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RESEARCH ARTICLE

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Identification, pathogenicity and distribution of the causal agents of dieback in avocado orchards in Spain

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Abstract

An increased incidence of dieback from branches in several avocado orchards in southern Spain was observed in 2014. Surveys were conducted from May to October 2014, sampling the affected branches to isolate the causal agents. A total of 68 fungal isolates, recovered from ten avocado orchards, were identified, by morphological characterisation and DNA sequencing, as belonging to the genera: *Neofusicoccum parvum* (50%), *Colletotrichum gloeosporioides* (17.6%), *Neofusicoccum luteum* (16.2%), *Neofusicoccum australe* (13.2%), *Neofusicoccum mediterraneum* (1.5%) and *Lasiodiplodia theobromae* (1.5%). A decreasing level of virulence in artificial inoculations on avocado plants was observed in *N. parvum, N. luteum, N. mediterraneum, N. australe, C. gloeosporioides* and *L. theobromae*, there were significant differences among *N. parvum* and the rest of species of this genus, and significant differences were only observed between *N. luteum* and *C. gloeosporioides*. The geographical distribution of *N. parvum* and *N. Luteum* covers different areas, while *C. gloeosporioides* and *N. australe* are located only in the areas around Benamocarra and Vélez-Málaga (southern Spain), while *N. mediterraneum* and *L. theobromae* appear only occasionally. This is the first study of avocado branch cankers in Spain which identifies the causal agents and establishes their pathogenicity groups, with *N. parvum* as the most important causal agent of avocado dieback in this area.

Additional keywords: *Botryosphaeriaceae*; *Lasiodiplodia*; *Neofusicoccum*; *Colletotrichum*; *Persea americana*.

Abbreviations used: AUDPC (Area Under Disease Progress Curve); ITS (Internal Transcribed Spacer); LSD (Least Significant Difference); PDA (Potato Dextrose Agar).

Authors' contributions: Conceived and designed the study (CJLH); performed the experiments (IAG); interpretation of data, wrote the paper (CJLH, IAG, DRR).

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Introduction

The avocado (*Persea americana* Mill.) is cultivated worldwide, but was commercially produced in Europe for the first time in Spain. In the 1970s, commercial avocado orchards were established in southern Spain (provinces of Málaga and Granada) because the microclimate in this area bears similarities to the conditions in different regions of America, such as Mexico, Peru and California, which have a long tradition of growing this crop, with high levels of production (http://faostat3.fao.org/browse/Q/QC/E).

However, avocado production is decreasing all over the world due to branch cankers and fruit stem-end rot. Symptomatic trees exhibit red-brown cankers and dieback on branches associated with a characteristic white exudate (McDonald & Eskalen, 2011). The first stages of infection are often caused by mechanical injuries, which allow the access of pathogens. Avocado dieback has been observed in different countries with tropical and subtropical climate, such as Chile (Auger *et al*., 2013) and Colombia (Burbano-Figueroa *et al*., 2018) in South America or Spain (Zea-Bonilla *et al*., 2007) in Europe, and many fungal agents have been identified, especially those belonging to the *Botryosphaeriaceae* family.

In Spain, other subtropical crops different to avocado, such as loquat (*Eribotrya japonica* Lindl.), are affected by species of *Botryosphaeriaceae*, among them, *Diplodia malorum* Fuckel, *Diplodia olivarum*

A.J.L. Phillips, Frisullo & Lazzizera, *Diplodia seriata* De Not., species of complex *Diplodia pseudoseriata/Diplodia alatafructa, Diplodia* sp., *Dothiorella sarmentorum* (Fr.) A.J.L. Phillips, Alves & Luque, *Neofusicoccum mediterraneum* Crous, Wingf & A.J.L. Phillips, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Philips, *Spencermartinsia plurivora* Abdollahz., Javadi & A.J.L. Phillips and *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous (González-Domínguez *et al*., 2017). In this crop, other pathogens, namely *Alternaria alternata* (Fr.) Keissl., *Penicillium expansum* Link, *Botrytis cinerea* Pers., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Pestalotiopsis clavispora* (G.F. Atk.) Steyaert and *D. seriata* caused post-harvest diseases (Palou *et al*., 2016). Other Mediterranean crops such as almond (*Prunus dulcis* Webb) are also affected by fungi *Botryosphaeriaceae* (Gramaje *et al*., 2012) and *N. mediterraneum* and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. have been isolated from olive (*Olea europaea* L.) branches (Moral *et al*., 2017) and *N. parvum* from mango (*Mangifera indica* L.) trees (Arjona-Girona & López-Herrera, 2016).

Colletotrichum gloeosporioides and *N. parvum* have been described as causal agents of anthracnose and stem end rot in avocado fruit (cv. Hass) in Turkey (Akgül & Awan, 2016). *N. parvum* and *D. seriata* caused dieback in grapevine (*Vitis vinifera* L.) (Spagnolo *et al*., 2017). Over the last two decades, significant losses have been recorded in citrus production in Portugal from anthracnose symptoms caused by *C. gloeosporioides* (Ramos *et al*., 2016).

Neofusicoccum parvum, *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips were identified in California (McDonald *et al*., 2009). *N. parvum* was also found in Mexico (Molina-Gayosso *et al*., 2012), mainly affecting fruits, and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. in Peru (Alama *et al*., 2006). Although the most important post-harvest disease in Chile is anthracnose, caused by *C. gloeosporioides*, a new disease caused by *N. australe* was also found (Montealegre *et al*., 2016).

The aims of this work were to study the distribution of avocado trees affected by branch cankers in commercial orchards in southern Spain, identify their fungal agents and establish their virulence groups.

Material and methods

Sampling and fungal isolation

Field surveys were carried out on avocado orchards from May to October 2014 in Málaga (southern Spain). Young twigs and branches of avocado trees from commercial orchards showing dieback symptoms were isolated (Fig. 1A-D).

The collected plant material was disinfested in 10 g L^{-1} sodium hypochlorite for 3 min and pieces of bark or internal wood showing lesions were plated onto potato dextrose agar (PDA) medium (Difco Laboratoires, Detroit, MI, USA) with lactic acid (0.2%). The cultures were kept on acidified PDA at 25°C in darkness for 3 days, and later, pure cultures were obtained by excising and transferring hyphal tips from the fungal colonies to fresh PDA plates.

Fungal identification

The pure cultures obtained were first identified based on colony morphology and conidial characteristics by comparing them with the previous studies by Phillips (2006).

To confirm the previous macroscopic fungal identification, DNA extractions from each isolate recovered with a similar colony morphology to *Botryosphaeriaceae* isolates were performed as described by Choi *et al*. (1992), and a sequence analysis of the internal transcribed spacer (ITS) nrDNA region using the primers ITS4 and ITS5, an analysis of partial β-tubulin gene BT2a and BT2b (Glass & Donaldsons, 1995) and a translation elongation factor 1-α gene regions (EF1-α) EF1-728F and EF1-986R (Carbone & Kohn, 1999) were performed. PCR products (600 bp for ITS, 340-495 bp for BT2 and 350 bp for EF-1α) were sequenced in the two ways (3′-5′and 5′-3′) by the Proteomic Department of SCAI (University of Córdoba, Spain). The sequences of each isolate were used to search for similar sequences in GenBank using BLAST (v. 2.0,

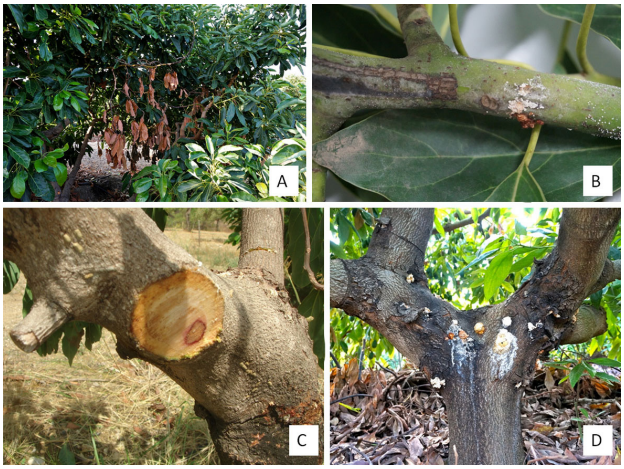

Figure 1. Disease symptoms on avocado tree branches associated with dieback. A, Dry branches. B, External necrosis in twigs. C, Internal lesions in branches. D, External lesions with exudates.

National Center for Biotechnology Information, US Nat Inst of Health, Bethesda, MD, USA).

PDA Petri dishes with pure cultures were incubated at 25 ºC in darkness and pycnidia formation was induced on water agar with pine needles and UV light; the length and width of the conidia were then measured.

Pathogenicity tests

Pathogenicity tests were carried out on stems of eighteen-month-old avocado plants of cv. Topa-Topa, growing on Laura substrate consisting of peat, coconut fiber and perlite at a ratio of 6:1:0.6 v/v/v. One plant per fungal isolate was used and five wounds were made on each stem. Five-mm mycelia plugs from the edge of a fresh fungal colony were placed onto the wounded stem and incubated in a greenhouse at 25 ºC ± 5 ºC. The necrotic lesions were measured after 3, 6 and 9 days of inoculation and the standardised area under disease progress curves (AUDPCs) was calculated. To fulfil Koch's postulates, pieces of necrotic tissue were taken from infected twigs and plated on PDA Petri dishes to identify the pathogen.

Statistical analysis

A completely randomised experimental design with 'Statistic 9' was used to study the virulence of the isolates. The treatment averages were compared using Fisher's Least Significant Difference (LSD) test to separate the means (p<0.05) (Steel & Torrie, 1985).

Results

Fungal identification

Amplified sequences from each of the 14 representative isolates were compared with isolates from GenBank (accession numbers: see Table 1). Based on a BLAST search of Gen-Bank nucleotide database, the closest hits of the isolates with the ITS, BT2 and EF1-α sequences are shown in Table 1. The identity percentages between nucleotides were very high – close to 100% in most cases. The number of insertions and deletions in one sequence relative to another were low because there were not many gaps (0-1%), and therefore it was not necessary to introduce a space into an alignment to compensate for the other.

After the macroscopic fungal identification of the morphological characters of 68 isolates and their subsequent confirmation by DNA sequence analyses, all the isolates were identified as belonging to the genera *Neofusicoccum, Colletotrichum* and *Lasiodiplodia*, with six different species in different proportions: *N. parvum* (50%), *C. gloeosporioides* (18%), *N. australe* (13%), *N. luteum* (16%), *N. mediterraneum* (1.5%) and *L. theobromae* (1.5%) (Table 2).

The width and length of the conidia (Fig. 2A-F) from 14 representative fungal isolates: *N. parvum* (AR1, AR7, AR10, AR17 CA, AR22 and AR33), *C. gloeosporioides* (AR14 CA and AR28 P), *N. australe* (AR31 and AR41-2), *N. luteum* (AR18 and AR46), *N. mediterraneum* (AR30) and *L. theobromae* (AR12 G) were then measured (Table 3). *L. theobromae* (21.99±1.43 µm × 13.18±0.5 µm) showed the greatest conidia size, although the shape of the conidia was not the most elongated. *N. parvum* (17.97±1.73 µm × 6.5±0.49 µm) and *N. mediterraneum* (22.66±1.57 µm × 7.03±0.18 µm) were of medium size but had a more elongated shape and the rest, *C. gloeosporioides* (18.14±1.29 µm × 5.23±0.49 µm), *N. luteum* (16.62±3.25 µm × 4.30±0.54 µm) and *N. australe* (16.89±1.3 µm × 4.09±0.39 µm) were the smallest, but had the greatest elongation.

Neofusicoccum parvum is the main pathogen causing branch dieback in avocado plants and it was present in all the locations sampled, with a total incidence of 50%. *N. luteum* was also common, while *C. gloeosporioides* and *N. australe* were located only at the biggest locations (Benamocarra and Vélez Málaga) with rates of incidence of 16.2%, 17.6% and 13.2%, respectively. *N. mediterraneum* and *L. theobromae* appeared occasionally, with low rates of incidence of around 1.5% (Table 4).

Pathogenicity test

The avocado plants artificially inoculated with fungal isolates showed necrotic stem lesions (Fig. 3) and the pathogenicity of all the genera of the fungal isolates was evaluated and compared (F=24.54; df=2, 342; p<0.05). *Neofusicoccum* was the most virulent genus, with significant differences with *Colletotrichum* and *Lasiodiplodia*, although no differences were observed between these last two genera. We also evaluated the pathogenicity of the different species of the *Neofusicoccum* genus (F=20.03; df=3, 276; p<0.05), with *N. parvum* being the most virulent, significantly different from *N. australe*, *N. mediterraneum* and *N. luteum*, with no differences observed among the latter. Finally, the pathogenicity of species from the different genera was also evaluated (F=26.33; df=5, 339; P<0.05) with the following results (in the decreasing order of pathogenicity): *N. parvum*, *N. luteum*, *N. mediterraneum*, *N. australe*, *C. gloeosporioides* and *L. theobromae*; there were also significant differences between *N. parvum* and the other species, and the

Table 1. Closest hits of the representative fungal isolates, with the ITS, BT2 and EF1-α sequences.

Species	Fungal isolate (FI)	GeneBank accession No. (GB)	Identities between nucleotides (FI/ GB)	Gaps
ITS sequence				
Neofusicoccum parvum	AR1	KX244812.1	540/542 (99 %)	0/542 (0 %)
	AR7	KX244812.1	541/541 (100 %)	0/541 (0 %)
	AR10 L	KT440949.1	556/560 (99 %)	3/560 (0 %)
	AR17 CB	GQ471820.1	548/554 (99 %)	2/554 (0 %)
	AR22	KX244812.1	364/415 (88 %)	0/415 (0 %)
	AR33	KX244812.1	441/478 (88 %)	0/478 (0 %)
Colletotrichum gloeosporioides	AR14 CA	KX197384.1	421/492 (99 %)	0/492 (0 %)
	AR28 P	KT184762.1	335/363 (92 %)	0/363 (0 %)
N. australe	AR31	KF702388.1	553/554 (99 %)	0/554 (0 %)
	AR41-2	KT440921.1	497/503 (99 %)	0/503 (0 %)
N. luteum	AR46	HQ392721.1	527/531 (99 %)	1/531 (0 %)
	AR18	JX869036.1	547/552 (99 %)	1/552 (0 %)
N. mediterraneum	AR30	KF778818.1	420/485 (87 %)	0/485 (0 %)
Lasiodiplodia theobromae	AR12 G	KC357277.1	507/507 (100 %)	0/507 (0 %)
BT2 sequence				
N. parvum	AR1	KU554658.1	308/313 (98 %)	2/313 (0 %)
	AR7	KU554658.1	293/295 (99 %)	0/295 (0 %)
	AR10 L	KU554658.1	265/267 (99 %)	1/267 (0 %)
	AR17 CB	KU554658.1	269/271 (99 %)	1/271 (0 %)
	AR22	KU554658.1	258/260 (99 %)	1/260 (0 %)
	AR33	KU554658.1	265/266 (99 %)	0/266 (0 %)
C. gloeosporioides	AR14 CA	KU534987.1	253/256 (99 %)	0/256 (0 %)
	AR28 P	KU534987.1	261/264 (99 %)	1/264 (0 %)
N. australe	AR31	KU836639.1	267/272 (98 %)	2/272 (0 %)
	AR41-2	KU836639.1	301/310 (97 %)	2/310 (0 %)
N. luteum	AR46	JX515686.1	288/294 (98 %)	3/294 (0 %)
	AR18	KP860768.1	259/260 (99 %)	1/260 (0 %)
N. mediterraneum	AR30	KF778903.1	311/315 (99 %)	2/315 (0 %)
L. theobromae	AR12 G	KR260829.1	291/298 (98 %)	1/298 (0 %)
EF1-α sequence				
N. parvum	AR1	KX648483.1	169/174 (97%)	0/174 (0%)
	AR7	KX464699.1	247/252 (98%)	0/252 (0%)
	AR10 L	KX464699.1	247/252 (98%)	0/252 (0%)
	AR17 CB	KP183182.1	252/252 (100%)	0/252 (0%)
	AR22	KX648481.1	246/247 (99%)	0/247 (0%)
	AR33	KP183189.1	87/93 (94%)	1/93 (1%)
C. gloeosporioides	AR14 CA	-	-	-
	AR28 P	-	-	-
N. australe	AR31	JF271793.1	245/251 (98%)	1/251 (0%)
	AR41-2	JF271793.1	194/209 (93%)	2/209 (0%)
N. luteum	AR46	HQ392740.1	246/248 (99%)	1/248 (0%)
	AR18	HQ392753.1	233/248 (94%)	3/248 (1%)
N. mediterraneum	AR30	JQ772083.1	203/216 (94%)	1/216 (0%)
L. theobromae	AR12 G	KU737510.1	169/182 (93%)	0/182 (0%)

Table 2. Identification of fungal isolates.

Isolate	Species	Origin*	Isolate	Species	Origin*
AR1	*Neofusicoccum parvum*	Vélez-Málaga	AR21	*N. parvum*	Cajiz
AR2	*N. australe*	Vélez-Málaga	AR22	*N. parvum*	Vélez-Málaga
AR3	*N. parvum*	Algarrobo-Costa	AR23	*N. luteum*	Vélez-Málaga
AR4	*N. parvum*	Benamocarra	AR24	*N. parvum*	Vélez-Málaga
AR5 L1	*N. australe*	Benamocarra	AR24 G	*N. parvum*	Vélez-Málaga
AR5 L2	*N. australe*	Benamocarra	AR25	*N. luteum*	Vélez-Málaga
AR6	*Colletotrichum gloeosporioides*	Benamocarra	AR26	*N luteum*	Vélez-Málaga
AR7	*N. parvum*	Benamocarra	AR27	*N. parvum*	Vélez-Málaga
AR8 L1	*N. parvum*	Benamocarra	AR28 G	*N. luteum*	Vélez-Málaga
AR8 L2	*N. parvum*	Benamocarra	AR28 P	*C. gloeosporioides*	Vélez-Málaga
AR9	*N. parvum*	Benamocarra	AR29	*N. australe*	Vélez-Málaga
AR10 C	*N. luteum*	Benamocarra	AR29 P	*N. luteum*	Vélez-Málaga
AR10 L	*N. parvum*	Benamocarra	AR30	*N. mediterraneum*	Vélez-Málaga
AR11 L1	*N. australe*	Benamocarra	AR31	*N. australe*	Vélez-Málaga
AR11 L2	*C. gloeosporioides*	Benamocarra	AR32	*N. australe*	Vélez-Málaga
AR12 P	*C. gloeosporioides*	Benamocarra	AR33	*N. parvum*	Algarrobo-Costa
AR12 G	*Lasiodiplodia theobromae*	Benamocarra	AR34	*N. parvum*	Iznate
AR13	*N. luteum*	Benamocarra	AR35 A	*N. parvum*	Iznate
AR14 CA	*C. gloeosporioides*	Benamocarra	AR35 G	*N. parvum*	Iznate
AR14 CB	*C. gloeosporioides*	Benamocarra	AR35 C	*C. gloeosporioides*	Iznate
AR15 G	*N. parvum*	Benamocarra	AR36	*N. parvum*	Iznate
AR15 C	*C. gloeosporioides*	Benamocarra	AR37	*N. parvum*	Iznate
AR15 L	*C. gloeosporioides*	Benamocarra	AR38	*N. parvum*	Iznate
AR16 G	*N. parvum*	Benamocarra	AR39 A	*N. luteum*	Iznate
AR16 L	*C. gloeosporioides*	Benamocarra	AR39 C	*N. luteum*	Iznate
AR16 P	*N. parvum*	Benamocarra	AR40	*C. gloeosporioides*	Benamocarrra
AR17 CA	*N. parvum*	Benamocarra	AR41-1	*C. gloeosporioides*	Benamocarrra
AR17 CB	*N. parvum*	Benamocarra	AR41-2	*N. australe*	Benamocarrra
AR17 G	*N. parvum*	Benamocarra	AR42	*N. parvum*	Benamocarrra
AR18	*N. luteum*	Vélez-Málaga	AR43	*N. australe*	Benamocarrra
AR19 CA	*N. parvum*	Vélez-Málaga	AR44	*N. parvum*	Benamocarrra
AR19 CB	*N. parvum*	Vélez-Málaga	AR45	*N. parvum*	Benamargosa
AR19 G	*N. parvum*	Vélez-Málaga	AR46	*N. luteum*	Benamargosa
AR20	*N. parvum*	Vélez-Málaga	AR47	*N. parvum*	Benamargosa

*Sampling were carried out in different locations in Málaga province, Spain.

differences between *N. luteum* and *C. gloeosporioides* were also observed.

Significantly different virulence groups were established within each species (from 68 isolates) with more than one representative isolate, and 10 groups for *N. parvum*, 6 for *N. luteum*, 3 for *N. australe* and 5 for *C. gloeosporioides* were obtained (Table 5). The virulence was homogenized for *N. australe* because it was defined in only three groups, although with a similar number of isolates for *N. luteum* and *C. gloeosporioides*, the virulence was defined in more groups (5 or 6).

Discussion

This study shows the incidence and diversity of fungal isolates associated with avocado dieback in commercial orchards in southern Spain. The list includes different genera (*Neofusicoccum, Colletotrichum* and *Lasiodiplodia*) and species (*N. parvum, N. luteum, N. mediterraneum, N. australe, C. gloeosporioides* and *L. theobromae*) that are also usually involved in dieback in avocado orchards in other countries (McDonald & Eskalen, 2011).

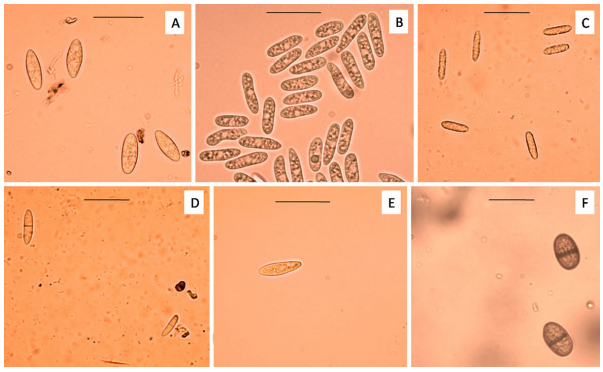

Figure 2. Conidia from representative isolates. A, *Neofusicoccum parvum*. B, *Colletotrichum gloeosporioides*. C, *Neofusicoccum austral*. D, *Neofusicoccum luteum*. E, *Neofusicoccum mediterraneum*. F, *Lasiodiplodia theobromae*. Scale bar= 20 µm.

Neofusicoccum parvum is the most abundant species, with an incidence of 50%, followed by *C. gloeosporioides* with 18%, *N. australe* and *N. luteum* with 13% and 16%, respectively, and finally, *N. mediterraneum* and *L. theobromae*, which appear only occasionally with an incidence of 1.5% in each case. Our results agree with the greater incidence of the species *N. parvum* and *N. australe* associated with almond orchards in other areas of Spain (Gramaje *et al*., 2012). *N. parvum* is considered the most common species associated with grapevine decline syndrome (Armengol *et al*., 2001; Aroca *et al*., 2006) and *N. mediterraneum* was also isolated from olive fruits in southern Spain, showing symptoms of Dalmatian disease (Moral *et al*., 2010).

In other countries, *N. parvum*, *N. luteum* and *N. australe* associated with avocado dieback have also been described in California (McDonald *et al*., 2009). Typical Dothiorella canker symptoms observed included darkened and friable bark showing a dry, white, powdery exudate. These studies concluded that the higher incidence of these pathogens is a consequence of the high-density planting frequent in Californian avocado crops and they recommend more suitable management strategies (McDonald & Eskalen, 2011). *N. luteum* was identified in California as the main cause of stem-end rot in harvested avocado fruit (65%), followed by *C. gloeosporioides* with an incidence of 35% (Twizeyimana *et al*., 2013). In the same way, the fungi detected in avocado branch cankers in Spain could also affect the fruit directly, leading to a fall in production. *L. theobromae* has been also described as the causal agent of avocado dieback in Peru (Alama *et al*., 2006). The symptoms of cankers and red-brown lesion with white exudates observed after artificial inoculation were similar to natural infection. There are other examples in which species of these genera have been associated

Table 3. Average measurements (width and length in µm of 120 conidia) from the different fungal isolates.

Isolates	Length (L)	Width (W)	L/W ratio
Neofusicoccum parvum	17.97 ± 1.73	6.5 ± 0.49	2.77
Colletotrichum gloeosporioides	18.14 ± 1.29	5.23 ± 0.49	3.23
N. australe	16.89 ± 1.3	4.09 ± 0.39	4.17
N. luteum	16.62 ± 3.25	4.30 ± 0.54	3.84
N. mediterraneum	22.66 ± 1.57	7.03 ± 0.18	3.23
Lasiodiplodia theobromae	21.99 ± 1.43	13.18 ± 0.5	1.67

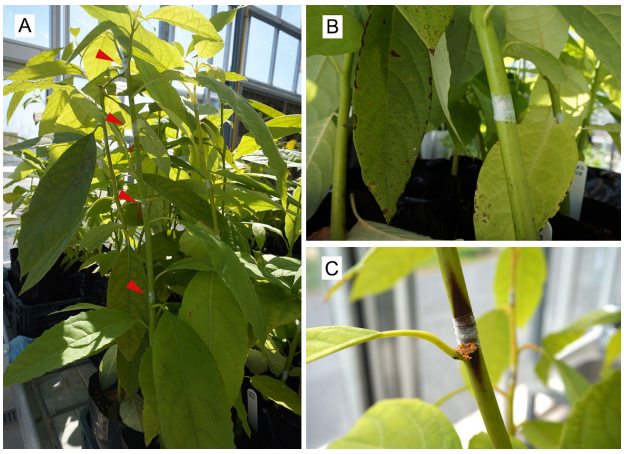

Figure 3. Artificial inoculations. A, Avocado plant with points of inoculations (red arrowheads). B, Mycelia plugs (5-mm in diameter) placed onto wounded stem and sealed with parafilm. C, Necrotic lesions on avocado stem.

Table 4. Number and percentage (in brackets) of isolates of each species by location.

Location	*N. parvum*	*C. gloeosporioides*	*N. australe*	*N. luteum*	*N. mediterraneum*	*L. theobromae*	Total
Vélez-Málaga	9 (43%)	1 (4.5%)	4 (19%)	6 (29%)	1 (4.5%)	0	21
Benamocarra	14 (44%)	10 (31%)	5 (16%)	2 (6%)	0	1 (3%)	32
Algarrobo-Costa	2 (100%)	0	0	0	0	0	2
Iznate	6 (67%)	1 (11%)	0	2 (22%)	0	0	9
Benamargosa	2 (67%)	0	0	1 (33%)	0	0	3
Cajiz	1 (100%)	0	0	0	0	0	1
Total	34 (50%)	12 (17.6%)	9 (13.2 %)	11 (16.2%)	1 (1.5 %)	1 (1.5 %)	68

Table 5. Virulence groups for *Neofusicoccum parvum*, *Neofusicoccum australe, Neofusicoccum luteum* and *Colletotrichum gloeosporioides* isolates. Comparison of data averaged of five repetitions using Fisher's LSD test to separate the means (p<0.05) (Steel & Torrie 1985). In each column, numbers followed by the same letter are not significantly different according to the LSD test.

N. parvum	**AUDPCs[1]**		***N. australe***	**AUDPCs[1]**	
AR10 L	3.55402	a	AR41-2	1.2620	a
AR27	3.0240	ab	AR5 L1	1.1440	ab
AR3	3.0140	ab	AR29	0.7480	abc
AR8 L2	2.9600	ab	AR11 L1	0.6780	bc
AR19 CB	2.9000	ab	AR2	0.6700	bc
AR17 G	2.4100	bc	AR32	0.6560	bc
AR4	2.3500	bcd	AR5 L2	0.6300	bc
AR17 CB	2.3240	bcd	AR43	0.3640	c
AR42	2.3040	bcd	AR31	0.2320	c
AR20	2.2280	bcde			
AR8 L1	2.1800	bcdef	***N. luteum***	**AUDPCs[1]**	
AR19 G	2.1080	bcdefg	AR18	1.8540	a
AR22	1.8660	cdefgh	AR10 C	1.4860	ab
AR19 CA	1.7900	cdefgh	AR26	1.0860	bc
AR24	1.6760	cdefghi	AR39 A	0.9260	cd
AR9	1.6420	cdefghi	AR13	0.8600	cde
AR17 CA	1.5280	cdefghij	AR29 P	0.7680	cdef
AR24 G	1.4280	cdefghij	AR23	0.7580	cdef
AR38	1.3980	cdefghij	AR28 G	0.6580	cdef
AR1	1.3580	defghij	AR25	0.6060	def
AR37	1.3420	defghij	AR39 C	0.4600	ef
AR44	1.2680	efghij	AR43	0.3480	f
AR21	1.2600	efghij			
AR15 G	1.2100	fghij	***C. gloeosporioides***	**AUDPCs[1]**	
AR16 G	1.1380	ghij	AR14 CA	0.8160	a
AR34	1.1160	ghij	AR6	0.7240	ab
AR7	0.9880	hij	AR14 CB	0.6740	abc
AR16 P	0.8780	hij	AR40	0.5160	bcd
AR35 G	0.7500	ij	AR15 C	0.4940	bcd
AR35 A	0.7320	ij	AR11 L2	0.4660	cd
AR47	0.6700	ij	AR16 L	0.4200	de
AR33	0.5860	j	AR15 L	0.3900	de
AR36	0.5800	j	AR12 P	0.3820	de
AR45	0.5260	j	AR35 C	0.3460	de
			AR41-1	0.2860	de
			AR28 P	0.1880	e

[1]AUDPCs: standardized area under disease progress curve for necrotic lesion observed along 9 days.

to avocado causing anthracnose and stem-end rot in Turkey (Akgül & Awan, 2016), or to other plants such as oak (*Quercus robur* L.) in Portugal (Barradas *et al*., 2013) and olive in Tunisia (Triki *et al*., 2015) causing dieback, or shoot blight and plant decay on pomegranate (*Punica granatum* L.) in Italy (Riccioni *et al*., 2017).

In our study, we conclude that *N. parvum* and *N. luteum* are widely extended, while *C. gloeosporioides* and *N. australe* are located only in the biggest areas, which could be due to the wider dispersion or higher production of conidia of *N. parvum* and *N. luteum*. Future experiments should be carried out using spore trapping (Eskalen *et al*., 2013) to confirm this theory. Our results, showing the greater virulence of isolates of *N. parvum* and *N. luteum* when compared with *C. gloeosporioides* and the greater virulence of *N. parvum* vs *N. australe*, coincide with the results of Eskalen *et al*. (2013), who reported that lesions caused by *N. parvum* and *N. luteum* were larger than those caused by *N. australe*. *N. mediterraneum* and *L. theobromae* appeared occasionally, but did not appear to be a great threat.

Although *N. parvum* has been previously described in avocado (Zea-Bonilla *et al*., 2007) and in other crops such as blueberry (*Vaccinium* spp.) (Castillo *et al*., 2013) and mango (Arjona-Girona & López-Herrera, 2016) on the southern coast of Andalusia, Spain, an increased incidence of dieback on branches in avocado orchards has been observed and this could constitute a serious threat, in a similar way to *N. luteum, N. australe* and *C. gloeosporioides*, to the yield of avocado orchards in this area. The fungal inoculum tends to increase due to the poor management of pruning remains, which are often shredded and mixed into the soil instead of being burned, as famers usually do in this area.

This is the first study of avocado cankers on branches in southern Spain which evaluates the causal agents and establishes its pathogenicity groups. *N. parvum* is the most abundant species observed, and is the most important causal agent of dieback avocado in this area. *N. luteum, N. australe* and *C. gloeosporioides* showed the lower incidence as causal agents of the disease.

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