (SNPs) were identified. Population structure analysis revealed relatively narrow genetic diversity amongst recently released Australian varieties and two groups of varieties separated by the level of AB resistance. Several regions of the chickpea genome were under positive selection based on Tajima's *D* test. Both Fst genome- scan and genome-wide association studies (GWAS) identified a 100 kb region (AB4.1) on chromosome 4 that was significantly associated with AB resistance. The AB4.1 region co-located to a large QTL interval of 7 Mb∼30 Mb identified previously in three different mapping populations which were genotyped at relatively low density with SSR or SNP markers. The AB4.1 region was validated by GWAS in an additional collection of 132 advanced breeding lines from the Australian chickpea breeding program, genotyped with approximately 144,000 SNPs. The reduced level of nucleotide diversity and long extent of linkage disequilibrium also suggested the AB4.1 region may have gone through selective sweeps probably caused by selection of the AB resistance trait in breeding. In total, 12 predicted genes were located in the AB4.1 QTL region, including those annotated as: NBS-LRR receptor-like kinase, wall-associated kinase, zinc finger protein, and serine/threonine protein kinases. One significant SNP located in the conserved catalytic domain of a NBS-LRR receptor-like kinase led to amino acid substitution. Transcriptional analysis using qPCR showed that some predicted genes were significantly induced in resistant lines after inoculation compared to non-inoculated plants. This study demonstrates the power of combining WGRS data with relatively simple traits to rapidly develop "functional makers" for marker-assisted selection and genomic selection.

**Keywords: association study, re-sequencing, ascochyta blight, disease resistance, selective sweep, Fst, QTL**

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****Correspondence:***
*Tim Sutton*
*tim.sutton@sa.gov.au*

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## INTRODUCTION

Chickpea (*Cicer arietinum*) is one of the world's most important grain legumes providing protein and micronutrients for millions of people in developing countries. Chickpea is an important commodity crop in Australia with a total production of 0.7 million ton in 2012 (FAO, 2012) and is an essential rotation component of farming systems providing nutritional benefits through nitrogen fixation and disease break. There are two market types of chickpea: kabuli and desi which difference in seed color, seed shape, and flower color. Following India, Australia is the world's second largest producer of chickpea; much of the annual harvest is exported to the Indian sub-continent.

Ascochyta blight (AB) is caused by the fungal pathogen *Ascochyta rabiei* (Pass.) Labr. AB symptoms can occur in any parts of the plant above the ground at any growth stage depending on the availability of the pathogen and the right environmental conditions. Infection can lead to necrotic lesions on leaves, stem breakage and eventual death of the plant as well as pod abortion and seed staining (Pande et al., 2005). The chickpea growing area in Australia reduced from 260,000 ha in 1998 to 110,000 ha in 2006 largely due to lack of durable AB resistance in commercial varieties and loss of growers' confident in growing chickpea (FAO, 2012). A similar decline in chickpea production caused by the outbreak of AB has also occurred in Canada (Chandirasekaran et al., 2009), USA (Kaiser et al., 1994), and Latin America (Kaiser et al., 2000). *A. rabiei* is spread by wind and rain splash, can survive on infected stem for up to 20 months (Kaiser and Hannan, 1987) and in artificial conditions has also been shown to be pathogenic on cowpea, lentil, and field pea (Pande et al., 2005). AB can be effectively controlled via intensive fungicide application, implementation of crop rotation strategies and seed treatment; however, using varieties with improved resistance remains one of the most cost-effective ways to manage AB in chickpea. The first Australian cultivar with improved resistance to AB compared to current varieties at the time, was Howzat released in 2001, followed by, Flipper, Yorker, and the most significant improvement with Genesis090 in 2005. As a result of selective breeding for AB resistance in chickpea, current varieties that make up the majority of annual chickpea production in Australia are rated as moderately resistant or resistant although loss of resistance was observed in a number of cultivars in 2016 (SA Sowing Guide 2017).

Using conventional breeding methods, considerable progress has been made towards the improvement of AB resistance in chickpea varieties (Pande et al., 2005). The application of marker-assisted breeding has recently gained in momentum due to the fast declining cost of genotyping/sequencing technologies and the emergence of high-throughput automatic technology. Using traditional bi-parental populations, several QTL for AB resistance have been identified on linkage groups LG2 (Udupa and Baum, 2003; Cho et al., 2004), LG3 (Tar'an et al., 2007), LG4 (Lichtenzveig et al., 2006; Tar'an et al., 2007; Sabbavarapu et al., 2013; Stephens et al., 2014), LG5 (Sabbavarapu et al., 2013), LG6 (Tar'an et al., 2007; Sabbavarapu et al., 2013), and LG8 (Lichtenzveig et al., 2006). One major QTL and/or one minor QTL have been repeatedly reported in a similar region of LG4 across several studies and therefore make this locus a good candidate region for improving AB resistance in chickpea (Lichtenzveig et al., 2006; Tar'an et al., 2007; Sabbavarapu et al., 2013; Stephens et al., 2014). Madrid et al., have identified an AB resistance gene, ethylene receptor (*ETR-1*), located near the peak of the major QTL in LG4 flanked by markers NCPGR91 and GAA47 (Madrid et al., 2013). Transcriptional profiling using 756 microarray features identified 95 candidate genes differentially expressed during *A. rabiei* infection in four chickpea genotypes (Coram and Pang, 2006). However, the *ETR-1* candidate characterized by Madrid et al. (2013) was not identified as being differentially expressed in the study by Coram and Pang (2006). A recent published study by Leo et al. (2016) examined the expression profiles of seventeen candidate genes. This work showed that six genes were differentially expressed across ten host genotypes under AB infection; however, their expression levels did not correlate well with the resistance classification of the lines suggesting that they might have a minor role in AB resistance and hence further research is warranted.

Next-generation sequencing technology can provide a relatively cheap and high-throughput genotyping option to discover genome variation and identify selection signatures in crop species such as chickpea (Varshney et al., 2013). Genotyping using molecular markers has been one of the cornerstone developments in modern plant breeding. There are now many methods that utilize NGS for genotyping, such as reduced genome representation sequencing methods like RAD-seq, GBS, and whole-genome resequencing (WGRS) (Davey et al., 2011; Elshire et al., 2011). These methods have different advantages and disadvantages and thus are suitable for different applications. Compared to WGRS, RAD-seq, and GBS are cheaper as they sample only a fraction of the genome. Thus, these methods are suitable for large scale genotyping of crops with large genome sizes, for example, genotyping a large number of F2 or advanced lines in a breeding program. In contrast, WGRS is more suited to pre-breeding activities where smaller number of key elite parents, landraces and wild species need to be studied carefully for genome variation (SNPs, CNV, structural variation) and association studies (Li et al., 2011). The cost of sequencing has dropped rapidly in the last decade, however, the major cost and time consuming part of NGS remaining is library preparation (Rohland and Reich, 2012). Fortunately, automation of library preparation using liquid-handling robotic stations is developing rapidly and now available from several commercial companies. Many WGRS studies have been reported in crop species such as rice (Huang et al., 2012; Wang et al., 2014), sorghum (Mace et al., 2013), tomato (Lin et al., 2014), and chickpea (Lake et al., 2016; Sadras et al., 2016; Thudi et al., 2016). One of the common findings in these studies is the marked reduction of genomic variation during domestication and/or breeder's selection.

The first objective of this study was to investigate the effect of selective breeding (AB resistance) on genetic diversity and population structure of the Australian chickpea breeding program over the last four decades using WGRS approaches. Secondly was to identify candidate genes involved in AB resistance associated with a major QTL on chromosome 4 using Fst genome-scan and genome-wide association mapping

approaches. Finally, results were validated using an independant set of chickpea germplasm and qPCR analysis.

# MATERIALS AND METHODS

## Plant Materials and Sequencing

In this study, the plant materials include 48 chickpea varieties released in Australia from 1978 to 2016, 16 advanced breeding lines, four landraces, and one wild chickpea *C. reticulatum* (Supplementary Table S1). The released varieties and advanced breeding lines are a good representation of the genetic diversity present in the Australian chickpea breeding program. The wild species *C. reticulatum* and landraces serve as a reference point for investigating genetic diversity. DNA was extracted from young leaf using Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. Pair-end sequencing libraries were constructed for each genotype with insert sizes of ∼500 bp using TruSeq library kit according to the Illumina manufacturer's instruction. Around 40 million 150 bp paired-end reads for each genotype were generated by the Australian Genome Research Facility in Brisbane, Australia using Illumina HiSeq 2000 platform. Sequence data is available from the NCBI Short Read Archive under BioProject accession PRJNA375953.

## Population Genomics Analysis

Paired-end reads for each genotype were trimmed, filtered, and mapped to the kabuli reference genome 1.0 using SOAP2 (Li et al., 2009). SNPs were called using the SGSautoSNP pipeline (Lorenc et al., 2012). The BAM files of each cultivar were separated into 16 AB resistant and 24 susceptible genotypes as two contrasting groups to obtain sample allele frequencies (SAF file) which is the probability of all read data given the sample allele frequency using the software ANGSD (Korneliussen et al., 2014). The resulting two SAF files of the two contrasting groups were used to estimate joint distribution of sample allele frequencies (2D-SFS) which was used as prior together with the two SAF files in Fst estimation using software ngsPopGen (Fumagalli et al., 2013). To reduce the effect of sampling error, Fst values of each site (SNPs) within a 100 kb non-overlapping window were averaged. The whole genome was scanned to identify regions with extreme population genetic differentiation (large Fst value compared to the surrounding region) which could be served as an indicator of selection signature. The rationale is that genetic differentiation between groups at a given neutral locus is determined by stochastic random factors such as genetic drift. If a locus is under natural or artificial selection, the pattern of genetic differentiation may change. For example, regions showing uncommon large amounts of genetic differentiation (difference alleles are fixed in different groups) may have undergone diversifying selection.

To correct errors in NGS data, allele frequencies were estimated using site frequency spectrum (SFS) as prior to improve inference of population genetic parameters ($\theta\pi$, $\theta w$, and Tajima's *D*) using the software ANGSD (Korneliussen et al., 2014). Nucleotide diversity ($\theta\pi$) was calculated separately for 16 AB resistant and 24 susceptible genotypes. The resistant genotypes were released after 2005 while the susceptible genotypes were released before 2005 except for GenesisKalkee and PBAPistol. To investigate directional and balancing selection in the chickpea genome, the SFS based neutrality test Tajima's *D* was calculated in 100 kb non-overlapping windows using the 69 genotypes (Korneliussen et al., 2013).

The relationship of the 69 genotypes was visualized using principal components analysis (PCA) implemented in ngsPopGen, a modification of Patterson's approach of PCA where SFS was incorporated to reduce uncertainty of genotype calling (Fumagalli et al., 2014).

Genome-wide association studies was performed using 59 genotypes with AB resistance data obtained from the Australian chickpea breeding program from evaluation over multiple years and locations. Mixed linear models (MLM) that implemented in the software GAPIT was used to evaluate the effects of each ∼250,000 SNPs (MAF > 5%) individually, adjusting for confounding effect such as population structure and kinship (Lipka et al., 2012). In order to speed up the computation time, the kinship matrix was compressed to its optimum groups and P3D method (population parameters previously determined) was used. The MLM can be written as:

$$y = 1\beta_1 + \boldsymbol{X}_{SNP}\beta_{SNP} + \boldsymbol{Q}_{PCA}V_{PCA} + \boldsymbol{Z}_{GENOTYPE}\gamma_{GENOTYPE} + \varepsilon, \quad (1)$$

where $y$ is the $n \times 1$ vector of AB scores, $1$ denotes a $n \times 1$ vector of 1s and $\beta_1$ is the intercept, $\boldsymbol{X}_{SNP}$ $(n \times p)$ is design matrix for the fixed effects of SNPs, $\boldsymbol{Z}_{GENOTYPE}$ $(n \times h)$ is the corresponding design matrix for the random effects of genotype, $\boldsymbol{Q}_{PCA}$ is design matrix for the fixed effects of population structure. The random genotype effect was similarly assumed to follow a normal distribution, $\gamma_{GENOTYPE} \sim N(0, \boldsymbol{K}\sigma^2_g)$, where $\boldsymbol{K}$ was the estimated kinship matrix and $\sigma^2_g$ the variance component due to genotype. To account for kinship in the estimation of random genotype effects, $\gamma_{GENOTYPE}$, the design matrix $\boldsymbol{Z}_{GENOTYPE}$ was multiplied by the cholesky-root of the kinship matrix. The residual error vector $\varepsilon$ $(n \times 1)$ was assumed to comprise independent and identically distributed random normal errors with mean of 0 and variance $\sigma^2$, $\varepsilon \sim N(0, \boldsymbol{I}\sigma^2)$.

The significant *p*-value cut-off was set as 3. 47E-04. Setting a *p*-value cut-off as 2.00E-07 (0.05/250,000) using the Bonferroni correction is too conservative for a pilot study with a relative small sample size like the current study. Besides, Bonferroni correction assumes the test variables are independent whereas SNPs are not independent due to Linkage disequilibrium (LD). Therefore, a modified Bonferroni correction was used in this study; an alpha level of 0.05 is divided by the number of independent segments of the genome (instead of the number of tested SNPs) which is calculated from the average decay of LD in this germplasm. The average decay of LD in this study ($r^2 = 0.2$) is 5,062 kb, given the chickpea genome size of 730,000 kb, the number of independent segments of the genome in this germplasm is 144. Therefore, the p-value cut-off was set as 0.05/144 which is 3. 47E-04. The circular representation of the chickpea genome was generated using software CIRCOS (Krzywinski et al., 2009)

**TABLE 1 | Summary of linkage disequilibrium (LD) and single nucleotide polymorphisms (SNPs) used to estimate LD.**

| Chromosome | No. SNPs in 69 genotypes[1] | No. SNPs in 68 genotypes[2] | No. SNP used to estimated LD | Mean $r^2$ | LD decay (kb) |
|---|---|---|---|---|---|
| Ca1 | 110,295 | 69,424 | 3,386 | 0.18 | 2,000 |
| Ca2 | 75,410 | 40,404 | 2,667 | 0.10 | 1,500 |
| Ca3 | 105,954 | 42,213 | 1,444 | 0.15 | 4,800 |
| Ca4 | 170,747 | 118,778 | 4,092 | 0.28 | 23,000 |
| Ca5 | 112,194 | 46,770 | 1,457 | 0.07 | 1,000 |
| Ca6 | 130,732 | 68,481 | 2,276 | 0.10 | 2,500 |
| Ca7 | 93,455 | 44,933 | 2,316 | 0.16 | 5,200 |
| Ca8 | 28,624 | 13,356 | 267 | 0.08 | 500 |
| Total/average | 827,411 | 444,359 | 17,905 | 0.14 | 5,062 |

[1]*68 Cicer arietinum plus one Cicer reticulatum*
[2]*68 Cicer arietinum*

**TABLE 2 | Genetic diversity of the 69 genotypes.**

| Germplasm | No. genotypes | No. SNPs | $\theta_\pi(10^{-4})$ |
|---|---|---|---|
| Varieties+advanced Lines+landraces+wild | 69 | 827,411 | 1.07 |
| Varieties+advanced Lines+landraces | 68 | 451,546 | 0.83 |
| Varieties+advanced lines | 64 | 429,810 | 0.81 |
| Varieties | 47 | 312,955 | 0.73 |
| –Released during 1978–2004 (AB susceptible predominantly) | 21 | 233,059 | 0.78 |
| –Released during 2005–2013 (AB resistant predominantly) | 16 | 162,748 | 0.59 |

## GWAS Validation

A panel of 132 advanced chickpea lines from diverse backgrounds was used for validation. In order to evaluate AB resistance, the 132 advanced lines were grown with RCBD design and replicated two times in pots with four plants in an open area enclosed by a net to avoid animal damage. The seedlings were inoculated to run off with a single conidium-derived *A. rabiei* isolate (FT 13092-1, at a concentration of $1 \times 10^6$ spores/ml) when plants were 5 weeks old. This isolate which belongs to pathotype IV was collected in 2013 from Genesis 090 chickpea (one of the tested lines) in a trial at Kingsford Research Station, South Australia. Plants were kept with an optimal moisture level by mist irrigation. Three weeks after inoculation, AB resistance scores were measured by carefully examining the level of damage on leaves and stems of each plant using with a disease rating scale of 1–9 modified from Singh et al. (1981). The 132 advanced chickpea lines were sequenced and SNPs were called in the same way as the 69 genotypes described above. GWAS was also performed in the same way as the 59 genotypes using GAPIT.

## Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed on six chickpea lines of differing AB resistance (PBAPistol, DICC8191, PBAMonarch, ICC3996, ICC12004, DICC8218) from the panel of 132 advanced lines under the condition of with and without (mock-treated) *A. rabiei* inoculation. Leaf tissues of the six genotypes (5 weeks old stage) were collected 24 and 48 h after inoculation with six biological replicates taken. RNA was isolated and purified using Direct-zol RNA Miniprep according to the manufacturer's instructions. cDNA synthesis was carried out using SuperScript® IV Reverse Transcriptase (Life Technologies). The cDNA samples were diluted 20 times in MQ H2O. Three replicate PCRs for each of the samples were included in every run containing: 2 μL of cDNA solution (or the diluted standard, or water), 5 μL Kapa Sybr Fast Universal 2X qPCR Master Mix (Geneworks), 1.2 μL of each of the forward and reverse primers (Supplementary Table S2) at 4 μM and 0.6 μL of water. The total volume of the PCR reactions was 10 μL. Reactions were performed in QuantStudio6 (Life Technologies): 3 min at 95°C followed by 40 cycles of 3 second at 95°C, 20 s at 60°C, fluorescent acquisition at 60°C. Followed by melt curve analysis: 15 s at 95°C, 1 min at 60°C then increase temperature from 60°C to 95°C with fluorescence readings acquired at 0.5°C increments. Three reference genes (HSP90, EF1a, GAPDH), determined to be expressed consistently previously, were used to normalize the expression level of candidate genes (Garg et al., 2010).

# RESULTS

## Genome Variation

Sixty-nine chickpea genotypes were sequenced using WGRS methods. They included 47 chickpea varieties released in Australia from 1978 to 2013, 17 advanced breeding lines, four landraces and one accession representing the wild chickpea species *Cicer reticulatum* (Supplementary Table S1). In total, approximately 0.9 billion Illumina paired-end reads (150 bp) from 69 genotypes were mapped to the kabuli reference genome 2.6.2. The mapping depth ranged from 0.64× to 10.37× with a mean of 3.35×. For the 69 genotypes, 827,411 SNPs ranging from 170,747 on Ca4 to 28,624 on Ca8 were discovered (**Table 1**). However, when the *C. reticulatum* accession PI48977 was removed from analysis the total number of SNPs dropped to 444,359, while θπ dropped from $1.07 \times 10^{-4}$ to $0.83 \times 10^{-4}$ (**Table 2**). Further, excluding the four landraces from the analysis (leaving 64 varieties and advanced breeding lines), the total number of SNPs only dropped from 451,546 to 429,810, while θπ

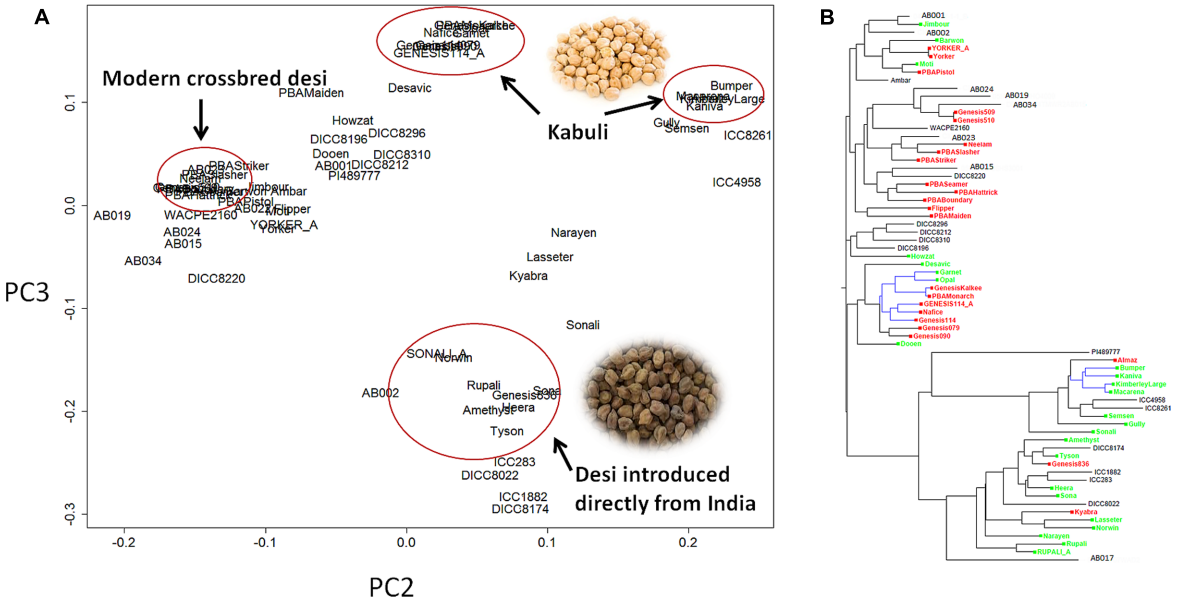


**FIGURE 1 | Population structure of 69 chickpea genotypes based on single nucleotide polymorphism (SNP) data. (A)** Principle component analysis (PCA). **(B)** Phylogenetic tree. Varieties released after 2005 [mainly ascochyta blight (AB) resistant] are highlighted in red, varieties released before 2005 (mainly AB susceptible) are highlighted in green, the remainder are advanced lines or landraces. The tree branches highlighted in blue are kabuli, whereas the rest are desi.

dropped from $0.83 \times 10^{-4}$ to $0.81 \times 10^{-4}$, which indicated that the varieties and advanced breeding lines represented most of the genetic diversity present in the landraces included in this study. When the collection of 47 varieties was grouped into release dates from 1978-2004 (predominantly AB susceptible) and 2005–2013 (predominantly AB resistant), it was shown that the latter represented a lower level of genetic diversity (**Table 2**). LD was estimated using 17,905 high-confident SNPs with minimum coverage of five reads. The $r^2$ on each chromosome ranged from 0.07 to 0.28 with an average of 0.14 (**Table 1**). Setting $r^2$ cut-off as 0.2, LD decay ranged from 500 to 23,000 kb with an average of 5,062 kb (**Table 1** and Supplementary Figures S1A–H).

## Population Structure

Principle component analysis (PCA) showed that the *C. reticulatum* accession PI48977 separated clearly from *C. arietinum* under PC1 vs. PC2 (Supplementary Figure S2). In PC2 vs. PC3, there were two distinct groups of kabuli chickpea whereas the relationship of desi chickpea was more complex (**Figure 1**). One group of kabuli mainly contained the Genesis$^{TM}$ series introduced to Australia from ICARDA (International Center for Agricultural Research in the Dry Areas). The other grouping of kabuli mainly contained older released varieties dating back to the 1980's, with unknown origin. The desi types were generally separated from the kabuli types with a few exceptions (Gully, Semsen). One group of desi type contains lines introduced directly from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) and their progeny. This group includes some old Australian cultivars such as Tyson, Amethyst, Sona, Heera, and Norwin. Rupali and Sonali, derived from Amethyst and Tyson, respectively, also belong to this group and have gone through a pollen selection process at low temperature aimed at developing chilling tolerant varieties. Another group of desi lines, containing modern variety releases from the Australia chickpea breeding program, cluster closely together and have very narrow genetic diversity (**Figure 1** and **Table 2**). In fact, most of the recently released desi varieties (PBAMaiden, PBAStriker, PBABoundary, PBASlasher, PBAHattrick, PBASeamer, Neelam, and Ambar) have their pedigree traced back to ICC3996, ICC14903, and ICC13729; three AB resistant lines from Iran. The Phylogenetic tree was in agreement with the PCA in general. Varieties released prior to and after 2005 were separated into two distinct groups. The significant outbreak of AB in Australia in the late 1990s that led to rapid decline in area sown to chickpea initiated the rapid prioritization of breeding for improved ascochyta resistance (Pande et al., 2005). As such, varieties released after 2005 were predominantly AB resistant and varieties released before 2005 were predominantly AB susceptible.

## Selection Signature and AB Resistance

Both natural and artificial selection shape the chickpea genome, and methods such as Tajima's D have been widely used to detect selection signatures in genomes (Qanbari and Simianer, 2014). To avoid biased estimation of allele frequency using low depth NGS data, Tajima's *D* was calculated using an empirical Bayes approach (Korneliussen et al., 2013). Tajima's *D* showed that 4.74% of the genome was under balancing selection ($D > 2$) while 0.66% of the genome was under purifying selection ($D < -2$, **Figure 2**). Chromosome 1 had the largest proportion (11.22%) of genome under

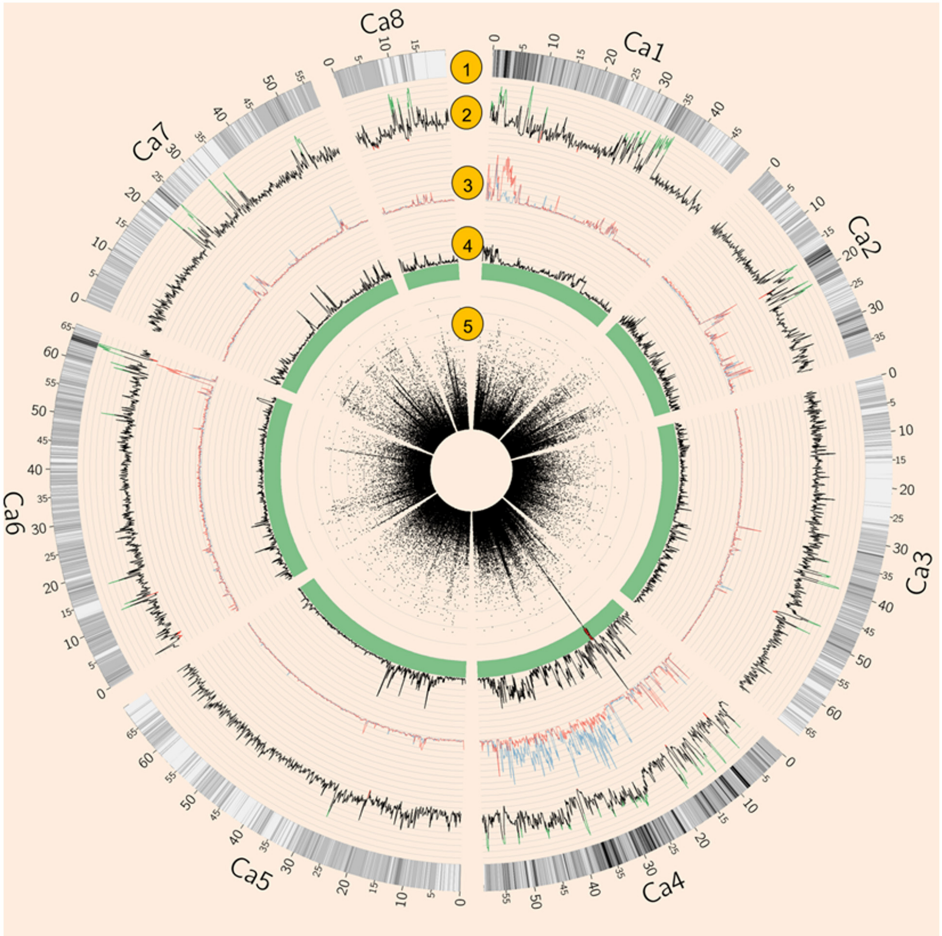


**FIGURE 2 | Summary of whole genome re-sequencing (WGRS) data.** (1) SNP density. (2) Tajima's *D* of 64 Australian varieties and four Indian landraces. Values above 2 are highlighted in green while values below –2 are in red. (3) Nucleotide diversity ($\theta\pi$) of AB susceptible (blue) and resistant (red) varieties. (4) Fst of AB susceptible versus resistant varieties. (5) Circular Manhattan plot of genome-wide association studies (GWAS) result. Each black dot represents a SNP, Red dots represent SNPs with *p*-values lower than 3. 47E-04 (equal to 0.05 with modified Bonferroni correction).

balancing selection whereas chromosome 5 had the least (0.14%). Chromosome 8 had the largest proportion (2.5%) of the genome under purifying selection, whereas none was detected on chromosome 7 (**Figure 2** and Supplementary Table S3).

There were 730 predicted genes under balancing selection ($D > 2$) of which 427 genes have been deposited in the KEGG database[1] and classified into ten functional categories: genetic information processing (210), environmental information processing (42), carbohydrate metabolism (26), enzyme families (18), amino acid metabolism (16), cellular processes (15), lipid metabolism (15), energy metabolism (12), other categories (49), and unclassified (24). We observed 21 NBS-LRR genes and 98 receptor-like kinases (RLK) under balancing selection, comprising 16.3% of total genes under balancing selection. NBS-LRR and RLK are well known classes of resistance genes in plants and a target of balancing selection (McDowell et al., 1998). However, we did not find any pathway enriched with genes under selection using the web-based software KOBAS (Wu et al., 2006).

There were 171 predicted genes under purifying selection ($D < -2$), of which 90 genes have been deposited in the KEGG database and classified into ten functional categories: genetic information processing (35), environmental information processing (10), carbohydrate metabolism (9), amino acid metabolism (5), cellular processes (9), nucleotide metabolism (3), Metabolism of terpenoids and polyketide (3), lipid metabolism (3), other categories (8), and unclassified (4). Three genes under purifying selection (beta-amyrin 11-oxidase, gibberellin 2-beta-dioxygenase, transcription factor *PIF3*) are involved in gibberellic acid biosynthesis and signal transduction. Additionally, two genes *AUX/IAA* and *JAZ* were involved in auxin and jasmonic acid signal transduction, respectively. However, we did not find any pathways enriched with genes under selection using the web-based software KOBAS (Wu et al., 2006). In contrast to the large proportion of NBS-LRR and RLK candidate genes observed

[1]http://www.genome.jp/kegg/

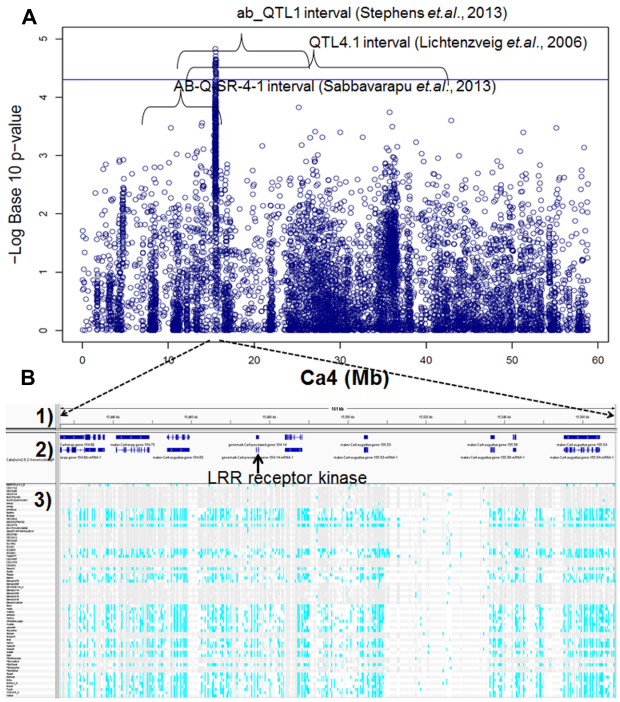


**FIGURE 3 | (A)** Manhattan plot of GWAS result on Ca4. Twenty SNPs, significantly associated with AB resistance, were located within three AB resistant QTL intervals discovered previously by bi-parental mapping. **(B)** Predicted genes and SNPs in AB4.1 associated with AB resistance. (1) Physical position. (2) Predicted genes. (3) SNPs of 69 genotypes in AB4.1, gray represents reference allele, light blue represents alternative allele, and white represents missing.

under balancing selection, a single NBS-LRR gene and four RLK genes were identified under purifying selection.

Genome-wide association studies identified 20 SNPs significantly ($p < 0.001$) associated with AB resistance explaining 19.8–21.8% phenotypic variation (**Figure 2** and Supplementary Table S5). These SNPs in high LD were all clustered into a peak on chromosome 4 (Ca4: 15,855, 018..15,980,584), called AB4.1 hereafter. In total, 12 predicted genes were located in the AB4.1 region including one LRR receptor-like kinase (Ca_05515), one wall-associated kinase (Ca_05520), one zinc finger protein (Ca_05511), one cysteine-rich receptor-like kinase (Ca_05516), four serine/threonine protein kinases (Ca_05517, Ca_05521, Ca_05522, and Ca_05523) and five uncharacterized proteins (Ca_05512, Ca_05513, Ca_05514, Ca_05518, and Ca_05519, **Figure 3** and Supplementary Table S4). One significant SNP (Ca4: 15,920,939), located in the conserved catalytic domain of the LRR receptor-like kinase (Ca_05515), led to an amino acid substitution (Gly/Ala, **Figure 4**). All other significant SNPs were located in non-coding regions of the genes. The chickpea genome was scanned to identify selection signatures of AB resistance using the Fst outlier-based approach. A sliding window of 100 kb was used to minimize the effect of sampling error. Fst compares the variance of allele frequencies within and between ABS and ABR groups. The distribution of Fst was highly skewed toward 0, but ranged from 0 to 0.84 across the whole genome (Supplementary Figure S3). Chromosome 4 had the largest average Fst (0.14) while Chromosome 6 had the smallest average Fst (0.03). The region with the largest Fst (0.84) was located on chromosome four spaning 100 kb (Ca4:15,801,345..15,901,345) which overlapped with the AB4.1 region detected with GWAS (**Figure 2**). Moreover, this region was found to be under balancing selection using Tajima's D statistic (**Figure 2**). The average nucleotide diversity ($\theta\pi$) of AB susceptible lines across the whole genome was similar to that of the 18 AB resistant lines. The pattern of nucleotide diversity distribution was similar in the two groups except on Ca4 where almost half of the chromosome (starting from 23 to 50 Mb) showed a remarkably reduced level of nucleotide diversity in AB resistant lines compared to AB susceptible lines (1.14E-05 vs. 4.5E-05). Additionally, the extent of LD observed on chromosome 4 was 23,000 kb ($r^2 = 0.2$ cut-off) which was approximately 50 times larger than that observed on chromosome 8 (**Table 1** and Supplementary Figure S2). This indicates the occurrence of selective sweeps, possibly resulting from selection of AB resistance in the Australian chickpea breeding program.

In order to validate the GWAS results based on 59 released varieties, we screened a distinct set of germplasm, comprised of 132 advanced lines for AB resistance. We observed large variation in AB resistance ($p < 0.0001$) in this germplasm, ranging from almost without damage to completely dead (Supplementary Figure S4). These 132 advanced lines were subjected to WGRS and ∼144,000 SNPs were discovered in the same manner described for the 69 genotypes. Combining SNP data with AB resistance data, GWAS identified one SNP, significantly ($p$-value = 2.40E-07) associated with AB resistance was located at a position (Ca4:15,768,013) approximately 87 kb from AB4.1 (**Figure 5**). The 20 significant SNPs present in the 59 varieties were not present in the 132 advanced lines probably due to lack of reads mapped to these 20 SNP regions. The LD surrounding the AB4.1 was very high ($r^2 > 0.9$), thus it is very likely that the significant SNP in the validation set was linked to AB4.1.

In order to study the function of the 12 predicted genes located in the AB4.1 region, transcriptome analysis using qPCR was performed on six chickpea lines of differing AB resistance (PBAPistol, DICC8191, PBAMonarch, ICC3996, ICC12004, DICC8218) from the panel of 132 advanced lines. Plants were grown both with and without *A. rabiei* inoculation. Eleven of the 12 predicted genes were successfully amplified. The expression level of the 11 predicted genes was generally induced by *A. rabiei* inoculation, with some lines induced more than others (Supplementary Figures S5–S15). In some predicted genes, a change of expression generally followed the resistance level of the lines. For example, for one serine/threonine receptor-like kinase (Ca_05521), expression increased approximately 6- and 3-fold in resistant lines ICC3996 and ICC12004, respectively, 24 h post inoculation compared to mock treated plants whereas there was no significant difference in susceptible and moderately susceptible lines PBAPistol, DICC8191, and PBAMonarch (**Figure 6**). Notably, the expression level of Ca_05521 in PBAPistol became significant 48 h after inoculation

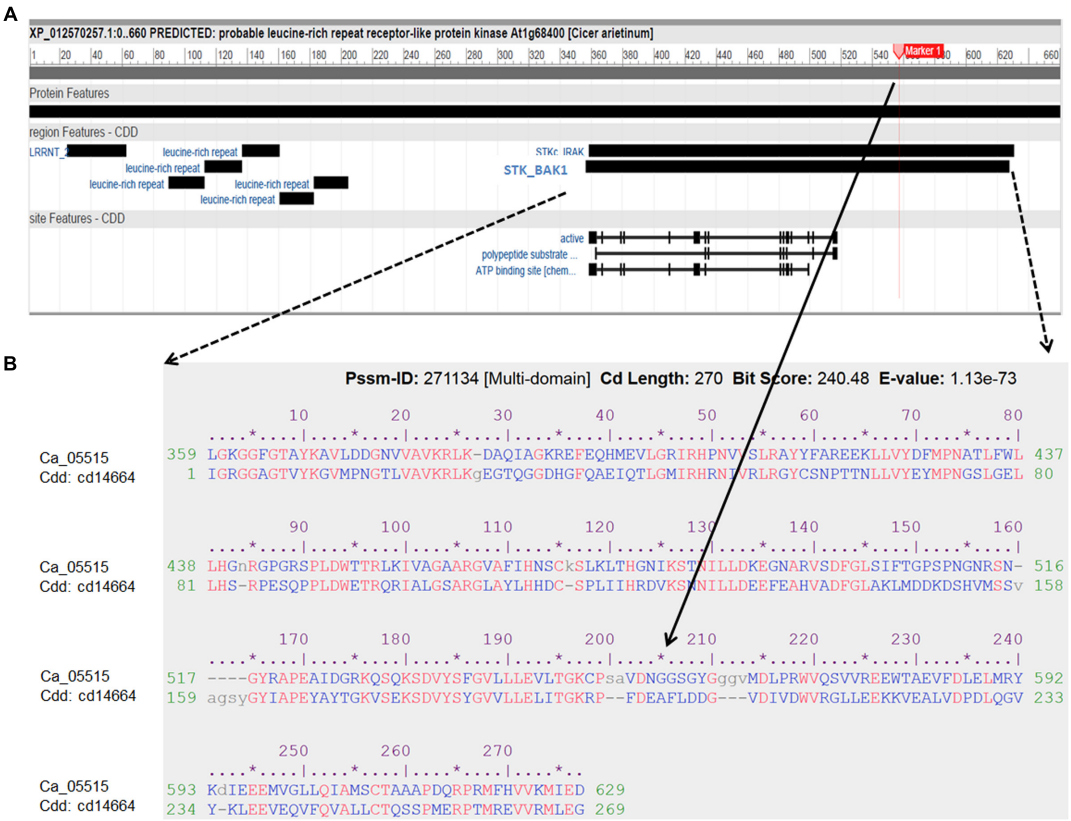


**FIGURE 4 | Characterization of the LRR receptor-like kinase (Ca_05515/XP012570257) and its conserved domains cd14664. (A)** Schematic diagram of the protein features of the LRR receptor-like kinase. Black boxes indicated specific conserved domain regions. LRRNT: N-terminal domain of LRR; STK_BAK1: kinases domain of brassinosteroid (BR)-associated kinase (BAK1). **(B)** Alignment of amino acid sequence of the LRR receptor-like kinase with the best hit conserved domain STK_BAK1. The SNP (Ca4: 15,920,939) with significant association with AB resistance, located in the STK_BAK1 leading to amino acid substitution (Gly/Ala), is highlighted as Marker 1 and indicated in the alignment.

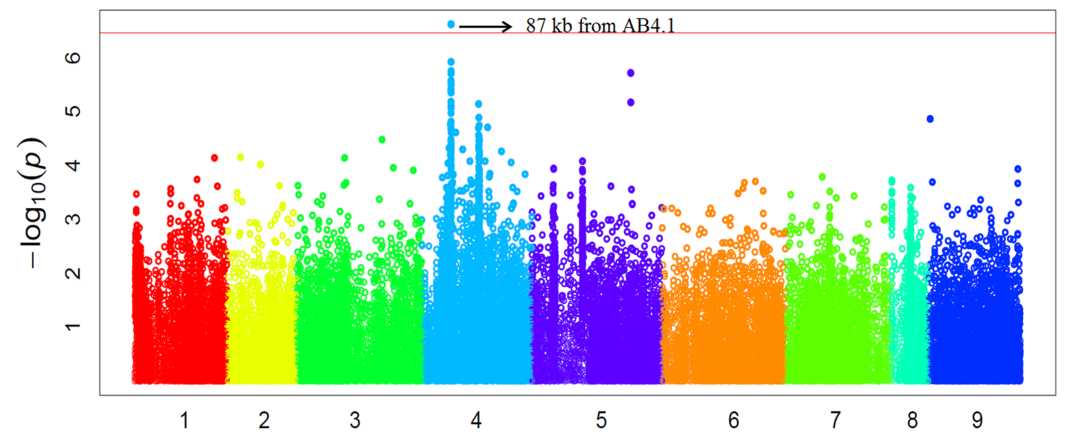


**FIGURE 5 | Manhattan plot of GWAS validation using 132 advanced lines.** The physical location of SNPs are in order according to chromosome number 1 to 8 while 9 represents all unassembled contigs. The red line is the significant threshold of *p*-value = 3.47E-07, equal to a level of 0.05 after Bonferroni correction. A SNP (Ca4:15,768,013) above the threshold located approximately 87 kb from AB4.1 is indicated.

while in DICC8191, and PBAMonarch the change remained not significant. Interestingly, the expression of a LRR receptor-like kinase gene (Ca_05515) was not significantly induced in all six lines 24 h after inoculation, whereas it was significantly induced in the three resistant and one susceptible line DICC8191 at 48 h after inoculation (**Figure 7**).

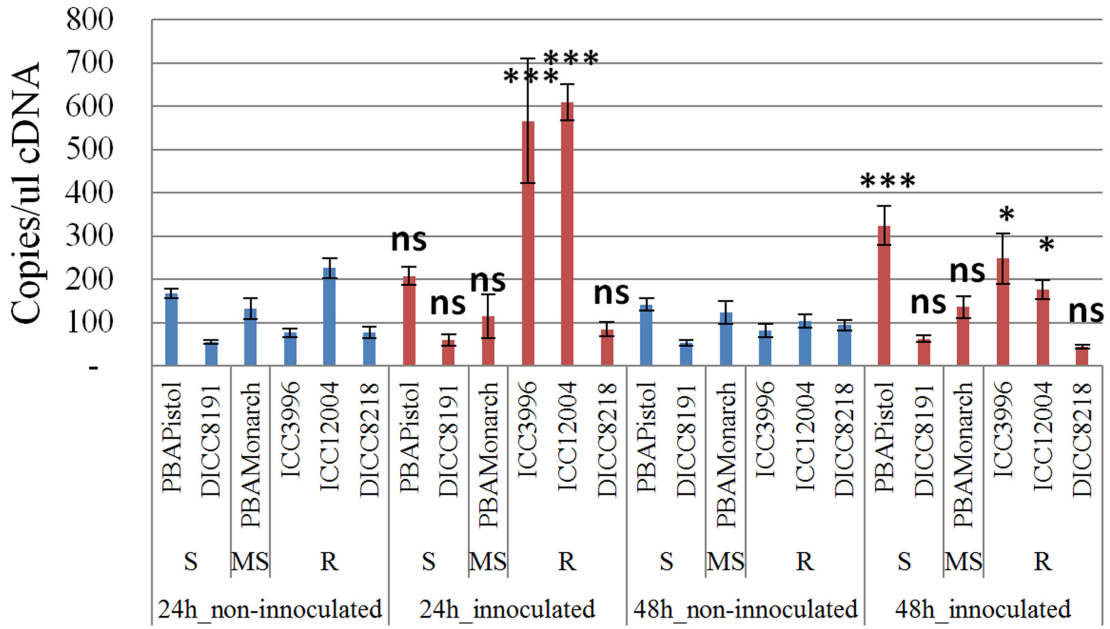


**FIGURE 6 | Transcription analysis (qPCR) of predicted gene serine/threonine receptor-like kinase (Ca_05521) in AB4.1 region with six chickpea lines at two time points.** Blue: non-inoculated; red: inoculated with *Ascochyta rabiei*. S: Susceptible; MS: Medium Susceptible; R: Resistant. Significant difference between inoculated and non-inoculated lines are shown as ***$p$-value <0.001, *$p$-value < 0.05. ns: non-significant

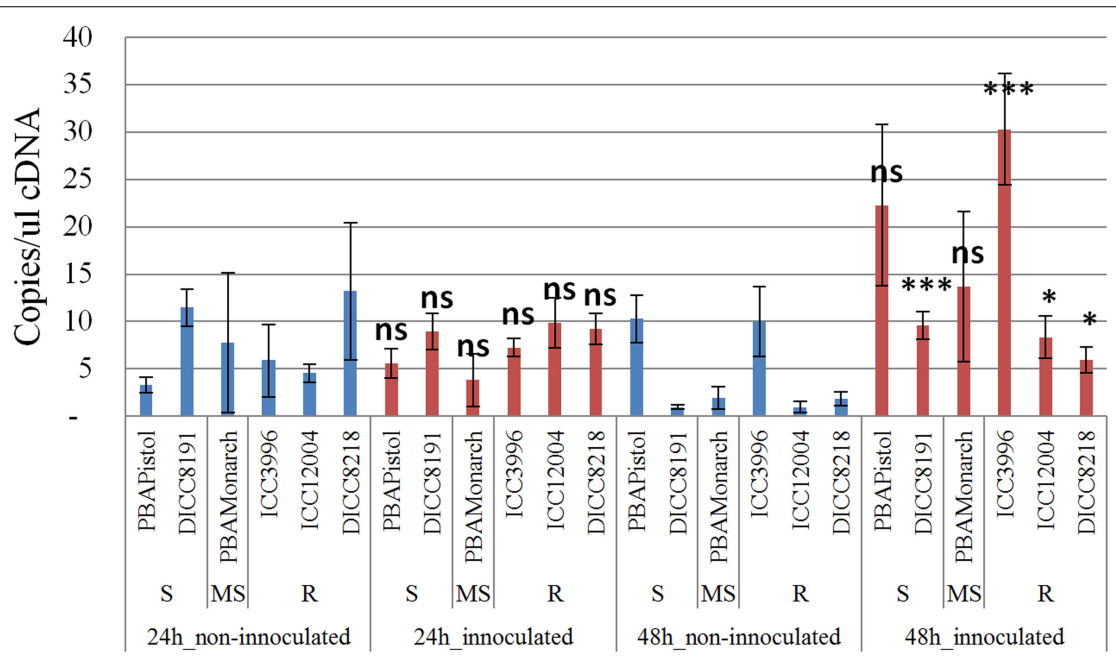


**FIGURE 7 | Transcription analysis (qPCR) of predicted gene LRR receptor-like kinase (Ca_05515) located in AB4.1 region with six chickpea lines at two time points.** Blue: non-inoculated; red: inoculated with *A. rabiei*. S: Susceptible; MS: Medium Susceptible; R: Resistant. Significant difference between inoculated and non-inoculated lines are shown as ***$p$-value < 0.001, *$p$-value < 0.05. ns: non-significant

## DISCUSSION

Ascochyta blight, caused by *A. rabiei* is a significant fungal disease of pulses worldwide. The outbreak of AB in Australia in the late 1990s reduced chickpea production significantly and drove a marked shift in the cultivation of chickpea from southern Australia into the northern Australian growing regions of NSW and southern Queensland. Similar to other fungal

diseases, AB can be managed using different strategies which include crop rotation, pre-sowing seed fungicide treatment, in crop foliar fungicide treatment and the adoption of AB resistant or moderately resistant varieties. The latter has been a focus of chickpea breeding in Australia since the AB epidemic of the late 1990s and AB resistance is now considered an essential trait for new variety development. Genotypic analysis revealed a low level of genetic diversity among recent varieties (post 2005), an observation explained in part by the relatively narrow genetic base of breeding material in Australia. In fact, most Australian desi varieties can have part of their pedigree traced back to three Iranian landraces ICC3996, ICC14903, and ICC13729. Genetic diversity is vital to all crop improvement programs and efforts to find new sources of AB resistance and develop molecular tools to support empirical breeding is a priority for chickpea breeding in Australia. Several past studies have utilized the genetic diversity of wild species in chickpea to improve AB resistance. *C. reticulatum*, a close relative wild species of *C. arietinum*, showed much higher genetic variability compared to *C. arietinum* in this study. Sources of resistance have been found in *C. bijugum*, *C. echinospermum*, and *C. reticulatum* (Collard et al., 2001). However, it can be challenging to efficiently incorporate these novel sources of resistance into breeding programs; using the latest technologies such as the NGS method employed in this study can help to improve the efficiency of this process.

The genetic basis of AB resistance in chickpea has been previously investigated and QTL explaining resistance identified in bi-parental mapping populations have been reported (Pande et al., 2005; Li et al., 2015). However, the large size of the QTL regions identified (up to 30 Mb physical size) has limited their application in maker-assisted selection due to disassociation of linked markers from the resistance locus through recombination, and linkage drag which can cause unexpected genetic background effects (Mackay, 2001; Jannink, 2007; Collard and Mackill, 2008). WGRS approaches have the advantage that they can unbiasedly identify hundreds of 1000s of sequences variants (SNPs, Indels, CNVs) in a cost-effective manner. This is particularly relevant in a species with a relative small genome such as chickpea. Compared to other marker technologies such as SSRs, the mapping resolution achieved with WGRS approaches can be reached to the QTN (Quantitative Trait Nucleotide) level, which can potentially result in the detection of genetic variants in the actual gene sequence controlling a trait of interest.

In this study, we have refined the physical size of an AB resistance QTL on chromosome 4 previously identified in three independent RIL populations, to approximately 100 kb (AB4.1) and containing 12 predicted genes (**Figure 3**). The first study, using 120 RIL lines (Hadas × ICC5810) and SSRs, identified an AB resistance QTL with 14.4% explained phenotypic variation spaning around 30 Mb between marker H3C041 and TA2 (Lichtenzveig et al., 2006); The second study, using 188 F2 individuals (C 214 × ILC 3279) and 69 polymorphic SSRs, likely identified the same AB resistance QTL with 31.9% explained phenotypic variation spaning around 7 Mb between marker STMS11 and TA130 (Sabbavarapu et al., 2013); The third study, using 150 RIL lines (Lasseter × ICC3996) and 504 polymorphic SSRs and SNPs, mapped a QTL to the same AB resistance locus with 14–45% explained phenotypic variation spaning around 13 Mb between markers SSR TA146 and SNP_40000185 (Stephens et al., 2014).

In this study, 12 predicted genes were located in the AB4.1 region, including one LRR receptor-like kinase, one wall-associated kinase, one zinc finger protein, one cysteine-rich RLK and four serine/threonine RLK. The nucleotide-binding site leucine-rich repeat (NBS-LRR) family of proteins is one of the largest classes of resistance (R-genes) genes in plants with documented roles in defense signaling and pathogen recognition (Afzal et al., 2008; Ameline-Torregrosa et al., 2008; Mace et al., 2014). The LRR domain, characterized by the consensus amino acid sequence LxxLxLxxNxLxx, is likely involved in interaction of pathogen elicitor whereas the NBS region (catalytic domain) may bind and hydrolyses ATP and GTP to activate downstream phosphorylation signaling and eventually target gene expressions (Tameling et al., 2002; DeYoung and Innes, 2006). In a recent study in sorghum, it was shown that NBS-LRR genes were significantly enriched in a genomic region containing QTL for northern leaf blight disease resistance (Mace et al., 2014). A LRR receptor-like kinase (Ca_05515) was detected in the AB4.1 region under selection for AB resistance using Fst genome-scan. Using GWAS, one significant SNP (Ca4: 15,435,288) was identified to be located in the exon of this gene which led to amino acid substitution (Gly/Ala). This substitution was located in a conserved catalytic domain which has been suggested to be under purifying selection due to the functional constraints in signal transduction (Afzal et al., 2008). This catalytic domain has high similarity to the brassinosteroid (BR)-associated kinase (BAK1). BAK1 was first identified as a positive regulator in brassinosteroid signaling and later discovered to play an important role in innate immunity in plants (Chinchilla et al., 2007). Additionally, transcripts of this gene were significantly induced by *A. rabiei* inoculation in all three resistant lines. Up-regulation of RLK (including LRR receptor kinases) under biotic stress is one of the common features in early defense responses (Lehti-Shiu et al., 2009). Further research should be pursued to understand the potential role that this LRR receptor-like kinase has in AB resistance in chickpea.

Serine/threonine RLK belong to the RLK class of proteins which are involved in plant development and disease resistance via phosphorylating serine or threonine residues (Afzal et al., 2008). The structure of RLK normally includes a C-terminal intracellular kinase domain, a transmembrane domain, and a N-terminal extracellular receptor domain (De Smet et al., 2009). A recent study in Arabidopsis showed that a serine/threonine receptor-like kinase, PBL13, was involved in plant disease response by enhancing ROS burst and increasing flagellin-induced activation of MAP kinases (Lin et al., 2015). In this current work, three serine/threonine RLK were located in the 100 kb AB4.1 region. Previous studies have shown that RLK are often duplicated to accommodate disease resistance response (Shiu et al., 2004). Duplicated receptor protein kinases might be

retained due to their diverse specificity in recognizing different pathogens or elicitors (Shiu and Bleecker, 2003).

A predicted cysteine-rich receptor-like kinase gene (CRK) was detected in the AB4.1 region under selection for AB resistance. CRK is a sub-family of plant RLKs with one or several repeats of unknown functional domains (DUF26) consisting of a C-X8-C-X2-C motif (Chen, 2001; Bourdais et al., 2015). Previous studies suggested that CRK was involved in biotic and abiotic stresses response; Overexpression of CRK in Arabidopsis led to hypersensitive response-like cell death (Chen et al., 2003, 2004) and increased tolerance to the pathogen *Pseudomonas syringae pv. Tomato* (Acharya et al., 2007). A large-scale study using 82 CRKs T-DNA insertion lines demonstrated that CRKs played an important role in regulating reactive oxygen species (ROS)-related stress responses such as stomatal closure caused by pathogen and abiotic factors (Bourdais et al., 2015). However, transcription level of this CRK gene was not correlated with the AB resistance ranking of the six lines examined indicating this gene might not be involved in AB resistance.

Wall associated-receptor kinases (WAK) are another sub-family of plant RLKs with epidermal growth factor (EGF) repeats in the extracellular domain that can bind to pectin generated by invading pathogens (Kohorn and Kohorn, 2012; Kohorn, 2016). Two recent studies in maize showed that WAK played an important role in response to head smut soil-borne disease caused by fungus *Sporisorium reilianum* (Zuo et al., 2015) and northern corn leaf blight caused by fungus *Exserohilum turcicum* (Hurni et al., 2015). One WAK gene was detected in the AB4.1 region under selection for AB resistance. Transcripts of this gene were significantly induced by *A. rabiei* inoculation in two resistant lines ICC3996 and ICC12004 but not in another resistant line DICC8218. A possible reason may be that DICC8218 has a different resistance mechanism that does not involve this particular gene, or that this gene is not involved in AB resistance.

To dissect genetic variation of genomes, many whole genome resequencing projects have been carried out in human (Abecasis et al., 2012; Auton et al., 2015), livestock (Daetwyler et al., 2014; Ai et al., 2015), and plant species using NGS technology (Huang et al., 2012; Varshney et al., 2013; Li et al., 2014). Although numerous genomes have been sequenced and hundreds of thousands of markers discovered, this information could not be transferred into breeding without high-throughput and accurate phenotyping technology. For complex traits, such as yield and drought tolerance controlled by numerous genes with small effect size and highly influenced by environment, a large number of samples precisely tested in different environments are needed to secure enough statistical power to discover marker-trait association. For simple traits with high heritability such as some disease resistance traits controlled by a few major genes with large effect size, a relatively small sample can have enough statistical power as demonstrated in wheat (Jordan et al., 2015) and Arabidopsis (Atwell et al., 2010). As shown in this study using only 59 genotypes yet with a large amount of maker information (∼250,000 SNPs), we have narrowed down a major AB resistance QTL interval (up to 30 Mb) to a 100 kb region containing only 12 predicted genes. Additionally, we have validated this result with a larger sample size.

## SUMMARY

Both natural and artificial selection processes have marked the chickpea genome with various selection signatures. One common signature is a selective sweep, characterized by an extensive genomic region with a decreased level of genetic diversity. The analytical power to discover these signatures has been improved using NGS technology and advances in statistical methods. By resequencing 69 diverse chickpea genotypes, we have detected a 100 kb genomic region containing 12 predicted genes under selection for AB resistance using GWAS and Fst genome-scan. A subsequent GWAS validation study has confirmed this finding. Transcriptional analysis using qPCR has shown that some predicted genes were significantly induced in resistant lines after inoculation compared to non-inoculated plants.

## AUTHOR CONTRIBUTIONS

YL conceived, designed, and interpret the study. YL and PR analyzed the data. JB contributed to sequencing; KH and JD contributed to phenotyping; DE supervised data analysis; TS supervised the study and edited the manuscript; All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00359/full#supplementary-material

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# Transcription Factor Repertoire of Necrotrophic Fungal Phytopathogen *Ascochyta rabiei*: Predominance of MYB Transcription Factors As Potential Regulators of Secretome

*Sandhya Verma†, Rajesh K. Gazara† and Praveen K. Verma**

*Plant Immunity Laboratory, National Institute of Plant Genome Research, New Delhi, India*

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***Correspondence:**
*Praveen K. Verma*
*pkv@nipgr.ac.in*

*† These authors have contributed equally to this work.*

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Transcription factors (TFs) are the key players in gene expression and their study is highly significant for shedding light on the molecular mechanisms and evolutionary history of organisms. During host–pathogen interaction, extensive reprogramming of gene expression facilitated by TFs is likely to occur in both host and pathogen. To date, the knowledge about TF repertoire in filamentous fungi is in infancy. The necrotrophic fungus *Ascochyta rabiei*, that causes destructive Ascochyta blight (AB) disease of chickpea (*Cicer arietinum*), demands more comprehensive study for better understanding of *Ascochyta*-legume pathosystem. In the present study, we performed the genome-wide identification and analysis of TFs in *A. rabiei*. Taking advantage of *A. rabiei* genome sequence, we used a bioinformatic approach to predict the TF repertoire of *A. rabiei*. For identification and classification of *A. rabiei* TFs, we designed a comprehensive pipeline using a combination of BLAST and InterProScan software. A total of 381 *A. rabiei* TFs were predicted and divided into 32 fungal specific families of TFs. The gene structure, domain organization and phylogenetic analysis of abundant families of *A. rabiei* TFs were also carried out. Comparative study of *A. rabiei* TFs with that of other necrotrophic, biotrophic, hemibiotrophic, symbiotic, and saprotrophic fungi was performed. It suggested presence of both conserved as well as unique features among them. Moreover, *cis*-acting elements on promoter sequences of earlier predicted *A. rabiei* secretome were also identified. With the help of published *A. rabiei* transcriptome data, the differential expression of TF and secretory protein coding genes was analyzed. Furthermore, comprehensive expression analysis of few selected *A. rabiei* TFs using quantitative real-time polymerase chain reaction revealed variety of expression patterns during host colonization. These genes were expressed in at least one of the time points tested post infection. Overall, this study illustrates the first genome-wide identification and analysis of TF repertoire of *A. rabiei*. This work would provide the basis for further studies to dissect role of TFs in the molecular mechanisms during *A. rabiei*–chickpea interactions.

**Keywords: plant–pathogen interaction, *Ascochyta rabiei*, necrotrophic fungi, transcription factors, *cis*-acting elements, secretome**

## INTRODUCTION

A very adaptable and economical source of protein available to mankind is legumes. Apart from having high nutritional value, legumes also serve as great natural soil fertilizers. They provide nitrogen to the crops by fixing atmospheric nitrogen, thereby, decreasing the use of artificial nitrogen fertilizers. This ultimately minimizes the side effects that artificial fertilizers impose on our environment. However, like all plants, legumes are also challenged by various biotic and abiotic stresses causing major yield loss worldwide. The defense responses of plants in combating these environmental stresses are crucial for completing their lifecycle successfully.

Chickpea is one of the important legume crops grown worldwide. Its global production is estimated to be 14 million metric tons (FAO, 2014). India is the largest producer with annual production of about 10 million tons that accounts for 70% of the total world production. However, chickpea faces Ascochyta blight (AB) as a major constraint to its production. It can result in 100% crop mortality and complete yield loss (Singh et al., 1984). Due to several epidemics of AB, substantial losses have been reported from India, Pakistan, Australia, Spain (Pande et al., 2005), Canada (Chandirasekaran et al., 2009), Latin America (Kaiser et al., 2000), and United States (Kaiser et al., 1994). AB is a foliar disease caused by the necrotrophic fungus *Ascochyta rabiei* (Pass.) Labrouse [teleomorph: *Didymella rabiei* (Kovachevski) v. Arx]. This fungus belongs to dothideomycetes class of filamentous fungi. It hibernates on crop residues and seeds between seasons and readily gets transmitted through infected seeds and airborne ascospores. The conidia present in the field sometimes leads to several cycles of infection in the same season when favorable conditions prevail. *A. rabiei* penetrates the host directly through the cuticle and sometimes through stomata as well, eventually resulting in necrosis (Jayakumar et al., 2005). Despite after several pathological and molecular studies, the pathogenicity mechanisms of *A. rabiei* are still poorly understood. However in recent years, an in-depth and path-breaking research has been done in *A. rabiei* that changed many of the conventional concepts. One such example is a report where it was revealed that the solanapyrones toxins of *A. rabiei* are not required for pathogenicity (Kim et al., 2015). This suggests that necrotrophic fungi exploit the cell death machinery of the host plant for pathogenesis, which abandons the earlier concept that necrotrophic fungi solely relies on lytic enzymes for causing necrosis. Recently, the transcriptome analysis of *A. rabiei* has been carried out and a number of transcripts and putatively secreted proteins coding transcripts up-regulated during infection were identified (Fondevilla et al., 2015).

In order to solve the mystery of plant–pathogen interaction, the intricate biological mechanisms of the pathogen needs to be well addressed. Various signaling components and downstream factors play crucial role in connecting the specific pathways into an interlaced regulatory network. Among such components, transcription factors (TFs) are vital for regulating numerous mechanisms and responses. TFs are proteins that have at least one DNA-binding domain (DBD) and an activation domain (AD). The DBD binds to the sequence-specific *cis*-acting elements present in promoters of the target genes. On the other hand, the AD triggers transcription from promoter by recruiting the transcriptional machinery. Besides these, other domains might occur to facilitate homo/hetero-dimerization or the binding with co-activators or co-repressors. The DBDs of TFs are usually highly conserved and are the basis of classification of TFs into superclasses and classes (Stegmaier et al., 2004). By contrast, the ADs are far less conserved.

Knowledge about the TFs repertoire in phytopathogenic fungi is still very limited. Most of the studies have been carried out in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Spitz and Furlong, 2012). With the rise in ease and availability of whole genome and transcriptome data, genome-wide identification of TFs prove to be highly beneficial to get insights of the TF repertoire present in an organism. Genome-wide search and functional identification of TFs has been carried out in the mycorrhizal fungus *Tuber melanosporum* (Montanini et al., 2011). A bioinformatics approach in combination with functional analysis in yeast and transcriptome profiling was performed to identify *T. melanosporum* TFs. Montanini et al. (2011) found that *T. melanosporum* consists of 102 homologs of previously characterized TFs, 57 homologs of hypothetical TFs, and 42 putative TFs apparently specific to *Tuber*. About one-fifth of the *in silico* predicted TFs of *T. melanosporum* were validated by yeast screen. Moreover, 29 TFs were up-regulated in ectomycorrhiza or fruiting bodies. There are other numerous studies and databases related to genome-wide search of TFs in plants (Guo et al., 2008; Mochida et al., 2010), mouse (Kanamori et al., 2004; Zheng et al., 2008), *Drosophila melanogaster* (Adryan and Teichmann, 2006), human (Lee et al., 2007; Zheng et al., 2008; Fulton et al., 2009), and rat (Zheng et al., 2008). Similarly, AnimalTFDB is a comprehensive TF database for 50 animal species ranging from *Caenorhabditis elegans* to human (Zhang et al., 2012). TRANSFAC database and its module TRANSCompel are available to study transcriptional gene regulation in eukaryotes (Matys et al., 2006). The DBD database consists of predicted TF repertoires for 930 completely sequenced genomes of eukaryotes and prokaryotes (Wilson et al., 2008). Still there are limited resources available to study TFs in case of phytopathogenic fungi.

With the available genome of *A. rabiei* (Verma et al., 2016), we have the unique prospect to identify and study a global view of putative TFs repertoire present in *A. rabiei*, for the first time. In the present study, the aim was to obtain the largest possible catalog of DBD-containing proteins which are the *bona fide* transcriptional regulators encoded by *A. rabiei* genome. The *A. rabiei* putative TF repertoire has been compared with that of several other filamentous fungi. Gene structure and phylogenetic analyses have been performed to shed light on the basic information about putative TFs of *A. rabiei*. In order to gain clues regarding expression profiles of these putative TFs and their relevance in regulation of *A. rabiei* secretome during infection, we explored the published transcriptomic profiles in the mycelium and infected chickpea leaves (Fondevilla et al., 2015). The results obtained herein gives us insight into the putative TF repertoire of *A. rabiei* and provides a significant basis for future studies on

functional characterization of TFs involved in various biological processes of the phytopathogenic fungi.

## MATERIALS AND METHODS

### Data Collection and Identification of *A. rabiei* Transcription Factors

The list of TFs of selected 30 fungal species was obtained from the publicly available databases: DBD release 2.0[1] and FTFD v1.2[2]. To acquire the protein sequences of TFs, the proteomes of these fungi were downloaded from JGI. Using the protein IDs, the corresponding protein sequences of TFs were retrieved for proteomes and were used as a database. A total of 10,596 protein sequences of *A. rabiei* (Supplementary Dataset S1) were used as queries to identify all possible TFs by performing BLASTP (*e*-value cutoff $\leq 1e^{-5}$) search against TFs of 30 fungal species as database. This resulted in 1,854 hits as subject sequences. InterProScan v5.21-60.0[3] was employed to identify TF DBDs belonging to 12 superfamilies and 37 PFAM families in fungi in the resultant 1,854 hits. A total of 392 protein sequences had DBDs and these sequences were used to perform BLASTP with FTFD at default parameters. Out of 392, 376 protein sequences returned significant hits, while 16 protein sequences did not show any hits. These 16 protein sequences were further used as queries to perform BLAST searches in NCBI and 5 of them showed significant similarity to fungal TFs in the database. Therefore, a total of 381 putative TFs were predicted.

### Gene Structure Analysis, Domain Organization, and Phylogenetic Analysis

The exon and intron structures of individual TF coding genes belonging to different families were illustrated with the help of Gene Structure Display Server v2.0[4] (Hu et al., 2015) by aligning the cDNA sequences with the corresponding genomic DNA sequences. The functional motifs or domains of putative TF protein sequences were analyzed using CDvist[5] (Adebali et al., 2015). For phylogenetic analysis, multiple sequence alignments of the full-length protein sequences were performed using MUSCLE v3.8.31[6] with maxiters set at 1000. The acquired alignment was used to carry out phylogenetic analyses using Bayesian Inference (BI) implemented in MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003). The protein sequence alignments were run over 3,000,000 generations under a mixed amino acid substitution model with two independent runs and each containing four Markov Chain Monte Carlo (MCMC) chains. To estimate the posterior probabilities for each node, a sampling frequency was set at 300 iterations with MCMC left at default settings. The consensus tree was finally generated with the help of Sumt function of MrBayes. By removing the burn-in generations for each run the posterior probabilities were estimated. All the phylogenetic trees were visualized using FigTree v1.4[7].

### *De Novo* Motif Discovery

The RSAT suite for fungi (Regulatory Sequence Analysis Tools[8]) was used to identify motifs enriched in the 1 kb promoter sequences of genes encoding secretory proteins using pleosporales as the background model and 6-, 7-, or 8-bp-long seeds on both the strands. Once we had the *cis*-regulatory elements identified across the promoter regions, annotation of *cis*-regulatory elements were carried out by scanning UniPROBE database[9] (Newburger and Bulyk, 2009).

### Identification of Differentially Expressed Genes

RNA-seq reads available for *A. rabiei* grown "*in medium*" and "*in planta*" at time points 12, 36, and 96 hours post inoculation (hpi) were downloaded from NCBI (Fondevilla et al., 2015). Reads were mapped on the genome of *A. rabiei* using TopHat2.1.0 software (Kim et al., 2013). Transcriptional levels were estimated with Cufflinks v2.1.1 (Trapnell et al., 2013) and normalized by fragments per kilobase of transcript per million mapped reads (FPKM). The differential expression between *in medium* and *in planta* at different time points was determined by Cuffdiff v2.2.1 (Trapnell et al., 2013). The transcripts with difference of at least two-fold change along with *P*-value $\leq 0.05$ were considered to be significantly differentially expressed. The *P*-value was generated by Benjamini–Hochberg correction for multiple tests running in the background of Cuffdiff v2.2.1.

### Culture Conditions and Plant Infection

Vegetative mycelia of *A. rabiei* isolate ArD2 (Indian Type Culture Collection No. 4638) were grown on potato dextrose agar (PDA; Difco Laboratories, United states) for 20 days in dark. For harvesting fungal tissue, *A. rabiei* spores were grown in potato dextrose broth (PDB; Difco Laboratories, United states) for 5 days in dark at 22°C in an incubator shaker at 120 rpm. Mycelial samples were collected, immediately frozen in liquid nitrogen and stored at −80°C. For plant infection, 21 days old chickpea seedlings (Pusa-362) were spray inoculated with *A. rabiei* spore suspensions of concentration $2 \times 10^5$ spores/ml. Infected leaves and stems were harvested at 12, 24, 72, and 144 hpi with three biological replicates and stored as above.

### RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from *A. rabiei* infected chickpea samples using the TRIzol® reagent (Invitrogen, United states). The contaminating genomic DNA was removed by treating samples with RNase-free RQ1 DNase (Promega, United states) as per the manufacturer's instruction. One microgram of total RNA primed with Oligo-dT was used for first-strand cDNA synthesis

[1] http://www.transcriptionfactor.org
[2] http://ftfd.snu.ac.kr/
[3] http://www.ebi.ac.uk/Tools/pfa/iprscan5/
[4] http://gsds.cbi.pku.edu.cn
[5] http://cdvist.utk.edu
[6] http://www.ebi.ac.uk/Tools/msa/muscle/
[7] http://tree.bio.ed.ac.uk/software/figtree/
[8] http://www.rsat.eu/
[9] http://thebrain.bwh.harvard.edu/uniprobe/

using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United states), according to given instructions in the manual. Primer pairs were designed from the untranslated regions (UTRs) of the target genes using Primer Express® (version 3.0) software with the default parameters. For the internal control, elongation factor1-alpha (*ArEF1a*) was used. Each primer combination gave specific amplification of single desired band. Moreover, only one melting temperature was observed for each primer pair while dissociation curve testing. Quantitative real-time PCR (qRT-PCR) was carried out using the PowerUp™ SYBR® Green Master Mix (ABI) in a 7900HT Real-Time PCR System. Each reaction mixture contained 10 µl of SYBR® Green PCR Master Mix, 2 µl of cDNA, and 18 pmol of each primer in a final volume of 20 µl. The thermal cycling parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 60°C. Each reaction was performed in triplicate. Transcripts of each gene and reference gene *ArEF1a* were amplified using the primers listed in Supplementary Table S1. The obtained values for each gene were then normalized according to the $C_T$ values of *ArEF1a* (Singh et al., 2012; Nizam et al., 2014b). Relative gene expression levels were calculated using the ΔΔCT method.

# RESULTS

## Genome-Wide Identification and Classification of *A. rabiei* Transcription Factors

There are no bioinformatics tools available to predict the putative TFs in filamentous fungi. Therefore, a systematic workflow was employed in order to identify the putative TFs encoded by *A. rabiei* genome (**Figure 1**). For this, first of all, a comprehensive list of 30 different fungal species was prepared (**Table 1**). These fungal species were selected on the basis of their ecology and lifestyle. The list consisted of 26 fungal species belonging to *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Sordariomycetes*, *Saccharomycetes,* and *Schizosaccharomycetes* classes of ascomycetes and also 4 fungal species from the phylum basidiomycetes. They represented different forms of lifestyle such as necrotrophy, hemibiotrophy, biotrophy, saprotrophy, symbiosis, and animal pathogens. These criteria were chosen to ensure maximum possible coverage while predicting the putative TFs of *A. rabiei*. The list of predicted TFs of these 30 fungal species was extracted from DBD: a TF prediction database (Wilson et al., 2008) and Fungal Transcription Factor Database (FTFD) (Park et al., 2008). Since these databases provided only the list of TFs and not their protein sequences, thus, the proteomes of the 30 fungal species were downloaded to obtain the corresponding protein sequences. Now these predicted TFs of the 30 fungal species was used as database to predict putative TFs of *A. rabiei*. BLASTP search was carried out using 10,596 protein sequences of *A. rabiei* as query against TFs of 30 species as database. A total of 1,854 hits were obtained. In fungi, 12 superfamilies and 37 PFAM families of TF DBDs are predicted to exist (Shelest, 2008). Considering this, SUPERFAMILY (Gough et al., 2001) and PFAM family (Finn et al., 2014) search were employed on 1,854 resultant hits using InterProScan (Jones et al., 2014). A total of 392 proteins were predicted to have TF DBDs from 12 superfamilies and 37 PFAM families. To minimize false predictions, each of the 392 putative TFs was employed for BLAST search in FTFD database. Sixteen putative TFs that failed to show any match in FTFD database were searched in NCBI database and were selected if they displayed significant homology with any known TF. From these analyses, a set of 381 proteins were obtained with significant hits (Supplementary Dataset S2) and were designated as the putative TFs of *A. rabiei*.

Among the 381 putative TFs of *A. rabiei*, 142 and 76 proteins showed significant matches in SUPERFAMILY and PFAM family databases, respectively, whereas 163 proteins were common in both (**Figure 2A**). These 381 putative TFs were then classified and annotated on the basis of 83 InterPro terms present in the InterPro database (Finn et al., 2017), which is also the basis of fungal TF annotation in FTFD database. It grouped 381 *A. rabiei* putative TFs into 32 families (Supplementary Table S2). The maximum number of putative TFs (150) belonged to zinc-cluster superfamily [$Zn(II)_2Cys_6$], which is the largest class of fungal-specific domains. This was followed by $C_2H_2$ type zinc finger domains having 61 putative TFs. The third abundant family of putative TFs in *A. rabiei* was nucleic acid-binding-OB-fold with 45 proteins.

## Comparison of *A. rabiei* Transcription Factors with Other Fungal Species

In order to compare *A. rabiei* putative TF repertoire with other fungal genomes, 13 fungal species were selected representing the major lifestyles. Following the same workflow as for *A. rabiei*, putative TFs were predicted for these 13 fungal species (**Table 1**). The number of predicted TFs in closely related necrotrophic fungi *Cochliobolus heterostrophus* and *Pyrenophora tritici-repentis* were 362 and 366, respectively, similar to the 381 putative TFs of *A. rabiei*. A set of 389 putative TFs were predicted in *Botrytis cinerea*. In *Parastagonospora nodorum* and *Sclerotinia sclerotiorum*, 435 and 431 putative TFs were predicted, respectively. Interestingly, the predicted putative TFs in selected biotrophic fungi, i.e., *Blumeria graminis* f. sp. *hordei* (224), *Puccinia graminis* f. sp. *tritici* (287), and *Ustilago maydis* (272) were comparatively fewer than the number of putative TFs predicted in necrotrophic fungi. Also, the hemibiotrophic fungi *Mycosphaerella graminicola* (623) and *Fusarium oxysporum* f. sp. *lycopersici* (566), and symbiont *Laccaria bicolor* (613) had significantly higher number of predicted TFs. However, the hemibiotrophic fungi *Magnaporthe oryzae* (378) and saprotroph *Neurospora crassa* (403) had putative TFs close to putative TFs predicted for *A. rabiei*.

The predicted TFs from 13 fungal species were classified and annotated on the basis of 83 InterPro terms (Supplementary Table S2). All the fungal species, except *B. graminis* f. sp. *hordei* and *P. graminis* f. sp. *tritici*, had the highest number of putative TFs belonging to zinc-cluster superfamily [$Zn(II)_2Cys_6$]. The HMG (High Mobility Group) and AraC type helix-turn-helix family TFs were abundant in *A. rabiei* similar to *M. graminicola* and *L. bicolor*. The Myb TFs were also more in *A. rabiei* as

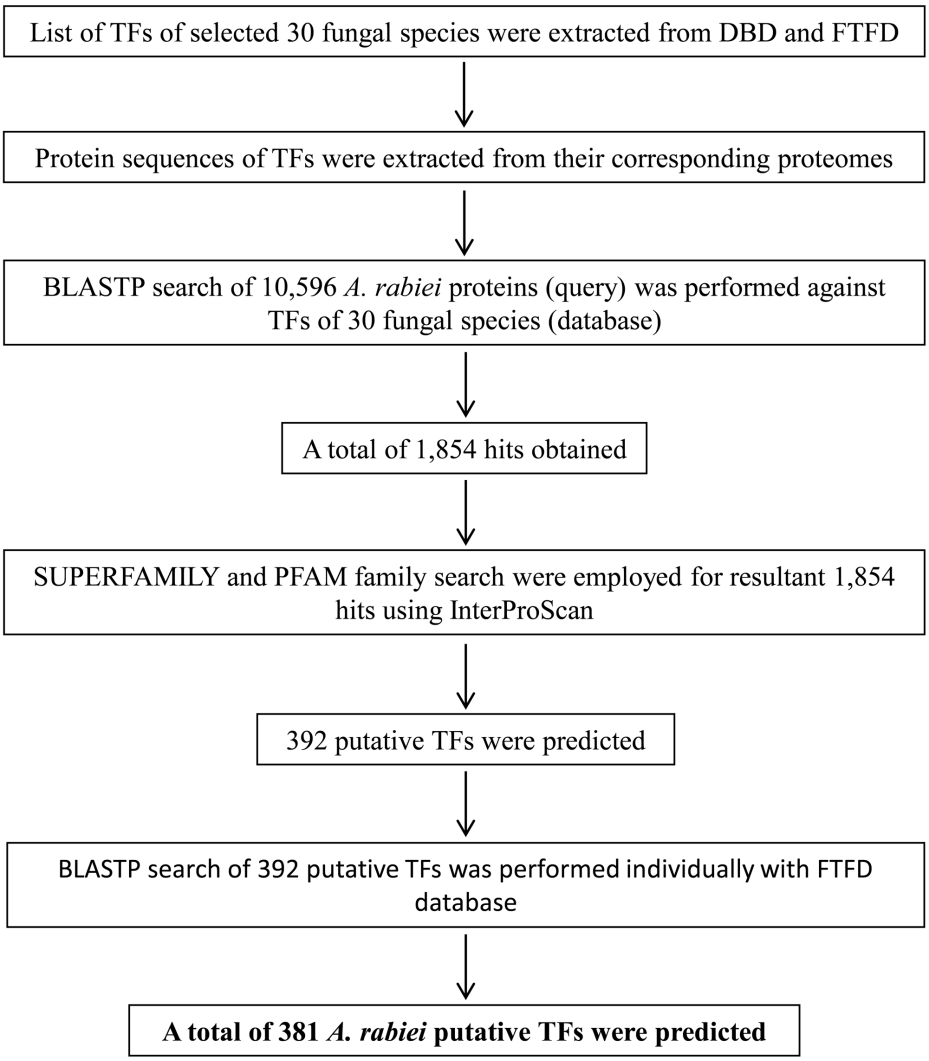


**FIGURE 1 |** Overview of the computational pipeline used to identify the putative TFs of *A. rabiei*. Based on sequence similarity with characterized TFs from other fungi and the presence of conserved DNA-binding domain regions, proteins were assigned as TFs. A set of 381 putative TFs were predicted in the genome of *A. rabiei*.

compared to other necrotrophs. Also, $C_2H_2$ zinc finger TFs were significantly higher in *A. rabiei* unlike most of the selected fungal species, particularly biotrophic fungi (**Figures 2B,C**). The winged helix repressor DNA-binding family was little underrepresented in *A. rabiei* compared to other fungi. This indicates that *A. rabiei* putative TFs are distributed among different classes where the total number of predicted TFs and their distribution into distinct classes differ marginally from those of the selected biotrophic, hemibiotrophic, and symbiotic fungi.

## Gene Structure Analysis of Most Prevalent *A. rabiei* Transcription Factor Families

To explore the structural diversity of most abundant putative TFs of *A. rabiei*, we analyzed their exon–intron organization. In the most prevalent zinc-cluster superfamily [$Zn(II)_2Cys_6$] having 150 putative TFs, 10 genes were intronless while 28 had single intron (Supplementary Figure S1). The intron phases for all the genes were also analyzed. There are three different phase classes to which introns can be assigned: phase 0, 1, and 2. Phase 0 intron locates between two codons while phase 1 intron splits codons between the first and second nucleotides, and intron is said to be phase 2 when it splits codons between the second and third nucleotides. Half of the single intronic genes encoding zinc-cluster [$Zn(II)_2Cys_6$] TFs had intron phase 0. In $C_2H_2$ zinc finger TFs, 13 genes were equally found intronless and single intronic with phase 1 intron as the most prevalent one (Supplementary Figure S2). The third most abundant putative TF family, nucleic acid-binding-OB-fold, had 6 intronless genes and 13 single intron genes with most of them having intron phase 1 (Supplementary Figure S3). Out of 28 winged helix repressor DNA-binding TFs, 2 were intronless and 6 were single intron genes with phase 1 as the most common intron phase (**Figure 3A**). In Myb TF family,

**TABLE 1 |** List of fungal species used in this study.

| S. No. | Species | Class; Order | Proteome link | Lifestyle | Predicted putative TFs |
|---|---|---|---|---|---|
| 1. | *Alternaria brassicicola* | *Dothideomycetes; Pleosporales* | http://genome.jgi.doe.gov/Altbr1/Altbr1.download.ftp.html | Necrotroph | n.a. |
| 2. | *Ashbya gossypii ATCC 10895* | *Saccharomycetes; Saccharomycetales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Ashgo1 | Biotroph | n.a. |
| 3. | *Aspergillus flavus* | *Eurotiomycetes; Eurotiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Aspfl1 | Necrotroph | n.a. |
| 4. | *Aspergillus fumigatus* | *Eurotiomycetes; Eurotiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Aspfu1 | Animal pathogen | n.a. |
| 5. | *Blumeria graminis f. sp. hordei DH14* | *Leotiomycetes; Erysiphaceae* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Blugr1 | Biotroph | 224 |
| 6. | *Botrytis cinerea* | *Leotiomycetes; Helotiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Botci1 | Necrotroph | 389 |
| 7. | *Candida albicans SC5314* | *Saccharomycetes; Saccharomycetales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Canalb1 | Animal pathogen | n.a. |
| 8. | *Coccidioides immitis* | *Eurotiomycetes; Onygenales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Cocim1 | Animal pathogen | n.a. |
| 9. | *Cochliobolus heterostrophus* | *Dothideomycetes; Pleosporales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=CocheC5_3 | Necrotroph | 362 |
| 10. | *Colletotrichum graminicola M1.00* | *Sordariomycetes; Glomerellales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Colgr1 | Hemibiotroph | n.a. |
| 11. | *Cryptococcus neoformans JEC2* | *Tremellomycetes; Tremellales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Cryne_JEC21_1 | Animal pathogen | n.a. |
| 12. | *Fusarium graminearu* | *Sordariomycetes; Hypocreales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Fusgr1 | Necrotroph | n.a. |
| 13. | *Fusarium oxysporum f. sp. lycopersici 4286* | *Sordariomycetes; Hypocreales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Fusox1 | Hemibiotroph | 566 |
| 14. | *Fusarium verticillioides 7600* | *Sordariomycetes; Hypocreales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Fusve1 | Hemibiotroph | n.a. |
| 15. | *Laccaria bicolor* | *Agaricomycetes; Agaricales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Lacbi2 | Symbiont | 613 |
| 16. | *Leptosphaeria maculans* | *Dothideomycetes; Pleosporales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Lepmu1 | Hemibiotroph | n.a. |
| 17. | *Magnaporthe oryzae 70-15* | *Sordariomycetes; Magnaporthales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Maggr1 | Hemibiotroph | 378 |
| 18. | *Mycosphaerella fijiensis* | *Dothideomycetes; Capnodiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Mycfi2 | Hemibiotroph | n.a. |
| 19. | *Mycosphaerella graminicola* | *Dothideomycetes; Capnodiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Mycgr3 | Hemibiotroph | 623 |
| 20. | *Neosartorya fischeri* | *Eurotiomycetes; Eurotiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Neofi1 | Animal pathogen | n.a. |
| 21. | *Neurospora crassa* | *Sordariomycetes; Sordariales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Neucr2 | Saprotroph | 403 |
| 22. | *Puccinia graminis f. sp. tritici* | *Pucciniomycetes; Pucciniales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Pucgr2 | Biotroph | 287 |
| 23. | *Pyrenophora tritici-repentis* | *Dothideomycetes; Pleosporales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Pyrtr1 | Necrotroph | 366 |
| 24. | *Saccharomyces cerevisiae S288C* | *Saccharomycetes; Saccharomycetales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Sacce1 | Saprotroph | n.a. |
| 25. | *Schizosaccharomyces pombe* | *Schizosaccharomycetes; Schizosaccharomycetales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Schpo1 | Saprotroph | n.a. |
| 26. | *Sclerotinia sclerotiorum* | *Leotiomycetes; Helotiales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Sclsc1 | Necrotroph | 431 |
| 27. | *Parastagonospora nodorum* | *Dothideomycetes; Pleosporales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Stano2 | Necrotroph | 435 |
| 28. | *Trichoderma virens* | *Sordariomycetes; Hypocreales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=TriviGv29_8_2 | Saprotroph | n.a. |
| 29. | *Ustilago maydis 521* | *Ustilaginomycetes; Ustilaginales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Ustma1 | Biotroph | 272 |
| 30. | *Verticillium dahliae VdLs. 17* | *Sordariomycetes; Glomerellales* | http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Verda1 | Hemibiotroph | n.a. |

*n.a., not analyzed.*

intronless genes were 6 and single intron genes were 5 and had intron phase 0 in most of them (**Figure 3B**). Altogether, this analysis reveals that single intron genes with intron phase either 0 or 1 were predominant in the abundantly found putative TFs of *A. rabiei*.

## Domain Organization and Phylogenetic Analysis of Myb, bHLH, and bZIP Transcription Factor Families

Owing to the significant biological functions of TFs, we studied their domain architecture and phylogenetic relationships to gain evolutionary insights. The three families of putative TFs, Myb, bHLH, and bZIP, showed presence of characteristic domains in their protein sequences (**Figure 4**). The Myb TFs had numerous low-complexity regions which are the regions containing little diversity in their amino acid composition (**Figure 4A**). Basic helix-loop-helix domain was present in each of the members of bHLH TFs; however, the size of domain was varying (**Figure 4B**). Similarly, all bZIP TFs of *A. rabiei* had a bZIP domain of uniform size with coiled coil regions overlapping with the bZIP domain (**Figure 4C**). Both these domains are important for the dimerization of the proteins.

Phylogenetic analysis of Myb TFs of *A. rabiei* with other closely related necrotrophic fungi, i.e., *C. heterostrophus*, *P. tritici-repentis* and *P. nodorum* was performed. It showed that two major clades were formed in *C. heterostrophus*, *P. tritici-repentis,* and *P. nodorum* (**Figure 5**). On the contrary, *A. rabiei* had Myb TFs grouped into single clade only. The bHLH TFs showed much conserved distribution in the selected fungal species. They were distributed into two major clades in all of the four fungi (**Figure 6**). Similarly, the bZIP TFs were also divided into two clades (**Figure 7**). The bHLH and bZIP TFs particularly showed very similar pattern of phylogenetic tree between *A. rabiei* and *C. heterostrophus*. In the same way, *P. tritici-repentis* and *P. nodorum* had identical phylogenetic relationship of these TFs. This suggests that these putative TFs have evolved in highly conserved manner in *A. rabiei* and other closely related necrotrophic fungi.

## *Cis*-Regulatory Elements in Promoter of *A. rabiei* Genes Encoding Secretory Proteins

We earlier reported the necrotrophic effector repertoire of *A. rabiei* (Verma et al., 2016). A set of 758 putative secretory proteins were predicted to constitute the secretome. Therefore, we investigated the *cis*-regulatory elements present in the promoter sequences of those *A. rabiei* genes that encode secretory proteins. This would aid in identifying the putative TFs that regulate the co-ordinated expression of *A. rabiei* secretome. For this, up to 1 kb 5′ flanking sequence of the genes encoding putative secretory proteins were selected and any other gene sequences occurring in these promoter regions were discarded. A scan in promoters of the 758 genes was performed using RSAT suite (Medina-Rivera et al., 2015) to obtain highly frequent DNA patterns known to be the binding sites of TFs. After scanning, the 10 most frequent motifs were identified (**Figure 8** and **Table 2**). The motif CATCAACACCAC was the most recurrent motif that was predicted to bind to the promoter regions of 234 genes out of 758 genes encoding secretory proteins and had 316 numbers of occurrences. This was followed by CATCTCCACCAC motif that was identified in the promoters of 222 genes with 303 instances of occurrences. The third most abundant motif was TCCTTCCCC, which was present at 216 promoter sequences and had 273 matches.

Once we identified the most frequent *cis*-regulatory elements across the promoter regions of the genes encoding secretory proteins of *A. rabiei*, the TFs known to bind on these sites were identified. For this, the motifs were scanned for their corresponding TFs using UniPROBE database (Newburger and Bulyk, 2009). It revealed that Myb TFs were the corresponding TFs that bind to CATCAACACCAC motif (**Table 2**). For the second most abundant motif CATCTCCACCAC, $[Zn(II)_2Cys_6]$ zinc cluster TFs were predicted to bind. The Myb TFs were also predicted to bind to the motif TCCTTCCCC. Among the 10 most frequent motifs identified, the corresponding TFs for 3 motifs were Myb TFs. In addition, two *cis*-regulatory elements were regulated by $C_2H_2$ zinc finger TFs. Likewise, the $[Zn(II)_2Cys_6]$ zinc cluster, bZIP, APSES, HMG, and homeobox TFs were predicted to bind on 1 of the 10 motifs identified. This suggests that the secretome of *A. rabiei* is regulated by an array of TFs, mainly Myb TFs.

## Differentially Expressed Transcription Factor Genes of *A. rabiei* during Host Infection

Recently, the transcriptome data of *A. rabiei* during plant invasion was reported (Fondevilla et al., 2015). We utilized this data in order to identify the differentially expressed genes (DEGs) encoding predicted TFs during host infection. The RNA-seq reads of "*in medium*" and "*in planta*" samples at time points 12, 36, and 96 hpi were mapped on *A. rabiei* genome and expression values were calculated in the terms of FPKM. A total of 13 putative TF encoding genes were found differentially expressed (**Figure 9**). Interestingly, seven genes were found to be expressed exclusively *in medium* and were not at all expressed during *in planta* conditions (**Figure 9**). Likewise six genes which were expressed during *in planta* conditions had no expression *in medium*. Four putative TF encoding genes were found expressed exclusively at 96 hpi whereas only one putative TF (bZIP) encoding gene was expressing at all the three time points during plant infection (**Figure 9**). The DEGs mainly belonged to $[Zn(II)_2Cys_6]$ zinc cluster, $C_2H_2$ zinc finger and nucleic acid-binding-OB-fold family of TFs.

We also investigated the expression of genes encoding secretory proteins of *A. rabiei* that we have predicted earlier (Verma et al., 2016). A set of 34 putative secretory protein coding genes were found differentially expressed (**Figure 10**). During *in planta* conditions, 27 genes showed differential expression at different time points and these genes were not expressed when the fungus was grown in *in vitro* condition. At 96 hpi, most of the secretory protein coding genes (26) were up-regulated and out of these, 23 genes were exclusively expressed at this time

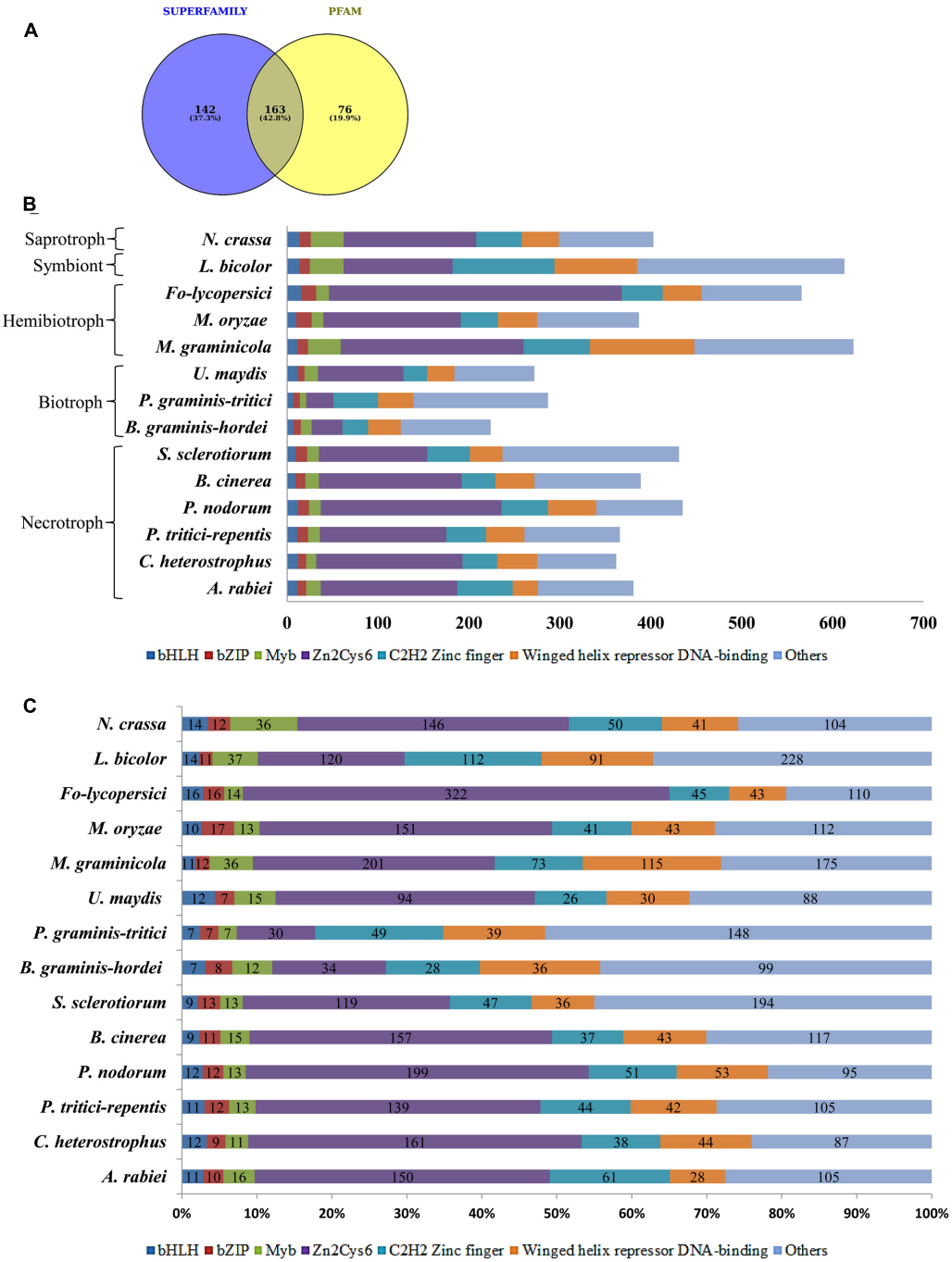


**FIGURE 2 |** Distribution of *A. rabiei* putative TFs among different structural categories. **(A)** Out of 381 putative TFs predicted, 142 had significant matches with fungi specific 12 superfamilies and 76 had similarities with fungi specific 37 PFAM families (Shelest, 2008). Whereas 163 proteins showed matches in both SUPERFAMILY and PFAM family database. **(B)** The *A. rabiei* putative TFs were compared with TF repertoire of other necrotrophs, biotrophs, hemibiotrophs, symbiont, and saprotroph. The DNA binding domains considered for comparison were winged helix repressor DNA-binding, $C_2H_2$ zinc finger, $[Zn(II)_2Cys_6]$, Myb, bZIP, and bHLH domains. The less represented DNA binding domains were categorized under others. **(C)** The relative abundance of each of the selected TF families across the fungal species is shown. The numbers inside the bars of graph are the absolute number of TFs in that family.

point of infection. On the other hand, two secretory protein coding genes were differentially expressed at all the three time points of infection. Interestingly, there were seven genes which were expressed only during *in medium* condition. This suggests that *A. rabiei* exhibits a much orchestrated spatial and temporal distribution of secretory proteins during its lifecycle.

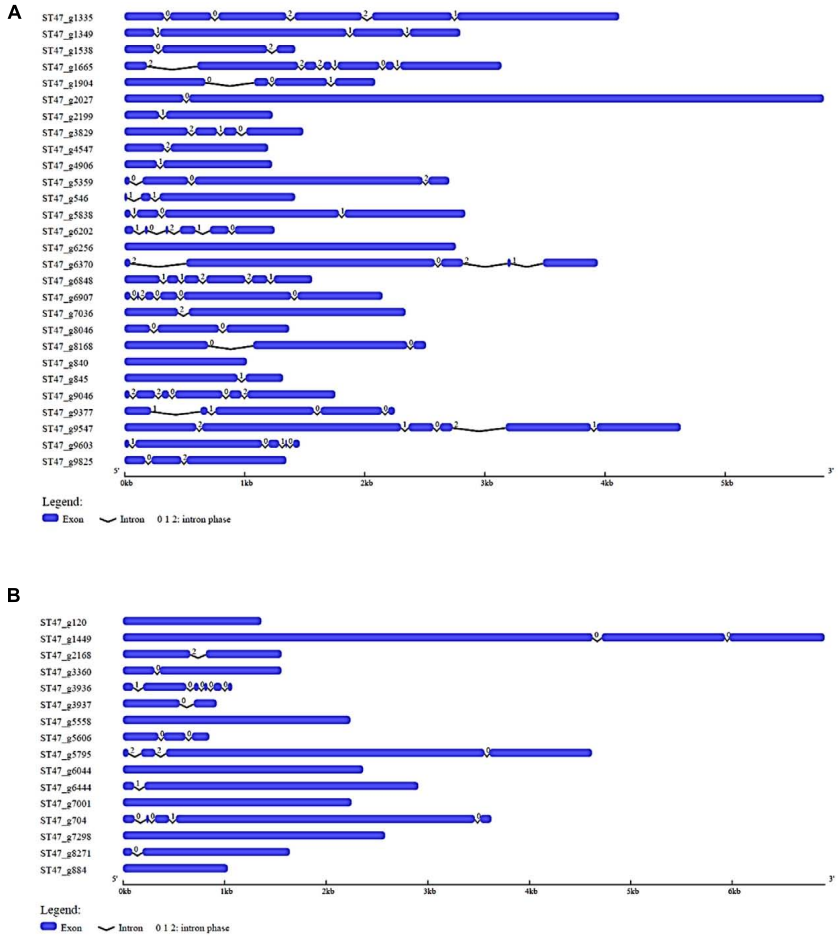


**FIGURE 3 |** Gene structure analysis. The exon–intron organization is shown for **(A)** winged helix repressor DNA-binding, and **(B)** Myb family of *A. rabiei* putative TFs. Exons and introns are represented by blue rectangles and black lines, respectively. The numbers 0, 1, and 2 represent the intron phase.

In order to get the insights of putative TFs that regulate the secretory proteins of *A. rabiei*, we searched for the presence of most frequent *cis*-regulatory elements in the promoters of secretory protein encoding genes which were found up-regulated during *in medium* and *in planta* conditions. Twelve secretory proteins had CATCAACACCAC motif in their promoters and this motif is the binding site for Myb TFs, particularly Rap1 (Supplementary Dataset S3). Similarly, CCTGCAT motif was found in promoters of 11 secretory proteins and is the binding site of APSES TF (Phd1). The bZIP and $C_2H_2$ zinc finger TFs appeared to regulate expression of 7 secretory proteins due to the presence of their binding sites on promoter sequences. It indicates that an array of putative TFs regulate secretory proteins during host invasion and *in vitro* condition.

## Expression Analysis of *A. rabiei* Transcription Factors by qRT-PCR

For transcriptomic studies of *A. rabiei*–chickpea pathosystem (Fondevilla et al., 2015), *A. rabiei* isolate P4 (originated from Kaljebrin, Syria) was used to inoculate susceptible chickpea cultivar "Blanco Lechoso." In our laboratory, we use ArD2 isolate of *A. rabiei* (obtained from Indian Agricultural Research Institute, New Delhi, India) representing pathotype 2 to infect highly susceptible chickpea cultivar Pusa-362. Therefore, we investigated whether the putative TFs exhibit similar expression pattern in *A. rabiei*–chickpea pathosystem when the pathotype of *A. rabiei* and the chickpea cultivar were different. For this, the expression profile of seven different putative TFs during host colonization was assayed by qRT-PCR at 12, 24, 72, and 144 hpi (**Figure 11**). All the selected genes were found differentially expressed during infection. In comparison with other putative TF genes, the abundance of *ST47_g4184* transcripts (nucleic acid-binding-OB-fold) was highest at almost all the time points. Its expression was highest at 12 hpi that drastically reduced at 24 hpi and then again gradually increased at 72 and 144 hpi suggesting a bi-phasic induction. Similarly, *ST47_g10012* ($C_2H_2$ zinc finger) also showed maximum expression at 12 and 144 hpi. In terms of expression pattern, the transcripts of *ST47_g120* (Myb), *ST47_g1688* (bZIP), *ST47_g3570* (C2H2 zinc finger), *ST47_g4184* and *ST47_g6471* (C2H2 zinc finger) showed

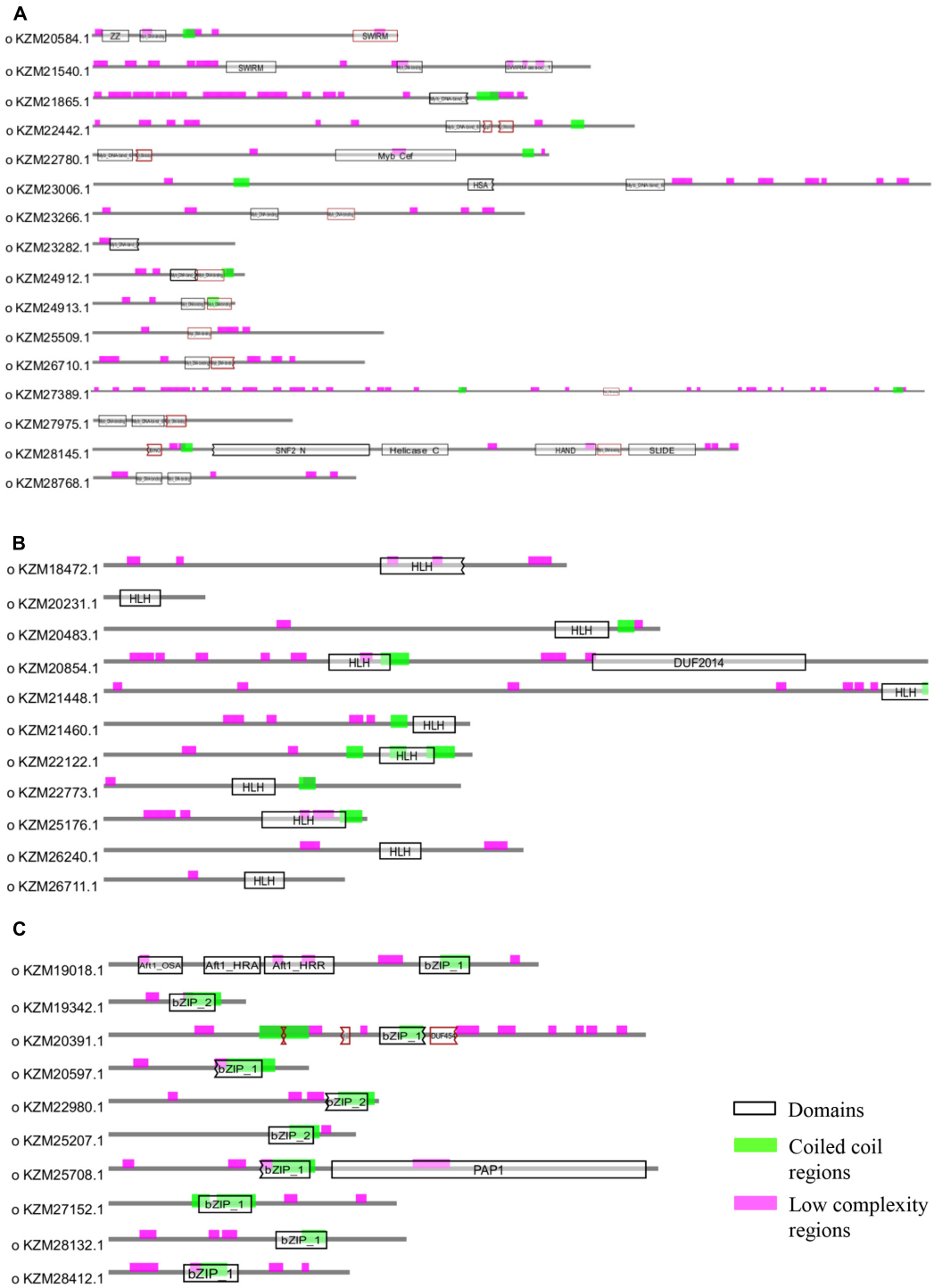


**FIGURE 4 |** Structural analysis of putative TFs. The protein domains of **(A)** Myb, **(B)** bHLH, and **(C)** bZIP family of *A. rabiei* putative TFs are shown. The domains are denoted by black outlined hollow rectangles, whereas coiled coil regions and low complexity regions are represented by green and pink rectangles, respectively.

maximum expression at 12 hpi followed by 144 hpi suggesting that these genes were getting induced during initial phases of pathogenesis. While *ST47_g3398* [$Zn(II)_2Cys_6$] and *ST47_g10012* appeared to play role during later stages of pathogenesis as they showed maximum transcript abundance at 144 hpi. Overall, these results indicate that TFs may exhibit differential expression in a temporal manner specific to the pathotype of *A. rabiei* and the cultivar of chickpea.

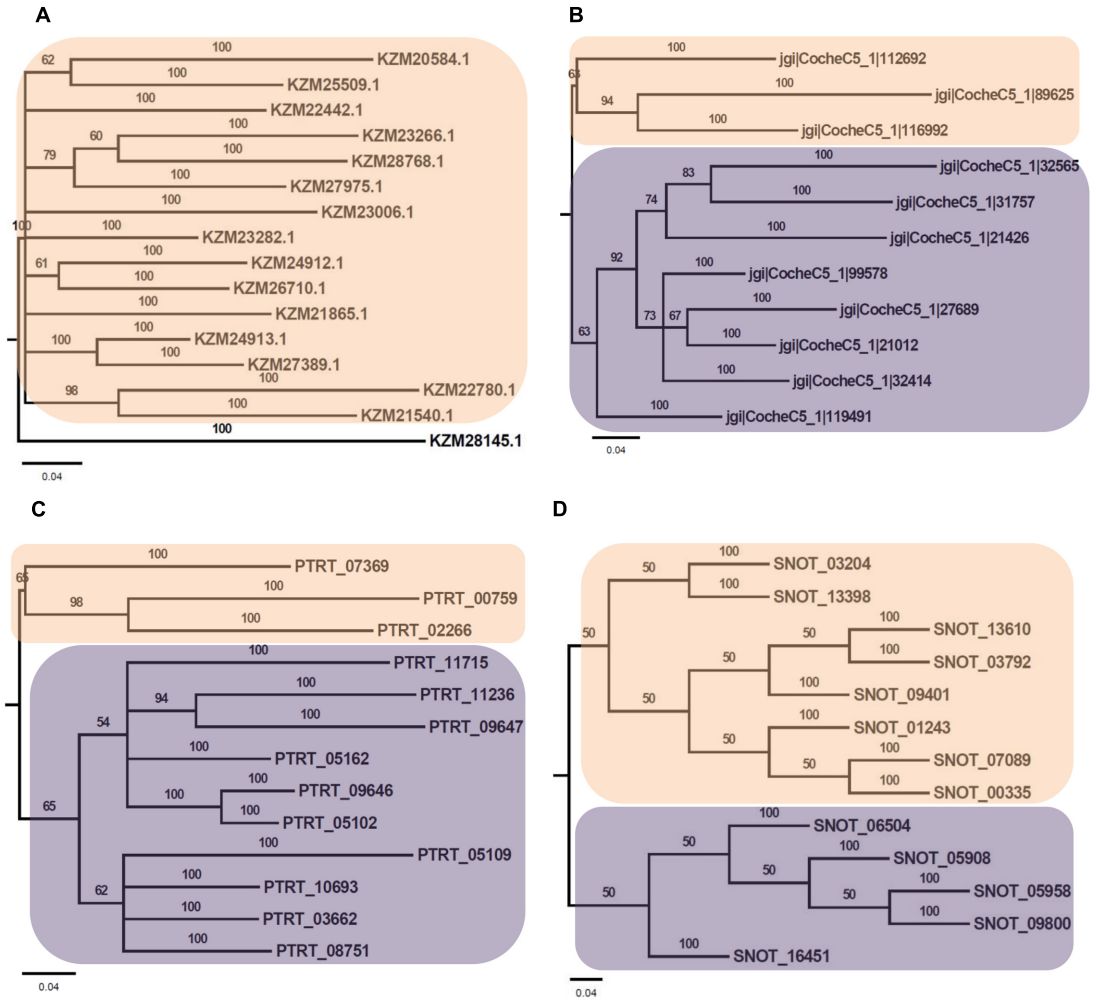


**FIGURE 5 |** Phylogenetic analysis of Myb family of putative TFs. **(A)** The evolutionary relationship of Myb family of *A. rabiei* putative TFs was compared to that of **(B)** *C. heterostrophus*, **(C)** *P. tritici-repentis*, and **(D)** *P. nodorum* based on Bayesian inference analysis. Each clade is highlighted by colored rectangular block. The Bayesian posterior probabilities are indicated at the nodes.

## DISCUSSION

Whenever a pathogen attacks host plant, extensive reprogramming of gene expression facilitated by TFs occur in both the host and pathogen (Verma et al., 2013). Several distinct reports suggest the crucial role played by TFs in the growth, development and virulence of filamentous fungi. Consequently, the attention has been recently focused to study TFs since they regulate an array of pathogenicity and stress responsive genes. In yeast and filamentous fungi, a basic leucine zipper (bZIP) TF Activating Protein 1 (AP-1) acts as a transcriptional activator in response to oxidative stress (Wu and Moye-Rowley, 1994; Toone and Jones, 1998; Reverberi et al., 2008) and carries out multiple functions as a redox regulator (Fernandes et al., 1997; Karin et al., 1997). The yeast AP-1 family of TFs consists of Yap1 in *S. cerevisiae*, Pap1 in *S. pombe*, Cap1 in *Candida albicans*, and Kap1 in *Kluyveromyces lactis* (Toone et al., 2001). Later, Yap1 orthologs were also identified in *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Aspergillus parasiticus* where they act as key players in cellular defense against oxidative stress (Asano et al., 2007; Reverberi et al., 2008, 2012). However, in necrotrophic phytopathogenic fungus *B. cinerea*, a bZIP TF (BcAtf1) regulates expression of catalase B but does not contribute to osmotic and oxidative stress tolerance (Temme et al., 2012). In *M. oryzae*, MST12, a homolog of the yeast TF Ste12, plays crucial role in host penetration and colonization (Park et al., 2002). Few other specific TFs, for instance *Cochliobolus carbonum* ccSNF1, regulates *in planta* expression of cell wall degrading enzymes (Tonukari et al., 2000). In *Fusarium graminearum*, the pathogenicity and sexual development is largely affected by a Myb-like transcription factor MYT3 (Kim et al., 2014). Similarly, the AbPf2 TF of *Alternaria brassicicola* is an important regulator of pathogenesis and it does not affect other cellular processes (Cho et al., 2013). Therefore, TFs are vital for survival and completion of the lifecycle.

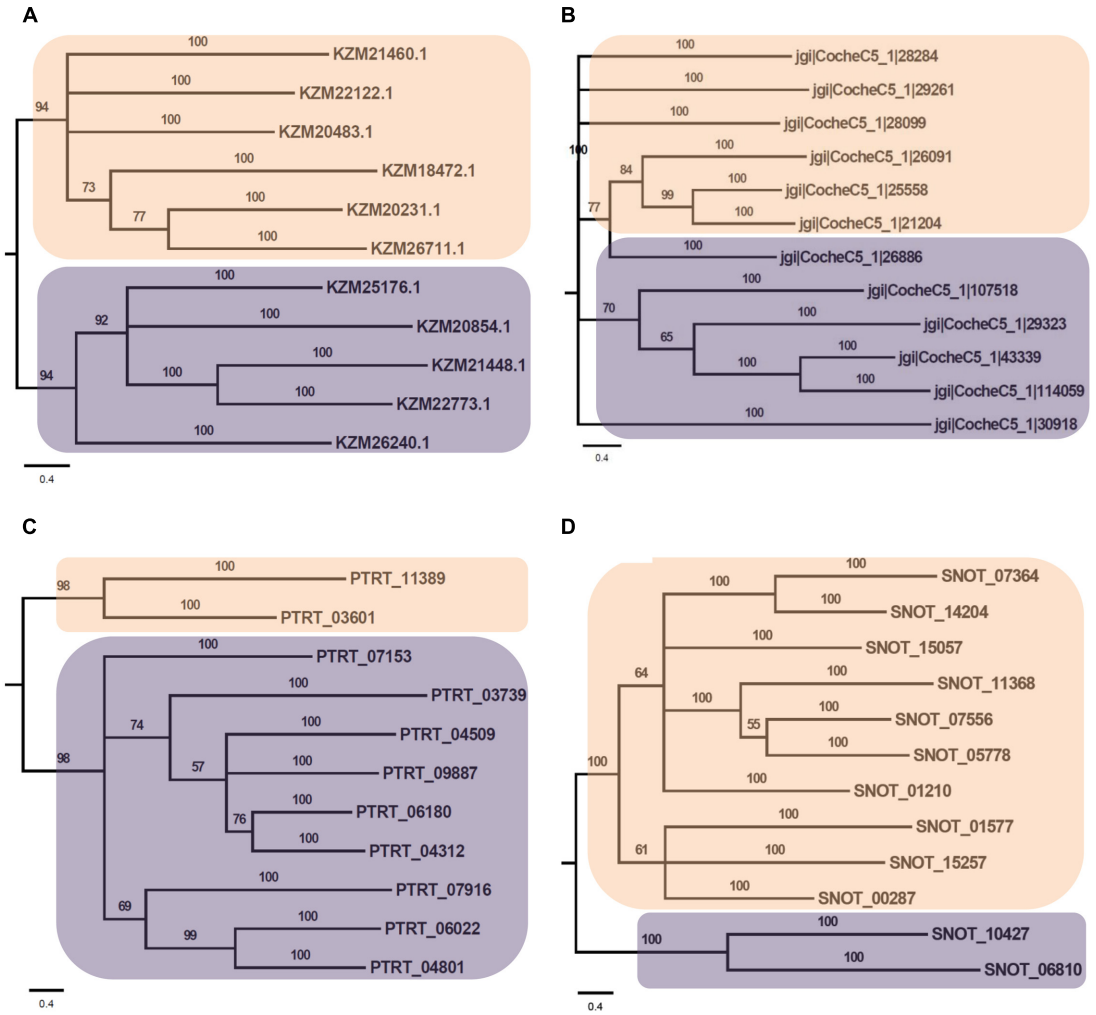


**FIGURE 6 |** Phylogenetic analysis of bHLH family of putative TFs. **(A)** The evolutionary relationship of bHLH family of *A. rabiei* putative TFs was compared to that of **(B)** *C. heterostrophus*, **(C)** *P. tritici-repentis*, and **(D)** *P. nodorum* based on Bayesian inference analysis. Each clade is highlighted by colored rectangular block. The Bayesian posterior probabilities are indicated at the nodes.

*Ascochyta* spp. are highly devastating pathogens causing severe losses in production of legumes worldwide. However, not much is known about the pathogenicity mechanisms of this necrotrophic fungus. There are only few reports available which are not quite sufficient to shed light on understanding of *A. rabiei*–chickpea pathosystem. Despite extensive pathological studies, the nature and extent of pathogenic variability in *A. rabiei* have not been clearly established. The mechanisms by which *A. rabiei* infects and colonizes chickpea plants remain poorly understood. Though, with the recent advances in genomics, transcriptomics, and molecular studies of *A. rabiei*, it is now possible to carry out in depth investigations of *A. rabiei* pathogenicity. The genetic manipulations/transformations of *A. rabiei* have made identification of gene functions possible (Nizam et al., 2010). Similarly, various comprehensive studies of *A. rabiei* gene families have been performed in order to gain the molecular and evolutionary insights (Nizam et al., 2014a,b; Kim et al., 2015).

With the present study, we have further expanded the understanding of *A. rabiei* as a pathogen. Since TFs have a major role in fungal development, pathogenesis and response to the environment, we have identified and classified the putative TF repertoire of *A. rabiei*. This detailed analysis was achievable mainly due to the availability of *A. rabiei* genome sequencing data. A comprehensive computational approach was implemented that resulted in identification of a set of 381 proteins as the putative TFs of *A. rabiei*. In fungi, usually the TFs comprise 0.5–8% of the gene content and can be classified on the basis of structure of their DBDs (Stegmaier et al., 2004; Wilson et al., 2008). In *A. rabiei*, TFs constitute 3.5% of the gene content. Among the *A. rabiei* putative TFs predicted, $[Zn(II)_2Cys_6]$ family of TFs was overwhelmingly predominated. The $[Zn(II)_2Cys_6]$ TFs are usually the most abundant TFs in fungi as revealed by

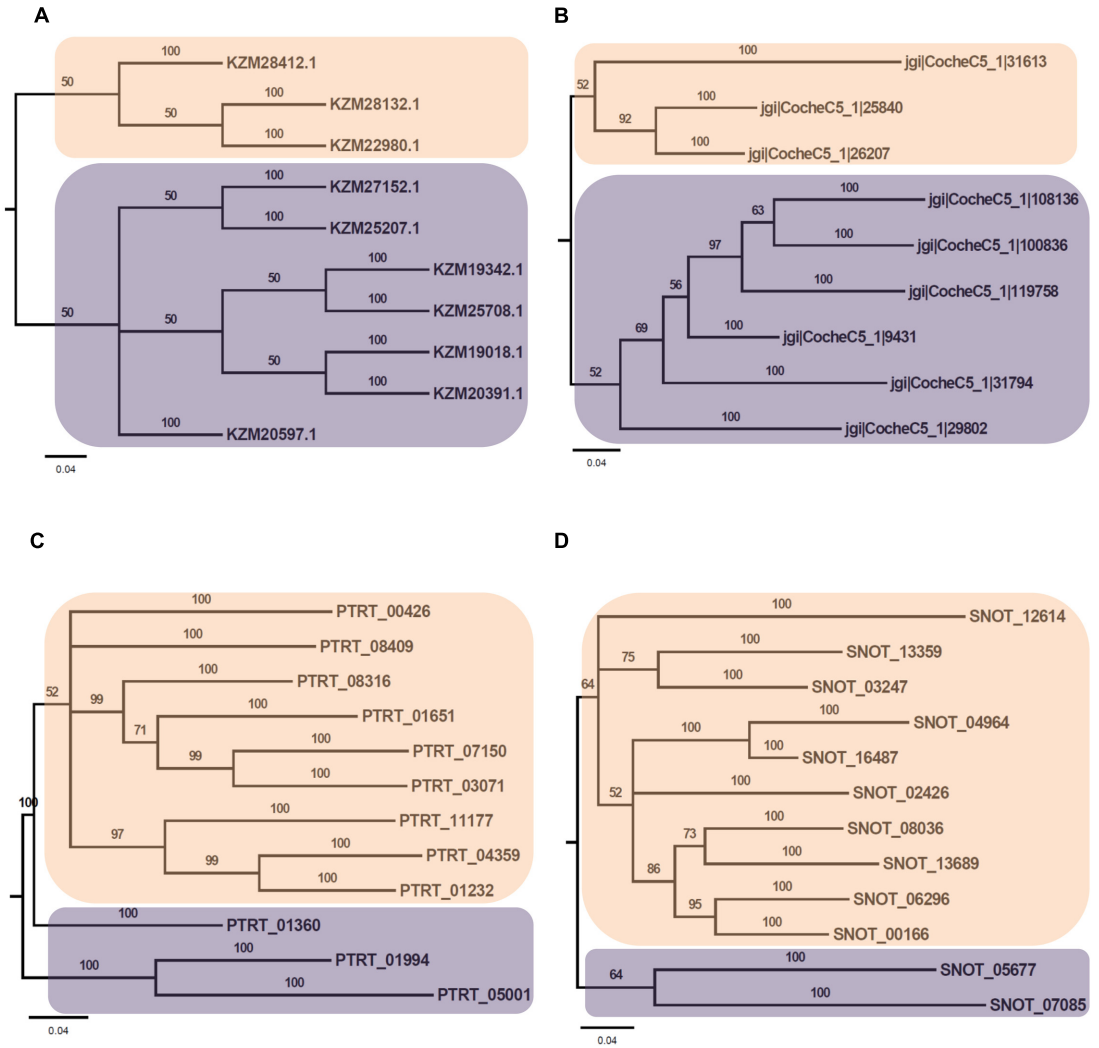


**FIGURE 7 |** Phylogenetic analysis of bZIP family of putative TFs. **(A)** The evolutionary relationship of bZIP family of *A. rabiei* putative TFs was compared to that of **(B)** *C. heterostrophus*, **(C)** *P. tritici-repentis,* and **(D)** *P. nodorum* based on Bayesian inference analysis. Each clade is highlighted by colored rectangular block. The Bayesian posterior probabilities are indicated at the nodes.

few studies analyzing 37 known fungal transcription regulator-related PFAM domains in several ascomycete and basidiomycete genomes (Shelest, 2008; Todd et al., 2014). A variety of cellular and metabolic processes are regulated by this class of TFs. In model filamentous ascomycete fungus *Podospora anserina*, the two zinc cluster proteins RSE2 and RSE3 regulates expression of genes encoding alternative oxidase, phosphoenolpyruvate carboxykinase, fructose-1,6-biphosphatase, alternative NADH dehydrogenase, a $[Zn(II)_2Cys_6]$ TF, a flavohemoglobin, and various hydrolases (Bovier et al., 2014). Necrotrophic fungus *B. cinerea* has a $[Zn(II)_2Cys_6]$ TF, BcGaaR that induces D-galacturonic acid (GalA)-inducible genes and promotes growth of *B. cinerea* on GalA (Zhang et al., 2015). In *F. graminearum*, $[Zn(II)_2Cys_6]$ TF EBR1 regulates virulence and apical dominance of the hyphal tip (Zhao et al., 2011). Furthermore, a high-throughput gene knockout of 104 $[Zn(II)_2Cys_6]$ TF genes in *M. oryzae* was performed that suggested their significance in growth, asexual and infection-related development, pathogenesis and response to nine abiotic stresses (Lu et al., 2014). It also revealed that $[Zn(II)_2Cys_6]$ TFs involved in pathogenicity frequently tend to function in multiple development stages. During hemibiotrophic *Colletotrichum lindemuthianum*-bean interaction, CLTA1 (a fungal zinc cluster TF) regulates biotrophic phase specific genes facilitating the biotrophy to necrotrophy switch (Dufresne et al., 2000). Therefore, the prevalence of $[Zn(II)_2Cys_6]$ family of TFs in *A. rabiei* suggests that they might play important role in survival and virulence of *A. rabiei*.

In *A. rabiei*, the $C_2H_2$ zinc finger class was second most abundant class which represents a smaller but major regulator class in the ascomycetes. These are involved in development (Kwon et al., 2010) and calcium signaling in *A. nidulans* (Hagiwara et al., 2008). Another $C_2H_2$ TF, MtfA, is a regulator

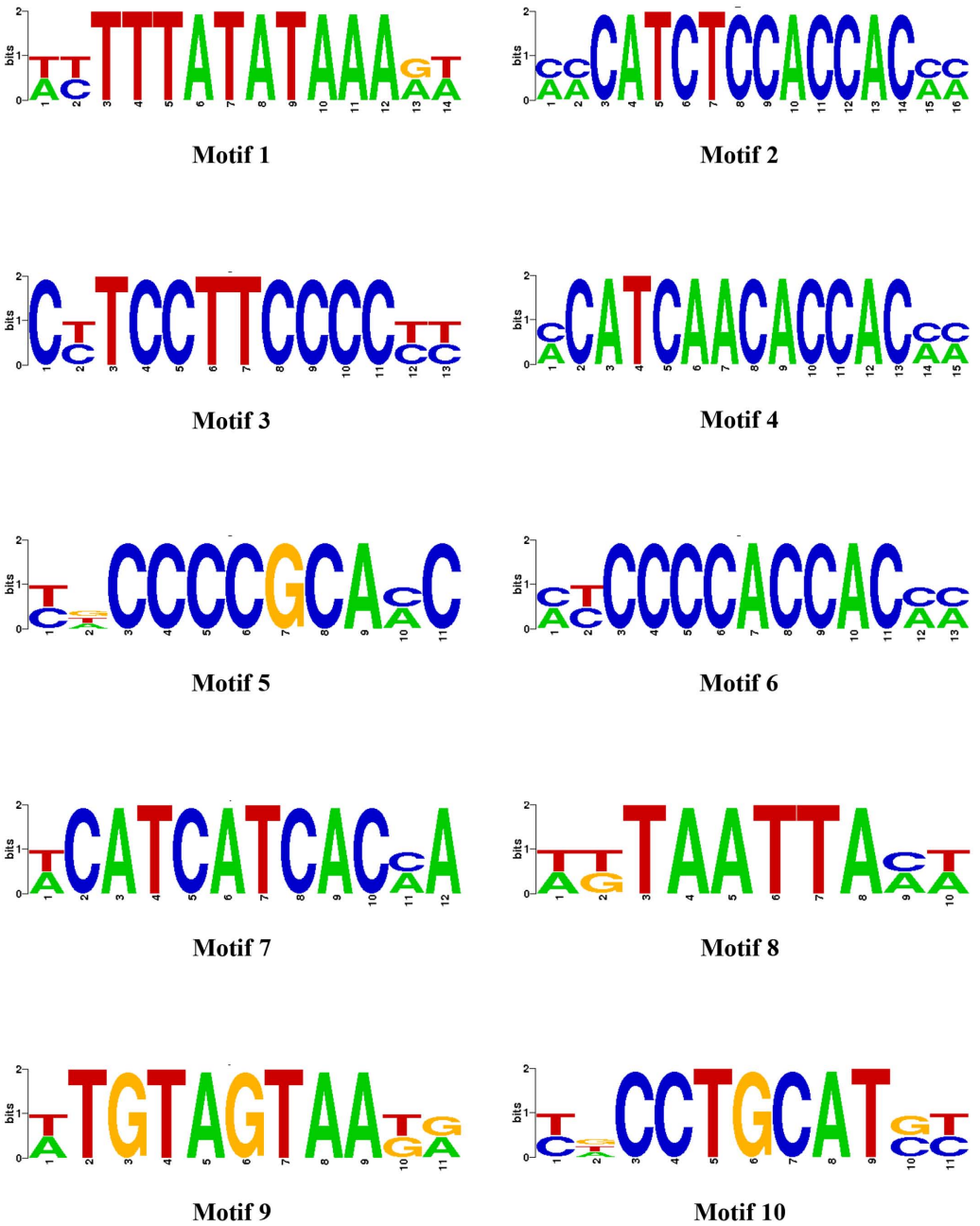


**FIGURE 8 |** The most enriched *cis*-regulatory elements in the promoter sequences of secretory protein coding genes. Ten most abundant motifs in the promoter sequences of genes encoding *A. rabiei* secretory proteins are shown, as identified by RSAT suite for fungi (Regulatory Sequence Analysis).

of secondary metabolism and morphogenesis in *A. nidulans* (Ramamoorthy et al., 2013). Moreover, a conserved $C_2H_2$ TF (RsrA) coordinates a NapA mediated oxidative response in *Aspergillus* fungi (Bok et al., 2014). In *A. fumigatus*, GipA induces production of immunosuppressive secondary metabolite gliotoxin (Schoberle et al., 2014). In *A. parasiticus* and *Aspergillus flavus*, deletion of *MSNA* resulted in enhanced production of conidia, ROS, aflatoxin, and kojic acid (Roze et al., 2004). In addition, the $C_2H_2$ TFs may use multiple recognition motifs to control gene expression (Han et al., 2016). This indicates that $C_2H_2$ zinc finger class of TFs in *A. rabiei* might be the major contributors in regulating the growth and developmental processes.

Comparison of *A. rabiei* putative TFs with that of other necrotrophic, biotrophic, hemibiotrophic, symbiotic, and saprotrophic fungi suggested a conserved as well as unique distribution of TFs among different classes in all the selected fungi. This indicates toward the evolutionary specificity of

**TABLE 2 |** Ten most enriched *cis*-regulatory elements identified in the promoter sequences of secretory protein coding genes.

| Name | Motifs | UniProbe Database match | *E*-value (UniProbe match) | Matches | No. of promoters |
|---|---|---|---|---|---|
| Motif_1 | wyTTTATATAAArw | Nhp6a (HMG factors) | 0.000002 | 303 | 123 |
| Motif_2 | mmCaTCTCCACCACmm | Cha4 (C6 zinc cluster factors) | 0.004379 | 303 | 222 |
| Motif_3 | cyTCCTTCCCcyy | Rap1 (Myb) | 0.21617 | 273 | 216 |
| Motif_4 | mcATCAACACCACmm | Rap1 (Myb) | 0.015551 | 316 | 234 |
| Motif_5 | ydCCCCGCAmc | YML081W (zf-C2H2) | 0.000008 | 200 | 167 |
| Motif_6 | myCCCCACCACmm | RPN4 (zf-C2H2) | 0.003615 | 243 | 184 |
| Motif_7 | wcATCATCACma | Pbf2 (Myb) | 0.002092 | 273 | 205 |
| Motif_8 | wkTAATTAmw | Yox1 (Homeobox) | 0 | 164 | 71 |
| Motif_9 | wtGTAGTAAkr | Sko1 (bZIP_1) | 0.008315 | 214 | 156 |
| Motif_10 | ydCCTGCATsy | Phd1 (HTH APSES-type) | 0.000388 | 157 | 141 |

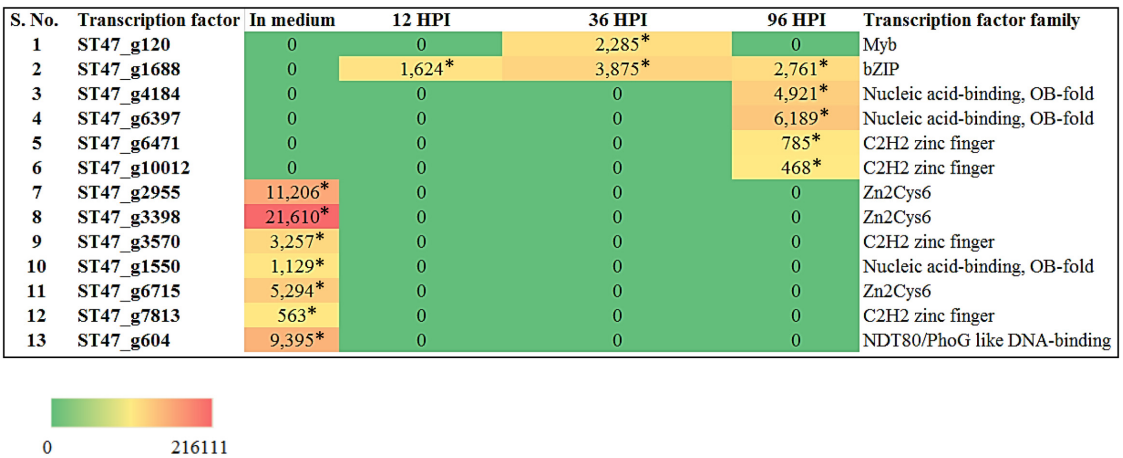


**FIGURE 9 |** Heat map representation of *A. rabiei* putative TFs. The expression of *A. rabiei* putative TF genes is shown during vegetative growth (in medium) and host invasion [12, 36, and 96 hours post inoculation (hpi)]. For this, *A. rabiei* transcriptome data was utilized (Fondevilla et al., 2015). Expression of putative TFs genes are denoted in the terms of FPKM values. Values with asterisk represent *P*-value < 0.005 at which differentially expressed genes (DEGs) were found.

TFs depending on the lifestyle and host of the fungi. Similar indications were also provided when phylogenetic analysis of *A. rabiei* Myb, bHLH and bZIP TF families was compared with other closely related necrotrophic fungi, i.e., *C. heterostrophus*, *P. tritici-repentis,* and *P. nodorum*. It suggested formation of well conserved clades in these TF families among the closely related necrotrophic fungi. The domain organization of Myb, bHLH, and bZIP TF families of *A. rabiei* showed presence of characteristic domains as well as numerous low-complexity regions. Various studies have suggested that these regions show significant divergence across protein families. Proteins containing low-complexity regions have more binding partners across different protein–protein interaction networks (Coletta et al., 2010). The low-complexity regions positioned at center of protein sequence are related to transcription-related gene ontology terms, whereas terminally located low-complexity regions are associated with translation and stress response-related terms (Coletta et al., 2010).

The TFs of bZIP family of *A. rabiei* had bZIP domains superimposed with coiled coil regions. Several TFs of bHLH and Myb family also had coiled coil regions. The architecture of a particular coiled-coil domain governs its oligomerization state, rigidity and ability to function as a molecular recognition system. In fungi, Myb TFs play an important role in cell differentiation, development and pathogenicity (Arratia-Quijada et al., 2012; Kim et al., 2014). Similarly, basic helix-loop-helix (bHLH) TFs are also known to be involved in development (Murre et al., 1994). The highly functionally conserved bZIP TFs ensure proper growth, development, sulfur metabolism, and iron homeostasis in fungi (Guo et al., 2011; Kong et al., 2015; Sun et al., 2016). They are highly stress responsive and are vital for pathogenicity in a reactive oxygen species-dependent manner. The gene structure of $[Zn(II)_2Cys_6]$, $C_2H_2$, nucleic acid-binding-OB-fold, winged helix repressor DNA-binding and Myb family of TFs suggested that the genes with single intron had intron phase either 0 or 1 predominantly. The three classes of intron phases are far from even distribution. Phase 0 introns are usually present in excess (Long et al., 1995) and the inferred evolution of intron phase distribution showed that the proportion of phase 0 introns increased over evolution (Nguyen et al., 2006). Therefore, the excess of phase 0 introns in *A. rabiei* putative TF genes indicates

| S. No. | Secretory protein | In medium | 12 HPI | 36 HPI | 96 HPI | InterPro | Description |
|---|---|---|---|---|---|---|---|
| 1 | ST47_g6821 | 0 | 1,783* | 3,172* | 0 | IPR018620 | Ubiquitin 3 binding protein But2 C-terminal domain |
| 2 | ST47_g8306 | 0 | 6,482* | 90,261* | 31,537* | IPR001938 | Thaumatin |
| 3 | ST47_g8923 | 0 | 2,522* | 1,451* | 743* | IPR017853 | Glycoside hydrolase superfamily |
| 4 | ST47_g5360 | 0 | 0 | 2,358* | 1,615* | IPR024655 | Uncharacterised protein family, glycosyl hydrolase catalytic domain |
| 5 | ST47_g438 | 0 | 0 | 0 | 734* | IPR011330 | Glycoside hydrolase/deacetylase, beta/alpha-barrel |
| 6 | ST47_g520 | 0 | 0 | 0 | 2,661* | | |
| 7 | ST47_g695 | 0 | 0 | 0 | 560* | IPR017774 | Bicupin, oxalate decarboxylase/oxidase |
| 8 | ST47_g1741 | 0 | 0 | 0 | 604* | IPR008922 | Uncharacterised domain, di-copper centre |
| 9 | ST47_g1932 | 0 | 0 | 0 | 19,057* | IPR001254 | Serine proteases, trypsin domain |
| 10 | ST47_g2005 | 0 | 0 | 0 | 1,530* | IPR008972 | Cupredoxin |
| 11 | ST47_g2527 | 0 | 0 | 0 | 1,404* | IPR012334 | Pectin lyase fold |
| 12 | ST47_g2544 | 0 | 0 | 0 | 503* | | |
| 13 | ST47_g3049 | 0 | 0 | 0 | 410* | IPR001842 | Peptidase M36, fungalysin |
| 14 | ST47_g4399 | 0 | 0 | 0 | 570* | IPR024655 | Uncharacterised protein family, glycosyl hydrolase catalytic domain |
| 15 | ST47_g4721 | 0 | 0 | 0 | 1,163* | IPR013781 | Glycoside hydrolase, catalytic domain |
| 16 | ST47_g5233 | 0 | 0 | 0 | 531* | | |
| 17 | ST47_g5489 | 0 | 0 | 0 | 1,404* | | |
| 18 | ST47_g6573 | 0 | 0 | 0 | 1,079* | | |
| 19 | ST47_g6595 | 0 | 0 | 0 | 1,044* | | |
| 20 | ST47_g6846 | 0 | 0 | 0 | 715* | IPR002937 | Amine oxidase |
| 21 | ST47_g6850 | 0 | 0 | 0 | 1,197* | | |
| 22 | ST47_g7695 | 0 | 0 | 0 | 564* | IPR016166 | FAD-binding, type 2 |
| 23 | ST47_g8289 | 0 | 0 | 0 | 1,149* | | |
| 24 | ST47_g8321 | 0 | 0 | 0 | 1,918* | | |
| 25 | ST47_g8740 | 0 | 0 | 0 | 695* | IPR000028 | Chloroperoxidase |
| 26 | ST47_g9909 | 0 | 0 | 0 | 8,380* | | |
| 27 | ST47_g10580 | 0 | 0 | 0 | 882* | IPR032382 | Alternaria alternata allergen 1 |
| 28 | ST47_g606 | 1,980* | 0 | 0 | 0 | | |
| 29 | ST47_g633 | 2,553* | 0 | 0 | 0 | IPR014720 | Double-stranded RNA-binding domain |
| 30 | ST47_g1803 | 2,244* | 0 | 0 | 0 | | |
| 31 | ST47_g3545 | 2,733* | 0 | 0 | 0 | | |
| 32 | ST47_g5565 | 4,279* | 0 | 0 | 0 | IPR029033 | Histidine phosphatase superfamily |
| 33 | ST47_g8454 | 1,661* | 0 | 0 | 0 | IPR008972 | Cupredoxin |
| 34 | ST47_g9382 | 1,462* | 0 | 0 | 0 | | |


**FIGURE 10 |** Heat map representation of *A. rabiei* putative secretory protein coding genes. The expression pattern of genes encoding *A. rabiei* putative secretory proteins is shown during vegetative growth (in medium) and host invasion (12, 36, and 96 hpi). For this, *A. rabiei* transcriptome data was utilized (Fondevilla et al., 2015). Expression of putative secretory protein coding genes are denoted in the terms of FPKM values. Values with asterisk represent *P*-value $< 0.05$ at which DEGs were found.

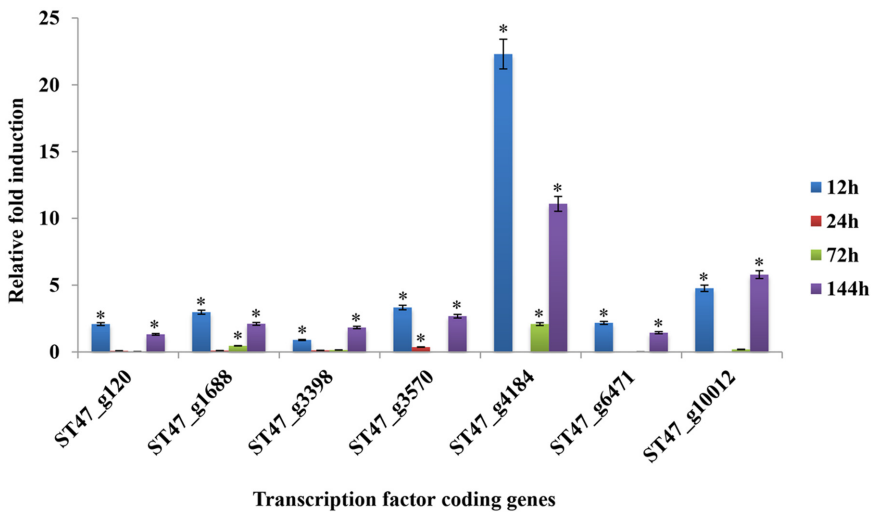


**FIGURE 11 |** Expression profiles of putative TF genes assayed by qRT-PCR during host colonization. Bar diagrams representing the expression pattern of seven putative TF genes are shown as the fold-change compared to the control. Expression was analyzed in *A. rabiei*-infected chickpea samples at 12, 24, 72, and 144 hpi. *ArEF1a* gene was used as an internal reference gene. Asterisks denote significant difference compared with the expression level at 0 hpi ($p < 0.05$).

toward the evolutionary development of this pathogen over the years.

Earlier, we predicted a set of 758 proteins in the secretome of *A. rabiei* (Verma et al., 2016). Since the secretory proteins are largely involved in the pathogenesis and deriving nutrition from the host, the *cis*-regulatory elements present in promoter sequences of secretory proteins coding genes of *A. rabiei* were identified. The secretome coding genes of *A. rabiei* were predicted to be significantly regulated by Myb TFs followed by $[Zn(II)_2Cys_6]$ zinc cluster TFs. Fungal genomes have at least 37 PFAM families of TF (Shelest, 2008). So far, 12 TFs from four families (Zinc finger, APSES, WOPR, and Fork head) have been found to regulate the gene expression of candidate effectors (Tan and Oliver, 2017). A central role for Zn-finger TF in effector expression was studied in *A. brassicicola* where a $[Zn(II)_2Cys_6]$ zinc cluster TF (AbPf2) regulates expression of 106 genes, out of which 33 genes encode secreted proteins, including eight putative effector proteins (Cho et al., 2013). Plants challenged with Δ*abpf2* mutants had elevated expression levels of photosynthesis, pentose phosphate pathway and primary metabolism related genes but decreased levels of defense-related genes. In *P. nodorum*, AbPf2 ortholog PnPf2 positively regulates the necrotrophic effectors *SnToxA* and *SnTox3* expression (Rybak et al., 2017). Likewise, PtrPf2 controls the expression of *P. tritici-repentis ToxA*, a near-identical copy of *SnToxA* (Rybak et al., 2017). This indicates that Pf2 TF exhibits a conserved role in regulating the effectors. Furthermore, SnStuA (an APSES bHLH TF) was found to regulate the expression of *SnTox3* (IpCho et al., 2010). In *Leptosphaeria maculans*, the expression of *AvrLm4-7*, *AvrLm1*, and *AvrLm6* is regulated by LmStuA (Soyer et al., 2015). The example of WOPR TFs was found in *Verticillium dahlia* and *Cladosporium fulvum*. The VdSge1 TF is required for full expression of the Cys-rich effector *Ave1* (Santhanam and Thomma, 2013). Similarly, CfWor1 TF primarily regulates development of *Cladosporium*, but also indirectly controls expression of a subset of effector genes (Ökmen et al., 2014). In *U. maydis*, a fork head TF named *Fox1* acts as a positive regulator of six candidate effector genes (Zahiri et al., 2010). Over all, these studies indicate the significance of TFs in regulating the effectors that ultimately govern the pathogenesis. The *A. rabiei* secretome was predicted to be mainly regulated by Myb TFs, therefore, it would be highly significant to identify the potential Myb TFs that controls the expression of *A. rabiei* effectors.

In order to understand significance of *A. rabiei* secretome and putative TFs during host colonization, the RNA-seq data of *A. rabiei* during *in medium* and *in planta* conditions were analyzed (Fondevilla et al., 2015). Thirty-four secretory protein coding genes and seven putative TF genes were differentially expressed. Most of these secretory protein coding genes had binding sites of Myb TFs (particularly Rap1) in their promoter sequences. This is consistent with our computational prediction suggesting Myb TFs as the major regulators of *A. rabiei* secretome. The qRT-PCR analysis of few *A. rabiei* putative TFs showed a lot of differences in expression profiling as compared to RNA-seq data. The putative TFs ST47_3398 and ST47_3570, which were not expressing during *in planta* conditions in RNA-seq data, showed expression in qRT-PCR analysis. Furthermore, RNA-seq data showed that majority of putative TFs were expressed during later stages of infection. By contrast, according to qRT-PCR analysis almost all selected putative TFs were expressed in early stages of infection as well. Biphasic kinetics of gene expression is commonly observed in both pathogen and host during infection (Schneider et al., 2011; Singh et al., 2012). This suggests that the expression profile of these putative TFs could be differential depending on the pathotype of *A. rabiei* and the cultivar of chickpea. Further investigation in this direction is a prerequisite to determine if TFs function discretely in different pathotypes of *A. rabiei*.

Since TFs as regulators play a crucial role in the life cycle of fungi, their presence or absence may offer opportunities or enforce limitations on the natural habitat of fungal species. The present study provides a platform to study *A. rabiei* TFs in detail. This will offer better insight into the evolution of regulatory mechanisms in *A. rabiei*. Comparative studies of *A. rabiei* TFs in other pathotypes will decipher their contribution in determining the pathotypes of this fungus. The extensive knowledge obtained will aid in designing successful strategies to control this devastating pathogen and prevent further crop losses.

## AUTHOR CONTRIBUTIONS

SV, RG, and PV designed the experiments; RG and SV performed bioinformatics analysis; SV performed the experiments; SV, RG, and PV analyzed data; and SV, RG, and PV wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01037/full#supplementary-material

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frontiers in Plant Science

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# Clarification on Host Range of *Didymella pinodes* the Causal Agent of Pea Ascochyta Blight

*Eleonora Barilli *, Maria José Cobos and Diego Rubiales*

*Institute for Sustainable Agriculture, CSIC, Córdoba, Spain*

*Didymella pinodes* is the principal causal agent of ascochyta blight, one of the most important fungal diseases of pea (*Pisum sativum*) worldwide. Understanding its host specificity has crucial implications in epidemiology and management; however, this has not been clearly delineated yet. In this study we attempt to clarify the host range of *D. pinodes* and to compare it with that of other close *Didymella* spp. *D. pinodes* was very virulent on pea accessions, although differences in virulence were identified among isolates. On the contrary, studied isolates of *D. fabae*, *D. rabiei,* and *D. lentil* showed a reduced ability to infect pea not causing macroscopically visible symptoms on any of the pea accessions tested. *D. pinodes* isolates were also infective to some extend on almost all species tested including species such as *Hedysarum coronarium*, *Lathyrus sativus*, *Lupinus albus*, *Medicago* spp., *Trifolium* spp., *Trigonella foenum-graecum,* and *Vicia articulata* which were not mentioned before as hosts of *D. pinodes*. On the contrary, *D. lentil* and *D. rabiei* were more specific, infecting only lentil and chickpea, respectively. *D. fabae* was intermediate, infecting mainly faba bean, but also slightly other species such as *Glycine max*, *Phaseolus vulgaris*, *Trifolium* spp., *Vicia sativa,* and *V. articulata*. DNA sequence analysis of the nuclear ribosomal internal transcribed spacer region (ITS) was performed to confirm identity of the isolates studies and to determine phylogenetic relationship among the *Didymella* species, revealing the presence of two clearly distinct clades. Clade one was represented by two supported subclusters including *D. fabae* isolates as well as *D. rabiei* with *D. lentil* isolates. Clade two was the largest and included all the *D. pinodes* isolates as well as *Phoma medicaginis* var. *pinodella*. Genetic distance between *D. pinodes* and the other *Didymella* spp. isolates was not correlated with overall differences in pathogenicity. Based on evidences presented here, *D. pinodes* is not specialized on pea and its host range is larger than that of *D. fabae*, *D. lentil,* and *D. rabiei*. This has relevant implications in epidemiology and control as these species might act as alternative hosts for *D. pinodes*.

**Keywords: pea, legume, ascochyta blight, dydimella pinodes, host range, disease management**

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****Correspondence:***
*Eleonora Barilli*
*ebarilli@ias.csic.es*

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## INTRODUCTION

Cool season legumes play an important role in farming systems worldwide (Siddique et al., 2012). They provide important services to societies as they are important sources of oil, fiber, protein-rich food and feed while supplying nitrogen (N) to agro-ecosystems via their unique ability to fix atmospheric $N_2$ in symbiosis with the soil bacteria rhizobia, increasing soil carbon content, and

stimulating the productivity of the crops that follow (Jensen et al., 2012). Among them, field pea (*Pisum sativum* L.) is widely grown across cooler temperate zones of the world on about 6.2 m ha annually with total production generally ranging between 10 and 11 m tons (FAOSTAT, 2015).

Ascochyta blight diseases represent serious limitations to legume production worldwide (Rubiales and Fondevilla, 2012; Khan et al., 2013). *Didymella fabae* Jellis and Punith. (anamorph *Ascochyta fabae* Speg.), *D. lentis* Kaiser, Wang and Rogers (anamorph *A. lentis* Vassiljevsky) and *D. rabiei* (Kovachevski) v. Arx (anamorph *A. rabiei* (Pass) Labr.) are the causal agents of ascochyta blights on faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), and chickpea (*Cicer arietinum* L.), respectively (Kaiser et al., 1997; Hernandez-Bello et al., 2006; Tivoli and Banniza, 2007). Yield losses caused by aschochyta blight are in order of 40% in lentil (Gossen and Derksen, 2003), but in severe cases losses higher than 90% have been reported in faba bean (Omri Benyoussef et al., 2012) and chickpea (Pande et al., 2005). In pea, this disease is caused by a complex of fungi formed by *Ascochyta pisi* Lib., *Didymella pinodes* (Berk and Blox) Petrak, *Phoma medicaginis* var. *pinodella* (L.K. Jones) Morgan-Jones and K.B. Burch and *Phoma koolunga* Davidson, Hartley, Priest, Krysinska-Kaczmarek, Herdina, McKay, and Scott (this last is, at the time, with limited presence in South and Western Australia; Tran et al., 2016). Of these, *D. pinodes* (formerly known as *Mycosphaerella pinodes* (Berk. and A. Bloxam) Vestergr., anamorph *Ascochyta pinodes* L.K. Jones) is the most predominant and damaging pathogen and under some conditions can cause yield losses up to 70% (Tivoli and Banniza, 2007).

*D. pinodes* remains an extremely difficult pathogen to control, primarily due to limited levels of host resistance available, and secondarily because fungicides are often uneconomic (Khan et al., 2013). Therefore, the main disease control strategy has been to avoid sowing close to infested field pea stubbles and/or to delay sowing of field pea crops for as long as possible in order to avoid the majority of ascospores, particularly those falling on emerging pea seedlings (Salam et al., 2011). Nevertheless, the late sowing is not an option in some countries due to the short crop season and this practice incurs unsustainable yield penalties in many instances (Khan et al., 2013). Other control measures involving crop rotation and intercropping have been also tested (Bailey et al., 2001; McDonald and Peck, 2009; Fernández-Aparicio et al., 2010) showing potential in disease reduction.

A better understanding of a pathogen's host range is critical to handle ascochyta blight and to break its cycle with more effectiveness, particularly in regions where pea is frequently grown and where the disease is endemic or where ascospores are an overriding primary source of initial infection. *D. pinodes* is known to be less specialized than other *Didymella* spp. (Sprague, 1929; Sattar, 1934; Le May et al., 2014), which increases the potential of this specie to survive. In fact, adjacent naturally infected alternative hosts could serve as important sources of inoculum to initiate disease epidemics on cultivated peas. So, the impact of alternative hosts on plant pathogen adaptation must be taken into account since they affect the survival of pathogen populations, and transmission opportunities to different components and ecological niches (wild/cultivated, cultivated/cultivated; Woolhouse et al., 2001), as recently showed for *D. rabiei* (Trapero-Casas and Kaiser, 2009). Nevertheless, despite its importance, the host range of *D. pinodes* on legume species other than *Pisum* spp. is poorly understood (Bretag, 2004; Taylor and Ford, 2007; Khan et al., 2013; Le May et al., 2014).

The aims of this study were therefore (i) to further refine the host range of *D. pinodes* within cultivated and wild legumes; (ii) to assess the susceptibility/resistance of different accessions within each of these legume species to nine isolates of *D. pinodes* from different geographical origin; (iii) to compare the host range of *D. pinodes* with that of other *Didymella* species; and (iv) to relate fungal isolates by ITS molecular markers.

# MATERIALS AND METHODS

## Fungal Isolates

Nine isolates of *D. pinodes*, two isolates of *D. fabae*, one of *D. lentil,* and one of *D. rabiei*, all from IAS-CSIC fungal collection, were used in the experiments (information reported in **Table 1**). Local *D. pinodes* isolate Dp-CO-99, as well as isolates Dp-FR-88, Dp-PO-03 and Dp-JAP-03 have previously shown to differ in aggressiveness toward pea accessions (Fondevilla et al., 2005). All isolates were monoconidial and were preserved in sterile cellulose filter papers.

## Plant Material

Disease responses were studied on accessions of 20 legumes species (**Table 2**): alfalfa (*Medicago sativa* L.), barrel medick (*M. truncatula* Gaertn.), button medick (*M. orbicularis* (L.) Bartal.), chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), common vetch (*Vicia sativa* L.), faba bean (*Vicia faba* L.), fenugreek (*Trigonella foenum-graecum* L.), grass pea (*Lathyrus sativus* L.), lentil (*Lens culinaris* Medik.), oneflower vetch (*Vicia articulata* Hornem.), pea (*Pisum sativum* ssp. *sativum* L.), prinkly scorpion's tail (*Scorpiurus muricatus* L.), red clover (*Trifolium pratense* L.), soybean (*Glycine max* (L.), subterranean clover (*T. subterraneum* L.), sulla (*Hedysarum coronarium* L.), tawny pea (*P. fulvum* Sibth. & Sm.), white clover (*T. repens* L.), and white lupin (*Lupinus albus* L.). From 1 to 6 accessions per species were tested (**Table 2**).

To ensure experiments with a uniform plant development stage, seeds were scarified by nicking with a razor blade and then germinated for 48 h on wet filter paper in a Petri dish at 4°C. The Petri dishes were then transferred to 20°C for 5–7 days. Germinated seeds were planted into plastic pots (6 × 6 × 10 cm) filled with a 1:1 mixture of sand and peat in a rust-free growth chamber. Plants were pre-germinated and sown at 3 days intervals in order to be able to select seedlings at the same growing stage at the time of inoculation. There were three independent replicates per fungal isolate, arranged in a complete randomized design. Each replicate consisted of 3 pots with 5 plants each per accession. Experiments were repeated three times. Pea cv. Messire was included in each replication as a common susceptible check. Plants were grown in a growth chamber at 20°C, under a photoperiod of 14/10 h day/night regime, with 148 $\mu$mol/m$^2$s irradiance at plant canopy for 3 weeks, until the plants reached the 4–5-leaf stage.

**TABLE 1 | Codes of reference, specie definition, collecting site, year and GenBank accession relative to the fungus isolates used in the study.**

| Fungal code | Fungal specie | Collecting site | Collecting year | GenBank n° |
|---|---|---|---|---|
| Dp-CO-99 | *Didymella pinodes* | Córdoba, Spain | 1999 | KR259388 |
| Dp-FR-88 | *D. pinodes* | Rennes, France | 2003 | KR259380 |
| Dp-PdT-03 | *D. pinodes* | Palmar de Troya, Spain | 2003 | KR259391 |
| Dp-PO-03 | *D. pinodes* | Wąsy, Poland | 2003 | KR259387 |
| Dp-JAP-03 | *D. pinodes* | Japan | 2003 | KR259392 |
| Dp-ANN-13 | *D. pinodes* | Annaba, Algeria | 2013 | KR259390 |
| Dp-M07-4 | *D. pinodes* | Perth, Australia | 2013 | KR259383 |
| Dp-Esc-13 | *D. pinodes* | Escacena del Campo, Spain | 2013 | KR259389 |
| Dp-KHM-13 | *D. pinodes* | Khemis Miliana, Algeria | 2013 | KR259386 |
| Df-AU04 | *D. fabae* | Gleisdorf, Austria | 2005 | KR259385 |
| Df-857 | *D. fabae* | France | 2005 | KR259384 |
| Dl-AL10 | *D. lentil* | Germany | 2010 | KR259381 |
| Dr-Pt04 | *D. rabiei* | Aleppo, Syria | 2010 | KR259382 |
| | *Ascochyta pisi* | Pullman, USA | 2007 | DQ383954 |
| | *D. pinodes* | Canberra, Australia | 2009 | EU338435 |
| | *Phoma koolunga* | Canberra, Australia | 2009 | EU338427 |
| | *P. medicaginis* var. *pinodella* | Palampour, India | 2008 | FJ032641 |

## Plant Inoculation

Plants with 4-5 leaves were inoculated as described by Fondevilla et al. (2005) with some modifications. Inoculum was prepared by multiplying spores of each isolate on PDA (Potato Dextrose Agar) medium with chloramphenicol (60 mg/l PDA) and ampicillin (50 mg/l PDA) at 20°C with 16 h light/8 h dark photoperiod. Spore suspensions were prepared by flooding the surface of 10-day-old cultures with sterile distilled water, gently scraping the colony with a glass rod and filtering the suspension through two layers of sterile cheesecloth. Concentration of pycnidiospores was determined with a haemocytometer and adjusted to $10^6$ spores/ml. Tween 20 (VWR) was added as wetting agent (two drops per 500 ml pycnidiospore suspension). The pycnidiospore suspensions were sprayed at the 4–5-leaf stage using a handheld sprayer at a rate of 1 ml per plant. After inoculation, plants were covered with a polyethylene sheet during the first 24 h in darkness, and high humidity was ensured by ultrasonic humidifiers operating for 15 min every 2 h. Later on, the polyethylene cover was removed and plants were maintained 9 more days in a growth chamber (under conditions described above). Every 2 days, water was added to the trays to maintain high relative humidity (95–100%).

## Disease Assessment

Plant response to infection was visually assessed 10 days after inoculation using two separate assessments. Disease severity (DS) was assessed by a visual estimation of the percent of diseased tissue per plant (Fondevilla et al., 2005). In addition, disease rating (DR) was visually assessed on leaves over the first, second and third nodes of each plant using a 0–5 scale defined by Roger and Tivoli (1996) were 0 = no lesions; 1 = a few scattered flecks; 2 = numerous flecks; 3 = 10–15% of the leaf area necrotic and appearance of coalescent necrosis; 4 = 50% of the leaf area dehydrated or necrotic; 5 = 75–100% of the leaf area dehydrated or necrotic. DR was then calculated as the average of values scored per node. Accessions displaying an average DR > 3 combined with DS > 35% were considered as highly susceptible, accessions displaying an average DR > 3 combined with DS values lower than 35% were considered as susceptible, accessions showing an average DR included between 2 and 3 combined with DS values < 35% were considered as moderately resistant and, finally, accessions displaying DR < 2 combined with DS values < 10% were considered as highly resistant.

## DNA Extraction and Its Amplification

Monoconidial cultures of the 13 isolates were grown in Petri dishes using PDA medium as described above. Mycelium was collected by flooding the surface of 5-day-old cultures with sterile distilled water (2 ml per Petri dishes), gently scraping the colony with a glass rod and filtering the suspension through two layers of sterile cheesecloth. Three Petri dishes per isolate were used, in order to ensure sufficient amount of fungal material. Suspension was centrifuged at maximum speed (14,000 rpm) and pellet was collected. DNA was extracted from ground mycelium using the DNeasy plant minikit (Qiagen, Ltd.). DNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to 20 ng μl/1 for PCR. Primers ITS1 and ITS2 were used to amplify the nuclear ribosomal internal transcribed spacer (ITS) region ITS1-5.8S-ITS2 following the protocol described by White et al. (1990). PCR products were extracted with a sterile scalpel and purified using the QIAquick Gel Extraction kit (Qiagen®) following the protocol of the manufacturer. The purified products were cloned using the pGEM-T Easy Vector Systems kit (Promega, Madison, WI, USA) following Barilli et al. (2011) protocol. Sequencing was carried out on an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Service, STAB VIDA GENOMICS LAB, Caparica, Portugal. For each isolate,

**TABLE 2 | Response of legume species to isolates of *D. pinodes* in seedling stage, measured 10 days after inoculation under controlled conditions.**

| Legume host | Code | Dp-M07-4 | | Dp-Esc-13 | | Dp-KHM-13 | | Dp-ANN-13 | | Dp-JAP-03 | | Dp-PO-03 | | Dp-CO-99 | | Dp-PdT-03 | | Dp-FR-88 | | Dl-AL10 | | Dr-Pt04 | | Df-AU04 | | Df-857 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DR[A] | DS[B] | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS |
| **PEAS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pisum sativum* | Messire[3] | 5 | 80bc* | 5 | 77b | 5 | 63b | 5 | 63ab | 4.7 | 66bc | 5 | 50a | 5 | 79ab | 5 | 50a | 5 | 50a | 0 | 0 | 0 | 0 | 2 | 2a | 0 | 0 |
| *P. sativum* | J20 | 5 | 87ab | 5 | 93a | 5 | 98a | 5 | 80ab | 5 | 100a | 5 | 62a | 5 | 87a | 5 | 43a | 4 | 35a | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0 |
| *P. sativum* | J4 | 5 | 95a | 5 | 90ab | 5 | 75ab | 5 | 83ab | 5 | 100a | 4.3 | 40a | 5 | 57bc | 5 | 25a | 4 | 15b | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0 |
| *P. sativum* | 6NIL | 5 | 83ab | 5 | 93a | 5 | 82ab | 5 | 92a | 5 | 77b | 5 | 70a | 5 | 47c | 5 | 25a | 5 | 30a | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0 |
| *P. fulvum* | IFPI3260 | 4 | 67c | 3.3 | 57c | 4.7 | 60b | 4 | 51.7b | 4 | 42c | 4.7 | 63a | 1.3 | 7d | 3 | 25a | 3 | 22b | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0 |
| AVERAGE | | | 82A | | 81A | | 76A | | 74A | | 78A | | 57A | | 54A | | 30A | | 26A | | 0B | | 0B | | 0.3C | | 0E |
| **WHITE LUPIN** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lupinus albus* | Giza 2[1] | – | – | 5 | 95a | – | – | 5 | 40a | 4 | 7b | 5 | 47a | 4 | 15a | – | – | 3 | 13a | – | – | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. albus* | Lup34[4] | 3 | 20a | 5 | 100a | 5 | 47a | 5 | 50a | 5 | 40ab | 4.7 | 43a | 5 | 25a | 3.3 | 40a | 3 | 5a | – | – | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. albus* | Lup35[4] | 4 | 35a | 5 | 22b | 5 | 37ab | 5 | 50a | 5 | 67a | 4 | 40a | 5 | 20a | 3 | 9b | 3 | 12a | 3.3 | 20a | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. albus* | Lup39[4] | 4.3 | 20a | 5 | 25b | 5 | 26b | 5 | 50a | 5 | 40ab | 4.7 | 44a | 5 | 25a | 5 | 46a | 3.3 | 15a | 0 | 0b | 0 | 0 | 0 | 0 | 0 | 0 |
| AVERAGE | | | 25CDE | | 53B | | 37B | | 47B | | 38B | | 43B | | 21.5BC | | 33A | | 9B | | 10B | | 0B | | 0C | | 0E |
| **CLOVERS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Trifolium pratense* | E07[5] | 4.7 | 47a | 4.3 | 30a | 4.7 | 37a | 5 | 58a | 4 | 40a | 4.7 | 38a | 3 | 20a | 4.7 | 30a | 3 | 10a | 2.7 | 12a | 0 | 0 | 3 | 13a | 3 | 8a |
| *T. subterraneum* | E08[5] | 4 | 20a | 3.3 | 20a | 4 | 20ab | 4 | 37a | 5 | 32a | 3.5 | 20a | 2 | 17a | 3 | 20a | 3 | 3a | 0 | 0b | 0 | 0 | 0 | 0b | 2.3 | 6a |
| *T. repens* | Anteria[1] | 3.7 | 26a | 4.3 | 30a | 4.3 | 17b | 4.7 | 30a | 4.2 | 38a | 3.3 | 15a | 2 | 16a | 5 | 32a | 3 | 8a | 0 | 0b | 0 | 0 | 0 | 0b | 2 | 7a |
| AVERAGE | | | 31BCD | | 27CDE | | 24BC | | 42BC | | 36B | 25BC | | | 17BC | | 27A | | 7B | | 4B | | 0B | | 4BC | | 8CD |
| **MEDICKS** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Medicago truncatula* | Parabinga[1] | – | – | 3 | 20ab | 2.7 | 10a | 2.3 | 35a | 5 | 40b | 2 | 5b | 2.7 | 25a | 2 | 5a | 1.3 | 5a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. truncatula* | Paraggio[1] | 2 | 20a | 2 | 12b | 4 | 20a | 2.3 | 27a | 3.7 | 37b | 2 | 5b | 2 | 30a | – | – | 2 | 6a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. truncatula* | M263[4] | 1.7 | 18a | 3.7 | 53a | 2.7 | 10a | 2 | 30a | 4.5 | 80a | 2.3 | 30a | 2 | 37a | 2 | 5a | 1.3 | 11a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. orbicularis* | M264[4] | 2 | 27a | – | – | 2.7 | 7a | 3 | 15a | 4.3 | 37b | – | – | 2.7 | 35a | 2 | 10a | – | – | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. orbicularis* | M281[4] | – | – | 4.3 | 47a | 3 | 10a | 3.3 | 21a | 4.5 | 60ab | 4.7 | 32a | – | – | 2 | 8a | 3 | 15a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AVERAGE | | | 22DEF | | 36BCD | | 12CD | | 25CD | | 38B | | 23C | | 34B | | 7BC | | 9B | | 0B | | 0B | | 0C | | 0E |
| **ONEFLOWER VETCH** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Vicia articulata* | BGE013376[6] | 4 | 37a | 3 | 37a | 4 | 27a | 4.3 | 45ab | 4 | 23a | 2.3 | 23a | – | – | 4.3 | 40a | 2.7 | 8a | 0 | 0 | 0 | 0 | 3 | 8a | 3 | 4a |
| *V. articulata* | BGE013984[6] | 4 | 43a | 4.3 | 60a | 3.7 | 30a | 4 | 43ab | 4 | 53a | 3 | 25a | 3 | 30a | 3.7 | 28a | 3 | 11a | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0a |
| *V. articulata* | BGE013985[6] | 4 | 40a | 3.7 | 63a | 4.3 | 33a | 4.3 | 53a | 5 | 53a | 3.7 | 33a | 2 | 15a | 4.3 | 30a | 3 | 8a | 0 | 0 | 2.3 | 3a | 0 | 0b | 2.3 | 3a |
| *V. articulata* | BGE018824[6] | 4 | 43a | 4 | 60a | 4.3 | 32a | 4 | 28b | 4 | 50a | 3 | 35a | 3 | 20a | 4.7 | 33a | 2.7 | 7a | 0 | 0 | 0 | 0 | 0 | 0b | 0 | 0a |
| AVERAGE | | | 41BC | | 55B | | 30B | | 43BC | | 48B | | 29BC | | 22BC | | 35A | | 9B | | 0B | | 0.8B | | 2BC | | 2DE |
| **CHICKPEA** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Cicer arietinum* | ILC72[2] | 3 | 15a | 4 | 13a | 2.7 | 6a | 4.3 | 8a | 1 | 1a | 4.3 | 30a | 0.7 | 2a | 4.3 | 7a | 1.3 | 3a | 0 | 0b | 0.3 | 5b | 0 | 0 | 0 | 0 |
| *C. arietinum* | M38[2] | 3.3 | 10a | 3.4 | 15a | 3.7 | 5a | 4.7 | 8a | 3.3 | 10a | 5 | 30a | 4.5 | 25b | 5 | 15a | 1.3 | 2a | 0 | 0b | 4.7 | 40a | 0 | 0 | 0 | 0 |

*(Continued)*

**TABLE 2 | Continued**

| Legume host | Code | Dp-M07-4 | | Dp-Esc-13 | | Dp-KHM-13 | | Dp-ANN-13 | | Dp-JAP-03 | | Dp-PO-03 | | Dp-CO-99 | | Dp-PdT-03 | | Dp-FR-88 | | Dl-AL10 | | Dr-Pt04 | | Df-AU04 | | Df-857 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DR[A] | DS[B] | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS |
| *C. arietinum* | AS18[2] | 1.7 | 9a | 3 | 8a | 3 | 4a | – | – | 3 | 12a | 4.7 | 21a | 3 | 5a | 4.3 | 7a | 1 | 3a | 3 | 30a | 5 | 37a | 0 | 0 | 0 | 0 |
| *C. arietinum* | AS19[2] | 2.7 | 10a | 2 | 13a | 3 | 5a | 5 | 5a | 3 | 12a | 5 | 28a | 3 | 7a | 5 | 8a | – | – | 0 | 0b | 3.8 | 35a | 0 | 0 | 0 | 0 |
| *C. arietinum* | AS23[2] | – | – | 3 | 10a | – | – | 5 | 7a | 2.7 | 5a | – | – | – | – | 5 | 10a | 1 | 1a | 0 | 0b | 2.6 | 40b | 0 | 0 | 0 | 0 |
| AVERAGE | | | 10EF | | 12EF | | 5D | | 7DE | | 8D | 28BC | | | 8C | | 10BC | | 2B | | 6B | | 31.4A | | 0C | | 0E |
| **LENTIL** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lens culinaris* | S17[2] | 4 | 29a | 4.3 | 62a | 4 | 47a | 4.7 | 53a | 5 | 48ab | 3.7 | 35a | – | – | 3.7 | 15a | 2 | 17a | 4.3 | 24a | 0 | 0 | 0 | 0 | 3 | 6a |
| *L. culinaris* | S23[2] | 4 | 57a | 3.7 | 60a | 4.7 | 47a | 5 | 67a | 5 | 60a | 2.7 | 25a | 2.3 | 10a | 4 | 33a | 2 | 8ab | 4.3 | 28a | 0 | 0 | 0 | 0 | 1.5 | 3ab |
| *L. culinaris* | R5[2] | 4 | 30a | 4.7 | 57a | 4 | 27b | 4.7 | 37a | 4 | 50ab | 3.3 | 30a | 3 | 22a | 4 | 17a | 2 | 4b | 4 | 45a | 0 | 0 | 0 | 0 | 0 | 0b |
| *L. culinaris* | R17[2] | 4 | 43a | 4 | 50a | 4.5 | 42ab | 4.7 | 45a | 4 | 32b | 3.7 | 20a | 1.3 | 7a | 3.3 | 7b | 0.7 | 2b | 4 | 48a | 0 | 0 | 0 | 0 | 1.3 | 1b |
| AVERAGE | | | 40BCD | | 58B | | 40B | | 49B | | 46B | | 27BC | | 13BC | | 18AB | | 6B | | 35A | | 0B | | 0C | | 3CDE |
| **SOYBEAN** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Glycine max* | PI08100[5] | 1.7 | 8 | 0 | 0 | 3 | 10 | 3.3 | 12 | 3 | 30 | 1 | 1 | 2 | 17 | 2 | 5 | 3 | 4 | 0 | 0 | 3 | 4 | 3.3 | 17 | 4 | 30 |
| AVERAGE | | | 8EF | | 0F | | 10CD | | 12DE | | 30BC | | 1D | 17BC | | | 5BC | | 4B | | 0B | | 4AB | | 17A | | 30A |

| Host specie | Code | Dp-M07-4 | | Dp-Esc-13 | | Dp-KHM-13 | | Dp-ANN-13 | | Dp-JAP-03 | | Dp-PO-03 | | Dp-CO-99 | | Dp-PdT-03 | | Dp-FR-88 | | Dl-AL10 | | Dr-Pt04 | | Df-AU04 | | Df-857 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DR[A] | DS[B] | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS |
| **COMMON VETCH** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Vicia sativa* | 3151[4] | 3 | 33a | 2 | 15a | 3.3 | 30a | 3 | 13a | 2 | 6a | 3 | 20a | 1.3 | 10a | 2.7 | 10a | 3 | 3a | 0 | 0b | 1 | 2a | 4 | 5a | 3.7 | 8a |
| *V. sativa* | 3154[4] | 3 | 37a | 2.3 | 28a | 3.3 | 35a | 3 | 23a | 2.3 | 18a | 3 | 20a | 2 | 10a | 2.7 | 8a | 3 | 11a | 1.7 | 7ab | 1.7 | 5a | 4 | 10a | 4 | 6a |
| *V. sativa* | 3155[4] | 3.7 | 33a | 2.3 | 27a | 3 | 35a | 3 | 15a | 2 | 17a | 2.3 | 20a | 1.7 | 10a | 2.3 | 13a | 1 | 3a | 0.9 | 2b | 0.5 | 2a | 4 | 8a | 4 | 8a |
| *V. sativa* | 3156[4] | 3 | 25a | 2 | 23a | 3.7 | 36a | 3 | 23a | 2 | 12a | 2.7 | 13a | 1.3 | 8a | 2 | 8a | 0.3 | 1b | 2 | 10a | 0 | 0a | 4 | 5a | 4 | 8a |
| AVERAGE | | | 32BCD | | 23DEF | | 34B | | 18DE | | 14CD | | 18CD | | 10C | | 10BC | | 5B | | 4B | | 2B | | 7B | | 7C |
| **GRASS PEA** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lathyrus sativus* | ILAT1[3] | 4 | 33a | 4.7 | 40a | 4 | 37a | – | – | 4.7 | 40a | – | – | – | – | 4.3 | 32a | 2.3 | 4a | 0 | 0b | 0 | 0 | 0 | 0b | 0 | 0 |
| *L. sativus* | ILAT10[3] | 3.7 | 50a | 4.3 | 57a | 3.7 | 17a | 5 | 63a | 4.7 | 37a | 4.3 | 48a | 2.3 | 17a | 4 | 20a | 3.5 | 12a | 1 | 2a | 0 | 0 | 4 | 5ab | 0 | 0 |
| *L. sativus* | ILAT16[3] | 4 | 43a | 5 | 53a | 4 | 28a | 5 | 50a | 4.3 | 50a | 3.7 | 33a | 1.7 | 10a | 3.7 | 17a | 3 | 7a | 0 | 0b | 0 | 0 | 4 | 10a | 0 | 0 |
| *L. sativus* | ILAT18[3] | 4.3 | 46a | 4 | 37a | 4.3 | 37a | 5 | 55a | 4.7 | 43a | 4.7 | 48a | 1 | 7a | 4.3 | 23a | 2.4 | 5a | – | – | 0 | 0 | 0 | 0b | 0 | 0 |
| *L. sativus* | BGE017184[6] | 4.3 | 42a | 5 | 70a | 2 | 27a | 5 | 47a | 4.7 | 45a | 4 | 37a | 0 | 0b | 4 | 30a | 3.7 | 11a | – | – | 0 | 0 | – | – | – | – |
| AVERAGE | | | 43B | | 51B | | 29B | | 54B | | 36B | | 42B | | 13AB | | 26A | | 8B | | 0.6B | | 0B | | 3BC | | 0E |
| **SULLA** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Hedysarum coronarium* | Sparacia[1] | 2.7 | 20a | 3.3 | 58a | 4 | 43a | 2.3 | 8 | 4.7 | 47a | 3 | 20b | 2 | 15a | 1.7 | 9b | 0.7 | 1a | 0 | 0b | 0 | 0 | 0 | 0 | 0 | 0 |
| *H. coronarium* | Grimaldi[1] | 1.5 | 10a | 2.7 | 33a | 4 | 45a | – | – | 5 | 60a | 3 | 42a | 1 | 5b | 1.7 | 5b | 1.3 | 10a | 0 | 0b | 0 | 0 | 0 | 0 | 0 | 0 |

*(Continued)*

**TABLE 2 | Continued**

| Host specie | Code | Dp-M07-4 | | Dp-Esc-13 | | Dp-KHM-13 | | Dp-ANN-13 | | Dp-JAP-03 | | Dp-PO-03 | | Dp-CO-99 | | Dp-PdT-03 | | Dp-FR-88 | | Dl-AL10 | | Dr-Pt04 | | Df-AU04 | | Df-857 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DR[A] | DS[B] | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS | DR | DS |
| *H. coronarium* | PI516575[5] | – | – | 3 | 55a | 3 | 35a | – | – | 4 | 50a | 3 | 50a | – | – | 2 | 9b | – | – | – | – | 0 | 0 | 0 | 0 | 0 | 0 |
| *H. coronarium* | PI516583[5] | – | – | 4 | 70a | – | – | – | – | – | – | 3 | 40a | – | – | 2.3 | 33a | 1 | 10a | 1 | 3a | 0 | 0 | 0 | 0 | 0 | 0 |
| AVERAGE | | | 15EF | | 54BC | | 41B | | 8DE | | 52.3B | | 36BC | | 10C | | 14BC | | 7B | | 1B | | 0B | | 0C | | 0E |
| **PRINKLY SCORPION'S TAIL** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Scorpiurus muricatus* | 44[4] | 1 | 2a | 3.3 | 35a | 3.3 | 30a | 1 | 1a | 3 | 38ab | 2 | 8a | 1 | 7a | 0.7 | 5a | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2a | 0 | 0 |
| *S. muricatus* | 52[4] | 0 | 0a | 1.7 | 10b | 0 | 0b | – | – | 2.5 | 27b | 1 | 6a | 1 | 5a | 0 | 0a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0a | 0 | 0 |
| *S. muricatus* | 63[4] | 0 | 0a | 2.3 | 15b | 1.5 | 10a | 1 | 1a | 3.7 | 47a | 1 | 1a | 0.5 | 1a | – | – | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0a | 0 | 0 |
| *S. muricatus* | 71[4] | 0.7 | 2a | 2.7 | 22b | 3.7 | 37a | 1.3 | 4a | 3.7 | 43ab | 0.3 | 1a | 1 | 7a | 2 | 5a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0a | 0 | 0 |
| AVERAGE | | | 1G | | 20DEF | | 34B | | 2E | | 40B | | 4D | | 5C | | 3C | | 0B | | 0B | | 0B | | 0.5C | | 0E |
| **FABA BEAN** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Vicia faba* | Baraca[1] | 1.3 | 3a | 1.7 | 8a | 2.7 | 13a | 1.3 | 2a | 0 | 0a | 1 | 1b | 1 | 1a | 0.7 | 1a | 0 | 0a | 0 | 0 | 0 | 0 | 4 | 16a | 4 | 23a |
| *V. faba* | Brocal[1] | 2 | 8a | 1.7 | 7a | 3 | 8a | 0 | 0b | 0 | 0a | 1.7 | 5a | 0 | 0a | 0.3 | 1a | 0 | 0a | 0 | 0 | 0 | 0 | 4 | 18a | 4 | 12a |
| *V. faba* | Navio6[1] | 1.7 | 2a | 2 | 10a | 3 | 13a | 0 | 0b | 0 | 0a | 0.7 | 1b | 1 | 2a | 0.3 | 1a | 0.3 | 1a | 0 | 0 | 0 | 0 | 4 | 15a | 4 | 25a |
| *V. faba* | Quijote[1] | 1.3 | 4a | 2 | 10a | 3 | 17a | 0 | 0b | 1 | 1a | 2 | 6a | 1 | 2a | 0.3 | 1a | 0.7 | 1a | 0 | 0 | 0 | 0 | 4 | 20a | 4 | 23a |
| AVERAGE | | | 4F | | 9EF | | 13CD | | 0.4E | | 0.3D | | 3D | | 1C | | 1C | | 0.5B | | 0B | | 0B | | 16A | | 19B |
| **FENUGREEK** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Trigonella foenum-graecum* | PI138685[5] | 4 | 10b | – | – | – | – | 4 | 70a | 1 | 5a | 5 | 60a | 1 | 5a | 1.5 | 8b | 1 | 1a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *T. foenum-graecum* | PI251281[5] | 4 | 13b | 2 | 17a | 2.5 | 10b | 4 | 25b | – | – | 4 | 30b | – | – | – | – | 1 | 1a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *T. foenum-graecum* | PI164140[5] | 3.3 | 35a | 4 | 25a | 4 | 30a | 4.5 | 38ab | 1 | 7a | 4 | 30b | 1 | 1a | 1.7 | 30a | 1 | 4a | 0 | 0 | 0 | 0 | – | – | – | – |
| AVERAGE | | | 20DEF | | 21DEF | | 20CD | | 44B | | 6D | | 40AB | | 3C | | 19B | | 2B | | 0B | | 0B | | 0C | | 0E |
| **COMMON BEAN** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Phaseolus vulgaris* | Dominguez[1] | 0 | 0 | 0 | 0 | – | – | – | – | 1 | 2a | 1 | 1a | 0 | 0 | 0.7 | 1a | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 5b | 3.3 | 7b |
| *P. vulgaris* | Krina[1] | 0 | 0 | 0 | 0 | 0.3 | 1a | 0 | 0 | 0 | 0 | 1.3 | 1a | 0 | 0 | 0.3 | 1a | 0 | 0 | 0 | 0 | 0 | 0 | – | – | – | – |
| *P. vulgaris* | BGE020024[6] | 0 | 0 | 0 | 0 | 0 | 0a | 0 | 0 | 0 | 0 | 1 | 1a | 0 | 0 | – | – | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 6b | 4 | 12a |
| *P. vulgaris* | BGE039971[6] | 0 | 0 | 0 | 0 | 0 | 0a | 0 | 0 | 0.7 | 2a | – | – | 0 | 0 | – | – | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 7a | 4 | 8b |
| AVERAGE | | | 0G | | 0F | | 0.3D | | 0E | | 1D | | 1D | | 0C | | 1C | | 0B | | 0B | | 0B | | 6BC | | 9C |

*[A]DR, Disease rating following 0–5 scale defined by Roger and Tivoli (1996). [B]DS, final disease severity (%) measured under controlled conditions. [1]Commercial varieties are named. [2]Belonging to IAS-CSIC collection. [3]Provided by ICARDA (Syria). [4]Collected by authors. [5]Provided by USDA (USA). [6]Provided by CRF-INIA (Spain). *Data followed with different letters, per column (lower letter types) and host species (capital letter type), are significantly different (LSD test, P = 0.01). – Not determined.*

two clones were sequenced. Both forward and reverse strands were sequenced for each clone. ITS sequences were submitted to GenBank.

In addition to this, sequences from *Ascochyta pisi*, *Didymella pinodes*, *Phoma koolunga,* and *P. medicaginis* var. *pinodella* (**Table 1**) retrieved from GenBank (http://www.ncbi.nih.gov; Davidson et al., 2007; Peever et al., 2007) were included in the analysis.

## Statistical Analysis

### Disease Responses

All isolate x species combinations (including several accessions per species) were arranged in a completely randomized design in a controlled condition growth chamber. For the whole data set, only final disease severity values were included in the statistical analysis. Disease severity was first analyzed by taking into account differences in pathogenicity between the 13 *Didymella* spp. isolates according to the species evaluated (by averaging disease severity among accessions within each species).

Disease severity was assessed for every *Didymella* spp. isolate between accessions within each species. The whole experiment was repeated three times. Before performing analyses of variance, the normality and equality of variances were checked using Shapiro–Wilk's (Shapiro and Wilk, 1965) and Bartlett's tests (Little and Hills, 1978) respectively. When necessary, DS percentage data were transformed to angles (y = arcsine (x/100)) and again checked before applying analysis of variance. Differences between isolates, species, or accessions within species were compared by analysis of variance (ANOVA) followed by a least significant difference (LSD) test, with values of $P < 0.01$ considered significant. Statistical analyses were performed with Statistix software (version 8.0; Analytical Software, Tallahassee, USA).

Disease rating (DR) was visually estimated as the mean disease score over the first, second and third leaves of each accession within each specie.

The entire data set was analyzed by Principal Component Analysis (PCA) using the web-based software PAST (Hammer et al., 2001), available at http://nhm2.uio.no/norlex/past/download.html, with the following settings: covariance matrix type, four principal components, 1-fold change threshold for clusters, and 0.3 correlation thresholds for clusters. PCA results were represented as a biplot, with accessions more susceptible to a specific *Didymella* spp. isolate (according to both DS and DR) located in the same area of the graph.

### ITS Sequence Analysis

Sequences were aligned and adjusted manually with Mega version 6 (Tamura et al., 2013) using the penalties of 15 for gap opening and 6.66 for gap extension. Estimates of genetic similarity (GS) were calculated for all possible pairs of genotypes according to Rho similarity coefficient (Posada and Crandall, 1998).

The evolutionary history was inferred using the unweighted pair-group method with arithmetic average (UPGMA; Sneath and Sokal, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and a dendrograms was constructed.

The trees were rooted using *P. koolunga* as outgroup. The scores between 50 and 74 bootstrap percentages (BS) were defined as weak support, scores between 75 and 89% BS as moderate support and scores > 90% BS as strong support. A likelihood ratchet employs multiple sequential truncated searches on different starting trees created by fast algorithmic searches on reweighed data, in the hope of exploring a larger proportion of tree space, analogous to the parsimony ratchet (Nixon, 1999). We ran 200 iterations with the general time reversible likelihood model of evolution with gamma distribution (GTR+G) and uniformly reweighing 15% of the data-set per iteration. Bootstrap support values from 1000 replicates were calculated using the heuristic search with random addition-sequence with 10 replicates limited to 10,000 tree rearrangements (branch swaps) imposed separately for each addition-sequence replicate (rearlimit = 10,000; limitperrep =yes). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are reported in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were excluded in analyses.

# RESULTS

The local *Didymella pinodes* isolate Dp-CO-99 caused different disease rating (DR) (**Table 2**) as well as significantly different disease severity (DS) values on the tested legume species ($P < 0.01$; **Figure 1**). The highest levels of susceptibility were found in *P. sativum* (DR = 5; DS = 67%) confirming expectations (Fondevilla et al., 2005), followed by *L. albus* (DR = 4.7; DS > 20%), *Trifolium* spp., *Medicago* spp., *V. articulata*, *C. arietinum,* and *L. culinaris* ($2 \leq$ DR < 3; DS > 15%). Some infection was also observed on *G. max*, *V. sativa*, *L. sativus*, *H. coronarium*, *P. fulvum*, *S. muricatus*, *V. faba,* and *T. foenum-graecum* although at the level of resistance (DR < 2; DS < 20%). *P. vulgaris* did not showed any symptoms of fungal infection (DS and DR = 0; **Figure 1**).

Results from cross inoculations performed with different *Didymella* spp. showed that the legume species under study displayed differential resistance/susceptibility to each isolate as indicated by significant specie x isolate interactions in ANOVA ($P < 0.01$; **Table 2**). Statistical analysis showed a significant effect of legume species (sum of squares = 353,064, $P < 0.001$), fungal isolates (sum of squares = 125,118, $P < 0.001$), and their interaction (sum of squares = 75,346, $P < 0.001$), indicating that not all *D. pinodes* isolates displayed the same infection pattern toward the legume species involved in this study.

*P. sativum* accessions showed DR values = 4 against all *D. pinodes* tested (**Table 2**), although level of infection varied greatly (DS from 15 to 100%). Isolates Dp-M07-4 (DS 80–95%), Dp-Esc-13 (DS 77–93.3%), Dp-JAP-03 (DS 66–100%), and Dp-KHM-13 (DS 63–98%) were the most aggressive on

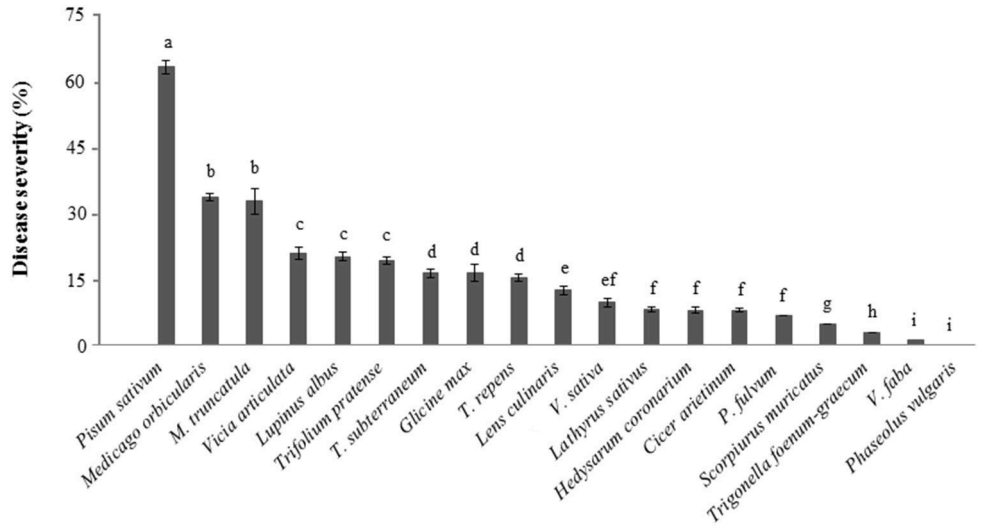


**FIGURE 1 | Percentage of disease severity (DS%) measured on foliar organs of different legume species in response to inoculation with *D. pinodes* isolate Dp-CO-99 under controlled conditions.** Averages per species are presented. The bars indicate the standard deviation; different letters indicate significant differences ($P = 0.01$).

cultivated peas (**Table 2**, **Figure 2A**). *P. fulvum* was generally more resistant than *P. sativum*, with DR ranging from 1.3 to 4.7 and DS from 7 to 67%. In particular, accession IFPI3260 confirmed here its high resistance against Dp-CO-99 (DR = 1.3, DS = 6.7; **Figure 2B**; Fondevilla et al., 2005). In addition, *P. fulvum* was also moderately resistant to isolates Dp-FR-88 and Dp-Esc-13 (DR = 3; DS < 25%). As for *P. sativum*, accession IFPI3260 was immune to other *Didymella* spp. isolates tested (**Table 2**).

Accessions from *L. albus* were also susceptible to *D. pinodes* (DR = 3), showing level of infection that varied depending on the isolate tested (average DS = 34%, range 9–100%). Isolates Dp-Esc-13 and Dp-ANN-13 were the most virulent (DR = 5; DS > 40%; **Table 2**, **Figure 2C**). By contrary, *L. albus* was resistant to both *D. rabiei* and *D. fabae*, while only accession Lup35 was moderately infected by *D. lentil* (**Table 2**).

*Trifolium* spp. showed responses to *D. pinodes* infections that were from moderately resistant to susceptible (averages ranging between DR 2.5–4.6 and DS 7–42%; **Table 2**). Isolate Dp-ANN-13 was the most virulent (DR > 4.7; DS > 30%) while Dp-CO-99 and Dp-FR-88 the lesser (DR < 3.7; DS < 20%; **Table 2**, **Figure 2D**). Accessions studied were not infected by *D. rabiei*, whereas *T. pratense* was slightly infected by *D. lentil* and *D. fabae* (**Table 2**).

*V. articulata* accessions were from highly susceptible to moderate resistant against *D. pinodes* inoculations (averages ranging between DR 2.6–4.3 and DS 9–55%), being differences significant among accessions and isolates ($P < 0.01$) (**Table 2**, **Figure 2E**). *V. articulata* was immune to *D. lentil*, whereas only certain accessions were slightly infected by *D. rabiei* or *D. fabae* (DR from 2.3 to 3.7, DS < 8%).

Similarly, *Medicago* spp. accessions showed from resistance to susceptibility to *D. pinodes* infections. Nevertheless, differences between plant species were not consistent (**Table 2**, **Figure 2F**). Isolate Dp-JAP-03 was the most virulent on all *Medicago* accessions studied inciting DR ranging from 3.7 to 5 and DS ranging from 37 to 80%. *Medicago* accessions were not affected by any other *Didymella* spp.

Response of *L. culinaris* accessions to *D. pinodes* varied greatly, depending on the isolate tested (averages ranging between DR 0.7–5 and DS 4–67%) (**Table 2**, **Figure 2G**). As for peas, isolates Dp-Esc-13, Dp-JAP-03, and Dp-M07-4 were highly virulent on all accessions tested (DR > 3.7, DS > 40%; **Table 2**). By contrary, lentils were less damaged by isolate Dp-FR-88 (DR $\leq$ 2, DS < 10%). As expected, all accessions tested were susceptible to *D. lentil*, with no significant differences between them (DR $\geq$ 4, DS $\geq$ 20%). By contrary, *D. rabiei* did not cause any symptoms on lentils and *D. fabae* was only slightly infective (DR < 3, DS $\leq$ 6%; **Table 2**).

Accession PI08100 from *G. max* showed from moderate to high resistance against *D. pinodes* infections (**Figure 2H**), being isolate Dp-JAP-03 the most virulent (DR = 3, DS = 30%). By contrary, no symptoms were found on PI08100 after Dp-PO-03 and Dp-Esc-13 inoculations. This accession was immune to *D. lentil*, slightly infected by *D. rabiei* (DR = 3, DS = 4%) and susceptible to *D. fabae* (DR > 3.3, DS > 17%; **Table 2**).

Similarly, responses from *V. sativa* varied greatly, being resistant to isolates Dp-CO-99 and Dp-FR-88 (averages DR < 1.8 and DS < 10%) and susceptible to Dp-KHM-13 (DR > 3, DS > 30%), with no significantly difference among accessions (**Table 2**, **Figure 2I**). *V. sativa* showed a fully compatible interaction with both *D. fabae* isolates in spite of a reduced severity (DS < 10%). Nevertheless, both *D. rabiei* and *D. lentil* caused foliar symptoms at reduced rates (DR < 2, DS $\leq$ 10; **Table 2**).

Except for local isolate Dp-CO-99, studied *L. sativus* accessions were moderately or highly susceptible to all *D. pinodes*

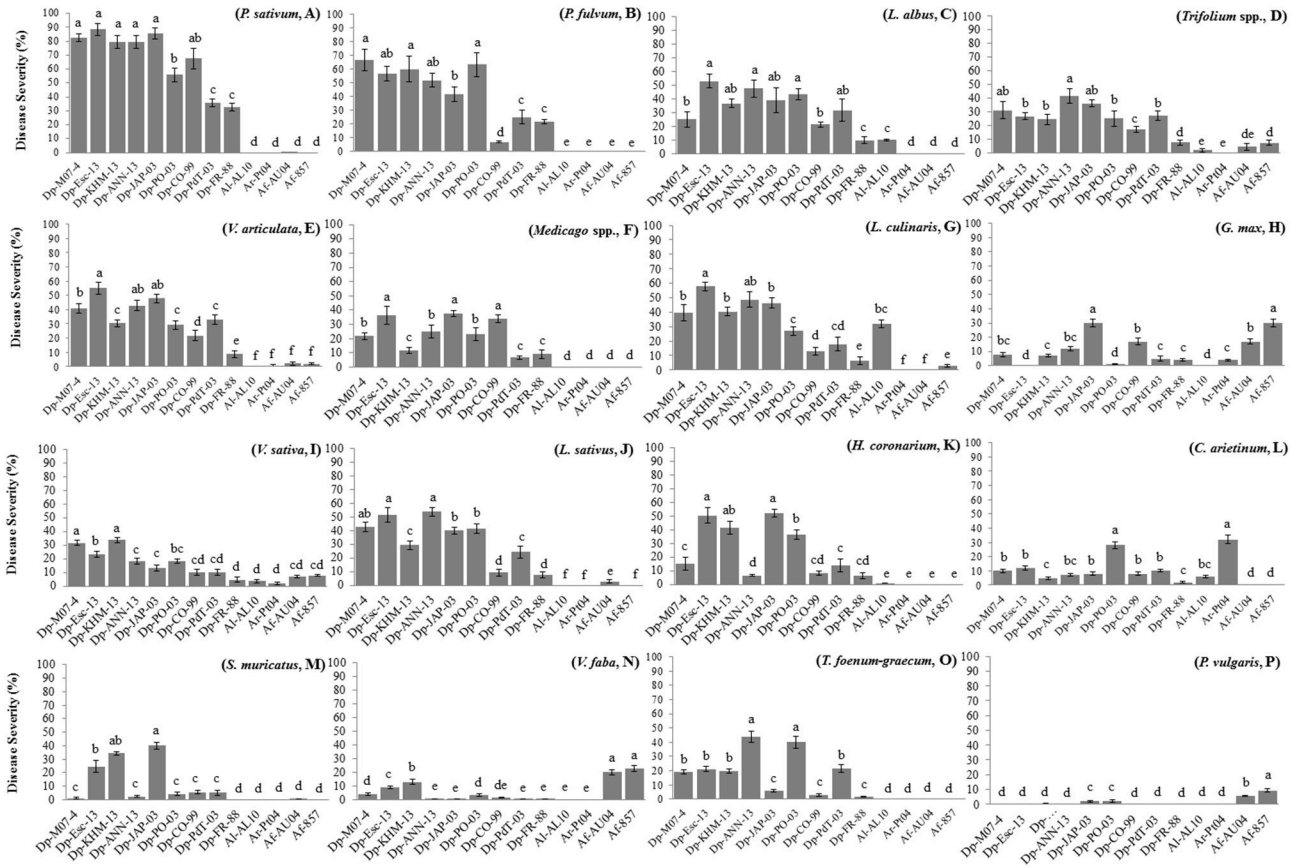


**FIGURE 2 | Disease severity (%) measured on whole plants of different legume species after infection by isolates of *Didymella* spp. under controlled conditions.** Averages per species are presented: **(A)** pea (*Pisum sativum*), **(B)** tawny pea (*P. fulvum*), **(C)** white lupin (*Lupinus albus*), **(D)** clovers (*Trifolium pratense*, *T. subterraneum*, *T. repens*), **(E)** oneflower vetch (*V. articulata*), **(F)** medicks (*Medicago orbicularis*, *M. truncatula*), **(G)** lentil (*Lens culinaris*), **(H)** soybean (*Glycine max*), **(I)** common vetch (*V. sativa*), **(J)** grass pea (*Lathyrus sativus*), **(K)** sulla (*Hedysarum coronarium*), **(L)** chickpea (*Cicer arietinum*), **(M)** prinkly scorpion's tail (*Scorpiorus muricatus*), **(N)** faba bean (*Vicia faba*), **(O)** fenugreek (*Trigonella foenum-graecum*), **(P)** common bean (*Phaseolus vulgaris*). The bars indicate the standard deviation. Different letters indicate significant differences ($P = 0.01$).

isolates studied, being isolates Dp-Esc-13 and Dp-ANN-13 the most virulent (DR > 4, DS > 37%; **Table 2**, **Figure 2J**). Accessions from *L. sativus* were immune or highly resistant to infection from *D. rabiei*, *D. lentil,* and *D. fabae* isolates (**Table 2**).

Responses of *C. arietinum* varied greatly depending both on the *D. pinodes* isolate employed as well as the accession tested (**Figure 2L**, **Table 2**), but infection was always reduced compared to pea accessions. Accessions showed DR from low to high, depending on the isolate, but always with low DS (<30 %). Isolate Dp-Po-03 was the most virulent on chickpea (DR > 4.3), while all accessions were resistant to isolate Dp-FR-88 (DR < 1.3, DS < 3%). Chickpea was resistant to both *D. fabae* isolates, while accession AS18 showed moderate susceptibility to Dl-AL10 infection. Chickpea showed a fully compatible interaction with *D. rabiei* isolate studied (Dr-Pt04) although significant differences between accessions were found (**Table 2**).

*H. coronarium*, *S. muricatus,* and *T. foenum-graecum* showed differential responses to *D. pinodes* inoculations depending principally on the isolate tested ($P < 0.01$; **Figures 2K,M–O,** respectively). In general, accessions showed symptoms that were significantly reduced comparing with *P. sativum*, also if some exceptions were found (e.g., *H. coronarium* and DP-JAP-03 or *T. foenum-graecum* and Dp-PO-03 with DR $\geq$ 4 and DS $\geq$ 30%; **Table 2**). With the exception of isolate Dp-KHM-13, *V. faba* was highly resistant against almost all *D. pinodes* studied (DR $\leq$ 2 and DS $\leq$ 10%; **Table 2**). Accessions belonging to *H. coronarium*, *S. muricatus,* and *T. foenum-graecum* were highly resistant or immune to infection with other *Didymella* spp. *V. faba* was highly susceptible to both *D. fabae* isolates with no significant differences among accessions, while no symptoms were found after Dr-Pt04 and Dl-AL10 inoculations (**Table 2**).

Finally, *P. vulgaris* was highly resistant to all *Didymella* spp. isolates since no or limited symptoms were foundflentils were less damaged by isolate on all accessions tested (DR $\leq$ 1.3, DS $\leq$ 2%) with exception of *D. fabae* that caused compatible interactions (DR $\geq$ 3.3) although with reduced DS values (**Table 2**, **Figure 2P**).

Among the isolates tested**,** Dp-KHM-13 was the most virulent being common bean the unique legume specie tested that was immune, while Dp-FR-88 was the lesser damaging

isolate (**Table 3**). Isolate Dl-AL10 (*D. lentil*) was only virulent on *L. culinaris* accessions, while isolate Dr-Pt04 (*D. rabiei*) showed symptoms on *C. arietinum* and, although limited, on *G. max*. Finally, *G. max*, *P. vulgaris*, *T. pratense*, *V. sativa,* and *V. faba* were susceptible to isolates from *D. fabae* (**Table 3**).

Principal component analysis (PCA) showed that two principal axes gave eigenvalues greater than 1, while the other axis all had eigenvalues lesser than 1 (**Table 4**). Hence, the first two principal components were considered important and contribute the most in the distribution of variation existing among the isolates. The component 1 had an eigenvalue of 2.8034, accounted for 40.62% of the overall variance in the data set (**Table 4**). Component 2 had an eigenvalue of 2.2101 and accounted for 31.1% of the total variance. Hence, the two principal components contributed for 71.69% of the total variability (**Table 4**). The first pc was more related to the level of aggressiveness expressed by *D. pinodes*, *D. lentil,* and *D. rabiei* isolates, while the second pc contributed for those expressed by *D. fabae* isolates to all cultivars tested (**Figure 3**). On the other hand, we can also appreciate certain host specificity between the legumes and fungal isolate species. The scattered diagram showed a major distance between isolates belonging to *D. fabae* and *D. rabiei* with the rest that were studied (**Figure 3**).

ITS analysis by MEGA6 originates an optimal tree with the sum of branch length = 0.06595538. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (**Figure 4**). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are reported in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 437 positions in the final dataset.

From the dendrogram generated, using UPGMA with the genetic distance coefficient, the 17 isolates could be classified into two main clusters that clearly separate all isolates belonging to *D. pinodes* from the others (**Figure 4**). Cluster 1 (bootstrap support [BS] = 91 from Maximum Composite Likelihood analysis) included all isolates from *D. pinodes* used for the study as well as the *D. pinodes* isolate from GenBank. *D. pinodes* isolates showed to be monophyletic since they were included in a unique well-supported branch ([BS] = 99). The isolate of *P. medicaginis* var. *pinodella* was also included in this clade although it was divergent and on a branch apart from the rest of the isolates included.

Clade II ([BS] = 71) comprised two isolates of *D. fabae, one* isolate from *D. lentil*, one isolate from *D. rabiei* and *one* isolate from *A. pisi*. *D. fabae* isolates showed to be monophyletic since they were included in a unique well-supported branch ([BS] = 99). By contrary, isolates from *D. lentil* and *D. rabiei* clustered together in other strongly supported branch ([BS] = 97) where *A. pisi* was apart ([BS] = 80). Finally, isolate from *P. koolunga* did not fit with any other isolates.

## DISCUSSION

Cool season legumes play an important role for human food and animal feed throughout the world. These crops are attacked by numerous aerial fungal pathogens that cause considerable losses in quality and quantity (Tivoli et al., 2006; Muehlbauer and Chen, 2007). The major necrotrophic fungal diseases are ascochyta blight on various grain legumes and *Didymella pinodes* was reported as the principal agent causing aschochyta blight on peas (Tivoli et al., 2006; Khan et al., 2013).

The aim of this current study was to analyse variations in the susceptibility of different legume species to *D. pinodes* compared to other *Didymella* spp., as well as to characterize the disease response of different cultivars within different legume species toward several *D. pinodes* isolates under controlled conditions. The results demonstrated that *D. pinodes* is able to cause disease in a number of legume species, that *D. pinodes* isolates from different geographical origin are differentially aggressive toward the legume species, and that cultivars within each legume species responded differentially to *D. pinodes*.

Infection of several host species is common in agrosystems leading to change in epidemic characteristics and pathogenicity. As a result, these processes will modify the survival of pathogen populations and their transmission (Woolhouse et al., 2001). In fact, variation in disease response can be significant at both the host species level as well as the host cultivar level, as was recently shown (Moussart et al., 2008; Le May et al., 2014). In the current study, cultivars from 20 different legume species were used to characterize the behavior of *D. pinodes* isolates sampled from pea. Visible symptoms caused by *D. pinodes* isolates were observed on all the legume species examined in this study, excepted with common bean. Large differences in susceptibility to *D. pinodes* were observed among the infected hosts, with *Pisum* spp. being the most susceptible, followed by *L. sativus*, *L. culinaris*, *L. albus*, *Medicago* spp., *Trifolium* spp., *T. foenum-graecum,* and *V. articulata*. In contrast to other *Didymella* species, *D. pinodes* appears to have the widest host range, since only accessions from lentil and chickpea were severely infected by *D. lentil* and *D. rabiei*, respectively, while *D. fabae* infected principally beans (common bean, faba bean, and soybean) and common vetch. Results for *D. lentil* and *D. rabiei* agreed with previous studies which demonstrated that artificial inoculations with Ascochyta fungi in the greenhouse and/or growth chambers are host-specific (Kaiser et al., 1997; Khan et al., 1999; Hernandez-Bello et al., 2006; Peever et al., 2007). In fact, it was previously found that *D. fabae*, *D. lentil,* and *D. rabiei* only diseased their respective hosts, while no visible symptoms were observed on any of the plant species other than faba bean, lentil and chickpea (Kaiser et al., 1997; Trapero-Casas and Kaiser, 2009). Nevertheless, for *D. rabiei*, Trapero-Casas and Kaiser (2009) also found that the fungus was able to survive on other leguminous or weeds, even though it did not show any visible symptoms and that this phenomenon could serve as secondary reservoirs in the absence of the natural host. In our study, isolates from *D. fabae* were highly virulent on faba bean but were also able to slightly infect other beans and vetch.

**TABLE 3 | Disease reaction of fourteen isolates of *Didymella* spp. from different geographical origin on fifteen leguminous species, performed under controlled growing conditions.**

| Host specie | N | Dp-M07-4 | Dp-Esc-13 | Dp-KHM-13 | Dp-ANN-13 | Dp-JAP-03 | Dp-PO-03 | Dp-CO-99 | Dp-PdT-03 | Dp-FR-88 | DI-AL10 | Dr-Pt04 | Df-AU04 | Df-857 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pisum sativum* | 4 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | – | – | – | – |
| *P. fulvum* | 1 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | – | + | + | – | – | – | – |
| *Lupinus albus* | 4 | +++/+* | ++++/+++ | ++++/+++ | ++++ | ++++/+++ | ++++ | +++ | ++++/+ | +/+++ | –/+++ | – | – | – |
| *Trifolium pretense* | 1 | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | + | +++ | + | + | – | + | + |
| *T. repens* | 1 | +++ | +++ | +++ | +++ | ++++ | +++ | + | +++ | + | – | – | – | + |
| *T. subterraneum* | 1 | +++ | +++ | +++ | ++++ | +++ | +++ | + | + | + | – | – | – | + |
| *Lathyrus sativus* | 5 | ++++/+++ | ++++ | +++/++++ | ++++ | ++++ | ++++/+++ | –/+ | +++ | +/+++ | – | – | –/+++ | – |
| *Lens culinaris* | 4 | ++++/+++ | ++++ | ++++/+++ | ++++ | ++++/+++ | +++/+ | +/- | +++ | –/+ | ++++/+++ | – | – | –/+ |
| *Vicia articulate* | 4 | ++++ | ++++ | +++ | ++++/+++ | ++++/+++ | +++/+ | + | +++/++++ | + | – | –/+ | –/+ | –/+ |
| *Trigonella foenum-graecum* | 3 | +++ | +++/+ | +++/+ | ++++/+++ | – | +++/++++ | – | –/+ | – | – | – | – | – |
| *Medicago orbicularis* | 2 | + | ++++ | + | +++ | ++++ | +++ | + | + | + | – | – | – | – |
| *M. truncatula* | 3 | + | +/+++ | +/+++ | + | ++++ | + | + | – | –/+ | – | – | – | – |
| *Cicer arietinum* | 5 | –/+++ | +++/+ | +/+++ | +++ | –/+++ | +++ | –/++++ | +++ | – | –/+ | ++++/– | – | – |
| *Hedysarum coronarium* | 4 | +/- | ++++/+ | ++++ | + | ++++ | ++++/+ | –/+ | –/+ | – | – | – | – | – |
| *Scorpiurus muricatus* | 4 | – | –/+++ | –/++++ | – | +++/+ | – | – | – | – | – | – | – | – |
| *Vicia sativa* | 4 | +++/++++ | + | +++/++++ | + | + | + | – | + | –/+ | – | – | +++ | +++ |
| *Glycine max* | 1 | – | – | + | +++ | +++ | – | + | – | + | – | + | +++ | +++ |
| *Vicia faba* | 4 | – | –/+ | + | – | – | – | – | – | – | – | – | +++ | +++ |
| *Phaseolus vulgaris* | 4 | – | – | – | – | – | – | – | – | – | – | – | +++ | +++ |

*Results correspond to the combination of Disease Rate (based on a 0–5 scale, Roger and Tivoli 1996) and the % of the whole plant covered by symptoms (% Disease Severity, DS %) as follows:*
*++++, highly susceptible (DR ≥ 3 and DS > 35%); +++, susceptible (DR ≥ 3 and DS < 35%); +, moderately resistant (2 < DR < 3 and DS < 35%); –,highly resistant (DR < 2 and DS < 10%). *When several classifications are reported means that genotypes from the same specie showed different responses to the same fungal isolate. Classification reported in first position is the most common.*

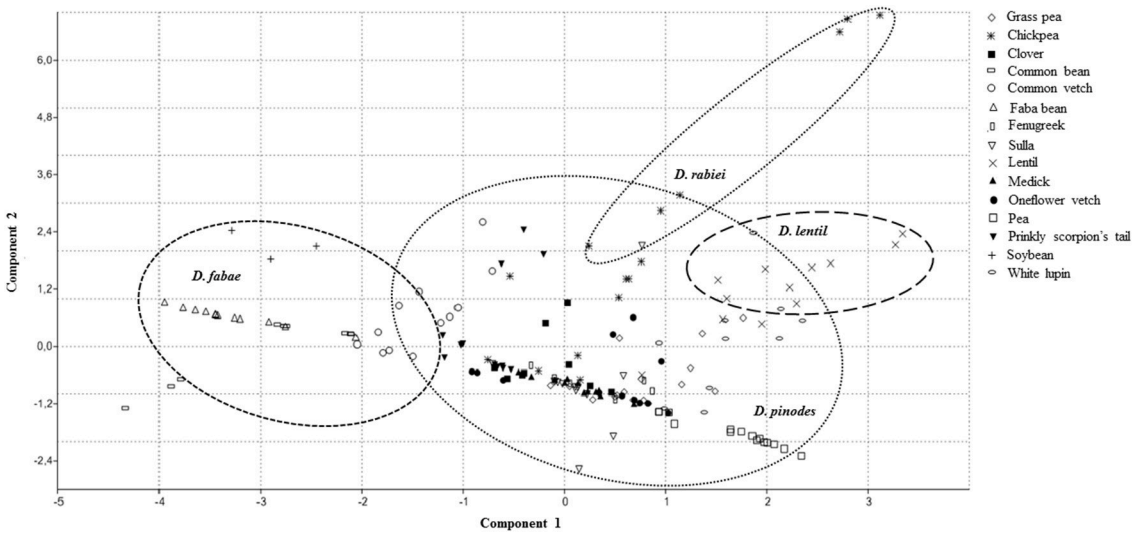


**FIGURE 3 | Scattered diagram generated by principal component analysis (PCA) showing associations between Disease Severity and Disease Rating response performed by 13 isolates of *Didymella* spp. on 15 leguminous species.** A short distance between plant accessions and fungal isolate in the component space is indicative in susceptibility of the plant/pathogen interaction.

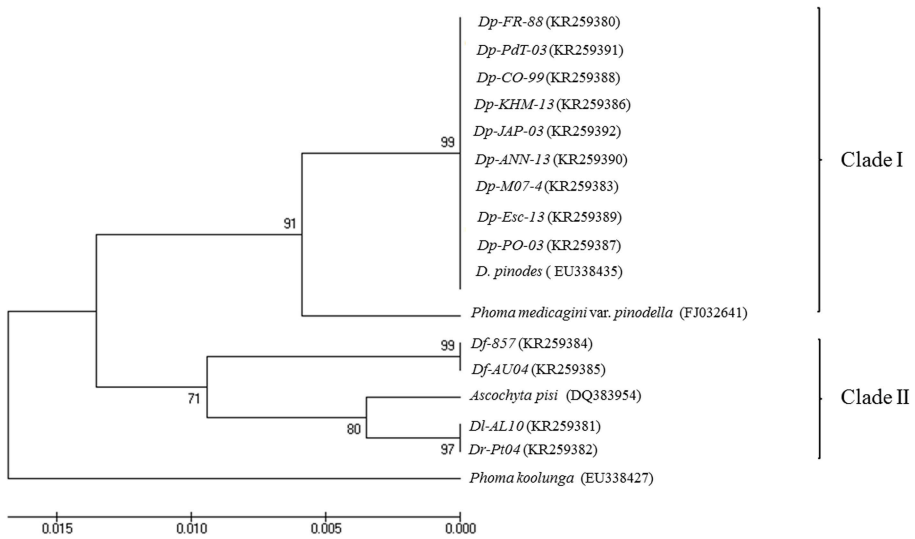


**FIGURE 4 | UPGMA dendrograms of 13 samples of *Didymella* spp. based on Dice distance for Internal Transcribed Spacer regions analysis.**

Regarding *D. pinodes* virulence, the results obtained with pea genotypes with very low levels of partial resistance were similar to those obtained by Fondevilla et al. (2005) and Le May et al. (2014) with common vetch and clover. All genotypes studied from *P. sativum* showed high susceptibility to all isolates tested, while accession IFPI3260 from *P. fulvum* (tawny pea) displayed a certain degree of partial resistance. These results confirms that only incomplete resistance is available for cultivated pea, while the highest levels of resistance are available in related *Pisum* species. In fact, sources of resistance to *D. pinodes* were recently found in accessions belonging to *P. fulvum*, *P. sativum* ssp. *syriacum,* and *P. sativum* ssp. *elatius* (Zhang et al., 2003; Fondevilla et al., 2005; Carrillo et al., 2013). Accession IFPI3260 showed from moderate to high resistance against 4 out of 9 *D. pinodes* isolates tested under controlled conditions. This accession was previously identified also as an important source of resistance against pea powdery mildew (*Erysiphe pisi* DC) and pea rust (*Uromyces pisi* (Pers.) Wint) (Fondevilla et al., 2007; Barilli et al., 2009) and is included in our department plant breeding programme.

*Lathyrus* has been reported as a resistant leguminous to *D. pinodes* infection firstly by Weimer (1947) who studied

**TABLE 4 | Principal components for disease rating (DR) and disease severity (DS) values of 13 isolates of *Didymella* spp.**

| | Component 1 | Component 2 |
|---|---|---|
| Eigenvalues | 2.8034 | 2.2101 |
| Proportion of variance | 40.623 | 31.067 |
| Cumulative variance | 40.623 | 71.690 |

accessions belonging to *L. tingitanus*, *L. sativus* and *L. hirsutus*, followed by another relevant report (Gurung et al., 2002) which confirmed resistance of *L. sativus*, and added *L. ochrus* and *L. clymenum* as species with high degree of resistance. Nevertheless, all accessions from *L. sativus* used in our study resulted to be highly susceptible to all *D. pinodes* isolates tested under controlled conditions. Susceptibility in white lupin (*L. albus*), lentil (*L. culinaris*), fenugreek (*T. foenum-graecum*) and oneflower vetch (*V. articulata*) is described here for the first time, expanding the current knowledge of *D. pinodes*'s host range.

The almost complete absence of symptoms in common bean (*P. vulgaris*) against several *D. pinodes* isolates may indicate that this species is a non-host species or that the fungus had invaded the host tissues internally although no visible symptoms were observed. This has been previously found for *D. rabiei*, which was recovered consistently from inoculated tissue of pea without causing any visible symptoms (Trapero-Casas and Kaiser, 2009). Future histological studies will be necessary to clarify this fact.

Unlike common bean, common vetch (*V. sativa*), faba bean (*V. faba*), and soybean (*G. max*) may be defined as a host plant under conditions of high inoculum pressure, but all genotypes studied displayed a very high level of partial resistance against the set of fungal isolates tested. As the conditions used in this study were very favorable for disease development on plants, the results would require confirmation by testing under different infection conditions such as in the field since growth habit, canopy morphology, lodging and precocity can affect *D. pinodes* development (Khan et al., 2013) and plant susceptibility since it was reported that plant symptoms were more severe at plant maturity than at the seedling stage (Zhang et al., 2003). In addition, plant seasonality might also be another factor that influenced plant susceptibility in the field. Common vetch and faba bean are cool season legumes, whereas common bean and soybean are summer crops. Influences of mean temperatures and humidity on host plant susceptibility during crop development needs to be further investigated, as on *Didymella* spp. the temperatures before and after the fungal infection period affected disease development and symptom expression (Trapero-Casas and Kaiser, 1992; Roger et al., 1999; Frenkel et al., 2008).

The use of faba bean has been previously tested in pea intercropped field as an alternative control measure to limit aschochyta blight (Fernández-Aparicio et al., 2010), leading to a fungal reduction by up to 60%. Introduction of species as common bean, common vetch, faba bean, soybean in pea rotation or intercropped may be tested in relation with a reduction of aerial spores during the cropping season and the survival of the pathogen into the soil residues by chlamydospore and sclerotium production. In fact it has been previously reported that introduction of plants with modified characteristics than pea imposes a non-host barrier, and as a consequence, less conidia are surviving and successfully transported to new developing host tissue (Zhang et al., 2003; McDonald and Peck, 2009; Fernández-Aparicio et al., 2010).

The existence of susceptible, partially and highly resistant genotypes within the same species (as in medicks, sulla, fenugreek, chickpea, prinkly scorpion's tail) suggest that the reaction may therefore be described as cultivar specific since the fungal ability to infect these other species depends on the susceptibility of the cultivar chosen (Moussart et al., 2008). *C. arietinum* accessions showed different degrees of susceptibility depending on the accession and the isolate tested, nevertheless cv. ILC72 was one of the lesser diseased after *D. pinodes* inoculation. ILC72 is a *D. rabiei* resistant line from ICARDA which showed a degree of resistance in the field and in controlled environments (Muehlbauer and Chen, 2007), as confirmed here. This accession has been thoroughly used in breeding programmes worldwide, as well in studies of the genetic of resistance to aschochyta blight (Cobos et al., 2006; Muehlbauer and Chen, 2007; Madrid et al., 2014). Susceptibility found here to certain *D. pinodes* isolates in cultivars belonging to *H. coronarium*, *Medicago* spp., *S. muricatus* and *T. foenum-graecum* is also described here for the first time. The susceptibility of these pasture legume species need to be tracked under field conditions before to become a serious agricultural problem. Thus, for each legume species, it should be interesting to enlarge the set of genotypes tested to make possible the identification of resistant genotypes.

In terms of pathogenicity, results on peas showed that the local isolate Dp-Co-99 was not always the most aggressive. In fact, disease severity measured on the primary host plants showed that isolates Dp-M07-4, Dp-Esc-13, Dp-KHM-13, and Dp-ANN-13 (from Perth, Australia, Escacena del Campo, Spain and both Khemis Miliana and Annaba from Algeria, respectively) were significantly more aggressive, hence dangerous if introduced in other fields. Migration of invasive organisms might lead to selective emergence of adapted isolates in novel geographic regions and on specific host genotypes (Leo et al., 2015). The evolutionary potential of pathogens may be increased and subsequently adapt to overcome host resistances (Linde et al., 2009). Available resistance to *D. pinodes* is partial and governed by multiple quantitative resistance loci (Rubiales and Fondevilla, 2012). Pathogen aggressiveness could incur a gradual evolution and adaptation that may lead to an "erosion" of resistance, especially if a monoculture farming system is applied (Gandon, 2002).

*D. pinodes* is a teleomorph of *A. pinodes* that reproduces asexually by pycnidia containing splash-dispersed pycnospores (Roger and Tivoli, 1996), and sexually by perithecia releasing wind-dispersed ascospores (Tivoli and Banniza, 2007). With the presence of sexual reproduction, new combination of genes could arise in the field, from one growing season to the next (Ali et al., 1994). The existence of pathotypes between *D. pinodes* isolates is still a matter of concern since there are numerous reports analyzing differential reaction of fungal isolate collection on various hosts leading to ambiguous conclusions (Ali et al., 1978; Zhang et al., 2003; Setti et al., 2009, 2011). Here, despite

their large geographical distance (Africa, Australia and Europe), we found a similarity between the host range pattern and the low genetic variability between the *D. pinodes* isolates used for the study. Both results from *D. pinodes* host range as well as molecular ITS analysis indicate a lack of pathotypes within the fungal collection used here.

## CONCLUSIONS

Knowledge of the host range is important to determine whether other crops could be affected. Understanding of population diversity and identification of pathogenic variation within plant species will assist in the management of ascochyta blight diseases.

If common bean is a non-host to *D. pinodes* as our results suggest, the use of this specie may have positive effect on soil infestation and subsequent disease development. Conversely, the use of grass pea, clover, lentil, oneflower vetch, white lupin might considerably increase the inoculum potential of the soil, having a deleterious effect on the subsequent pea crop. Ascospores produced in pseudothecia on overwintered debris of alternative hosts may serve as important sources of primary inoculum and/or inoculum necessary for secondary infections later in the growing season, as other aschochyta species did (Trapero-Casas et al., 1996; Trapero-Casas and Kaiser, 2009). Infected alternative hosts also may aid in the pathogen's survival from one growing season to the next, as do pea debris and infected seeds (Kaiser, 1990, 1992, 1997).

The use of chickpea, medick, sulla or fenugreek cultivars with qualitative resistance could be considered, but studies on the risk of resistance breakdown are required. As well, it would be important to determine if and which species could act as bridging hosts allowing for the crossing of *D. pinodes* isolates from one legume with those from another, as demonstrated with *Ascochyta* spp. by Hernandez-Bello et al. (2006) for *A. pisi* and *A. fabae* isolates. This is especially important in light of the plasticity of *D. pinodes* which is highly adaptable under the influence of biotic and abiotic factors (Le May et al., 2014).

## AUTHOR CONTRIBUTIONS

EB designed experiments. Together with MC carried out the experimental work. EB carried out most of the data analysis and contributed to the writing of the manuscript. DR contributed to the interpretation of results and writing of the manuscript. MC also contributed to critical reading.

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# Ultrastructural and Cytological Studies on *Mycosphaerella pinodes* Infection of the Model Legume *Medicago truncatula*

*Tomoko Suzuki[1,2], Aya Maeda[1], Masaya Hirose[1], Yuki Ichinose[1], Tomonori Shiraishi[1†] and Kazuhiro Toyoda[1]**

[1] Laboratory of Plant Pathology and Genetic Engineering, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan, [2] Faculty of Science, Japan Women's University, Bunkyo-ku, Japan

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***Correspondence:**
*Kazuhiro Toyoda*
*pisatin@okayama-u.ac.jp*

**†Present address:**
*Tomonori Shiraishi, Research Institute for Biological Sciences, Okayama, Okayama, Japan*

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Ascochyta (Mycosphaerella) blight on cultivated peas is primarily caused by infection through asexual spores (pycnospores) of *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren [recently renamed *Peyronellaea pinodes* (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley]. Using a model pathosystem involving *Medicago truncatula* and *Mycosphaerella pinodes* strain OMP-1, we examined the histology and ultrastructure of early infection events and fungal development including penetration by appressoria, vegetative growth of infection hyphae, and host responses. On the susceptible ecotype R108-1, pycnospores germinated and grew over the surface of the epidermis, then formed an appressoria and penetrated the cuticle. Beneath the cuticle, the infection peg expanded into a hyphae that grew within the outer wall of the epidermis. Subsequently, the hyphae penetrated down within mesophyll cells and proliferated vigorously, eventually, forming asexual fruiting bodies (pycnidia). In contrast, successful penetration and subsequent growth of infection hyphae were considerably restricted in the ecotype Caliph. Detected by its reaction with cerium chloride ($CeCl_3$) to generate electron-dense cerium perhydroxides in transmission electron micrographs, hydrogen peroxide ($H_2O_2$) accumulated in epidermal and mesophyll cells of Caliph challenged with pycnospores of *M. pinodes*. This intracellular localization was confirmed by energy-dispersive X-ray spectroscopy. Our observations thus indicate that the oxidative burst reaction leading to the generation of reactive oxygen species is associated with a local host defense response in Caliph, since no clear $H_2O_2$ accumulation was detectable in susceptible R108-1. Indeed, aberrant hyphae such as intrahyphal hyphae and dead hyphae, probably due to a local defense elicited by the fungus, were abundant in Caliph but not in R108-1. Our results on the cellular interactions between the fungus and host cells provide additional insights to understand foliar infection by *M. pinodes* on cultivated peas.

**Keywords: transmission electron microscopy (TEM), disease resistance, energy-dispersive X-ray (EDX) spectroscopy, hydrogen peroxide ($H_2O_2$), intrahyphal hyphae, susceptibility**

## INTRODUCTION

Ascochyta (Mycosphaerella) blight of pea, caused by *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren [syn. *Peyronellaea pinodes* (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley], is one of the most important diseases of grain legumes worldwide, especially in Europe, North America, Australia, and New Zealand (Moussart et al., 1998; Bretag and Ramsey, 2000). The disease annually causes 10% yield losses and occasionally reach 50% yield losses (Wallen, 1965, 1974; Xue et al., 1997). Despite the economic impact and numerous studies on this disease, little is known about the cytological features during infection by *M. pinodes*, especially in resistant interactions. One reason is due to the lack of resistant cultivars of pea (*Pisum sativum* L.) as well as the available resources in the *Pisum* germplasm collection with strong resistance to this disease (Prioul-Gervais et al., 2007).

*Mycosphaerella pinodes* is a hemibiotrophic pathogenic fungus that directly penetrates host epidermal cells. This disease is normally initiated by asexual pycnospores, which germinate to develop non-melanized appressoria that penetrate host cuticles directly (Clulow et al., 1991; Nasir et al., 1992). Clulow et al. (1991) reported that a pycnospore of *M. pinodes* germinated to form a germ tube which differentiates into an appressorium that directly penetrates the host cuticle. Subsequently, the infection peg formed an infection hyphae, which grows through the outer wall of the epidermis without killing the epidermal cells, frequently penetrating cells directly. Based on microscopic observations, they suggested that the early stage of infection, lasting at least 48 h after inoculation, is biotrophic and is then followed by the typical necrotrophic phase involving progressive necrosis.

In regard to fungal virulence and disease development, we found that this fungus secretes both an elicitor and a suppressor for plant defense during germination, mainly before the actual penetration (Shiraishi et al., 1978, 1992). Shiraishi et al. (1992) successfully determined the chemical structures of two suppressors, named supprescins A and B. These are small mucin-type glycopeptides containing *N*-acetylgalactosamine (NAcGal) attached to the serine residue in the peptide moiety. Interestingly, the supprescin B exhibits a "V-shaped structure" with a strong positive charge, which readily facilitates targeting of the host protein(s) (Shiraishi et al., 1992, 1997; Toyoda et al., 2016). In fact, the purified supprescins can severely inhibit the proton-pumping activity of host plasma membrane ATPase (Yoshioka et al., 1990; Shiraishi et al., 1991; Amano et al., 1995) and the related signal transduction pathway dependent on phosphatidylinositols and related lipids (Toyoda et al., 1992), temporarily reducing the capability of the host cell to defend itself (Yamada et al., 1989). Actually, the suppressor treatment renders the host cells susceptible even to unrelated (non-pathogenic) pathogens (Shiraishi et al., 1997; Toyoda et al., 2011, 2016), indicating that the suppressors are required for conditioning susceptibility of host cells.

The oxidative burst is one of the earliest defense responses to pathogen attack which leads to a transient accumulation of reactive oxygen species (ROS), including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (Bradley et al., 1992; Mehdy, 1994; Lamb and Dixon, 1997). ROS produced during the oxidative burst not only protect against invading pathogens, but also act as signaling molecules that initiate plant defense responses (Bradley et al., 1992; Mehdy, 1994; Lamb and Dixon, 1997). In some cases, ROS can inhibit pathogen growth by strengthening host cell walls through oxidative cross-linking of glycoproteins, such as proline-rich protein (Bradley et al., 1992; Deepak et al., 2007). Previously, we demonstrated that the oxidative burst in pea leaves elicited with an elicitor preparation from *M. pinodes* is effectively inhibited or delayed by a suppressor from the same fungus (Kiba et al., 1996, 1997; Toyoda et al., 2012; Amano et al., 2013). On the basis of these findings, it is likely that a rapid and effective production of ROS is a hallmark of resistance response to the fungal infection. However, subcellular aspects of host defense responses to *M. pinodes* infection, especially in relation to resistance have been studied little. The purpose of this study was thus to observe infection behavior after germination of pycnospores of *M. pinodes* as well as the host cell responses to the infection, using a recently developed model pathosystem involving *Medicago truncatula* (Toyoda et al., 2013a). Specifically, we aimed to observe the ultrastructural features during symptom development and differences in host responses between *M. truncatula* ecotypes. Fungal strategies resulting in successful colonization are also discussed.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Previously, we evaluated disease susceptibility of 19 *Medicago truncatula* ecotypes to *Mycosphaerella pinodes* strain OMP-1 and selected ecotype R108-1 as a highly susceptible ecotype and Caliph as having the lowest susceptibility (Toyoda et al., 2013a). Seeds of *M. truncatula* ecotype R108-1 and Caliph were scarified by treatment with anhydrous sulfuric acid for 5 min, then washed thoroughly with tap water (Toyoda et al., 2013a). Seeds were then germinated on wetted filter paper, then grown on water-swelled Jiffy-7 peat pellets (AS Jiffy Products, Oslo, Norway) in a growth room at 22°C, with a 10 h light/14 h dark cycle at 11.8 $W \cdot m^{-2}$ as described previously (Toyoda et al., 2013a). Detached leaves of 4- to 6-week-old seedlings were used for all experiments.

### Pathogens and Inoculations

*Mycosphaerella pinodes* strain OMP-1 (NBRC 30342, ATCC 42741), isolated in Akaiwa City, Okayama Prefecture, Japan in 1978, was cultured onto V8 juice agar medium at 23°C for 7 days as described previously (Shiraishi et al., 1978). Pycnospores that formed were suspended in sterile distilled water, and the concentration was adjusted to $1 \times 10^4$, $10^5$ or $10^6$ spores/ml for each inoculation. To perform a detached leaf assay, trifoliate leaves were excised from 4- to 6-week-old seedlings and maintained alive on moist cotton in a plastic tray filled with wetted paper towel. For inoculation, 10 μl of pycnospore suspensions containing 0.02% (v/v) Tween 20 was carefully dropped on the adaxial surface of detached leaves. Each

tray was then covered with a clear plastic wrap to maintain a high humidity and kept in a dew chamber at 22°C, with 14-h illumination per day at 11.8 $W \cdot m^{-2}$.

## Light Microscopy

Pycnospore germination and subsequent formation of infection hyphae were observed with a light microscope (Olympus BX60, Olympus, Tokyo, Japan). The inoculated leaves were fixed with a mixture of ethanol and acetic acid (24:1, v/v) at 3, 6, 9, 12, and 24 hour post inoculation (hpi), and decolorized with the same mixture at room temperature for 6 h and stained with 0.5% (w/v) aniline blue (Nacalai Tesque, Tokyo, Japan) in 0.1 M potassium phosphate buffer (pH 8.5) for 15 min. The samples were then rinsed with 70% ethanol and distilled water and viewed with a bright field microscope (Olympus BX60, Olympus).

## DAB Staining

The inoculated leaves were soaked in 1 mg/ml 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, St. Louis, MO, United States) in distilled water at 18 or 24 hpi, and then vacuum-infiltrated for 30 s three times and incubated for an additional 8 h at room temperature. The leaves were then fixed and decolorized with the 24:1 mixture of ethanol and acetic acid at room temperature for 6 h. In most cases, the fungal structures were stained with aniline blue as described already, then viewed with the light microscope.

## Light and Transmission Electron Microscopy (TEM)

At 1, 3, 5 and 7 days post inoculation (dpi), the inoculated leaves were cut into small pieces (2 mm × 3 mm) with a razor blade. Samples of R108-1 and Caliph at 1 and 3 dpi were cut from the region beneath the inoculated site. Samples of R108-1 were prepared at 5 and 7 dpi from the region surrounding the inoculated site. The specimens were prefixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C overnight and postfixed with 1% (w/v) buffered osmium tetroxide at room temperature for 1 h. The fixed specimens were dehydrated in a graded ethanol series (30, 50, 70, and 90% v/v ethanol; 20 min each change and then three 30-min changes of 100% ethanol) and infiltrated with Quetol 651 resin mixture (Nissin EM, Tokyo, Japan). Semithin sections (700 nm) were cut from resin blocks using a diamond knife. The sections were mounted on a glass slide and stained with 0.6% (w/v) toluidine blue including 1% (w/v) sodium tetraborate. After washing with distilled water, the stained sections were observed with the light microscope (Olympus BX60). Ultrathin sections (70–90 nm) were cut from the resin blocks and mounted on copper grids. The sections were stained with 1% (w/v) uranyl acetate for 30 min and lead solutions for 30 min as described previously (Suzuki et al., 2003). Sections were observed with a transmission electron microscope (TEM) (H-7500, Hitachi, Tokyo, Japan) at 80 kV. Fixatives, cacodylate buffer and osmium tetroxide used here are biohazard, so all procedures including weighing and solution preparation of chemicals were performed in a fume hood using protective clothing and gloves. Handling and waste disposal were carried out according to the Guidelines for the Management of Chemical Substances issued by the Japanese government.

## TEM Observation for $H_2O_2$ Accumulation

We used a histochemical analysis to detect hydrogen peroxide ($H_2O_2$) *in situ*, based on the generation of cerium perhydroxides as described by Bestwick et al. (1998). The inoculated portions of the leaves were cut into to small pieces (2 mm × 3 mm), then the leaf pieces were soaked in 50 mM MOPS/KOH (pH 7.2) containing 5 mM $CeCl_3$ for 1 h after the vacuum infiltration. Leaf pieces were prefixed in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (CAB) at room temperature for 4 h, and postfixed with 1% (w/v) buffered osmium tetroxide for 1 h. After fixation, leaf pieces were washed three times for 10 min in CAB and dehydrated with ethanol and infiltrated in Quetol 651 resin mixture as described above. The resin blocks were sectioned (100–120 nm) using a diamond knife, and the unstained sections were observed with the Hitach H-7500 TEM at 60 kV. Uninoculated leaf pieces were used as the control. For the negative control, inoculated leaf pieces were treated with MOPS as a substitute for $CeCl_3$.

## Elemental Analysis for $H_2O_2$-Reactive Deposits

Intracellular localization of cerium perhydroxides resulting from reaction of $H_2O_2$ with $CeCl_3$ was assessed cytochemically by energy-dispersive X-ray (EDX) spectroscopy. Unstained sections were subjected to EDX point microanalysis and EDX elemental mapping using a TEM and STEM system (H-7510, Hitachi) equipped with an EDX detector operated at 80 kV and analytical software (EMAX 5770W, Horiba, Kyoto, Japan).

# RESULTS

## Infection Behavior of *M. pinodes* on *M. truncatula* Ecotype R108-1 and Caliph

To observe infection and disease symptoms on leaves of two *M. truncatula* ecotypes (highly susceptible ecotype R108-1 and least susceptible ecotype Caliph), the leaves of both ecotypes were inoculated with pycnospores of *M. pinodes*. Repeated inoculation trials indicated that the percentage of pycnospore germination on both ecotypes was similar and that 80% of the pycnospores had germinated by 6 hpi (**Figure 1A**, dotted line). The rate of forming infection hyphae differed significantly between the two ecotypes. In particular, unlike on the R108-1, successful penetration was significantly suppressed on the Caliph, when assessed at 24 hpi (**Figure 1A**, solid line). Disease symptoms differed remarkably between the ecotypes especially when inoculated with a high concentration of pycnospores ($1 \times 10^6$ spores/ml). Brown lesions on R108-1 expanded from the inoculation sites, whereas those on Caliph never expanded (**Figure 1B**). Initial symptoms appeared as small, slightly raised spots on leaves, when R108-1 was inoculated with a low concentration of pycnospores ($1 \times 10^5$ or $1 \times 10^4$ spores/ml) and the lesions were surrounded by yellow halos (**Figure 1B**, left). Roger et al. (1999) reported that spore

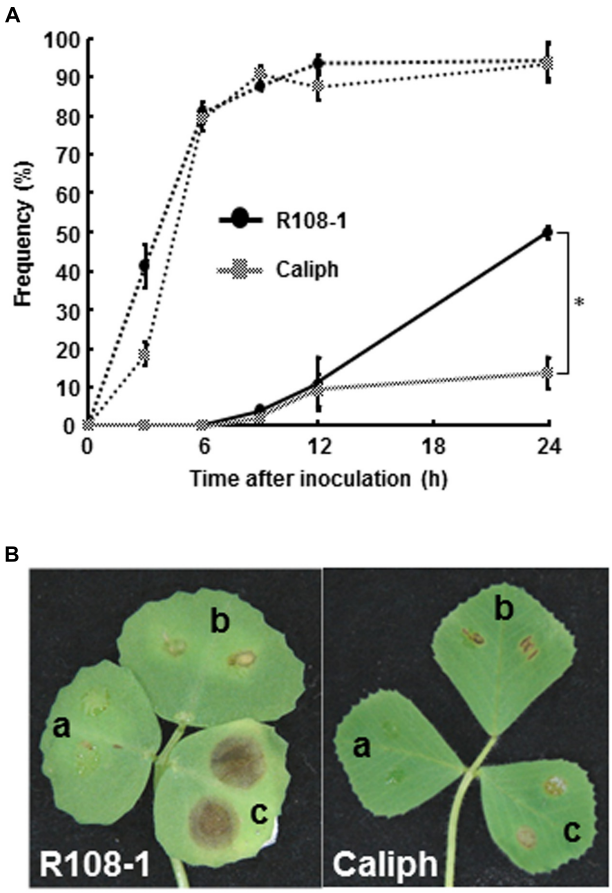


**FIGURE 1 | (A)** Percentage germination of pycnospores (dotted line) and formation of infection hyphae (solid line) on/in detached leaves of *Medicago truncatula* ecotypes Caliph and R108-1. Values are means ± standard deviations from three independent experiments. Asterisk indicates a significant difference as revealed by a Student's *t*-test. **(B)** Lesion formation on detached leaves of each ecotype after inoculation with pycnospores of *Mycosphaerella pinodes* (a = $1 \times 10^4$, b = $1 \times 10^5$, c = $1 \times 10^6$ spores/ml) and incubation at 22°C for 3 days.

germination, appressorial formation, and subsequent penetration were initiated on pea, by 2, 6 and 8 h after inoculation, respectively. Since spores germinated by 3 hpi and subsequent penetration through the wall of the epidermal cells occurred by 9 hpi on both R108-1 and Caliph of *M. truncatula* (**Figure 1A**), early infection events on the *M. truncatula* were quite similar to development on pea, except for the rate of forming infection hyphae in Caliph.

## Invasion Behavior of *M. pinodes* in Leaf Tissues of Both Ecotypes

Leaf sections showed striking differences in the extension of infection hyphae into leaf tissues between ecotypes R108-1 and Caliph. In susceptible R108-1, *M. pinodes* penetrated the adaxial epidermal surface (inoculated site) with appressoria and formed infection hyphae in the epidermal cells, and then grew intercellularly and eventually reached the abaxial epidermis (**Figure 2A**, arrowheads) just 1 dpi. At this stage, fungal structures were stained equally well as seen in healthy chloroplasts with toluidine blue, indicating that almost all plant cells retained their fine structure. At 3 dpi in R108-1 leaf tissues, infection hyphae remained entirely under the inoculated sites, and immature pycnidia often formed inside the mesophyll tissue (**Figure 2B**). The outline of the plant cells was amorphous, and the stained chloroplasts had disappeared as the result of plant cell disruption (**Figure 2B**). At 5 dpi, host cell organelles were completely disintegrated, and most pycnidia matured (**Figure 2C**). In R108-1, infection hyphae grew prolifically and reached the abaxial side of epidermis by 24 hpi, and subsequently formed pycnidia by 3 dpi (**Figures 2B,C**). The latent period for developing pycnidia on pea is 3–4 days (Roger et al., 1999), hence infection behavior of *M. pinodes* on the ecotype R108-1 is almost the same as seen on its natural host, pea.

Unlike on R108-1, the growth of infection hyphae after penetration in Caliph was restricted. At 1 dpi, infection hyphae had invaded only the adaxial epidermis, and cytoplasm in cells with hyphae were granulated (**Figure 2D**). At 3 dpi, although infection hyphae occasionally extended into palisade parenchyma cells, most host cells that contained hyphae had shrunk (**Figure 2E**, especially note the epidermal cells). Infection hyphae finally reached the abaxial epidermis (**Figure 2F**, arrowhead) at 7 dpi, and host tissues were entirely collapsed (**Figure 2F**). Toyoda et al. (2013a) indicated that at the inoculation site on Caliph leaves, *M. pinodes* induced scattered flecking or small necrotic lesions, which are probably associated with a local resistance response. Additionally, pycnidia were not found on Caliph (Toyoda et al., 2013a). Moussart et al. (2007) reported that 34 *Medicago* accessions, including Caliph, exhibited a high level of resistance to *M. pinodes* infection, and the lesions were limited to the inoculation site. Thus, our present observation agrees well with previous findings that extension of infection hyphae and induced lesions are confined in Caliph (Moussart et al., 2007; Toyoda et al., 2013a). Therefore, we reconfirmed again that ecotype Caliph is resistant to *M. pinodes* strain OMP-1.

## Ultrastructure of Interaction Sites between Plant and Fungus

### Susceptible Ecotype

**Figure 3** shows fine structures of the ecotype R108-1 challenged with pycnospores of *M. pinodes* at 3 dpi, which corresponds to **Figure 2B**. Penetration pegs, which emerged from the basal part of appressoria, never directly invaded the cytoplasm of plant cells; infection hyphae (subcuticular hyphae) formed and grew in the epidermal cell walls (**Figure 3A**, arrows). Following formation of subcuticular hyphae, the hyphae proliferated in the mesophyll and abaxial epidermal cells (**Figures 3B,C**, arrows). In the invaded cells, cell organelles such as chloroplasts were entirely degraded (**Figures 3B,C**), the same as in susceptible pea epicotyls (Clulow et al., 1991). Clulow et al. (1991) observed that infection pegs of *M. pinodes* were unusually broad, and infection hyphae extended horizontally into the outer wall of epidermal cells of the epicotyls. Similarly, infection hyphae tunneled through the outer wall, creating ridges on leaf surfaces of *M. truncatula* that were

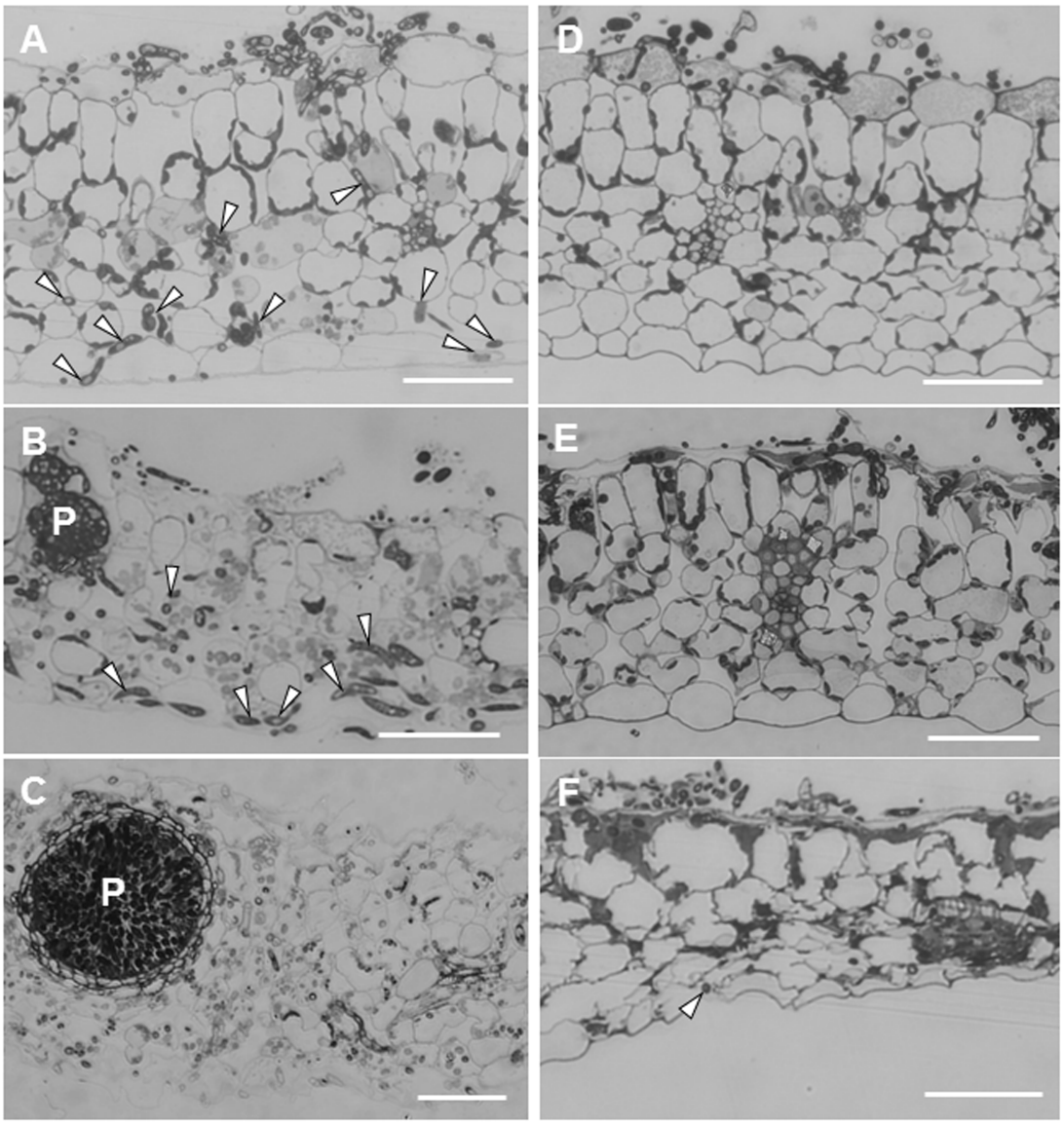


**FIGURE 2 |** Transverse sections cut from leaves of *M. truncatula* ecotype R108-1 **(A–C)** and Caliph **(D–F)** inoculated with pycnospores of *M. pinodes*. All sections were stained with toluidine blue. **(A)** Infection hyphae (arrowheads) had penetrated the adaxial epidermis and grown to the abaxial epidermis by 1 day after inoculation (dpi). **(B)** At 3 dpi, infection hyphae proliferated within tissue underneath the inoculated region, and immature pycnidia (P) had formed. **(C)** At 5 dpi, infection hyphae had grown from the inoculated region, and a mature pycnidium (P) can be seen in the disintegrated leaf tissue. **(D)** At 1 dpi, infection hyphae had penetrated the adaxial epidermis but had not grown into mesophyll cells. **(E)** At 3 dpi, epidermal cells with infection hyphae collapsed, and infection hyphae had grown into certain mesophyll cells. **(F)** At 7 dpi, mesophyll cells as well as the abaxial epidermis was deformed, and cells were shrunken where sparse infection hyphae were observed at the abaxial epidermis. Bars = 50 μm.

visible with scanning electron microscopy (Toyoda et al., 2013a). Although many pathogenic fungi that grow subcuticularly can directly penetrate the wall of epidermal cells and grow in the cytoplasm or periplasmic space (Hohl and Stossel, 1976; Mims et al., 2000; Wharton et al., 2001), not all pathogens do. For example, as infection hyphae of *Alternaria alternata* Japanese pear pathotype or *Venturia nashicola* begin to develop, they grow into pectin layers (Park et al., 2000; Suzuki et al., 2003). Our results thus showed that infection pegs emerged from appressoria, differentiated to form an infection vesicle and the infection hyphae then subsequently grew within the outer wall of epidermis to get nutrients from the host cells. (**Figure 3A**, arrows). Similar observations were reported for *M. pinodes* by Clulow et al. (1991) and Nasir et al. (1992), respectively, indicating that infection hyphae generated from the infection vesicle tunneled through the wall of the epidermis, when the susceptible pea cultivars were challenged with the fungus. Thus, *M. pinodes* likely requires the formation of infection vesicles

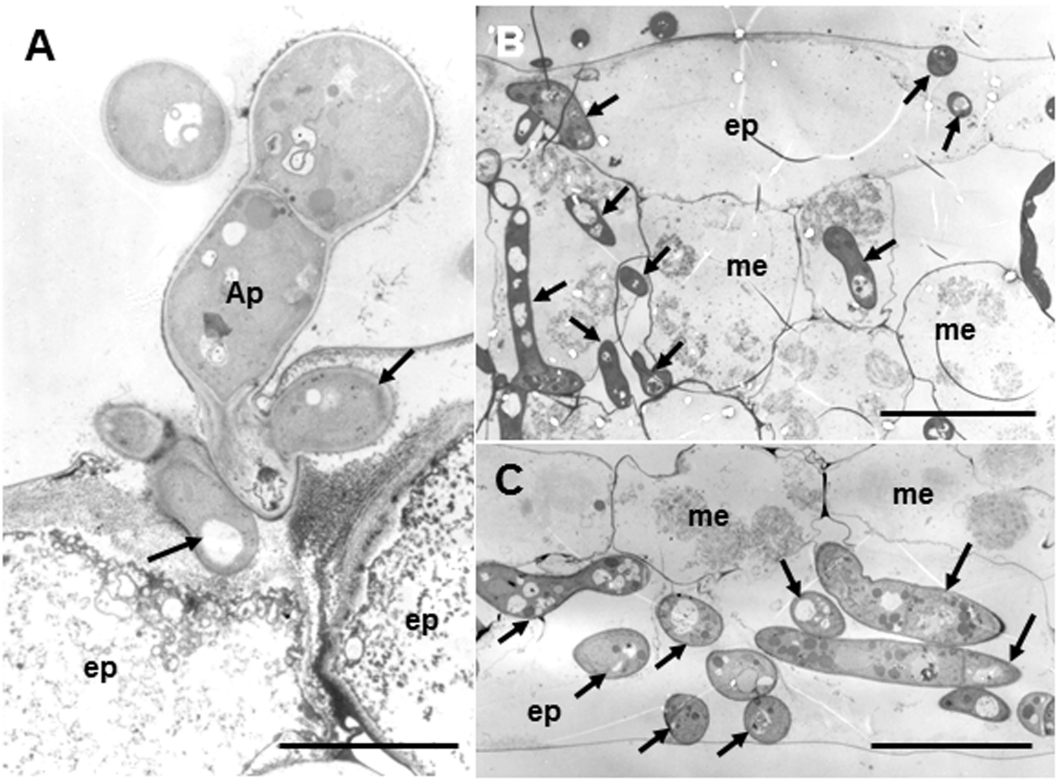


**FIGURE 3 |** Transmission electron micrographs of susceptible R108-1 leaves at 3 days after inoculation with *M. pinodes*. **(A)** Infection hyphae (arrows) that look like infection vesicles formed in the cell wall of the adaxial epidermal (ep) cell. Appressorium (Ap). Bar = 5 μm. **(B)** Adaxial epidermis and mesophyll cells (me) invaded by hyphae. Host cell organelles were degraded. Bar = 20 μm. **(C)** Extensive hyphae in abaxial epidermal cells. Bar = 20 μm.

within the outer cell wall to enable subsequent extension of infection hyphae into the rest of the leaf tissues. During the penetration into the cuticle, the host cell wall became degraded and/or swollen near infection hyphae. This degradation and swelling of the cell wall are likely ascribed to the action of cell wall-degrading enzymes secreted by the growing hyphae. Our results also showed cell wall degradation and collapsed organelles in the leaf tissues with hyphae of R108-1. These ultrastructural changes probably result from degradative enzymes and fungal toxins known as ascochitine (Oku and Nakanishi, 1966), which are released by *M. pinodes*.

The suppressors (supprescins A and B) produced by the pycnospores of *M. pinodes* have been shown to be the major determinants of host specificity, and they certainly suppress and/or delay host defense responses (Shiraishi et al., 1992, 1997; Toyoda et al., 2016). Since the susceptibility responses of pea caused the suppressor from *M. pinodes* are well reproducible in ecotype R108-1 of *M. truncatula* (Toyoda et al., 2013a), the pycnospores also use the same strategy to establish infection in R108-1.

### Resistant Ecotype Caliph

Regardless of host ecotypes, the pycnospores of *M. pinodes* germinated and grew on the host cuticle, then penetrated the epidermal cell wall, and infection hyphae formed in the epidermal cell walls (**Figure 4A**). Although infection hyphae had extended into the palisade parenchyma by 3 days after inoculation, aberrant hyphae such as dead hyphae and intrahyphal hyphae were only found in resistant ecotype, indicating the altered fungal growth in resistant tissues (**Figure 4B**). Occasionally, hyphae were observed to pass from one epidermal cell to the next mesophyll cells, but the cytoplasmic degeneration and organelle disruption occurred in both host and fungal cells (**Figures 4C,D**).

## $H_2O_2$ Accumulation at Interaction Sites

To verify resistance responses especially in Caliph, accumulation of $H_2O_2$ was analyzed *in situ* at the interaction sites using LM and TEM. Reaction of DAB with $H_2O_2$ rapidly generates insoluble, reddish-brown precipitates. In resistant ecotype Caliph, the epidermal cells where the pycnospores of *M. pinodes* attempted to penetrate or formed infection hyphae had strong reddish-brown staining (**Figure 5A**). To date, $H_2O_2$ accumulation in infected tissues has also been shown in different plant species using a cytochemical analysis with cerium chloride and TEM (Shinogi et al., 2003; Hyon et al., 2010). In the present study, the active oxygen species hydrogen peroxide ($H_2O_2$) was thus detected cytochemically through its reaction with cerium chloride to generate electron-dense deposits of cerium perhydroxides. In Caliph, the most remarkable electron-dense deposits of cerium

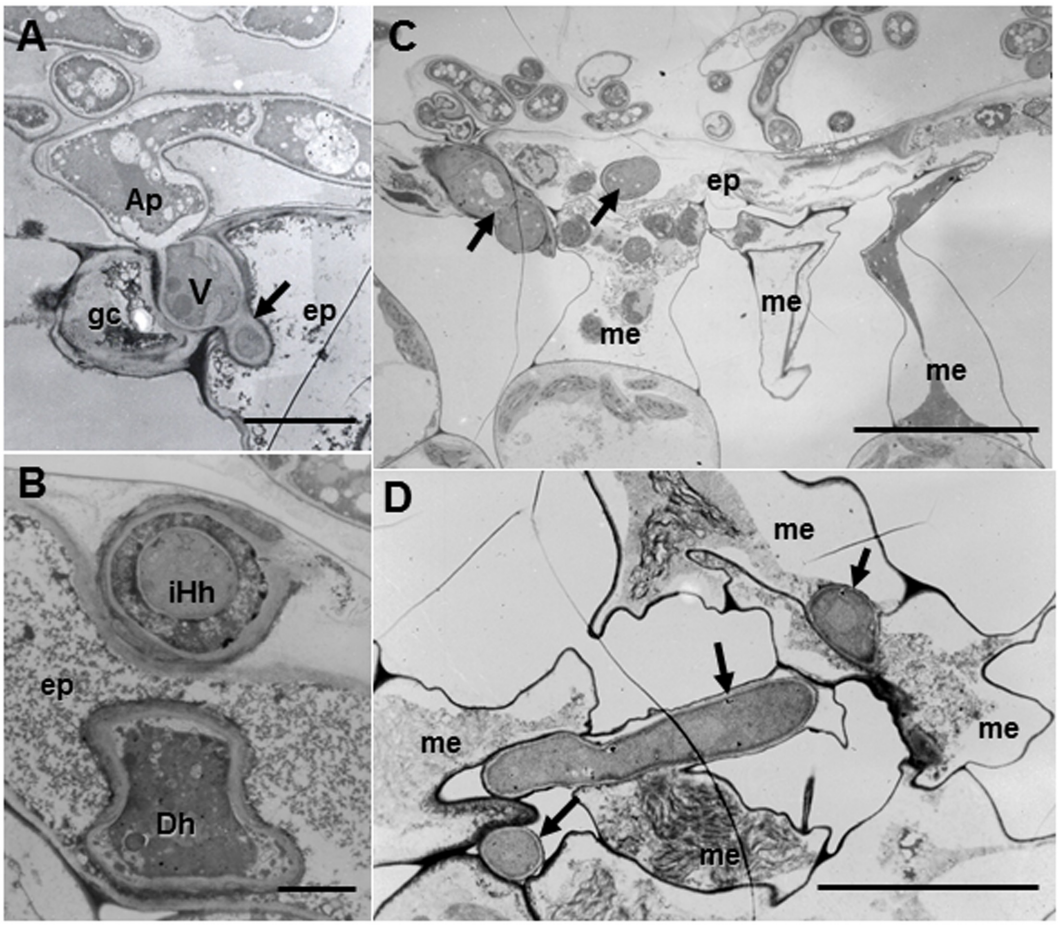


**FIGURE 4 |** Transmission electron micrographs of resistant Caliph leaves at 3 days after inoculation with *M. pinodes*. **(A)** Infection vesicle (V) emerged from the tip of an appressorium (Ap) to cell walls between guard cell (gc) and epidermal cell (ep), and infection hyphae extended into epidermal cell (ep). Bar = 5 μm. **(B)** Aberrant hyphae in epidermal cell; intrahyphal hyphae (iHh) and dead hypha (Dh). Bar = 2 > μm. **(C)** Epidermis invaded by hyphae and adjoining mesophyll cells had shrunk. Bar = 20 μm. **(D)** Mesophyll cells in contact with the hyphae had shrunk. Bar = 10 μm.

perhydroxides were observed in epidermal cells where the pathogen invaded, and the deposits were often seen at the plasma membrane of the mesophyll cell adjacent to the epidermal cells, and at the base of the appressorium such as at the septum (**Figure 5B**, arrows). In fact, the deposits appeared in the apposition next to the host epidermal cell wall (i.e., papilla) that formed underneath the infection hyphae (**Figure 5C**, asterisk), but not in R108-1 (**Figure 5F**, asterisk). In mesophyll cells adjacent to the epidermal cells of Caliph, positive deposits appeared at the contact point between the host plasma membrane and infection hyphae and between the fungal cell wall and edge of the host cell wall (**Figure 5D**).

In contrast to Caliph, the epidermal cells where the pathogen penetrated or formed infection hyphae stained a very pale brown when the R108-1 was inoculated with the fungus (**Figure 5E**). At 24 hpi on R108-1, none of the epidermal cells had the strong reddish-brown staining that was present in Caliph (data not shown). Although electron-dense deposits were rarely observed in host epidermal cells, the deposits appeared only at the base of the appressorium and, sometimes at the plasmalemma of the mesophyll cell adjacent to the epidermal cell (**Figure 5F**, arrows). However, the deposits in the mesophyll plasmalemma were absent when infection hyphae were present in mesophyll cells (**Figure 5G**). These deposits were validated by STEM/EDX analysis as being from cerium perhydroxides (**Figure 6**). Highly localized production of $H_2O_2$ was found in the cytoplasm of epidermal cells penetrated by the pathogen in ecotype Caliph, but not in ecotype R108-1. In addition, $H_2O_2$ was also localized at plasma membranes and tiny deposits were found at the walls of mesophyll cells adjacent to the cell walls where infection hyphae and wall appositions were present. Taken together, our present observations indicate that a localized, intensive $H_2O_2$ accumulation is likely associated with the host defense response in the Caliph challenged with pycnospores of *M. pinodes*.

Although a small area of $H_2O_2$ was localized at the plasma membrane of mesophyll cells near the penetrating infection hyphae in R108-1 by 18 hpi, it was not present where infection hyphae had penetrated. There are many reports that fungal

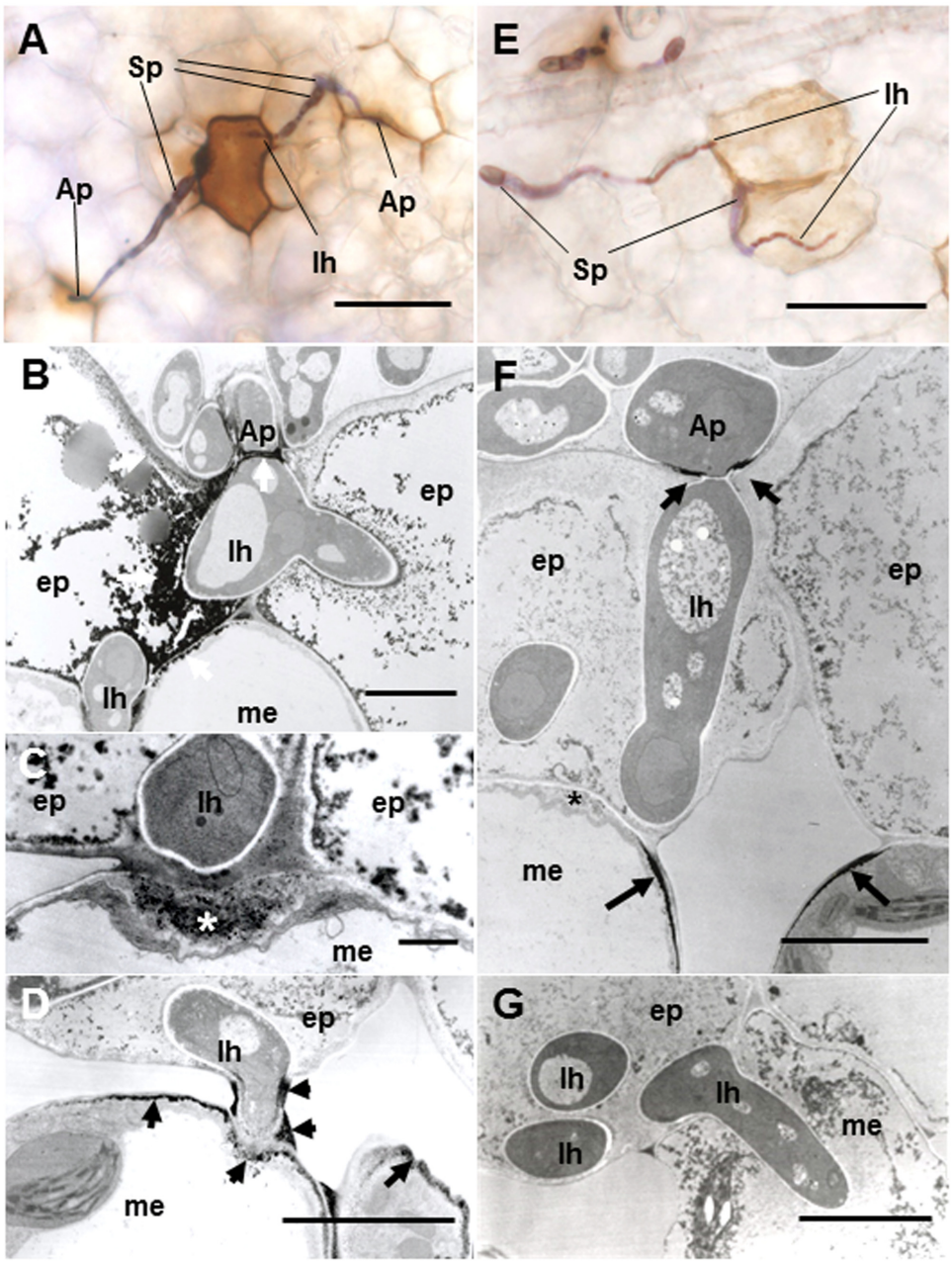


**FIGURE 5 |** Detection of $H_2O_2$ accumulation using diaminobenzidine (DAB) **(A,E)** and cerium chloride **(B–D,F,G)** in leaves of Caliph **(A–D)** and R108-1 **(E,F)** invaded by *M. pinodes* at 18 hpi. In resistant Caliph, reddish-brown reaction product was observed where infection hyphae had formed and at the sites around appressoria **(A)**. Cerium perhydroxides deposits in cytoplasm and plasma membranes of the adaxial epidermis penetrated by hyphae, plasma membranes of mesophyll cells that abut the epidermal cells and the tip of fungal appressorium **(B)**. Cerium perhydroxides deposits inside wall apposition (asterisk, **C**), and mesophyll plasmalemma penetrated by hyphae and around hypha and its wall **(D)**. In susceptible R108-1, epidermal cells with infection hyphae were stained pale brown **(E)**. Cerium perhydroxides in the appressorial wall in contact with the epidermal wall and mesophyll plasmalemma adjacent to epidermis penetrated by hyphae **(F)**. No accumulation of cerium perhydroxides was observed where hyphae penetrated mesophyll cells **(G)**. **A,E** bars: 50 μm. **B,D,F,G** bars: 5 μm. **C** bars: 1 μm.

pathogens produce ROS and antioxidant proteins such as superoxide dismutase and catalase (Witteveen et al., 1992; Gil-ad and Mayer, 1999; Gil-ad et al., 2000; Bussink and Oliver, 2001; Mayer et al., 2001; Egan et al., 2007). Therefore, pycnospores of *M. pinodes* can likely eliminate ROS during hyphal development or suppress host defense reactions in the susceptible interaction. The ability of the fungus to generate antioxidants agrees well with our previous findings that a suppressor from *M. pinodes* effectively suppresses elicitor-stimulated $O_2^-$ and $H_2O_2$ generation, which are catalyzed by

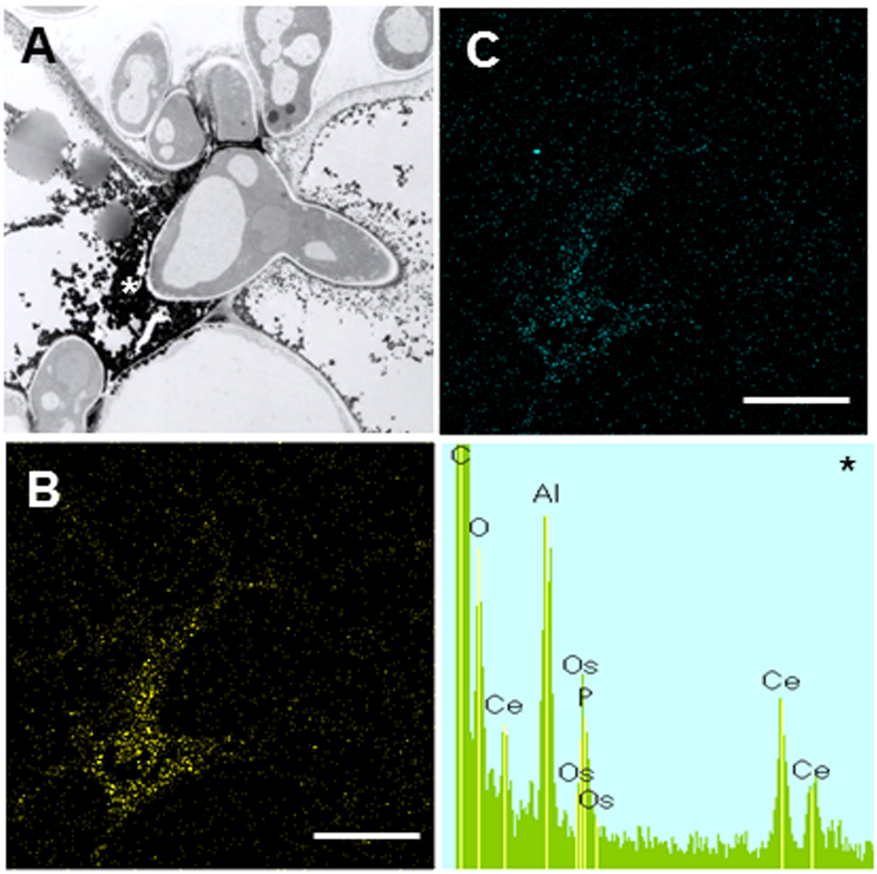


**FIGURE 6 |** Elemental maps and energy-dispersive X-ray (EDX) spectrum showing the deposition of cerium perhydroxides at fungal invasion sites in Caliph epidermis. **(A)** Transmission electron microscopy (TEM) image is the same as **Figure 5B**. Ce **(B)** and O **(C)** mapping images, and analytical point spectrum (asterisk).

a cell wall peroxidase (Kiba et al., 1996, 1997) and copper amine oxidase (Toyoda et al., 2012), respectively. In susceptible R108-1, a tiny accumulation of $H_2O_2$ was localized underneath appressoria and around penetration pegs, probably in response to the development of these infection structures. This $H_2O_2$ generation was probably associated with fungal growth such as development of infection-related structures. In the necrotrophic Japanese pear pathotype of *A. alternata*, $H_2O_2$ accumulation was also detected at the cell walls in contact with these infection structures in both, compatible and incompatible interactions (Shinogi et al., 2003; Hyon et al., 2010). The role of ROS for fungal virulence and development has been shown in different phytopathogenic fungi (Heller and Tudzynski, 2011). Collectively, the small amount of ROS detected in R108-1 beneath the appressorium is a common phenomenon, especially in fungal pathogens that infect through the cuticle.

## DISCUSSION

In this study, we observed infection behavior after germination of pycnospores of *M. pinodes* as well as the host cell responses to the infection, using susceptible and resistant ecotypes of *M. truncatula*. Microscopic analysis revealed that the germination and subsequent appressorial formation were commonly observed regardless of susceptible or resistant ecotype of *M. truncatula*, although the rate of formation of infection hyphae varied depending on the ecotypes (**Figure 1A**). In the susceptible ecotype R108-1, the fungus initially developed infection hyphae that grew within the outer wall of the epidermis. At 3 days after inoculation, the fungus grew intercellularly and intracellularly in subepidermal tissues of the susceptible leaves, eventually forming asexual pycnidia. Histopathological observation suggested that cell degeneration, especially mesophyll dissolution around invading hyphae is associated with expansion of disease symptoms in susceptible leaves (**Figures 2A–C**). In contrast, the rate of forming infection hyphae was considerably reduced on the resistant Caliph (**Figure 1A**), indicating an arrest in fungal growth probably due to host's defense-related factors. Occasionally, hyphae were observed to pass from one epidermal cell to the next mesophyll cells of the Caliph (**Figures 4C,D**), but the cytoplasmic degeneration and organelle disruption occurred presumably due to a local defense elicited by the fungus. In fact, extensive production of hydrogen peroxide ($H_2O_2$) in epidermal and mesophyll cells was observed in the resistant interaction as

detected by the reaction with cerium chloride to produce cerium perhydroxides under transmission electron microcopy (**Figure 4**). Our observations thus indicate that the oxidative burst leading to the generation of ROS including $H_2O_2$ is likely associated with a local defense response elicited in Caliph, since no obvious $H_2O_2$ accumulation was detectable in the susceptible R108-1.

Another striking feature of the resistant interaction was the observation of aberrant hyphae in or around the epidermal cells. Because these structures were not observed in the susceptible leaves, these aberrations may not be caused only by the structural defense. Rather, certain defense-related factors may be involved in these morphological changes of hyphae. Intrahyphal hyphae or the growth of hyphae within existing hyphae have been demonstrated in a number of phytopathogenic fungi and are considered to be survival forms under stressed conditions (Lim et al., 1983; Kim et al., 2001, 2004, 2012). Kim et al. (2001) reported that aberrant hyphal structures such as intrahyphal hyphae were found only in the resistant apple fruit tissues infected with *Botryosphaeria dothidea*. Since the intrahyphal hyphae were observed only in the Caliph, the structural modifications may not be caused solely by the structural defense. Rather, certain biochemical factors or defense-related compounds during the active defense may be involved in the hyphal morphological modifications, although the precise stimuli triggering the development of intrahyphal hyphae remain unclear. Taken together, we conclude that the structural aberrations likely are common mechanisms of fungi to be protected from a hostile environment in a resistant host by being enclosed by another hyphae. The structural differences between susceptible and resistant responses as well as the host responses will therefore provide information on defense-related characteristics of *M. pinodes* and the model host *M. truncatula*.

*Mycosphaerella pinodes* secretes a suppressor to avoid host defense responses (Shiraishi et al., 1992; Toyoda et al., 2016). In fact, the suppressor delays or suppresses elicitor-induced accumulation of *PR10-1*-mRNA in susceptible pea (Amano et al., 2013) and *M. truncatula* (Toyoda et al., 2013a). Recently, using the susceptible *M. truncatula* (ecotype R108-1), we showed the suppressor rapidly induces accumulation of mRNAs encoding almost all enzymes involved in jasmonic acid (JA) synthesis (Toyoda et al., 2013b). The application of exogenous JA to *M. truncatula* leaves evidently suppressed the elicitor-induced accumulation of *PR10-1* mRNA (Toyoda et al., 2013b). These observations indicate that a JA-mediated process(es) is probably involved in promoting susceptibility to *M. pinodes*. Plants have two major signaling molecules regulating plant immunity. Salicylic acid (SA), mediating resistance to biotrophic pathogens and JA/ethylene mediates resistance to necrotrophic pathogens (Glazebrook, 2005). In most cases, the molecules JA and SA interact with each other. Given that *M. pinodes* employs biotrophic and necrotrophic phases in its infection cycle, our results indicate that the fungus may use the JA-mediated signaling pathway through the secretion of a suppressor, to avoid the SA-regulated, elicitor-induced defenses during the early stage of infection. In our separate study with the pea, we showed that disease susceptibility to infection by *M. pinodes* was considerably reduced when *LOX* (*lipoxygenase*), *AOS* (*allene oxide synthase*), *AOC* (*allene oxide cyclase*) or *OPR* (*12-oxophytodienoic acid reductase*) were silenced (Toyoda et al., 2013b). Taken together, our results suggest that *M. pinodes* may manipulate the physiology of host cells, in particular JA synthesis, to colonize and promote disease susceptibility in the susceptible pea and *M. truncatula*.

## CONCLUSION

Our cytological studies on the infection process of *M. pinodes* on a susceptible and resistant ecotypes of the model plant *M. truncatula* suggested the role of the oxidative burst in host resistance. Indeed, fungal growth appears to be restricted through $H_2O_2$ production and/or associated defense-related factors. This model pathosystem involving *M. pinodes* and *M. truncatula* may assist in better understanding pathogenesis of the fungus on pea, thus providing information on the breeding of the resistant cultivars of pea.

## AUTHOR CONTRIBUTIONS

TSu, AM, MH, TSh, and KT planned and performed the experiments. The corresponding author KT discussed the research with all authors, and TSu and KT wrote the manuscript. The final manuscript was approved by all authors.

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# Fine Mapping of QTLs for Ascochyta Blight Resistance in Pea Using Heterogeneous Inbred Families

*Ambuj B. Jha, Krishna K. Gali, Bunyamin Tar'an and Thomas D. Warkentin**

*Crop Development Centre – Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK, Canada*

Ascochyta blight (AB) is an important disease of pea which can cause severe grain yield loss under wet conditions. In our previous study, we identified two quantitative trait loci (QTLs) abIII-1 and abI-IV-2 for AB resistance and these QTLs were consistent across locations and/or years in an inter-specific pea population (PR-19) developed from a cross between Alfetta (*Pisum sativum*) and P651 (*P. fulvum*). The objectives of this study were to fine map the abIII-1 and abI-IV-2 QTLs using a high density single nucleotide polymorphism (SNP)-based genetic linkage map and analyze identified markers in heterogeneous inbred family (HIF) populations. Selective genotyping of 51 PR-19 recombinant inbred lines was performed using genotyping-by-sequencing (GBS) and the resulting high density genetic linkage map was used to identify eight new SNP markers within the abI-IV-2 QTL, whereas no additional SNPs were identified within the abIII-1 QTL. Two HIF populations HIF-224 (143 lines) and HIF-173 (126 lines) were developed from $F_6$ RILs PR-19-224 and PR-19-173, respectively. The HIF populations evaluated under field conditions in 2015 and 2016 showed a wide range of variation for reaction to AB resistance. Lodging score had significant positive ($P < 0.001$) correlation with AB scores. HIFs were genotyped using SNP markers within targeted QTLs. The genotypic and phenotypic data of the HIFs were used to identify two new QTLs, abI-IV-2.1 and abI-IV-2.2 for AB resistance within the abI-IV-2 QTL. These QTLs individually explained 5.5 to 14% of the total phenotypic variation. Resistance to lodging was also associated with these two QTLs. Identified SNP markers will be useful in marker assisted selection for development of pea cultivars with improved AB resistance.

**Keywords: ascochyta blight, genotyping-by-sequencing, heterogeneous inbred family, quantitative trait loci, *Pisum fulvum*, *P. sativum***

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****Correspondence:***
*Thomas D. Warkentin*
*tom.warkentin@usask.ca*

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## INTRODUCTION

Ascochyta blight (AB), caused by *Peyronellaea pinodes* (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley (Aveskamp et al., 2010), is the most important pea (*Pisum sativum*) disease which can severely affect grain yield under wet conditions in most pea growing regions in the world (Lawyer, 1984; Xue et al., 1997; Kraft et al., 1998). The impact of the disease under field conditions is greatly affected by agronomic traits including lodging and plant height (Tar'an et al., 2003; Banniza et al., 2005; Le May et al., 2009; Jha et al., 2013, 2016). Genetic resistance is the optimal approach to reduce the disease impact (Zimmer and Sabourin, 1986). More than 3500 cultivated pea accessions were evaluated for their reaction to the disease resulting in the identification of a few lines with

low to moderate levels of resistance (Kraft et al., 1998; Zhang et al., 2006). In contrast, a higher level of resistance was identified in wild pea (*P. fulvum*) accessions (Clulow et al., 1991; Wroth, 1998; Fondevilla et al., 2005; Jha et al., 2012). Further, Fondevilla et al. (2005) reported the highest level of resistance in accession P651 (*P. fulvum*) compared to other wild peas, P670 (*P. sativum* ssp. *elatius*) and P665 (*P. sativum* ssp. *syriacum*). Promising accessions (*P. fulvum* and *P. sativum* ssp. *elatius*) were identified upon evaluation of 44 wild pea accessions which had the potential for improvement of AB resistance (Jha et al., 2012). Among them, the most promising accession, P651 (*P. fulvum*) was utilized for resistance breeding (Sindhu et al., 2014; Jha et al., 2016).

Previously, more than 30 quantitative trait loci (QTLs) were identified for resistance to AB in *P. sativum* mapping populations on all seven linkage groups (LGs) under field or controlled conditions (Timmerman-Vaughan et al., 2002, 2004; Tar'an et al., 2003; Prioul et al., 2004). QTLs were also identified in a cross involving wild pea, *P. sativum* subsp. *syriacum* (Fondevilla et al., 2008, 2011; Carrillo et al., 2014). Co-localization of QTLs for disease resistance with candidate genes including *RGAs* (resistance gene analogs), *PsDof1* (a putative transcription factor) and *DRR230-b* (a pea defensin) involved in defense responses to *P. pinodes* was reported in pea (Timmerman-Vaughan et al., 2002, 2016; Prioul-Gervais et al., 2007). Further, Jha et al. (2015) reported significant association of SNPs detected within candidate genes *PsDof1* (PsDof1p308) and *RGA-G3A* (RGA-G3Ap103) with AB scores. Most recently, nine QTLs were identified for AB resistance in an inter-specific pea population (PR-19) developed from a cross between Alfetta (*P. sativum*) and wild pea accession P651 (*P. fulvum*) (Jha et al., 2016). These QTLs individually explained 7.5 to 28% of the phenotypic variation.

Quantitative trait loci mapping studies in several pea crosses have resulted in the identification of genomic regions associated with AB resistance, however, these QTLs cover large regions which may not be effective for marker-assisted selection (MAS). Though several markers linked to resistance genes have been identified, even the closest markers are not necessarily tightly linked to the gene of interest (reviewed by Michelmore, 1995). Recombination could occur between a marker and QTL if markers are not tightly linked to genes (Collard et al., 2005). High-resolution or fine mapping of QTLs can be used to identify more tightly-linked or perfect markers within the gene sequence that can be efficiently utilized for MAS (reviewed by Mohan et al., 1997). Development of an advanced population, such as near isogenic lines (NILs), is required for fine mapping. Conventional consecutive backcrossing method was the original method for NIL development. Tuinstra et al. (1997) proposed development of heterogeneous inbred family (HIF) populations, an alternative, more efficient method than the NILs. This approach has been widely used in several species including Arabidopsis, soybean and maize for fine mapping of QTLs (Meng et al., 2008; Bai et al., 2010; Todesco et al., 2010; Coles et al., 2011; Dwiyanti et al., 2011; Watanabe et al., 2011; Bouteillé et al., 2012).

Among the nine AB resistance QTLs identified in PR-19 population, two QTLs abIII-1 and abI-IV-2 were consistent across locations and/or years (Jha et al., 2016). The objectives of this research were to identify additional SNP markers within abIII-I and abI-IV-2 QTLs and to fine map them using HIF populations for identification of closely linked markers for AB resistance in pea.

# MATERIALS AND METHODS

## Plant Material

Previously, PR-19 recombinant inbred line (RIL) population was generated from a cross between Alfetta (*P. sativum*) and P651 (*P. fulvum*) (Sindhu et al., 2014). P651 (original code IFPI3232) was first identified in Syria, then characterized by Consejo Superior de Investigaciones Científicas (Cordoba, Spain). For fine mapping of QTLs abI-IV-2 and abIII-1, HIF populations HIF-224 and HIF-173 were developed from $F_6$ RILs of PR-19-224 and PR-19-173, respectively.

## Selection of PR-19 Lines for HIF Populations

RILs PR-19-57, PR-19-132, PR-19-176, and PR-19-224 segregated for marker loci associated with the QTL abI-IV-2, and PR-19-04, PR-19-65, PR-19-115, and PR-19-173 segregated for marker loci associated with the QTL abIII-1. Three seeds of each of these RILs were sown in 2 gallon pots in a greenhouse with 22 $\pm$ 3°C day/20 $\pm$ 3°C night temperature under an 18-h photoperiod with approximately 60% relative humidity. Genomic DNA was extracted from freeze-dried leaf tissue collected from each plant using DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA) and used for Kompetitive Alelle Specific PCR (KASP) assays to validate heterozygous alleles for SNP loci within the QTLs. Allele-specific primers were designed for SNP loci PsC8780p118 (abIII-1) and PsC6805p316 (abI-IV-2) (Supplementary Table S1) using Primer-Picker software (LGC Genomics, Beverly, MA, USA). A total reaction volume of 10 μl was prepared by adding 20 ng of template DNA, 5 μl of KASP 2X Reaction Mix and 0.14 μl of KASP assay mixture (LGC Genomics, Beverly, MA, USA) in a 96-well plate format. Amplifications were performed using StepOnePlus Real-Time PCR system (Applied Biosystems, USA) according to the program described in Jha et al. (2015). Genotypic data were analyzed using SNPViewer software (LGC Genomics, Beverly, MA, USA).

## Development of HIF-224 and HIF-173

Ten $F_6$ seeds each for PR-19-224 and PR-19-173 were grown under greenhouse conditions and tested for heterogeneity by KASP assays as described earlier. Based on these assays, five seeds for PR-19-224 and seven seeds for PR-19-173 had heterozygous alleles for markers associated with abI-IV-2 and abIII-1, respectively. Seeds were bulked from five plants of PR-19-224 and seven plants of PR-19-173. Using single seed descent, self-pollination and bulking of seeds were conducted for $F_7$ to $F_8$ generation. Progenies at $F_8$ were represented HIF-224 and HIF-173 for PR-19-224 and PR-19-173, respectively (**Figure 1**).

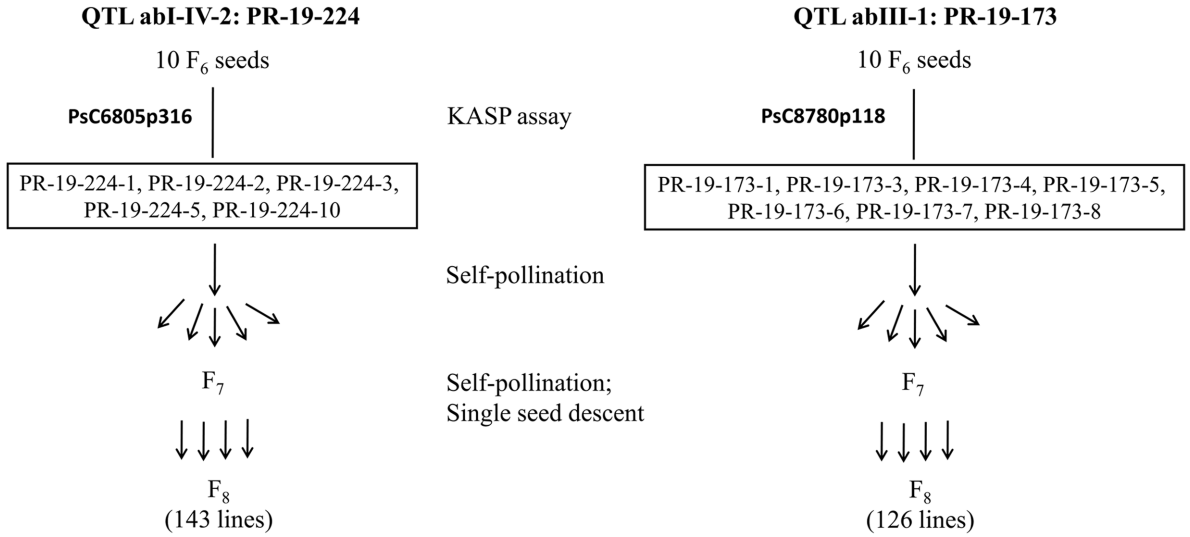


**FIGURE 1 | Development of heterogeneous inbred family (HIF) populations, HIF-224 and HIF-173 from lines PR-19-224 and PR-19-173, respectively.**

## Assessment of AB Resistance and Other Agronomic Traits Under Field Conditions

HIF-224 (143 lines) and HIF-173 (126 lines) along with parental checks (Alfetta and P651) were evaluated for reaction to AB and other agronomic traits including days to flower (DTF), plant height, lodging, days to maturity (DTM), and grain yield on a plot basis under field conditions in 2015 at Saskatoon with two replicates, and in 2016 at Saskatoon and Rosthern with three replicates at each location. The experimental design was a randomized complete block design with three-row plots of 1.0 m × 1.0 m, a plant density of 75 plants $m^{-2}$ and row spacing of 0.25 m. Plants were inoculated at the start of the flowering stage with approximately 3 g per plot of pea straw that had been naturally infected by *P. pinodes* in the previous season, air dried, and chopped into approximately 2-cm pieces. HIFs were evaluated for AB severity at pod filling and physiological maturity stages (80% of pods in the plot turned brown) using a scale of 0 (no disease) to 9 (whole plant severely blighted) based on Xue et al. (1996). Lodging was assessed on a 1 (upright) to 9 (completely lodged) scale. Plant height was measured from the soil level to the tip of the central stem at physiological maturity. DTF and DTM were calculated as the number of days from planting to 50% bloom and physiological maturity, respectively.

## Identification of Additional SNPs in QTLs

A high density genetic linkage map of PR-19 based on selective genotyping of the RIL population was developed for identification of additional SNP loci within the two targeted QTLs. Fifty-one $F_7$ RILs of PR-19 including PR-19-224 and PR-19-173 along with the parents (Alfetta and P651) were genotyped using genotyping-by-sequencing (GBS) method as described by Elshire et al. (2011). Twenty ng/μL DNA of each RIL as quantified using picogreen was digested with restriction enzymes *Pst*I and *Msp*I. Digested DNA of individual RILs was ligated with a unique 4 to 8 base pair barcode adapter. At this stage the DNA samples were pooled for construction of a single library for sequencing. Paired-end sequencing of the library was done in a single lane of an Illumina Hiseq sequencer using V4 sequencing chemistry.

The raw sequence reads were assigned to individual RILs based on the ligated barcode adapter. Following this deconvolution, barcode sequences were removed from the sequence. The reads were then trimmed for quality with Trimmomatic-0.33, and mapped to the draft genome assembly provided through the pea genome sequencing consortium (Madoui et al., 2016) using Bowtie2-2.2.5. SNP variants were identified and converted to VCF format using Samtools-1.1 and BCFtools-1.1.

After filtering for missing values and heterozygosity, 6160 SNP markers were selected for linkage analysis. Segregation data of these markers were combined with 733 polymorphic SNP markers previously genotyped using Illumina GoldenGate 1536 SNP array (Jha et al., 2016). Combined SNP marker segregation data were used for linkage analysis using MstMap. SNP markers from the GoldenGate assay served as anchor markers to identify additional SNP loci within the targeted QTLs. All the SNP markers identified within QTLs were converted to KASP assays (Supplementary Table S1) and used for genotyping of the complete set of 144 RILs of PR-19 for cross-validation of their genetic linkage positions.

## Genotyping of HIF-224 and HIF-173

Genomic DNA was extracted from freeze dried leaf tissues collected from single plants of HIF-224 (143 lines) and HIF-173 (126 lines) progenies using DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California, USA). HIF-224 lines were genotyped using 20 SNP markers (Supplementary Table S1) representing the QTL abI-IV-2 and the region adjacent to the QTL by KASP assays. HIF-173 population segregating for QTL

abIII-1 was genotyped with three SNP markers, PsC22609p103, PsC8780p118, and PsC23317p284, each representing a unique locus within this QTL.

## Linkage Mapping and QTL Analysis in HIF Populations

The linkage map was constructed separately for PR-19, HIF-224, and HIF-173 using MAPMAKER (Lander et al., 1987). QTL mapping was performed by composite interval mapping (CIM) using Windows QTL Cartographer 2.5 (Wang et al., 2012). The significance threshold ($P < 0.05$) was used to declare the presence of QTLs by performing 1000 permutations of the data (Churchill and Doerge, 1994). MapChart 2.2 was used for graphical presentation of linkage maps (Voorrips, 2002).

## Statistical Analysis

PROC MIXED implemented in SAS®9.3 (SAS Institute Inc. Cary, NC, USA) was used for data analysis. Line was treated as a fixed effect whereas replication was treated as a random effect across the HIFs. Homogeneity of variance test (HOVTEST) was used to assess the homogeneity of variance among replications.

# RESULTS

## Selection of PR-19 Lines for HIF Populations

Four RILs each tested for abI-IV-2 (PR-19-57, PR-19-132, PR-19-176, and PR-19-224) and abIII-1 (PR-19-04, PR-19-65, PR-19-115, and PR-19-173) had heterozygous alleles within QTLs, i.e., these lines were segregating for markers associated with AB, which is a prerequisite for HIF development. On the basis of KASP assays and AB scores of lines, PR-19-224 and PR-19-173 were selected for development of HIF-224 and HIF-173, respectively.

## Assessment of AB Resistance and Other Agronomic Traits under Field Conditions

HIF-224 and HIF-173 showed a wide range of variation for reaction to AB, plant height, lodging, and grain yield under field conditions in 2015 at Saskatoon and in 2015 and 2016 at Saskatoon and Rosthern locations in Saskatchewan (**Tables 1**, **2** and **Figures 2**–**4**). Data from different station years could not be combined for analysis of variance due to significant effect of locations and years in the HOVTEST. In general, the effect of line was significant ($P < 0.05$) for AB scores, plant height, lodging, and grain yield. AB scores of HIF-224 ranged from 2 to 7 at pod filling, and 2 to 8 at physiological maturity (0–9 scale), whereas for HIF-173, scores ranged from 1 to 7 at pod filling, and 2 to 9 at physiological maturity. Alfetta had disease score of 3 to 4 at pod filling and 4 to 5 at physiological stage, whereas P651 had disease score of 2 to 3 and 3 to 4 at pod filling and physiological maturity stage, respectively. Lodging scores varied from 1 to 9 for HIF-224, whereas for HIF-173, scores varied from 1 to 7 on the 1–9 scale. Alfetta had 1 to 3 lodging score whereas P651 had 8 to 9 score. HIFs had a small range of variation for DTF and DTM at different station years, while plant height and grain yield had a wide range of variation among tested HIF lines. For both HIFs, AB scores were positively correlated with lodging ($P < 0.001$) and negatively correlated with plant height ($P < 0.001$) and grain yield ($P < 0.01$) (**Tables 3**, **4**).

## Identification of Additional SNP Markers within QTLs

Overall, 10,985 SNPs were identified at a read depth of 10 by selective genotyping of 51 PR-19 RILs using GBS method. After filtering for allele distribution, 6160 SNPs along with 733 previously genotyped SNPs were used for construction of a high density genetic linkage map to identify markers within QTLs. Based on the high density genetic linkage map, 12 SNP markers

**TABLE 1 | *F*-values, coefficients of variations (CV) of statistical analyses, and means with standard deviations (SD) of ascochyta blight (AB) scores and other agronomic assessments for 143 lines of heterogeneous inbred family (HIF) population, HIF-224 evaluated under field conditions in 2015 at Saskatoon and in 2016 at Saskatoon and Rosthern, Saskatchewan.**

| | | AB1 (0–9 scale) | AB2 (0–9 scale) | Days to flower (DTF) | Plant height (cm) | Lodging (1–9 scale) | Days to maturity | Grain yield (Kg/ha) |
|---|---|---|---|---|---|---|---|---|
| Saskatoon 2015 | Line | 1.4* | 1.9*** | 0.7NS | 2.6*** | 1.8*** | 1.0NS | 0.9NS |
| | Range | 2–6 | 2–8 | 36–39 | 29–54 | 2–8 | 75–79 | 45–1154 |
| | Mean ± SD | 3.4 ± 0.8 | 4.4 ± 1.1 | 37.4 ± 0.7 | 44.5 ± 5.0 | 5.6 ± 0.9 | 77.6 ± 1.4 | 416 ± 218 |
| | CV (%) | 23.8 | 25.0 | 2.0 | 11.2 | 15.8 | 1.8 | 52.4 |
| Saskatoon 2016 | Line | 1.9*** | 5.1*** | 0.9NS | 1.9*** | 3.5*** | 1.3NS | 2.7*** |
| | Range | 2–6 | 2–8 | 38–41 | 28–57 | 1–9 | 71–74 | 37–2912 |
| | Mean ± SD | 3.2 ± 0.7 | 4.5 ± 1.0 | 39.6 ± 0.9 | 44.2 ± 8.5 | 4.5 ± 1.8 | 72.6 ± 1.1 | 864 ± 66 |
| | CV (%) | 22.6 | 22.2 | 2.4 | 19.2 | 39.1 | 1.4 | 52.1 |
| Rosthern 2016 | Line | 2.3*** | 2.0*** | 1.1NS | 2.9*** | 5.1*** | 1.2NS | 3.2*** |
| | Range | 2–7 | 3–8 | 40–44 | 28–60 | 1–9 | 80–84 | 58–2487 |
| | Mean ± SD | 4.2 ± 0.8 | 5.7 ± 0.9 | 41.7 ± 1.2 | 43.1 ± 6.4 | 5.1 ± 1.4 | 82.1 ± 1.2 | 1051 ± 59 |
| | CV (%) | 20.0 | 15.8 | 2.6 | 14.8 | 27.4 | 1.4 | 38.7 |

*NS- not significant; *P < 0.05; **P < 0.01; ***P < 0.001; AB1 and AB2 denote AB scores at pod filling and physiological maturity stages, respectively.*

**TABLE 2 | *F*-values, coefficients of variations (CV) of statistical analyses, and means with standard deviations (SD) of AB scores and other agronomic assessments for 126 lines of heterogeneous inbred family (HIF) population, HIF-173 evaluated under field conditions in 2015 at Saskatoon and in 2016 at Saskatoon and Rosthern, Saskatchewan.**

| | | AB1 (0–9 scale) | AB2 (0–9 scale) | DTF | Plant height (cm) | Lodging (1–9 scale) | Days to maturity | Grain yield (Kg/ha) |
|---|---|---|---|---|---|---|---|---|
| Saskatoon 2015 | Line | 1.4* | 1.5* | 1.0NS | 5.9*** | 1.3NS | 1.3NS | 6.5*** |
| | Range | 1–6 | 2–7 | 37–41 | 28–64 | 2–7 | 85–92 | 205–2886 |
| | Mean ± SD | 3.3 ± 0.7 | 4.1 ± 1.0 | 38.4 ± 1.0 | 54.0 ± 6.6 | 4.6 ± 0.7 | 89.3 ± 2.6 | 980 ± 507 |
| | CV (%) | 32.6 | 26.5 | 2.5 | 12.2 | 14.3 | 2.9 | 51.7 |
| Saskatoon 2016 | Line | 6.4*** | 8.6*** | 1.5* | 14.7*** | 3.7*** | 1.1NS | 5.41*** |
| | Range | 2–7 | 3–8 | 35–39 | 29–76 | 2–6 | 83–88 | 95–4126 |
| | Mean ± SD | 4.5 ± 1.2 | 5.7 ± 1.3 | 37.0 ± 1.2 | 62.4 ± 11.3 | 3.7 ± 1.1 | 85.1 ± 1.4 | 2008 ± 113 |
| | CV (%) | 26.4 | 22.3 | 3.1 | 18.2 | 27.5 | 1.6 | 38.4 |
| Rosthern 2016 | Line | 4.6*** | 7.6*** | 1.0NS | 11.3*** | 1.5** | 1.4* | 3.3*** |
| | Range | 3–7 | 4–9 | 37–42 | 27–74 | 1–6 | 90–95 | 40–4858 |
| | Mean ± SD | 4.9 ± 1.1 | 6.6 ± 1.3 | 39.9 ± 1.2 | 55.3 ± 13.4 | 3.2 ± 0.8 | 92.6 ± 1.3 | 1602 ± 104 |
| | CV (%) | 20.4 | 19.2 | 2.7 | 24.3 | 25.1 | 1.4 | 44.3 |

*NS- not significant; *P < 0.05; **P < 0.01; ***P < 0.001; AB1 and AB2 denote AB scores at pod filling and physiological maturity stages, respectively.*

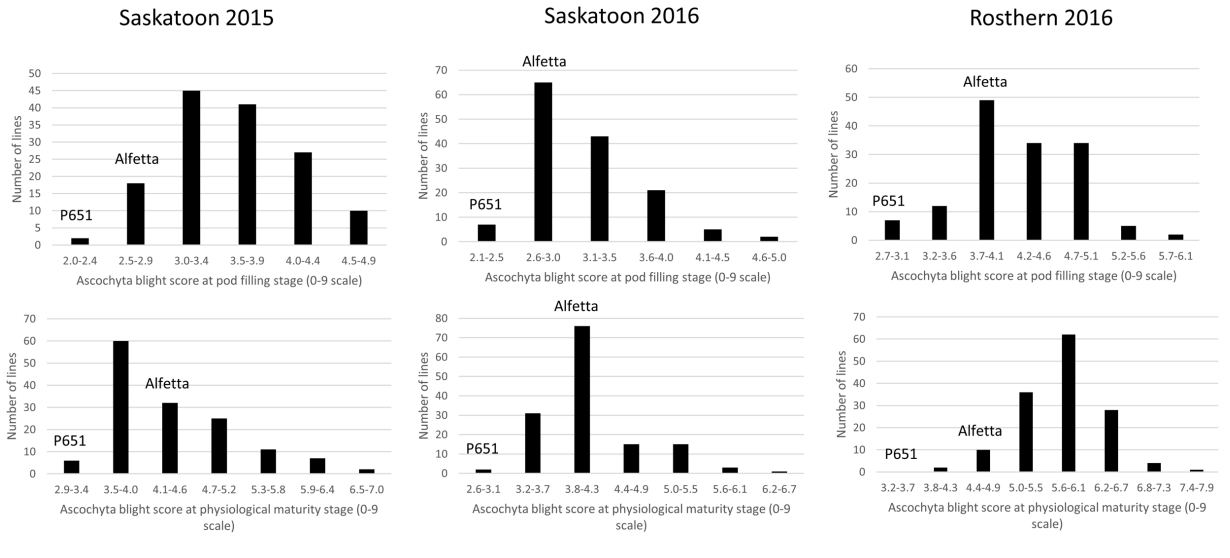


**FIGURE 2 | Frequency distribution of 143 lines of HIF population, HIF-224 using least square means of Saskatoon 2015, Saskatoon 2016 and Rosthern 2016 for the reaction to ascochyta blight (AB) resistance at pod filling and physiological maturity stages under field conditions.**

were identified within abI-IV-2 QTL. Ten of the 12 markers along with previously identified SNP markers from an Illumina GoldenGate array were used for genotyping of a complete set of PR-19 RILs (144) to re-confirm their position and order within the QTL. Following linkage analysis of markers of this QTL, the eight SNP markers identified from the high density genetic linkage map were confirmed to localize within the existing QTL flanked by SNP markers PsC943p541/PsC4233p498 and PsC8970p349/PsC7884p449, whereas two SNPs were located to the region adjacent to the QTL. Mapping of eight additional SNPs within the QTL has increased the map distance of the QTL from 13.4 to 17.1 cM (**Figures 5A,B**).

In the case of abIII-1, based on the high density genetic linkage map, no additional SNP marker was identified within the QTL (**Figure 6B**). Two flanking markers of the QTL were converted to KASP assays and were used for genotyping the complete set of RILs. Linkage analysis of this region based on these two flanking markers and known existing markers within the QTL reconfirmed the order of SNP markers on the high density genetic linkage map.

## Fine Mapping of QTLs for AB Resistance

For fine mapping of abI-IV-2 QTL, 143 lines of HIF-224 segregating for this QTL were genotyped with 20 SNP markers using KASP assays. This set of 20 SNP markers included 10 previously known SNP markers and 10 markers currently identified through the high density genetic linkage map. Of

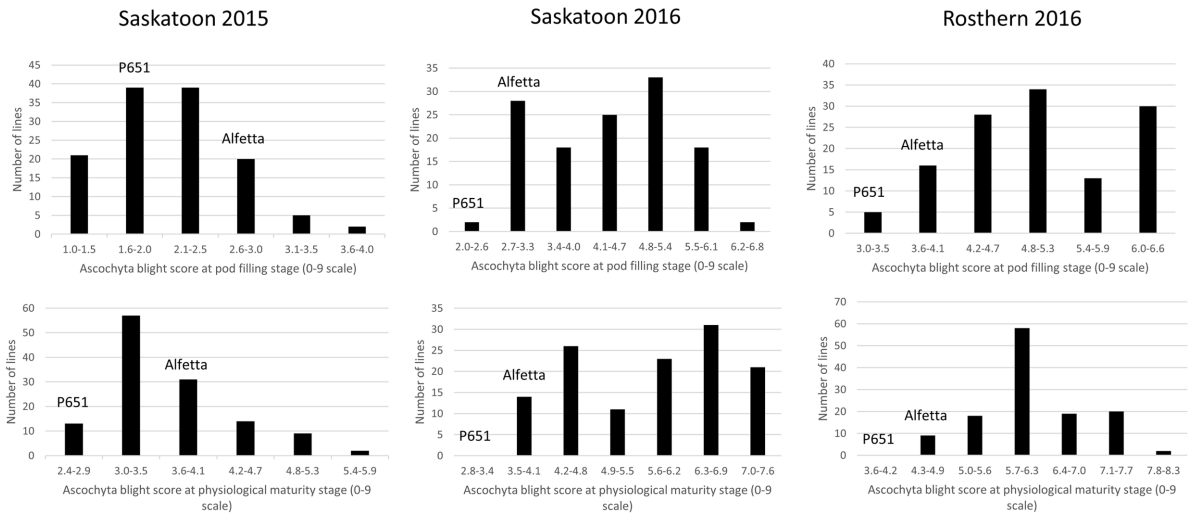


**FIGURE 3 | Frequency distribution of 126 lines of HIF population, HIF-173 using least square means of Saskatoon 2015, Saskatoon 2016 and Rosthern 2016 for the reaction to AB resistance at pod filling and physiological maturity stages under field conditions.**

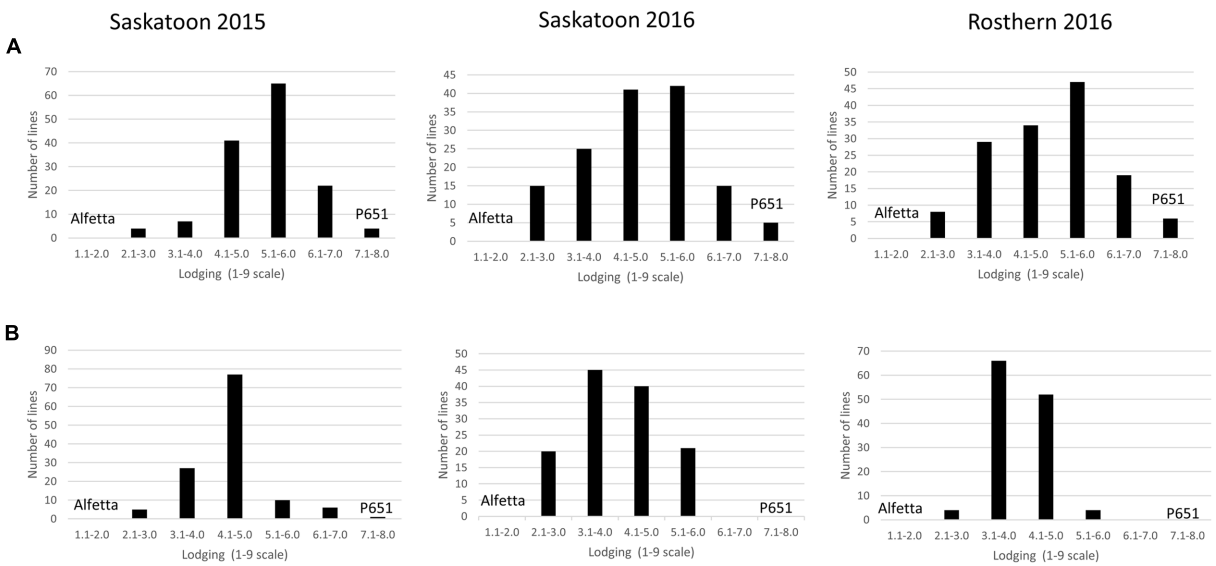


**FIGURE 4 | Frequency distribution of 143 lines of HIF population, HIF-224 (A)** and 126 lines of HIF-173 **(B)** using least square means of Saskatoon 2015, Saskatoon 2016 and Rosthern 2016 for lodging at physiological maturity stage under field conditions.

the total genotyped, 17 SNP markers were used for linkage analysis to verify the marker order and distance in the HIF population. The 17 SNP markers represented a map distance of 86.3 cM in HIF-224 population (**Figure 7**). Based on the field evaluation of HIF-224 population in 2015 and 2016 trials, two new QTLs, abI-IV-2.1 and abI-IV-2.2 were identified for AB resistance within the abI-IV-2 QTL (**Table 5** and **Figure 7**). QTL abI-IV-2.1 explained 5.5 to 14% of the total phenotypic variation, whereas abI-IV-2.2 explained 7 to 10% of the total variation. QTLs for lodging resistance were also associated with these two QTLs. Alfetta contributed alleles for AB resistance as well as for lodging resistance. Fine mapping with HIF lines has confirmed the occurrence of AB resistance QTLs within the previously reported QTL ab-IV-2, and provided additional markers for MAS of this QTL in breeding populations.

Additional SNP makers within the abIII-1 QTL were not identified using the high density genetic linkage map. The extreme distortion of allele segregation determined based on the existing three SNP markers within this QTL did not allow for the determination of the linkage order of these markers in HIF-173 population. Additionally, significant recombination within this QTL was not identified in the HIF family to continue with other tests to determine the significance of these markers.

**TABLE 3 | Pearson correlation coefficients for traits of 143 lines of heterogeneous inbred family (HIF) population, HIF-224 evaluated under field conditions in 2015 at Saskatoon and in 2016 at Saskatoon and Rosthern, Saskatchewan.**

| HIF-224 | DTF | Plant height | Lodging | Days to maturity | Grain yield | AB1 |
|---|---|---|---|---|---|---|
| Plant height | −0.01NS | | | | | |
| Lodging | −0.11NS | −0.52*** | | | | |
| Days to maturity | 0.92*** | 0.01NS | −0.11NS | | | |
| Grain yield | 0.04NS | 0.69*** | −0.62*** | 0.04NS | | |
| AB1 | −0.23** | −0.53*** | 0.67*** | −0.23** | −0.64*** | |
| AB2 | −0.24** | −0.40*** | 0.59*** | −0.23** | −0.49*** | 0.86*** |

*NS-not significant; **P < 0.01; ***P < 0.001; AB1 and AB2 denote AB scores at pod filling and physiological maturity stages, respectively.*

**TABLE 4 | Pearson correlation coefficients for traits of 126 lines of heterogeneous inbred family (HIF) population, HIF-173 evaluated under field conditions in 2015 at Saskatoon and in 2016 at Saskatoon and Rosthern, Saskatchewan.**

| HIF-173 | DTF | Plant height | Lodging | Days to maturity | Grain yield | AB1 |
|---|---|---|---|---|---|---|
| Plant height | −0.25** | | | | | |
| Lodging | 0.18* | −0.48*** | | | | |
| Days to maturity | 0.77*** | −0.12NS | 0.16NS | | | |
| Grain yield | −0.09NS | 0.56*** | −0.44*** | −0.12NS | | |
| AB1 | 0.28** | −0.65*** | 0.46*** | 0.23* | −0.28** | |
| AB2 | 0.34*** | −0.69*** | 0.49*** | 0.24** | −0.33** | 0.96*** |

*NS-not significant; *P < 0.05; **P < 0.01; ***P < 0.001; AB1 and AB2 denote AB scores at pod filling and physiological maturity stages, respectively.*

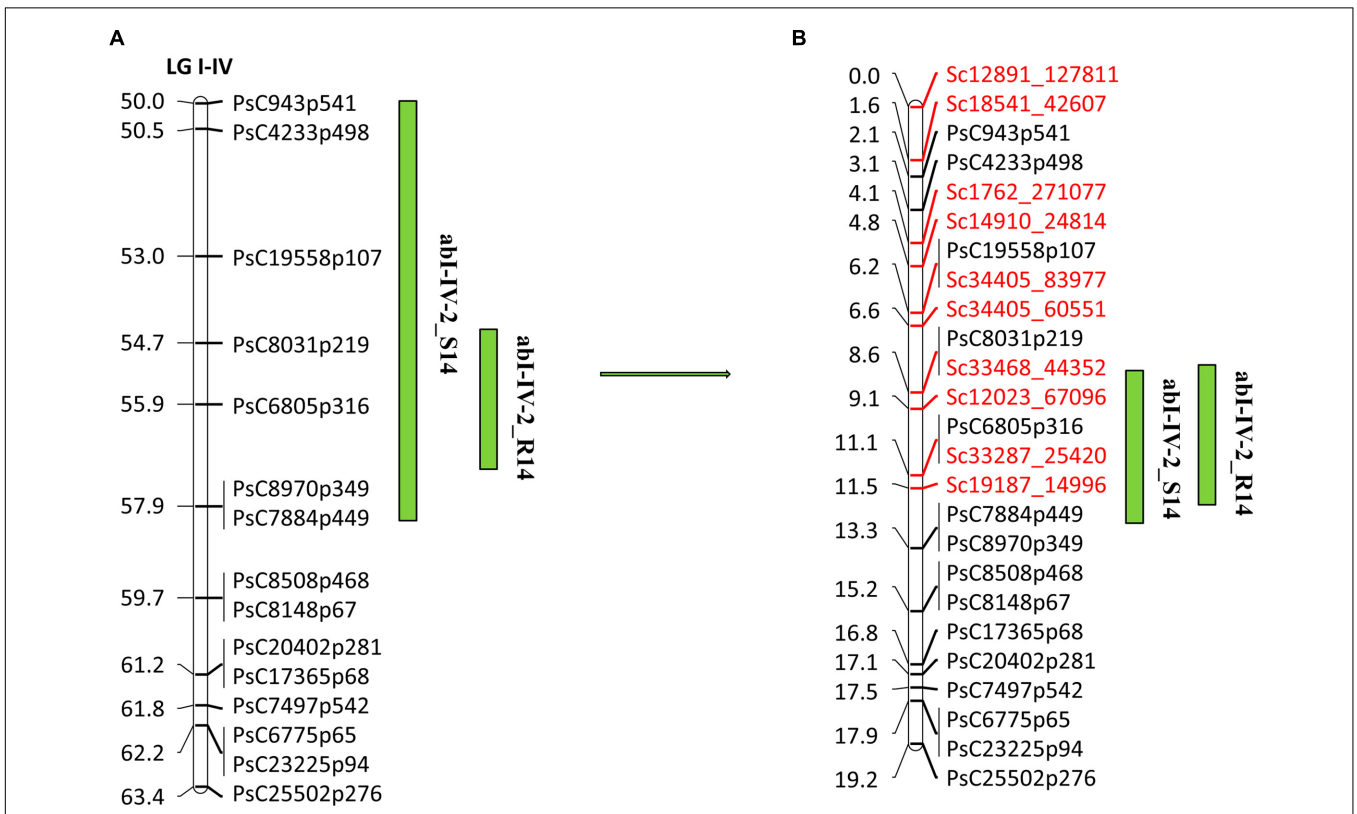


**FIGURE 5 | Quantitative trait locus (QTL) abI-IV-2 based on SNP linkage map in PR-19 (Jha et al., 2016) (A)**, additional SNPs identified by fine mapping using genotyping-by-sequencing (GBS) method in PR-19 **(B)**. Locations of QTLs for AB are shown by vertical bars. S14 and R14 associated with QTLs name denote 2014 Saskatoon and 2014 Rosthern, respectively.

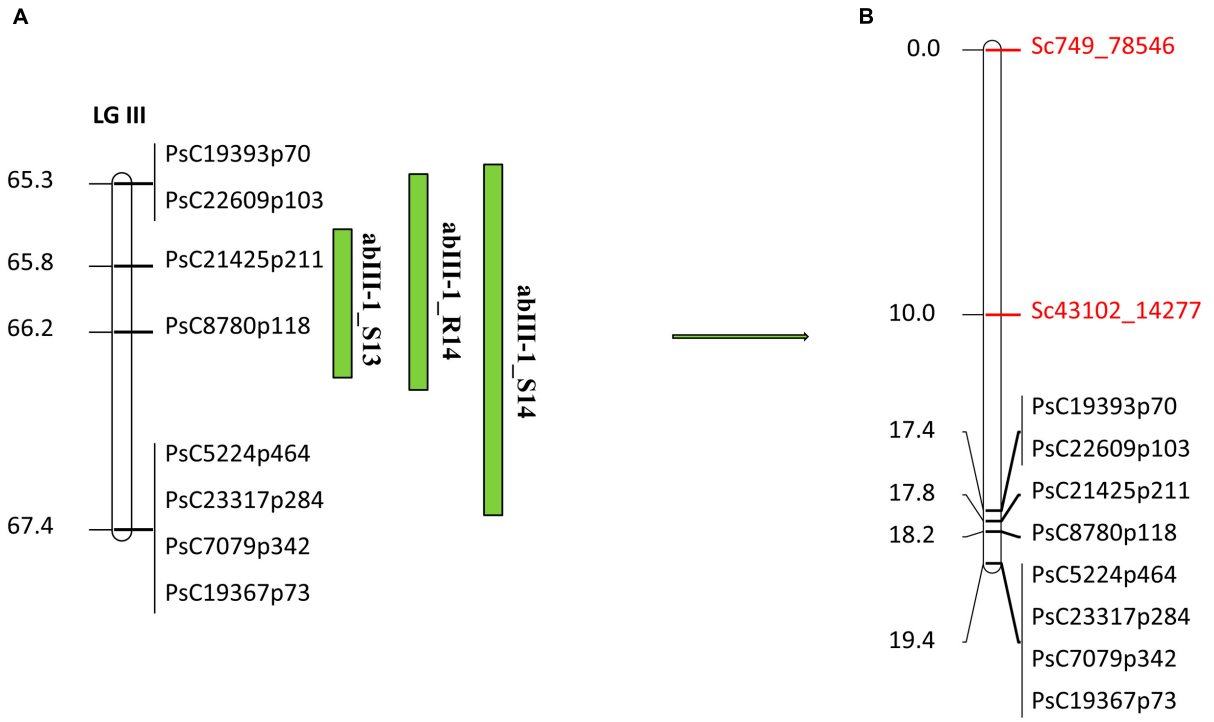


**FIGURE 6 | Quantitative trait locus abIII-1 based on SNP linkage map in PR-19 (Jha et al., 2016) (A)**, additional SNPs identified by fine mapping using GBS method in PR-19 **(B)**. Locations of QTLs for AB are shown by vertical bars. S13, S14, and R14 associated with QTLs name denote 2013 Saskatoon, 2014 Saskatoon, and 2014 Rosthern, respectively.

**TABLE 5 | Quantitative trait loci (QTLs) detected for reaction to AB resistance and lodging in abI-IV-2 QTL in HIF population, HIF-224 evaluated under field conditions in 2015 at Saskatoon and in 2016 at Saskatoon and Rosthern.**

| QTL | Trait | Year | Location | Locus[a] | Max. LOD value | % Variation[b] | Additive genetic effect[c] |
|---|---|---|---|---|---|---|---|
| abI-IV-2.1 | AB1, AB2 | 2015 | Saskatoon | Sc1762_271077 | 6.6 | 14.0 | −0.6 |
| | AB1 | 2016 | Saskatoon | PsC943p541 | 4.3 | 5.5 | −0.3 |
| lodgI-IV-1 | Lodging | 2015 | Saskatoon | PsC943p541 | 4.4 | 9.9 | −0.4 |
| | Lodging | 2016 | Saskatoon | Sc14910_24814 | 3.7 | 5.8 | −0.3 |
| abI-IV-2.2 | AB1, AB2 | 2016 | Saskatoon | PsC8970p349 | 6.4 | 9.7 | −0.4 |
| | AB1, AB2 | 2016 | Rosthern | Sc33287_25420 | 5.0 | 6.6 | −0.4 |
| lodgI-IV-2 | Lodging | 2016 | Saskatoon | PsC8970p349 | 6.8 | 24.6 | −0.1 |

*AB1 and AB2 denote AB scores at pod filling and physiological maturity stages, respectively.* [a]*Closest marker to the identified QTL with maximum LOD value;* [b]*Percentage of total variability explained by the QTL detected for the trait;* [c]*The value associated with the Alfetta allele; a negative value means that the Alfetta allele decreases the value of the trait.*

## DISCUSSION

ABs are the most important diseases of pulse crops (Tivoli et al., 2006; Muehlbauer and Chen, 2007). Resistance breeding is considered the most effective method of control; however, this process is slow due to the complex nature of resistance (Muehlbauer and Chen, 2007; Rubiales and Fondevilla, 2012). Significant progress has been made in resistance breeding with the advancement of innovative tools including next generation sequencing. Several QTLs have been reported for AB resistance in pea (Timmerman-Vaughan et al., 2002, 2004; Tar'an et al., 2003; Prioul et al., 2004; Fondevilla et al., 2008, 2011; Jha et al., 2016), chickpea (Udupa and Baum, 2003; Lichtenzveig et al., 2006; Tar'an et al., 2007; Sabbavarapu et al., 2013), lentil (Sudheesh et al., 2016), and faba bean (Atienza et al., 2016).

Sudheesh et al. (2016) reported validation of previously reported QTLs for AB resistance in lentil on genetic maps based on SNP and SSR markers developed from three RIL populations. Further, they identified two common genomic regions for disease resistance in two out of three maps that could provide validated markers associated with disease for lentil improvement. Similarly, Atienza et al. (2016) studied validation and stability of major QTLs located on chromosomes II and III for AB resistance in faba bean under field and controlled conditions and reported

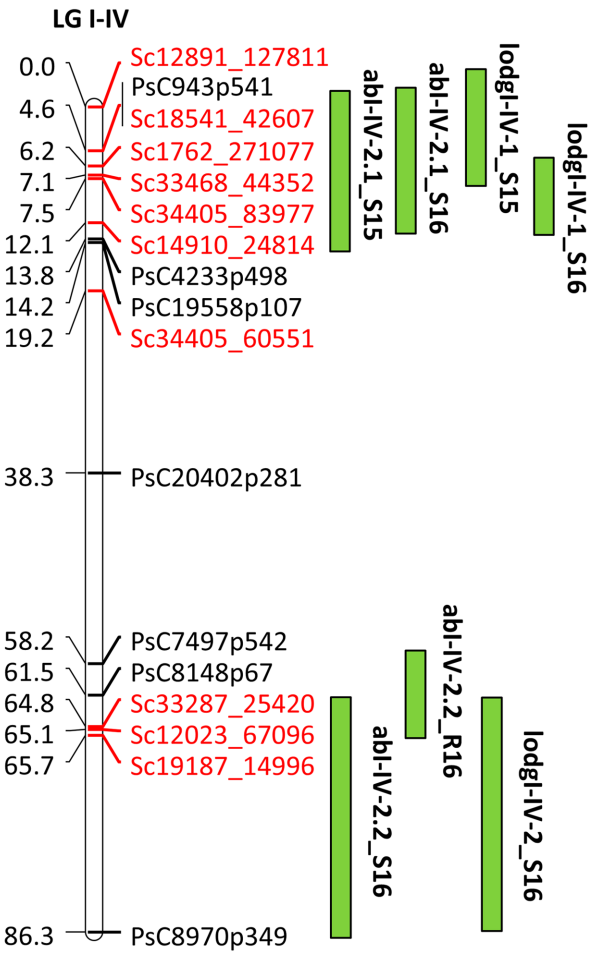


**FIGURE 7 | Quantitative trait locus abI-IV-2 based on GoldenGate and GBS markers in HIF population, HIF-224.** Locations of QTLs for AB and lodging (lodg) are shown by vertical bars. S15, S16, and R16 associated with QTLs name denote 2015 Saskatoon, 2016 Saskatoon, and 2016 Rosthern, respectively.

that QTL Af2 located on chromosome II was the same QTL reported previously by other researchers. In chickpea, QTLs were identified for AB resistance on LGs 2, 3, 4, 5, 6, and 8 (Udupa and Baum, 2003; Lichtenzveig et al., 2006; Tar'an et al., 2007; Anbessa et al., 2009; Sabbavarapu et al., 2013). Among them, one major QTL on LG 4 was reported by several researchers under different conditions (Lichtenzveig et al., 2006; Tar'an et al., 2007; Anbessa et al., 2009; Sabbavarapu et al., 2013). Most recently, Li et al. (2017) identified a 100 kb genomic region containing 12 candidate genes for disease resistance associated with a major QTL on chromosome 4 of chickpea using Fst genome-scan and genome-wide association mapping.

Grain yield loss due to AB is a major cause for concern in pea growing regions. Several studies have been conducted to identify improved sources of resistance for pea breeding. Many QTLs were reported for AB resistance in pea (Timmerman-Vaughan et al., 2002, 2004; Tar'an et al., 2003; Prioul et al., 2004; Fondevilla et al., 2008, 2011; Carrillo et al., 2014). Under field conditions, Timmerman-Vaughan et al. (2002, 2004) reported several QTLs for resistance on LGs I, II, III, IV, V, VII, and Group A in two pea mapping populations, whereas Tar'an et al. (2003) identified three QTLs on LGs II, IV, and VI. Prioul et al. (2004) reported six QTLs on LGs III, Va, VI, and VII and 10 QTLs on LGs II, III, Va, and VII under controlled and field conditions, respectively. In *P. sativum* ssp. *syriacum*, six QTLs were reported on LGs II, III, IV and V by Fondevilla et al. (2008), whereas three additional QTLs were identified by Fondevilla et al. (2011) on LGs III and VI. Carrillo et al. (2014) identified four new QTLs on LGs II, III, and V controlling cellular mechanisms involved in AB resistance in *P. sativum ssp. syriacum*. A comparative analysis showed that QTL MpIII.1 (Fondevilla et al., 2008) was located on the same distal part of LG III where Prioul et al. (2004) identified mpIII-1. Fondevilla et al. (2011) indicated that QTLs MpIII.1, MpIII.3, and MpIII.2 detected in *P. sativum* ssp. *syriacum* corresponded to the QTLs mpIII-1, mpIII-3, and mpIII-5 identified in *P. sativum* by Prioul et al. (2004).

With the long-term objective to develop disease resistant pea cultivars, P651 (*P. fulvum*) a wild accession with improved resistance was identified and utilized for the development of an inter-specific pea population (PR-19) (Jha et al., 2012; Sindhu et al., 2014). Nine QTLs were identified for AB resistance in PR-19 and these QTLs individually explained 7.5 to 28% of phenotypic variation (Jha et al., 2016). Among these QTLs, abI-IV-2 and abIII-1 were consistent across locations and/or years with greater effects (16 to 28% of the total phenotypic variation) and P651 contributed alleles for disease resistance. Based on shared anchored markers, none of the identified QTLs were located in the regions of previously reported QTLs for AB resistance in pea (Jha et al., 2016).

In this research, abI-IV-2 and abIII-1 were selected for fine mapping to develop closely linked markers associated with AB resistance. For this purpose, four RILs each were identified in the abI-IV-2 and abIII-1 QTLs, for development of HIF populations. Among these RILs, lines PR-19-224 and PR-19-173 were selected for development of HIF-224 and HIF-173, respectively, on the basis of presence of heterozygous alleles as determined by KASP assay and AB scores. These HIFs served as segregating populations for fine mapping.

To find additional markers within QTLs, selective genotyping of 51 PR-19 RILs was performed using GBS. Based on linkage map construction from these RILs, 12 SNPs were identified in regions next to the highly linked markers within QTL abI-IV-2. Ten of the 12 markers were further genotyped on the complete set of PR-19 RILs (144) to determine the exact position and order of the tested markers in the QTL. Eight out of 10 SNPs from GBS were mapped within QTL abI-IV-2. Three markers (Sc34405_60551, Sc33468_44352, and Sc12023_67096) were located within the closest flanking markers (PsC6805p316 and PsC19558p107) located on either side of marker (PsC8031p219) having highest LOD in the QTL. The presence of QTL abI-IV-2 was validated on linkage map of PR-19 lines enriched with additional GBS markers. GBS marker Sc33287_25420 was the closest marker to the identified QTL with maximum LOD value and co-located with PsC6805p316. HIF-224 lines were

genotyped using 20 SNPs including 10 GBS markers. A linkage map was constructed from 17 markers which covered 86.3 cM distance. The order and distance of markers were different compared to abI-IV-2 QTL obtained for PR-19. This could be due to recombination between the nearest markers within the QTL, or with markers near this QTL. In the case of PR-19, markers PsC20402p281 and PsC7497p542 were adjacent to the abI-IV-2 QTL and distant (around 7 cM) from the closest marker (PsC8031p219) to the QTL. However, in HIF-224, these markers were present within the QTL abI-IV-2 and covered more than 40 cM distance out of 86.3 cM. The larger map distance in HIF population compared to PR-19 RIL population could be due to the possibility that RIL PR-19-224 selected for HIF development was not heterozygous for the entire QTL. This RIL was fixed for alleles from Alfetta at several loci and was the best line that could be selected for maximum heterozygosity within this QTL based on genotyping of the $F_6$ generation. Further, line PR-19-224 selected for HIF development might also contain positive alleles at other ascochyta resistance QTLs which might have affected determining the true effect of this QTL on disease resistance, thus there was no spike observed in LOD value in the HIF population.

Two new QTLs, abI-IV-2.1 and abI-IV-2.2 were identified within abI-IV-2 QTL due to additional SNP markers identified and these QTLs individually explained 5.5 to 14% of the total phenotypic variation. In general, improvement in LOD value was observed in comparison to previously identified QTL. QTLs for lodging resistance were co-located with QTLs associated with AB resistance. The parent Alfetta contributed the alleles for AB resistance as well as for lodging resistance. In this research, it was observed that the difference in AB score was relatively narrow between the parents under field conditions. On a 0-9 scale, Alfetta had 3 to 4 and 4 to 5 disease scores at pod filling and physiological maturity stage, respectively, whereas P651 had 2 to 3 at pod filling stage and 3 to 4 at physiological maturity stage. Further, Alfetta (1–3) had very low lodging score compared to P651 (8–9) on the 1–9 scale. Previous studies reported positive correlation between AB and lodging scores (Tar'an et al., 2003; Banniza et al., 2005; Jha et al., 2013, 2016). Under field conditions, lodging might play an important role in the disease progression and AB score could be related to disease avoidance rather than resistance *per se*. Our previous study (Jha et al., 2016) reported that three out of six QTLs identified under field conditions could account for disease avoidance as these loci were also associated with traits including lodging or plant height. Alternatively, resistance under field conditions could be due to physiological resistance (Khan et al., 2013) as canopy architecture features including branching, lodging resistance, and leaf area index could affect the impact of disease by affecting the microclimate within the canopy and splash dispersal of *P. pinodes* conidia (Schoeny et al., 2008; Le May et al., 2009). In case of abIII-1, no additional marker was identified within the QTL (2.1 cM). Five and three additional SNP markers identified in QTLs abI-IV-2.1 and abI-IV-2.2, respectively, by fine mapping can be used for marker assisted selection. Further, promising HIF lines harboring QTLs for disease resistance can be utilized as donors for development of cultivars with improved AB resistance.

## AUTHOR CONTRIBUTIONS

AJ, BT, and TW conceived and designed the experiments. AJ and TW were involved in the development of HIFs and the multi-year field trials. GBS markers were developed by KG. AJ and KG were involved in the genotyping of PR-19 and HIFs and data analysis. AJ wrote the manuscript with input from KG, TW, and BT. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00765/full#supplementary-material

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