Characterization and pathogenicity of *Cylindrocarpon*-like asexual morphs associated with black foot disease in Algerian grapevine nurseries, with the description of *Pleiocarpon algeriense* sp. nov.

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Summary: During a survey of black foot disease in Algerian grapevine nurseries, a collection of 79 *Cylindrocarpon*-like isolates were obtained. Based on morphology and DNA sequence data of histone H3 (*his3*), three species of *Dactylonectria* were identified including *Dactylonectria torresensis* (40 isolates), *D. macrodidyma* (24 isolates) and *D. novozelandica* (14 isolates). In addition, one isolate belonging to the genus *Pleiocarpon* was found and it is described here as a new species, *Pleiocarpon algeriense*, based on morphological features and DNA sequence data of the internal transcribed spacer region (ITS), translation elongation factor 1-alpha (*tef1*), β-tubulin (*tub2*), large subunit nrDNA (LSU) and histone H3 (*his3*). This is the first time that these species are reported in Algeria. Pathogenicity tests, were conducted with representative isolates from each species. All of them were able to induce typical necrosis symptoms on grapevine cuttings. These results emphasize the urgent need to implement an integrated management strategy for black foot disease in Algerian grapevine nurseries in order to reduce the incidence of this disease on grapevine planting material and to prevent that it spreads to new grapevine production areas.

Key words: black foot, *Dactylonectria*, *Pleiocarpon*, taxonomy, phylogeny, *Vitis vinifera*.

Introduction

Currently, viticulture occupies an important place in Algerian agriculture, consisting of diversified varieties and cultivars which are threatened by several diseases including Grapevine Trunk Diseases (GTDs) (Levadoux et al. 1971; Berraf-Tebbal et al. 2011). GTDs reefers to a complex of different fungal diseases affecting the perennial organs of grapevine, leading to the death of the plant in the most of cases (Mugnai et al. 1999; Armengol et al.

2001; Úrbez-Torres 2011; Bertsch et al. 2013). Thus, GTDs compromise vineyards productivity and longevity, resulting in considerable economic losses (Larignon 2012; Viret and Gindro 2014; Hofstetter et al. 2017).

Black foot disease of grapevines is one of the main GTDs affecting young plants (Halleen et al. 2003; Chaverri et al. 2011; Agustí-Brisach and Armengol 2013; Carlucci et al. 2017). The disease was given this name due to the presence of brown to black necrosis on the base of the rootstock (Badour 1969) and its incidence has increased significantly in most grapevine production areas of the world over the last two decades (Gramaje and Armengol 2011; Agustí-Brisach and Armengol 2013; Agustí-Brisach et al. 2016; Carlucci et al. 2017). Black-foot disease has been reported in many vineyards around the world, such as Australia (Sweetingham 1983; Whitelaw-Weckert et al. 2007), Brazil (Garrido et al. 2004), Canada (O'Gorman et al. 2009; Petit et al. 2011), France (Maluta and Larignon 1991), Italy (Carlucci et al. 2017), Portugal (Rego 1994; Rego et al. 2000), South Africa (Fourie and Halleen 2001), Spain (Alaniz et al. 2007; Agustí-Brisach and Armengol 2013), United States (Petit and Gubler 2005), and many other countries.

Grapevine plants affected by black foot disease show reduced vigour, shortened internodes and small leaves with interveinal chlorosis and necrosis (Halleen et al. 2006b; Reis et al. 2013). Longitudinal sections made through the trunk bases of the affected vines show brown to black vascular streaks and large blackened sectors (Oliveira et al. 2004; Alaniz et al. 2007; Abreo et al. 2010; Carlucci et al. 2017). Black foot pathogens have been isolated from symptomatic or asymptomatic rootstock mother plants (Fourie and Halleen 2004), rooted rootstock cuttings (Halleen et al. 2003; Aroca et al. 2006; Dubrovsky and Fabritius 2007), young grafted vines (Oliveira et al. 2004; Rumbos and Rumbou 2001) and also mature grapevines (Agustí-Brisach et al. 2014).

Black foot disease was firstly described as caused by "*Cylindrocarpon*" species. *Campylocarpon* was the first genus segregated from *Cylindrocarpon* (Halleen et al. 2004; Lombard et al. 2014). Following that, Chaverri et al. (2011) identified three new genera namely *Ilyonectria, Rugonectria* and *Thelonectria* with *I. radicicola*, *R. rugulosa* and *T. discophora* as type species, respectively. However, studies based on multi-gene phylogeny and morphological comparison performed by Cabral (2012a,b) and Lombard et al. (2014) re-evaluated the genera with *Cylindrocarpon*-like asexual morphs and highlighted the paraphyletic nature of *Ilyonectria,* leading to the introduction of the genus *Dactylonectria* to accommodate *Ilyonectria* species from grapevine. Nowadays, black foot disease of grapevine is known to be associated with fungal species from the following genera: *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria, Ilyonectria*, *Neonectria* and *Thelonectria* (Lombard et al. 2014; Carlucci et al. 2017).

Recently, *Pleiocarpon,* a new *Nectriaceae* monotypic genus, phylogenetically closely related to the genus *Thelonectria*, was described in Italy (Aiello et al. 2017). The name of the genus *Pleiocarpon* is derived from the highly variable conidial shapes this fungus produces in culture. The type species was named *Pleiocarpon strelitziae* due to the host, *Strelitzia reginae*, from which this fungus was isolated in an ornamental nursery in Italy (Aiello et al. 2017). The species *P. strelitziae* is characterized by simple conidiophores or aggregated to form sporodochia, abundant microconidia, aseptate, hyaline, ellipsoid to ovoid or subcylindrical, straight to slightly curved, with clearly laterally displaced hilum. The macroconidia are cylindrical to subcylindrical, hyaline, straight to curved, 1−5-septate. The chlamydospores are not observed (Aiello et al. 2017).

In Algeria, cases of grapevine apoplexy were reported by Debray (1892), but the description of the associated symptoms does not allow knowing if it was indeed caused by GTDs. In 1905, Ravaz also reported high plant mortality rates in many wine-growing regions of Algeria. In 2005 GTDs were first described in Algeria affecting

mature grapevines, and currently they seem to be the main cause of the dieback and mortality observed in young and adult Algerian vineyards during the last decade (Berraf and Peros 2005; Berraf-Tebbal et al. 2011; Ammad et al. 2014; Berraf-Tebbal et al. 2014). Nevertheless, black foot disease and the associated pathogens have not yet been reported on grapevine.

Thus, the aim of the present study was to characterize a large collection of *Cylindrocarpon*-like asexual morph isolates associated with black foot disease, which were recovered from grapevine nurseries in different regions of Northern Algeria, based on morphology and comparison of DNA sequence data. Moreover, a pathogenicity test was performed to determine the virulence of each species to grapevine.

Materials and methods

Fungal isolation

One hundred and ninety symptomatic one-year-old grapevine grafted plants and rootstocks, including the cultivars Muscat d'Alexandrie, Vitroblack, Chasselat, Ora, and SO4 rootstock, were randomly sampled from 2015 to 2017 at three commercial grapevine nurseries located in three different provinces of Algeria: Skikda (n=90), Blida (n=70) and Ain Temouchent (n=30) (Table 1). Transverse and longitudinal sections were made at three areas of each plant; the grafting point, the basal part in the crown and the middle part between the grafting point and the basal part, to reveal internal symptoms of GTDs. From each area of the plant, ten pieces of wood of approximately 5 mm^2 diameter, were cut by a sterile scalpel and surface-disinfected with 8% sodium hypochlorite for 10 min, rinsed twice with sterile distilled water and dried on sterile absorbent paper. These wood pieces were transferred onto Petri dishes containing potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France), amended with 0.5 g l^{-1} of streptomycin sulphate (PDAS). The plates were incubated in darkness at 25 C° and examined daily. Fungal colonies were subcultured on fresh PDA plates in order to obtain pure cultures. From these primary isolations, single spore isolates were obtained, and stored in 15% glycerol solution at -80ºC into 1.5 ml cryovials.

Morphological identification

To determine the morphological characteristics of the isolates, they were plated onto three different types of media: PDA (BD Difco, Sparks, MD, USA), oatmeal agar (OA), and synthetic nutrient-poor agar (SNA; Nirenberg 1976), with or without the addition of two 1 cm2 pieces of sterile filter paper on the medium surface (Crous et al. 2009). Then, they were incubated at 25ºC during 5 weeks with under mixed white and near-UV light and a 12 h photoperiod. Colony characters and pigment production on PDA and OA were noted after incubation at 25ºC in darkness for 10 days. Colony colours (surface and reverse) were rated according to Rayner (1970). Measurements of the fungal structures were performed in an agar square that was removed from the SNA plates and placed on a microscope slide, a drop of water and a cover slip were added. Observations were done in Leica DM2500 microscope with differential and images were captured using a Leica DFC295 digital camera using the software Leica Application Suite (LAS) version 3.3.0. For each informative structure, 30 measurements were obtained. The 95% confidence intervals were determined and the extremes of the conidial measurements are shown in parenthesis, while for the other structures, only the extremes are presented. Cardinal growth temperatures were

determined by inoculating 90 mm diameter PDA plates with a 3 mm diameter plug cut from the edge of an actively growing colony. Colony growth was recorded after 7 days in two orthogonal directions. Temperature growth experiments were performed at 5 to 35ºC, with 5ºC intervals, with three replicates per isolate at each temperature.

Molecular identification

DNA extraction and sequencing

Total genomic DNA was extracted from 6-d-old single-spore cultures grown on potato dextrose agar for 6 days at 25°C in darkness using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA), according to the manufacturer's instructions. A previous mycelia disruption was perform with 4 tungsten carbide beads of 3 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, California, USA) at 5m/s for 20 s twice. Partial gene sequences were determined for the histone H3 (*his3*) using the primers and protocols of Cabral et al. (2012a), in order to identify the species involved. Additionally, internal transcribed spacer and intervening 5.8S gene (ITS) region, partial 28S nrRNA gene (LSU), partial regions of the β-tubulin (*tub2*), translation elongation factor 1-α (*tef1*) and RNA polymerase II second largest subunit (*rpb2*) region genes were sequenced for some isolates to better resolve their identification. Primers used were CYLH3F and CYLH3R (Crous et al. 2004b) for *his3*, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS, LR0R (Moncalvo et al. 1995) and LR5 (Vilgalys and Hester 1990) for LSU, T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for *tub2*, CylEF-1 (5'- ATG GGT AAG GAV GAV AAG AC-3'; J.Z. Groenewald, unpublished) and CylEF-R2 (Crous et al. 2004b) for *tef1*, and RPB2-5F2 and RPB2-7cR (O'Donnell et al. 2007) for *rpb2*. The cycle conditions in a Peltier Thermal Cycler-200 (MJ Research) were: 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, elongation at 72ºC for 45 s, and a final extension at 72ºC for 10 min. PCR products were sequenced by Macrogen Inc., Sequencing Center (The Netherlands, Europe). Integrity of the sequences was ensured by sequencing the amplicons in both directions using the same primer pairs used for amplification. Consensus sequences for all isolates were assembled and compiled into a single file (Fasta format), using Sequencher software v. 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA), and compared to those disposed in the NCBI Genbank database, using the Basic Local Alignment Search Tool (BLAST).

Phylogenetic analyses

Phylogenetic analyses were conducted with the isolates for which it was not possible to infer species level, using the four loci ITS, LSU, *tub2* and *tef1*. The *his3* and *rpb2* gene regions could not be included in the phylogenetic inference due to the limited sequence data available for *Cylindrocarpon*-like fungi. Analyses were based on Bayesian inference (BI) and Maximum Likelihood (ML) and were performed firstly with single-locus alignment, and successively, with a combined alignment of the four loci. GenBank sequences from different species were selected in consonance with their high similarity with our query sequences, according to Aiello et al. (2017) (Table 2). These were added to the sequences obtained, aligned and edited manually, if necessary, using MEGA 7.0.26 (Kumar et al. 2015). Incomplete portions at either end of the alignments were excluded prior to analyses. SequenceMatrix 1.8 program (Vaidya et al. 2011) was used to combine the alignments of each locus in a single

file. Bayesian analyses were performed with MrBayes v. 3.2.1 (Ronquist et al. 2012) on the CIPRES Science Gateway V 3.3 (Miller et al. 2010) according to Mora-Sala et al. (2018). The Maximum Likelihood analysis (ML) was performed with MEGA 7.0.26 (Kumar et al. 2015), determining the best nucleotide substitution model settings for each locus. Both analyses were performed, rooting the trees to *Rugonectria rugulosa* (CBS 126565) (Table 2). Sequences derived in this study were lodged in GenBank (accession numbers listed in Table S1). Alignments and phylogenetic trees were lodged in TreeBASE under study number 23154 (http://purl.org/phylo/treebase/phylows/study/TB2:S23154) and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous et al. 2004a).

Pathogenicity tests

Representative isolates from each phylogenetically resolved species namely, *D. macrodidyma* (WAM8, WAM63), *D. novozelandica* (WAM95, WAM186), *D. torresensis* (WAM124, WAM163) and *P. algeriense* (WAM6), were selected to determine their pathogenicity to grapevine wood. Trials were conducted on one-year-old cuttings of grapevine cv. Cardinal. Before inoculation, these cuttings were subjected to hot water treatment at 53 °C for 30 min, to eliminate the presence of any fungal GTDs pathogens (Gramaje et al. 2009; Carlucci et al. 2017). Eighty dormant cuttings were cut into equal length (35 cm), containing 3 to 4 buds. Then, the cuttings were wounded between two nodes with and scalpel and a 5 mm mycelial plug from a 10 days old colony of each isolate grown on PDA was placed in the wound. Negative controls were inoculated with fresh, no-colonized, PDA plugs. The inoculated cuttings were wrapped with wet sterile cotton and parafilm around the inoculation point to prevent desiccation. There were 10 replicates per isolate, and the same number of cuttings were used as controls. All cuttings were immediately transplanted into pots containing sterilized water as a growth substrate (10 cuttings per pot), which were incubated in a phytotron at 25°C. One month after inoculation the cuttings were examined by removing the bark and measuring the length of the lesions in both directions from the inoculation point. Small pieces (0.2 to 0.5 cm) of necrotic tissue from the edge of each lesion were cut and placed on PDAS in an attempt to recover the inoculated fungi and complete Koch's postulates. Fungi were identified as described above. Lesion length data were subjected to statistical analysis to determine the homogeneity of the variance of the dataset by performing analysis of variance (ANOVA) and means comparison by Fisher's Least Significant Difference (LSD) test at $P \leq 0.05$.

Results

During the survey, internal wood necrosis were revealed by cross-sections on the grapevine nursery plants. The necrosis consisted of different brownish discolorations around the pith more consistent at the basal part and less important at the medium part. The isolation from these symptomatic tissues yielded to 79 *Cylindrocarpon*-like asexual morph isolates. The colour of the colonies on PDA varied from white to yellow or light to dark brown, with cottony mycelium. Based on the microscopic observations, all the isolates produced macroconidia and microconidia, as described by Cabral et al. (2012a) and Halleen et al. (2006a).

Isolates, molecular identification and phylogenetic analysis

The identification of 79 isolates was performed using the primers CYLH3F and CYLH3R. DNA sequence data showed high similarities (≥99%) with the reference sequences disposed in the NCBI Genbank database, and confirmed 78 isolates belonging to the genus *Dactylonectria*: *D. torresensis* (40 isolates), *D. macrodidyma* (24 isolates) and *D. novozelandica* (14 isolates), and one isolate belonging to the genus *Pleiocarpon*. ITS, LSU, *tef1*, *tub2* and rpb2 genes were sequenced for the isolates WAM104, WAM158, WAM168, WAM180, and WAM186, which showed 99% of similarity with *D. novozelandica* (three nucleotide differences), and for the isolate WAM6, which showed a similarity of 99% with *P. strelitziae* (four nucleotide differences). Similarity values of 100% were observed when WAM104, WAM158, WAM168, WAM180 and WAM186 were compared with *D. novozelandica* sequences thus, they were not considered a new species. WAM6 showed similarity values of 99% when compared with *P. strelitziae* sequences of ITS (three nucleotide differences and a gap), *tef1* (six nucleotide differences) and *tub2* (three nucleotide differences). Therefore, phylogenetic analysis were performed using ITS, *tef1*, *tub2* and LSU sequences of WAM6 and 15 more taxa, including *Rugonectria rugulosa* (CBS 126565) as an outgroup taxa. The four loci alignment (including the outgroup) contained 2103 aligned characters (including gaps), from which 500 corresponded to ITS sequences, 803 to LSU, 515 to *tef1* and 285 to *tub2*. Of the 2103 characters used in the analysis, 379 were parsimony-informative, 163 were variable and parsimony-uninformative and 1561 were constant. BI and ML trees had similar topology and only the ML tree is presented with posterior probability values (PP) and bootstrap support values (BS) (Fig. 1). The Maximum Likelihood tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The obtained phylogeny placed the isolate WAM6 in a different clade than the other eight isolates of *P. strelitziae* with a posterior probability value of 1 and a bootstrap support value of 100%, confirming the isolate WAM6 as a novel species (Fig. 1) belonging to the genus *Pleiocarpon*, which is described below as new species, *P. algeriense* (Fig. 2).

Taxonomy

Based on the phylogenetic analysis and morphological characters, one new species of *Pleiocarpon* is described (Fig. 2).

***Pleiocarpon algeriense* sp. nov.** W. Aigoun-Mouhous**,** A. Cabral and A. Berraf-Tebbal. MycoBank MB827378**.**

Etymology: Named after Algeria, where the species was first discovered.

Type: **Algeria:** Blida, Larbaa, isolated from the basal part of rootstock SO4 in a one-year-old nursery plant (cv. Vitroblack grafted on SO4), May 2017, coll./isol. W. Aigoun-Mouhous (CBS H-23695 – holotype; CBS 144964 = WAM6 – ex-type culture).

Description: *Ascomata* not observed. *Conidiophores* simple or aggregating to form sporodochia. *Simple conidiophores* solitary, arising laterally or terminally from aerial mycelium, to loosely aggregated, unbranched or sparsely branched, 1−3-septate, 50−110 µm long, bearing one to three conidiogenous cells. *Conidiogenous cells* monophialidic, cylindrical, tapering slightly towards the apex, 16−40 µm long, 2−3 µm wide at the base, 1.5−2

µm near the apex. *Sporodochia* consisting of a pulvinate mass of short conidiophores, the conidiogenous cells monophialidic, cylindrical, tapering towards the apex, 14−32 µm long, and 2−3.5 µm wide at the base and 1.5−2 µm near the apex. *Microconidia* aseptate, with a minute or clearly laterally displaced hilum, ellipsoid to ovoid or subcylindrical, straight to slightly curved, (6−)8−8.5(−10,5) × (2−)3−3.3(−4)µm (av. 8.3 × 3.2 µm) with a length:width ratio of (1,8−)2,5−2,8(−3,8), formed in heads on simple conidiophores. *Macroconidia* formed on sporodochia on SNA around and over the filter paper pieces, hyaline, straight to curved, 1−5-septate, predominantly 3-septate, apex or apical cell typically slightly bent to one side and minutely beaked, base with sometimes visible, centrally located or laterally displaced hilum; 1-septate (17−)25−30(−42) × (4.5−)6−6.5(−8) µm (av. 27.4 × 6.2 µm) with a length:width ratio of (3−)4−5(−6.7); 2-septate (28−)35−39.5(−51) × (5.5−)6.7−7.3(−8) µm (av. 37.2 × 7 µm) with a length:width ratio of (3.7−)5−5.7(−7.3); 3-septate (28−)41.5−46(−57) × (5−)7−7.5(−8.5) µm (av. 43.7 × 7.1 µm) with a length:width ratio of (4.2−)5.9−6.4(−8.3); 4-septate (47.5−)53−55.5(−61) × (7−)7.5−8(−9) µm (av. 54.3 × 7.7 µm) with a length:width ratio of (5.7−)6.9−7.3(−8.2); 5-septate (50−)57−59.5(−65.5) × (6.5−)7.5−7.8(−8.5) µm (av. 58.2 × 7.6 µm) with a length:width ratio of (6.5−)7.4−7.9(−9.3). *Chlamydospores* rarely observed, globose to subglobose, 7–12 × 6–10 µm, smooth but often appearing rough due to deposits, thick-walled, formed intercalary in chains, hyaline.

Culture characteristics: Mycelium cottony with average density on PDA and low to average on OA. Surface on PDA cinnamon to honey, with buff aerial mycelium, on OA light cinnamon to buff, with aerial mycelium white. Zonation absent, transparency homogeneous, margin uneven on PDA and even on OA. Reverse on PDA and OA similar to surface.

Cardinal growth temperatures: No growth was observed on PDA at 10 °C, while at 15 ºC colonies grew 12 mm diam. after 7 days. Optimum temperature for growth is 25-30°C, when colonies reach 32–36 mm diam, after 7 days. Colony diam was 14–16 mm at 35°C, after 7 days.

Habitat: Basal part of rootstock of nursery grafted plants of *Vitis* spp.

Known distribution: Northern Algeria, Blida.

Genetic identification: LSU and *rpb2* do not separate *P. algeriense* from *P. strelitziae.* Both species can be distinguished *by* three differences in ITS positions 180 (T/A), 290 (G/A) and 433 (C/T); four differences in *his3* positions 95 (A/G), 295 (C/T), 323 (C/T), 434 (C/T), six differences in *tef1* on positions 317 (T/C), 323 (G/A), 334 (T/C), 375 (A/G), 527 (T/C), 651 (A/G) and three differences in *tub2* positions 364 (T/C), 376 (G/C) and 409 (C/T).

Notes: *P. algeriense* is closely related with *P. strelitziae* based on the phylogenetic inference in this study. Morphologically can be distinguished by having longer (50–110 µm) solitary conidiophores when compared to *P. strelitziae* (50−85 µm; Aiello et al. 2017). The 2-septate (28−)35−39.5(−51) × (5.5−)6.7−7.3(−8) µm (av. 37.2 × 7 µm); 3-septate (28−)41.5−46(−57) × (5−)7−7.5(−8.5) µm (av. 43.7 × 7.1 µm); 4-septate (47.5−)53−55.5(−61) × (7−)7.5−8(−9) µm (av. 54.3 × 7.7 µm) and 5-septate (50−)57−59.5(−65.5) × (6.5−)7.5−7.8(−8.5) µm (av. 58.2 ×

7.6 µm) macroconidia are larger and wider than those of *P. strelitziae* (23−29(−31) × 5 µm (av. 26 × 5 µm), (28−)30−40(−46) × 5−6 µm (av. 35 × 6 µm), (36−)37−41 × 6−7 µm (av. 39 × 6 µm) and (41−)42−47(−50) × 5−7 µm (av. 44 × 6 µm), respectively; (Aiello et al. 2017). Chlamydopores were observed in *P. algeriense* and were not present in *P. strelitziae* (Aiello et al. 2017).

Frequency and localization of the species

A total of 79 isolates were obtained by sampling from commercial grapevine nurseries located in three regions of North Algeria: Ain Temouchent (20 isolates), Blida (8 isolates) and Skikda (51 isolates). Most of the isolates (77) were obtained from the basal area of the plants and only two isolates were obtained from the medium area, whereas no isolates were found in the grafting point. *Cylindrocarpon*-like asexual morph were detected in all grapevine nurseries surveyed.

Concerning the geographical distribution of the different species (Fig. 3), in Ain Temouchent province 80% of the isolates obtained belong to *D. torresensis* and 20% to *D. macrodidyma.* In Bilda province, 75% of the isolates obtained belong to *D. torresensis*, 12.5% *D. macrodidyma* and 12.5% to *P. algeriense*. In the Skikda province, *D. macrodidyma* (23 isolates) was the most frequently isolated species (45%), followed by *D. torresensis* (35%) and *D. novozelandica* (20%) .

Pathogenicity tests

All the *Dactylonectria* and *Pleiocarpon* isolates used in the pathogenicity test were pathogenic to the grapevine cuttings. Within 30 days after inoculation irregular black to brown necrosis starting from the point of inoculation developed on the wood tissue under the bark. The negative control plants did not develop any symptoms (Fig. 4). Percent recovery of the pathogens was higher than 95% from all inoculated cuttings and reisolated species were confirmed to be the same inoculated previously. No isolates were obtained from the negative control.

The size of the necrotic lesions varied among the isolates studied. The most virulent isolate was WAM124 (*D. torresensis*), which produced the longest lesion size (4,20 ± 2,36 cm) and differed statistically from all the other isolates. It was followed by the isolate WAM95 (*D. novozelandica*) (2,89 ± 1,37 cm), which was statistically different from the other isolates, except WAM8 (*D. macrodidyma*). The smallest lesion size were produced by the isolate WAM163 (*D. torresensis*) (1,65 ± 0,35 cm), which was found to be the less virulent isolate, but not statistically different from the isolates WAM6, WAM8, WAM63 and WAM186. The necrotic lesion produced by the isolate WAM6 (*P. algeriense*) (1,93 ± 0,25 cm) was not statistically different from the *Dactylonectria* isolates WAM8, WAN63, WAM163 and WAM163 (Fig. 5).

Discussion

This is the first study evaluating the relevance of black foot disease and the associated pathogens in Algerian grapevine nurseries. Our results confirm the presence of *Dactylonectria* spp. as causal agents of internal wood necrosis of grapevine rootstocks and adds the genus *Pleiocarpon* to the list of genera currently associated with this complex disease worldwide. The integration of morphological characters and DNA sequences allowed the

identification of four species, belonging to two genera; namely *D. macrodidyma, D. novozelandica, D. torresensis*, and *P. algeriense.* This later has been described here as a new species and the four species are reported for the first time on grapevine in Algeria.

Overall, the results obtained in the survey of Algerian grapevine nurseries fit with the relative importance of the different *Dactylonectria* species reported in other similar studies worldwide. Our results showed that *D. torresensis* was the most frequent species. Isolates of this pathogen were found in the five cultivars and on the three provinces surveyed. This pathogen was first described as *Ilyonectria torresensis* by Cabral et al. (2012a) in Portugal from grapevine. Later it was renamed as *D. torresensis* after a re-evaluation of the *Ilyonectria* species by Lombard et al. (2014). *Dactylonectria torresensis* is considered the most frequent pathogen associated with black foot disease of grapevine. According to Larignon (2016), this species has been reported in Australia (Cabral et al. 2012a), Canada (Cabral et al. 2012a, Úrbez-Torres et al. 2014), Italy (Carlucci et al. 2017), New Zealand (Cabral et al. 2012a), Portugal (Cabral et al. 2012a, Reis et al. 2013), Spain (Agustí-Brisach et al. 2013), South Africa (Cabral et al. 2012a) and United States (Cabral et al. 2012a).

Dactylonectria macrodidyma was the second most common species found. This species was first described as *Cylindrocarpon macrodidyma* by Halleen et al. (2004) from grapevine in South Africa, latter Chaverri named it as Ilyonectria macrodidyma (2011) and introduced as *D. macrodidyma* by Lombard et al. (2014). This pathogen has been reported on grapevine in Australia (Whitelaw-Weckert et al. 2007), Brazil (Santos et al. 2014), USA (Petit and Gubler 2005), Canada (Petit et al. 2011, Úrbez-Torres et al. 2014), Chile (Auger et al. 2007), New Zealand (Halleen et al. 2004), Portugal (Cabral et al. 2012a; Reis et al. 2013), South Africa (Halleen et al. 2004), Spain (Alaniz et al. 2009), Switzerland (Hofstetter et al. 2009), Turkey (Özben et al. 2012) and Uruguay (Abreo et al. 2010).

Regarding *D. novozelandica,* this pathogen was first described as *I. novozelandica* by Cabral et al. (2012b) from grapevine, being classified as *D. novozelandica* by Lombard et al. (2014). This species has been isolated from grapevine in New Zealand (Cabral et al. 2012a), Peru (Alvarez et al. 2012, Munive et al. 2013), Portugal (Reis et al. 2013), South Africa (Cabral et al. 2012a), Spain (Agustí-Brisach et al. 2013) and USA (Cabral et al. 2012a).

The description of *P. algeriense* in our study adds a second species to the *Pleiocarpon* genus, which to date was only represented by one species, *P. strelitziae*, isolated from the ornamental plant species *Strelitzia reginae* in Italy (Aiello et al. 2017). Moreover, *P. algeriense* introduces *Vitis* spp. as a new host plant for this genus. Additional surveys are needed to better understand the role of *Pleiocarpon* genus as plant pathogens, its host range and distribution.

The preliminary pathogenicity tests with isolates of *D. macrodidyma, D. novozelandica*, *D. torresensis* and *P. algeriense* on grapevine cuttings confirmed that all of them were able to develop irregular black to brown necrosis on the wood tissue under the bark. These findings confirm previous studies, in which severe disease symptoms were reproduced by artificial inoculation of one-year-old grapevine rootstock shoots with *D. torresensis* (Carlucci et al. 2017). Moreover, grapevine plants inoculated with *D. macrodidyma* showed necrosis of the leaf ribs, reduction in root mass, root and crown necrosis, browning of vessels, drying of shoots, and death (Santos et al. 2014). By comparing the lesions produced by *P. algeriense,* there were no statitical differences with some of the isolates of the three *Dactylonectria* spp. included in our study. In the survey performed by Aiello et al. (2017) on ornamental nurseries in Italy, the plants of *Strelitzia reginae* inoculated with representative isolates of *P. strelitziae* showed dry basal stem rot symptoms similar to those observed in the nursery. Future new pathogenicity tests with

additional isolates of *P. algeriense* will be useful to understand the role of this species in the black foot disease complex.

It is well known that soils in grapevine nurseries and vineyards are an important inoculum source for black-foot pathogens (Agustí-Brisach et al. 2013; 2014; Berlanas et al. 2017). Moreover, several studies have led to the conclusion that planting material can be already infected by black foot pathogens in young vineyards, either from infected mother vines (Ridgway et al. 2002; Halleen et al. 2003; Gramaje and Armengol 2011) or by contamination during the propagation process in nurseries (Gramaje and Armengol 2011; Gramaje and Di Marco 2015). Taking into account this information, our results emphasize the urgent need to implement an integrated management strategy for black foot disease in Algerian grapevine nurseries (Gramaje et al. 2018) in order to reduce the incidence of this disease on grapevine planting material and to prevent that it spreads to new grapevine production areas.

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579 **Figure captions**

580 **Figure. 1** Maximum Likelihood tree inferred from the combined ITS, *tef1*, *tub2*, and LSU sequence alignments
581 and determined by using Tamura Nei model with 1,000 rapid bootstrap inferences. A discrete Gamma distribution
582 was used to model evolutionary rate differences among sites 5 categories (+*G*, parameter = 0.2428). Posterior
583 probability and bootstrap support values (PP/BS) are indicated near the corresponding nodes, where asterisk
584 indicates not supported and the symbol – indicates that the value was lower than 0.70 or 70, respectively. The scale
585 bar indicates the expected changes per site. Newly described species are indicated by grey boxes. The tree is rooted
586 to *Rugonectria rugulosa* (CBS 126565).
587
588 **Figure. 2** *Pleiocarpon algeriense* (A−C) Simple, sparsely branched conidiophores of the aerial mycelium. (D)
589 *Sporodochia* over the filter paper pieces on SNA. (E−F) Complex conidiophores. (G−I) Micro- and macroconidia.
590 (J) Chlamydospores. Bars A−C, F−G, I−J = 10 µm; D = 200 µm and E, H = 20 µm. All from isolate CBS 144964.
591
592 **Figure. 3** Spatial distribution in North Algeria of the species associated to black foot disease found in this study.

Figure. 4 Necrotic lesions induced by the *Dactylonectria* and *Pleiocarpon* species on grapevine cuttings. **C**: control; *D. macrodidyma* (**1**:WAM8; **2**: WAM63); *D. novozelandica* (**3**: WAM95; **4**: WAM186); *D. torresensis* (**5**: WAM124; **6**: WAM163); **7**: *P. algeriense* (WAM6).

Figure. 5 Means lesion length (cm) caused by *Dactylonectria macrodidyma* (WAM8; WAM63); *D. novozelandica* (WAM95; WAM186); *D. torresensis* (WAM124; WAM163) and *Pleiocarpon algeriense* (WAM6) associated to black foot disease in Algerian grapevine nurseries. Error bars represent the standard error of means. Significant differences are represented with different letters above columns according to Fisher's Least Significant Difference test ($P \leq 0.05$).

606 **Table 1** Sampling regions and samples characteristics

Region	Latitude	Longitude	Grapevine plants		Age (months)	Number of plants
			Scion	Rootstock		
Skikda	36°52′34″ N	6°54′33″ E	Muscat d'Alexandrie	41B	12	30
			Ora	41B	12	30
			Chasselat	41B	12	30
Blida	36°28'12"N	6°54′33″ E	Muscat d'Alexandrie	SO4	12	40
			Vitroblack	SO4	12	30
Ain Temouchent	35°17′50″ N	1°08′25″ O	-	SO4	12	30
Total						**190**

607

Table 2 Isolation details and GenBank accession numbers of the isolates included in the phylogenetic analysis

Species	Isolate number[1]	Origin	Host	Collector	GenBank accession numbers[2]					
					LSU	**ITS**	***β-tub2***	***tef1***	***Rpb2***	***his3***
Pleiocarpon strelitziae	**CBS 142251** ST1; CPC 27628	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304672	KY304644	KY304750	KY304722	KY304697	KY304616
P. strelitziae	CBS 142252 ST20	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304688	KY304663	KY304769	KY304741	KY304713	KY304635
P. strelitziae	ST10	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304678	KY304653	KY304759	KY304731	KY304705	KY304625
P. strelitziae	ST11	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304679	KY304654	KY304760	KY304732	KY304706	KY304626
P. strelitziae	ST12	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304680	KY304655	KY304761	KY304733	KY304707	KY304627
P. strelitziae	ST13	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304681	KY304656	KY304762	KY304734	-	KY304628

P. strelitziae	ST17	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304685	KY304660	KY304766	KY304738	KY304710	KY304632
P. strelitziae	ST18	Italy	*S. reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304686	KY304661	KY304767	KY304739	KY304711	KY304633
Pleiocarpon algeriense	**CBS 144964** WAM6	Algeria	*Vitis vinifera*	W. Aigoun-Mouhous	MH587321	MH587320	MH587324	MH587323	MH587322	MH587296
Rugonectria rugulosa	CBS 126565	Venezuela	Dead tree	L. Lombard, N.A. van der Merwe, J.Z. Groenewald and P.W. Crous	KM231615	KM231749	KM232007	KM231873	-	-
Thelonectria discophora	**CBS 134034** AR 4742	Chile	*Tepualia stipularis*	C. Salgado, A.Y. Rossman and P. Chaverri	KC121440	KC153714	KC153779	KC153843	-	-
T. olida	CBS 215.67 ATCC 16548 IMI 116873	Germany	*Asparagus officinalis*	C. Salgado	KJ022058	KJ021982	KM232024	-	-	-
T. olida	CBS 142255	Italy	*Strelitzia reginae*	D. Aiello, G. Polizzi, P.W. Crous and L. Lombard	KY304684	KY304659	KY304765	KY304737	KY304709	KY304631

T.rubi	**CBS 113.12** IMI 113918		*Rubus idaeus*	C. Salgado, A.Y. Rossman and P. Chaverri	KC121444	KC153718	KC153783	KC153847	-	-
T. trachosa	**CBS 112467** IMI 352560	Scotland	Bark of conifer	P. Chaverri, C. Salgado, Y. Hirooka, A.Y. Rossman and G.J. Samuels	HM364312	AY677297	AY677258	KM231896	-	-
T. veuillotiana	CBS 132341 AR 1751	Azores	*Eucalyptus* sp.	C, Salgado, A. Rossman, G.J. Samuels, M. Capdet and P. Chaverri	JQ403345	JQ403305	JQ394698	JQ394734	-	-

Ex-type strains are shown in bold. [1] AR: Amy Y. Rossman working collection; ATCC: American Type Culture Collection, Virginia, USA; CBS: Westerdijk Fungal Biodiversity Institute , Utrecht, The Netherlands; CPC: Pedro Crous working collection housed at CBS; IMI: International Mycological Institute, CABI- Bioscience, Egham, Bakeham Lane, UK; ST: D. Aiello personal culture numbers; WAM: Wassila Aigoun-Mouhous collection, LBSM, ENS Kouba, Algeria.[2]ITS = internal transcribed spacers and intervening 5.8S rDNA, LSU = 28S large subunit ribosomal rDNA, *his3* = histone H3, *rpb2* = RNA polymerase II largest subunit, *tef1* = translation elongation factor 1-alpha, *tub2* = β-tubulin**.**

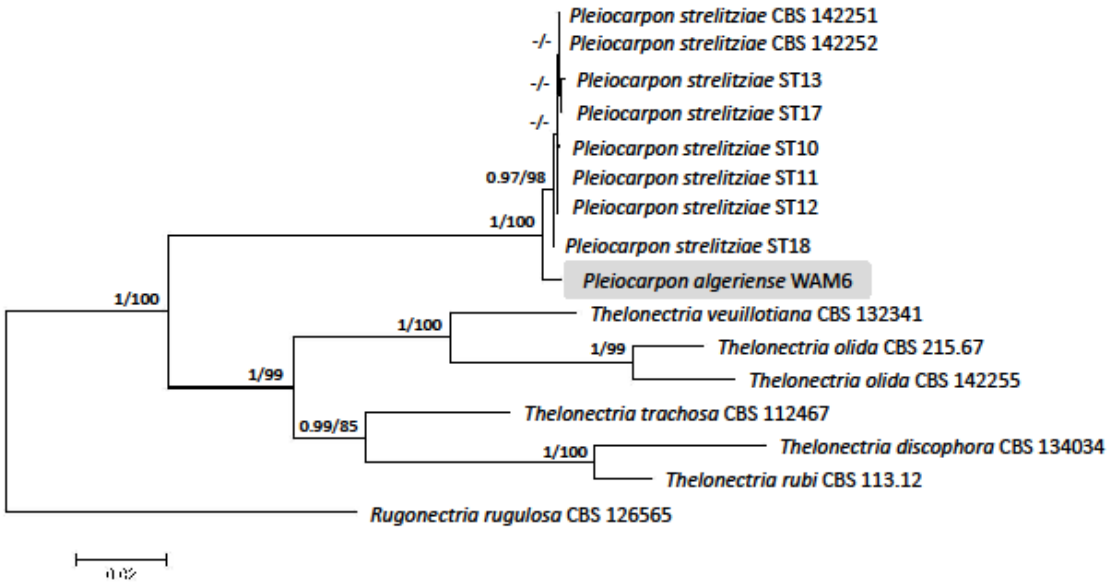
Pleiocarpon strelitziae CBS 142251
-/-
Pleiocarpon strelitziae CBS 142252
-/-
Pleiocarpon strelitziae ST13
-/-
Pleiocarpon strelitziae ST17
Pleiocarpon strelitziae ST10
0.97/98
Pleiocarpon strelitziae ST11
Pleiocarpon strelitziae ST12
1/100
Pleiocarpon strelitziae ST18
Pleiocarpon algeriense WAM6
1/100
Thelonectria veuillotiana CBS 132341
1/100
1/99
Thelonectria olida CBS 215.67
Thelonectria olida CBS 142255
1/99
Thelonectria trachosa CBS 112467
0.99/85
1/100
Thelonectria discophora CBS 134034
Thelonectria rubi CBS 113.12
Rugonectria rugulosa CBS 126565

FIGURA 1

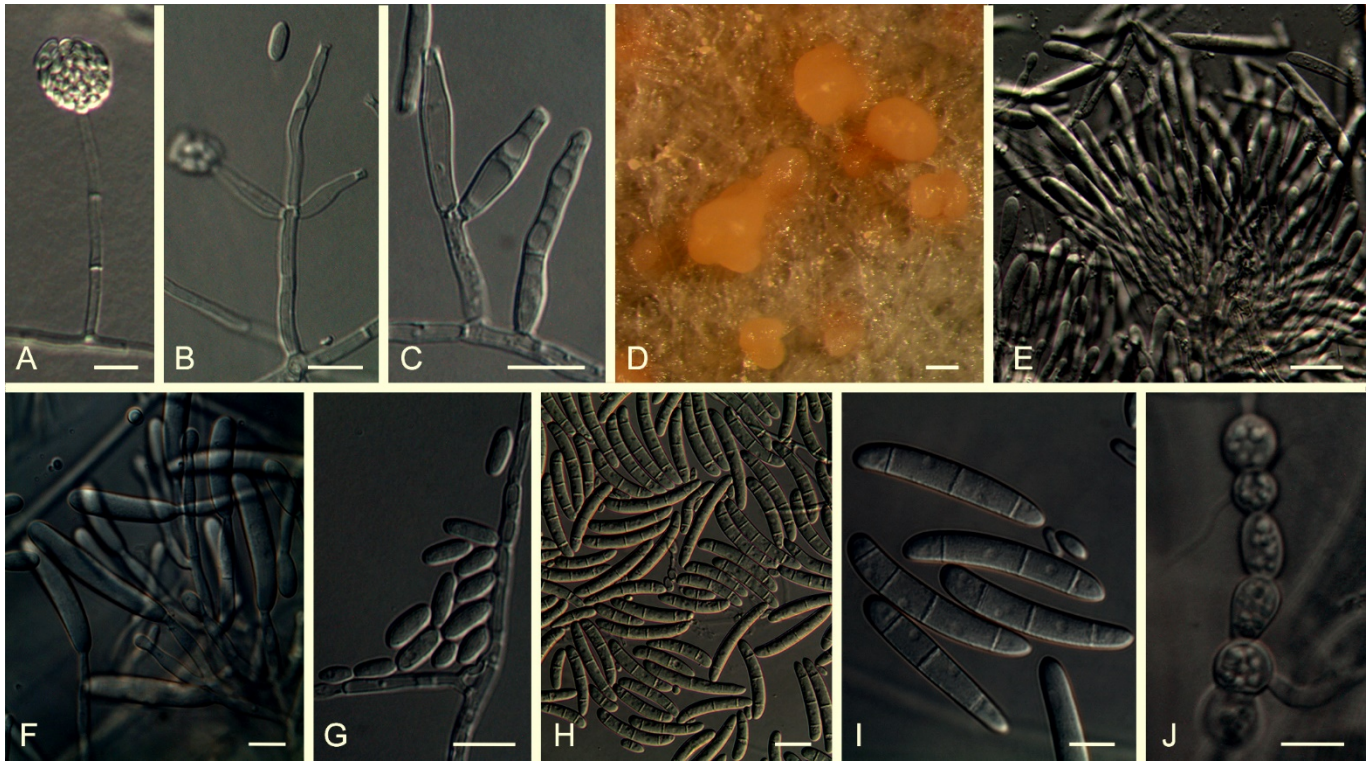

FIGURA 2

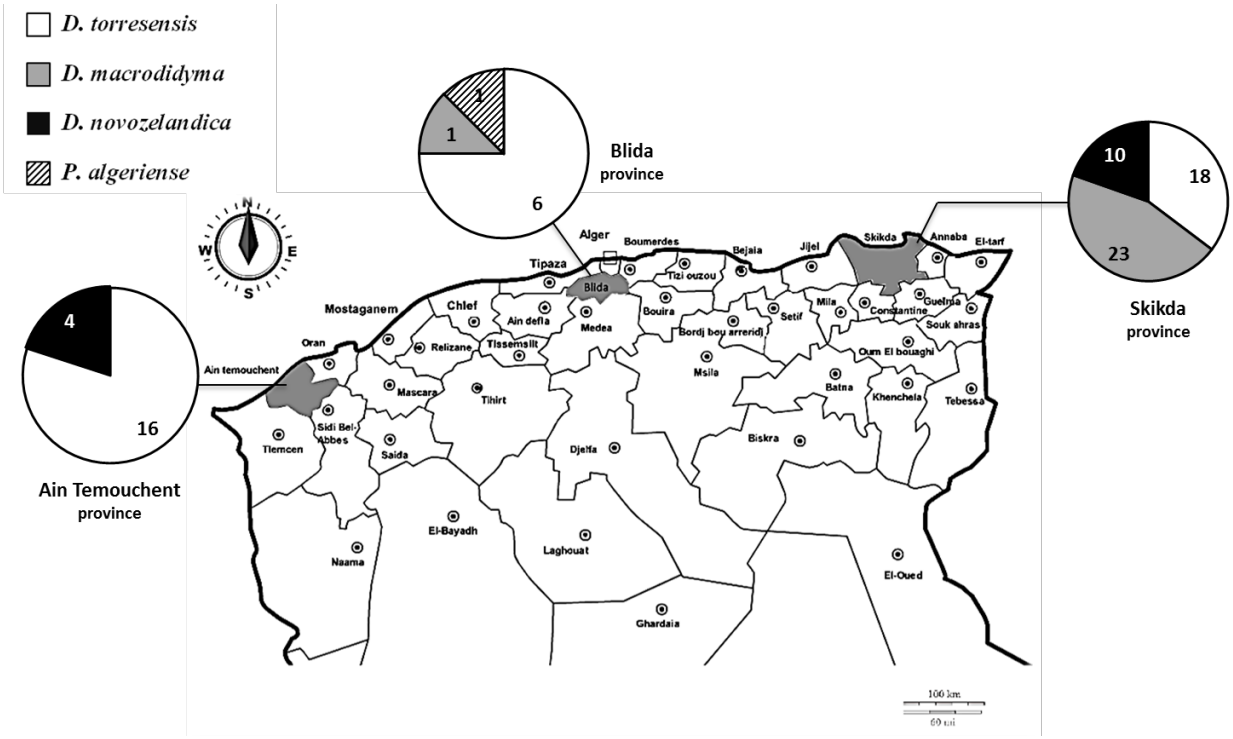
D. torresensis
D. macrodidyma
D. novozelandica
P. algeriense
1
1
6
Blida
province
10
18
23
Skikda
province
4
16
Ain Temouchent
province
N
W
E
S
Alger
Boumerdes
Bejaia
Jijel
Skikda
Annaba
El-tarf
Tipaza
Blida
Tizi ouzou
Mostaganem
Chlef
Ain defla
Medea
Bouira
Setif
Mila
Constantine
Guelma
Souk ahras
Oran
Relizane
Tissemsilt
Bordj bou arreridj
Oum El bouaghi
Ain temouchent
Msila
Batna
Khenchela
Tebessa
Mascara
Tihirt
Sidi Bel-Abbes
Tlemcen
Saida
Djelfa
Biskra
El-Bayadh
Laghouat
Naama
El-Oued
Ghardaia
100 km
60 mi

FIGURA 3

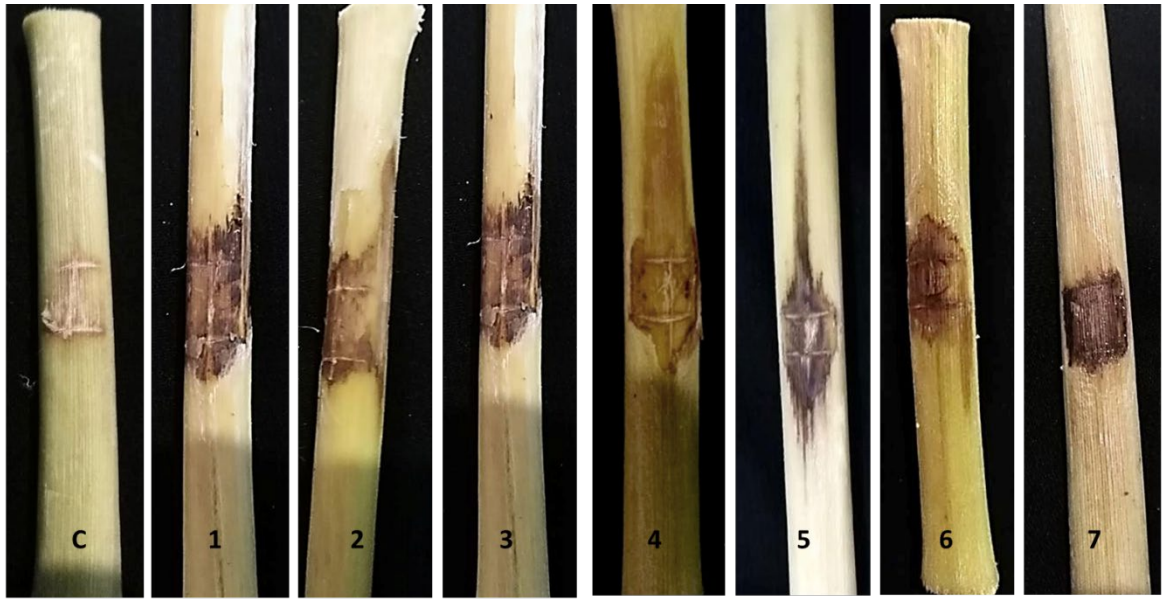

FIGURA 4

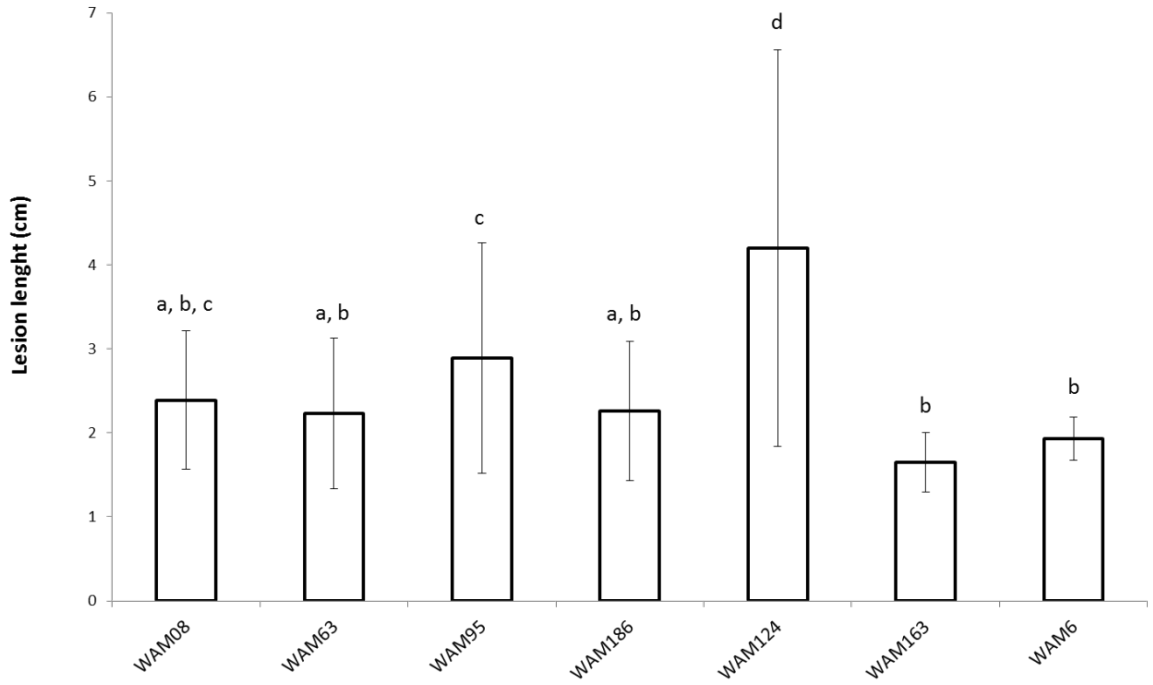
Lesion lenght (cm)
7
6
5
4
3
2
1
0
a, b, c
a, b
c
a, b
d
b
b
WAM08
WAM63
WAM95
WAM186
WAM124
WAM163
WAM6

FIGURA 5