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Cross pathogenicity of *Neofusicoccum australe* and *Neofusicoccum stellenboschiana* on grapevine and selected fruit and ornamental trees

Kabo MOJEREMANE[1], Palesa LEBENYA[2], Ihan L. DU PLESSIS[2,3], Marieta VAN DER RIJST[4], Lizel MOSTERT[3], Josep ARMENGOL[1], Francois HALLEEN[2,3,*]

[1] *Instituto Agroforestal Mediterráneo (IAM), Universitat Politècnica de València (UPV), Camino de Vera S/N, 46022-Valencia, Spain*
[2] *Plant Protection Division, ARC-Infruitec Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa*
[3] *Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*
[4] *ARC Biometry Unit, Private Bag X5026, Stellenbosch, 7599, South Africa*
*Corresponding author. E-mail: halleenf@arc.agric.za

Summary. *Neofusicoccum australe* is one of the most important *Botryosphaeriaceae* pathogens occurring on fruit and vine crops. This fungus was recently taxonomically reassessed, identifying *N. stellenboschiana* as a separate species. Previous pathogenicity studies used *N. stellenboschiana* and *N. australe* isolates as *N. australe*, so assessment of the pathogenicity of these two species on grapevine and other hosts was required. A pathogenicity trial was conducted on detached shoots of grapevine, plum, apple, olive and Peruvian pepper tree. Shoots were individually inoculated with 11 *N. australe* and eight *N. stellenboschiana* isolates originally isolated from grapevine, plum, apple, olive, Peruvian pepper and fig. Both species formed lesions on all five hosts and were re-isolated 5 weeks post-inoculation. In general, the largest lesions were formed on plum and smallest on Peruvian pepper. Isolate host origin did not influence ability to cause lesions on other hosts. Isolates of *N. australe* and *N. stellenboschiana* differed in virulence on the various hosts, ranging from those that caused the largest lesions, a group causing intermediate lesions, and another causing lesions similar to uninoculated controls. The study demonstrates that *N. australe* and *N. stellenboschiana* isolates originating from various fruit hosts can infect alternative hosts including grapevine and other major fruit crops.

Keywords. *Botryosphaeriaceae*, pome fruit, stone fruit, olive, virulence.

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INTRODUCTION

Grapevine and fruit industries play major roles in the South African economy, in particular in the Western Cape Province. Approximately

125,800 ha are planted with grapevine, 36,421 ha with pome fruit and 17,680 ha with stone fruit (Hortgro, 2017). In recent years, several other fruit crops, including olive, have also become of major importance. Olive plantings increased by 135% to 3190 ha during the last 11 years, of which 92% are planted in the Western Cape (Hortgro, 2020). Fungi in the *Botryosphaeriaceae* are well-recognized trunk pathogens of grapevine, pome and stone fruit trees, as well as olives (Slippers *et al*., 2007; Úrbez-Torres and Gubler, 2009; Cloete *et al*., 2011).

Twenty-six species from *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinisa* have been reported as pathogens of grapevine (Billones-Baaijens and Savocchia, 2019). Although pathogenicity and virulence vary between species, numerous studies have shown that these fungi are capable of colonising grapevine wood causing disease, and are recognised as important grapevine trunk pathogens. Some of the most virulent grapevine trunk pathogens include *L. theobromae*, *N. australe*, *N. luteum, N. parvum, B. dothidea, D. mutila* and *D. seriata* (Van Niekerk *et al*., 2004; Úrbez-Torres and Gubler, 2009; Qiu *et al*., 2016; Billones-Baaijens and Savocchia, 2019). *Botryosphaeriaceae* reported from or associated with trunk diseases on apple include *B. dothidea*, *D. bulgarica*, *D. intermedia*, *D. malorum*, *D. mutila*, *D. seriata*, *N. australe*, *N. parvum*, *N. stellenboschiana, N. algeriense*, *N. vitifusiforme, N. viticlavatum*, and *N. ribis* (Slippers *et al*., 2007; Cloete *et al.,* 2011; Phillips *et al*., 2012; Lopes *et al*., 2016; Zafari and Soleiman, 2017; Havenga *et al*., 2019). In South Africa, several *Botryosphaeriaceae* have been associated with Botryosphaeria dieback in stone fruit including plum. These pathogens include *D. africana*, *D. mutila*, *D. pinea*, *D. seriata*, *D. viticola*, *L. plurivora*, *N. australe*, and *N. vitifusiforme* (Damm *et al.,* 2007, Slippers *et al.,* 2007). Some of the species associated with olive trunk diseases worldwide include *B. dothidea*, *D. corticola*, *D. mutila, D. seriata*, *Dothiorella iberica*, *L. theobromae*, *N. luteum, N. mediterraneum*, *N. parvum*, *N. vitifusiforme, N. australe*, and *N. ribis* (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al.,* 2010; 2017; 2019; Kaliterna *et al*., 2012; Carlucci *et al*., 2013; Úrbez-Torres *et al.,* 2013; Triki *et al*., 2014; Lopes *et al*., 2016; Spies *et al*., 2020).

Botryosphaeriaceae can infect grapevines and fruit trees through wounds, especially through those caused by pruning (Van Niekerk *et al*., 2010; Kotze *et al*., 2011; Havenga *et al*., 2019). Symptoms associated with Botryosphaeria dieback in grapevine include shoot dieback, wood necroses and perennial cankers (*Úrbez-Torres,* 2011). These symptoms are usually observed in the field 1-2 years after infection, and mainly occur in mature vineyards that are 8 years and older (Úrbez-Torres *et al.,* 2008). In cross sections through affected plant parts, symptoms are wedge- and arch-shaped lesions, watery discolouration of the wood, and sometimes also necrotic spots. Other symptoms include moderate chlorosis of the leaves caused by some species of *Botryosphaeriaceae* (Van Niekerk *et al.,* 2006; White *et al*., 2011). As trunk pathogens of pome and stone fruit trees, *Botryosphaeriaceae* are associated with stem and branch cankers, dieback, twig blights, wood rots, bark discolouration, gummoses, scaling-off of the host bark, and tree death in severe cases (Slippers *et al.,* 2007; Cloete *et al.,* 2011; Zafari and Soleimani, 2017). In olive trees, *Botryosphaeriaceae* cause cankers, twig dieback and branch dieback (Romero *et al.,* 2005; Moral *et al.,* 2017). Cankers are usually observed in stem, branch or trunk cross sections, and these reduce water and nutrient movement through host vascular tissues (Úrbez-Torres *et al.,* 2013).

In the Western Cape, vineyards are usually located in close proximity to fruit orchards and other woody plants used as ornamentals and windbreaks. In recent years, many unproductive vineyards have been removed and replanted with fruit orchards, and *vice versa*.

Within the South African grapevine and fruit industries, *N. australe* is recognized as one of the most important *Botryosphaeriaceae* pathogens. In pathogenicity studies conducted with detached green and mature grapevine shoots, as well as mature field grown vines, *N. australe* consistently produced the largest lesions or lesions within the largest lesion group (Van Niekerk *et al*., 2004). Pathogenicity studies conducted on detached green nectarine and plum shoots, also showed that *N. australe* caused lesions within the largest lesion group (Damm *et al*., 2007). Furthermore, on detached woody apple and pear shoots, the pathogen caused lesions within the largest group (Cloete *et al*., 2011).

Neofusicoccum australe recently underwent taxonomic reassessment (Yang *et al*., 2017) and South African isolates from fruit trees and grapevine previously identified as *N. australe* now also include *N. stellenboschiana*. Some of the previous pathogenicity studies (e.g. Van Niekerk *et al*., 2004) used *N. stellenboschiana* and *N. australe* isolates identified as "*N. australe*". Therefore, it is important to conduct pathogenicity tests with these two species on grapevine, as well as multiple hosts representative of the woody host diversity found close to vineyards, to determine whether these plants can be alternative hosts. In the present study, *N. australe* and *N. stellenboschiana* cultures previously isolated from grapevine and other woody hosts were subjected to cross pathogenicity studies on grapevine, apple, plum, olive and Peruvian pepper, to determine if these hosts could be alternative hosts to each other.

MATERIALS AND METHODS

Fungal inoculum and inoculations

The pathogenicity trials were conducted on 1-year-old detached shoots of *Vitis vinifera* cv. Cabernet Sauvignon, *Olea europaea* subsp. *europaea* cv. Frantoio, *Prunus salicina* cv. Sun Kiss, *Malus domestica* cv. Granny Smith and *Schinus molle* (Peruvian pepper tree). For each of the five inoculated hosts, 200 shoots were independently cut from different plants. Eleven *N. australe* and eight *N. stellenboschiana* isolates (Table 1), and uncolonized agar plugs for negative controls, were used for the inoculations, resulting in a total of 20 treatments. These isolates were originally isolated from grapevine (four isolates), olive (four), plum (two), apple (three), Peruvian pepper (four) and fig (two isolates), all from plants showing characteristic dieback symptoms and internal wood necroses associated with trunk diseases. The *Neofusicoccum* isolates are maintained in the fungal culture collection of the Department of Plant Pathology, University of Stellenbosch (STE-U) as well as the Agricultural Research Council (ARC) Infruitec - Nietvoorbij, Stellenbosch, South Africa. Before inoculations, isolates were subcultured in Petri dishes onto Potato Dextrose Agar (PDA, Biolab) amended with 250 mg L^{-1} chloromycetin (PDA-C). The cultures were grown for 3-7 d at 23-24°C, with daily cycles of approx. 12 h daylight and 12 h of darkness. Fungal plugs (4 mm diam.) were cut from the margins of these colonies with a cork-borer for use as inoculum. The shoots were cut into uniform size (25 cm), and were surface sterilized by immersing in 70% ethanol for 30 sec, followed by sodium hypochlorite (3.5%) for 1 min, and ethanol for 30 sec. The shoots were then air dried in a laminar flow cabinet. Each shoot was then wounded with an electric drill with a 4 mm drill bit. The wound was made deep enough to reach into the xylem tissue but not deep enough to reach the pith. The 4 mm agar plugs were then inserted into the wounds, mycelium side first, immediately after wounding, and the inoculated wounds were wrapped with parafilm to prevent drying. The inoculated shoots were put on moist sterile paper towels inside plastic boxes measuring 29 × 23.5 × 5.5 cm, were covered with a plastic lid, and left on a lab bench for 5 weeks at 22-24°C. The boxes were opened daily to check for fungal contamination on shoots or paper towels and to ensure that the paper towels remained moist. The moist paper towels were replaced weekly. After 5 weeks the shoots were removed from the moist chambers. The shoots were each split longitudinally with a meat saw through the point of inoculation, and the vascular discolouration on both sides of the point of inoculation (lesion lengths) were measured using digital callipers.

Not all inoculations of the different test hosts could be completed simultaneously. Therefore, the different inoculated hosts were assessed as separate trials. For each host, the experimental design was a randomised complete block with the 20 treatments replicated at random in five plastic boxes. Each plastic box contained 20 shoots of a specific host, each inoculated with one of the 20 fungal isolates. The trial for each inoculated host was repeated on a second occasion using new inoculum for each isolate.

Table 1. *Neofusicoccum stellenboschiana* and *Neofusicoccum australe* isolates used in cross pathogenicity experiments in this study.

Isolate identification number (STEU)	Original host	Area obtained	Species
9129	Fig	Durbanville	*N. stellenboschiana*
9130	Peruvian pepper	Durbanville	*N. stellenboschiana*
9131	Fig	Durbanville	*N. australe*
9132	Peruvian pepper	Durbanville	*N. australe*
9133	Peruvian pepper	Durbanville	*N. stellenboschiana*
9134	Olive	Durbanville	*N. stellenboschiana*
9135	Olive	Durbanville	*N. australe*
9136	Olive	Hermanus	*N. stellenboschiana*
9137	Olive	Hermanus	*N. australe*
9138	Peruvian pepper	Hermanus	*N. australe*
9139	Grapevine	Constantia	*N. australe*
9140	Grapevine	Constantia	*N. australe*
9141	Grapevine	Constantia	*N. stellenboschiana*
9142	Grapevine	Constantia	*N. stellenboschiana*
8302	Apple	Riviersonderend	*N. australe*
8307	Apple	Riviersonderend	*N. stellenboschiana*
8852	Plum	Clanwilliam	*N. australe*
8851	Plum	Clanwilliam	*N. australe*
8301	Apple	Riviersonderend	*N. australe*

Fungus re-isolation and identification

After taking lesion measurements, two pieces of each shoot were surface sterilized as described above. For each plant sample, 12 pieces of tissue (each ≈ 1 × 1 x 2 mm) were cut, with the first piece from the margin of healthy and necrotic tissue and the other 11 pieces from the entire length of each lesion. The tissue pieces were then placed onto PDA-C in three Petri dishes. The Petri dishes were incubated at 23-24°C for 4 weeks. The dishes were monitored daily, and fungal growth resembling *Neofusicoccum* was hyphal tip sub-cultured onto PDA-C

Petri plates. Morphological characteristics were initially used to compare re-isolated cultures with those of the originally inoculated isolates. Representative subsamples, one culture from each of the 19 isolates were also subjected to DNA extraction and molecular identification, to satisfy Koch's postulates.

DNA extraction, PCR amplification and DNA sequencing

Mycelia of the different isolates were scraped directly from the Petri dishes and transferred into Eppendorf tubes. DNA was isolated using a CTAB extraction method (Damm *et al.*, 2008). To confirm the species identity, the β-tubulin region was amplified using primers Bt2a and Bt2b (Glass and Donaldson, 1995). PCR amplifications were performed in 20 µL reactions containing 1× KAPA Taq Ready Mix (KAPA Biosystems), 0.08 µM of each primer, 2 µL DNA and the remaining volume was filled with double-distilled H_2O. PCR conditions consisted of initial denaturation step at 94°C for 5 mins, followed 36 cycles each of 45s at 94°C, 45s at 55°C and 90s at 72°C, with final extension step at 72°C for 6 min. An applied Biosystems 2700 PCR machine (Carlsbad) was used to carry out PCR reactions. Gel electrophoresis was performed on a 1% (w/v) agarose gel in TAE running buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.5) after staining with ethidium bromide. To visualize the gel under the ultraviolet (UV) light, the GeneGenius Gel Documentation and Analysis System (Syngene) alongside a 100-bp DNA ladder (GeneRuler, Thermo Fischer Scientific) were used. PCR products were then purified using the MSB Spin PCRapase kit (Invitek), and were prepared for reverse and forward sequencing. Thermocycler conditions were set for 1 min at 95°C, 30 cycles each of 10s at 95°C, 5s at 50°C and 4 min at 60°C, with a final extension of 30s at 60°C. The nucleotide samples were then sent to the DNA Sequencing Unit at the Central Analytical Facility of Stellenbosch University for sequencing.

Data analyses

For each inoculated host, analysis of variance (ANOVA) was performed per trial on the lesion length data obtained, according to the randomized block experimental design, using General Linear Models (GLM) procedure of SAS statistical software (Version 9.4; SAS Institute Inc.). Trial results were also combined in one analysis of variance after testing for trial homogeneity of variance using Levene's test (Levene, 1960). For the inoculated hosts apple, olive, and Peruvian pepper trial, variances were not equal; so weighted analyses of variance were performed as described by John and Quenouille (1977). The Shapiro-Wilk test (Shapiro and Wilk, 1965) was performed to test for deviation from normality. Fisher's least significance was calculated at the 5% level to compare isolate means (Ott, 1998). A probability level of 5% was considered statistically significant for all tests.

RESULTS

According to the ANOVA's (Supplementary Tables S1 to S5) there were no Isolate × Experiment interactions between the two repeat trials for all five hosts ($P > 0.05$), so combined analyses could be performed for each host. There were statistically significant ($P < 0.05$) differences between *Neofusicoccum* isolates for all the hosts (Supplementary Tables S1 to S5). Although the hosts data were analysed separately, the largest lesions developed on plum (mean lesion length = 154.4 mm) followed by grapevine (128.3 mm), apple (113.2 mm), olive (94.0 mm) and Peruvian pepper (43.2 mm) (Tables 2 to 6). The lesion lengths could be grouped into three categories: those that did not differ significantly from the experimental controls, those that grouped together with the largest lesions, and an intermediate group.

Lesion formation by Neofusicoccum australe *and* Neofusicoccum stellenboschiana *isolates within hosts*

Apple

Neofusicoccum australe (isolate STEU 9131) was the most virulent isolate to apple shoots (mean lesion length = 113.2 mm: Table 2). *Neofusicoccum stellenboschiana* isolate STEU 9141 caused the largest lesions among the *N. stellenboschiana* isolates (mean lesion length = 107.1 mm). These two isolates and isolates STEU 9140, STEU 8852, STEU 8301, STEU 9134, STEU 9138, STEU 9139, STEU 9129, and STEU 8307 caused lesions which were significantly larger than those from the controls, and were the group causing the largest lesions. This group included six *N. australe* and four *N. stellenboschiana* isolates, which were originally isolated from fig, grapevine, plum, apple, olive or Peruvian pepper. *Neofusicoccum australe* STEU 8851 caused the smallest lesions (mean = 21.5 mm). Isolates STEU 8851, STEU 9136, STEU 9132, STEU 8302, STEU 9135, STEU 9137, STEU 9130, STEU 9133, and STEU 9142 caused lesions not significantly different from the non-inoculated controls (mean = 11.9 mm). This group included five *N. australe* and four *N. stellenboschiana* isolates, originally from grapevine,

Table 2. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old apple shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9131	*N. australe*	Fig	113.2a	1.15	84
9141	*N. stellenboschiana*	Grapevine	107.1ab	1.25	97
9140	*N. australe*	Grapevine	92.9abc	0.75	83
8852	*N. australe*	Plum	87.5abc	1.12	91
8301	*N. australe*	Apple	83.8abcd	1.10	85
9134	*N. stellenboschiana*	Olive	76.1abcde	1.44	96
9138	*N. australe*	Peruvian pepper	74.7abcdef	0.96	67
9139	*N. australe*	Grape	73.1abcdef	0.93	80
9129	*N. stellenboschiana*	Fig	72.9abcdef	0.94	75
8307	*N. stellenboschiana*	Apple	68.2abcdef	1.00	79
9142	*N. stellenboschiana*	Grape	57.3bcdefg	1.25	70
9133	*N. stellenboschiana*	Peruvian pepper	55.0bcdefg	1.15	73
9130	*N. stellenboschiana*	Peruvian pepper	49.9cdefg	0.75	88
9137	*N. australe*	Olive	48.9cdefg	0.87	80
9135	*N. australe*	Olive	42.7cdefg	0.95	73
8302	*N. australe*	Apple	30.8defg	0.54	62
9132	*N. australe*	Peruvian pepper	28.2efg	0.81	68
9136	*N. stellenboschiana*	Olive	24.9efg	0.59	76
8851	*N. australe*	Plum	21.5fg	0.60	36
Control	Non-inoculated		11.9g	0.50	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 53.45.

plum, apple, olive or Peruvian pepper. Two of the three isolates originally from apple (STEU 8301 and STEU 8307) caused lesions in the largest lesion group, whereas the third (STEU 8302) caused lesions not significantly different from the non-inoculated controls. All the *Neofusicoccum* isolates were re-isolated from inoculated tissues, with re-isolation percentages ranging from 67 to 97% for the group causing large lesions, and 35 to 88% for the group for which lesion lengths did not differ from the non-inoculated controls.

Grapevine

All the isolates caused lesions in grapevine that were significantly larger than for the non-inoculated controls (mean = 0.0 mm: Table 3). *Neofusicoccum stellenboschiana* isolate STEU 9134 caused the largest lesions (mean = 128.3 mm) and together with *N. australe* STEU 9131 and STEU 8852 were the group causing the largest lesions. These isolates were originally from, respectively, olive, fig or plum. The rest of the *Neofusicoccum* isolates caused lesions in the intermediate size group (mean = 40.3 to 95.1 mm), although there was an upper intermediate lesion size group (STEU 9133, STEU 9132, STEU 8307, STEU 9141, STEU 9135, STEU 9138, and STEU 9130) and lower intermediate size group. The upper intermediate group included four *N. stellenboschiana* and three *N. australe* isolates, whereas the lower intermediate group included three *N. stellenboschiana* and six *N. australe* isolates. One of the four isolates originally from grapevine (STEU 9141) caused lesions in the upper intermediate lesion size group, whereas the other three were in the lower intermediate size group. The re-isolation percentages ranged from 68 to 77% for the large lesion group, 61 to 94% for the upper intermediate group, and 28 to 79% for the lower intermediate lesion size group.

Olive

Neofusicoccum stellenboschiana (STEU 9133) caused the largest lesions on olive (mean = 94.0 mm: Table 4). However, *N. stellenboschiana* isolates STEU 9142, STEU 9130, STEU 9141 and STEU 9129 caused lesions that

Table 3. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old grapevine shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9134	*N. stellenboschiana*	Olive	128.3a	1.42	77
9131	*N. australe*	Fig	110.7ab	1.04	70
8852	*N. australe*	Plum	103.8abc	1.00	68
9133	*N. stellenboschiana*	Peruvian pepper	95.1bcd	0.59	61
9132	*N. australe*	Peruvian pepper	92.0bcde	1.04	78
8307	*N. stellenboschiana*	Apple	79.6bcdef	1.06	80
9141	*N. stellenboschiana*	Grapevine	79.3cdef	0.95	61
9135	*N. australe*	Olive	78.7cdef	0.88	94
9138	*N. australe*	Peruvian pepper	66.2defg	0.94	80
9130	*N. stellenboschiana*	Peruvian pepper	65.4defg	0.45	65
8301	*N. australe*	Apple	63.3efg	0.62	69
9139	*N. australe*	Grapevine	62.9efg	1.08	69
9140	*N. australe*	Grapevine	62.1efg	0.91	73
9136	*N. stellenboschiana*	Olive	59.2fg	0.45	70
8302	*N. australe*	Apple	55.5fg	1.05	28
8851	*N. australe*	Plum	52.2fg	0.31	79
9129	*N. stellenboschiana*	Fig	49.8fg	0.23	77
9137	*N. australe*	Olive	43.6g	0.39	78
9142	*N. stellenboschiana*	Grapevine	40.3g	0.72	77
Control	Non-inoculated		0.0h	0.00	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD= 31.20.

were not significantly different from isolate STEU 9133, hence in the group causing the largest lesions. These isolates were originally from Peruvian pepper, fig or grapevine. *Neofusicoccum australe* STEU 9132, STEU 9137, STEU 9140, STEU 9139, STEU 8851, STEU 8301, and STEU 8302 formed lesions (mean = 23.5 to 44.2 mm) that were not significantly different from the controls (23.8 mm). *Neofusicoccum stellenboschiana* isolates STEU 8307, STEU 9136, and STEU 9134, and *N. australe* STEU 9131, STEU 9138, STEU 9135, STEU 8852, and STEU 9132 produced lesions that were significantly smaller than those from isolate STEU 9133, and were in the intermediate lesion size group. All the isolates in the large lesion group were *N. stellenboschiana*. Isolates of this pathogen were only in the large or intermediate lesion group, whereas all the lesions that did not differ from the controls were from *N. australe* inoculations. Three of the isolates originally from olive caused lesions in the intermediate group, and one was in the group where lesions were not different from the controls. The re-isolation percentages were from 27 to 100% for all the isolates. In the two cases where 100% re-isolations were obtained, both the isolates originated from olive.

Peruvian pepper

All the isolates caused lesion lengths significantly greater than those for the non-inoculated controls (mean = 9.9 mm: Table 5). *Neofusicoccum stellenboschiana* isolate STEU 9142 caused the largest lesions (mean = 43.2 mm). However, this was not significantly different from the lesions caused by 14 other isolates, six of *N. stellenboschiana* and eight of *N. australe*. These isolates were originally from olive, grapevine, Peruvian pepper, apple, fig or plum. *Neofusicoccum australe* isolates STEU 9135, STEU 8851 and STEU 9138 and *N. stellenboschiana* STEU 9136 caused lesions that were significantly shorter (mean lengths = 24.8 to 28.3 mm) than STEU 9142, and were in the intermediate lesion size group. Three of the four isolates originally from Peruvian pepper caused lesions in the large lesion group. The re-isolation percentages from Peruvian pepper shoots ranged from 59 to 93% in the large lesion group and 76 to 91% in the intermediate group.

Table 4. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old olive shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9133	*N. stellenboschiana*	Peruvian pepper	93.8 a	0.69	73
9142	*N. stellenboschiana*	Grapevine	91.8 ab	0.96	90
9130	*N. stellenboschiana*	Peruvian pepper	87.5 abc	0.50	89
9141	*N. stellenboschiana*	Grape	78.0 abcd	0.52	90
9129	*N. stellenboschiana*	Fig	76.2 abcde	0.76	80
8307	*N. stellenboschiana*	Apple	70.8 bcdef	0.62	73
9136	*N. stellenboschiana*	Olive	69.3 cdef	0.74	100
9131	*N. australe*	Fig	63.5 defg	0.81	93
9134	*N. stellenboschiana*	Olive	61.1 defg	0.84	90
9138	*N. australe*	Peruvian pepper	56.2 efg	0.55	90
9135	*N. australe*	Olive	51.7 fgh	0.84	100
8852	*N. australe*	Plum	45.8ghi	0.96	83
9132	*N. australe*	Peruvian pepper	44.2 ghij	0.69	83
9137	*N. australe*	Olive	43.3 ghijk	0.69	97
9140	*N. australe*	Grapevine	34.6 hijk	0.78	80
9139	*N. australe*	Grapevine	32.1 hijk	0.49	83
8851	*N. australe*	Plum	29.4 ijk	0.87	43
Control	Non-inoculated		23.8 jk	0.99	0
8301	*N. australe*	Apple	23.5 jk	0.75	36
8302	*N. australe*	Apple	17.5 k	0.50	28

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 7.05.

Plum

All the isolates caused lesion lengths that were significantly greater than the controls (mean = 17.1 mm: Table 6). *Neofusicoccum australe* isolate STEU 9138 caused the largest lesions (mean = 154.4 mm). However, mean lesion size was not significantly different from the lesions caused by 15 other isolates, seven of *N. stellenboschiana* and eight of *N. australe*. These isolates were originally from Peruvian pepper, apple, grapevine, olive, fig or plum. *Neofusicoccum stellenboschiana* isolate STEU 9130 and *N. australe* STEU 8302 and STEU 8851 caused lesions which were significantly smaller (mean lengths = 70.1 to 105.6 mm) than those caused by STEU 9138, and were in the intermediate lesion size group. One of the isolates originally from plum (STEU 8852) caused lesions in the large lesion group, whereas the second isolate from plum caused lesions in the intermediate size group. The re-isolation percentages from plum shoots ranged from 60 to 100%, and were 70 to 100% in the large lesion group and 60 to 90% in the intermediate group.

Lesion formation from Neofusicoccum australe *and* Neofusicoccum stellenboschiana *isolates across different hosts*

Five isolates, *N. australe* STEU 8852 and STEU 9131, *and N. stellenboschiana* STEU 9134, STEU 9129, and STEU 9141, caused lesions within the large lesion size group on four hosts, and lesions on one host in the intermediate size group. None of these isolates caused lesions similar to the non-inoculated controls on any of the tested hosts. Isolates STEU 8852, STEU 418 and STEU 9134 formed lesions within the large size group on apple, grape, plum and Peruvian pepper, whereas intermediate size lesions were formed on olive. Isolates STEU 9129 and STEU 9141 formed lesions within the large lesion group on apple, plum, Peruvian pepper and olive, whereas intermediate-sized lesions were formed on grape. These five isolates were therefore virulent across all the hosts tested.

Isolate STEU 8307 of *N. stellenboschiana* formed lesions within the large lesion group on three hosts, apple, plum, and Peruvian pepper, intermediate lesions on grape and olive, and no lesions similar to the non-inoculated controls on any host. This isolate was, there-

Table 5. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old Peruvian pepper shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion length (mm)[a]	Std dev	Re-isolation (%)
9142	*N. stellenboschiana*	Grapevine	43.2 a	1.23	90
9130	*N. stellenboschiana*	Peruvian pepper	40.2 ab	1.28	89
8307	*N. stellenboschiana*	Apple	39.9 ab	1.04	63
8302	*N. australe*	Apple	39.1 abc	0.91	91
9129	*N. stellenboschiana*	Fig	39.0 abc	0.85	91
9131	*N. australe*	Fig	37.8 abc	0.73	93
9137	*N. australe*	Olive	36.8 abc	1.59	66
9141	*N. stellenboschiana*	Grapevine	36.1 abc	0.98	90
9134	*N. stellenboschiana*	Olive	34.7 abc	1.02	76
8852	*N. australe*	Plum	34.7 abc	0.99	90
9133	*N. stellenboschiana*	Peruvian pepper	33.5 abc	1.16	73
9140	*N. australe*	Grapevine	33.0 abc	0.81	59
8301	*N. australe*	Apple	30.5 abc	0.78	90
9139	*N. australe*	Grapevine	30.2 abc	1.28	84
9132	*N. australe*	Peruvian pepper	29.1 abc	0.83	93
9135	*N. australe*	Olive	28.3 bc	0.79	91
9136	*N. stellenboschiana*	Olive	26.7 bc	0.90	87
8851	*N. australe*	Plum	26.5 bc	0.47	76
9138	*N. australe*	Peruvian pepper	24.8c	0.27	89
Control	Non-inoculated		9.9d	0.63	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments. LSD = 14.30.

fore, virulent across all the hosts tested. Five isolates, including STEU 9133 and STEU 9142 of *N. stellenboschiana*, and STEU 9140, STEU 8301, and STEU 9139 of *N. australe*, also formed lesions within the large lesion size group on three hosts each, plum, Peruvian pepper and olive for STEU 9133 and STEU 9142, and plum, Peruvian pepper and apple for STEU 9140, STEU 8301 and STEU 9139. These five isolates also produced intermediate sized lesions on grape, and lesions that did not differ from the non-inoculated controls in apple (STEU 9133 and STEU 9142) and olive (STEU 9140, STEU 8301 and STEU 9139). These isolates were, therefore, virulent on at least four of the hosts tested.

Four isolates, three of *N. australe* (STEU 9138, STEU 9132, STEU 9137) and one of *N. stellenboschiana* (STEU 9129), formed large lesions on two hosts each, but then differed in the number of hosts where they caused intermediate-sized lesions or lesions similar to the non-inoculated controls. Isolate STEU 9138 produced large lesions on plum and apple and intermediate-sized lesions on grape, olive and Peruvian pepper. This isolate was, therefore, virulent across all the tested hosts. Isolate STEU 421 formed large lesions on olive and pepper, intermediate-sized lesions on grape and plum, and lesions similar to the non-inoculated controls on apple. Isolates STEU 9132 and STEU 9137 formed large lesions on plum and Peruvian pepper, intermediate-sized on grapevine, and lesions similar to the non-inoculated controls on apple and olive. Although isolates STEU 9129, STEU 9132 and STEU 9137 were virulent on three to four hosts, these isolates showed more variation in virulence across the different hosts than other isolates.

Isolates STEU 9136 of *N. stellenboschiana* and STEU 9135 and STEU 8302 of *N. australe* produced large lesions only on one host each, plum in the case of STEU 9136 and STEU 9135 and Peruvian pepper for STEU 8302. Isolates STEU 9136 and STEU 9135 produced intermediate-sized lesions on grape, olive and Peruvian pepper, with lesions similar to the non-inoculated controls on apple. Isolate STEU 8302 produced intermediate-sized lesions on plum and grape, so displayed high virulence variation. Isolate STEU 8851 was by far the weakest pathogen, not being able to form large lesions on any of the tested hosts, intermediate-sized lesions on grapevine, plum and Peruvian pepper, and lesions not

Table 6. Mean lesions sizes obtained from a pathogenicity study conducted with detached 1-year-old plum shoots artificially inoculated with *Neofusicoccum australe* or *Neofusicoccum stellenboschiana*.

Isolate identification number (STEU)	Fungus	Original host	Mean lesion Length (mm)[a]	Std dev	Re-isolation (%)
9138	*N. australe*	Peruvian pepper	154.4a	0.73	100
8301	*N. australe*	Apple	151.7a	0.85	97
9136	*N. stellenboschiana*	Olive	150.0a	1.18	96
9141	*N. stellenboschiana*	Grapevine	148.4a	0.61	98
9134	*N. stellenboschiana*	Olive	146.8ab	0.71	85
8307	*N. stellenboschiana*	Apple	146.5ab	0.74	90
9135	*N. australe*	Olive	145.0abc	0.89	73
9129	*N. stellenboschiana*	Fig	144.9abc	0.85	77
9133	*N. stellenboschiana*	Peruvian pepper	143.0abc	0.88	76
9139	*N. australe*	Grapevine	141.5abc	1.03	92
9137	*N. australe*	Olive	140.7abc	1.13	89
9132	*N. australe*	Peruvian pepper	128.0abc	1.09	70
8852	*N. australe*	Plum	128.0abc	0.70	87
9142	*N. stellenboschiana*	Grapevine	124.4abc	1.25	77
9131	*N. australe*	Fig	121.8abc	0.71	100
9140	*N. australe*	Grapevine	112.3abcd	0.96	87
8851	*N. australe*	Plum	105.6bcd	0.60	7
8302	*N. australe*	Apple	103.7cd	0.65	90
9130	*N. stellenboschiana*	Peruvian pepper	70.1d	0.91	60
Control	Non-inoculated		17.1e	0.35	0

[a] Means followed by the same letter are not significantly different ($P = 0.05$). Means were calculated from 20 measurements, from ten replicates per isolate repeated over two experiments.LSD= 42.67.

significantly different from the non-inoculated controls in apple and olive.

DISCUSSION

All the *Neofusicoccum australe* and *N. stellenboschiana* isolates evaluated in this pathogenicity study were able to infect, colonise and cause necrosis on apple, grapevine, olive, Peruvian pepper and plum. Lesions extended upward and downward from the points of inoculation, with variable lengths depending on the pathogen-host combination. The inoculated fungi were re-isolated from each of these hosts and together with the characteristic lesions produced, *N. australe* and *N. stellenboschiana* can therefore be regarded as pathogens of these hosts. This is the first report of *N. australe* and *N. stellenboschiana* as pathogens of Peruvian pepper. *Neofusicoccum ribis* is the only *Botryosphaeriaceae* species that had previously been associated with Peruvian pepper, in Hawaii (Stevens and Shear, 1929). Although *N. stellenboschiana* was associated with trunk disease symptoms on nursery apple trees, no pathogenicity studies were conducted (Havenga *et al*., 2019). Furthermore, only two previous studies have linked *N australe* with olive plants showing canker and dieback symptoms. However, the study by Lopes *et al*. (2016), conducted in Portugal, did not perform pathogenicity studies, whereas the study by Triki *et al*. (2014) in Tunisia conducted a pathogenicity study showing formation of a brown colour on stems of 2-year-old plug-inoculated potted olive trees. Triki *et al*. (2014) did not disclose whether the symptom was external or internal, nor was the extent of the lesion formation provided. Pathogenicity studies were conducted by Lazzizera *et al*. (2008) with *N. australe*, however, on olive drupes in Italy where the pathogen was regarded as highly virulent.

Although the data for inoculation of different hosts could not be compared statistically, the present study has shown that the largest lesions resulting from inoculations were formed on plum, followed by grapevine, apple, olive and Peruvian pepper. This indicates that plum could be more susceptible to infection by *N. australe* and *N. stellenboschiana* than the other four hosts. The fact that all 19 isolates evaluated caused lesions on plum shoots that were larger than those from

the non-inoculated controls indicates that plum was highly susceptible to these pathogens. This was also the case in Peruvian pepper, for which none of the evaluated isolates caused lesions similar to those from the non-inoculated controls, confirming that all 19 isolates were pathogenic to this host. The high re-isolation percentages obtained from Peruvian pepper is further confirmation of the susceptibility of this host. Peruvian pepper trees often grow in close proximity to vineyards and fruit orchards in the Western Cape of South Africa, confirming that they are probably alternative hosts of *Neofusicoccum* spp., and are sources of inoculum to closely associated vineyards and fruit orchards.

Considering the virulence of *N. australe* and *N. stellenboschiana* isolates within the different hosts evaluated, no clear patterns were identified in most of the hosts. The exception was olive, where all the isolates producing large lesions were *N. stellenboschiana*, and all the isolates where lesions were not different to the non-inoculated controls were *N. australe*. Significant virulence differences within the same species was observed for all the tested hosts, except for Peruvian pepper inoculated with *N. australe*, where the lesion lengths did not differ significantly. For example, *N. australe* isolate STEU 9131 produced lesions that were five times longer than those from *N. australe* STEU 8851 in apple shoots. Also in apple, *N. stellenboschiana* isolate STEU 9141 caused lesions that were four times longer than those from *N. stellenboschiana* STEU 9136. High virulence diversity within *N. australe* has been documented on several fruit trees and other woody hosts (Cloete *et al*., 2011; Jami *et al*., 2015; Baskarathevan *et al*., 2017; Aćimović *et al*., 2018; Arjona-Girona *et al*., 2019).

Lesion formation from *N. australe* and *N. stellenboschiana* isolates across the tested hosts showed that within both fungi there were isolates that were more virulent than others. Some isolates caused large lesions on four hosts and lesions different from the non-inoculated controls in a fifth (isolates STEU 8852, STEU 9131, STEU 9129, STEU 9141 and STEU 9134). Some of the isolates of *N. australe* (i.e. STEU 8302) and *N. stellenboschiana* (i.e. STEU 9136) had variable virulence across hosts, forming lesions in all three size categories. Only *N. australe* isolate STEU 8851 was unable to cause large lesions in on any of the hosts. This isolate was therefore a weak pathogen, although it could infect, colonise and cause lesions significantly different from the non-inoculated controls in three of the five test hosts.

The isolates of *N. australe* and *N. stellenboschiana* used in the present study were originally obtained from different woody hosts, but this did not affect their abilities to cause lesions on different hosts. None of the isolates originally from a specific host caused large lesions on that host. The two isolates originally from fig (STEU 9131 and STEU 9129) produced large lesions on apple, Peruvian pepper and plum, but on grape and olive, one isolate caused large lesions and the other caused intermediate-sized lesions. These results are similar to those of Amponsah *et al*. (2011), where isolates of *N. luteum, N. parvum* and *N. australe* from grapevine and non-grapevine hosts were able to infect grapevine shoots and produce lesions. Also, Sessa *et al*. (2016) showed in Uruguay, that *N. australe* isolates from apple branches showing papyraceous cankers caused large lesions when 1-year-old apple, pear or peach shoots were inoculated with six Botryosphaeriaceous species, in a field trial. *Neofusicoccum australe* is known from at least 46 hosts from 18 plant families (Sakalidis *et al*., 2011). Based on microsatellite data, native populations *N. australe* in Australia were highly diverse, with no discernible host or habitat restrictions, but rather geographic distribution and life-strategies controlled by environmental factors (Sakalidis *et al*., 2011). Even in a different pathosystem, *N. australe* originally isolated from avocado and blueberry caused leaf lesions on macadamia and blueberry leaves when the petioles of detached leaves were inoculated with the pathogen (Liddle *et al*., 2019). *Neofusicoccum australe* isolates isolated from healthy leaves and branchlets of *Acacia karroo*, a native tree with wide distribution in South Africa, were shown to be highly virulent when 1-year-old *A. karroo* seedling stems were inoculated with the fungus (Jami *et al*., 2015).

In blueberries in Portugal, Hilário *et al*. (2020) showed that *N. australe* occurred alone in branches with dieback, but also from dead branches in association with other *Botryosphaeriaceae* and from the same asymptomatic branch, as endophytes or latent pathogens co-existing with *N. parvum*, which was the more pathogenic of the two species on blueberry. Moyo *et al*. (2016) surveyed trunk pathogens and associated symptom types on *Diospyros kaki* (persimmon) in South Africa. *Neofusicoccum australe* co-existed in 11 trunk pathogen combinations in various symptom types. Co-inoculation of *Seimatosporium vitifusiforme* with *D. seriata*, previously isolated from one canker in a vineyard, caused an increase in lesion length (Lawrence *et al*., 2018). Co-infection by *N. australe* and other trunk pathogens has not been investigated previously, and, due to its very wide host range and virulence on many hosts, this is likely to be a fruitful study. Detached shoot assays would be good starting points, but field trials are also highly recommended.

The present study has showed that *N. australe* and *N. stellenboschiana* isolates originating from different

fruit tree hosts can cross-infect alternative hosts, causing lesions. These infections are likely to be inoculum sources for infestation of grapevine and other major fruit crops. In the South African fruit and vine industries, only one previous study (Van Niekerk *et al*., 2010) investigated spore release patterns associated with *Botryosphaeriaceae* species in vineyards, but spore release in fruit orchards has not been investigated. Little is known about the inoculum sources and plant-to-plant dispersal of *Botryosphaeriaceae* species within vineyards and fruit orchards. Moyo *et al*. (2014) reported *Botryosphaeriaceae* species (including *N. australe*) on the exoskeletons of arthropods in close proximity to grapevine pruning wounds. To effectively manage Botryosphaeria canker and dieback of grapevine and fruit trees, growers will have to be cognizant of the importance of appropriate sanitation practices for vineyards and orchards, but also for other closely associated woody hosts. Results of the present study have shown that in addition to *N. australe*, *N. stellenboschiana* is likely to be an important trunk pathogen of grapevine and fruit trees. Future research should investigate the biology of *N. stellenboschiana* to support appropriate disease management strategies. Most importantly, testing of pruning wound protectants against *N. stellenboschiana* infections is urgently required.

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