

Estimation of viable propagules of black-foot disease pathogens in grapevine cultivated soils and their relation to production systems and soil properties

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Abstract

Aims The study aimed to assess comparatively the accuracy and efficiency of three culture media protocols for estimating black-foot disease pathogens populations in soils and to examine how shifts in the abundance and composition of black-foot pathogens correspond to changes in specific soil properties.

Methods Firstly, culture media were compared by evaluating the mycelial growth of selected black-foot pathogens and by estimating the population of *Dactylonectria torresensis* from artificially infested soils. Secondly, the most efficient culture medium was selected for estimating the viable propagules of black-foot disease pathogens in eight naturally infested soils. An analysis of the soil physicochemical properties was conducted. Data were statistically analyzed in order to explore possible relationships between the studied variables.

Results Glucose-Faba Bean Rose Bengal Agar (GFBRBA) was selected as the most efficient culture medium. All naturally infested soils tested positive for the presence of black-foot pathogens. *D. torresensis* was the most frequently isolated species, followed by

Dactylonectria alcacerensis and *Ilyonectria liriodendri*. A positive relationship between calcium carbonate and the Colony-Forming Units (CFUs) level of black-foot pathogens in soil was obtained.

Conclusions In this study, we provide an early, specific, and accurate detection of viable propagules of black-foot pathogens in soil, which is critical to understand the ecology of these fungi and to design effective management strategies.

Keywords *Dactylonectria torresensis* · Fungal ecology · Grapevine trunk disease · Soilborne inoculum

Abbreviations

CEC	Cation Exchange Capacity
CFU	Colony-Forming Unit
EC	Electric conductivity
GFBA	Glucose-Faba Bean Agar
GFBRGA	Glucose-Faba Bean Rose Bengal Agar
ITS	Internal Transcribed Spacer
MRBA	Modified Rose Bengal Agar
NMDS	Non-Metric Multidimensional Scaling Analysis
LSD	Least Significant Difference
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDAC	Potato Dextrose Agar supplemented with 250 mg l ⁻¹ of chloramphenicol
SNA	Spezieller Nährstoffarmer Agar
SOM	Soil Organic Matter

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Introduction

Black-foot disease is one of the main soilborne fungal diseases affecting grapevine production worldwide, being particularly important in grapevine nurseries and new plantations, where it causes the young vine decline syndrome (Halleen et al. 2006; Gramaje and Armengol 2011; Agustí-Brisach and Armengol 2013). Young vines affected by black-foot disease generally appear normal at planting, but differences in vigour become marked: reduced trunk caliper, shortened internodes, reduced foliage/canopy fill, and reduced leaf area. During the first 3 to 5 years after planting, foliar symptoms may appear as small leaves with interveinal chlorosis, followed by necrosis and early defoliation (Agustí-Brisach and Armengol 2013). Removal of rootstock bark of declining plants reveals black discoloration and necrosis of wood tissues which develop from the base of the rootstock. Below ground, symptoms include reduced total root biomass, low numbers of feeder roots, and black, sunken, necrotic root lesions (Agustí-Brisach and Armengol 2013).

Cylindrocarpon-like anamorphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria* and *Thelonectria* have been associated with black-foot disease (Agustí-Brisach and Armengol 2013; Lombard et al. 2014; Carlucci et al. 2017), with *Dactylonectria torresensis* the most common species associated with diseased vines in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016). The disease cycle of black-foot pathogens on grapevines is not completely known, but the behaviour of *Cylindrocarpon*-like anamorphs on other hosts has been studied in more detail (Booth 1966; Brayford 1993), and provided information about the likely disease cycle of associated genera on grapevines. *Cylindrocarpon*-like anamorphs readily produce conidia and some species also chlamydospores in culture, which indicates that these propagules are likely to be produced on the diseased roots and stem bases of infected vines. The conidia are dispersed in soil water and the chlamydospores can allow these fungi to survive in the soil for extended periods of time (Petit et al. 2011). They infect grapevines through natural openings or wounds, such as the non-callused part of the lower trunk. Infection can also occur through wounds in canes, such as disbudding wounds, from which the infection progresses downward to the base of the trunk (Halleen et al. 2006). The occurrence of black-foot disease

pathogens during the nursery propagation process (Halleen et al. 2003, 2007; Agustí-Brisach et al. 2013a; Cardoso et al. 2013; Reis et al. 2013) and in the vineyard (Agustí-Brisach et al. 2013b, 2014), has resulted in a number of hypotheses on the sources of primary inoculum and their dissemination.

The detection, identification and quantification of black-foot pathogens from soil samples have been mainly focused on the use of DNA based molecular techniques (Damm and Fourie 2005; Probst et al. 2010; Cardoso et al. 2013; Agustí-Brisach et al. 2014). Although these methods are very useful, it is important to consider that the mere presence of DNA does not indicate whether viable pathogen propagules are present and active (England et al. 1997; Demanèche et al. 2001).

The use of traditional methods to identify fungal pathogens in soil environments is challenging. These methods rely mainly on the dilution plating technique together with the use of selective media, and microscopy to identify sporulating fungal structures. The advantages in the use of these methods reside in their simplicity and low cost. They have provided quite a sensitive detection of soil fungi and have been widely used in diversity studies in different habitats (Elmholt et al. 1999; Cho et al. 2001; Cabello and Arambarri 2002). By contrast, one of the major constraints with cultural studies is that fungal diversity may be quite high in soil, processing of cultures can be time consuming and laborious when a large number of isolates has to be handled, and the risk of culture contamination is always high and in most cases fast growing fungi will overgrow others and fill the plate (Jeewon and Hyde 2007). In addition, there can be many species that behave similarly under cultural conditions and exhibit similar morphology but are in fact different species.

The alternative to isolate black-foot disease pathogens from soil is to bait for them using susceptible hosts (Agustí-Brisach et al. 2013b), which is an efficient method, but usually takes time, since this method involves growing grapevine seedlings and planting them in target soils till recovery and isolation after a long time (Agustí-Brisach et al. 2013b). In addition, baiting introduces a potential bias because of host adaptation, so these isolates might not be valid for use in further studies aimed to determine and compare the genetic composition of black-foot pathogen populations strictly living in soil. The effect of resident plants on fungal soil populations has been previously reported for *Fusarium oxysporum* (Edel et al. 1997).

The development of an improved medium for detecting viable propagules of black-foot pathogens would allow us (i) to improve our knowledge on the ecology of these fungi in soil, (ii) to alert nurseries and growers to the presence of the pathogens in soil, (iii) to use disease risk as a factor in making management decisions, (iv) to characterize the diversity, genetic structure and pathogenicity of black-foot pathogen populations in soil and compare them with those collected from grapevine roots and rootstock and from asymptomatic secondary hosts (rotational crops in nursery fields and weeds), and (v) to compare the results of the soil plating assays using different types of field soil with existing quantitative molecular protocols (Probst et al. 2010; Agustí-Brisach et al. 2014).

With this aim, the goal of this study was (i) to compare modified culture media already published in the literature for the detection of *Cylindrocarpon*-like anamorphs or other soilborne pathogens phylogenetically related to black-foot genera, (ii) to assess the usefulness of the most efficient culture medium for estimating black-foot disease pathogens populations in naturally infested soils, and (iii) to examine how shifts in the abundance and composition of black-foot pathogens correspond to changes in specific soil properties.

Material and methods

Culture media preparation

Three media were prepared in order to compare their efficiency for growing selected black-foot pathogens and the recovery of *Dactylonectria torresensis* from sterile and non-sterile soil: (i) a modification of Rose Bengal Agar (MRBA; Reedler et al. 2003) medium consisted of 32 g of Rose Bengal Agar (Difco, Detroit, MI, USA) (5 g Bacto peptone, 10 g dextrose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g Bacto agar), 30 mg streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA), 250 mg ampicillin (Sigma-Aldrich, St. Louis, MO, USA), 10 mg rifampicin (Sigma-Aldrich, St. Louis, MO, USA) (dissolved in 1 ml ethanol), 500 mg pentachloronitrobenzene, 500 mg dicloran, and 1 l distilled water; (ii) the Glucose-Faba Bean Rose Bengal Agar (GFRBA), modified from Hunter et al. (1980), consisted of a faba bean infusion (boiling 62.5 g of fresh faba beans in 1 l distilled water for 5 min, keeping them in water for 12 h and filtering through cheesecloth, saving 0.75 l effluent), 32 g of Rose Bengal Agar (Difco) (5 g Bacto peptone, 10 g dextrose,

1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g Bacto agar), 90 g of glucose (Scharlab S.L., Barcelona, Spain), 50 mg of chloramphenicol (Sigma-Aldrich), 30 mg streptomycin sulphate (Sigma-Aldrich), 250 mg ampicillin (Sigma-Aldrich), 10 mg rifampicin (Sigma-Aldrich) (dissolved in 1 ml ethanol), and 0.25 l distilled water; and (iii) the Glucose-Faba Bean Agar (GFBA), modified from Singleton et al. (1993), consisted of a faba bean infusion as described above, 15 g of Bacteriological agar (Panreac AppliChem, Barcelona, Spain), 100 g of glucose (Scharlab S.L.), 50 mg of chloramphenicol (Sigma-Aldrich), 30 mg streptomycin sulphate (Sigma-Aldrich), 250 mg ampicillin (Sigma-Aldrich), 10 mg rifampicin (Sigma-Aldrich) (dissolved in 1 ml ethanol), and 0.25 l distilled water.

In all media, antibiotics and fungicides were added and thoroughly mixed after autoclaving at 121 °C for 15 min and cooling to 55 °C. The pH of all media was measured using a pH meter (Fisher Scientific, Santa Clara, CA) when the media temperature ranged between 39 and 45 °C after each batch was prepared.

Evaluation of mycelial growth

Seven single spore isolates (one isolate of the species *Campylocarpon fasciculare*, *Dactylonectria alcaerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis* and *Ilyonectria liriodendri*) obtained from different geographic locations and grapevine rootstock–scion combinations in Spain, and one isolate each of the species *Fusarium oxysporum* and *Rhizoctonia solani* were evaluated for mycelia growth on the media described above (Table 1). Isolates were obtained from the culture collection of the Instituto Agroforestal Mediterráneo at the Universitat Politècnica de València (Spain). Three plates of each medium were inoculated centrally with a 5-mm diameter agar disk from the advancing margins of 2-week-old cultures of each isolate on PDA. All plates were incubated at 25 °C. Colony diameters were measured after 10 days of incubation. The experiment was conducted twice.

Preparation of artificially infested soil and recovery of *Dactylonectria torresensis*

Bulk soil was collected from a 10-year old vineyard in La Grajera (Logroño, Spain), with a shovel at 10 to 30 cm depth (field code C) (Table 2). Four subsamples were randomly taken from the interrow space

Table 1 Fungal isolates used in this study and radial growth (in mm after 10 days at 25 °C) on different culture media

Species	Isolate	Year	Location	Host	GenBank accessions ^a	Radial growth on MRBA ^b	Radial growth on GFRBA ^c	Radial growth on GFBA ^d
<i>Campylocarpon fasciculare</i>	GIHF-102	2015	Aielo de Malferit (Valencia)	Grapevine rootstock 110 Richter	KY887653	23.2 ^b b B ^c	26.7 b A	45.0 a BC
<i>Dactylonectria alcaerensis</i>	DA-1	2016	Aielo de Malferit (Valencia)	Grapevine rootstock 110 Richter	KY887654	17.7 c D	24.2 b B	46.2 a B
<i>D. macrodichyma</i>	057	2016	Aielo de Malferit (Valencia)	Grapevine rootstock 110 Richter	KY887655	15.2 c E	26.5 b A	41.2 a D
<i>D. novozelandica</i>	058	2016	Aielo de Malferit (Valencia)	Grapevine rootstock 110 Richter	KY887656	23.7 b B	24.5 b B	38.7 a E
<i>D. pauciseptata</i>	GIHF-100	2016	Madrid	Common box	KY887657	19.5 b C	21.7 b C	41.2 a D
<i>D. torresensis</i>	BV-149	2015	Mendavia (Navarra)	Grapevine rootstock 110 Richter	KY887658	16.7 c D	20.7 b C	38.5 a E
<i>Ilyonectria liriodendri</i>	IL-1	2016	Aielo de Malferit (Valencia)	Grapevine rootstock 110 Richter	KY887659	24.0 c B	26.5 b A	43.7 a C
<i>Fusarium oxysporum</i>	GIHF-125	2016	El Perelló (Valencia)	Tomato	KY887660	26.2 c A	28.2 b A	72.0 a A
<i>Rhizoctonia solani</i>	GIHF-140	2016	Calasparra (Murcia)	Spinach	KY887661	25.5 c A	28.0 b A	72.0 a A

^a GenBank accessions correspond to histone (black-foot pathogens), elongation factor (*F. oxysporum*) and internal transcribed spacer (*R. solani*) sequences

^b MRBA Modification of Rose Bengal Agar, GFRBA Glucose-Faba Bean Rose Bengal Agar, GFBA Glucose-Faba Bean Agar

^c Average of six replicates

^d Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$). Capital letters are for comparison of means in the same column. Small letters are for comparison of means in the same row

of asymptomatic grapevine plants (about 0.5 kg of soil/sample) in May 2016. Samples were mixed well, air-dried for one week and sieved (2-mm to 5-mm mesh size) prior to soil physicochemical analyses and culture media test. One half-soil sample was sterilized twice by autoclaving at 121 °C for 1 h at one-day interval.

Mycelium of one isolate of *D. torresensis* (BV-048) was produced on sterilised wheat grains. The wheat grains (200 g) were placed in a 1 L conical flask containing 500 mL of tap water and heated to boiling. The grains were left to settle for 10 min then washed three times with tap water and the excess drained off. The grains were autoclaved at 121 °C for 15 min and left for 24 h, after which the process was repeated. Each flask of wheat grains was inoculated with ten 8 mm mycelium plugs of the BV-048 isolate taken from the edge of the *D. torresensis* cultures grown on PDA plates and incubated at 25 °C for three weeks. The flasks were incubated at 20 °C in the dark for one month, during which they were shaken daily by hand (5 s) to assist colonisation by mycelium, which was confirmed visually.

Sterile and non-sterile soil (10 g, fresh weight) was mixed in a 1:1 proportion with 10 g of *D. torresensis* inoculum produced in wheat grains. Each mixture was incubated for three weeks at 25 °C in the darkness. After this period, mixtures (20 g) were diluted in 100 mL of 0.1% water agar (dilution 1). The mixture was homogenised by shaking the bottle in a reciprocating shaker for 10 min at 250 rpm. One mL aliquots were serially diluted 100 times in 100 mL of 0.1% water agar (dilution 2) and then 10 times in 9 mL falcon tubes with sterile water (dilution 3). Sterile pipettes were used to transfer 150 µl each of the last two dilutions onto the surface of an agar plate. The soil suspension was spread evenly across the surface of the plate by the use of sterile glass spreading rods. Nine plates were prepared for each of the four soil samples, dilution and culture media (MRBA, GFRBA and GFBA). The experiment was repeated three times. Plates were incubated at 25 °C in the dark for 10 days, and examined daily for development of *D. torresensis* colonies using a stereomicroscope at 7.5 x magnification. Colony counts were converted to colony-forming units (CFU) per gram of dry soil.

Detection of black-foot pathogens in naturally infested soils

Eight fields located in different regions in Spain were surveyed in June 2016: four established vineyards (A,

Table 2 Field sites used for soil sampling

Field code	Geographical origin		Field type
	Location	Province	
A	Aldeanueva del Ebro	La Rioja	27-year-old vineyard (Tempranillo / 110 Richter)
B	Olite	Navarra	7-year-old vineyard (different scion/rootstock combinations)
C	Logroño	La Rioja	10-year-old vineyard (Tempranillo / 110 Richter)
D	Logroño	La Rioja	2-year-old vineyard (Tempranillo / 110 Richter)
E	Canals	Valencia	Field nursery (Tempranillo / 110 Richter)
F	Canals	Valencia	Field nursery (Tempranillo / 110 Richter)
G	Mendavia	Navarra	Field nursery during third year of rotation (bare fallow land)
H	Canals	Valencia	Field nursery during first year of rotation (fallow land with weeds)

B, C and D), two nursery fields (E and F), and two nursery fields in rotation (G and H) (Table 2). Each field had a sampling area marked by a “V” pattern that included four interrow vineyard spaces, except for fields G and H, for soil sampling. Four bulk soil samples were taken with a shovel at 10 to 30 cm depth in each field (about 0.5 kg of soil/sample), and then processed and analysed for physicochemical properties in the laboratory. Samples were diluted as described before and soil suspensions were spread at dilution 2 onto GfBRBA medium, which was selected as the most efficient dilution/medium combination in the previous experiment. Four plates were prepared for each sub-sample. The experiment was repeated three times (48 plates per soil sample). Plates were incubated at 25 °C in the dark for 10 days, and examined daily for development of fungal colonies using a stereomicroscope at 7.5 x magnification. Colonies emerging from the agar were transferred to PDA for further morphological and molecular identification, and those identified as black-foot pathogens were converted to CFU per gram of dry soil.

Fungal identification

Fungal isolates resembling black-foot pathogens were identified morphologically by macroscopic characters, including colony texture, colour, and the shape of the growing margin on PDA. Conidia were observed and measured from colonies growing on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1 × 1 cm piece of filter paper to the colony surface (Alaniz et al. 2007; Chaverri et al. 2011).

For DNA extraction, fungal mycelium and conidia from single spore isolates grown on PDA for 2 to 3 weeks at 25 °C in the dark were scraped and mechanically

disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer’s instructions. DNA was visualized on 1% agarose gels stained with RedSafe (iNtRON Biotechnology, Lynnwood, WA, USA) DNA stain and was stored at –20 °C.

All fungal species were identified by analysis of the Internal Transcribed Spacer (ITS) region of DNA amplified using the fungal universal primers ITS1F (Gardes and Bruns 1993) and ITS4. The identification of black-foot pathogens was confirmed by sequencing part of the histone H3 gene using CYLH3F and CYLH3R primers (Crous et al. 2004; Cabral et al. 2012a, b). Polymerase chain reaction (PCR) products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). The sequences obtained were then blasted in GenBank.

Soil physicochemical properties analysis

Soils sampled were tested for pH and electric conductivity (EC) in water with a soil solution ratio of 1:5, soil organic matter (SOM) by dichromate oxidation (Nelson and Sommers 1982), soil texture by laser diffraction particle size (Diffractometer LS 13320, Beckman Coulter Inc., Brea, Calif.), total carbonate by infrared (Equilab CO-202; Equilab, Jakarta, Indonesia), cation exchange capacity (CEC) by the cobaltihexamine method (Orsini and Remy 1976), assimilable calcium and magnesium by the cobaltihexamine method and by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA) and P, K, S, Mg, Mn, Fe, Ca and Na by ICP and Mehlich method

(Mehlich 1984). Analyses were conducted in the official Regional Laboratory of La Grajera (Logroño, Spain).

Data analyses

For the mycelial growth and the culture media assays, data were analysed using one-way ANOVA. Student's *t* test least significant difference (LSD) was calculated at the 5% significance level to compare mean mycelial growth between the different media and fungal species, and to compare mean CFUs between the different media, respectively.

Due to a non-uniformity of variance, black-foot populations CFU counts from naturally infested soils were transformed to $\log_{10}(x + 1)$, where *x* is the number of CFUs per g of soil per petri dish. This transformation was better at achieving homogeneity of variance than several others tried. Data were analysed using one-way ANOVA. LSD test was used to determine the differences between black-foot total counts in different fields. Data from all experiments were analysed using the Statistix 10 software (Analytical Software).

Soil physicochemical variables were subjected to principal component analysis (PCA) in order to group the different fields tested and to reduce the observed variables into a smaller number of principal components (artificial variables) that will account for most of the variance in the observed variables. PCA analysis was performed using the R version 3.3.2 (The R Foundation for Statistical Computing) (R Core Team 2016) packages *stats* (R Core Team 2016) and *devtools* (Wickham and Chang 2016). Non-metric multidimensional scaling analyses (NMDS) based on dissimilarities calculated using the Bray–Curtis index after Hellinger standardization were run with *MetaMDS* functions within the *vegan* package (Oksanen et al. 2008) using R and soil physicochemical variables vectors were fitted using the *envfit* routine. Correlations with CFU (R^2) were obtained by fitting linear trends to the NMDS ordination obtained for each variable and significance (*P*) was determined by permutation (*nperm* = 1000).

Results

Effect of media on mycelial growth

The pH of GFBA medium was the lowest (pH = 5.9 ± 0.04), MRBA medium was the highest

(pH = 7.0 ± 0.06) and GFBRBA medium was intermediate (pH = 6.3 ± 0.09). Colony diameters of black-foot isolates as well as *F. oxysporum* and *R. solani* isolates in GFBA were significantly greater than on the other two tested media (*P* < 0.05) (Table 1). In the MRBA and GFBA media, colony diameters of *F. oxysporum* and *R. solani* isolates were significantly greater than black-foot pathogens (*P* < 0.05). In the GFBRBA medium, colony diameters of *C. fasciculare*, *D. macrodidyma* and *I. liriodendri* did not show significant differences with mycelial growth of *F. oxysporum* and *R. solani* isolates (*P* > 0.05). Most of the species of black-foot produced a brown or yellow pigment on the agar of the media MRBA and GFBRBA.

Detection of *Dactylonectria torresensis* in artificially infested soils

Recovery of *D. torresensis* from sterile soil samples was affected by the type of media used (Table 3). In dilution 2, the CFUs/g soil in GFBRBA and GFBA media were significantly greater than in MRBA medium. In dilution 3, recovery of *D. torresensis* on GFBRBA medium was increased over MRBA and GFBA media. Regarding the non-sterile soil samples, no significant differences were found among media when measuring the CFUs/g soils in dilution 2 (Table 3). In dilution 3, the CFUs/g soil in GFBRBA and GFBA media were significantly greater than in MRBA medium.

Detection of black-foot pathogens in naturally infested soils

All fields were positive for the presence of black-foot pathogens. *D. torresensis* was isolated from all soil samples evaluated with an inoculum density ranging from 2.793 to 0.631 \log_{10} CFU/g soil (Fig. 1). Significant differences were found in the CFU of *D. torresensis* among soils (*P* < 0.01) (Fig. 1). This species was isolated most frequently from field B (2.824 ± 0.207 \log_{10} CFU/g soil), with no significant differences with field A (2.699 ± 0.250 \log_{10} CFU/g soil). In general, established vineyards (A, B, C: 2.523 ± 0.201 \log_{10} CFU/g soil, D: 2.224 ± 0.220 \log_{10} CFU/g soil) and nursery fields (E: 2.399 ± 0.097 \log_{10} CFU/g soil, F: 2.399 ± 0.028 \log_{10} CFU/g soil) had higher inoculum density of *D. torresensis* than nursery fields in rotation (G: 1.925 ± 0.035 \log_{10} CFU/g soil, H: 2.224 ± 0.211 \log_{10} CFU/g soil). The inoculum density of

Table 3 Comparison of media for recovery efficiency of *Dactylonectria torresensis* from artificially infested soils

Media ^a	Sterile soil		Non-sterile soil	
	Dilution 2	Dilution 3	Dilution 2	Dilution 3
MRBA	Mean estimated population density (CFU / g soil) 7407.4 ^b ± 79.1 b ^c	Mean estimated population density (CFU / g soil) 1538.5 ± 148.1 b	Mean estimated population density (CFU / g soil) 8592.6 ± 1002.3 a	Mean estimated population density (CFU / g soil) 1481.5 ± 148.1 b
GFRBA	9037.0 ± 120.4 a	4444.4 ± 326.2 a	8740.7 ± 1328.2 a	1538.5 ± 148.1 a
GFBA	9185.2 ± 135.1 a	1481.5 ± 148.1 b	8888.9 ± 1405.4 a	1538.5 ± 148.1 a

^a MRBA: Modification of Rose Bengal Agar; GFRBA: Glucose-Faba Bean Rose Bengal Agar; GFBA: Glucose-Faba Bean Agar

^b Average of 3 replicates of 9 plates each

^c Least significant difference: means followed by the same letter do not differ significantly ($P < 0.05$)

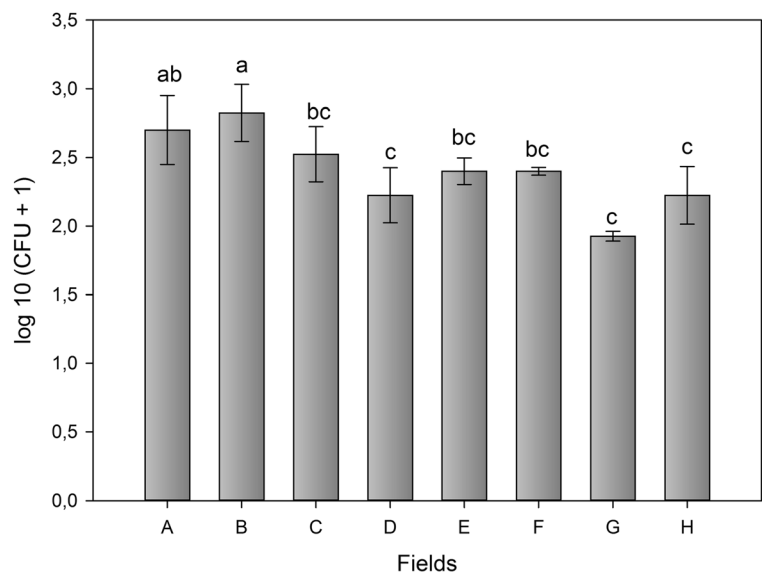
D. torresensis in nursery fields (E and F) was not significantly different from established vineyards (A and C). *Dactylonectria alcacerensis* was also isolated from fields B ($1.262 \pm 0.21 \log_{10}$ CFU / g soil) and F ($2.621 \pm 0.02 \log_{10}$ / g soil), while *I. liriodendri* was only detected in field F ($0.631 \pm 0.18 \log_{10}$ / g soil).

Physicochemical properties of the soil

The soil physicochemical properties varied between the experimental fields (Table 4). The PCA analysis showed the ordination of experimental fields according to soil physicochemical properties as well as the relationships among studied variables (Fig. 2). The first coordinate explained 44.7% of the variation, the second coordinate explained 36.7% of the variation, and the third coordinate explained 11.4% of the

variation. In the plot of the first and second coordinates, axis 1 was clearly associated with soil texture (percentages of clay, sand and silt), as well as with CaCO_3 , P, S and Fe concentrations. The percentage of clay, silt and CaCO_3 increased to the positive side of the axis, whereas the percentage of sand, P, S and Fe increased to the negative side, thus separating field B (high CaCO_3 concentration and percentage of silt) from field E (high K concentration and percentage of sand). The second PCA axis was associated mainly with K concentration and moderately with CEC (increasing to the positive side of the axis), and moderately with pH and Ca (increasing to the negative side of the axis). This separated the fields in two groups: soils with medium-high Ca concentration (fields A, C and G), and those with high percentages of K concentration (fields F and H).

Fig. 1 Mean \log_{10} (Colony Forming Units (CFU) + 1) of *Dactylonectria torresensis* isolated from eight field soils. Results are reported as mean values of 48 plates from four soil samples per field. Means followed by the same letter are not significantly different ($P < 0.05$). Bars represent standard error of the mean



Relationships between CFU of black-foot pathogens and physicochemical properties of the soil

Non-metric multidimensional scaling analysis (NMDS) grouped the variable CFU of black-foot pathogens per g of soil with CaCO₃ concentration (Fig. 3). Moreover, CFU of black-foot pathogens per g of soil correlated positively with CaCO₃ concentration ($r = 0.61$; $p < 0.05$).

Discussion

Early, specific, and accurate detection of black-foot pathogens in soil is essential to prevent the infection of grapevine planting material by these pathogens in field nurseries and also during the first years after planting. In this study, the accuracy and efficiency of three modified culture media protocols published in the literature for the detection of *Cylindrocarpon*-like anamorphs or other soilborne pathogens phylogenetically related to black-foot genera based on plating dry soil samples was assessed comparatively (Hunter et al. 1980; Singleton et al. 1993; Reedler et al. 2003). We selected a robust protocol, modified from Hunter et al. (1980), that was efficient in detecting black-foot pathogens in naturally infested soils. The culture medium was selected based on the ability of most of the black-foot species to produce a brown or yellow pigment on the agar and the good performance obtained in the recovery of *D. torresensis* from both sterile and non-sterile soil samples. In addition, the mycelial growth of other relevant soilborne fungi (*F. oxysporum* and *R. solani*) was slowed down on GFBRBA and no significant differences were observed with the mycelial growth of some black-foot species. The use of this culture medium would avoid the overgrowing of black-foot species by fast-growing fungi such as *F. oxysporum* and *R. solani* in soil plating assays. Although the emphasis of this study was put on viticulture, other agricultural systems could equally benefit from our optimised protocol.

Detection of *D. torresensis* using the selected protocol confirmed this species as the most frequent black-foot pathogen isolated from the studied soils. Previous research aimed to determine the incidence of black-foot pathogens in grapevine plants in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013) and Spain (Tolosa-Almendros et al. 2016) also identified *D. torresensis* as the most frequent black-foot pathogen species. *D. torresensis* was the species with the widest

occurrence in the study carried out by Cabral et al. (2012a), being present on four continents, and associated with *Vitis vinifera*, *Abies nordmanniana*, *Fragaria* sp., and *Quercus* sp. This species is also able to affect other woody hosts such as *Protea* sp. in South Africa (Lombard et al. 2013), *Viburnum tinus* in Italy (Aiello et al. 2015) and *Actinidia chinensis* in Turkey (Erper et al. 2013), and has been reported as the most frequently isolated species from necrotic lesions of loquat roots (Agustí-Brisach et al. 2016). Our findings are in agreement with the results obtained by Cardoso et al. (2013), who reported *D. torresensis* as the predominant species in rhizosphere soil samples collected from rootstock mother fields and nursery fields in Portugal.

Recent studies in rhizosphere research on grapevine (Zarraonaindia et al. 2015) and other crops (Turner et al. 2013) suggested that plant species has a clear, significant influence on the microbiota composition in rhizosphere soil. In addition, these studies recognized changes in the microbiota composition between rhizosphere and bulk soil. Active selection of the root microbiota, and in particular plant pathogens, as a consequence of the use of specific plant genotypes has been studied extensively in other pathosystems (Lannou and Mundt 1997; Gandon and Michalakis 2002; Brown and Tellier 2011). Thus, more research is needed to explore the effects of rootstock selection on black-foot pathogen populations in rhizosphere soil and the changes of these populations between bulk soil and rhizosphere samples.

The inoculum density of *D. torresensis* (CFU per gram of soil) varied among field soils, with this species most often found from bulk soil of 7-year-old and 27-year-old vineyards versus a 2-year-old vineyard and nursery fields during third year of rotation. The richness of black-foot species found in a field nursery was greater than in the other field samples tested. This confirms nursery fields are an important source of soilborne inoculum of black-foot pathogens, which agrees with previous research (Halleen et al. 2003, 2007; Agustí-Brisach et al. 2013a, b, 2014; Cardoso et al. 2013; Reis et al. 2013). Black-foot disease pathogens cause necrosis in the basal end of the rootstock, leading to the early decline and the death of vines exclusively in nurseries and young vineyards (<8 years old) (Agustí-Brisach and Armengol 2013). In this study, we found that *D. torresensis* inoculum is also present in a 27-year-old mature vineyard. However, at this stage, plants are usually less susceptible to black-foot infection than those planted in grapevine nurseries or recently

Table 4 Physicochemical properties of the eight soils examined in this study. Values represent the mean \pm SE

Soil	pH	P mg/100 g	K mg/100 g	S mg/100 g	Mg mg/100 g	Mn mg/100 g	Fe mg/100 g	Ca mg/100 g	Na mg/100 g	SOM%
A	8.42 \pm 0.02	3.47 \pm 0.27	15.52 \pm 0.59	4.37 \pm 0.38	25.57 \pm 0.29	9.31 \pm 0.87	7.5 \pm 0.44	1570.92 \pm 220.8	6.08 \pm 0.19	0.74 \pm 0.02
B	8.1	1.8 \pm 0.11	17.3 \pm 0.44	0.9 \pm 0.27	15.0 \pm 0.26	9.23 \pm 0.11	3.27 \pm 0.07	1862.15 \pm 12.6	1.46 \pm 0.05	1.75 \pm 0.01
C	8.12 \pm 0.05	1.91 \pm 0.20	19.97 \pm 0.42	7.95 \pm 0.11	23.3 \pm 0.45	2.44 \pm 0.12	3.18 \pm 0.062	3437.9 \pm 79.6	1.64 \pm 0.02	1.15
D	8.12 \pm 0.02	8.07 \pm 1.88	33.05 \pm 2.57	15.65 \pm 1.26	49.37 \pm 3.52	9.62 \pm 0.66	9.6 \pm 0.38	2512.05 \pm 231.1	3.31 \pm 0.36	1.61 \pm 0.13
E	8.3	23.92 \pm 0.73	23.17 \pm 0.16	14.02 \pm 0.33	44.57 \pm 0.83	15.6 \pm 0.07	7.71 \pm 0.46	534.3 \pm 15.1	12.57 \pm 0.16	0.67 \pm 0.01
F	8.0	10.42 \pm 0.08	61.55 \pm 0.66	13.6 \pm 0.36	60.65 \pm 0.69	11.1 \pm 0.04	5.91 \pm 0.15	1359.02 \pm 12.2	9.95 \pm 0.15	2.26 \pm 0.04
G	8.27 \pm 0.02	2.5 \pm 0.20	26.55 \pm 1.83	13.87 \pm 0.29	32.75 \pm 1.11	6.37 \pm 1.49	4.95 \pm 0.49	2173.97 \pm 282.7	2.22 \pm 0.17	1.12 \pm 0.02
H	8.27 \pm 0.02	9.01 \pm 0.10	50.92 \pm 0.63	2.42 \pm 0.05	66.2 \pm 0.51	20.37 \pm 0.22	4.42 \pm 0.10	993.9 \pm 10.7	10.17 \pm 0.19	0.99 \pm 0.01

Clay%	Sand%	Silt%	CO ₃ Ca	CEC mekv/100 g	EC mS/cm	Assim. Ca mekv/100 g	Assim. Mg mekv/100 g
21.6 \pm 0.25	37.25 \pm 0.29	41.12 \pm 0.05	15.05 \pm 0.03	9.7 \pm 0.23	0.15	10.2 \pm 0.19	1.47 \pm 0.03
29.42 \pm 0.28	21.62 \pm 0.66	49.0 \pm 0.41	18.67 \pm 0.23	21.25 \pm 0.10	0.16	20.4 \pm 0.15	0.8 \pm 0.04
24.97 \pm 0.27	32.75 \pm 0.62	42.27 \pm 0.33	14.97 \pm 0.06	11.3 \pm 0.18	0.15	11.92 \pm 0.14	0.87 \pm 0.01
15.6 \pm 0.26	38.27 \pm 0.45	46.1 \pm 0.37	11.02 \pm 0.56	10.45 \pm 0.51	0.22 \pm 0.01	8.92 \pm 0.37	2.33 \pm 0.21
14.37 \pm 0.07	61.47 \pm 0.08	24.12 \pm 0.12	5.77 \pm 0.18	8.75 \pm 0.43	0.28	7.92 \pm 0.08	2.32 \pm 0.02
27.47 \pm 0.20	26.67 \pm 0.67	44.85 \pm 0.53	16.32 \pm 0.42	21.55 \pm 0.22	0.31	19.05 \pm 0.15	3.71 \pm 0.04
26.45 \pm 0.43	29.6 \pm 1.09	43.95 \pm 0.71	7.0 \pm 0.71	10.67 \pm 1.03	0.12	14.35 \pm 0.13	1.42 \pm 0.01
26.3 \pm 0.28	27.72 \pm 0.25	42.17 \pm 3.86	7.35 \pm 1.01	18.45 \pm 0.18	0.16	14.3 \pm 0.33	3.47 \pm 0.17

^a Average of 4 replicates

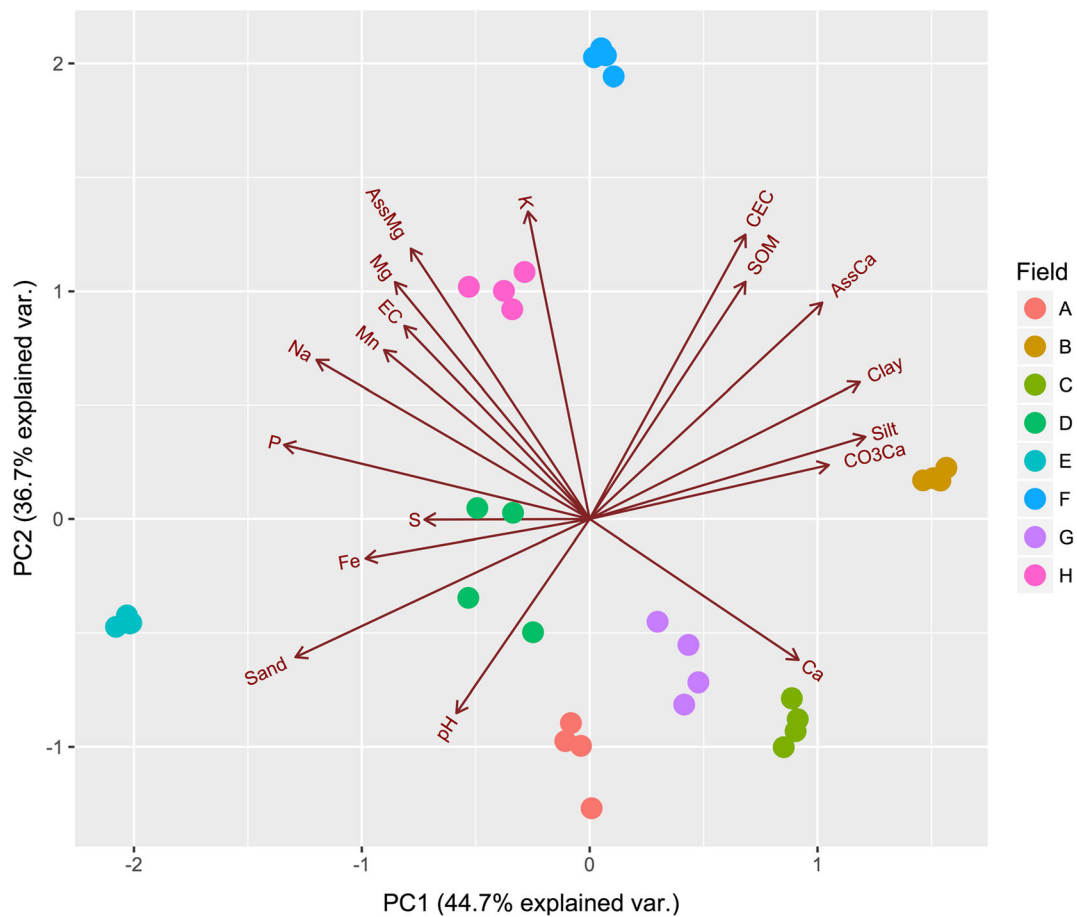


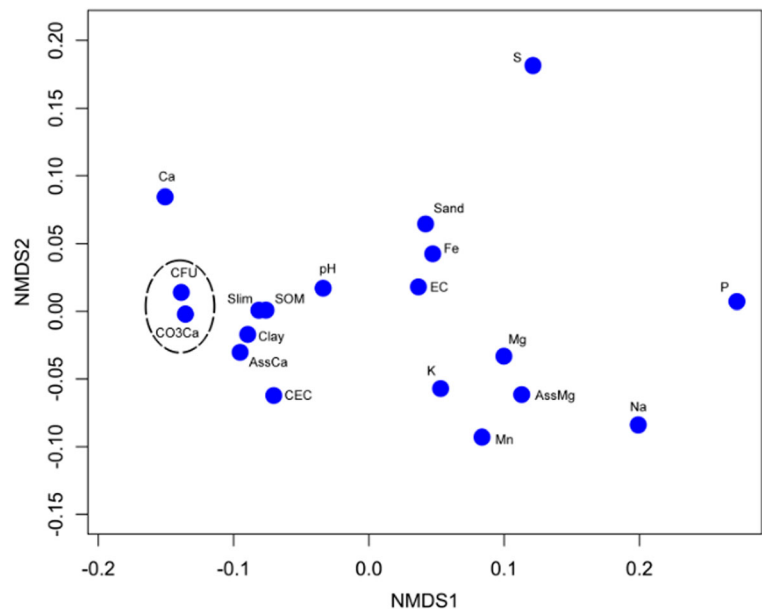
Fig. 2 Principal component analysis (PCA) of the eight studied fields in terms of the physicochemical properties of the soil. The percentage value given within parentheses corresponds to the variance explained by each principal component

established vineyards. This decrease in susceptibility to black-foot pathogens with host age may be linked to the development of defence mechanisms by older plants that inhibit disease development, such as changes in root exudates, or changes in the composition and structure of the root system that make it less vulnerable to soilborne pathogen infections.

It is thought that most of the species associated with black-foot disease survive in soil for extended periods due to the production of chlamydospores (Schroers et al. 2008; Chaverri et al. 2011), which can survive for multiple years in absence of suitable hosts. In our study, we demonstrated that viable inoculum of *D. torresensis* is still present during the rotation cycle as conidia or chlamydospores, which agrees with the results obtained by Cardoso et al. (2013) in Portugal. In Spain, according to the current nursery legislation, nursery fields used for the rooting phase cannot be planted for more than two

consecutive growing seasons, and must have not been used for grapevine cultivation at least for the previous 12 years. These periods could be reduced to one and 6 years respectively if the nursery field is previously disinfested against nematodes. Planting material is usually planted the first year, followed by three years of rotation with other crops such as wheat and/or barley, and land in a bare fallow strategy. Periodic bare fallow treatments (every third year) have proven effective in reducing pathogen populations in bareroot conifer nurseries (Jones and Benson 2003). This strategy works by removing the host and by decreasing the amount of organic matter in the soil, thereby eliminating or decreasing the food base for soilborne pathogens. However, bare fallow is less likely to work as well for certain soilborne pathogens, such as black-foot pathogens, which produce resistant structures that survive for long periods in soil (Schroers et al. 2008; Chaverri et al. 2011).

Fig. 3 Non-Metric Multidimensional Scaling Analysis (NMDS) based on similarity matrices among soil physicochemical properties and Colony Forming Units (CFU) of black-foot pathogens in soil



Despite the impressive success of crop rotation for managing numerous plant diseases, it has had mixed results for some soilborne pathogens with broad host ranges and long-lived inoculum (Easton et al. 1992; Davis et al. 1999). The unsuccessful results of rotation in some cases raise the possibility that asymptomatic or “hidden” hosts used in rotational programs in grapevine nurseries may be maintaining the inoculum bank. We therefore hypothesize that asymptomatic secondary hosts, specifically rotational crops and weeds, maintain populations of black-foot pathogens in grapevine nurseries and young vineyards. Some species of black-foot pathogens are able to colonize weeds, even though these hosts do not show symptoms of decline (Agustí-Brisach et al. 2011). In South Africa, Halleen et al. (2003) already suggested that the standard nursery practice of a 2-year rotation system, alternated with a cover crop, might have led to a build-up of black-foot pathogens in these soils. Pathogen diversity maintained by asymptomatic hosts may have a detrimental long-term consequence for disease management.

Our results showed a relationship between calcium carbonate and the CFUs of black-foot pathogens in soil. Calcium carbonate is a widely used amendment to neutralize soil acidity and to supply calcium for plant nutrition. In general, previous research has showed that calcium carbonate amendments have reduced infection by soilborne diseases, such as *Fusarium* wilt (Woltz and Jones 1981) and those caused by *Phytophthora* spp. (Boughton et al. 1978), but excessive calcium may

increase disease severity (Schmitthenner and Canaday 1983; Spiegel et al. 1987). Soil is essential for the production of healthy vines and nursery stock. It provides physical support for roots and supplies mineral nutrients and water necessary for growth. The soil is also the environment in which plant roots interact with soilborne pathogens. Therefore, an understanding of how soil properties affect both plant and pathogen health is critical for making effective pest management decisions. The physiochemical characteristics of the soil may favour certain microbial groups over others, thereby leading to shifts in the composition of microbial communities (Brant et al. 2006; Kaiser et al. 2010). More research is needed to elucidate interactions of primary and secondary macronutrients, micronutrients and other physicochemical properties, with disease in enhancing or minimizing black-foot disease incidence in grapevine.

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