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ABSTRACT

Ascochyta blight (AB) is an important seed-borne and foliar disease of field peas and is widely recognised as a major productivity barrier. The disease is caused by several necrotrophic fungal pathogens, including *Didymella pisi*, *D. pinodes*, *D. pinodella* and *Ascochyta koolunga*. These pathogens can exist independently or together (known as the Ascochyta blight disease complex) within a pea field and even on single plants. Different AB pathogens constantly interact with each other on host plants. The interspecific interactions can remarkably influence disease development and outcome. Understanding the interactions of different AB pathogens on disease development will help us better control this serious disease. In this study, we investigated the interaction of the three AB pathogens, *D. pisi*, *D. pinodes* and *D. pinodella*, in single pea plants. A biocontrol agent, *Pseudomonas protegens* (Pf-5), was also included in this study to understand the impact of plant-beneficial microbes on the interspecific interactions of the AB pathogens. The results revealed a dual interaction with both mutualism and competition among the AB species over time. The mutualistic interaction among the three species allowed *D. pisi* to establish growth in a previously immune cultivar, Fallon. Meanwhile, *D. pinodes* became more aggressive and reached up to 60% of the abundance in the population. These findings provide a knowledge base for effective integrated management of pea AB disease, highlighting the importance of *Ascochyta* species inspection during seed exchange, target species for resistance breeding, and *Pseudomonas protegens* Pf-5 as a potential biocontrol agent.

1 | Introduction

Biological interaction refers to the effects that co-existing organisms have on each other in an ecosystem. The nature of these interactions can be diverse and vary depending on the environmental conditions in which the interactions take place. Consequently, the ecological relationships between individual organisms and entire species are often challenging to define and quantify and are frequently influenced by the scale and context of the interactions (Brooker et al. 2009; Harrison and Cornell 2008; Ricklefs 2008). There are three possible

types of interaction that could occur among living species in a specific niche: competition, mutualism and commensalism. Competition is the process by which organisms directly or indirectly interact with each other, resulting in a change in their fitness when they compete for the same finite resource. The outcome typically has adverse effects on the more vulnerable competitors. Mutualism is a form of symbiotic relationship in which all organisms involved derive benefits from the interaction relationship. Commensalism is a type of interaction where one organism gains an advantage while the other is not affected either positively or negatively. These effects

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can be attributed to varying interaction mechanisms, including competition for resources or space, changes in the host's susceptibility through induced resistance, or the production of toxins by one species that inhibits the growth of the other (Le May et al. 2009). Interactions among pathogens play a significant role in shaping the structure of pathogen communities and, consequently, the dynamics, severity and impact on crop output (May et al. 2005; Savary et al. 1997; Savary and Zadoks 1992; Tivoli et al. 1996). The presence and quantity of pathogen species on a particular host plant might vary depending on whether the initial infecting species cause modifications in the host plant and impact the establishment and growth of subsequent infesting species (Ristaino et al. 2021). To enhance our ability to manage the disease, it is crucial to gain a deeper understanding of the impact of interspecific interaction on the host (Chen et al. 2022).

Field pea (*Pisum sativum*) is a legume grown worldwide as an annual crop throughout the cool season. The nutritional and environmental benefits of field peas for both humans and animals are extremely significant. Pea seeds contain approximately 15%–35% of the essential amino acids, minerals and carbohydrates required by the body (Dahl et al. 2012; Iqbal et al. 2006; Mudryj et al. 2014; Wu et al. 2023). Field peas serve as hosts to a diverse community of pathogens and may often be simultaneously infected by more than one pathogen species. Ascochyta blight (AB) is a seed-borne and foliar disease caused by a complex of necrotrophic pathogenic fungi, including *Didymella pisi* (synonym: *Ascochyta pisi*), *D. pinodes* (synonym: *A. pinodes*, *Peyronellaea pinodes*), *D. pinodella* (synonym: *A. pinodella*) and *A. koolunga* (synonym: *Phoma koolunga*) (Chen et al. 2015; Chilvers et al. 2009; Davidson and Kimber 2007; Hou et al. 2020; Khan et al. 2013; McDonald and Peck 2009; Onfroy et al. 1999). Historically, *D. pinodes* has been the focus of the disease complex, as it is widely regarded as the most aggressive pathogen among the four (Bretag et al. 2006; Khan et al. 2013; Le May et al. 2012; Moussart et al. 1998; Tivoli and Banniza 2007). *D. pinodes* is the dominant pathogen in the Canadian prairies (Gossen et al. 2011) and is also recognised as the primary pathogen of the complex across southern Australia (Bretag et al. 2006; Khan et al. 2013). Despite *D. pisi* co-occurring with *D. pinodes* in southern France (Tivoli and Banniza 2007), *D. pinodes* is given the most attention and is considered the most destructive one.

Cropping practices influence the ecological balance among species involved in the AB complex (Barbetti et al. 2021). Management decisions in a particular location can favour and establish the dominance of any species. During the mid-1960s in Canada, *D. pinodes* emerged as the prevailing species on field pea seeds following the introduction of resistance to *D. pisi* (Tivoli and Banniza 2007). The pathogen population in Western Australia has varied across time and geographic locations (Tran et al. 2014), to the extent that *A. koolunga* can be more virulent than *D. pinodella* on stems and *D. pinodes* on both stems and leaves of field pea (Tran et al. 2015). *D. pisi* is the predominant pathogen in Europe and Asia and has been associated with recent disease outbreaks in Georgia and Spain (Chilvers et al. 2007; Kaiser et al. 2008), despite being the least aggressive in the complex (Wallen 1965). The likelihood of one or more species in the

complex disappearing or reappearing is extensively documented (Li et al. 2011, 2012; Tran et al. 2014), emphasising the extremely variable nature of the disease complex.

Disease complexes causing similar symptoms in a single host plant species are of particular interest. The temporal sequence of epidemic development and overall disease severity and reproductive success of each pathogen species within the complex are important factors (Le May et al. 2009). They play a crucial role in designing effective control methods within a cropping season and ensure the persistence of the inoculum over successive years. Though the symptoms, life cycles, and taxonomic relationships of individual species of AB are well understood (Bretag et al. 2006), the impact of co-infection by the complex on their respective developments and severity is still unknown. The distribution of species within the AB complex varies significantly over time and geographic regions (Tran et al. 2014). This poses a significant challenge for the process of field screening and selection for varietal resistance, as the identification of resistance is reliant on the species found at each specific field screening site. When a resistant variety specific to one species is used, it changes the balance of species in the complex toward other species that are less limited by that resistance. Previous reports have highlighted the difficulties in developing resistance to multiple species of the AB complex (Fondevilla et al. 2005; Fondevilla, Almeida, et al. 2011; Wroth 1998). Specifically, studies have shown that defence proteins down-regulate against *A. koolunga* and up-regulate against *D. pinodes* (Fondevilla, Küster, et al. 2011; Prioul-Gervais et al. 2007; Tran et al. 2018). Furthermore, significant interactions exist among the species within the Ascochyta blight complex not only in terms of the consequent overall severity of disease (Le May et al. 2009) but also the differential disease severities across the different plant components, including the leaf, stem, epicotyl and root (Tran et al. 2015, 2016).

In this study, we characterise the interactions of three *Ascochyta* species and use the categories of interactions as a framework to predict the inherent processes and the impact of the interspecific interactions on the host. Our objective is to estimate the risk of disease severity when different species of the complex are present together in the field, predict the ultimate population composition of AB in the complex, and provide knowledge bases for developing robust resistant cultivars and implementing effective strategies to manage the pathogens.

2 | Materials and Methods

2.1 | Plant Materials and Growth Conditions

Two spring pea varieties, Fallon and Greenwood, were used in the study. The seeds were provided by Dr. Kevin McPhee, the Department of Plant Sciences and Plant Pathology at Montana State University (MSU), USA. These varieties were chosen because Fallon is immune to *D. pisi* but susceptible to *D. pinodes* and *D. pinodella*, whereas Greenwood is susceptible to all three species.

Pea plants were grown in a greenhouse with a day/night temperature of 22°C/18°C, 22% humidity and 16 h of light at the MSU

Plant Growth Centre. The seeds were sown in pots containing a mixture of peat and Sunshine Mix 1 (Sun Gro Horticulture) at a ratio of 1:1.

2.2 | Pathogens and Cultural Conditions

The study used three species of the AB complex: *D. pinodes* isolate C6, *D. pisi* isolate 22-P2033 and *D. pinodella* isolate ab. The pathogens were isolated from AB-contaminated seed lots submitted by growers to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) at MSU. The three fungal species were isolated and subsequently verified as pure cultures of each species by PCR using species-specific primers (Table 1) by Dr. Bernard Nyamesorto, formerly at the Department of Plant Sciences and Plant Pathology, MSU. The isolates were cultured on a pea meal agar (PMA) comprising 25g pulverised dry pea using a blender and 15g agar (Thermos Scientific) in 1 L of double-distilled water (ddH₂O) at 22°C to facilitate the growth of conidia spores.

2.3 | Inoculum Preparation

To study the interactions and population composition of the three *Ascochyta* species over 8 days when co-existing, we mixed the three species with an equal spore density (referred to as mixed-inoculum) to inoculate pea plants. At the same time, the two varieties were also inoculated with single-species inoculum containing only conidia of a single, pure species of the three AB species. In order to be able to compare the growth rate of each species in peas inoculated with mixed-inoculum and corresponding single-species inoculum, the two types of inocula were prepared from the same stocks.

In detail, three single-species stocks were prepared by flooding the cultured surface of each species with 30 mL sterile distilled water (SDW) and gently scraping the inundated culture surface with a glass rod for 10 min to dislodge conidia. Conidia were then collected after filtration through a layer of cheesecloth and used as stocks. The spore density of the stocks was counted by pipetting 10 µL of the inoculum onto a haemocytometer under a light microscope. The inocula were prepared by diluting a calculated amount of each stock with SDW + Tween 20 (two drops per 200 mL) to achieve a spore density of 10⁵ spores/mL in a single-species inoculum. To prepare a mixed inoculum, if a 200 mL single-species inoculum of *D. pinodes*, *D. pisi* and *D. pinodella* used the stocks of 40, 50 and 30 mL, respectively, then 40, 50 and

30 mL of the same amount corresponding to the species stock were taken and topped up to 200 mL with SDW + Tween 20. The spore density of each species in the mixture was equivalent to that of the single inoculum, but the total spore density in the mixed inoculum was 3 × 10⁵ spores/mL.

2.4 | Inoculation of the Fungal Pathogens

2.4.1 | Inoculation for the Interaction and Growth Rate Study

Twenty plants of each variety (five seeds/pot, four pots/variety) were grown for the mixed inoculum. Meanwhile, 20 plants/variety were prepared similarly for the three single-species inocula. Mixed inoculum or single-species inoculum was applied to whole plants of Fallon and Greenwood at the four-node stage via spray-inoculation. In the spraying process, the inoculum was uniformly applied as a fine mist from the top of the seedlings in all directions to ensure every part of the plant was covered with inoculum. All inoculated plants were transferred into an I-60D dew chamber (Percival Scientific Inc.) preconditioned to 20°C–22°C for 24 h in the dark and then moved to the same greenhouse room and kept in a plastic tent with mists produced by humidifiers 6 h a day to achieve 100% humidity. After inoculation, three plants from the same pot were sampled for one of the four time points for the time-course study. Three inoculated leaves per variety at each time point were sampled by placing each leaf in between the cap and the top of a 1.5-mL tube to obtain the same size of leaf disc. Three biological replicates were conducted for the study.

2.4.2 | Inoculation for the Detached-Leaf Assays

Three pairs of stipules from the third node of pea plants were detached and placed on PMA in Petri dishes. For the results in Section 3.3, the detached leaves were treated with three treatments. The naming of the treatments was based on the sequence of actions. In the MP treatment, the detached Fallon stipules were applied with a mixed inoculum of *D. pinodes* and *D. pinodella* and then *D. pisi* was inoculated onto the leaves after 24 h. In the MUP treatment, the detached Fallon stipules were applied with a mixed inoculum of *D. pinodes* and *D. pinodella*; 24 h later, the leaves were placed under UV radiation for 30 min and then *D. pisi* was inoculated onto the leaves. The UV treatment was to kill all pre-existing microbes. In the

TABLE 1 | Species-specific primers for the *Ascochyta* species identification.

Name	Sequence (5'–3')	Tm (°C)	Target
BNpisiF	CTGCTCCTCGTCATGATC	52.4	<i>Didymella pisi</i>
BNpisiR	TGACGTACCTTGAAGAAGTA	50.3	
BNdellaF	CGCCATGATCGGAACACATA	55.1	<i>Didymella pinodella</i>
BNdellaR	ACGCGTATCCCTTCGAATTAC	54.8	
BNnodesF	TCCTCGATGTCAATATCAC	48.7	<i>Didymella pinodes</i>
BNnodesR	GACGATTCTGCCTGTTATG	50.7	

UP treatment, *D. pisi* was spray inoculated onto the detached stipules pretreated with 30 min of UV light. The UV treatment was done inside a PCR/UV workstation (COY Laboratory Products Inc). All the pathogen inoculations were spray inoculated.

2.4.3 | Inoculation for the Detached-Leaf Assay (Results in Section 3.4)

Three pairs of stipules from the third node of pea plants were detached and placed on PMA in Petri dishes. The pathogens were inoculated by pipetting 10 μ L of each species inoculum on a designated side of the leaf.

2.4.4 | Inoculation for Artificial Nutrient Study

A square piece of each previously cultured species was inoculated on three sides of PMA in Petri dishes. The plates were kept at room temperature.

2.5 | Inoculum Preparation and Inoculation of Beneficial Bacterium

Pseudomonas protegens (Pf-5) is a well-characterised plant-beneficial bacterium (Yan et al. 2017) and was used in this study to understand the possible influence of symbiotic bacteria on the interspecific interactions of the AB complex.

To prepare the bacterial inoculum, Pf-5 was cultured overnight on King's B medium plates at 28°C. The cells were harvested from the plates and washed three times using sterilised water into a 300 mL suspension with a concentration of 10⁹ cells/mL. The bacterial suspension was spray inoculated onto pea leaves until the leaf surfaces were fully coated and appeared uniformly wet. The inoculated plants were kept in a humid growth chamber at 28°C for 24 h before inoculating the fungal pathogens described above.

2.6 | Inhibition Assays of the AB Fungal Pathogens by *P. protegens* Pf-5

The inhibition assays were conducted on PMA plates. A 5 mm agar plug from a 2-week-old culture of the fungal pathogens was placed onto the centre of the PMA plates. Bacterial cells of Pf-5 were washed three times using SDW into a 1 mL suspension with a concentration of 10⁹ cells/mL. A drop of 3 μ L of the bacterial cell suspension was placed on the plate about 2.5 cm away from the centre of the fungal plug. The plates were air-dried and incubated at 28°C without light. At least three replicates were used for each tested fungal pathogen in one experiment. The inhibition zone was measured at 4 days after inoculation. The experiment was repeated at least two times independently. The fungal hyphal growth distances (d_1 and d_2 , with and without Pf-5 inhibition) were measured

4 days after the bacterial inoculation. The inhibition rate was calculated by the equation

$$\text{Inhibition rate (\%)} = [(d_2 - d_1) / d_2] \times 100$$

2.7 | Sample Collection, DNA Extraction and Quantitative PCR

Three leaf disc samples were collected from inoculated leaves at 24 h post-inoculation (hpi) and 3, 6 and 8 days post-inoculation (dpi), frozen in liquid nitrogen, and kept at -80°C for DNA extraction. For each treatment, leaf discs of identical dimensions were collected from three single plants per time point, with three biological replicates. DNA was isolated using a DNeasy Plant Mini Kit (Qiagen) based on the manufacturer's instructions. The concentration of total DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.). The integrity of the DNAs was checked via 1% agarose gel electrophoresis with 1 μ L of the sample, 4 μ L water and 1 μ L loading buffer (98% formamide, 10 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanole) stained by GelRed (Bio-Rad) at 90 V for 30 min.

To monitor the population composition of the three *Ascochyta* species on pea leaves over time when co-existing, the DNA abundance of each species was tested from the same DNA sample at each time point with species-specific primers via quantitative real-time PCR (qPCR). A qPCR was performed in a CFX96 real-time PCR detection system (Bio-Rad). PCR was set up using the iTaq Universal SYBR Green Supermix (Bio-Rad) in a 20 μ L total reaction mix volume containing 2 μ L of a mixture of forward and reverse primers (1 pmol/ μ L), 10 μ L of iTaq, 2 μ L of DNA (25.5 ng/ μ L) and 6 μ L of RNase-free water. The qPCR was performed with an initial incubation at 50°C for 10 min, followed by denaturation at 95°C for 5 min. The amplification was carried out for 39 cycles, each consisting of denaturation at 95°C for 10 s and annealing/extension at 55°C for 30 s. Pathogen-specific primer sequences for qPCR are listed in Table 1. The relative abundance was presented as a fold change of 2^{- Δ Ct}. The relative ratio was measured using DNA abundance referenced to the species with the least abundance in the same DNA sample at each time point. Three biological replications were tested, and three technical repeats were set for each reaction.

The relative growth rate of each pathogen over time in the mixed inoculum was referenced to the abundance at 1 dpi of the same species. The relative growth rate between the mixed inoculum and the corresponding single inoculum was measured as differences in relative abundance, with the C_t value of the single inoculum as a reference per time point.

2.8 | Statistical Analysis

Statistical analysis was performed using a combination of Microsoft Excel and R-software (R v. 4.2.0). All the experiments were conducted in controlled conditions. qPCR data are presented as the mean of absolute quantification number (C_q) $\pm$ SD of three biological replicates. Statistical differences between mean values were determined by the Student's *t* test. Pie charts

and line graphs were generated for species inoculum/cultivar and visualised in Excel.

3 | Results

3.1 | Mutual and Competitive Relationships Among the Three *Ascochyta* Species

To investigate how the three *Ascochyta* species interact with each other when they co-exist on the same host plants, we measured the relative DNA abundance of each species from the same leaf discs during pathogenesis at 1, 3, 6 and 8 dpi. The three pathogens were mixed in the same spore density (referred to as a mixed inoculum) and inoculated onto two pea varieties, Greenwood, which is susceptible to all three *Ascochyta* species tested, and Fallon, which is immune to *D. pisi* but susceptible to the other two species. At 1 dpi, the relative ratio of *D. pinodes*:*D. pinodella*:*D. pisi* was 1:16:3 in Greenwood (Figure 1a) and 1:6:2 in Fallon (Figure 1b). This result showed that *D. pinodella* was the most abundant species detected in both varieties at 1 dpi. We observed shifts in the relative ratio of the three species over time from the initial 1:16:3 at 1 dpi to 7:3:1 at 8 dpi in Greenwood (Figure 1a). *D. pinodella* gradually lost its advantage; *D. pinodes* became the most abundant species and *D. pisi* was the least abundant at 6 and 8 dpi (Figure 1a). Different shifts were observed in Fallon. *D. pinodella* maintained the advanced growth rate at 1 and 3 dpi, and then *D. pinodes* gradually caught up, forming a community with a similar amount of *D. pinodella* and *D. pinodes* at 8 dpi (Figure 1b). Noticeably, *D. pisi* was the second most abundant species at 3 dpi in Fallon, a cultivar immune to this species (Annan et al. 2023), suggesting that *D. pisi* did benefit from the interaction and supported a mutual relationship. However, the three species did not proliferate at the same ratio in either variety, showing a competitive relationship simultaneously, with *D. pinodes* being the most dominant of the

three. When we compared the relative growth of each pathogen over time, a similar growth pattern was observed for all pathogens (Figure 2a,b). The relative abundance of *D. pinodes* increased significantly at 6 dpi, followed by *D. pisi*. However, the abundance of *D. pinodella* did not increase significantly over the time course in Greenwood (Figure 2a), but a significant increase was detected in Fallon at 8 dpi (Figure 2b). Collectively, the study revealed that population composition resulted from both mutualistic and competitive relationships among the three species and the different dynamic changes influenced by the host background.

3.2 | Enhanced Aggressiveness When the Three Species Co-Exist

To understand the impact of such mutualism and competition on a host plant, we monitored and compared the growth rate of each species on the same cultivars inoculated with a mixed inoculum to their corresponding single-species inoculum. The relative abundance of each species from the two treatments was measured and compared to ascertain if the pathogens increased their growth rate or became more aggressive when co-existing. Equal amounts of spore density of each species were inoculated on Greenwood and Fallon under two treatments (mixed vs. single) prepared from the same stock culture. The differences in relative abundances were presented as a fold change of $2^{-\Delta C_t}$ between the mixed-inoculated and corresponding single-species-inoculated same variety at the same time point. When the ΔC_t is negative, suggesting more abundance from the mixed-inoculated plants, this is referred to as enhanced aggressiveness of the species when co-existing. The differences in the relative abundances of *D. pinodes* in leaves of both Greenwood (Figure 3a) and Fallon (Figure 3b) inoculated with mixed inoculum were negative at 3 and 6 dpi and closer to zero at 1 and 8 dpi. Similarly, the difference in relative

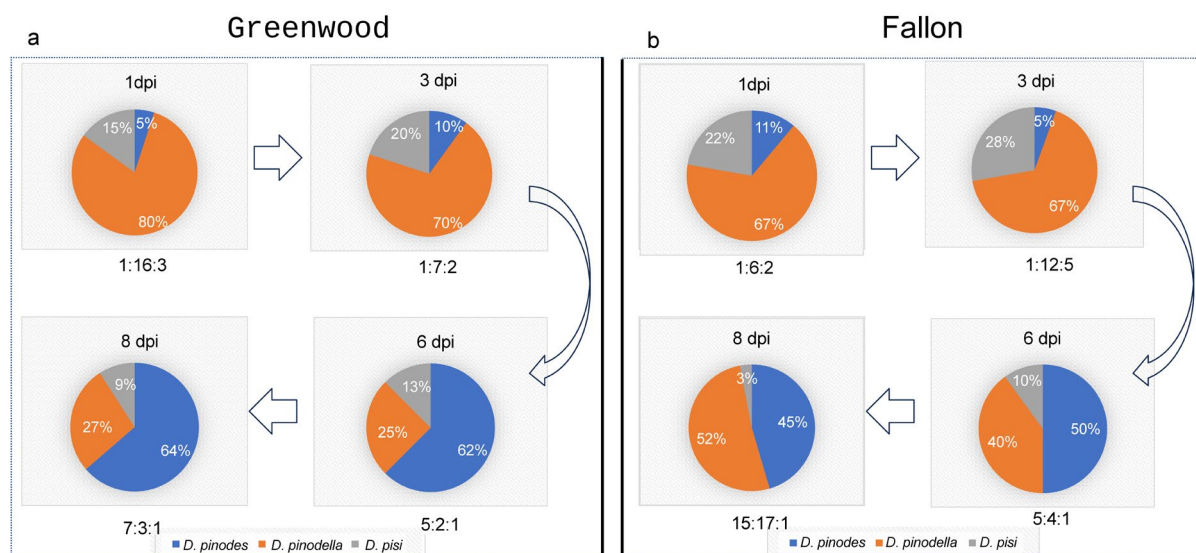


FIGURE 1 | Population compositions of the three species *Didymella pisi*, *D. pinodes* and *D. pinodella* on two pea cultivars over time. The relative DNA abundance of each species from the same leaf discs with mixed inoculum at 1, 3, 6 and 8 days post-inoculation (dpi) are presented as percentages and relative ratios on (a) cv. Greenwood and (b) cv. Fallon. The ratio among the species at each time point was computed relative to the pathogen species with the lowest abundance at each time point. The relative ratios among the species are depicted as percentages in the pie chart. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/jpp.14136)]

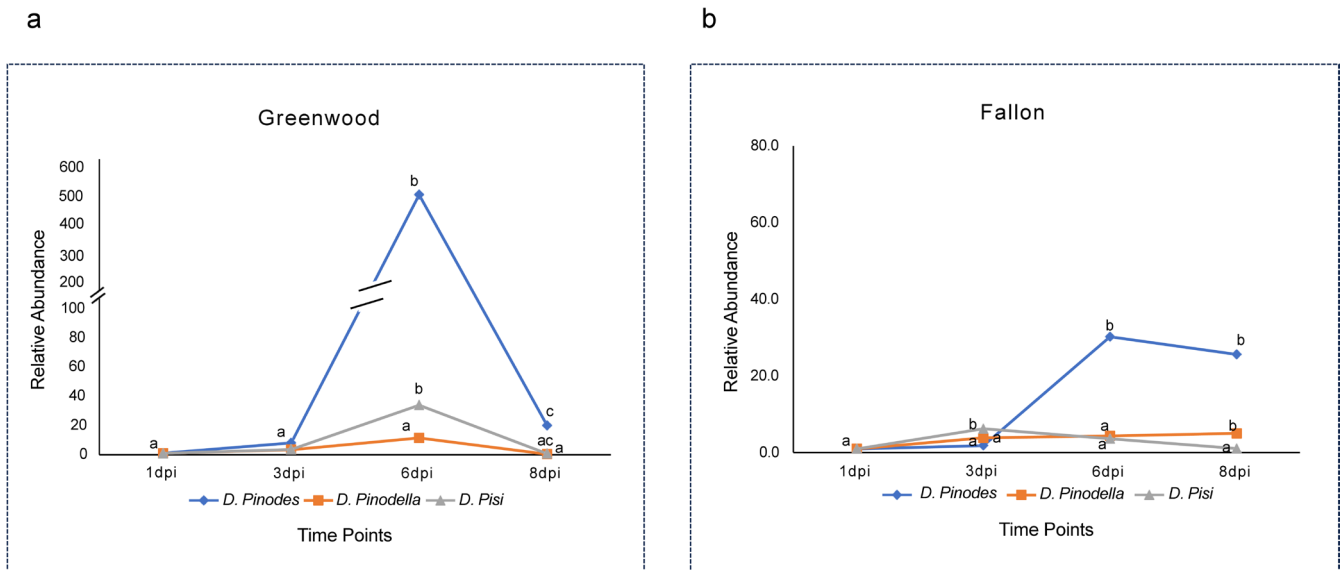


FIGURE 2 | Relative growth of *Didymella pisi*, *D. pinodes* and *D. pinodella* on two pea cultivars with a mixed inoculum via the relative abundance (quantity of DNA) among pathogen species at 1, 3, 6 and 8 days post-inoculation (dpi) on (a) cv. Greenwood and (b) cv. Fallon. The abundance of each species was computed relative to the level of their corresponding species at 1 dpi. Different letters denote statistical significance at the $p \leq 0.05$ level calculated between each time point and their corresponding species at 1 dpi. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

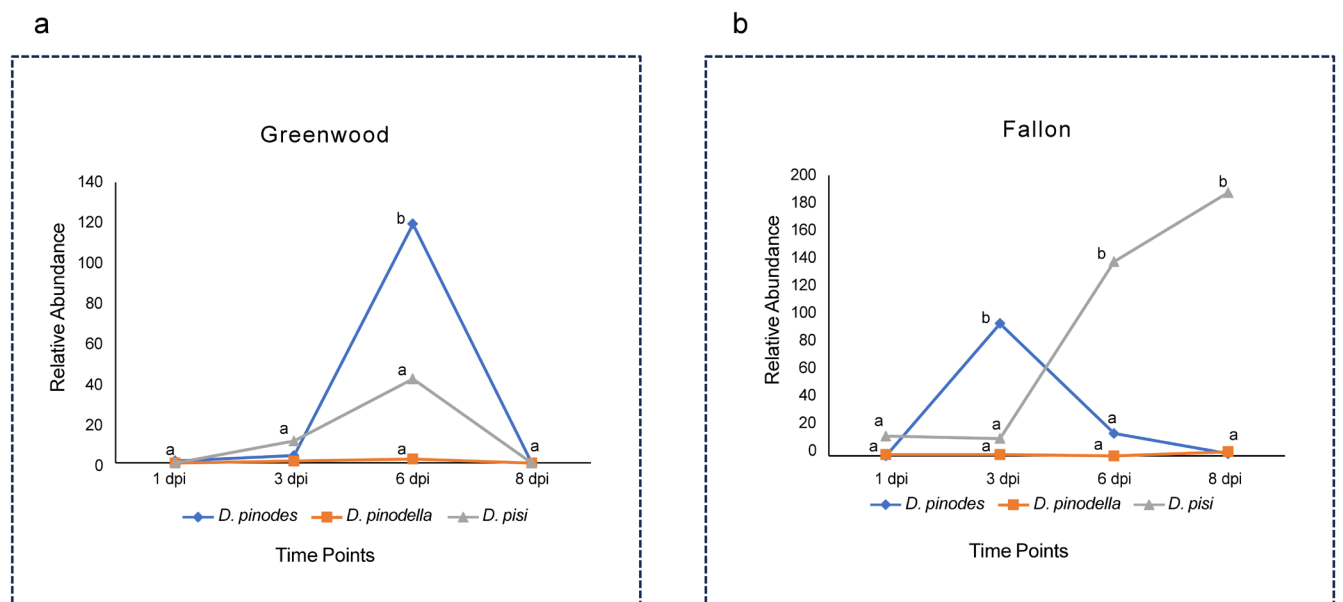


FIGURE 3 | Comparison of the growth rate of *Didymella pisi*, *D. pinodes* and *D. pinodella* on two pea cultivars between mixed versus single inoculum. The difference in the relative abundance (quantity of DNA) of each pathogen species in mixed-inoculum treatment as against their corresponding single-inoculum treatment on (a) cv. Greenwood and (b) cv. Fallon. The difference in relative abundance at each time point was calculated for each pathogen species in mixed inoculum relative to their corresponding single inoculum. Different letters denote statistical significance at the $p \leq 0.05$ level calculated between each time point relative to the difference of each pathogen in mixed-inoculum treatment as against their corresponding single-inoculum treatment. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

abundances of *D. pisi* on Greenwood inoculated with mixed inoculum was negative at 3 and 6 dpi and closer to zero at 1 and 8 dpi (Figure 3a). However, we observed a difference in Fallon. *D. pisi* was undetectable in a *D. pisi* single-species-inoculated plant, but it had a significant amount of growth from 3 dpi to 8 dpi when co-inoculated with the other two *Ascochyta* species (Figure 3b). The results suggest that *D. pisi* can benefit from the competitors for growth. In contrast, there were no

differences in the relative abundance of *D. pinodella* in both treatments on Greenwood (Figure 3a) and Fallon (Figure 3b). To summarise, *D. pinodes* became much more aggressive when co-existing with the other two species. Compared to single-species inoculation, mixed-species inoculation benefited the growth of *D. pisi* at the early stages of disease development. This benefit decreased and eventually disappeared (Figure 3a) at the late stages of disease development. Another

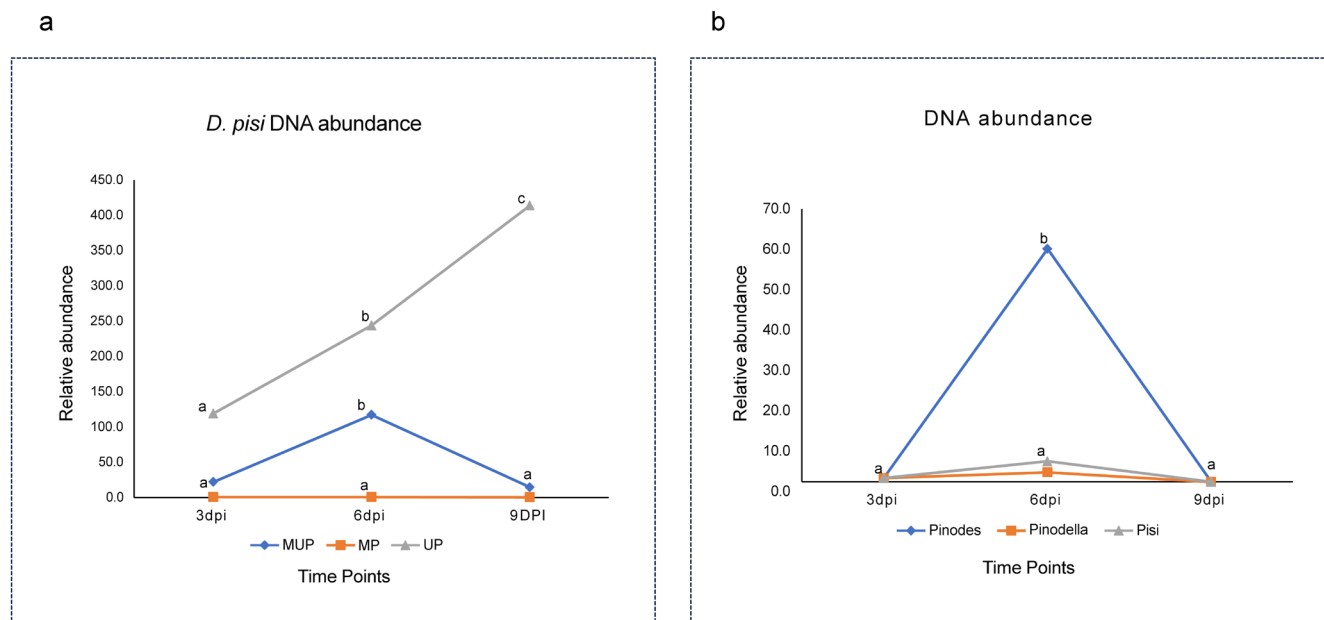


FIGURE 4 | (a) DNA relative abundance of *Didymella pisi* in three treatments. Detached Fallon pea leaves were treated with *D. pinodes* and *D. pinodella* 24 h before (MP treatment), with *D. pinodes* and *D. pinodella* then 30 min UV-treated (MUP treatment), and 30 min UV treatment only (UP treatment) prior to inoculation with *D. pisi*. The abundance of *D. pisi* in all treatments was computed relative to the level at 3 days post-inoculation (dpi) of the MP treatment. (b) Relative abundance of each species under the MUP treatment over time. The relative abundance of each species was computed relative to the corresponding species at 3 dpi. Different letters denote statistical significance at the $p \leq 0.05$ level calculated between each time point and treatment. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

interesting discovery is that *D. pisi* gained a higher and longer-lasting level of growth benefit from the mixed inoculation on the resistant host Fallon (Figure 3b) than on the susceptible host Greenwood (Figure 3a).

3.3 | Limited Benefits From Other Competitors

Fallon's immunity to *D. pisi* has already been established, and the immunity does not confer resistance to the other species (Annan et al. 2023). To further study how *D. pisi* benefited from the competitors, we set up an experiment to measure the relative abundance (quantity of DNA) of *D. pisi* in three treatments at three different time points. In the MP treatment, *D. pisi* was inoculated on the detached Fallon stipules applied with *D. pinodes* and *D. pinodella* 24 h prior, and no *D. pisi* DNA was detected from the stipules over the time points (Figure 4a); this is in contrast to the mixed-species inoculation where *D. pisi* was inoculated with the other two species at the same time. However, in the MUP treatment, a significant amount of *D. pisi* DNA was detected at 6 dpi on the Fallon leaves colonised by *D. pinodes* and *D. pinodella* but treated with UV light for 30 min, but the amount of *D. pisi* DNA decreased to none at 9 dpi (Figure 4a). In a UV-only control, *D. pisi* had a continuously increased growth over the time points on Fallon stipules (Figure 4a). The unexpected growth pattern of *D. pisi* in the MUP treatment led us to investigate what inhibited the growth of *D. pisi* at 9 dpi. We proceeded to ascertain the DNA of the other two species at each time point and observed a similar trend in the relative abundance of each species, all increased at 6 dpi and decreased at 9 dpi (Figure 4b). The results suggested that *D. pinodes* and *D. pinodella* were

not completely killed by the 30-min UV radiation. Once they recovered, the growth of *D. pisi* was suppressed on the same stipules.

Disease symptoms were also documented under each treatment at the three time points. The brown lesions progressed much faster in the MP treatment (Figure 5a), and there were no visible disease symptoms in the MUP and UP treatments until 9 dpi (Figure 5b,c). This was expected because, in the MUP and UP treatments, *D. pisi* was the majority or the only pathogen on the stipules. *D. pisi* has a 7- to 9-day latent period, but *D. pinodes* and *D. pinodella* only have a 24-h and a 2- to 4-day latent period respectively. At 9 dpi, more severe disease symptoms were observed under both MUP and MP treatments. Overall, the results suggested that *D. pisi* could benefit and obtain nutrients from an immune host after the immunity was compromised by the competitors, but only before the competitors were fully established.

3.4 | Territorial Colonisation Among the *Ascochyta* Species

A significant shift in the composition of the three species in pea plants raised the question: Do the species have different growth rates? To answer this question, we set up an experiment using two different nutrient sources (artificial culture and in planta). Square pieces of the three species were placed on three sides of an artificial medium (Figure 6), and 10 μ L inoculum of each species was pipetted at the centre of the Fallon stipule (Figure S1) and Greenwood stipule (Figure S2). Their growth rates were observed and documented over time

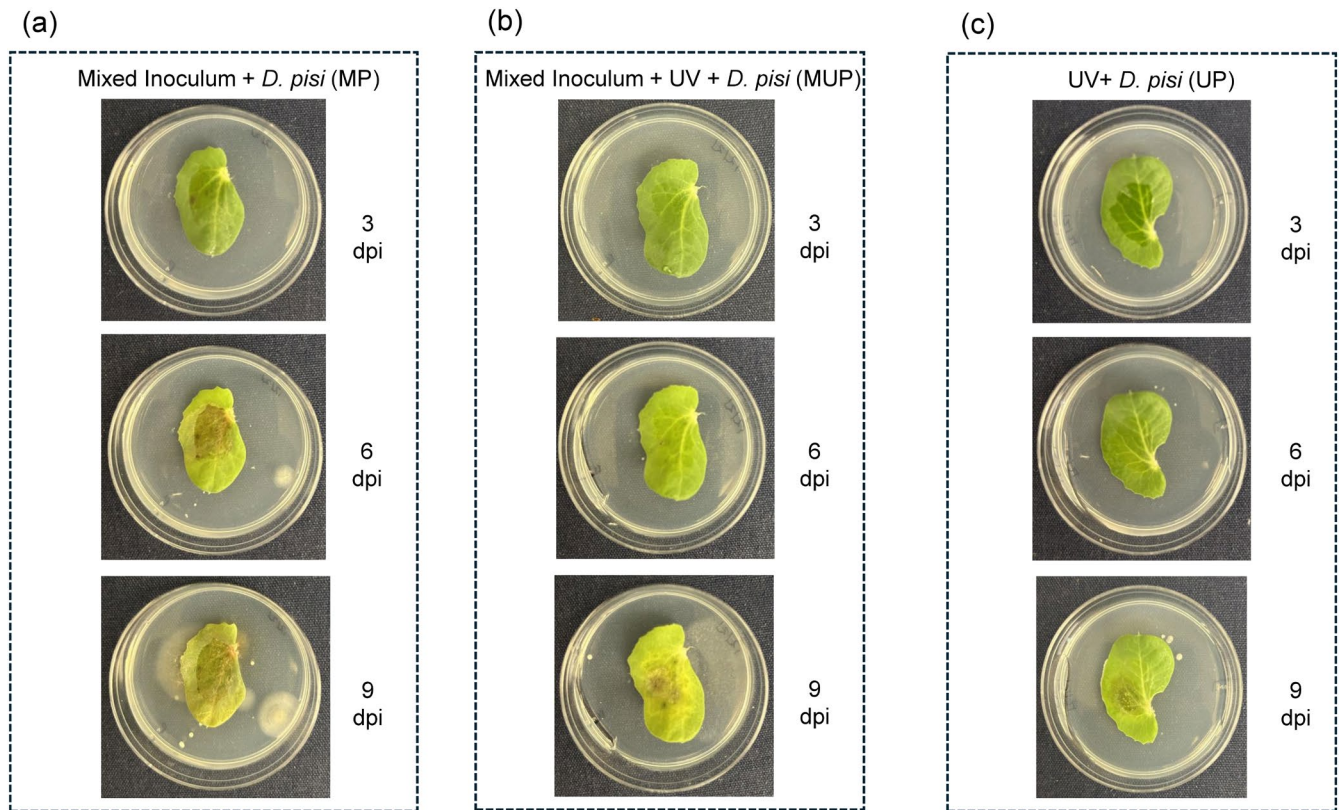


FIGURE 5 | Disease symptoms observed at different time points under three different treatments. Detached Fallon pea leaves were treated with (a) *Didymella pinodes* and *D. pinodella* 24 h before (MP treatment), with (b) *D. pinodes* and *D. pinodella* then 30 min UV treated (MUP treatment), and (c) 30 min UV treatment only (UP treatment) prior to inoculation with *D. pisi*. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

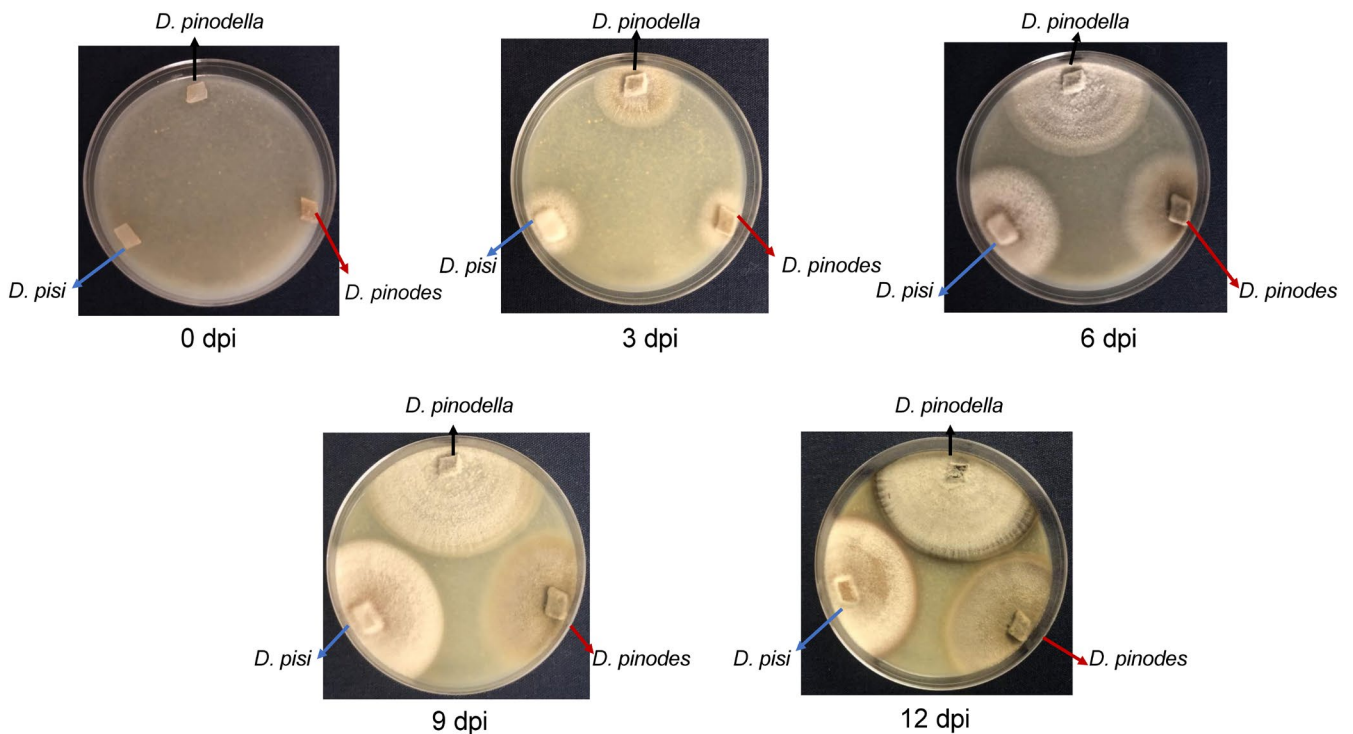


FIGURE 6 | Growth rates of the three *Ascochyta* species *Didymella pisi*, *D. pinodes* and *D. pinodella*, on an artificial medium over time. Square pieces of each species were placed on three sides of a pea meal agar plate. Their growth rates were photographed over 12 days at 0, 3, 6, 9 and 12 days post-inoculation (dpi). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

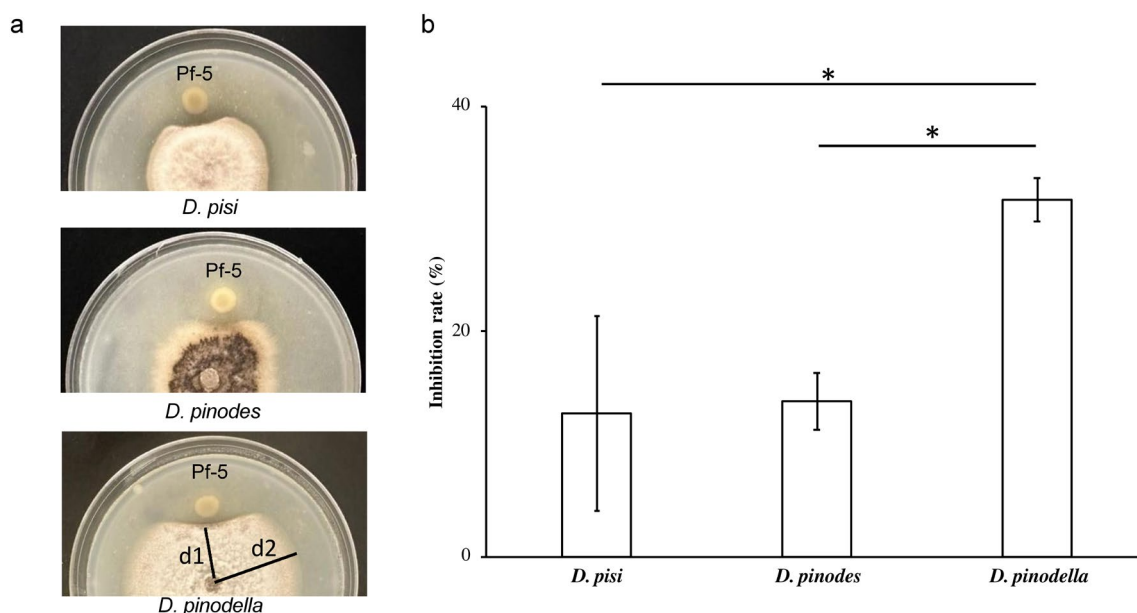


FIGURE 7 | Inhibition by *Pseudomonas protegens* Pf-5 against *Didymella pinodes*, *D. pisi* and *D. pinodella*. (a) Fungal pathogens were inoculated at the centre of pea meal agar (PMA) plates. Three days after inoculation with *D. pinodella* and 7 days after inoculation with *D. pisi* and *D. pinodes*, Pf-5 was inoculated on the PMA plates 2.5 cm from the fungal growth centre. Four days after bacterial inoculation, fungal hyphal growth distances were measured as d_1 and d_2 , indicating the fungal growth with and without Pf-5 inhibition, respectively. (b) The inhibition rate was calculated by the equation: Inhibition rate (%) = $[(d_2 - d_1) / d_2] \times 100$. The graph shows the average and standard deviation of four repeats. * indicates a significant difference ($p < 0.05$) analysed by the Student's t test. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

(Figure 6, Figure S3). *D. pinodella* was the fastest-growing species among the three species in artificial medium, but *D. pinodes* was the fastest-growing species in planta in both cultivars. In addition, none of the pathogens crossed each other's boundaries on the same plate (Figure 6); a similar phenomenon was observed from an in-planta assay when we pipetted 10 μ L inoculum of each species at three corners of a detached Fallon stipule (Figure S3a) and Greenwood stipules (Figure S3b). The results suggested that the *Ascochyta* species have different growth rates from different nutrient sources. Furthermore, the three species showed territorial colonisation; once an area is colonised by a species, it is protected from colonisation by other *Ascochyta* species.

3.5 | Growth of the *Ascochyta* Species Inhibited by *P. protegens* Pf-5

The above study revealed that host background can influence the interactions of *Ascochyta* species. What would the population dynamics be if an additional species were introduced? To answer this question, we introduced *P. protegens* Pf-5, a well-characterised plant-beneficial bacterium, into the interspecific interactions of the *Ascochyta* blight pathogen complex. On PMA plates, Pf-5 inhibited all the *Ascochyta* species when inoculated alongside (Figure 7a). The highest inhibition rate was for *D. pinodella*, suggesting this species was inhibited the most among the three (Figure 7b).

To study the influence of Pf-5 on the dynamics of the *Ascochyta* population in planta, the three species were mixed in the same spore density and inoculated onto Pf-5-treated cultivars of Greenwood and Fallon 24 h prior. The relative abundance of

each species was measured at 1, 3, 6 and 8 dpi. At 1 dpi, the relative ratio of *D. pinodes*:*D. pinodella*:*D. pisi* was 1:2:1 in Greenwood (Figure S4) and 3:4:1 in Fallon (Figure S5). The ratio shifted from the initial 1:2:1 at 1 dpi to 4:18:1 at 8 dpi on Greenwood (Figure S4), with *D. pinodella* the most abundant, followed by *D. pinodes*, during the whole course of the experiment. The result was in stark contrast with the observation of the plate assay, where *D. pinodella* was the most inhibited. We observed different shifts in Fallon, from the initial 3:4:1 at 1 dpi to 9:4:1 at 8 dpi (Figure S5), with *D. pinodella* the most abundant within the first three time points but then losing its advantage at 8 dpi to *D. pinodes*. *D. pisi* was second most abundant in Fallon at 3 dpi, but the least abundant among the three in both cultivars at 8 dpi (Figures S4 and S5). Clearly, the addition of Pf-5 changed the population dynamic from the most abundant *D. pinodes* to *D. pinodella* on the same pea cultivar.

When we compared the relative growth of each species over time, there were no significant increases or decreases in relative abundance on either Greenwood (Figure S6a) or Fallon (Figure S6b), suggesting that neither species increased its biomass and Pf-5 suppressed the growth of the *Ascochyta* species in planta. The disease symptoms were much less severe on the plants pretreated with Pf-5 (Figure S7). The results suggested that Pf-5 may be an effective biocontrol agent against the pathogens.

4 | Discussion

We studied the interactions of *Ascochyta* species and the factors that influenced the interactions, aiming to understand the outcomes and risks when the species of the complex

are present together. Our study revealed a dual relationship among the three *Ascochyta* species. Mutualism is the keynote: *D. pisi* reproduced on the immune host Fallon with the help of the other two virulent species; in addition, each species had a higher growth rate from a mixed inoculum. Meanwhile, significant shifts in population composition indicated a competitive relationship. *D. pinodes* had the fastest growth rate over time in both cultivars, followed by *D. pisi* and *D. pinodella*, in that order. Nonetheless, their growth patterns remained consistent throughout time. This confirms reports of *D. pinodes* as the most aggressive and economically important among the three species (Bretag et al. 2006; Khan et al. 2013; Tivoli and Banniza 2007). The competitive interaction increased *D. pinodes* aggressiveness, and *D. pisi* grew on the immune cultivar Fallon. Based on these findings we could project a much higher disease severity when the three species co-exist than when present independently. Although *D. pisi* is the most predominant species in Montana according to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) (Owari et al. 2020), once *D. pinodes* appears it will quickly replace the old population, namely 'selective sweep' (Zhan and McDonald 2013). If seeds are exchanged without inspection for other *Ascochyta* species, we will risk the pathogen population being toppled by the more aggressive species of *D. pinodes*, similar to in Western Australia; the dynamic shifts of *A. koolunga* replacing *D. pinodes* in field peas (Tivoli and Banniza 2007) indicated that all species in the complex need to be targeted for sustainable control of the disease. We cannot afford to take a lax approach to AB disease management in Montana, even though *D. pisi* is currently the most prevalent species. Thus, a good understanding of the mechanisms, outcomes and effects of such competitive interaction would be useful for managing AB disease sustainably via the efficient use of pesticides and resistance genes (Zhan and McDonald 2013).

Plant-pathogenic fungi possess some characteristics that make them particularly well-suited for researching how competition affects individual fitness (Roughgarden 1979), such as discrete lesions that allow for the measurement of easily quantifiable fitness components like latent period, infectious period, sporulation rate and infection frequency (Zhan and McDonald 2013). The degree of competitiveness among the *Ascochyta* species also depends on their genetic makeup, reflected by the incubation period, pathogenicity compounds produced and adaptation to the environment. Among the three, *D. pinodes* has the shortest incubation period of 24 h, followed by *D. pinodella* (2–4 days) and *D. pisi* (7–9 days). This gives *D. pinodes* a competitive advantage over its rivals. We also noticed the differences in pathogen growth rates on different nutrient sources. *D. pinodella* was the fastest-growing species when cultured in an artificial medium when pre-existing resources were available; in contrast, *D. pinodes* was the most plentiful and fastest-growing species in planta. Meanwhile, compromised host cells by the competitors enabled *D. pisi* to grow to a certain degree on a previously immune cultivar before the competitors were fully established. Noticeably, species did not cross their boundaries of growth/colonisation into territories of other species in both artificial culture and in planta; they cease growing and never cross each other's boundaries once they reach the junction, even when the available food sources and space are gone. Thus, we surmise that different

compounds were used to kill the host cells and to establish territories and were released at different times during colonisation. Histology investigations on lesions on different parts of infected pea plants suggested phytotoxic compounds released by the pathogens are the primary cause because plant cells were damaged and disintegrated before direct contact with the fungus (Höhl et al. 1990). The primary phytotoxic compounds from *D. pisi* culture extracts are PRI, PRII (Abouzeid and El-Tarabily 2003) and ascosalitoxin (Evidente, Capasso, et al. 1993). Ascochitine can cause electrolyte leakage on susceptible cultivars when a purified form of the compound is directly applied to leaf discs (Smith et al. 1994). In contrast, *D. pinodes* and *D. pinodella* produce pinolidoxin, which exhibits strong phytotoxicity (Cimmino et al. 2012; Evidente, Lanzetta, et al. 1993; Kim et al. 2016) that inhibits the plant phenylpropanoid pathway (Evidente et al. 1998). Though not as effective as pinolidoxin, *D. pinodes* also produces herbarumin II, pinolide and putaminoxin (Agudo-Jurado et al. 2023; Cimmino et al. 2012). Thus, we speculate that *Ascochyta* species mark/protect their colonisation area by producing toxins to deter other species. We noticed discernible variations in the dynamics of the three species over time on the two susceptible cultivars, suggest that *D. pinodes* was better than *D. pinodella* at establishing territories early in colonisation. The production of distinct toxins suggests that the host possesses distinct receptors for each toxin. Mutations on receptors to all corresponding phytotoxins produced by one species will be insensitive to the toxin cocktail and, therefore, immune/resistant to the pathogen. This explains why Fallon's immunity to *D. pisi* cannot be transferred to the other species (Annan et al. 2023). Therefore, AB resistance breeding requires resistance to each species and pyramiding them all to achieve a sufficient level and longevity of a resistant variety (Annan et al. 2023).

We also investigated the impact of such competition on a host. An enhanced aggressiveness among species was observed when they were present simultaneously on a host compared to when present independently. The trade-off hypothesis posits that the most effective method for a pathogen to exploit a host, when just one strain of the pathogen infects the host, is to exhibit a moderate level of aggressiveness. This allows for the maximum transmission of the disease among hosts (Read 1994; Zhan and McDonald 2013). Based on our understanding, when a single pathogen species infects a host, its behaviour is characterised by an intermediate level of aggressiveness in acquiring nutrients. This is owing to the access to an abundance of resources available and the pathogen bidding its time for the occurrence of a favourable environment to transmit to a new host. In contrast, when multiple pathogen species co-infect hosts, the trade-off hypothesis might not hold true, and within-host competition might result in increased pathogen aggressiveness in our scenario (Alizon et al. 2009; Brown et al. 2002; Schürch and Roy 2004). We believe in a mixed inoculum; each species has a primary objective of competing for limited resources and establishing territorial domination with transmission to new secondary hosts. In this study, we also investigated how the plant-associated bacterium *P. protegens* Pf-5 influenced the population dynamics of the *Ascochyta* species and the disease developments in pea plants. We found that Pf-5 inhibited all three species, although at different rates, in the artificial medium and in planta. *D. pinodella* was inhibited the most in artificial culture, with *D. pinodes* being

the most inhibited in planta. Interestingly, *D. pinodella* and *D. pinodes* were the fastest-growing species in artificial culture and in planta, respectively. These results suggest that the degree of Pf-5-mediated inhibition of the fungal pathogens is probably related to the growth habit of the species in the two varying nutrient sources: the species that grows faster tends to be inhibited more by the antagonistic bacterium. The inhibition of growth of AB pathogens by Pf-5 in an artificial culture medium is consistent with the observation that this bacterium reduced the disease severity in greenhouse pot assays. To our knowledge, this is the first report that Pf-5 has been used in controlling *Ascochyta* species in field peas. The mechanism of Pf-5-mediated disease control against AB was not investigated in this work. However, based on the result that Pf-5 inhibited pathogen growth in the culture medium and in planta, it is likely that antibiotic production of Pf-5 plays a role in the disease control. Pf-5 produces a broad spectrum of antibiotics, including hydrogen cyanide, pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, rhizoxin, orfamide A and rhizoxin analogues (Yan et al. 2017, 2018). Many of these antibiotics have antifungal and/or anti-oomycete activity and were found to be required for Pf-5 to control the diseases. For example, we recently reported that 2,4-diacetylphloroglucinol inhibited the growth of *Aphanomyces euteiches* in the culture medium and mitigated the pea *Aphanomyces* root rot disease in greenhouse conditions (Lai et al. 2022). It will be interesting to identify if any of the known antibiotics produced by Pf-5 are required for the bacterium to inhibit *Ascochyta* pathogens and control pea AB disease complex.

Our study revealed territorial colonisation and synergistic and antagonistic relationships among the three *Ascochyta* species when co-existing in the same pea host. The outcomes of the interactions include changes in the population composition, enhanced aggressiveness of *D. pinodes* and more severe disease in infected peas. The findings suggest the importance of diagnosis during seed exchanges to prevent the introduction of new *Ascochyta* species and the preliminary targeting of species for resistance breeding in areas where the three *Ascochyta* species are present.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.