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Double trouble: Co-infection of potato with the causal agents of late and early blight

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Abstract

Global potato production is plagued by multiple pathogens, amongst which are *Phytophthora infestans* and *Alternaria solani*, the causal agents of potato late blight and early blight, respectively. Both these pathogens have different lifestyles and are successful pathogens of potato, but despite observations of both pathogens infecting potato simultaneously in field conditions, the tripartite interactions between potato and these two pathogens are so far poorly understood. Here we studied the interaction of *A. solani* and *P. infestans* first in vitro and subsequently in planta both in laboratory and field settings. We found that *A. solani* can inhibit *P. infestans* in terms of growth in vitro and also infection of potato in both laboratory experiments and in an agriculturally relevant field setting. *A. solani* had a direct inhibitory effect on *P. infestans* in vitro and compounds secreted by *A. solani* had both an inhibitory and disruptive effect on sporangia and mycelium of *P. infestans* in vitro. In planta infection bioassays revealed that simultaneous co-inoculation of both pathogens resulted in larger necrotic lesions than single inoculations; however, consecutive inoculations only resulted in larger lesions when *A. solani* was inoculated after *P. infestans*. These results indicate that the order in which these pathogens attempt to colonize potato is important for the disease outcome and that the influence of plant pathogens on each other should be accounted for in the design of future disease control strategies in crops such as potato.

KEYWORDS
Alternaria solani, early blight, field, late blight, *Phytophthora infestans*, tripartite interactions

1 | INTRODUCTION

The simultaneous occurrence of multiple diseases in crops is an understudied area in plant pathology; however, studies of animal and human diseases have shown that the presence of multiple pathogens can impact virulence and the course of disease development. Furthermore, they also present an important driver of epidemiological dynamics. Worldwide, the production of potato, *Solanum tuberosum*, is affected by several plant pathogens causing diseases. The notorious late blight pathogen *Phytophthora infestans*, that infamously destroyed potato production in many countries in the 19th century and launched the study of phytopathology, is still the number one potato pathogen (Judelson & Blanco, 2005; Savary et al., 2019). Early blight, caused by the fungus *Alternaria solani*, has

the potential to reduce yields by half if left uncontrolled (Leiminger & Hausladen, 2012). Currently, both potato diseases are mainly controlled by repeated applications of synthetic fungicides. Yet fungicide resistance, new regulations concerning fungicide use and a changing climate are all factors that challenge current disease control strategies (Elad & Pertot, 2014; Odilbekov et al., 2019; Schepers et al., 2018).

Plant pathogens such as *P. infestans* and *A. solani* are devastating and notorious pathogens plagueing potato production individually (Savary et al., 2019); however, in an agricultural field multiple pathogens are present or can arrive at any time. Yet the effect and interactions of unrelated fungal and oomycete pathogen species infecting the same host plants is largely unknown and understudied. Nonetheless, the infection of multiple unrelated pathogen species has been shown to result in strong effects in animal and human pathology (Tollenaere et al., 2016). The fact that multiple pathogens can infect a host plant or host population has been identified as one of the 13 challenges in modelling plant diseases (Cunniffe et al., 2015). Additionally, it has been suggested that co-infected plants should be targeted in disease control programmes because co-infection can be an important driver of epidemiological dynamics (Susi et al., 2015).

A. solani is classified as a necrotrophic pathogen but, according to previous studies, forms appressoria (Dita et al., 2007). The formation of an appressorium to infect the plant without causing considerable damage to the plant tissue is a strategy commonly used by biotrophic and hemibiotrophic pathogens but also employed by necrotrophic pathogens (Mendgen & Hahn, 2002; Prins et al., 2000). A study using hyperspectral spectroscopy, collecting reflectance data between 400 and 2400 nm for the detection of both *P. infestans* and *A. solani* in potato leaves, was able to detect distinct changes in the leaf reflectance spectra for both *A. solani* and *P. infestans* before lesions appeared (Gold et al., 2020), indicating that *A. solani* and *P. infestans* trigger different responses in the plant during early infection before the development of characteristic lesions specific to early or late blight. *P. infestans* is a hemibiotrophic pathogen that in a susceptible interaction suppresses plant defences by the use of effector proteins, allowing biotrophic growth; however, at a later stage, the switch to necrotrophy is made and necrosis-inducing effectors, and other molecules, are excreted (Lee & Rose, 2010).

When multiple pathogens require similar resources from the plant, this could influence co-infection/cohabitation dynamics. For example, if one of the pathogens triggers a response in the plant, this could be beneficial or negative for infection by the other pathogen (Tollenaere et al., 2016). When *Arabidopsis thaliana* is inoculated with *Pseudomonas syringae* and subsequently challenged with *Alternaria brassicicola* on the same leaf, larger *A. brassicicola* lesions were observed than for the control. However, inoculation of *P. syringae* and *A. brassicicola* on different leaves did not lead to significant differences in lesion size (Spoel et al., 2007), even though competition between pathogens with a similar lifestyle might be expected. *P. infestans* is not usually able to infect *A. thaliana*, which is therefore classified as a nonhost for this pathogen. However, Belhaj et al. (2017) showed that when *A. thaliana* was first infected and colonized by the oomycete *Albugo laibachii*, this rendered the plants susceptible to subsequent *P. infestans* infection. Moreover, both pathogens were observed to form haustoria in the same cells, indicating that the formation of a feeding structure by one pathogen did not limit formation of a feeding structure by the second pathogen. Furthermore, the *A. laibachii*-infected *A. thaliana* plants were not found to be more susceptible to *Blumeria graminis* f. sp. *hordei* and *Phakopsora pachyrhizi*, two other pathogens that are not adapted to *A. thaliana*. The effect of multiple pathogens present in a host plant at the same time is thus dependent on the specific combination of pathogens. Studies on these compatible or incompatible tripartite or multiparty interactions between plants and pathogens help to further elucidate susceptibility and resistance mechanisms, offering important insights into how disease dynamics may play out in an agricultural field setting where multiple biotic or abiotic stresses may affect plants.

In our potato field trials, natural infection by *P. infestans* infection is normally observed earlier in the season than that of *A. solani* (data not shown). When *P. infestans* is already present in a field, this does not appear to hinder subsequent *A. solani* infection. However, in 2017 *A. solani* infection occurred early, and subsequently *P. infestans* infection appeared, with late blight severity up to 0.1% observed. Despite the presence of *P. infestans* inoculum in the field, a few days later late blight was no longer visible, yet the early blight severity reached above 20% (Figure S1). Based on this field observation, we hypothesized that *P. infestans* was prevented from infection either by a direct interaction with *A. solani*, by *A. solani*-derived compounds or by the plant response triggered by *A. solani* infection.

Therefore, in this study we aimed to elucidate the effects of these two pathogens on each other. We hypothesized that the presence of *P. infestans* may benefit *A. solani* and thus promote more severe disease in potato, but that the presence of *A. solani* may inhibit *P. infestans*. Furthermore, the timing and order of the arrival of each of these pathogens may impact the outcome of the disease. We therefore investigated the effect of sequential and simultaneous inoculation of *P. infestans* and *A. solani* in vitro and in planta in both laboratory and field settings. To observe infections of both pathogens in planta at the microscopic level, we transformed *A. solani* with the green fluorescent protein (GFP) reporter, and co-inoculated potato with the *P. infestans* 88069 Td-tomato strain (McLellan et al., 2013) that expresses a red fluorescent protein (RFP) reporter, to distinguish each pathogen using confocal laser scanning microscopy.

2 | MATERIALS AND METHODS

2.1 | *P. infestans* maintenance and inoculum preparation

P. infestans strains 88069 (Pieterse et al., 1991) and pink6 (Cooke et al., 2012) were maintained on rye sucrose agar (RSA). *P. infestans* 88069 Td-tomato (expressing RFP; McLellan et al., 2013) was maintained on RSA supplemented with 5 μg/mL geneticin (G418). Sporangium inoculum was prepared by flooding

14-day-old cultures with 10 mL sterile tap water and gently rubbing the surface with a plastic L-shaped spatula and filtering the liquid through a 40 µm cell strainer to remove potential hyphae. The concentration of sporangia was determined using a Fuchs Rosenthal haemocytometer. Zoospore inoculum was prepared by flooding 14-day-old cultures with 10 mL sterile 4°C tap water and subsequently leaving them at 4°C for 2–4 h to induce zoospore release. The liquid was strained through 40-µm cell strainers, and the concentration of zoospores was determined using a Fuchs Rosenthal haemocytometer.

2.2 | *A. solani* maintenance and inoculum preparation

A. solani strains AS112 (Odilbekov et al., 2014) and NL03003 (CBS 143772; Iftikhar et al., 2017) were maintained on potato dextrose agar (PDA) and V8 juice solid medium, respectively. The plates were kept in the dark at room temperature (22°C) for 4 days and subsequently placed in an 18°C incubator equipped with UV-C light bulbs (model OSRAM HNS15G13 with dominant wavelength 254 nm) supplying 8 h of UV-C light per day for 10 days to induce sporulation. *A. solani* pCT74 GFP #2 was maintained on 20% PDA supplemented with 34 µg/mL hygromycin B and kept in the dark at room temperature for 7 days. Multiple small agar plugs were subsequently transferred to sporulation medium (30 g $CaCO_3$, 20 g Bacto agar, 1 L Milli-Q water, pH 7.4; Shahin & Shepard, 1979) and covered with 2 mL sterile Milli-Q water. The plates were incubated in the dark for 5 days at 18°C. Conidial inoculum was prepared by flooding the plates with 10 mL sterile tap water and gently rubbing of the growth using plastic L-shaped spatulas. The concentration of conidia was determined using a Fuchs Rosenthal haemocytometer.

2.3 | Transformation of *A. solani*

To allow us to follow the interactions of *A. solani* and *P. infestans* at the microscopic level within infected leaf tissue using fluorescent microscopy, we transformed *A. solani* with constitutively expressed GFP protein. Small agar plugs containing 7-day-old *A. solani* NL03003 mycelium were cut from a PDA plate and inoculated in 15 mL PDB medium. The liquid culture was grown for 24 h on a rotary shaker (170 rpm) at 28°C and mycelium was collected from 2 mL of culture through centrifugation at 6000 *g* for 10 min. The mycelium was washed twice with 0.7 M NaCl and collected again through centrifugation at 6000 *g* for 10 min. One millilitre of kitalase solution (10 mg/mL in 0.7 M NaCl) was added to digest the mycelium for 2 h at 28°C, while shaking at 170 rpm. The resulting protoplasts were filtered through two layers of Miracloth and collected through centrifugation for 10 min at 700 *g* at a temperature of 4°C. The protoplasts were washed in cold STC buffer (1 M sorbitol, 50 mM Tris–HCl, pH 8.0 and 50 mM $CaCl_2$) and finally aliquoted in STC buffer to obtain 5×10^6 protoplasts in 70 µL buffer. Five micrograms of pCT74 plasmid (10 µL; Nova Lifetech Ltd; Lorang et al., 2001) was added to the protoplasts, gently mixed and the mix was placed on ice for 10 min. The sample was incubated for 5 min at 42°C and then placed back on ice for 10 min. A total of 1.5 mL of 40% polyethylene glycol (PEG) 4000 (in STC buffer and at room temperature, 22°C) was added and 400 µL of the transformed protoplasts were added to 50 mL molten (40–45°C) regeneration medium (5 g yeast extract, 5 g casamino acid, 1 M sucrose, 0.8% agar). The regeneration medium was then divided over two deep Petri dishes and incubated at room temperature (22°C) overnight. The next day, the plates were overlaid with 25 mL of PDA containing 34 µg/mL hygromycin B. Emerging colonies were transferred to fresh PDA plates containing 34 µg/mL hygromycin B. In total, more than 15 transformants were obtained, of which the most fluorescent ones were maintained.

2.4 | Plant material

Solanum tuberosum 'Désirée' was maintained in vitro in MS20 medium. Two-week-old in vitro plantlets were planted in 2 L commercial soil (Exclusiv Blom & Plantjord; Emmaljunga Torvmull AB) supplemented with 15 mL of Osmocote Exact 3–4 months fertilizer beads (containing nitrogen-phosphorus-potassium 16-9-12 + 2MgO + trace elements). The plants were maintained in an artificial light plant chamber at 20°C and 65% RH, receiving 14 h of 160 µmol/s/m^2 light.

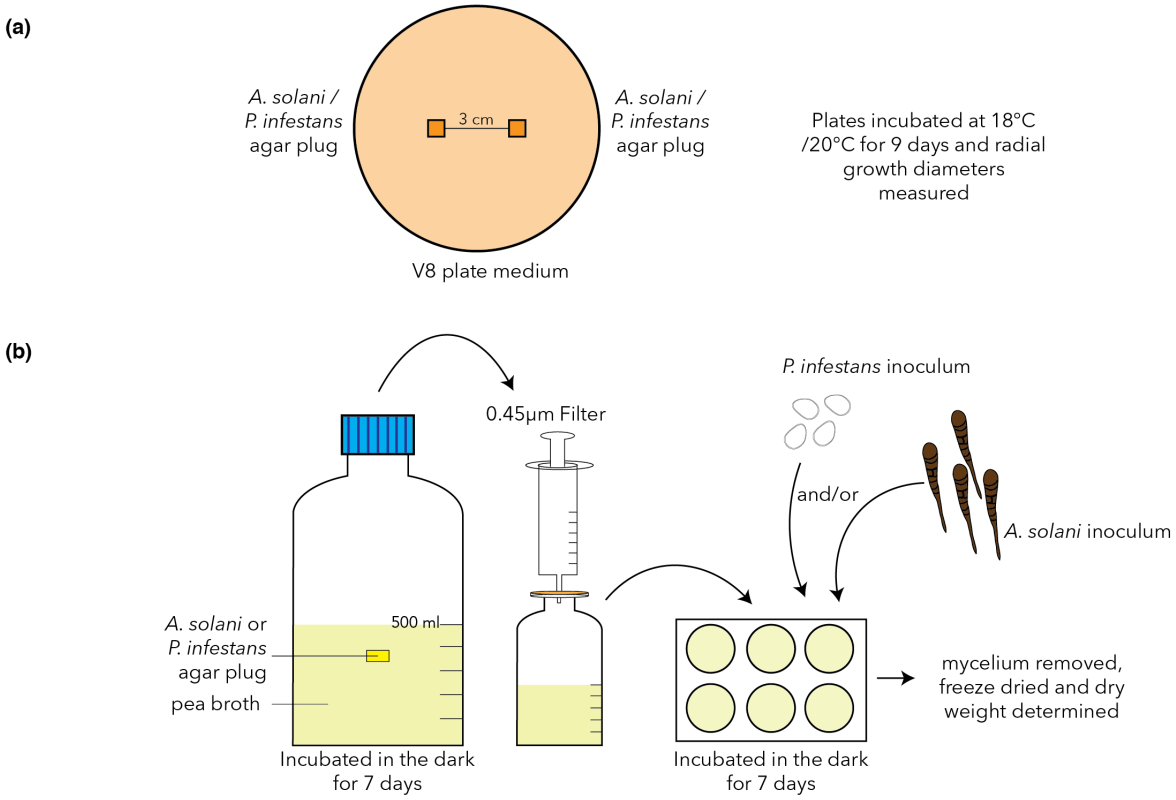
2.5 | Co-inoculation on solid medium

Two 5 × 5 mm agar plugs of 14-day-old sporulating cultures of *P. infestans* 88069 and/or *A. solani* NL03003 were inoculated onto V8 juice solid medium 3 cm apart. The prepared combinations were as follows: two *P. infestans* agar plugs, two *A. solani* agar plugs or one *P. infestans* and one *A. solani* plug plated together. Additionally, plates containing only one agar plug were prepared for *A. solani* and *P. infestans*. Two sets of five plates per combination were prepared. One set was incubated at 20°C and one set at 18°C for 9 days (Figure 1a). The average radial growth diameter was determined every 3 days. The experiment was repeated three times. Statistical analysis comparing the radial growth was performed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) pairwise comparison with 95% confidence in Minitab v. 18 statistical software package (www.minitab.com). ANOVA was performed with the treatments *A. solani*, *P. infestans* and *A. solani* + *P. infestans*. Temperature and experimental replicate were included as factors in the analysis.

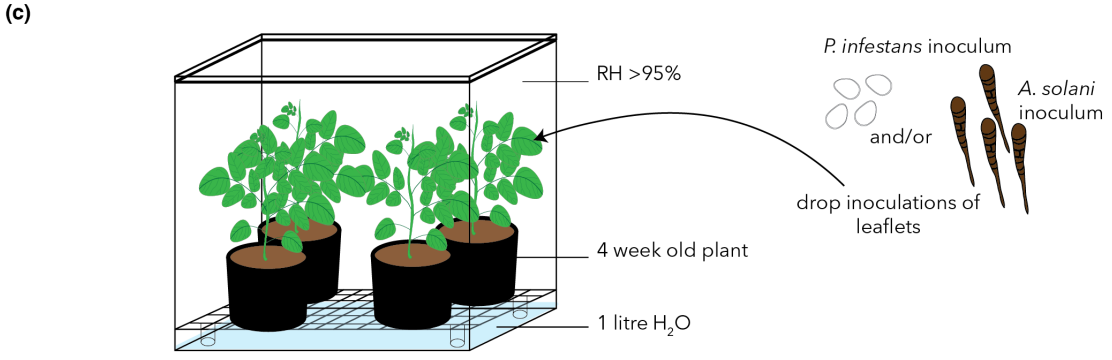
2.6 | Co-inoculation in liquid medium

Two 10 × 15 mm agar plugs of sporulating cultures of *P. infestans* 88069, *P. infestans* Pink6, *A. solani* NL03003 or *A. solani* NL03003 were added to 500 mL pea broth in 1 L Duran bottles and incubated

in vitro



in planta



in agro

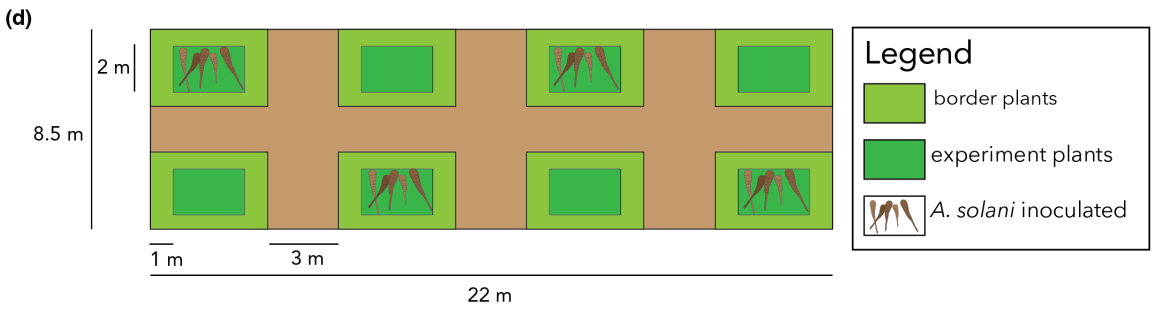


FIGURE 1 Schematic representation of the experimental set up and the field trial design used in this study. In vitro studies were performed on solid media (a) and in liquid cultures (b). Studies in planta were performed by inoculating whole plants contained in humidity boxes grown under controlled conditions in a growth chamber (c) and the field experiment *in agro* was performed according to the experimental plan depicted in (d).

in the dark at room temperature (22°C) for 7 days. Pea broth was used to ensure that non-sporulating mycelia were produced, and additionally multiple types of media were tested to ensure that the results of the experiments were the result of pathogen interactions with each other and not artefacts of in vitro preparation in different media. The *A. solani* liquid cultures were incubated on a shaking incubator at 50 rpm, whereas *P. infestans* cultures were incubated without shaking. After 1 week of growth the hyphae were removed, and the broth was filter-sterilized using 0.45 µm filters. An aliquot of 4 mL filtered medium was added to 6-well plates to which *P. infestans* 88069 inoculum (1 mL containing 10,000 sporangia) was subsequently added. A further 5 mL of the filtered medium was added to 6-well plates to which *A. solani* AS 112 inoculum (10 µL containing 100 conidia) was subsequently added. Filtered medium without previous pathogen growth was used as a control. Dual inoculation plates containing both pathogens were also prepared as described above. The cultures were incubated at room temperature in the dark for 7 days (Figure 1b). After 7 days, the mycelium was removed from the broth using an inoculation loop spatula and placed in preweighed 1.5 mL microcentrifuge tubes. The tubes were placed at −80°C overnight and subsequently lyophilized for 4 days. The dry weight was determined and statistical analysis comparing the dry weight was performed using one-way ANOVA followed by post hoc Tukey test. Separate ANOVAs were performed for the *P. infestans* dry weight and the *A. solani* dry weight. Statistical analysis comparing the *A. solani* dry weight and *P. infestans* plus *A. solani* dry weight, or the *P. infestans* dry weight and the *P. infestans* plus *A. solani* dry weight, were determined using two-sample *t* tests. All statistical analyses were performed in Minitab v. 18. The experiment was repeated three times.

2.7 | Whole plant infections and lesion scoring

Four-week-old potato plants were placed in custom-made acrylic boxes inside versatile environment incubators as described by Brouwer et al. (2020) (Figure 1c). From three fully expanded leaves in the middle of the plant canopy, top leaflets were drop-inoculated with two 10 µL droplets per plant, on either side of the mid vein. Conidial spore suspension (25,000/mL) of *A. solani* NL03003, 50,000 *P. infestans* 88069 zoospores/mL or inoculum containing both was used. We determined the optimal level of inoculum for each pathogen to try to maximize the chances of infection from each, rather than choosing a standard number to apply for both pathogens. Because *P. infestans* inoculum was from motile spores that require substantial energy to locate an infection site and to build a cell wall prior to germination (Judelson & Blanco, 2005), we settled on 50,000 zoospores/mL, which has been optimized in our previous experiments (Resjö et al., 2017). Inoculum of *A. solani* in the form of conidia was optimized by Brouwer et al. (2020). For the sequentially double-inoculated plants, inoculation droplets containing one of the pathogens were placed on the leaflets and 24 h later inoculation droplets containing the second pathogen were placed on the same spot. Per treatment, 24 leaflets were inoculated with two droplets, resulting in 48 lesion measurements per treatment. The lesion diameter for all plants was determined 5 days after the first inoculation. Statistical analysis comparing the lesion diameters was performed using one-way ANOVA followed by post hoc Tukey test in Minitab v. 18. The experiment was repeated three times.

2.8 | Confocal laser scanning microscopy of pathogen infection

Small leaf punches containing the inoculation spot were cut and mounted on microscopy slides. When the inoculation droplet was still present, no additional water was added; however, at the later time points when the inoculation droplet had dried out, sterile tap water was added on top of the leaf disc. All samples were analysed using an LSM 880 (Zeiss) and excited with a 488 nm argon laser for GFP and a 561 nm DPSS laser for td-tomato RFP, collecting emissions between 493 and 556 nm for GFP, 566 and 637 nm for td-tomato RFP, as well as transmission data. All images were collected as 1024 × 1024 pixel frames and were processed in ZEN 3.1(blue edition) software (Zeiss) and ImageJ v. 1.50i (Schneider et al., 2012). The number of fluorescent pixels for GFP and RFP was determined in ImageJ v. 1.50i by loading the raw data CLSM files, splitting the channels, removing salt and pepper type background noise, binarizing the image and using the histogram list function to obtain the number of pixels containing fluorescence.

2.9 | Inoculated field experiment

A field trial was carried out in 2020 at Helgegården (coordinates 56.02°N, 14.05°E), about 5 km west of Kristianstad, Sweden. The trial was planted and managed by the Swedish Rural Economy and Agricultural Societies (Hushållningssällskapet). The size of the trial was 8.5 × 22 m and it contained eight smaller plots separated by 3 m soil borders (Figure 1d). The field was fertilized with nitrogen (N), phosphorus (P) and potassium (K) according to common practice for potato: 200, 80 and 215 kg/ha for N, P and K, respectively. *S. tuberosum* 'Kuras' was planted in mid-June. Each plot contained five rows, of which the middle three were used in the experiment and the rows either side were considered to be border plants and not scored for disease symptoms. The trial had four replicate blocks. In each block

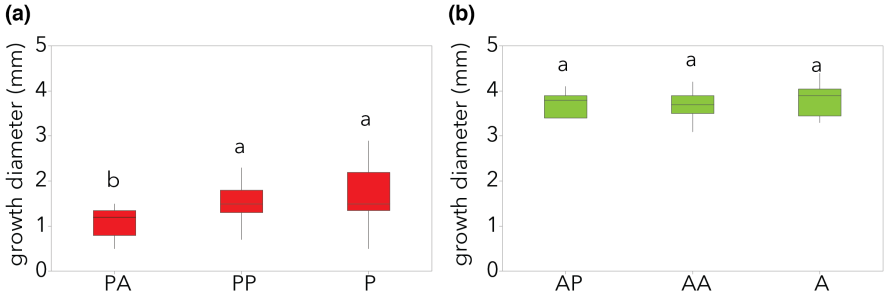


FIGURE 2 Radial growth after 9 days on V8 solid medium at 20°C of (a) *Phytophthora infestans* 88069 (P) and (b) *Alternaria solani* NL03003 (A) either plated with each other (PA and AP), plated with themselves (PP and AA) or alone (P and A). $N = 13$ and the letter labels correspond to the Fisher LSD grouping determined by one-way analysis of variance (ANOVA) followed by Fisher LSD pairwise comparison with 95% confidence. Two separate ANOVAs were performed, one for the *P. infestans* growth and one for the *A. solani* growth.

one of the two plots was inoculated with *A. solani* AS112-infected barley kernels (Euroblight protocol described by Metz et al., (2019)) (Figure 1d). Two applications of 30 g of infected kernels were spread in each plot. The first inoculation occurred on 15 July and the second on 5 August. To ensure optimal infection by *A. solani* before the occurrence of late blight, and because late blight will occur in almost every potato field in Sweden at the end of June/beginning of July, all plants were treated weekly with late blight fungicides (Revus, a.i. mandipropamid and Ranman Top, a.i. cyazofamid, alternated). These fungicides have no or negligible effect on early blight. The protection lasts a maximum of 10 days on leaves that are hit, according to common experience. In between the fungicide treatments, the potato plants had a period of active growth of 10–20 cm in height. This new growth is not protected against infection as the fungicides used are contact fungicides that are not systemically spread in the plant. The last late blight fungicide treatment was carried out on 10 August. Eleven days later, all plots were inoculated with *P. infestans* from naturally occurring infection from our other field trials in Mosslunda (coordinates 55.98°N, 14.11°E), 5 km south of Kristianstad, Sweden. *P. infestans* inoculation was achieved by brushing infected material over the plants in each plot and leaving the infected material in between the border plants. This inoculation resulted in good late blight infection after inoculation; we thus concluded that the contact fungicide treatments did not hamper late blight infection. The disease symptoms for both early blight and late blight were repeatedly scored for 5 weeks by visual inspection of lesion morphologies. Field plots adjacent to the trial site were planted but not used in the experiment. Planting consisted of four replicates lying parallel to and about 20 m from our experiment, were not inoculated with either *A. solani* or *P. infestans* but were initially treated against late blight in the same way. These plots were also scored for disease and may be considered as noninoculated controls. The disease severity, expressed as the percentage of infection per plot, was determined using the methods described by Duarte et al. (2013) and Liljeroth et al. (2016) for early blight and late blight, respectively. The relative area under the disease progress curve (rAUDPC) during the assessment period, as described by Odilbekov et al. (2020), was calculated for both late blight and early blight. The relation between late and early blight disease severity was investigated using regression analysis with Pearson correlation coefficient in Minitab v. 18.

3 | RESULTS

In this study, we aimed to investigate whether *A. solani* and *P. infestans* impact each other when grown together in vitro, in planta or in the field. Based on our previous field observations, we hypothesized that the presence of *A. solani* may hinder subsequent establishment of *P. infestans* in potato. Given the different mechanisms of pathogenicity employed by these two potato pathogens, we also hypothesized that the presence of *P. infestans* may aid subsequent establishment of *A. solani*. We tested the growth and pathogenicity of these two pathogens when co-inoculated together in vitro and when co-inoculated or sequentially inoculated in planta under controlled and field conditions.

3.1 | Radial growth experiments

In order to study whether *A. solani* and *P. infestans* growth on agar is affected by the presence of the other pathogen, a growth experiment of both pathogens cultured on the same medium plate either alone, with a second plug of the same pathogen or with the other pathogen was performed. The radial growth of *A. solani* was not influenced by the presence of *A. solani* or *P. infestans* (Figure 2). However, the radial growth of *P. infestans* was significantly negatively affected by the presence of *A. solani* on the same plate. This result was obtained both when the plates were incubated at 20°C (Figure 2) and 18°C (Figure S2).

3.2 | Pathogen growth in liquid medium previously harbouring *A. solani* or *P. infestans*

Because the growth of *P. infestans* was negatively affected by *A. solani* on solid medium, the influence of both pathogens on each

other's growth was further analysed in a liquid medium experiment. Both pathogens were grown in filtered control medium that previously contained *A. solani* or *P. infestans* cultures, respectively. Additionally, both pathogens were also added together to control liquid medium (Figure 3). When *P. infestans* was grown in medium that previously contained *A. solani*, the dry weight of hyphae was significantly lower than when *P. infestans* was grown in control medium or medium that previously contained *P. infestans* either of the same strain or another strain (Figure 3a,d). *A. solani* growth was also reduced compared to the control when grown in medium that previously contained *P. infestans* or *A. solani* of another strain and even more when grown in medium that previously harboured of *A. solani* of the same strain (Figure 3b). However, these reductions were much smaller than the reduction observed for *P. infestans* grown in medium that previously harboured *A. solani*. When the pathogens were added together in control medium, growth visually looked like *A. solani* growth (Figure S3). However, the resulting dry weight was significantly higher than the growth of *A. solani* alone in control medium ($p = 0.026$; Figure 3c).

3.3 | *P. infestans* sporangial and hyphal tip leakage in liquid that previously harboured *A. solani*

In order to determine whether the decrease in growth of *P. infestans* in liquid medium that previously harboured *A. solani* was caused by delayed germination or something else, the growth progression was visualized using inverted bright field microscopy (Figure 4). During the first 6 h of inoculation the sporangia started to germinate; however, a significant proportion of the sporangia displayed bursting on the side and subsequent leakage of sporangial cytoplasm (Figure 4a). Additionally, for the sporangia that germinated, swelling and subsequent leakage of cytoplasm from the hyphal tip was also observed (Figure 4b). Eight days after inoculation, some *P. infestans* growth occurred, but many of the sporangia displayed the burst phenotype and never germinated. Additionally, the hyphal growth that was established sometimes displayed hyphal tip bursting. The bursting of sporangia and hyphal tips was not observed for *P. infestans* grown in control medium or medium that previously harboured *P. infestans* growth.

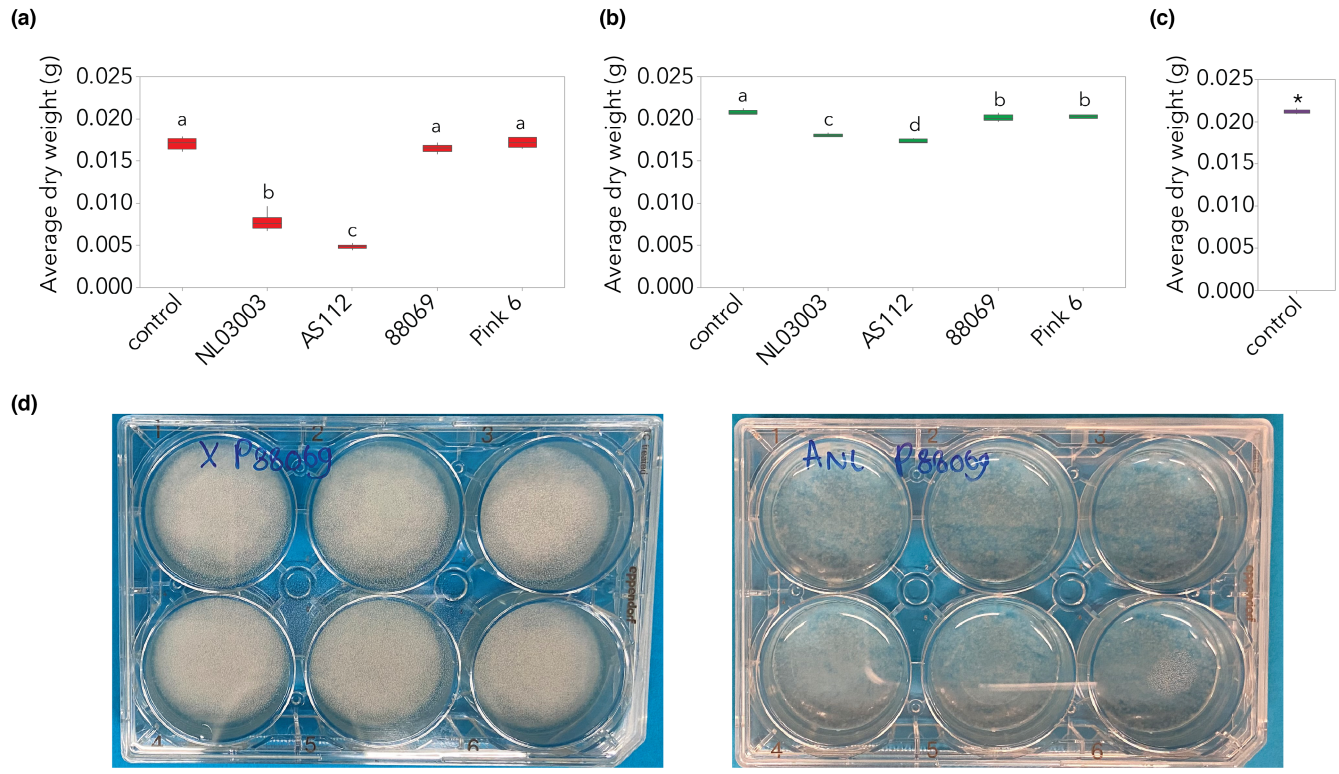


FIGURE 3 Average dry weight of hyphae produced in different liquid medium after 7 days. (a) Dry weight of hyphae of *Phytophthora infestans* 88069 grown in control medium or medium that previously contained *Alternaria solani* NL03003, *A. solani* AS112, *P. infestans* 88069 or *P. infestans* Pink 6. (b) Dry weight of hyphae of *A. solani* AS112 grown in control or medium that previously contained *A. solani* NL03003, *A. solani* AS112, *P. infestans* 88069 or *P. infestans* Pink 6. (c) Dry weight of hyphae of *P. infestans* 88069 and *A. solani* AS112 co-inoculated in control medium. All media were filtered through 0.45 μm filters before addition of either *P. infestans* 88069 or *A. solani* AS112 spores. $N = 6$ and the letters represent the grouping based on one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Two separate ANOVAs were performed, one for the *P. infestans* dry weight and one for the *A. solani* dry weight. Asterisk indicates the significant difference between growth in graph C and the growth of *A. solani* in control medium in graph B. (d) Photographs of the growth of *P. infestans* 88069 in control medium (left) and medium previously harbouring *A. solani* NL03003 (right).

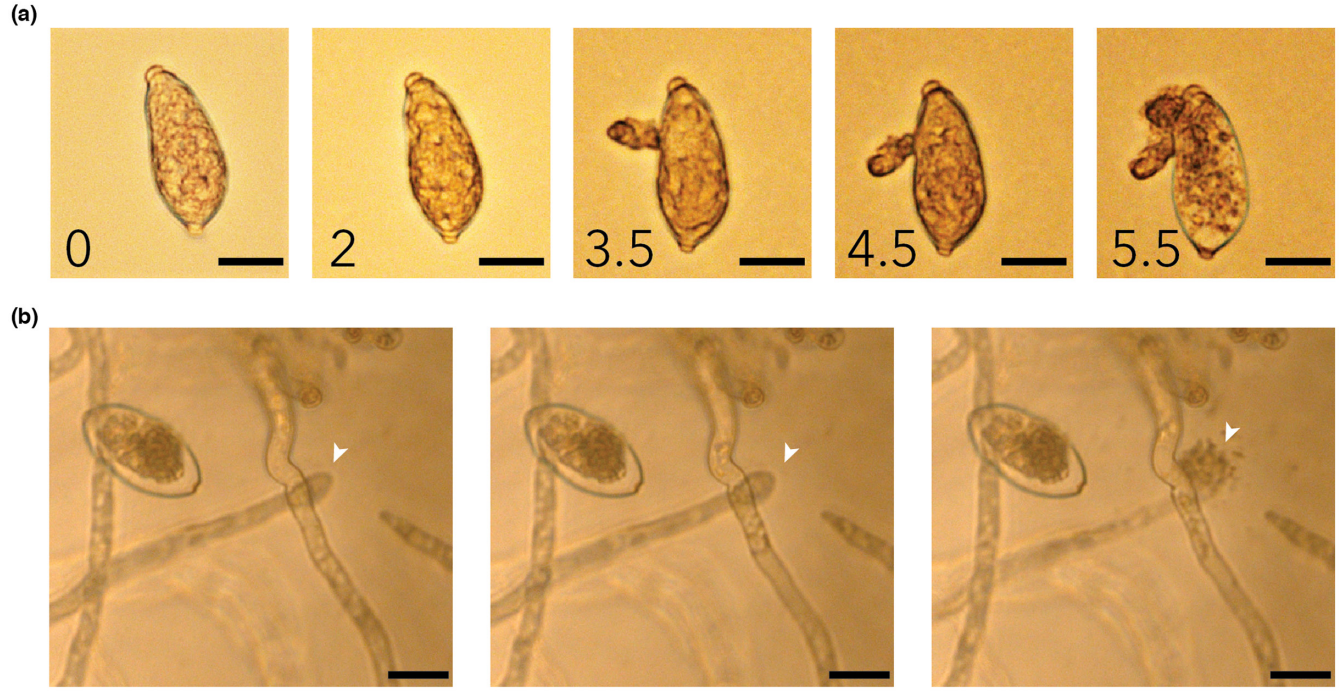


FIGURE 4 Micrographs of growth of *Phytophthora infestans* 88069 in liquid medium that previously contained *Alternaria solani* NL03003. The black scale bar for all images corresponds to 20 μm. (a) Sporangium at 0, 2, 3.5, 4.5 and 5.5 h postinoculation in medium. (b) Bursting of hyphal tips (indicated with arrow) observed 4 days postinoculation. The displayed images were collected with 30 min between each image.

3.4 | Necrotic lesion size in simultaneous and sequential co-infections in planta

With the aim of studying the effect of co-infection of potato leaflets with both pathogens, a growth chamber bioassay with simultaneous and sequential inoculation was performed. The lesion size was determined 5 days postinoculation (dpi) for leaflets inoculated with either *P. infestans*, *A. solani*, both *P. infestans* and *A. solani* simultaneously, *P. infestans* followed by *A. solani* 24 h later, or *A. solani* followed by *P. infestans* 24 h later (Figure 5). The simultaneous inoculation of both pathogens in the same spot resulted in the largest lesions that were necrotic and resembled *A. solani* lesions (Figure 5b). With sequential inoculations, the addition of *P. infestans* did not result in a difference in lesion size compared to *A. solani* inoculation only (Figure 5b). However, the addition of *A. solani* 24 h after *P. infestans* resulted in a significant increase in lesion size compared to *P. infestans* only (Figure 5b).

3.5 | Microscopy of simultaneous and sequential inoculation with *A. solani* and *P. infestans*

Using the same inoculation conditions for which the lesion size was determined, the infection process of drop inoculations on whole plants on the adaxial side of leaves was followed and imaged. Using confocal laser scanning microscopy, colonization of the mesophyll and emergence through the stomata at the abaxial surface was determined. In the single inoculated conditions, germ tube formation was observed at 1 h postinoculation (hpi) and colonization between the mesophyll cells by *P. infestans* was observed at 24 hpi and continued further (Figure 6a,b). Germinating *A. solani* conidia resulted in extensive growth in both directions of the conidia, both towards and away from the leaf surface (Figure 6c). The extensive growth of *A. solani* on top of the epidermal cells in the simultaneous co-inoculated conditions, to a large extent obstructed scanning of tissue layers underneath the network of hyphae. However, imaging at 48 hpi of the abaxial side of the leaves revealed colonization of the mesophyll cell layers by *A. solani* (Figure 6d).

In the simultaneously co-inoculated samples, even though germination of *P. infestans* cysts and/or sporangia was initially observed, colonization of the potato leaf was rarely observed (Figure 7a). When sequential inoculation of *A. solani* and *P. infestans* was performed with 24 h in between the inoculations, established *P. infestans* infection did not inhibit *A. solani* growth. After 48 h, colonization of both *P. infestans* and *A. solani* was observed on the abaxial side of the leaves (Figure 7b). Contrastingly, when *A. solani* inoculation was followed with *P. infestans* inoculation 24 h later, only *A. solani* colonization of the leaf was observed (Figure 7c). However, autofluorescence of necrotic cells, especially epidermal cells, was visible in the RFP channel (Figure 7c, Figure S4).

3.6 | Inoculation under field conditions

To determine whether the observed results from the growth chamber bioassays also apply in the field (i.e., under agriculturally

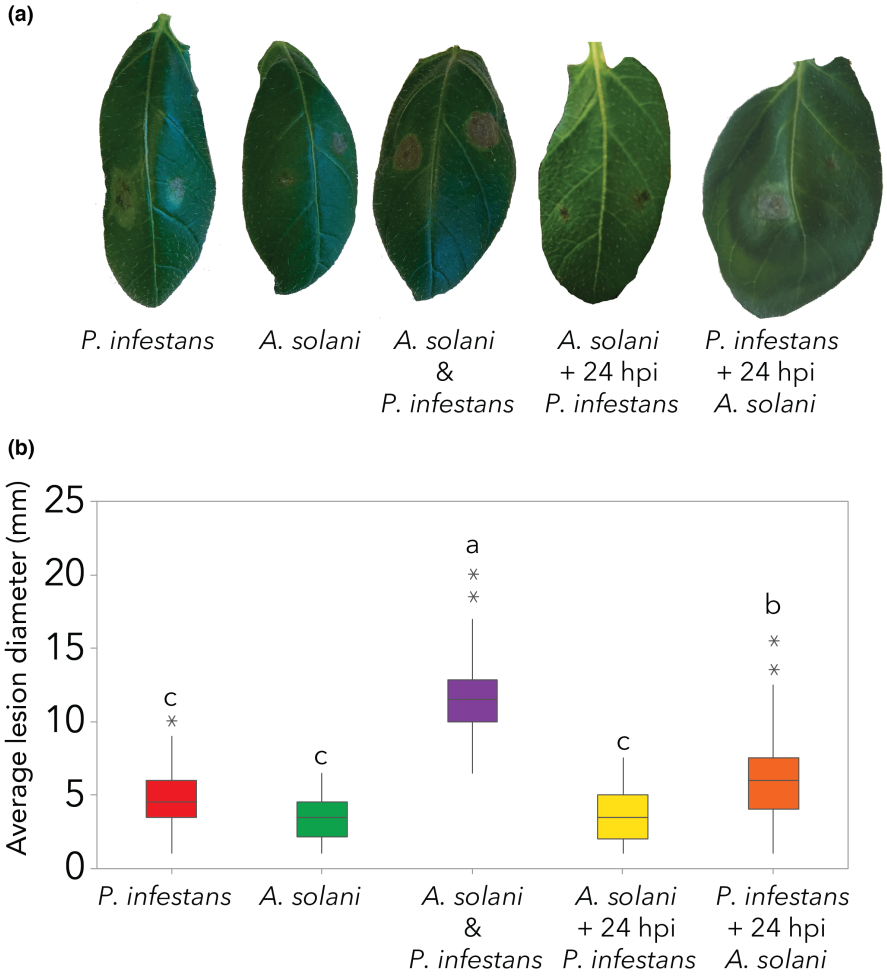


FIGURE 5 Co- and sequential inoculations of potato leaflets with *Phytophthora infestans* and *Alternaria solani*. (A) Representative photographs. (B) Average lesion diameter at 5 days postinoculation for plants inoculated with *P. infestans*, *A. solani*, *P. infestans* + *A. solani*, *A. solani* followed by *P. infestans* 24 h later, and *P. infestans* followed by *A. solani* 24 h later. $N = 48$ and the letters represent the grouping based on one-way analysis of variance followed by Tukey post hoc test.

relevant conditions), an inoculated field experiment was performed (Figure 8). Potato cv. Kuras was planted in mid-June and plants in half of the test plots were inoculated with *A. solani* AS112 in mid-July and early August, while also being treated with contact fungicides to prevent natural *P. infestans* infection. When clear early blight symptoms appeared in the *A. solani*-inoculated blocks, all the blocks were inoculated with *P. infestans*. The inoculation with *P. infestans* occurred 11 days after the last contact fungicide treatment. The first early blight symptoms appeared in the *A. solani*-inoculated plots in the beginning of August and then the infection increased gradually with time (Figure 8b). Later *A. solani* also spread, in the direction of the prevailing wind, to the noninoculated control plots. However, the rate of early blight infection was lower in all the noninoculated plots. In one of the noninoculated plots, away from the prevailing wind, the *A. solani* infection rate stayed much lower than in the other plots, indicating that the spread of *A. solani* from our inoculated plots to those plots was much lower or negligible. On 24 August, the *A. solani* disease scores in inoculated plots varied between 5% and 8% infection per plot, while in the noninoculated control plots the *A. solani* disease scores ranged from 0.1% to 1.5% (Figure 8b). After inoculation with *P. infestans* the disease symptoms of both diseases (Figure 8a) were scored over 5 weeks, to determine the influence of *A. solani* presence in the field on the occurrence of *P. infestans*-induced late blight. In general, the late blight disease development rate was slower in plots inoculated with *A. solani*. The presence of *A. solani* was shown to limit the development of late blight disease over the whole assessment period, because the rAUDPC of late blight was negatively correlated with the rAUDPC of early blight ($R^2 = 0.7865$, $p = 0.003$; Figure 8d). Additionally, the percentage of late blight disease present at the end of the assessment period was highly significantly negatively correlated ($R^2 = 0.9199$, $p = 0.00017$) with the percentage of early blight present (Figure 8e). Precipitation and temperature were, on average, similar during our field trial to the values for 2008–2021, although there was slightly lower precipitation in 2020 compared with the other years, particularly at the start of the season.

4 | DISCUSSION

In an agricultural setting, plants and plant pathogen interactions do not occur as simple binary interactions. An agricultural field contains a plethora of microbial species, both beneficial and pathogenic. However, many plant-pathogenic species and their host plant interactions are studied as binary interactions. In this study, we investigated the interactions of two common pathogens of potato from

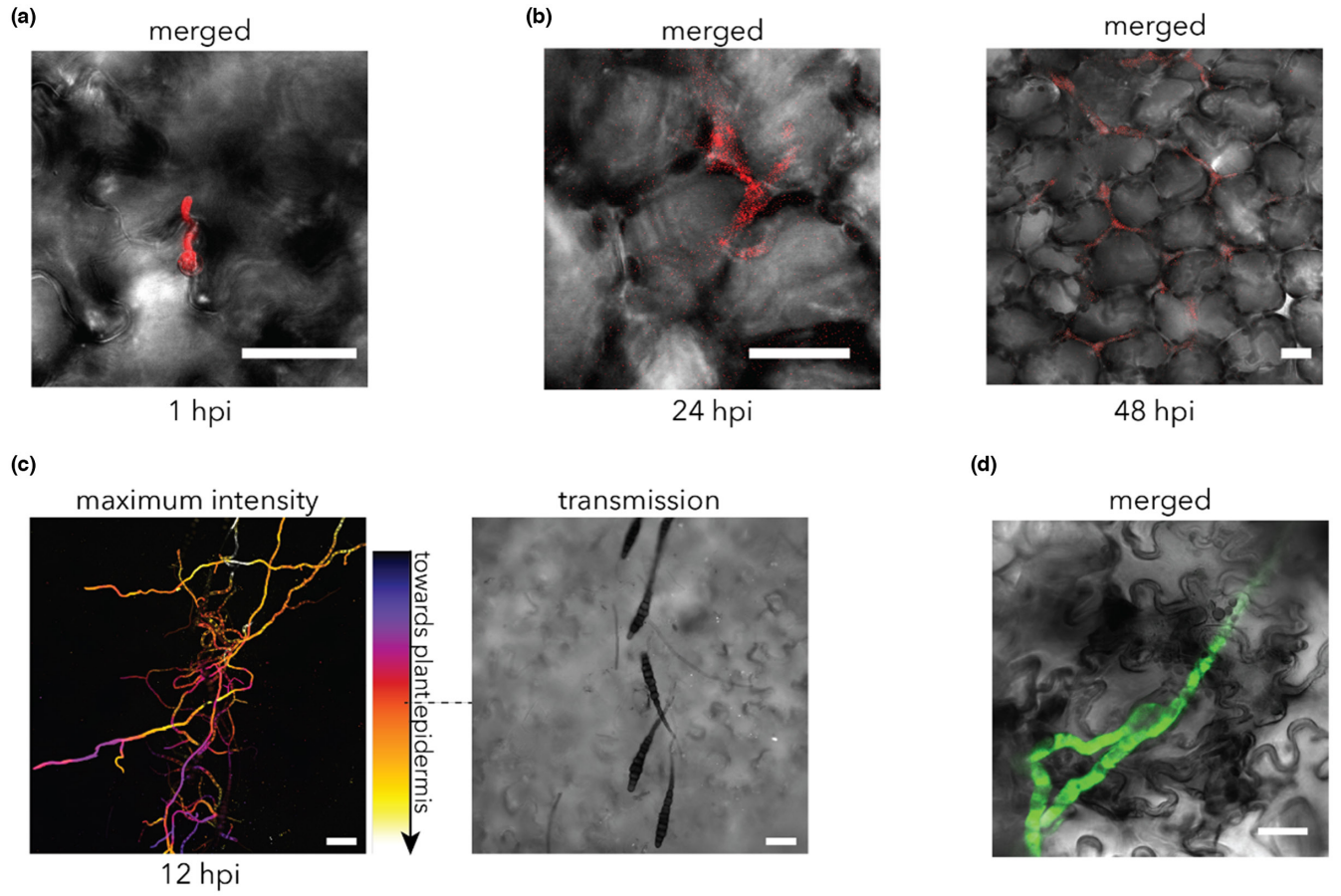


FIGURE 6 Representative confocal laser scanning micrographs obtained of single infection of potato leaves inoculated with *Alternaria solani* or *Phytophthora infestans* on the adaxial side of the leaf. (a) Germinated *P. infestans* zoospore 1 h postinoculation (hpi). Scale bar corresponds to 50 μm (b) *P. infestans* colonization within the mesophyll cell layer observed at 24 hpi and 48 hpi. Merged red fluorescent protein (RFP) and transmission micrographs of *P. infestans* td-tomato. Scale bars correspond to 20 μm. (c) Depth colour-coded maximum intensity projection micrograph of germinating *A. solani* conidia, displaying growth both in the direction towards (white) and away (purple) from the plant cells at 12 hpi in a leaf inoculated with *A. solani*. Transmission micrograph displayed from the middle of the *z*-stack shows the orientation of the *A. solani* conidia. Scale bar corresponds to 50 μm. (d) Merged green fluorescent protein (GFP) and transmission micrographs of *A. solani* GFP growth in green and the transmission channel, displaying emergence of *A. solani* from the plant mesophyll on the abaxial side of an *A. solani*-inoculated leaf at 48 hpi. Scale bar corresponds to 20 μm.

in vitro interactions to interactions in the field. We discovered that the late blight pathogen *P. infestans* is limited in growth in the presence of the early blight pathogen *A. solani* both in vitro and in planta depending on which pathogen is present first. In the field, the establishment of *A. solani* infections limits the potential for *P. infestans* to establish subsequent infections.

The in vitro growth rate of *P. infestans* on agar plate medium was reduced when it was plated together with *A. solani*. Because this *A. solani* isolate had a faster overall growth rate, it was difficult to determine whether the reduction in growth of *P. infestans* was due to a space limitation on the plate medium or other competing factors, such as the production of antagonistic metabolites by *A. solani*. To understand this relationship further, we grew each pathogen in a liquid culture system, using media that previously contained the other pathogen. Thus, we were able to measure the growth of each pathogen individually. When growing *P. infestans* in medium that previously contained *A. solani* we observed cytoplasmic leakage from both sporangia and hyphae. *Alternaria* species are known to produce secondary metabolites toxic to both plants, animals and humans (Meena & Samal, 2019). Several host-specific toxins have been characterized and described for *Alternaria* species causing plant diseases, and although the site of action differs for these toxins, their aim is to trigger cell death in the host (Meena & Samal, 2019). Yet there is limited knowledge on the specific toxins produced by *A. solani* and the toxin targets. In addition to host cell death, mycotoxins of phytopathogenic fungi are reportedly able to target other phytopathogenic species (Venkatesh & Keller, 2019), for example the maize pathogens *Ustilago maydis* and *Fusarium verticilliodes*. Jonkers et al. (2012) showed that *F. verticilliodes* secretes toxins and increases expression of cell wall-degrading enzymes when grown together in liquid with *U. maydis*. After initial accumulation of biomass for both species when grown

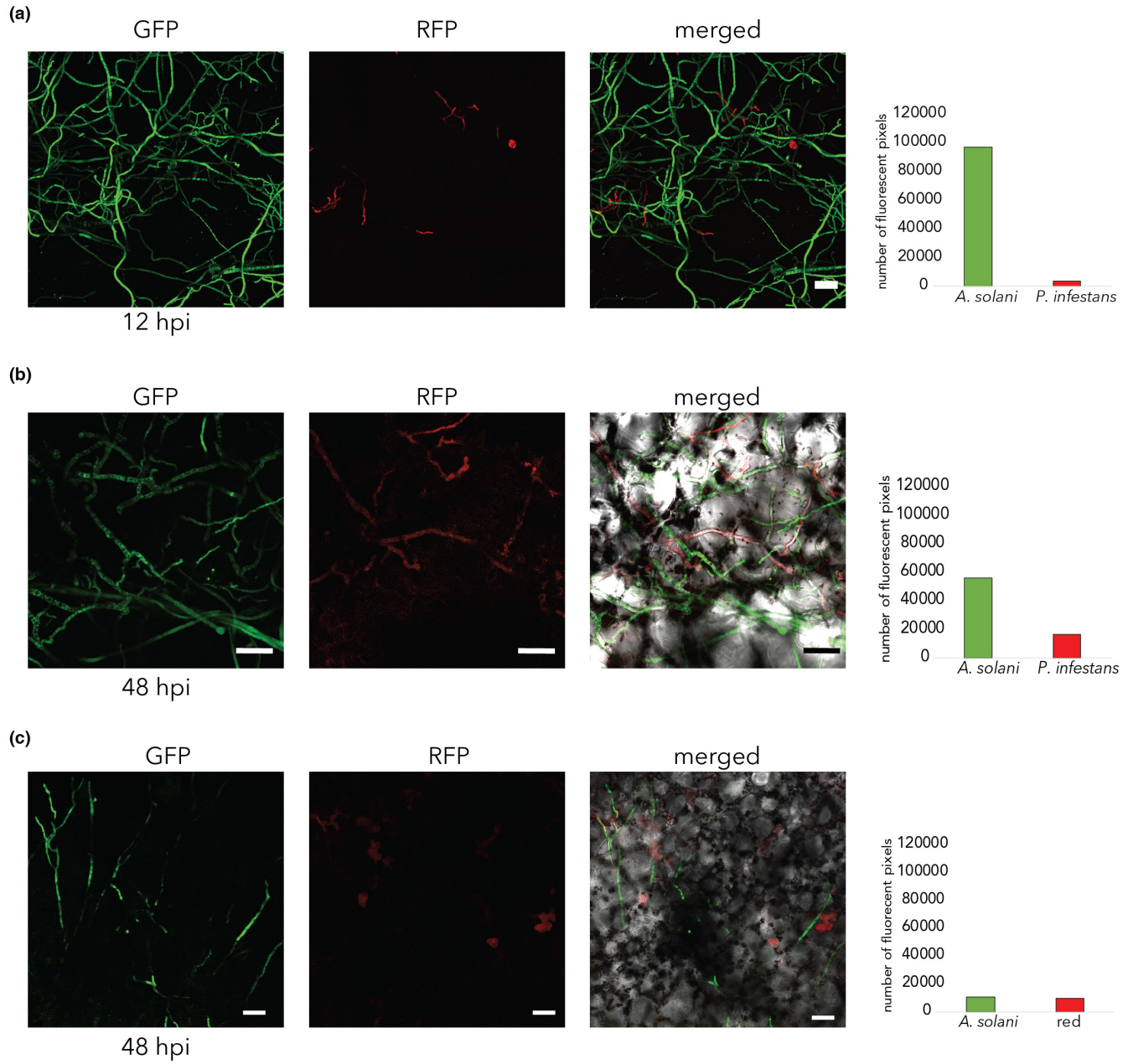
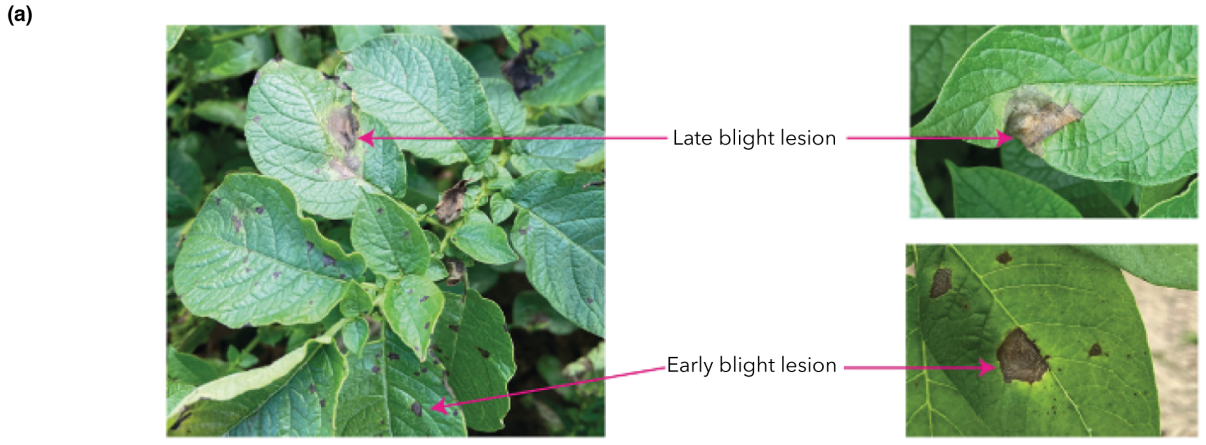
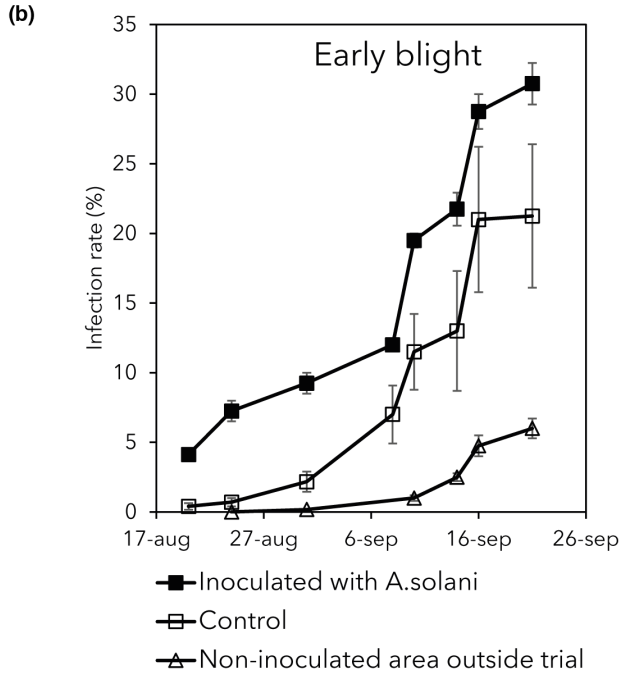


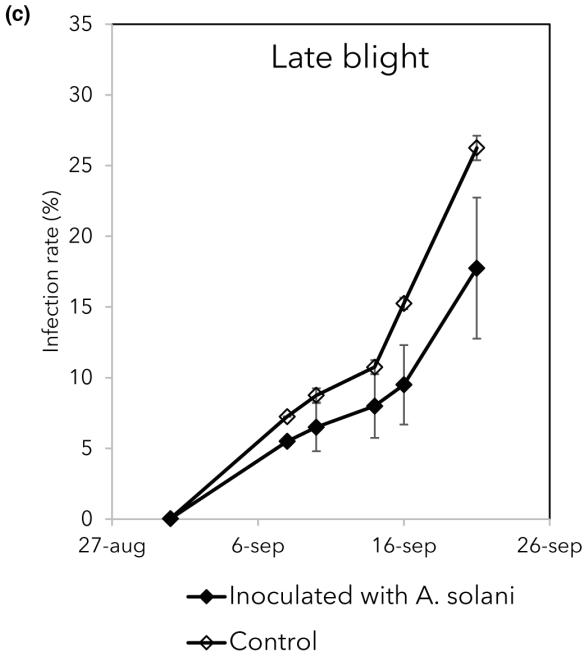
FIGURE 7 Representative confocal laser scanning micrographs obtained of infection of potato leaves inoculated with *Alternaria solani* and *Phytophthora infestans* on the adaxial side of the leaf. GFP, *A. solani* with green fluorescent protein; RFP, *P. infestans* td-tomato with red fluorescent protein; merged, for image (b) and (c) the merged image includes the transmission channel. All scale bars correspond to 50 μm. Bar graphs indicate number of pixels containing GFP (*A. solani*) and RFP (*P. infestans*) or red autofluorescence signals from dead cells (c). (a) Maximum intensity projection micrograph of simultaneous inoculation of *A. solani* and *P. infestans* imaged at 12 h postinoculation (hpi). (b) Micrographs of sequential inoculation with *P. infestans* followed by *A. solani* 24 h later, imaged on the abaxial side 48 hpi with *A. solani*. (c) Micrographs of sequential inoculation with *A. solani* followed by *P. infestans* 24 h later, imaged on the abaxial side of the leaf 48 hpi with *P. infestans*.

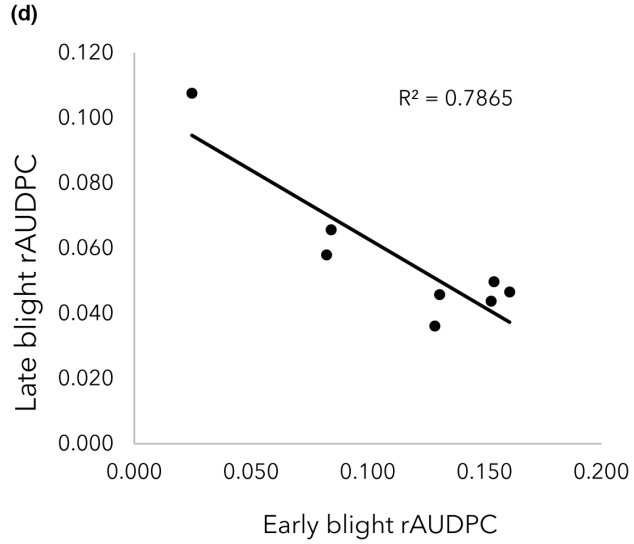
together, the biomass of *U. maydis* declined most severely over time, indicating efficacy of the *F. verticilliodes* toxins on *U. maydis*. It is possible that the reduction in *P. infestans* growth and the bursting phenotype we observed is due to mycotoxins produced by *A. solani*.

Simultaneous co-inoculation of *P. infestans* and *A. solani* in planta resulted in larger necrotic lesions than single inoculation of either *P. infestans* or *A. solani*. Microscopic analysis revealed that simultaneously co-inoculated lesions mainly displayed successful colonization of the host tissue by *A. solani*. Although *P. infestans* spores germinated, established infection of the host tissue was rarely observed. Yet the macroscopic lesions observed in the simultaneous co-inoculation were larger than the lesions of *A. solani* inoculation only. The presence of *P. infestans* thus seems to somehow enhance









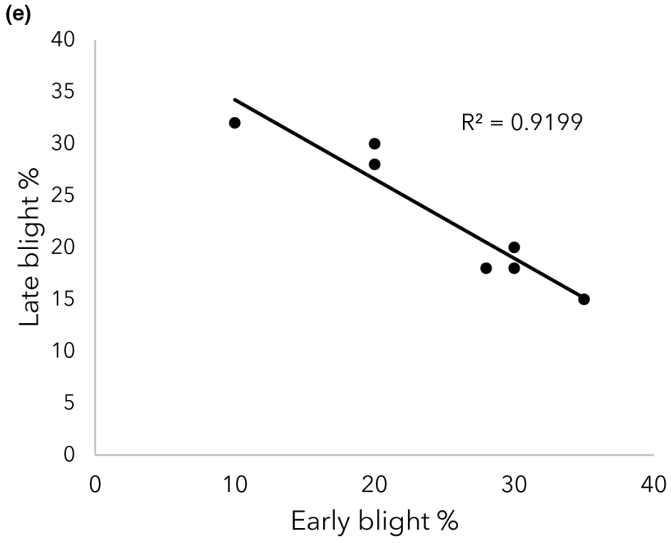


FIGURE 8 Field experiment on the effect of *Alternaria solani* infection on development of *Phytophthora infestans*. (a) Photographs of characteristic early and late blight lesions. (b) Percentage of early blight and (c) late blight infection and disease development curves for the assessment period in the field trial. Means of four replicate plots. All plots were inoculated with *P. infestans*. (d) Relative area under the disease progress curve (rAUDPC) for both late blight and early blight plotted against each other. Linear regression line displayed ($R^2 = 0.7865$, $p = 0.003$). Late blight and early blight disease percentage on the last scoring date (21 September) plotted against each other. Linear regression line displayed ($R^2 = 0.9199$, $p = 0.00017$).

the virulence of, or promote host susceptibility to, *A. solani*. It is possible that *P. infestans* has a direct positive effect on *A. solani*, or, alternatively, that the presence of *P. infestans* renders the host more susceptible to infection by *A. solani*. In a previous study, we observed similar larger lesion development in salicylic acid (SA)-deficient *NahG* plant lines (Brouwer et al., 2020). This indicates a role of intact SA signalling for potato defences against *A. solani*; during the biotrophic phase of *P. infestans* infection, levels of SA increase (Zhou et al., 2018). Interestingly, exogenous application of SA to detached tomato leaves rendered the leaves more susceptible to *A. solani*, with increased lesions compared to SA-untreated plants (Rahman et al., 2012). Contrastingly, exogenous application of SA to whole tomato plants grown in hydroponics resulted in increased *A. solani* resistance (Spletzer & Enyedi, 1999). These experiments indicate different roles of SA in defence against *A. solani*, increased susceptibility locally and induced resistance by systemically acquired resistance signals. The co-inoculation of both *A. solani* and *P. infestans* at the same spot on the leaf might result in local increased levels of SA that render the plants more susceptible to *A. solani* infection and result in the observed larger lesions. Additionally, *A. solani* could benefit from the plethora of effectors secreted by *P. infestans* that manipulate host immune responses, along with cell wall-degrading enzymes and other secreted enzymes such as isochorismatases that could interfere with SA signalling (Leesutthiphonchai et al., 2018). Even though *P. infestans* rarely established infection of the host tissue in the co-inoculated samples, it was previously shown that pre-infection germinated cysts and in vitro-created appressoria already secrete proteins such as RXLR effectors and cell wall-degrading enzymes (Resjö et al., 2017). This indicates that even the presence of germinated *P. infestans* could provide effectors and enzymes that could potentially aid the infection of *A. solani*.

A. solani was able to colonize potato when it was inoculated in planta at the same site used to inoculate *P. infestans* 24 h earlier. Larger lesions were observed when *A. solani* was inoculated after *P. infestans* compared to the controls where *A. solani* was inoculated after *A. solani*. However, when *P. infestans* was inoculated 24 h after *A. solani*, this did not significantly change the lesion size. Microscopic analysis revealed that the germination of *P. infestans* in the presence of *A. solani* was not inhibited, yet a network of fungal hyphae was observed in which the germinated *P. infestans* spores appeared entangled. It is thus possible that the decrease in successful colonization by *P. infestans* is due to the formation of a physical barrier in the form of *A. solani* hyphae. However, the in vitro experiments indicate the potential for a direct effect of *A. solani* metabolites or secreted proteins such as antimicrobial effectors on *P. infestans*. Additionally, the establishment of *A. solani* infection in the host could trigger plant responses that negatively influence the potential for *P. infestans* to establish infection. *P. infestans* establishes successful infection of potato by employing effectors to suppress host immune responses but also to limit the damage to the plant, because it requires living tissue for its biotrophic life stage (Leesutthiphonchai et al., 2018). However, the necrotroph *A. solani* triggers cell death to acquire nutrients. When *A. solani* is inoculated before *P. infestans*, this might thus ensure the conditions required for the biotrophic phase of the *P. infestans* infection are not met, and hence the successful colonization by *P. infestans* is blocked.

In line with the results obtained for the sequential inoculation of whole plants in the laboratory setting, in the field we observed a reduction of late blight symptoms when the establishment of early blight occurred before the inoculation with *P. infestans*. In the laboratory experiments, both pathogens were inoculated at the same spot, therefore a physical effect of the presence of the *A. solani* growth or triggered cell death could not be excluded. However, in the field experiment the inoculation spots on the leaves were not controlled but occurred in a similar way to natural infections. Yet, the presence of early blight caused by *A. solani* had a negative effect on the establishment of late blight caused by *P. infestans*. This was found both in an observation of a late blight trial that unintentionally became infected with *A. solani* and later in a designed field trial where plots were inoculated first with *A. solani* and later with *P. infestans*. The results indicate that the negative effect of *A. solani* on the ability of *P. infestans* to infect cannot solely be explained by a physical barrier but probably also involves antagonistic metabolites and induction or hampered suppression of defence responses in the host plant. In field settings, it is difficult to have noninoculated controls because after inoculation disease spread can influence those controls. Within our field experiment *A. solani* seemed to spread from inoculated plots to noninoculated neighbouring plots. However, in parallel noninoculated plots outside the experiment, the infection rate of early blight was much lower (Figure 8). Therefore, they may be considered as a noninoculated control. Still, we can conclude that the initial early blight infection clearly lowered the subsequent late blight infection, and without the spread of *A. solani* we expect that the difference in late blight infection between *A. solani*-inoculated and noninoculated plots would have been larger.

In order to successfully continue protection of food production in the future, new knowledge to improve our understanding of plant pathogen interactions, especially in agriculturally relevant settings, is required. In this study we showed that the disease-causing efficiency of the late blight pathogen is negatively correlated with the presence of early blight in the same field. Multipartite interactions between host and multiple pathogens

such as those observed in this study present interesting opportunities to study the direct and indirect interactions of multiple organisms that result in differences in disease establishment and severity. These data can also be used towards the development of control strategies that exploit the plant responses triggered by one pathogen in combatting another pathogen. Additionally, compounds such as toxins and proteins secreted by one pathogen might be used as an "antibiotic" against another pathogen or at least give clues to targets for novel control agents. To gain a deeper understanding of the interactions in this study, careful analyses of gene expression changes and metabolomics in multipartite interactions are required. Of special interest would be the further study of multipartite interactions under agriculturally relevant field conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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