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Pathogenicity of Single and Combined Inoculations of *Alternaria* spp. on Potato

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Abstract

In Europe, early blight has become an increasingly severe disease in potato cultivation. We investigated the lesion sizes caused by infection with different *Alternaria* spp. predominantly from the UK in detached-leaf assays and whole-plant assays of different potato cultivars. The analysis reveals that whilst there is no significant interaction between the potato cultivars used and the *Alternaria* spp. tested, statistically significant differences were observed between the group means of small-spored vs. large-spored *Alternaria* spp. The small-spored species grouped together with no significant differences amongst them, whilst large-spored species were able to cause large expanding lesions in all tests, with *Alternaria solani* exhibiting the most extensive lesion growth. We also explored the effect of co-inoculation treatments of *Alternaria* spp., revealing no significant difference between the inoculation with *Alternaria solani* alone and co-inoculation with *Alternaria solani* and *Alternaria alternata* or *Alternaria infectoria.* The findings contribute to our understanding of the behaviour of *Alternaria* spp. and their impact on potato cultivars.

Keywords *Alternaria alternata* · *Alternaria solani* · Co-inoculation · Detached-leaf assay · Early blight · Whole-plant assay

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Introduction

Alternaria species can be air-borne human allergens, post-harvest plant pathogens, plant pathogens, and general saprophytes, and have a remarkably broad host range (Thomma 2003). They are ubiquitous in the plant kingdom, causing infections on various plant organs. This widespread nature of *Alternaria* diseases leads to crop losses that rank amongst the highest caused by any plant pathogen (Agrios 2005).

Within the *Alternaria* genus, *Alternaria solani* Sorauer is perhaps one of the most damaging species. It is known for causing substantial losses in potato, pepper, and tomato crops. In potato, *A. solani* is thought to be the main causal agent of early blight. This disease primarily affects the foliage but can also cause symptoms on tubers. The fungus overwinters on crop debris, some weed plants, and seed tubers, with survival rates influenced by weather conditions, soil type, and factors like microbial agents, moisture, and solar radiation. The latter plays a role only when the inoculum is directly exposed (Rotem 1994). Other species of large-spored *Alternaria* have been identified as causing early blight, including *Alternaria grandis* and *Alternaria tomatophila* (Rodrigues et al. 2010), and *Alternaria protenta* (Ivanović et al. 2022; Woudenberg et al. 2014).

Most authors recognise a separate disease of potato (brown spot or leaf spot) caused predominantly by *Alternaria alternata* (Choi et al. 2023; Ding et al. 2019; Fairchild et al. 2013; Kirk & Wharton 2012; Leiminger et al. 2014; Soleimani & Kirk 2012; Vandecasteele et al. 2018). A number of other short-spored species of *Alternaria* have also been found in lesions on potato leaves, including *Alternaria tenuissima* (Zheng and Wu 2013), *Alternaria arborescens* (Tymon et al. 2015), and *Alternaria infectoria* (Sandoval et al. 2014).

Pathogenicity studies with the various *Alternaria* species on potato have revealed conflicting results. Turkensteen et al. (2010) concluded from extensive survey data that *A. alternata* was a very poor invader of healthy green tissue, but could more readily infect damaged or necrotic tissue. Spoelder et al. (2014) inoculated three isolates of *A. solani* and three of *A. alternata* separately on detached leaves of seven potato cultivars, and found no lesion development with *A. alternata*. In contrast, Zheng et al. (2015) found large lesions caused by both *A. tenuissima* and *A. alternata*, which were not significantly smaller than those caused by *A. solani*. Vandecasteele et al. (2018) showed that *A. alternata* and *A. arborescens* produced very limited symptoms of small black dots and vein necrosis compared to large spreading lesions of *A. solani* in a leaf disc assay. Kapsa and Osowski (2012) found that *A. alternata* produced larger lesions on leaves than *A. solani*, but that the reverse occurred on a tuber disc assay. Tymon et al. (2016) found that *A. solani* was significantly more agressive than *A. arborescens*, and another species, *Alternaria arbusti*, found in previous survey work (Tymon et al. 2015). Ding et al. (2019) showed that mean disease severity was significantly less for *A. alternata* isolates than it was for *A. solani* across three potato genotypes.

Most pathogenicity studies have been carried out by inoculating single species of *Alternaria* for comparative purposes. Spoelder et al. (2014) inoculated

A. alternata onto lesions already established by *A. solani*, but found no greater lesion expansion. However, given the diversity of species found on potato crops, the question remains whether different species act together to create more aggressive lesions, and thus whether agrochemical control methods and plant breeding programmes should be targeted at one or multiple species.

The objective of this work was to examine the pathogenicity of isolates of *Alternaria* species obtained from experimental trials and field crops in the UK, where early blight has been seen as an increasing issue (Lees et al. 2019), in comparison to a range of isolates from culture collections elsewhere. Both detached-leaf and whole-plant assays have been used. In addition, the potential for interaction between different *Alternaria* species in co-inoculation experiments was investigated. Molecular sequencing of single spore isolates of each culture used in the tests was used to aid identification.

Materials and Methods

Isolates of *Alternaria* Species Used in Detached-Leaf Assays (DLAs) and Whole-Plant Assays (WPAs)

Field isolates were obtained from two experimental field trials cropped with potato (early blight trials), conducted at the NIAB trial ground, Cambridge, UK, in 2015 and 2016. Isolates were initially classified as large-spored and small-spored species and later identified based on molecular sequences. Isolation and culturing were performed from single spores of each isolate, obtained from early blight symptomatic lesions of potato plants. Additional isolates were obtained from samples of early blight-symptomatic leaves, sent to NIAB from various UK potato growers, to give a total of 38 field and grower isolates, including a small number from UK sourced tubers or grown-on tubers in growth rooms (Table 1).

Eleven isolates from collections, including several *Alternaria* spp. from Wageningen University and Research (provided by Prof. Bart Thomma, Laboratory of Phytopathology) and the Westerdijk Fungal Biodiversity Institute (CBS), The Netherlands, were used.

For molecular identification, each isolate was cultured in 40 ml of Potato Dextrose Broth medium, incubated in a rotary-shaker incubator for 7–10 days, at 27 °C and under dark conditions. Mycelium was harvested and washed off with sterilised demi-water then freeze-dried for 48 h and ground using liquid nitrogen to obtain a fine powder. DNA was extracted using the Qiagen Puregene® DNA Purification Kit with minor modifications to increase DNA yield. Eight hundred microliter cell lysis solution, 6 µl Proteinase K solution, and 800 µl isopropanol were used instead of the typical amounts used in the standard protocol. The rest of the protocol was used as given in the kit's instructions. Each DNA sample's concentration and quality were quantified using a Thermo Scientific NanoDrop spectrophotometer. The ratio of absorbance at 260 nm and 280 nm for each sample was used to assess the DNA quality.

Table 1 Species designation of 49 isolates after identification with molecular markers; '*Year*' is the year of acquiring the isolate and '*Collection site*' refers to the location of acquisition. DLA and WPA designation refers to isolates used in different experiments

No	Species	Isolate ID	Year	Collection site	Use in assays
1	*A. solani*	sol 1	2015	Inoculated trial field	DLA 1, 2, 3, 4, 5, 6 WPA 1,2
2	*A. solani*	sol 2	2015	UK grower sample	
3	*A. solani*	sol 3	2016	Inoculated trial field	
4	*A. solani*	sol 4	2016	Inoculated trial field	
5	*A. solani*	sol 5	2016	Inoculated trial field	
6	*A. solani*	sol 6	2016	Inoculated trial field	
7	*A. solani*	sol 7	2016	Inoculated trial field	
8	*A. solani*	sol 8	2016	Inoculated trial field	
9	*A. solani*	sol 9	2016	Inoculated trial field	
10	*A. solani*	sol 10	2015	UK grower sample	DLA 1, 2, 3, 4
11	*A. solani*	sol 11	2015	UK grower sample	DLA 1, 2
12	*A. solani*	sol 12	2015	NIAB collection isolate	
13	*A. grandis*	grand 1	2015	WUR collection isolate	DLA 1, 2
14	*A. grandis*	grand 2	2017	CBS collection isolate	
15	*A. linariae*	lin 1	2017	CBS collection isolate	
16	*A. linariae*	lin 2	2017	CBS collection isolate	
17	*A. linariae*	lin 3	2015	Inoculated trial field	DLA 1, 2
18	*A. linariae*	lin 4	2015	NIAB collection isolate	DLA 1, 2
19	*A. protenta*	prot 1	2017	CBS collection isolate	DLA 1, 2
20	*A. protenta*	prot 2	2017	CBS collection isolate	
21	*A. protenta*	prot 3	2015	WUR collection isolate	
22	*A. infectoria*	infect 1	2016	UK grower sample	DLA 3, 4
23	*A. infectoria*	infect 2	2016	CBS collection isolate	DLA 1, 2, 5, 6
24	*A. infectoria*	infect 3	2016	UK grower sample	DLA 3, 4
25	*A. infectoria*	infect 4	2016	Inoculated trial field	DLA 3, 4
26	*A. infectoria*	infect 5	2016	Non-inoculated trial field	DLA 3, 4
27	*A. infectoria*	infect 6	2016	UK grower sample	DLA 3, 4
28	*A. infectoria*	infect 7	2015	Inoculated trial field	DLA 3, 4
29	*A. infectoria*	infect 8	2016	Non-inoculated trial field	DLA 3, 4
30	*A. infectoria*	infect 9	2016	UK grower sample	DLA 3, 4
31	*A. infectoria*	infect 10	2016	UK grower sample	DLA 3, 4
32	*A. infectoria*	infect 11	2016	UK grower sample	DLA 3, 4
33	*A. arborescens*	arb 1	2017	CBS collection isolate	
34	*A. arborescens*	arb 2	2017	CBS collection isolate	DLA 1, 2
35	*A. arborescens*	arb 3	2015	Markies tuber isolate	DLA 3, 4
36	*A. arborescens*	arb 4	2015	Markies tuber isolate	DLA 3, 4
37	*A. arborescens*	arb 5	2015	Inoculated trial field	DLA 3, 4
38	*A. arborescens*	arb 6	2015	Inoculated trial field	
39	*A. alternata*	alt 1	2017	CBS collection isolate	DLA 3, 4
40	*A. alternata*	alt 2	2015	Inoculated trial field	DLA 1, 2, 5, 6, WPA 1, 2
41	*A. alternata*	alt 3	2015	Inoculated trial field	DLA 1, 2, 3, 4

Table 1 (continued)

No	Species	Isolate ID	Year	Collection site	Use in assays
42	*A. alternata*	alt 4	2015	Inoculated trial field	
43	*A. alternata*	alt 5	2015	Inoculated trial field	DLA 3, 4
44	*A. alternata*	alt 6	2015	Sample from growth room	DLA 3, 4
45	*A. alternata*	alt 7	2015	Markies tuber isolate	DLA 3, 4
46	*A. alternata*	alt 8	2015	Inoculated trial field	DLA 3, 4
47	*A. alternata*	alt 9	2015	Non-inoculated trial field	DLA 3, 4
48	*A. alternata*	alt 10	2015	Markies tuber isolate	
49	*A. alternata*	alt 11	2015	UK grower sample	DLA 1, 2, 3, 4

All isolates were identified based on the genetic sequences of their ITS and RPB2 regions. The ITS region was amplified using ITS1 and ITS4 primers (Martin and Rygiewicz 2005). The RPB2 region was amplified using the RPB2–5F2 (Sung et al. 2007) and fRPB2–7cR (Liu et al. 1999) primers. Each PCR mixture consisted of a total volume of 25 µl, containing 1 µl DNA sample, 0.5 µl of forward-primer, 0.5 µl of reverse-primer, 2.5 µl of 2 µM dNTPs, 5 µl buffer, 0.1 µl goTAQ, and 15.4 µl Milli-Q water. Control reaction mixtures had the same composition, substituting the 1 µl DNA sample for 1 µl Milli-Q water. The PCR for the ITS region was performed on a Bio-Rad series 1000 Thermal cycler with an initial denaturation step of 95 °C for 10 min; 32 cycles of 95 °C for 1 min; annealing at 55 °C for 1 min; elongation at 72 °C for 90 s; and a final elongation step at 72 °C for 10 min. The PCR for the RPB2 region was performed on the same equipment with an initial denaturation step of 95 °C for 5 min; 32 cycles of 94 °C for 1 min; annealing at 50–53 °C for 30 s; elongation at 72 °C for 2 min; and a final elongation step at 72 °C for 2 min.

Identification by such sequences as ITS or RPB2 cannot give a completely certain identification of the isolates tested, because many isolates and sequences that have been submitted to various gene-banks come from isolates that have been identified only on the basis of their morphological characteristics, which can be erroneous (Simmons 2007).

Inducing Sporulation on *Alternaria* spp. Isolates

A custom medium was developed by adding 26 g Potato Dextrose Broth (PDA) and 8 g Technical Agar to a total volume of 1000 ml ddH_2O. To induce sporulation, isolates were grown in an incubator at 25 °C, under continuous fluorescent white light from 13-W Philips lamps, for 7 days. At this point in time, all small-spored isolates were readily sporulating with no further changes in conditions necessary.

For the large-spored isolates, the light in the incubator was turned off after day 7. This induced sporulation on all large-spored isolates, with the optimal time being between 18 and 24 h. Left longer than this, conidiospores started germinating and spore acquisition became more difficult since the conidiospores were covered with newly grown mycelium.

Sterilised water was added to the Petri dish and moved around gently to cover the entire surface of the culture. The spore suspension was collected in 50-ml sterilised plastic tubes and left undisturbed for 15–20 min to allow conidiospores to settle to the bottom. Supernatant was discarded and the final spore suspension was adjusted to 10^4 conidiospores/ml.

Plant Material and Isolates Used for Detached-Leaf Assays and Whole-Plant Assays

Seed tubers of class S (size category of 35–55 mm) of the cultivars Arsenal, Bintje, Markies, and Jelly were obtained from Dutch growers and stored in cold storage in the Unifarm facilities of Wageningen University and Research.

Seed tubers were selected for uniformity and left in a cold store in the dark to break dormancy and then planted in 5-l pots filled with peat-soil. Plants were grown in a glasshouse with an approximate 16-h natural light photoperiod. Day temperature varied between 19 and 27 °C and night temperature between 16 and 17 °C. Plants were watered daily and pot placement with the different cultivars was fully randomised. Two weeks after planting, plants were pruned to two similar stems per pot, to minimise variation between the detached leaves for subsequent experiments. Thirty-two days after planting, compound leaves were harvested for the DLAs. Harvesting was done on the day of the inoculation; only leaves of the 3rd, 4th, and 5th leaf rank, counting from the bottom of the plant, were included. Leaflets were cut off their respective compound leaf at the time of inoculation to minimise water loss. The apical leaflet from the compound leaf was not used in any of the experiments. The apical leaflets were prone to showing necrosis on the tip and were most often bigger in size than the rest of the leaflets of the same compound leaf.

For the WPAs, plants were pruned to one stem per pot, 2 weeks after planting, to minimise natural variation between plants caused by different numbers of stems and variation in individual stem growth within a plant. Plants were inspected daily during the experiment and any developing secondary stems were removed. Five weeks after planting, pots with the single-stem plants were moved from the glasshouse to a controlled climate glasshouse. Plants were selected for uniformity and for number of compound leaves.

Due to limitations on space, it was not possible to test all isolates collected in the various experiments, as indicated in Table 1. The numbers of small-spored isolates tested reflected the overall frequency that they were found in UK samples; i.e., *A. alternata* and *A. infectoria* were isolated more often than *A. arborescens*. For the large-spored species, *A. solani* was most frequent, and other large-spored species from culture collections were included for comparisons in DLA1 and 2. *A. linariae*, usually recognised as a tomato leaf pathogen, from the NIAB culture collection was included because two field isolates from UK were unexpectedly identified as *A. linariae*.

Inoculation of Leaflets for DLAs

Leaflets of each potato cultivar were randomised before inoculation. A unilateral wound was made in equidistant positions from the midrib on the adaxial surface, halfway between the midrib and each edge of the leaf. The wound was made by using a 25-gauge sterile syringe needle, scraping gently on the leaf cuticle to allow for easy penetration by the fungus. On each of the wounds, a 5-µl spore suspension with a concentration of 10^4 conidiospores/ml was deposited using a transfer pipette.

The co-inoculation treatments contained two drops, one for each of the co-inoculated species tested, each drop being 5 µl of spore suspension with a concentration of 10^4 conidiospores/ml. For all experiments, a mock inoculation with water was also included as control. The same protocol was used for all DLA experiments.

DLA1 was conducted in 2016 using cultivars Markies and Jelly. DLA2 was conducted in 2017 using the same cultivars. The final lesion growth analysis of both cultivars in both runs was included in the analyses. DLA1 and DLA2 tested all the *Alternaria* spp. that this study focused on, predominantly using material from CBS or WUR culture collection: three isolates of *A. alternata*, one isolate of *A. arborescens*, one isolate of *A. infectoria*, three isolates of *A. solani*, two isolates of *A. linariae*, one isolate of *A. grandis*, and one isolate of *A. protenta*.

Leaflets of each cultivar were arranged in a randomised complete block design, with isolate positions randomised within each block and five replications for the 2016 experiment run (DLA1) and six replications for the 2017 experiment (DLA2). Each block comprised a plastic box containing wet filter paper, covered with a plastic mesh. The inoculated leaflets were placed on top of the plastic mesh to avoid direct contact with water. Each box contained all the tested isolates inoculated on leaflets. Boxes were covered with plastic covers to ensure high relative humidity and placed in a controlled climate chamber with 14-h photoperiod, day temperature 20 °C, and night temperature of 15 °C. The experiment lasted 7 days post-inoculation.

DLA3 and DLA4 tested all the small-spored *Alternaria* spp. that this study focused on using material predominantly from UK grower or experimental fields: eight isolates of *A. alternata*, three isolates of *A. arborescens*, and ten isolates of *A. infectoria*, including two isolates of *A. solani* as control treatments. Leaflets of each cultivar were arranged in a randomised complete block design, with isolate positions randomised within each block and four replications. Each block comprised rectangular Petri dishes, into which two leaflets of the same cultivar were placed and each leaflet was inoculated with a random isolate. The Petri dishes contained wet filter paper, covered with a plastic mesh. The inoculated leaflets were placed on top of the plastic mesh to avoid direct contact with water. Petri dishes were covered with plastic lids to ensure high relative humidity and placed in a controlled climate chamber with the same conditions as DLA1 and DLA2 for 7 days.

DLA5 and DLA6 compared how co-inoculation of *A. alternata*+*A. solani* and *A. infectoria*+*A. solani* differed in their final lesion size to inoculations with *A. alternata*, *A. solani*, and *A. infectoria* individually in detached-leaf assays. In addition, a 'double' treatment of *A. solani* was added to the experiment. Leaflets of Markies and Arsenal cultivars were arranged in a randomised complete block design, with treatment positions and cultivar positions randomised within each block

and ten replications. Plastic boxes were used with the same experimental conditions as other DLAs.

Inoculation of Leaflets on Whole Plants

WPA1 and WPA2 compared how co-inoculation of *A. alternata*+*A. solani* differs in their final lesion size from inoculations with *A. alternata* and *A. solani* separately, when performed on whole plants. Cultivars Markies and Arsenal were both used in each experiment. Plants were transferred to benches in a climate-controlled room in Wageningen University and Research, 1 week before the inoculation to allow for acclimatisation and monitoring for any stress symptoms.

Plants were arranged in a randomised complete block design with ten replications. Each pot was considered as a replicate, giving one data point by averaging the size of the lesions in all the inoculated leaflets. Screening experiments tested for the significance of the leaf-rank position and found it to have no effect for the selected leaf-rank range of 5th to 8th compound leaves, and these leaf positions were therefore used for inoculations.

For each plant, two to three compound leaves were selected and two to four leaflets per compound leaf were tagged for inoculation depending on availability and uniformity, trying to select for leaves that were phenotypically as similar as possible. A point inoculation was made at equidistant positions from the midrib, halfway between the midrib, and each edge of the inoculated leaflet. There was no wounding performed.

On each inoculation site, a 10-µl spore suspension with a concentration of 10^4 conidiospores/ml for *A. solani* treatments and 10^5 conidiospores/ml for *A. alternata* was deposited using a transfer pipette. The co-inoculation treatments contained two drops, one for each of the co-inoculated species tested, each drop being 10 µl of spore suspension with the mentioned concentrations for each *Alternaria* spp. used. The spore suspension was supplemented with PDA with a dilution factor of 5. The addition of PDA was shown to aid in inoculum adherence on the leaflet surface, aid in spore germination, and in subsequent entry of the pathogen in the leaf tissue. Moreover, supplementation with diluted PDA prevented the inoculation site drying out (personal communication: Jaap Wolter, Wageningen University & Research, 2018). For both experiments, a water mock inoculation treatment was used as control.

Two cages with plastic panels were constructed around the plants to help maintain high relative humidity for assisting in infection establishment and lesion development. Care was taken so the plants would not touch each other or the plastic material during the experiment (Fig. 1). Two mist generators were placed inside the two cages and were set for an operation cycle of 5-min on and 2-min off. The on–off cycles were taking place during 22:00 to 05:00 each day after inoculation to ensure high RH during the night which was measured at 90%. Temperature was set to 21 °C during the day and 16 °C during the night, following the natural day-night cycle of between 13 and 17 h days during the

Fig. 1 Potato plants for WPA growing under controlled conditions. A custom plastic enclosure was built around the tables to ensure high relative humidity

experimental period and RH was maintained at 70%. The experiment lasted for 12 days post-inoculation (DPI). On the final day, compound leaves were collected and lesion area was measured.

Data Acquisition, Measuring Lesion Size

The protocol for measuring lesion sizes was almost identical for DLAs and WPAs with minor adjustments for each. For the DLAs, boxes containing leaflets were photographed at daily intervals, using a digital camera starting 1 DPI until the end of the experiment at 7 DPI. A scale ruler was placed next to the boxes. For the WPA, each compound leaf that contained inoculated leaflets was harvested 12 DPI, placed on scaled grid paper, with each square side 0.5 mm long, along with a scale ruler at the side. The setup was photographed using a digital camera.

The images were subsequently analysed using the measuring tools of the Photoshop CC software. The ruler scales in the pictures were used to set the scale for the measuring tools and gave high-accuracy measurements in cm^2. From the calculated area of each lesion, the radius of each lesion was calculated, with the assumption of each lesion being circular.

Data Analysis and Plotting

Data input was performed in Microsoft Excel and subsequent data clean-up and statistical analysis were performed with the R language (R Core Team 2017) using the 'nlme' package (Pinheiro et al. 2017) for the analyses and the 'ggplot2' package (Wickham 2009) for creating the graphical plots.

For analysing the results of each DLA and WPA experiment, a linear mixed effects model was fitted to the corresponding data using the 'lme' function from the 'nlme' package. The response variable in the model was the lesion radius of each inoculation treatment. The fixed effects in the model were the inoculum species and potato cultivar, which were included to account for the systematic variation in lesion radius due to these two factors.

The statistical model also included a random effects for the day post-inoculation nested within the unique identification code of each leaf, which was included to account for the random variation in radius that was associated with different days within each unique leaf. This random effects structure allowed for different slopes for the relationship between radius and day for each leaf identification code, thereby accounting for the fact that the rate of change in radius over time might not have been the same for all leaves. For experiments that relied on non-repeating measures (only final lesion measurement performed), the model did not include a day variable.

The statistical model was fitted using maximum likelihood estimation. The model also included a variance function structure that allowed for different variances for each level of the inoculation isolate factor. This was included to account for the possibility that the variability in radius might not have been the same for all isolates. Tables showing statistical outputs from the model are shown in supplementary information (online resource 1) for each experiment. Graphical plots and summaries of statistical differences are shown in the 'Results'.

Results

In these experiments, multiple isolates of different *Alternaria* species were inoculated onto detached leaves in the same plastic box to maximise the probability of uniform conditions for each isolate. The droplet inoculation, rather than a spray, ensured there was no chance of liquid inoculum contaminating leaf parts away from the point of inoculation. Lids on the boxes ensured that no movement from box to box could occur in the growth room. Symptoms were confined to the area around the inoculation point in all the inoculations carried out, and no symptoms were seen on the water mock inoculation points. These observations confirm that the comparison between isolates was valid, with no cross-contamination occurring. In the whole-plant assays, there was more chance of contamination as plants were exposed to small air currents which could have moved inoculum from the points of inoculation; nevertheless, no random lesion development was observed and symptoms were confined to inoculation points, again confirming that between isolate comparisons were valid.

Detached-Leaf Assays (DLA1, DLA2) Comparing Lesion Size Between Small- and Large-Spored *Alternaria* spp.

Analysis of variance showed that there was no significant interaction between the potato cultivars used and the *Alternaria* species tested, so the interaction term was removed, and the analysis performed again for the main effects of cultivar and species. The cultivar effect was shown to be significant only in the first run ($F(1,112)=14.12$, $p<0.05$) with Markies showing a marginally higher mean value of lesion radius compared to Jelly over all the tested *Alternaria* species (Markies, 0.112 cm; Jelly, 0.105 cm).

There were statistically significant differences in the group means of different *Alternaria* species attributed to the effect of species ($F(6,112)=644.69$, $p<0.05$ and $F(6,155)=97.17$, $p<0.05$), for DLA1 and DLA2, respectively. All small-spored species grouped together with no significant differences amongst them. The small-spored species gave significantly less lesion growth than all of the large-spored species. Large-spored species were always able to cause large expanding lesions, with *A. solani* consistently exhibiting the largest lesion growth (Fig. 2).

The final lesion radii for 7 days post-inoculation (7 DPI) for DLA1 and DLA2 are presented in Figs. 3 and 4.

Detached-Leaf Assays Comparing Lesion Size Between Small-Spored *Alternaria* spp. (DLA3, DLA4)

This experiment used four cultivars, Arsenal, Bintje, Jelly, and Markies, and additional isolates of small-spored *Alternaria* species, compared to two isolates of *A.*

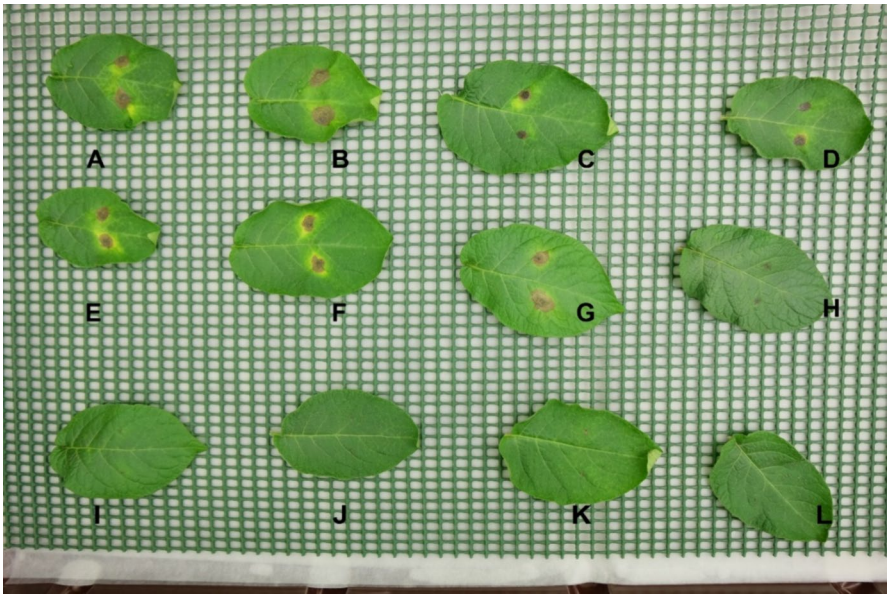

Fig. 2 DLA1. Characteristic early blight lesions 5 days after inoculation on detached leaves. **A**, **B**, **F** *A. solani*; **C**, **D** *A. linariae*; **E** *A. grandis*; **F**, **G** *A. protenta*; **H**, **I**, **J** *A. alternata*; **K** *A. infectoria*; **L** *A. arborescens*

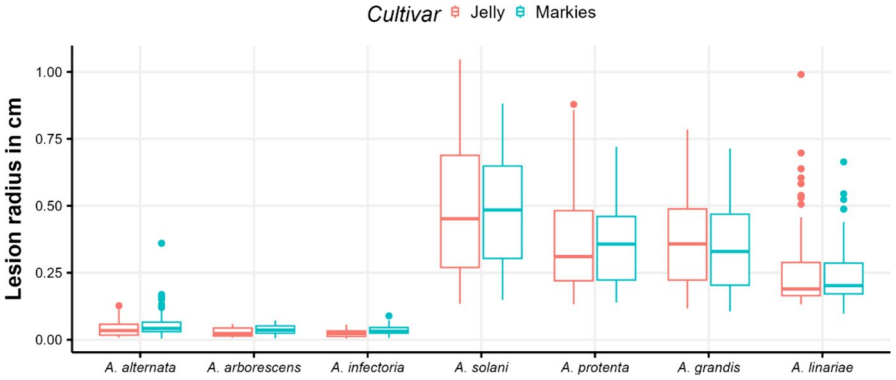

Fig. 3 Boxplot of final lesion radius in cm, on day 7 after inoculation in DLA1. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species treatment for easy comparison. Any dots appearing above or below the boxes represent outliers in the data

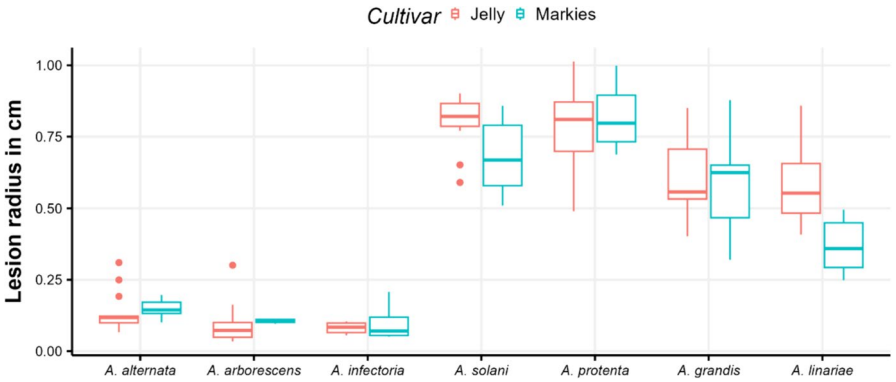

Fig. 4 Boxplot of final lesion radius in cm, on day 7 after inoculation in DLA2. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species treatment for easy comparison. Any dots appearing above or below the boxes represent outliers in the data

solani. The small-spored species again resulted in very small final lesion sizes when compared to *A. solani* (Figs. 5 and 6).

Analysis of variance showed that there was no significant interaction between the potato cultivar used and the *Alternaria* species tested so the interaction term was removed, and the analysis performed again for the main effects of cultivar and species. Analysis of DLA3 showed the cultivar effect to be non-significant ($F(3,354) = 2.3482$, $p = 0.0724$), and the same was true of DLA4 ($F(3,358) = 4.1340$, $p = 0.0067$). In DLA3, *A. infectoria* showed a statistically significantly smaller lesion radius compared to *A. arborescens* and *A. alternata* although this was not repeated in DLA4. The *A. solani* lesion radius was

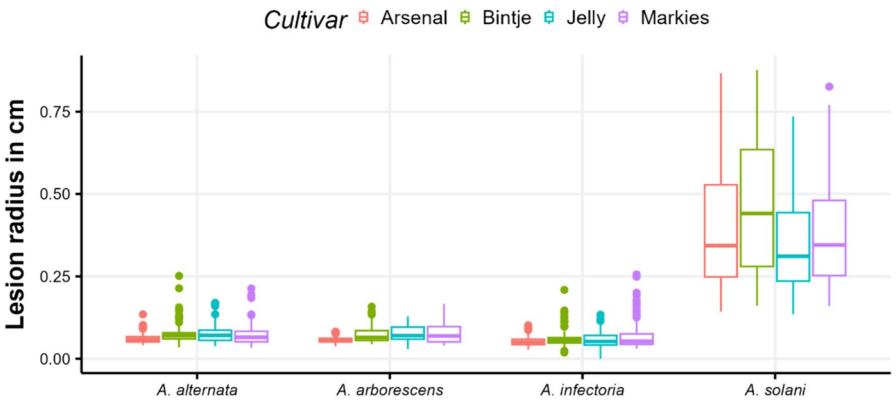

Fig. 5 Boxplot of final lesion radius in cm, on day 7 after inoculation in DLA3. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species treatment for easy comparison. Any dots appearing above or below the boxes represent outliers in the data

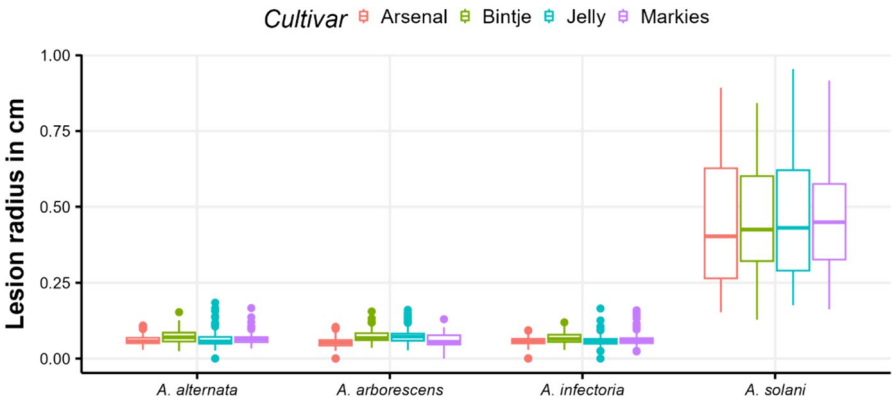

Fig. 6 Boxplot of final lesion radius in cm, on day 7 after inoculation in DLA4. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species treatment for easy comparison. Any dots appearing above or below the boxes represent outliers in the data

statistically significantly larger than that of all small-spored species in both experiments. The final lesion radii for 7 days post-inoculation (7 DPI) for DLA3 and DLA4 are presented in Figs. 5 and 6.

Detached-Leaf Assays Comparing Lesion Size of Co-inoculation Treatments of *Alternaria* spp. (DLA5, DLA6)

Both experiments showed similar results. There was no statistically significant effect of cultivar. Analysis of variance showed that there was a significant difference between the

final radii of lesions caused by the various *Alternaria* spp. inoculations ($F(5,114)=18.60$, $p<0.0001$) for DLA5 and ($F(5,234)=89.175$, $p<0.0001$) for DLA6. The differences in degrees of freedom are the result of the monitoring frequency between DLA5 and DLA6: for DLA5, lesion size was recorded at 3 time points (4, 8, 12 DPI) and for DLA6 at 6 time points (4, 5, 6, 7, 8, 9 DPI). All the co-inoculations, and *A. solani* alone, produced significantly larger lesions than the two small-spored species alone in both experiments. None of the co-inoculations differed significantly from each other, or *A. solani* alone in DLA 5, but the co-inoculation with two droplets of *A. solani* in DLA 6 did produce slightly larger lesions than *A. solani* co-inoculated with *A. alternata* or *A. infectoria.* Figures 7 and 8 show the final lesion radius for DLA5 and DLA6.

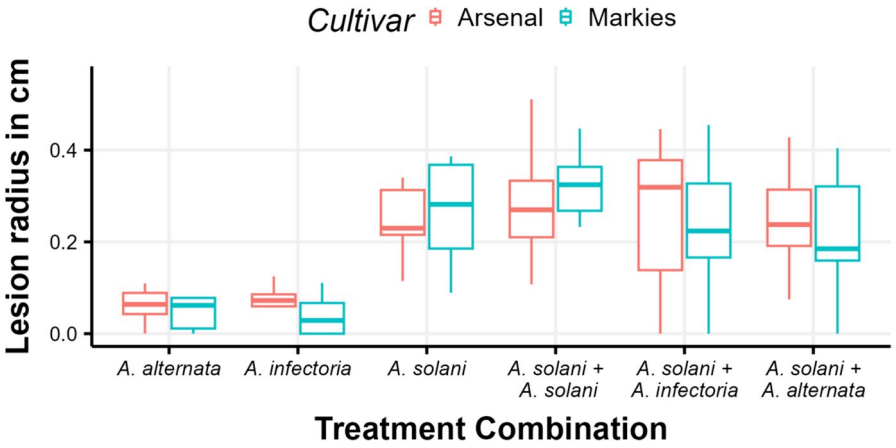

Fig. 7 Detached-leaf co-inoculations (DLA5). Boxplot of final lesion radius in cm, 12 DPI. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to specific cultivar. The cultivars are arranged adjacently for each species combination for easy comparison

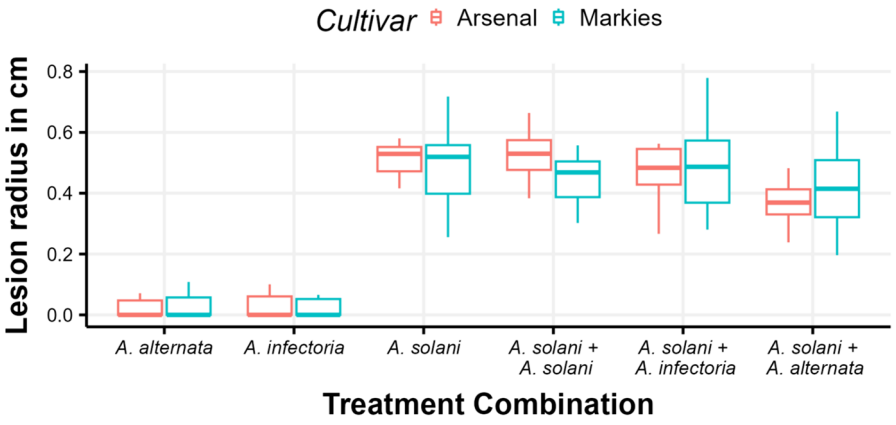

Fig. 8 Detached-leaf co-inoculations (DLA6). Boxplot of final lesion radius in cm, 9 DPI. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species combination for easy comparison

Whole-Plant Inoculations, Comparing Lesion Size of Co-inoculation Treatments of *A. solani* and *A. alternata* (WPA1, WPA2)

In this experiment, *A. solani* and *A. alternata* were used to inoculate leaves on whole plants, with each species used separately as a treatment and a third treatment that comprised the co-inoculation of both species. The experiment was repeated twice with similar results. *A. alternata* was unable to cause any lesions in both experiments (Figs. 9 and 10). Cultivar and the interaction of Treatment*Cultivar had no significant effect and were not included as factors in the final analysis of variance. Furthermore, the analysis of variance indicated that there was no significant difference between the two groups that caused visible lesions (*A. solani* + *A. alternata* vs. *A. solani*) ($F(1,18) = 1.05$, $p = 0.32$).

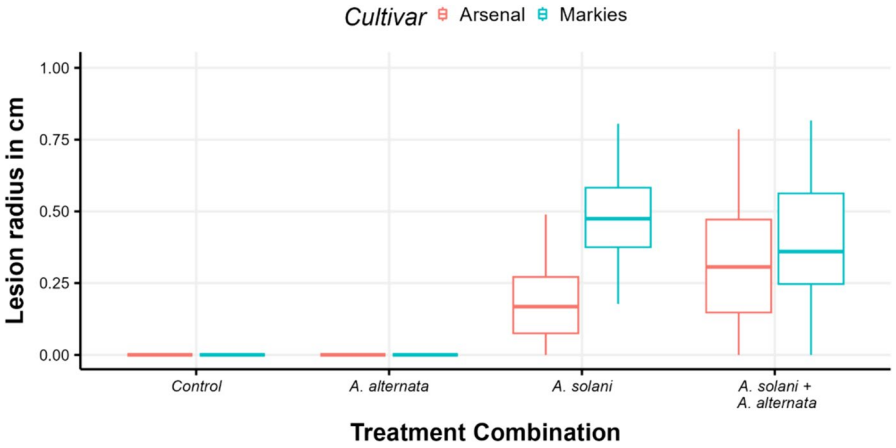

Fig. 9 Whole-plant co-inoculations, WPA1. Boxplot of final lesion radius in cm, 12 DPI. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species combination for easy comparison

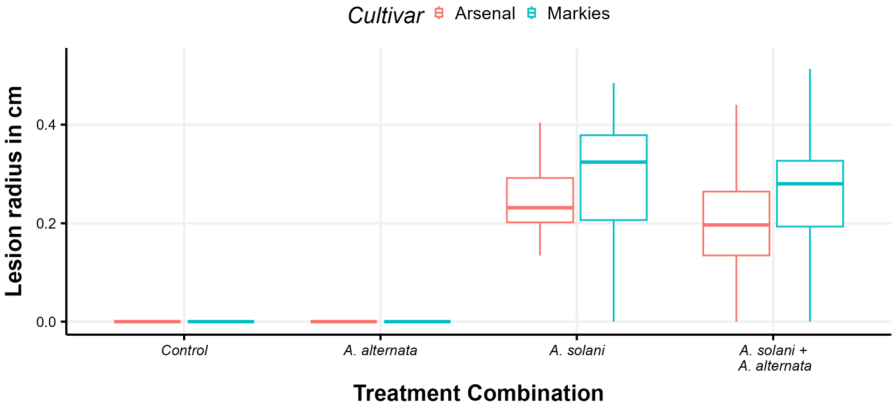

Fig. 10 Whole-plant co-inoculations, WPA2. Boxplot of final lesion radius in cm, 12 DPI. In the graph, individual cultivars are denoted by distinct colours, with each colour corresponding to a specific cultivar. The cultivars are arranged adjacently for each species combination for easy comparison

Discussion

In the detached-leaf experiments, the difference in pathogenicity of the large-spored *Alternaria* spp. vs. the small spored ones was evident. All of the small-spored *Alternaria* spp. tested had very small or negligible lesion growth over the course of the experiments and this was observed on all of the cultivars used. Totals of 9, 4, and 11 individual isolates of *A. alternata*, *A. arborescens*, and *A. infectoria* respectively were used in the DLA experiments, mostly from UK field and grower samples. Maximum lesion diameters observed were approximately 3 mm, with most being less than this. Although this relatively small number of isolates does not represent a survey, the results are in agreement with most data available for various short-spored species from northern European countries (Vandecasteele et al. 2018) and the USA (Tymon et al. 2016; Ding et al. 2019), but contrast with data from Spoelder et al. (2014) where no lesion growth at all was observed of *A. alternata* from the Netherlands, albeit with a low number of isolates. Two reports, Kapsa and Osowski (2012), using isolates from Poland, and Zheng et al. (2015), with isolates collected in China, found no significant differences in lesion size between short-spored and long-spored species. It is possible that differences within short-spored species in their ability to infect and colonise potato tissue do exist depending on populations tested. Budde-Rodriguez et al. (2022), using a tomato leaf assay with isolates from potato from 12 potato growing states in the USA, found significant differences in aggressiveness across a total of 18 isolates from *A. alternata*, *A. tenuissima*, and *A. arborescens*, but this was independent of species. Vandecasteele et al. (2018) found significant differences in virulence within a short-spored *Alternaria* species, but not between three species tested. Variations in inoculation methods employed in different studies may also influence outcomes, particularly wounding vs. non-wounding, whole plant vs. detached leaf, and tissue type used. Zheng et al. (2015) used apical detached leaflets of potato compound leaves in contrast to lateral leaflets from the rachis which were used here, and Kapsa and Osowski (2012) found contrasting results when their isolates were inoculated on tubers. Although only a single isolate of *A. alternata* from a symptomatic field lesion was used in whole, non-damaged plant assays in the present study, the absence of any lesion development confirms most other studies indicating that *A. alternata* is a weak pathogen, requiring stressed or damaged plants to produce small lesions.

There was evidence of significant variation between long-spored isolates in the size of lesions produced. *A. solani* and *A. protenta* produced larger lesions than *A. grandis* and *A. linariae* (syn. *Alternaria tomatophila*). Only *A. solani* and *A. linariae* were isolated from UK samples and whilst the latter was less aggressive, the implications for management strategies are as yet unknown. More comprehensive surveillance may reveal additional long-spored species, as reported by Ivanović et al. (2022) in Serbia and Landschoot et al. (2017) in Belgium.

The ubiquitous nature of *A. alternata* and other short-spored species in potato fields, and their isolation from potato leaves, may signify a role in an *Alternaria* disease complex, despite their weak pathogenicity in isolation. In simultaneous

co-inoculation experiments with *A. solani* coupled with either *A. alternata* or *A. infectoria*, there was no evidence of enhanced lesion growth. This supports other results where *A. alternata* inoculated onto previously established lesions of *A. solani* did not lead to larger lesion development (Spoelder et al. 2014). Vandecasteele et al. (2018), investigating the temporal dynamics of *Alternaria* spp. in potato fields, found that short-spored types predominated early in the growing season, followed by *A. solani*, and prior inoculation with different short-spored isolates at intervals before inoculation with *A. solani* still requires investigation.

This study has confirmed that short-spored *Alternaria* isolates isolated from UK potato crops produced typical small, brown spot type lesions, with limited expansion on damaged leaf tissue. *A. arborescens* and *A. infectoria* were identified in field isolates as well as *A.alternata*. *A. solani* and *A. linariae* produced larger characteristic early blight lesions on damaged or non-damaged leaves. Further work is necessary to explore whether specific control measures for brown spot are necessary.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11540-024-09739-8.

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Data Availability Data available on request to the corresponding author.

Declarations

Competing Interests Paul C. Struik is editor-in-chief of *Potato Research*.

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