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Pathogenic variation of *Alternaria* species associated with leaf blotch and fruit spot of apple in Australia

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Abstract Four *Alternaria* species groups (*A. longipes*, *A. arborescens*, *A. alternata*/*A. tenuissima* and *A. tenuissima*/*A. mali*) are associated with leaf blotch and fruit spot of apple in Australia. There is no information on the variability of pathogenicity among the species and isolates within each species causing leaf blotch or fruit spot. We used a detached leaf assay and an *in planta* fruit inoculation assay to determine the pathogenicity and virulence of the four *Alternaria* species. Our results showed that isolates within the same species were not specific to either leaf or fruit tissue and showed great variability in pathogenicity and virulence, indicating cross-pathogenicity, which may be isolate dependent rather than species dependent. Generally, virulence of *A. tenuissima* and *A. alternata* isolates on leaf and fruit was higher than other species. Isolates of all species groups were pathogenic on leaves of different cultivars, but pathogenicity on fruit of different cultivars varied among isolates and species. Implications of our findings on prevalence of the diseases in different apple-producing regions in Australia and the development of targeted disease management of the diseases are discussed.

Keywords Apple · *Alternaria* · Leaf blotch · Fruit spot · Pathogenicity · Aggressiveness

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Introduction

Alternaria leaf blotch and fruit spot of apple (*Malus x domestica* Borkh.) caused by *Alternaria* spp**.** causes yield losses of 15–25 % in high value cultivars to the Australian apple industry (Horlock 2006). Alternaria leaf blotch was first reported from the USA in 1924 (Roberts 1924) and now occurs worldwide (Filajdic and Sutton 1995; Rotondo et al. 2012; Sawamura 1962). In Australia, leaf blotch was first reported in Queensland in the 1990s and now occurs in all Australian apple-growing regions (Horlock 2006). The initial symptoms appear as brown blotches on the leaves and significant coverage of the leaves results in premature defoliation of the trees later in the season (Persley and Horlock 2009). Premature defoliation may give rise to reduced tree vigour and yield in the following seasons (Cordes 1987). Alternaria fruit spot of apple was first reported in Japan in the 1950s (Sawamura 1962) and recently in Italy (Rotondo et al. 2012) and Australia (Horlock 2006). In Australia, fruit spot was first reported to cause yield losses in the Queensland apple-growing region in 2002 (Horlock 2006) and there are anecdotal reports that fruit spot now occurs in all Australian apple-growing regions. Fruit spot is characterized by small, slightly sunken brown spots on the skin of fruit (Persley and Horlock 2009). Fruit showing symptoms are downgraded from premium fresh fruit to juice, resulting in a significant financial penalty to the growers.

Alternaria mali, also called *A. alternata* apple pathotype (Johnson et al. 2000; Kusaba and Tsuge 1994) or *A. alternata* f. sp. *mali* (Wu et al. 1999; Yoon et al. 1989) is the most frequently reported pathogen

associated with both leaf blotch and fruit spot of apple (Filajdic and Sutton 1991; Sawamura 1962). Recently, multiple *Alternaria* spp. have been associated with both diseases in Italy (Rotondo et al. 2012) and Australia (Harteveld et al. 2013). Rotondo et al. (2012) reported that *A. arborescens*, *A. tenuissima* and *A. alternata* are associated with both diseases in Italy, whereas in Australia, Harteveld et al. (2013) reported that four *Alternaria* species groups; *A. arborescens*, *A. alternata*/*A. tenuissima* intermediate, *A. tenuissima*/*A. mali* and *A. longipes*, are associated with both diseases. In this paper, *A. alternata* represents the *A. alternata*/*A. tenuissima* intermediate and *A. tenuissima* represents the *A. tenuissima*/*A. mali* clusters as described by Harteveld et al. (2013).

A. arborescens was the most frequently isolated and most prevalent species group related to leaf blotch in all Australian apple-growing regions, whereas the *A. tenuissima* and *A. alternata* species groups were mainly isolated from fruit in Queensland and New South Wales (Harteveld et al. 2013). The reasons for this are not fully understood. There is no information concerning the pathogenicity, defined as the ability to cause apple leaf blotch and/or fruit spot and virulence as the amount of damage caused to the host tissue, of the four *Alternaria* species groups nor is it known if pathogenicity of isolates is specific to the host tissue from which they were originally derived.

In New South Wales, severity of Alternaria leaf blotch and fruit spot is high in cultivars Royal Gala, followed by Red Delicious, then Pink Lady™ and Fuji; whereas in Queensland, Royal Gala, followed by Pink Lady™ and then Red Delicious are the most affected cultivars (Horlock 2006). Management of both diseases with fungicide is erratic and the efficacy of different fungicides varies among different states in Australia for unknown reasons. We hypothesize that this is due to pathogenic variation and variability in virulence among the *Alternaria* species groups. Therefore, the overall aim of this study was to determine if there is variation in pathogenicity and virulence among and within the *Alternaria* species groups causing leaf blotch and fruit spot of apples in Australia. Specifically, this study aimed to determine if: (*i*) all four *Alternaria* species groups are pathogenic on apple fruit and leaves, (*ii*) pathogenicity of the isolates is specific to the host tissue from which they were originally derived, (*iii*) isolates of *A. arborescens*, as the most prevalent species group associated with leaf blotch, are more virulent on apple leaves and fruit than other species groups, and (*iv*) the four *Alternaria* species groups are pathogenic on leaf and fruit of different apple cultivars. An enhanced understanding of host-pathogen interactions of the four *Alternaria* species groups associated with leaf blotch and fruit spot of apple in Australia will provide better understanding of their epidemiology and distribution in the Australian ecosystem and underpin development of targeted disease management for Alternaria leaf blotch and fruit spot of apple.

Materials and methods

Sources of isolates and inoculum preparation

Four isolates of each of the four *Alternaria* species groups were selected arbitrarily from a subset of 51 isolates described by Harteveld et al. (2013) and used for this study (Table 1). A mycelial agar plug of each isolate was placed on ½ strength potato dextrose agar (PDA, Difco Laboratories Inc.) and incubated at 25 °C in darkness for 14 days. Spore suspensions were prepared by flooding the culture dish with sterile distilled water and disrupting the spores using a sterile scalpel. The suspension was poured through two layers of cheesecloth to remove mycelial fragments. The spore concentration was determined using a haemocytometer and adjusted to a concentration of 10^5 spores/ml.

Pathogenicity to apple leaves and fruit of Royal Gala

To test if all isolates were pathogenic on apple leaves, detached leaf inoculation assays were performed using leaves obtained from young potted trees of the apple cultivar Royal Gala. Potted trees were maintained in a shade house, fertilized and watered daily. Plants were monitored for presence of other pathogens and arthropods, such as aphids and mites, which were controlled with oil (Biopest Paraffinic Oil, SACOA Pty Ltd.) and insecticidal soap spray (Natrasoap, Agrobest Australia Limited) when needed. The shoots of the plants were pruned to regenerate fresh shoots of 30–40 days old for each experiment. The first five fully expanded leaves on the terminal shoots were detached, rinsed with deionised water and air-dried. Three replicate leaves per isolate were used. Two 0.5-cm^2 square sterile filter paper discs soaked in spore suspension were placed on the abaxial side of each leaf, one filter paper on each side of the midrib of the leaf blade. A control experiment was

Table 1 Source of *Alternaria* isolates and results of pathogenicity assays for leaf blotch and fruit spot on apple leaves and fruits of cultivar Royal Gala

Accession number[a]	*Alternaria* species group[b]	Source of isolation				Pathogenicity[d]	
		Host tissue	Cultivar	Location[c]	Date collected	Leaf	Fruit
46452	*A. arborescens*	Fruit	Pink Lady	WA	May-05	+	-
46571	*A. arborescens*	Leaf	Fuji	SA	May-05	+	-
46872	*A. arborescens*	Leaf	Fuji	Vic	May-05	+	-
46512	*A. arborescens*	Leaf	Pink Lady	WA	May-05	+	-
46398	*A. alternata*	Fruit	unknown	NSW	Apr-05	+	+
46550	*A. alternata*	Fruit	Fuji	Qld	Apr-05	+	-
46590	*A. alternata*	Leaf	Fuji	Tas	May-05	+	+
46545	*A. alternata*	Leaf	Royal Gala	Qld	Mar-05	+	-
46361	*A. tenuissima*	Leaf	Royal Gala	NSW	Apr-05	+	+
46574	*A. tenuissima*	Leaf	Royal Gala	SA	May-05	+	+
46414	*A. tenuissima*	Leaf	Braeburn	NSW	Apr-05	+	-
54639	*A. tenuissima*	Fruit	Pink lady	NSW	May-11	+	+
46356	*A. longipes*	Fruit	Fuji	Qld	Apr-05	+	-
46455	*A. longipes*	Leaf	Pink Lady	WA	May-05	+	-
47966	*A. longipes*	Leaf	unknown	Qld	Dec-05	+	+
46899	*A. longipes*	Leaf	unknown	Vic	May-05	+	-

[a] Accession numbers represent the BRIP codes of the isolates as coded by the Queensland Plant Pathology Herbarium, Brisbane, Australia

[b] Identity of the isolates as described by Harteveld et al. (2013)

[c] NSW: New South Wales Qld: Queensland; WA: Western Australia; Vic.: Victoria; SA: South Australia; Tas.: Tasmania

[d] +represents infection and disease symptoms; − represents no disease symptoms

performed by soaking the filter paper in sterile water. The inoculated leaf (including the filter paper) was placed in a sealed plastic bag with wet cotton wool to provide continuous high relative humidity (>90 %) and incubated at 25ºC in darkness. After 5 days, pathogenicity on the leaves was recorded, where pathogenic was indicated with a+and not pathogenic with a -. The experiment was repeated once.

To test the pathogenicity of the same set of isolates on fruit, *in planta* fruit inoculation assays were performed on nonwounded apples on trees of cultivar Royal Gala in an experimental orchard at the Applethorpe Research Station of the Department of Agriculture, Fisheries and Forestry in Applethorpe, Queensland. To prevent natural infection, fruit were arbitrarily selected in the trees and bagged with white waterproof T20 paper bags (Palmwoods Farm and Garden Supplies, Queensland) 2 months before inoculation. Maturity of fruit was determined by starch and sugar content tests as described by Chennell et al. (2002) and fruit were inoculated near maturity, 2 to 3 weeks before harvest. Three replicate fruit were used per isolate. Inoculations were performed by soaking two 0.5-cm^2 filter paper discs in a spore suspension which was then placed on the fruit surface. Filter paper discs were held in place using sterile tape. Filter paper discs soaked in sterile water served as control experiment. The fruit (including the soaked filter paper) were bagged with plastic seal bags containing wet cotton wool, to maintain high humidity conditions for 24 h, thereafter, the plastic sealed bags were replaced with white waterproof T20 paper bags. Each inoculated fruit was assessed for fruit spot symptoms 2 weeks post inoculation. The experiment was performed in the 2011–12 production season and repeated in the 2012–13 production season.

Host tissue specificity of *Alternaria* isolates

To test if host tissue specificity existed based on the tissue where the isolates were originally derived from, five isolates obtained from symptomatic fruit and 11 isolates obtained from symptomatic leaves were inoculated on

leaves and fruit as described above (Table 1). Cross-pathogenicity of the isolates on leaves and fruit of Royal Gala was examined in relation to the source host tissue of the isolates.

Virulence on apple leaves and fruit of Royal Gala

To test if isolates of the prevalent *A. arborescens* species are more virulent than isolates of the other three species present, detached leaf inoculations were performed as described above. When inoculating, a 1-cm diameter circle was drawn centered by the middle of the filter paper disc using a permanent marker. After 5 days, blotch development on the leaves was recorded and the virulence of the isolates was measured as the severity of the leaf blotch affected tissue within the circled area. The experiment was repeated once.

In planta fruit inoculations were performed as described above. To indicate the inoculated area, a 1-cm diameter circle was drawn around the filter paper disc using a permanent marker. Virulence of the isolates was defined by the number of fruit spots that appeared within the circled area on the fruit and recorded as spots/fruit. The experiment was performed in the 2011–12 production season and repeated in the 2012–13 production season.

Pathogenicity of *Alternaria* species to different apple cultivars

To determine if pathogenicity varied among the *Alternaria* species towards different apple cultivars, detached leaf inoculation assays were performed on leaves of cultivars Royal Gala, FB22-47, Galaxy, Red Delicious and Pink Lady™. Virulence was measured as described above and the experiment was repeated once.

In planta fruit inoculation assays were performed as described above on nonwounded fruits on trees of cultivars Royal Gala and FB22-47 in experimental orchards at the Applethorpe Research Station of the Department of Agriculture, Fisheries and Forestry in Applethorpe, Queensland. Virulence was recorded as described above. The experiment was performed in the 2011–12 and 2012–13 production seasons.

Data analysis

Statistical analysis was performed using GenStat 14th edition software (VSN International, Hertfordshire, UK). Virulence data for each experiment were analyzed using the general analysis of variance (ANOVA) procedure in GenStat. Significant variation in virulence of the isolates within each species group on leaves and fruit of each cultivar and their interactions was examined. Significant treatment means were compared using Fisher's protected Least Significant Difference (LSD), $P=0.05$. To further examine the pathogenic variation on leaves of five cultivars, multivariate analysis using principal component analysis (PCA) was performed and a correlation biplot of the first two PCA factors was produced using XLSTAT software version 2013.4 (Addinsoft, Paris, France). Isolates were treated as observations and the virulence (disease severity) on the cultivars was treated as variables for analysis. Associations of the virulence and cultivars were evaluated using the Pearson χ^2 statistic test. XLSTAT was used to calculate the eigenvalues of the components and the eigenvectors of the variables of the three most significant components.

Results

Pathogenicity on apple leaves and fruit of cultivar Royal Gala

All four species groups caused leaf blotch in the detached leaf assay (Table 1). There was variation between experiment 1 and 2. In Expt. 1 all the 16 isolates caused leaf blotch, whereas in Expt. 2 only 12 isolates caused distinct leaf blotch symptoms (Table 2). *A. tenuissima* isolates 46574 and 46361 and *A. arborescens* isolates 46452 and 46872 did not cause leaf blotch symptoms in Expt. 2 (Table 2). No disease symptoms appeared in the control experiments.

In the fruit inoculation assays *in planta*, *A. alternata*, *A. tenuissima* and *A. longipes* species groups caused fruit spot symptoms whereas, *A. arborescens* did not cause fruit spot symptoms (Table 1). Of the four isolates of each species group used, only two *A. alternata* isolates, three *A. tenuissima* isolates and one *A. longipes* isolate caused distinct fruit spot symptoms (Table 1). No disease symptoms were observed in the control experiments. Variation was observed between the two seasons. In the 2011–12 season, five isolates were pathogenic including two *A. alternata* isolates (46398 and 46590) and three *A. tenuissima* isolates (46361, 46574 and 54639), whereas in the 2012–13 season, four isolates were pathogenic, one *A. alternata* isolate (46398), two

Table 2 Pathogenicity of *Alternaria* isolates on leaves of five different cultivars based on detached leaf inoculation assays

Accession number[a]	*Alternaria* species group[b]	Experiment 1					Experiment 2				
		Royal Gala	Galaxy	FB22-47	Red Delicious	Pink Lady™	Royal Gala	Galaxy	FB22-47	Red Delicious	Pink Lady™
46452	*A. arborescens*	+[c]	+	+	-	+	-	-	-	-	-
46571	*A. arborescens*	+	+	+	+	+	+	+	-	-	-
46872	*A. arborescens*	+	+	+	+	+	-	-	-	-	+
46512	*A. arborescens*	+	+	+	+	+	+	-	+	+	+
46398	*A. alternata*	+	+	+	+	+	+	+	+	-	-
46550	*A. alternata*	+	+	+	+	-	+	+	-	-	-
46590	*A. alternata*	+	+	+	+	+	+	+	+	+	+
46545	*A. alternata*	+	+	+	+	+	+	+	-	-	-
46361	*A. tenuissima*	+	+	+	+	+	-	-	-	+	+
46574	*A. tenuissima*	+	+	+	+	+	-	-	-	-	-
46414	*A. tenuissima*	+	+	+	+	+	+	+	+	+	+
54639	*A. tenuissima*	+	+	+	+	+	+	-	+	-	+
46356	*A. longipes*	+	+	+	+	+	+	-	+	-	+
46455	*A. longipes*	+	+	+	+	+	+	+	+	+	+
47966	*A. longipes*	+	+	+	+	+	+	-	-	-	+
46899	*A. longipes*	+	+	+	+	+	+	-	-	-	+

[a] Accession numbers represent the BRIP codes of the isolates as coded by the Queensland Plant Pathology Herbarium, Brisbane, Australia

[b] Identity of the isolates as described by Harteveld et al. (2013)

[c] +represents infection and disease symptoms; − represents no disease symptoms

A. tenuissima isolates (46361 and 54639) and one *A. longipes* isolate (47966).

Host tissue specificity of *Alternaria* isolates

Isolates were not specific to the host tissue from which they were derived. Isolates from both leaves and fruit were cross-pathogenic (Table 1). All the isolates obtained from fruit spot and leaf blotch symptoms caused leaf blotch, whereas only 40 % of the isolates obtained from leaf blotch (four of 11 isolates) or fruit spot (two of five isolates) caused fruit spot symptoms in the pathogenicity assays (Table 1).

Virulence on apple leaves of Royal Gala

There was no significant difference in the mean disease severity caused by the different species groups, but significant variation in virulence was observed among isolates within the species groups ($P<0.001$). *A. alternata* isolate 46590 and *A. tenuissima* isolate 46414 were the most virulent isolates with the highest mean disease severity of 75 %, which was significantly higher than to the other isolates (Fig. 1). *A. arborescens* isolate 46571 was the least virulent isolate, but was only significantly lower than 50 % of the isolates (Fig. 1). The virulence of the isolates on the leaves was not significantly ($P=0.599$) affected by the host tissue from which the isolates were derived.

Variation between the two repeats of the experiment was significant ($P<0.001$), this variation was caused by a significantly higher disease severity in Expt. 1 than in Expt. 2. This variation was consistent for all isolates, and there was no significant interaction among isolates and both experiments. Therefore, further analysis was performed combining the data of the two experiments.

Virulence on apple fruit of Royal Gala

There was a large significant ($P<0.001$) variation in virulence among species and among isolates within species group causing fruit spot. Fruit spot severity

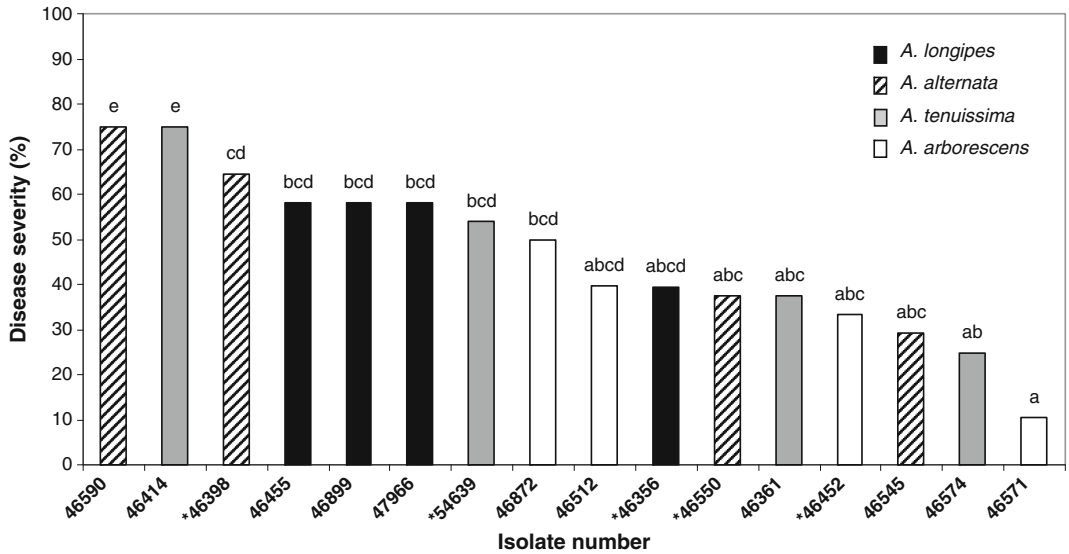

Fig. 1 Mean disease severity of *Alternaria* isolates of four species on apple leaves of cultivar Royal Gala. Isolates are ranked from the most virulent isolate at the top to least virulent at the bottom. * indicates isolates obtained from fruit tissue. Bars with the same letter are not significantly different according to Fisher's Protected Least Significant Difference at $P=0.05$

caused by the isolates was significantly higher in the 2011–12 season than the 2012–13 season (Table 3). In the 2011–12 season, the *A. alternata* and *A. tenuissima* species group each caused an average of 2.0 fruit spots, whereas *A. arborescens* and *A. longipes* did not cause fruit spot. In the 2012–13 season the mean disease severity caused by *A. longipes* (0.02), *A. alternata* (0.04) and *A. tenuissima* (0.08) was not significantly different ($P=0.361$). Isolates within species groups showed significant ($P<0.001$) variation in virulence in both seasons (Table 3). Based on the mean disease severity caused on fruit, *A. tenuissima* isolate 54639 was the most virulent in both seasons followed by *A. alternata* isolate 46398 and *A. tenuissima* isolate 46361 (Table 3).

The isolates obtained from symptomatic fruit caused a mean disease severity of 2.2 spots in the 2011–12 and 0.2 spots in the 2012–13 season, which was significantly higher than the disease severity of mean of 0.6 spots and 0.03 spots caused by leaf isolates during the two seasons ($P<0.001$).

Table 3 Mean severity of fruit spot of the six *Alternaria* isolates that were pathogenic on apple fruit of the cultivars Royal Gala and FB22-47 under field conditions

Isolate[a]	Species group	Host tissue	2011–12 season		2012–13 season	
			Royal Gala	FB22-47	Royal Gala	FB22-47
54639	*A. tenuissima*	Fruit	4.3 b[bc]	0.2	0.5 b	0
46398	*A. alternata*	Fruit	3.2 b	0	0.3 ab	0
46361	*A. tenuissima*	Leaf	3.0 b	0.2	0.2 ab	0
46590	*A. alternata*	Leaf	0.8 a	0	0 a	0
46574	*A. tenuissima*	Leaf	0.2 a	0	0 a	0
47966	*A. longipes*	Leaf	0 a	0	0.2 ab	0

[a] BRIP number as coded by the Queensland Plant Pathology Herbarium, Brisbane, Australia

[b] Mean disease severity in spots/fruit

[c] Values with the same letter are not significantly different according to Fisher's protected Least Significant Difference at $P = 0.05$

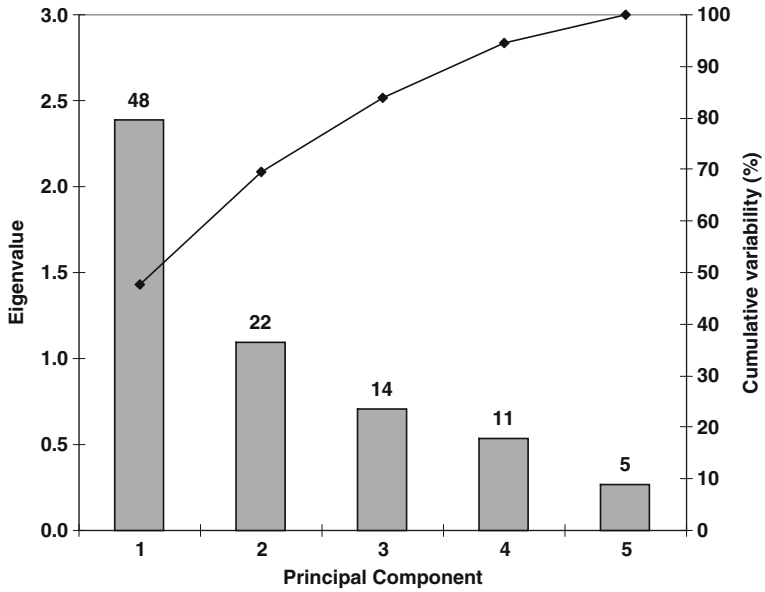

Fig. 2 Plot of principal components and the cumulative percentage of the variance explained for each component testing pathogenic variation of 16 *Alternaria* isolates on five apple cultivars. Values indicate percentage variance explained for each principal component

Pathogenicity of *Alternaria* isolates on leaves of different apple cultivars

All four species groups were pathogenic on the five apple cultivars tested. Significant variation was observed in pathogenicity of the isolates among the five cultivars between the two experiments. In Expt. 1 all isolates were pathogenic on the five cultivars tested, except *A. arborescens* isolate 46452 on Red Delicious and *A. alternata* isolate 46550 on Pink Lady™ (Table 2). Whereas, in Expt. 2, of the 16 isolates, 12 caused leaf blotch on Royal Gala, 10 on Pink Lady™, 7 on Galaxy and FB22-47 and five on Red Delicious caused leaf blotch (Table 2). *A. tenuissima* isolate 46414, *A. alternata* isolate 46590 and *A. longipes* isolate 46899 were the most pathogenic isolates producing disease symptoms on all the five cultivars tested in both experiments.

The principal component analysis of the disease severity data of the 16 *Alternaria* isolates on the five cultivars showed that the first three components explained 84 % of the total variance (Fig. 2). The first principal component showed a positive contribution of each of the cultivars (Table 4) and represented the variance in disease severity caused by the cultivars. The second component showed a strong positive coefficient for Pink Lady™ and negative for Red Delicious and FB22-47 (Table 4). The third component demonstrated a strong positive coefficient for Royal Gala whereas all others showed negative associations (Table 4). These two components account for the variance associated with cultivar resistance to the isolates. The correlation biplot of the two most significant factors showed that the isolates did not aggregate based on pathogenic variation of each species group or on pathogenicity to a specific cultivar. *A. longipes* isolates clustered around the axis of Pink Lady™ and Galaxy, showing that these isolates may be more pathogenic to these cultivars than the other three cultivars, whereas *A. tenuissima* and *A. alternata* isolates aggregated around FB22-47, Red Delicious and Royal Gala, indicating preferential pathogenicity to these cultivars. *A. arborescens* isolates did not show strong association with any of the cultivars and were further separated from the other isolates, indicating low levels of pathogenicity compared to the other isolates on the cultivars tested (Fig. 3).

Table 4 Weighting (eigenvectors) of the first three principal components of pathogenic variation of leaf blotch of 16 *Alternaria* isolates on five apple cultivars

Cultivar	Principal Component		
	1	2	3
Pink Lady™	0.17	0.89	−0.13
Royal Gala	0.42	0.10	0.90
Red Delicious	0.48	−0.26	−0.19
Galaxy	0.53	0.20	−0.33
FB22-47	0.54	−0.31	−0.16

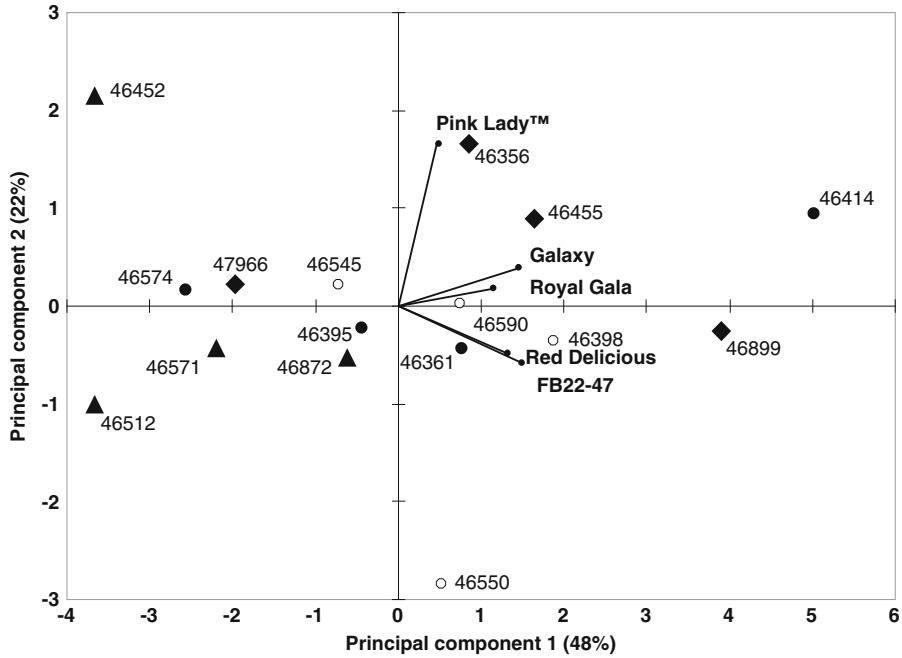

Fig. 3 Correlation biplot of the first two principal components that explained a total of 70 % of the variance showing the pathogenic variation of leaf blotch of 16 *Alternaria* isolates constituting four isolates of four different species groups (*A. arborescens* (▲), *A. tenuissima* (●), *A. alternata* (○) and *A. longipes* (cpfcpy)) on five apple cultivars: Royal Gala, Galaxy, FB22-47, Red Delicious and Pink Lady™

Pathogenicity of *Alternaria* isolates on fruit of different apple cultivars

Comparison of the pathogenicity of the isolates on inoculated fruit of cultivars Royal Gala and FB22-47 showed that six of the 16 isolates, representing *A. alternata*, *A. tenuissima* and *A. longipes* were pathogenic on fruit of Royal Gala (Table 3) and two isolates of the *A. tenuissima* group (46361 and 54639) were pathogenic on fruit of the cultivar FB22-47 only in the 2011–12 season (Table 3).

Discussion

In this study we used detached leaf and *in planta* fruit inoculation assays to demonstrate the pathogenic variation of *Alternaria* isolates of four species groups associated with leaf blotch and fruit spot of apple. The results showed that all four species groups described by Harteveld et al. (2013) are pathogenic to leaves and mainly two species groups *A. tenuissima* and *A. alternata* were pathogenic to fruit. Considerable variation was shown in pathogenicity and virulence among isolates within the species groups. Pathogenicity of isolates was not restricted by the origin of the host tissue where the isolates were obtained as the isolates showed cross-pathogenicity to both leaves and fruit. These findings indicate the possible development of fruit spot in the regions where only the leaf blotch currently occurs and fruit spot has not been reported.

This study clearly demonstrated that multiple *Alternaria* species groups; *A. arborescens*, *A. longipes*, *A. tenuissima* and *A. alternata* are responsible for leaf blotch and fruit spot of apple in Australia. Our findings are congruent with the result of Rotondo et al. (2012) in Italy where *A. arborescens*, *A. tenuissima* and *A. alternata* were similarly associated with both diseases. Multiple *Alternaria* spp. have been reported to cause similar diseases in various crops, such as Alternaria late blight of pistachio (Pryor and Michailides 2002), Alternaria leaf spot of almond (Teviotdale et al. 2001), brown apical necrosis and grey necrosis in hazelnut and walnut (Belisario et al. 2004) and Alternaria diseases in citrus (Peever et al. Peever et al. 2004). The reasons for multiple species causing the same disease on one host are not understood and species delineation within the genus *Alternaria* needs careful attention. Further research is also

needed to determine the role of genetic recombination in speciation and pathogenicity of the *Alternaria* spp. affecting fruit and nut trees.

A. arborescens was the most frequently isolated species from leaves with leaf blotch symptoms, constituting 51 % of all *Alternaria* isolates obtained from all apple-growing regions in Australia (Harteveld et al. 2013). However this study showed that the widespread prevalence of the species is not due to its pathogenicity or greater virulence over the other species groups on leaves. Whether *A. arborescens* has other competitive or ecological fitness advantages over the other species groups, which could explain the high prevalence of the species, is not known. Biological or ecological fitness of a pathogen is a combination of pathogenic fitness traits, such as aggressiveness and infection efficiency (Leach et al. 2001) with other fitness traits, such as mating behaviour, sporulation and growth rate (Pringle and Taylor 2002).

The general high virulence of the *A. tenuissima*, *A. alternata* and *A. longipes* isolates, which were mainly obtained from the Queensland and New South Wales growing regions, may account for the higher leaf blotch and fruit spot severities commonly observed in these growing regions compared to other regions in Australia. However, the difference in environmental conditions between these regions is significant and needs to be given due consideration. Isolates of *A. tenuissima*, *A. alternata* and *A. longipes* were also obtained from other apple orchards in Tasmania, Western Australia, Victoria and South Australia (Harteveld et al. 2013) indicating that under favourable conditions, severe fruit spot may occur in these regions as well.

All four species groups were pathogenic on leaves of Royal Gala, Galaxy, Red Delicious, FB22-47 and Pink Lady™. The findings indicate that there is little, or no, cultivar specificity in the species groups and signify the potential of the species to cause leaf blotch disease in all the five cultivars in regions where leaf blotch has been reported. The results of the fruit inoculations using fruit of the two cultivars Royal Gala and FB22-47 showed a lower level of pathogenicity on FB22-47. Although the *A. tenuissima* isolates 54639 and 46361 infected fruit of FB22-47 shows that the cultivar is susceptible and may indicate that these isolates are more pathogenic than the other isolates. Further research using more cultivars is needed to fully understand the putative cultivar specificity among the *Alternaria* species groups causing fruit spot of apple. The infection process of the different *Alternaria* species groups affecting apple fruit has not been investigated. It was observed that the skin of FB22-47 fruit has a smoother skin texture than Royal Gala fruit. The fruit skin texture may play an important role in the infection of apple pathogens (Konarska 2012) which may warrant further research.

The *Alternaria* isolates showed considerable variation in their pathogenicity and virulence to leaves and fruit within species groups. Similar observations on *Alternaria* isolates causing leaf blotch and fruit spot in Italy have been reported (Rotondo et al. 2012). Our study supports the conclusion that pathogenicity of *Alternaria* isolates affecting leaves and fruit of apple may be acquired independently (Rotondo et al. 2012). In addition, although the species groups of *A. arborescens* and *A. longipes* are mostly related to the leaf blotch variant of the disease, occasional isolates show pathogenicity to fruit (Harteveld et al. 2013). It is currently unclear how *Alternaria* isolates acquire their pathogenicity. The involvement of specific metabolites has been proposed, for example the three species groups *A. tenuissima*, *A. arborescens* and *A. alternata* produce different metabolites (Logrieco et al. 2009) and *A. tenuissima* isolates produce more toxic compounds than the other two species groups (Greco et al. 2012). The involvement of isolate specific metabolites has also been indicated (Andersen et al. 2002), which may explain the variability observed among isolates within species groups. More recently, recombination and horizontal gene transfer between *Alternaria* isolates have been reported, which may be responsible for the exchange of pathogenicity genes between isolates and species (Hu et al. 2012; Stewart et al. 2013) and may explain the lack of a clear definition between species groups with regards to their pathogenicity.

There was significant variation between Expt. 1 and 2 of the detached leaf inoculation assays. Disease severity was consistently lower in Expt. 2 in all the isolates. Although attempts were made to use leaves of similar age and kept in the same conditions in both experiments, this variation in disease severity could be due to the physiology or phenology of the potted trees. The potted trees were kept in a shade house and, although under netting, were subjected to weather and seasonal changes, which may have affected the status of the leaves and their susceptibility. In addition, the oil and soap sprays to control mites may have influenced the state of the leaves. In the fruit inoculation trial in the orchard, the conditions for artificial inoculation were performed as

similarly as possible, nevertheless large variation was observed in disease severity between the two seasons, with very low disease levels in the second season which may be due to weather and seasonal variation between the two seasons. Another possible explanation for the variation could be loss of pathogenicity due to culturing or storage of *Alternaria* isolates (Lloyd, 1969).

In conclusion, this study provides a general indication of the disease risks imposed by each of the *Alternaria* species groups. Due to the variability in pathogenicity and aggressiveness among *Alternaria* species and isolates within the species, care must be taken when selecting isolates and specific species groups to evaluate apple germplasm for resistance to leaf blotch and fruit spot. Our results will serve as a foundation for further research towards improved, targeted control strategies. These findings are of great epidemiological importance for the regions where fruit spot has not yet been reported. Under conducive conditions fruit spot may potentially affect all apple orchards in Australia. Hence, the development of disease management strategies for Alternaria leaf blotch should include fruit spot.

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