Isolation, pathogenicity and storage of New Zealand *Alternaria* species from *Malus × domestica*

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Abstract The pathogenicity of *Alternaria* spp. on *Malus × domestica* in New Zealand has not been investigated fully. *Alternaria* spp. were isolated from necrotic spots on leaves and fruit rots from various apple cultivars. Pathogenicity of 31 *Alternaria* spp. isolates was tested on excised 'Royal Gala', 'Golden Delicious' and 'Scifresh' leaves (wounded and unwounded) by measuring lesion diameters. Viability of *Alternaria* spp. grown from infected leaf material was assessed to compare various storage conditions. *Alternaria* spp. were consistently isolated from the central brown area of leaf lesions but could not be isolated from outside the leading edge or from the purple region surrounding the lesion. All isolates produced lesions on wounded leaves. Most isolates showed some pathogenicity on at least one cultivar, with lesions on unwounded leaves. Lesions on leaf material caused by *Alternaria* spp. can be stored for at least 11 weeks by freeze-drying or air-drying leaf material and storing at 0.5°C or room temperature. Some New Zealand *Alternaria* spp. isolates on *Malus × domestica* are pathogenic on fruit and leaves. Further studies are needed to better understand the biology of this pathogen in New Zealand and determine a suitable control programme.

Keywords *Alternaria*, isolation, storage, pathogenicity.

INTRODUCTION

Alternaria alternata is one of the most cosmopolitan fungal species and is generally considered saprophytic (Li et al. 2013). *Alternaria alternata* was first recorded as a fruit rot on apple in New Zealand as *Alternaria tenuis* in 1929 (Kidd 1929) and was later described as *Alternaria alternata* in 1969 (Dingley 1969).

Different *Alternaria* species on apple can cause symptoms on leaves, young shoots and fruit (Li et al. 2013). Leaf symptoms first appear as small round blackish spots which gradually enlarge in circular zonate rings to become a lesion consisting of a brown centre with a brownish-purple border.

The taxonomy of *Alternaria* species associated with *Malus* is complicated. A number of *Alternaria* species can cause similar symptoms

and often identification to species level is not carried out or only partially completed. *Alternaria* species associated with apple and other tree crops are small-spored species including *A. mali*, *A. infectoria*, *A. arborescens*, *A. alternata* and *A. tenuissima* (Simmons 1999; Serdani et al. 2002; Hong et al. 2006; Cannibal et al. 2008; Rotondo et al. 2012). *Alternaria mali* is the causal agent of alternaria leaf blotch which causes spotted leaves and severe defoliation. Other *Alternaria* species cause leaf blotch and fruit spot symptoms. (Sawamura & Yukita 2014). Rotondo et al. (2012) found that three *Alternaria* species, *A. arborescens*, *A. alternata* and *A. tenuissima* were identified to cause alternaria leaf blotch and fruit spot in Italy. Hartevelde et al. (2014a) suggest there are multiple phylogenetic *Alternaria* species-groups in Australia associated with

leaf blotch and fruit spot on apple including *A. arborescens*, *A. tenuissima*, *A. mali*, *A. alternata*, and *A. longipes*.

Alternaria leaf blotch has been found to cause up to 95% defoliation in the USA in the late growing season, reducing tree vigour, fruit quality and yield in the subsequent seasons (Suzuki et al. 1987; Filajdic & Sutton 1991, 1995; Hartevelde et al. 2014b;). In Australia, up to 50% defoliation was found to occur in New South Wales and Queensland production areas early in the growing season (Hartevelde et al. 2014b). Little information is available about the impact of *alternaria* fruit spot worldwide, but in Australia, fruit losses to individual growers in Queensland and New South Wales have been estimated to be 15–25% (Hartevelde et al. 2014b). Until very recently, there has been little published information on the incidence and pathogenicity of *Alternaria* spp. on *Malus* spp. in New Zealand. Toome-Heller et al, 2018 investigated the pathogenicity of two New Zealand *Alternaria* spp. isolates and found these were weak pathogens. The work reported here investigated whether leaf lesions collected in New Zealand and tentatively identified as *Alternaria* spp. were correctly diagnosed, whether collected isolates were saprophytic or pathogenic, and how to successfully store infected leaf material to establish an isolate collection at a later date.

MATERIALS AND METHODS

Isolation of *Alternaria* spp.

A wide range of necrotic spots on leaves from the apple cultivars ‘Koru’, ‘Royal Gala’, ‘Braeburn’, ‘Scifresh’ and ‘Cox’s Orange Pippin’ were collected by horticultural consultants in December 2016 from orchards in the Nelson region of New Zealand. Receipted samples were photographed and labelled. Initially, fungal isolations were made by surface-disinfesting fragments of necrotic spots on leaves in 0.5% NaOCl, double-rinsing in sterile water, plating onto half-strength potato dextrose agar amended with 5 ppm ampicillin and 10 ppm streptomycin (PDA+) and grown at 20°C under natural light. Subsequently, leaf lesions typical of those previously yielding *Alternaria*

spp. were dissected longitudinally into three sections (outside leading edge, edge of lesion, and central brown area of lesion) and plated onto half strength PDA+. Pure cultures were obtained using the hyphal tip method. Isolates were grown at 20°C under natural light and identified by spore morphology as being *Alternaria* spp.. Fruit rots from ‘Scifresh’ apples were also photographed, dissected, plated and grown onto half-strength PDA amended with antibiotics as described above.

Pathogenicity of isolates

Excised leaves, either wounded or unwounded, of different apple cultivars (‘Golden Delicious’, ‘Royal Gala’ and ‘Scifresh’) were placed into a plastic chamber. Each chamber had a moist paper towel in the bottom to maintain high relative humidity. ‘Golden Delicious’ and ‘Royal Gala’ leaves (unwounded and wounded using a scalpel to make a 1-mm cut) were challenged with one of 31 different *Alternaria* spp. isolates (four from fruit and 27 from leaves) by placement of a 2-mm diameter plug of a 6-week-old *Alternaria* spp. culture containing spores. ‘Scifresh’ leaves (unwounded and wounded as described above) were challenged with one of eighteen different *Alternaria* spp. isolates (four from fruit and 14 from leaves). Control treatments were challenged as described above using a 2-mm plug of half strength PDA agar. There were six replicate leaves for each isolate × cultivar × wounded/unwounded combination. Leaves were kept moist, incubated at 20°C and lesion diameters measured after 4, 6 and 8 days post-inoculation. Data were analysed using a binomial generalised linear model in Genstat (version 17, 2014, VSNi Ltd, Hemel Hempstead, UK).

Storage of *Alternaria* spp. isolates

Five 2-cm² segments of ‘Royal Gala’ leaves containing characteristic spots caused by *Alternaria* spp. were stored under the various conditions as outlined in Table 1. After 11 weeks, each of the five leaf pieces in each storage treatment were plated onto PDA+ and grown at 20°C. Culture plates were assessed for *Alternaria* spp. growth after 5 days.

Table 1 Isolation success of *Alternaria* spp. from leaves putatively infected with *Alternaria* spp. following 11-weeks' storage under various conditions. Data are number of successful isolations out of five plated leaf lesions.

Treatment	Positive isolations
Room temperature	5/5
Freeze dried then placed in desiccator at 0.5°C	5/5
Freeze dried then held at room temperature	5/5
Leaf samples directly frozen at -80°C	0/5
Leaf samples frozen at -20°C	0/5
Leaf samples suspended in Luria broth + 20% glycerol (LB+) and frozen at -20°C	0/5
Leaf samples suspended in LB+ and frozen at -80°C	0/5
Leaf samples suspended in LB+ and stored at 0.5°C	0/5
Leaf samples suspended in LB+ and held at 1°C for 10 h followed by -1°C for 10 h followed by -10°C for 24 h then put into -80°C freezer	0/5
Leaf samples held at 1°C for 10 h followed by -1°C for 10 h followed by -10°C for 24 h then put into -80°C freezer	0/5

RESULTS

Isolation of *Alternaria* spp.

Alternaria spp. were consistently isolated from leaf lesions that had a purple margin with a brown centre (Fig. 1) but rarely from other lesion types. Many submitted leaf samples that were thought to be infected with *Alternaria* spp. had lesions that did not have a distinctive purple margin. In most cases, *Alternaria* spp. were not isolated from lesions that did not have the purple margin. Micro-organisms isolated from these lesions included *Phoma* spp. and *Colletotrichum* spp.. When purple-margined leaf lesions were dissected longitudinally, *Alternaria* spp. were isolated from the central necrotic portion of the lesions but not from outside the leading edge of lesions or from within the characteristic purple region.

Alternaria spp. were readily isolated from fruit rots where lesions were typically firm, dark brown and sunken (Fig. 2). In cross-section, the lesions were shallow and black. The appearance of isolates from infected material on PDA agar was grey to black with concentric rings. Growth rates varied between isolates. Conidia were a tan colour with walls and septa a darker tan colour. Conidia were short-ovoid to ellipsoid in shape

Figure 1 Lesion on apple leaf caused by *Alternaria* spp. showing characteristic purple brown margin.

Figure 2 Fruit rot on 'Scifresh' apple caused by *Alternaria* spp..

with 1–4 transepta. Sometimes a longitudinal septum was observed. Primary conidiophores were long and subsequent conidiophores were sometimes geniculate.

Pathogenicity of isolates

The percentage of leaves that formed lesions when challenged with the different *Alternaria* spp. isolates is shown in Figure 3. The sterile control did not cause lesions on wounded or unwounded leaves, whereas all isolates caused lesions on some excised leaves. The lesions on the excised leaves were chocolate brown in colour. Sometimes the leading edge of the lesion was a lighter colour. However, this was not consistent. The purple margin observed on the field-collected leaves was not observed on the inoculated leaves. The cultivar, isolate and leaf wounding all had a significant effect on the incidence of leaf lesions (Table 2) with wounding, followed by isolate having the most significant effects. In addition, the two factor interactions were also significant where the different isolates performed differently with different cultivars, the difference between wounded and unwounded leaves varied between cultivars, and the difference between wounded and unwounded varied between isolates. However, the interaction between the three factors was not significant.

All isolates caused lesions on some of the wounded leaves. However, many of the isolates did not cause lesions on unwounded leaves. Diameter of lesions in the various wounded or unwounded apple cultivar leaves are shown in Figure 4. Lesions were generally larger on wounded than unwounded leaves. However, a few isolates had lesions of similar size on wounded and unwounded leaves, but this was not consistent across cultivars. Overall, 'Golden Delicious' had smaller lesions compared with 'Scifresh' and 'Royal Gala'.

Storage of *Alternaria* spp. isolates

Alternaria spp. viability after various storage conditions is given in Table 1. Leaf samples containing putative *Alternaria* spp. infections, which were stored at room temperature or freeze

dried and stored at either room temperature or 0.5°C, remained viable for at least 11 weeks. Samples which were suspended in a liquid media and/or frozen did not grow when plated onto agar.

DISCUSSION

Alternaria spp. were readily isolated from leaf lesions but usually when they exhibited the characteristic purple margin. Dissection and plating of the leaf region in and around the lesion indicates that the purple portion of the lesion may be a plant response to the infection as no *Alternaria* spp. were isolated from within the purple coloured tissue. The absence of a purple margin on lesions on the inoculated excised leaves also suggests that it may be a plant response. This could be due to the leaf being detached from the tree or the coloured margin may take time to develop. The frequent submission by technical consultants of leaf samples with old lesions thought to be infected with *Alternaria* spp. but not exhibiting the classical symptoms suggests that better education is needed to identify lesions on leaves caused by *Alternaria* spp..

Alternaria spp. are generally considered saprophytic fungi so it is not surprising that damage to leaves had a significant effect on the subsequent incidence of lesions of the inoculated leaves. However, many *Alternaria* spp. isolates caused lesions on unwounded leaves in the current study, indicating pathogenic ability. While *Alternaria* spp. may infect uninjured leaves, the presence of alternaria leaf spot in New Zealand orchards is commonly associated with trees that are stressed, through weather or water. A New Zealand study by Toome-Heller et al. in 2018 found that the pathogenicity of two New Zealand *Alternaria* spp. isolates were weak pathogens which is consistent with the findings in this research. Some isolates which were derived from fruit lesions readily infected leaves, suggesting that isolates can infect both fruit and leaves. Similarly, research in Australia using detached leaf assays and fruit inoculations showed that isolates within each of the species groups *A. arborescens*, *A. tenuissima*/*A. mali*, *A.*

Figure 3 Percentage of lesions on wounded (o) and unwounded (x) excised apple leaves (cultivars 'Golden Delicious', 'Royal Gala' and 'Scifresh') challenged with up to 31 different *Alternaria* spp. isolates 8 days post inoculation. Isolate 0 is sterile control.

Table 2 Analysis results of the effect of apple cultivar (apple cv), wounding and isolate on the incidence of *Alternaria* spp. lesions on excised apple leaves using a binomial generalised model.

	4 days		6 days		8 days	
	Deviance	P	Deviance	P	Deviance	P
Apple_cv (2 df)	8.7	0.013	21.4	<.001	28.9	<.001
Wound (1 df)	519.3	<.001	517.4	<.001	480.3	<.001
Isolate (31 df)	192.7	<.001	163.0	<.001	162.6	<.001
Apple_cv x Wound (2 df)	15.0	<.001	16.4	<.001	23.0	<.001
Apple_cv x Isolate (49 df)	82.3	0.002	94.1	<.001	85.0	0.001
Wound x Isolate (31 df)	83.1	<.001	69.6	<.001	73.5	<.001
Apple_cv x Wound x Isolate (49 df)	33.7	0.953	18.8	1.000	25.4	0.998

alternata/A. *tenuissima* and A. *longipes* were not specific to either fruit or leaf tissue (Harteveld et al. 2014b). The investigators also found a high level of variability in pathogenicity and aggressiveness which was isolate-specific rather than species-specific (Harteveld et al. 2014b). The conidial and conioophore morphology from the isolates in this study suggests that they may belong to the *Alternaria arborescens* complex (Simmons 2007), which is a similar finding to the Toome-Heller et al. (2018) work. There was some evidence in the current study for an interaction between isolate pathogenicity and cultivar, but this would need to be confirmed in repeated experiments. If confirmed, it would show a potential to identify resistance genes that could be incorporated into an apple breeding programme.

'Golden Delicious' is very susceptible to *Alternaria* spp. infection (Li et al. 2011), yet the results presented here showed that 'Scifresh' and 'Royal Gala' had a greater lesion size compared to 'Golden Delicious', which suggests that these new cultivars are even more susceptible to *Alternaria* spp. infection. Both 'Scifresh' and 'Royal Gala' have 'Golden Delicious' in their parentage, with 'Royal Gala' derived from a cross between 'Kidd's Orange' and 'Golden Delicious' (Brooks & Olmo 1972) and 'Scifresh' derived from a cross between 'Royal Gala' and the cultivar 'Braeburn' (Volz et al. 2004). Presumably, the susceptibility of 'Golden Delicious' has been inherited by 'Royal Gala' and 'Scifresh' as 'Braeburn' is considered relatively resistant to *Alternaria* spp..

The purpose of the leaf lesion storage trials was to be able to store infected leaf material long term without the need to produce isolates immediately. Laboratories can be busy during the spring period and unable to carry out isolations from large sample numbers within a short space of time. In addition, it was hoped that the ability to store leaf lesions rather than cultures long term may help maintain pathogenicity within isolates (not included in this work). This research showed that *Alternaria* spp. could be stored and remain viable on leaf material for up to 11 weeks by freeze drying or air-drying material and storing at 0.5° C or room temperature. *Alternaria* spp. did not remain viable when leaf material was stored in Lauria broth with glycerol and frozen to either -20 or -80°C. This result was surprising as many fungal spores remain viable when suspended in glycerol and frozen at either -20 or -80°C (Hartveld et al. 2012).

Findings from this work suggest that some New Zealand *Alternaria* spp. isolates on *Malus* spp. are pathogenic on fruit and leaves. Further studies may help to better understand its biology in New Zealand and determine a suitable control programme.

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Figure 4 Lesion diameter (mm) on wounded (o) and unwounded (x) excised apple leaves (cultivars 'Golden Delicious', 'Royal Gala' and 'Scifresh') challenged with up to 31 different *Alternaria* spp. isolates 8 days post inoculation. Isolate 0 is sterile control.

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