Lasiodiplodia mitidjana sp. nov. and other Botryosphaeriaceae species causing branch canker and dieback of Citrus sinensis in Algeria

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Full Title: Lasiodiplodia mitidjana sp. nov. and other Botryosphaeriaceae species causing branch canker and dieback of Citrus sinensis in Algeria

Short Title: Botryosphaeriaceae causing dieback on citrus.

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Keywords: Citrus cultivation, trunk diseases, fungi, identification, taxonomy, pathogenicity

Abstract: Several Botryosphaeriaceae species are known to occur worldwide, causing dieback, canker and fruit rot on various hosts. Surveys conducted in ten commercial citrus orchards in the northern region of Algeria revealed five species in Botryosphaeriaceae belonging to three genera associated with diseased trees. Morphological and cultural characteristics as well as phylogenetic analyses of the internal transcribed spacer (ITS) region and the translation elongation factor 1-alpha (tef1-α) identified Diplodia mutila, Diplodia seriata, Dothiorella viticola, Lasiodiplodia mediterranea and Lasiodiplodia mithidjana, which is described in this paper as a new species. Of these, L. mithidjana (14.1%) and L. mediterranea (13%) were the most widespread and abundant species. Pathogenicity tests revealed that L. mediterranea and D. seriata were the most aggressive species on citrus shoots. This study highlights the importance of Botryosphaeriaceae species as agents of canker and dieback of citrus trees in Algeria.

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Response to Reviewers: Answers to the reviewer 1
General comments: Akila Berraf-Tebbal and collaborators present in this manuscript the results of a survey on Botryosphaeriaceae diversity and pathogenicity affecting symptomatic sweet orange (Citrus sinensis) in Algeria. Botryosphaeriaceae species were identified in every orchard tested (n=10) and a total of five different species have been isolated from symptomatic samples, with frequencies ranging from 5.4% to 14.1% of the samples. One species appears to form a distinct monophyletic group - not described before - and is claimed by the authors to be a new species. The pathogenicity of two representative isolates for every species isolated (n=5) was tested experimentally on Citrus shoot and Koch’s postulate was verified for all of them. Differences in pathogenicity were observed between isolates.
Overall, the manuscript is well written and there is no doubt about the efforts made by the authors to produce this work. The results presented are interesting and alert about the potential spread and threats brought by these pathogens, as mentioned by the authors.
My main comments to improve this manuscript is that authors often goes on
conclusions that are not completely supported by the results. I suggest therefore to reconsider these conclusions or change the way they are presented to stick more on the facts.

We took into consideration all the comments regarding the conclusions and we made sure to do all the required modifications.

Furthermore, the GenBank accession for the Tef1-α sequences are not available which hamper my reviewing conclusions. This is even more problematic because the genetic difference observed to discriminate the new species are brought by this marker. The ITS and the tef1-α sequences have been deposited into GenBank; However, the tef1-α sequences are not automatically deposited into GenBank after being accessioned. Each sequence record is individually examined and processed by the GenBank annotation staff to ensure that it is free of errors or problems.

In this sense, a new species (Lasiodiplodia mitidja) is introduced in this study. This introduction is based on a two loci phylogeny, as well as morphological observations. I’m not a taxonomist myself, but are two SNPs (which I could not verified, and that is not illustrated by an alignment neither in the manuscript) and a bootstrap of 80 enough to consider the organism a new species? Concerning the morphological differences, as the authors mentioned, conidia “tend” to be larger and L/W ratio is different but for both measurements, no statistical significance is brought to the observations to confirm the difference. Can it represent a subpopulation of L. citricola? I presume it is not possible to test if those “species” outcross but if we have to be more rigorous, I would recommend to stay more prudent about the “new species” terminology and presented it more as a suggestion, or inform the readers that all the criteria to say it’s a new species are not completely fulfil.

We agree that this may be debatable. In fact, we have discussed this previously within the team. However, it is clearly aligned with the current trend for introduction of novel Lasiodiplodia species.

In the future it may well be proven that in fact it is not a new species different from L. citricola. But taxonomy is dynamic and hence frequently changing. For the moment we would like to introduce the new species. The fact is that eventually it will be described as a new species, if not by our group, then by someone else.

As an example of a case which is similar to ours, here are the nucleotide differences for the following mentioned species: L. chinensis vs L. lignicola vs L. pseudotheobromae. For all 3 species ITS is 100 % identical

As for Tef1:
- L. chinensis vs L. lignicola: 1 nucleotide difference
- L. chinensis vs L. pseudotheobromae: 3 nucleotides differences

My second concern is the ambiguity made between the types of wood alteration/symptoms observed in Citrus trees and the presence and implication of Botryosphaeriaceae. Botryosphaeriaceae species can be isolated from certain types of alteration and yet not being responsible of these alterations. Knowing the "opportunist" behaviour of these pathogens, I would not be surprised if they take over the habitat after a disequilibrium was induced into the microbiome of trees following another pathogen attack.

We agree with the reviewer. After considering your other comments on the same part and your suggestion of removing it and given that it is not relevant for the paper we decided to delete it.

The fact that the isolates were able to provoke symptoms experimentally does not necessarily mean they are responsible of the ones observed on the diseased trees, especially since the symptoms observed after artificial inoculation are not correlated to the ones observed on fields.

We agree with the reviewer, however the goal was to test pathogenicity of the isolates and this is the way to do it. Of course we cannot be sure that they will behave the same way in the field but they have the potential to do so.

Similarly, the presence of basidiocarps on heavily symptomatic Citrus is confusing for me, at least the way it is presented. What is the link between the Botryosphaeriaceae and the basidiocarps emergence, which species correspond to this basidiocarp?

We described the health status of the orchards where the sampling has been carried out (branch and shoot cankers, abnormal growth of epicormic shoots; defoliation and leaf chlorosis). Basidiocarps are the fruiting bodies of the decay-causing fungi. Their presence on the trunk means that it is an already rotting trunk and that some
ascomycetes (Botryosphaeriaceae, Diatrypaceae…) and Basidiomycetes (Fomitiporia, Phellinus….) have already colonized the trunk. In a similar fashion, Figure 4 is confusing as my conclusion on this figure is that Botryosphaeriaceae can be isolated from different types of symptoms and not that one species is more isolated from one type of symptoms than another as the authors tend to say. There is no statistic proving so, and a quick interpretation (but false) from a hurried reader would be that such species is responsible of such symptoms. At this point, those results are more detrimental that beneficial to the study. I either recommend to delete this part or erase those ambiguities by a deeper discussion and a clearer result presentation. What do we know about the multifactorial aspects of dieback diseases? Is there only one pathogen involved? I think study conducted on Botryosphaeriaceae and grapevine trunk disease can be related to this case. Furthermore, if this part is conserved, more insights on what is known about the different symptoms that can cause Botryosphaeriaceae could/should be presented in the introduction.

We agree with the reviewer and his comments. After pointing out these remarks we thoughtfully considered them and decided to delete the figure 4 as well as the paragraphs related to it.

Finally, the statistical methods used to test pathogenicity differences is either not well presented or the conclusions are not correct. This part needs to be improve. Have you tested species effect? Isolate effect? What are the p-value attributed to each ANOVA test, which factor has been tested by the ANOVA? Is LSD method (which is not described by the way) the more appropriate in your case?

We took into consideration your valuable comments and we made sure to change this part and we removed all the ambiguities.

Minor Comments:

L30: 14.1% percent of the samples and 13% of the samples
R: Revised as recommended

L31: what is the difference between widespread and abundant?
R: Widespread means that it is found or distributed over a large area. However, abundant means that it is existing or available in large quantities (it could have the same meaning as plentiful)

L42: I would erase (pomelos)
R: Revised as recommended

L43: Despite the high adaptation capacity of citrus trees to different climates (reference is missing)
R: Revised as recommended

L47: Citrus diseases are numerous and diverse, and are caused by phytopathogenic agents belonging to viruses, viroids, phytoplasmas, bacteria, and fungi (reference is missing).
R: Revised as recommended

L57: reference is missing
R: Revised as recommended

L63: colonize or affect?
R: We deleted ‘affect’

This part on Botryosphaeriaceae should be more documented: classification of Botryosphaeriaceae, how many genera, endophytes with symptomless period, etc…
R: Revised as recommended

L82: Surveys were conducted in ten commercial orchards in the northern region of Algeria, specifically, in the Mitidja plain at the base of the Tell Atlas Mountains (Table 1).

Table 1: I would add coordinates of the orchards and years of sampling.
R: Revised as recommended

L94: was the scalpel sterilized?
R: Yes, the scalpel was sterilized. This detail has been added to the manuscript.
In this paragraph, can you add more info about the PCR conditions?
R: Revised as recommended

L120-121: pyrosequencing?
R: the company used Sanger sequencing method.

L123: Newly generated sequences were deposited in GenBank (Table 2): the Tef1-α isn’t accessible.
R: The sequences are available in GenBank.

L124: Sequences for both DNA regions were retrieved in BLAST searches from GenBank [34].
Check the meaning of this sentence. For example: “Homologous sequences of the newly sequenced ones were retrieved from the GenBank by Blast.”
R: Revised as recommended

L125: Table 2: Can you add more info on this table, like type of tissue (trunk/branches), type of symptoms (under your classification), Orchard, etc….
R: Revised as recommended

L132: Please specify the request you made, or put the sequence in the supplemental files.
R: Revised as recommended (the sequences are in the supplementary files)

L136: what kind of adjustments?
R: The ITS and tef1-α sequences were initially aligned separately using ClustalX v. 1.83. The alignments were manually optimized by coding the missing sequences as “?” Ambiguous sequences at the start and the end were deleted and gaps were adjusted in BioEdit.

L164: this part need to be more precise/improve. Which threshold to accept the significance of the ANOVA, which factor tested, what LSD means for, which soft did you use?
R: Revised as recommended
Detailed responses:
Which threshold to accept the significance of the ANOVA
R: when the P value is below the threshold (0.05), the difference between the means is considered as significant.

Which factor tested?
R: We tested the lesions produced by each fungal isolate of the different species.

What LSD means for?
R: We changed the statistical test by using Tukey’s honestly significant difference (HSD) test.
Which soft did you use?
R: The R v. 3.5.1 statistical software was used to perform the statistical analysis.

L171: nothing is said about the distribution
R: We removed the word ‘distribution’ from the title

L172-173 and L174-175 could be fused for clarity purposes
R: We removed this paragraph as recommended.

L175: the total number of samples… Samples = branches?
R: The samples mean the different necrotic lesions found in the branches and the trunks of the 80 trees.
L176 – 177: this measurement is completely arbitrary and according to me abusive. If one pathogen would have occurred at 80% frequency, the difference between 11%(very frequent) and 4%(infrequent) would be meaningless. My advice, stick to the numbers and do not try to interpreted it in a frequent or infrequent way, that’s too
subjective.
R: We removed the paragraph related to the frequency of occurrence, as recommended.

L186: On heavily infected trees, basidiocarps emerged: that’s ambiguous, as said before.
R: We removed the description of the basidiocarps from the photoplate as well as from the text, as recommended.

L189: why wedge-shaped necrosis is not name WSN?
R: Revised as recommended

L191: similar BCN instead of BCN and YSW instead of NCC.
R: Revised as recommended

L196: the "e." is missing on the picture, but maybe see in it as a sign for not putting this picture...
R: Revised as recommended

L214: Why MP tree is not shown? At least in supplemental file?
R: Revised as recommended (the tree is in supplementary file)

L218: why 23 isolates and not 24 (10+14)
R: The sequence of one isolate was not good enough to use it for the phylogenetic analysis.

L228: The phylogenetic tree of only one method is presented, although the bootstrap values of the two methods are shown. Can we see the tree constructed with the second method in the supplemental files?
R: Revised as recommended

L279: can you have the sequence accessible please?
R: The sequence has been submitted to GenBank. It will be available online after verification of the annotation. We have included the accession number into the table 2.

L311: from what I’ve read, LSD test is not recommended anymore as sensitive to multiple comparison. Furthermore, as mentioned above, this test is not well conducted and presented. A histogram would be better, with a sign for significant difference, either at the isolate level and species level, with threshold use for significance. The 100% of re-isolation frequency for every isolates are not necessary in the table according to me, if you say it in the text.
R: Revised as recommended.

L317: Ambiguous: are you speaking of distribution in the wood? If yes, cut this paragraph in two: Frequency of occurrence / Distribution of Botryosphaeriaceae in the wood.
R: Revised as recommended

L320: I comment already the frequent and very frequent ranking, that’s abusive according to me.
R: We deleted this paragraph.

L323: For each orchard, at least two different species were isolated, average per orchard?
R: In the paragraph L323, we described the widespread of the species in the orchards. It was only to mention their presence on each orchard.
For more details, here is a table containing all the information about species distribution among the surveyed orchards.
Region
Oued El AlleugChiffa BoufarikStaoueli
Species/ Orchards12345678910Total
D. seriata----21--3410
D. mutila----32----5
L. mediterr.2231--22--12
L. mitidjana0333--22--13
Doth. vitic.1-1-1-12--117
L328: new paragraph or no paragraph at all as mentioned above, I found this part confusing.
R: Revised as recommended (We removed the paragraph).

L370-371: According to these authors, L. mediterranea has been found only on V-shaped necrotic sectors of grapevine while it has been isolated from all the lesion types of citrus trees in this study.
R: Revised as recommended

L373: what the results of Andolfi et al. bring to your results?
R: Andolfi et al. (2016) isolated and characterized the main secondary metabolites produced by L. mediterranea. They also, evaluated its phytotoxic and antifungal activities. These findings support our results, which show the ability of this fungal species to colonize and cause damages in the wood.

L374-375: maybe if you had isolated only the 4 or 5 isolates that were from brown central necrosis, would you have said that the species was exclusively found in brown central necrosis? This part of the discussion is not really constructive.
R: we removed the paragraph, as well as the figure 4.

L387: More interesting that this, it confirms its wide geographical range.
R: revised as recommended

L402: Wedge shaped lesion?
R: The paragraph is about the pathogenicity trial and the lesions produced by each Botryosphaeriaceae species. We did not consider the shape of the lesions, for the pathogenicity test.

L403-405: this part of the discussion goes beyond what your data show, either improve your statistical analysis or moderate the message.
R: Revised as recommended

L408-415: similar, hard to have this kind of discussion with two isolates per strains, with one phenotyping trial.
R: Revised as recommended

L446: References have to be reformatted: species not in italic, capital letter on every first word letter for some references, etc.; some other problem like L508 Phililips is written bizarrely.
R: Revised as recommended

Answers to the reviewer 2
Reviewer #2: This is a nice study of Botryosphaeriaceae which are important plant pathogens, including fruit trees. Algeria is unexplored both from mycological and pathological perspective and it is nice to see collaborations like this resulting in a good piece of work. I agree with authors regarding Lasiodiplodia mediterranea and L.vitis situation, especially because PCR artifacts introduced by primer sequences are unfortunately a common thing these days (my personal experience). I have small suggestions that would improve the paper.

Abstract, line 25-of Botryosphaeriaceae
R: Revised as recommended

Abstract, line 29- Delete which, add Lasiodiplodia mitidjiana is described in this paper as a new species
R: Revised as recommended

Abstract, line 62- delete effect
R: Revised as recommended

Materials-line 98-dried on sterilized paper (towels, filter paper?)
R: Revised as recommended

Materials, line 100-The mycelium emerging from wood pieces was transferred…
R: Revised as recommended

Materials, line 105-Isolates that lacked pycnidia production…
R: Revised as recommended

Materials, line 150-How did you select representative isolates?
R: We selected two isolates, from each phylogenetically resolved species.

Materials, line 151-Shoots? But above you mentioned branches (line 148)
R: Revised as recommended (We have standardized using shoot instead of branch).

Materials, line 157-“… well watered and maintained under favorable conditions” What do you mean by this? Were the cuttings in soil or in water? How many times per week did you change the water? Ambient temperature? Light?
R: The inoculated cuttings were wrapped with wet sterile cotton to avoid the desiccation of the agar plug. The shoots were immediately transplanted into pots containing sterilized water as a growth substrate (10 shoots per pot), which were incubated at the ambient room temperature, under daily photoperiod. The water of the container was changed twice a week.

Results, line 182-Degrees of intensity? Where are they?
R: The degrees of intensity refer to the different levels of the dieback symptoms observed in the orchards.

Results, line 186-Basidiocarps of which species or genera?
R: We did not identify the basidiocarps. We described all the symptoms related to the citrus trees dieback, including the fruiting bodies emerged from the trunks.

Results, line 309-What about control plants?
R: We did not isolate any of the tested species from the negative control.

Results, line 313-Now you mention branches again
R: revised as recommended

Discussion, line 360-and seriously affected trees can become
R: revised as recommended

Discussion, lines 403-404- “However, D. seriata was significantly different compared to the rest of the isolates” But previously you said that both D. seriata and L. mediterranea were most aggressive species (based on lesion lengths). So which species was in fact most aggressive? Also, what about differences in aggressiveness between different isolates of the same species?
R: The significant difference was made based on a comparison between all the tested isolates. It was not about the pairwise comparisons that take one isolate and compare it with each of the rest of isolates. D. seriata and L. mediterranea were the most aggressive species when compared to the rest of the species. However, D. seriata was the most aggressive species, considering the length of the lesion for each isolate, separately.

Also, what about differences in aggressiveness between different isolates of the same species?
R: Significant variation in aggressiveness can occur within and among isolates from the same species. This aggressiveness refers to the quantitative variation of pathogenicity on the susceptible host infection efficiency, the latent period, the spore production rate and the infectious period of each strain. These components are closely related to the genetic variability within the strains of the same species.

Discussion, lines 408-412-Was this previous study also about Bot on citrus trees?
R: the study was about Lasiodiplodia species (Botryosphaeriaceae) on grapevine.

Discussion, lines 409 and 411-In line 404 you are talking about aggressiveness. Now about virulence. In line 413 you talk again about aggressiveness. Virulence and
aggressiveness don’t mean the same thing. Replace the term virulence with aggressiveness in lines 409 and 411.  
R: Revised as recommended

Answers to the academic editor
L.273 ..we have shown...  
R: Revised as recommended
L. 279 “…these 2 nt are not real.” Are not real is confusing, maybe instead use “ were correctly determined”?  
R: Revised as recommended
L. 335 “The remaining species…” better mention the species’ name here.  
R: Revised as recommended
L. 351 A similar situation has been…  
R: Revised as recommended
L. 363 wortLd  
R: Revised as recommended
L. 373 compared instead of comparing  
R: Revised as recommended
L. 389 “…known for targeting economically important plants…” targeting sounds as if they are selecting the hosts based on the economical value. Maybe better say: …known to cause damage on several economically important species…”  
R: Revised as recommended
L. 393 “The later…”, better say “This latter species..” or just “It...”  
R: Revised as recommended
L. 408 …with a previous study…  
R: Revised as recommended
I would suggest to add some research perspectives at the end of the discussion. What kind of studies could help to better understand and develop management recommendations against citrus dieback?  
R: Revised as recommended

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|-----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
**Lasiodiplodia mitidjana** sp. nov. and other Botryosphaeriaceae species causing branch canker and dieback of *Citrus sinensis* in Algeria

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**Abstract**

Several *Botryosphaeriaceae* species are known to occur worldwide, causing dieback, canker and fruit rot on various hosts. Surveys conducted in ten commercial citrus orchards in the northern region of Algeria revealed five species of *Botryosphaeriaceae* belonging to three genera associated with diseased trees. Morphological and cultural characteristics as well as phylogenetic analyses of the internal transcribed spacer (ITS) region and the translation elongation factor 1-alpha (*tef1*-α) identified *Diplodia mutila*, *Diplodia seriata*, *Dothiorella viticola*, *Lasiodiplodia mediterranea* and a novel species which is here described as *Lasiodiplodia mitidjana* sp. nov.. Of these, *L. mitidjana* (14.1% of the samples) and *L. mediterranea* (13% of the samples) were the most widespread and abundant species.

Pathogenicity tests revealed that *L. mediterranea* and *D. seriata* were the most aggressive species on citrus shoots. This study highlights the importance of *Botryosphaeriaceae* species as agents of canker and dieback of citrus trees in Algeria.

**Key words:** Citrus cultivation, trunk diseases, fungi, identification, taxonomy, pathogenicity.
Introduction

Citrus cultivation is one of the major contributors to Algerian wealth and is part of the traditional agriculture of the country. Many types of citrus are grown in Algeria, including oranges (48 400 ha), clementine (10 817 ha), mandarins (2 347 ha), lemons (4 409 ha) and grapefruits (83 ha) [1]. Despite the high adaptation capacity of citrus trees to different climates [2], a number of unfavourable factors has led to a decrease of the total citrus yield in Algeria. Among these factors, ageing trees, droughts, inappropriate cultural practices and the effects of various pests and pathogens are the most important [2, 3]. Citrus diseases are numerous and diverse, and are caused by phytopathogenic agents belonging to viruses, viroids, phytoplasmas, bacteria, and fungi [2] Some pathogens cause very serious diseases, predisposing to, and inciting dieback, while others are less serious [2-5].

Recently, trunk diseases have become a growing threat in both, old and newly established orchards of citrus, worldwide. Symptoms include leaves that become yellow and fall early, shoots and twigs die, increasing the risk of citrus decay as the damage expands to the trunk [2, 6-8]. To date, among the fungi that impact citrus, Diaporthe species are well known for causing stem-end rot and melanose of fruits, young leaf and shoot gummosis and blight of perennial branches and trunks, in Greece, Italy, Malta, Portugal, Spain, China, Korea, New Zealand, and the USA [8-11]. Fusarium and Neocosmospora have also been reported causing canker and dieback diseases of citrus, in Tunisia, Greece, Italy and Spain [12-14]. The Diatrypaceae are other canker and dieback pathogens impacting citrus orchards [15]. Several Eutypella spp. have been reported from Citrus sp. In southern California desert, three distinct species of Eutypella are found associated with citrus branch canker, namely: Eutypella citricola, E. microtheca and a Eutypella sp. [15-17].

In addition to the above fungal pathogens that compromise citrus crops, several Botryosphaeriaceae species are known to colonize citrus trees. The Botryosphaeriaceae family is recognized as an important and widely distributed plant pathogen, which impacts on a variety of economically important hosts. It comprises 24 genera encompassing 222 species, living as endophytes, saprobes, or plant pathogens [18, 19]. Recent studies carried out in California, Italy and Tunisia have highlighted the Botryosphaeriaceae as the most prevalent fungi that cause cankers, vascular necrosis and dieback of citrus trees [7, 15, 16, 20]. Adesemoye et al. [21] recovered various Botryosphaeriaceae species from necrotic tissues of citrus branch canker and rootstock, including, Diplodia seriata, D. mutila, Dothiorella viticola, D. iberica, Lasiodiplodia parva, Neofusicoccum australe, N. luteum, N. mediterraneum, N. parvum and Neoscytalidium dimidiatum. In Iran, Abdollahzadeh et al. [22], described Lasiodiplodia citricola from citrus trees showing symptoms of branch dieback.

In Algeria, members of the Botryosphaeriaceae have been reported to cause diseases on Vitis vinifera [23-25], Quercus suber [26] and Cupressus macrocarpa [27]. Linaldeddu et al. [28] isolated and described Lasiodiplodia mediterranea from a cankered branch of Citrus sinensis trees in northern Algeria. However, the impact of Botryosphaeriaceae species on citrus trees has not been studied in detail. Therefore, the aim of this study was to investigate
and determine the incidence of the *Botryosphaeriaceae* species associated with branch canker and dieback in the major citrus-growing region of Algeria.

**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies. This study did not involve endangered or protected species.

**Field survey and sampling**

Surveys were conducted in ten commercial orchards in the northern region of Algeria. Specifically, in the Mitidja plain at the base of the Tell Atlas Mountains. The sampling was done in four municipalities; namely Oued El Aleug (4 orchards), Chiffa (2 orchards), Boufarik (2 orchards) and the coastal town, Sidi Fredj, located within the territory of the Staoueli municipality, situated by the Mediterranean Sea (2 orchards). The field diagnosis and sampling were performed between April 2013 and March 2015. Samples were collected from the orchards with permission of landowners. Trunks and branches showing symptoms such as dead shoots, defoliation, cankers, wood necrosis, and dieback were collected, randomly. A total of 80 symptomatic sweet orange (*Citrus sinensis*) trees were sampled (Table 1).

**Table 1. Citrus orchards surveyed and number of samples collected.**

| Locality     | GPS coordinates    | Orchards | Area (ha) | Number of trees sampled | Number of samples processed |
|--------------|--------------------|----------|-----------|-------------------------|----------------------------|
| Oued El Alleug | 36°33'21"N 2°47'22"E | a        | 18        | 5                       | 9                          |
|              |                    | b        | 16        | 5                       | 6                          |
|              |                    | c        | 28        | 5                       | 7                          |
|              |                    | d        | 6.8       | 5                       | 6                          |
| Chiffa       | 36°27'44"N 2°44'27"E | a        | 25        | 10                      | 13                         |
|              |                    | b        | 18        | 10                      | 10                         |
| Boufarik     | 36°34'31"N 2°54'46"E | a        | 43        | 10                      | 10                         |
|              |                    | b        | 27        | 10                      | 11                         |
| Staoueli     | 36°45'12"N 2°53'17"E | a        | 32        | 10                      | 10                         |
|              |                    | b        | 15        | 10                      | 10                         |
Fungal isolation and morphological characterization

In the laboratory, all samples were processed by peeling the outer bark surface with a sterilized scalpel. Longitudinal and transversal cuts were made to reveal the type and localization of the internal necrosis. From each lesion detected, ten pieces of wood, approx. 5 mm², were cut from the margins between necrotic and healthy tissues. These pieces were submerged in 4 % sodium hypochlorite for 15 min, washed thrice with sterile distilled water, dried with sterilized filter paper and placed onto the surface of potato dextrose agar (PDA, Difco Laboratories). Plates were incubated at 25 °C until growth was detected. The mycelium emerging from wood pieces were transferred onto fresh PDA plates and incubated under the same conditions.

Preliminary identifications to genus and tentative species level were based on colony and conidial morphology (colony colour, colony growth pattern, conidial size, shape, colour, striation, septation, conidiogenous cells, and presence of paraphyses) according to Phillips et al. [18]. Isolates that lacked pycnidia production on PDA were placed on autoclaved pine needles in ¼ strength PDA within 2–3 weeks, incubated at 25 °C under mixed near-UV and cool-white fluorescent light in a 12 h light 12 h dark regime for 2–6 weeks, to enhance fruiting body production. Conidiogenous layer and conidia were mounted in 100 % lactic acid and observed with a Nikon 80i light microscope.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 7 days old axenic cultures, grown on PDA at 25 °C, following Santos and Phillips [29]. PCR reactions were carried out with Taq DNA polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania). PCR reaction mixtures were prepared as previously described by Alves et al. [30], with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. The ITS region plus D1/D2 domain of the LSU was amplified with the primer pair ITS1 [31] and NL4 [32]. The amplification conditions were initial denaturation of 5 min at 95 °C, followed by 29 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1.5 min at 72 °C, and a final extension of 10 min at 72 °C. Part of the translation elongation factor 1 alpha gene (tef1-α) was amplified with primers EF1-688F and EF1-1251R [33]. The amplification conditions were: initial denaturation of 5 min at 95 C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, 1½ min at 72 °C, and a final extension period of 10 min at 72 °C. ITS and tef1-α regions were sequenced in both directions by STAB Vida Lda (Portugal), using the Sanger method.

The nucleotide sequences were read and edited with BioEdit Alignment Editor V.7.0.9.0 [34]. Newly generated sequences were deposited in GenBank (Table 2). Homological sequences of the newly sequenced ones were retrieved from the GenBank using the Basic Local Alignment Search Tool (BLAST) [35].
| Species                  | Isolate number | Host/ Substrate       | Origin                        | Collector                  | GenBank accession numbers |
|--------------------------|----------------|-----------------------|-------------------------------|----------------------------|----------------------------|
| *Lasiodiplodia* mediterranea | ALG77          | *Citrus* / wood canker | Algeria, Boufarik             | Akila Berraf-Tebbal        | MN104094, MN159093         |
| *L. mediterranea*        | ALG76          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104095, MN159094         |
| *L. mediterranea*        | ALG40          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104096, MN159095         |
| *L. mediterranea*        | ALG78          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104097, MN159096         |
| *L. mediterranea*        | ALG41          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104098, MN159097         |
| *L. mediterranea*        | ALG36          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104099, MN159098         |
| *L. mediterranea*        | ALG80          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104100, MN159099         |
| *L. mediterranea*        | ALG73          | *Citrus* / wood canker | Algeria, Boufarik             | Akila Berraf-Tebbal        | MN104101, MN159100         |
| *L. mediterranea*        | ALG74          | *Citrus* / wood canker | Algeria, Boufarik             | Akila Berraf-Tebbal        | MN104102, MN159101         |
| *L. mediterranea*        | ALG75          | *Citrus* / wood canker | Algeria, Boufarik             | Akila Berraf-Tebbal        | MN104103, MN159102         |
| *L. mediterranea*        | CBS 124060     | *Vitis*, wood fragment | Italy, Sicily                 | S. Burrano                 | KX464148, MN938928         |
| *L. mitidjana*           | ALG81          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104104, MN159103         |
| *L. mitidjana*           | ALG44          | *Citrus* / wood canker | Algeria, Oued El Alleug       | Akila Berraf-Tebbal        | MN104105, MN159104         |
| L. mitidjana | ALG39 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104106 | MN159105 |
|------------|-------|-------------------|------------------------|---------------------|---------|---------|
| L. mitidjana | ALG42 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104107 | MN159106 |
| L. mitidjana | ALG38 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104108 | MN159107 |
| L. mitidjana | ALG43 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104109 | MN159108 |
| L. mitidjana | ALG37 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104110 | MN159109 |
| L. mitidjana | ALG34 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104111 | MN159110 |
| L. mitidjana | ALG82 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104112 | MN159111 |
| L. mitidjana | ALG72 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104113 | MN159112 |
| L. mitidjana | ALG71 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104114 | MN159113 |
| L. mitidjana | ALG70 = MUM 19.90 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104115 | MN159114 |
| L. mitidjana | ALG69 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104116 | MN159115 |
| Diplodia seriata | ALG93 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104117 | MN159116 |
| D. seriata | ALG94 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104118 | MN159117 |
| D. seriata | ALG98 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104119 | MN159118 |
| D. seriata | ALG91 | Citrus/wood canker | Algeria, Chiffa | Akila Berraf-Tebbal | MN104120 | MN159119 |
| D. seriata | ALG92 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104121 | MN159120 |
| Species               | Accession No. | Disease            | Location         | Author          | National Collection Code | GenBank Collection Code |
|----------------------|---------------|--------------------|------------------|-----------------|--------------------------|-------------------------|
| *D. seriata*         | ALG90         | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104122                 | MN159121                |
| *D. seriata*         | ALG89         | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104123                 | MN159122                |
| *D. seriata*         | ALG96         | Citrus/wood canker | Algeria, Staoueli | Akila Beraf-Tebbal | MN104124                 | MN159123                |
| *D. seriata*         | ALG95         | Citrus/wood canker | Algeria, Staoueli | Akila Beraf-Tebbal | MN104125                 | MN159124                |
| *D. seriata*         | ALG97         | Citrus/wood canker | Algeria, Staoueli | Akila Beraf-Tebbal | MN104126                 | MN159125                |
| *D. mutila*          | ALG99         | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104127                 | MN159126                |
| *D. mutila*          | ALG103        | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104128                 | MN159127                |
| *D. mutila*          | ALG100        | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104129                 | MN159128                |
| *D. mutila*          | ALG102        | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104130                 | MN159129                |
| *D. mutila*          | ALG101        | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104131                 | MN159130                |
| *Dothiorella viticola* | ALG83        | Citrus/wood canker | Algeria, Staoueli | Akila Beraf-Tebbal | MN104087                 | MN159086                |
| *Doth. viticola*     | ALG35         | Citrus/wood canker | Algeria, Oued El Alleug | Akila Beraf-Tebbal | MN104088                 | MN159187                |
| *Doth. viticola*     | ALG84         | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104089                 | MN159188                |
| *Doth. viticola*     | ALG85         | Citrus/wood canker | Algeria, Staoueli | Akila Beraf-Tebbal | MN104090                 | MN159189                |
| *Doth. viticola*     | ALG86         | Citrus/wood canker | Algeria, Oued El Alleug | Akila Beraf-Tebbal | MN104091                 | MN159190                |
| *Doth. viticola*     | ALG87         | Citrus/wood canker | Algeria, Chiffa  | Akila Beraf-Tebbal | MN104092                 | MN159191                |
| *Doth. viticola* | ALG88 | Citrus/wood canker | Algeria, Chiffa | Akila Berraf-Tebbal | MN104093 | MN159192 |
Phylogenetic analysis

Sequences of all *Lasiodiplodia* species known from culture were retrieved from GenBank (S1 Table) and aligned with sequences of the isolates obtained in this study. Alignments were done with ClustalX v. 1.83 [36] using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Alignments were checked and manual adjustments made if necessary using BioEdit v. 7.2.5 [34]. Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were performed using MEGAX [37]. The best fitting DNA evolution model was determined also by MEGAX. ML analysis was performed on a Neighbour-Joining starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference. MP analysis was done using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The robustness of the trees (ML and MP) was evaluated by 1000 bootstrap replications.

Pathogenicity test

The ability of isolates to cause cankers was assessed *in vivo* on detached shoots collected from symptomless citrus trees. From each phylogenetically resolved species, two representative isolates were selected. Pathogenicity of each selected strain was tested on 1-year-old shoots of *Citrus sinensis*. The shoots with 25 mm in diameter were cut into equal length (25 cm long). They were then surface disinfected with 70% ethanol and wounded on an intermediate internode, with a scalpel. From each strain, a 5 mm diameter mycelial plug taken from a 5-day old colony growing on PD A was placed into the wound. Negative controls were inoculated with fresh, non-colonized, PDA plugs. Subsequently, the cuttings were wrapped with wet sterile cotton and sealed with Parafilm® to prevent the desiccation of the agar plug. The shoots were immediately transplanted into pots containing sterilized water as a growth substrate. They were incubated at the ambient room temperature, under daily photoperiod. The water of the container was changed twice a week. There were 10 replicates per isolate, and the same number of cuttings was used as controls. One month after inoculation, lengths of lesions produced by each strain were measured. In an attempt to recover the inoculated fungi and complete Koch’s postulates, necrotic tissue from the margin of the lesions was taken and placed onto PDA.

Statistical analyses

Data of lesion lengths caused by the fungal isolates belonging to the different species was subjected to one-way ANOVA (analysis of variance) with P≤0.05. Prior to analysis data were checked for normality, then, significance of differences between means was determined by
Turkey’s honestly significant difference (HSD) test. Statistical analyses were performed on the software R v. 3. 5. 1 and a significance level of 0.05 was used.

Nomenclature

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

Results

Disease symptoms

Citrus dieback was detected in all the orchards and regions investigated, with different degrees of intensity. Various external symptoms, including partial or complete dieback of the tree, branch and shoot cankers, abnormal growth of epicormic shoots; defoliation and leaf chlorosis were observed. Moreover, in certain orchards, bark cracking of the trunk and the branches was also noticeable (Fig 1).

The analysis of the 80 symptomatic sweet orange (Citrus sinensis) trees sampled to carry out the isolations, revealed the existence of 92 necrotic lesions in the trunks and the branches. They belonged to four types of wood alteration, including: wedge-shaped necrosis (WSN), that was the most prevalent lesion (n=30) of the total samples collected. The brown central necrosis (BCN) (n=26) was the second most prevalent lesion, followed by the black spots in the xylem (BS) (n=24) and yellow soft wood rot (YSW) (n=12).

Fig 1. Citrus tree with dieback symptoms (a), bark cracking of the trunk and gummosis (b), main internal symptoms of sectioned branches and trunks (c–f).

Fungal isolation and identification
Isolation carried out from ninety-two samples yielded a total of forty-seven fungal colonies belonging to Botryosphaeriaceae. On the basis of morphological characteristics, it was possible to distinguish three morphological groups according to colour and shape of conidia. Twenty-five isolates with brown sub-globose and striate conidia were grouped as Lasiodiplodia-like fungi. Fifteen isolates with brown oblong to ovoid conidia were considered as Dothiorella-like fungi. A further seven isolates with brown, ovoid thick walled and 1-septate conidia were considered as Diplodia-like fungi. The identification of the isolates was confirmed by analysis of ITS and tef1-α sequences, which distinguished five separate species. The BLAST searches in GenBank showed 99–100% identity with reference sequences of representative isolates including that of the ex-type. The identified species were: Diplodia seriata (10 isolates), Diplodia mutila (5 isolates), Dothiorella viticola (7 isolates), Lasiodiplodia mediterranea (10 isolates) and a Lasiodiplodia sp. (14 isolates) that could not be assigned to any of the currently known species.

Phylogenetic analysis

Phylogenetic analysis was performed using ITS and tef1-α sequences. Fragments of approximately 500 and 300 bases were determined for ITS and tef1-α regions, respectively. The ML and the MP trees are presented in figure 2 and figure S1, respectively. The combined ITS and tef1-α dataset of Lasiodiplodia consisted of 23 isolates aligned with sequences of 69 isolates retrieved from GenBank, representing a selection of all known Lasiodiplodia and 2 outgroup taxa (Diplodia seriata CBS 112555 and Diplodia mutila CBS 112553). In the ML phylogenetic tree (figure 2), the isolates obtained in this study grouped in two clades. The first clade comprised 10 isolates, which clustered together, with the ex-type strain of Lasiodiplodia mediterranea (CBS 137783) and the ex-type strain of Lasiodiplodia vitis (CBS 124060) (Table S1), forming a single monophyletic group. The second group contained 14 isolates, which formed a distinct clade, with a high bootstrap support (ML/MP = 80/94), was considered to represent a distinct species, which is described here as Lasiodiplodia mitidjana sp. nov. (Fig 2).

Fig 2. Maximum likelihood tree generated from the combined analysis of ITS and tef1-α sequence data. ML/MP bootstrap values are given at the nodes. Support values less than 50 % are omitted or indicated with ‘−’. The tree was rooted to Diplodia mutila and Diplodia seriata.

Taxonomy

Lasiodiplodia mitidjana A. Alves, A.E. Mahamedi & A. Berraf-Tebbal sp. nov. (Fig 3) [urn:lsid:mycobank.org:names: MB 832823]. Algeria, Mitidja, isolated from a branch canker of Citrus sinensis, June 2015, Akila Berraf-Tebbal, HOLOTYPE AVE-F-7, a dried culture sporulating on pine needles twigs deposited in the Herbarium Universitatis Aveirensis
(AVE), culture ex-holotype MUM 19.90 (=ALG70). Other isolates examined are listed in Table 2.

**Etymology**: named after Mitidja where the fungus was discovered.

**Sexual state**: Not seen. Asexual state: Conidiomata stromatic, pycnidial, produced on pine needles on ¼ strength PDA within 2–3 wks, dark brown to black, covered with dense mycelium, superficial or immersed in the host becoming erumpent when mature, mostly uniloculate, solitary, globose, thick-walled. Paraphyses hyaline, cylindrical, thin-walled, initially aseptate, becoming septate when mature, rounded at apex. Conidiogenous cells holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, sometimes slightly swollen at the base. Conidia subovoid to ellipsoid-ovoid, apex rounded, occasionally tapering to truncate base, widest in middle to upper third, thick-walled, with granular content, initially hyaline and aseptate, remaining so for a long time, becoming dark brown and 1-septate, with longitudinal striations, \((22.6–27.7(−31.9) × (13.5–16.7(−19.6)) \mu m, 95 \% \text{ confidence limits} = 27.3–28 × 16.5–16.9 \mu m (\text{av. of 125 conidia} ± SD = 27.7 ± 1.9 × 16.7 ± 1.1 \mu m, \text{L/W ratio} = 1.7)\).

**Cultural characteristics**: Colonies on PDA with moderate to dense aerial mycelium, initially white to smoke-grey, turning greenish grey on the surface and reverse, becoming dark slate blue with age.

**Cardinal temperatures for growth**: Minimum <10 °C, maximum < 40 °C and optimum 25 – 35 °C, covering the medium surface (90 mm) before 7 days at 25 °C in the dark.

**Habitat**: Twigs and branches of *Citrus sinensis*.

**Known geographic distribution**: Algeria.

**Notes**: Phylogenetically it is very closely related to *L. citricola* being distinguished by three bp in the *tef1*-α locus. Conidia tend to be larger than those of *L. citricola*, 95 % confidence limits = 24.1–24.9 × 15–15.7 μm (av. ± S.D. = 24.5 ± 0.2 × 15.4 ± 1.8 μm) and have a lower L/W ratio = 1.6.

*Lasiodiplodia mediterranea* Linaldeddu, Deidda & Berraf-Tebbal sp. nov. (Linaldeddu et al. 2015. Fungal diversity 71:207)

**MycoBank**: MB 808356

**Synonym**: *Lasiodiplodia vitis* Yang & Crous, sp. nov. (Yang et al. 2017. Fungal Biology 121)

**MycoBank**: MB817635

**Notes**: Yang et al. [38] described *L. vitis* as a novel species, clearly distinct from the species recognised on *Vitis vinifera* in Italy. However, in their study Yang et al. [38] did not include any representative of *L. mediterranea* which was described by Linaldeddu et al. [28] from
several hosts, including *V. vinifera* in Italy. However, we have shown that *L. vitis* is phylogenetically indistinguishable from *L. mediterranea*. Their ITS sequences are 100% identical and the *L. vitis* tefl-α sequence deposited in GenBank differs from the tefl-α of *L. mediterranea* in 2 nt positions (1 missing G and a C instead of a T in the EF-986R primer binding region). We re-sequenced the tefl-α region of *L. vitis* CBS124060 (*Table 2*) using primers EF1-688F and EF1-1251R [33] which span a larger region than primers EF1-728F and EF-986R used by Yang et al. [38] and verified that these 2 nt are not real. There is no missing G in the *L. vitis* sequence and the C instead of a T is an artefact in *L. mediterranea* sequence introduced by the EF-986R primer sequence. Thus, the tefl-α region of *L. vitis* CBS124060 is 100% identical to the tefl-α sequence of *L. mediterranea*.

![Fig 3. Lasiodiplodia mitidjana. (a-b). Pycnidia formed on pine needles. (c).](image)

Conidiogenous layer with conidia developing on conidiogenous cells. (d). Conidia developing on conidiogenous cells and paraphyses. (e,f,i). Hyaline aseptate conidia. (g,h). Hyaline aseptate brown 1-septate conidia in two focal planes showing the striations on the inner surface of the wall. (j). Aseptate conidia, one becoming brown. (k,l). Brown 1-septate conidia in two focal planes to show the striations in the inner surface of the wall. Scale bars: e = 20 μm, c,d,f–j = 10 μm; k–l = 5 μm.

**Pathogenicity test**

All the *Botryosphaeriaceae* isolates tested in the pathogenicity test were pathogenic to the citrus shoots. On the wood tissue under the bark, black to brown lesions developed, upward and downward from the inoculation point, within 30 days. The control plants did not develop any symptoms. Lesion lengths varied between the species and among the isolates of each species tested, with a significant difference (F=10.874; P < 0.001) (*Table 3*).

The most aggressive isolates were ALG91 (*D. seriata*) and ALG36 (*L. mediterranea*), which produced the longest lesions (5.49±2.65 cm and 4.39±1.31 cm, respectively) with a statistically significant difference recorded between ALG91 and the rest of the species, except for *L. mediterranea*. No significant difference in lesions size was observed between the isolates ALG40 (*L. mediterranea*) and ALG39 (*L. mitidjana*), which presented intermediate lesion lengths (3.83±0.97 and 3.88±1.24 cm, respectively). However, the smallest lesion size was produced by *Doth. viticola* ALG84 with 2.1±0.67 cm and both *D. mutila* isolates ALG102 (2.04±0.54) and ALG103 (2.05±0.4 cm). *D. seriata* was the only species that showed significant difference in lesion length between its two isolates (*Table 3*).

Koch’s postulates were confirmed by a successful re-isolation of all tested fungal species from the necrotic tissues (*Table 3*).

**Table 3. Mean lesion lengths (cm) caused by Doth. viticola, D. mutila, D. seriata, L. mediterranea and L. mitidjana species implicated in citrus dieback in northern Algeria,**
30 days after inoculation of detached green branches with mycelium-colonized agar plugs.

| Species          | Isolate | Mean lesion length (cm) ± SD |
|------------------|---------|-----------------------------|
| *D. seriata*     | ALG91   | 5.49±2.65 a                 |
| *D. seriata*     | ALG98   | 2.35±0.55 cd                |
| *L. mediterranea*| ALG36   | 4.39±1.31 ab                |
| *L. mediterranea*| ALG40   | 3.83±0.97 abc               |
| *Doth. viticola* | ALG86   | 2.82±0.86 bcd               |
| *Doth. viticola* | ALG84   | 2.1±0.67 d                  |
| *D. mutila*      | ALG102  | 2.04±0.54 d                 |
| *D. mutila*      | ALG103  | 2.03±0.29 d                 |
| *L. mitidjana*   | ALG39   | 3.88±1.24 abc               |
| *L. mitidjana*   | ALG34   | 2.2±0.67 cd                 |

The same letter after numbers refers to the isolates that do not differ significantly according to Turkey’s HSD test at P ≤ 0.05.

The same letter after numbers refers to the isolates that do not differ significantly according to Turkey’s HSD test at P ≤ 0.05.

Distribution of *Botryosphaeriaceae* species

Overall, the *Botryosphaeriaceae* species occurred in 42 of the 80 citrus trees showing canker and dieback symptoms. Five distinct *Botryosphaeriaceae* species were obtained in this study. Each species was found with its respective frequency, as follow: *L. citricola* (14.1%), *L. mediterranea* (13%) and *D. seriata* (10.9%), *Doth. viticola* (7.6%) and *D. mutila* (5.4%).

At least, two different species were found in each orchard. *L. mediterranea* and *Doth. viticola* were found in six of the ten surveyed orchards. They were followed by *L. mitidjana*, recorded from five orchards of two municipalities. *D. seriata* was found in four sampling sites; whereas, *D. mutila* was recovered from only two orchards of the same municipality.

Discussion

This study aimed to evaluate and characterize the diversity of the *Botryosphaeriaceae* species associated with dieback of *Citrus sinensis*. It represents the first survey and preliminary investigation of these species in the main citrus orchards in northern Algeria. Citrus canker and dieback were detected in all regions surveyed. Several external symptoms, including partial or complete dieback of the tree, branch and shoot cankers were observed. Over time, the disease can increase and seriously affected trees can become barren and eventually, die. Similar situation has been described in several citrus orchards, worldwide [7, 15, 20, 21, 39]. According to some authors, abiotic factors, including drought, severe sunburn or freezing predispose the trees to xylem dysfunction, leading to these diseases [2, 3, 39].
In this study, five species belonging to three different genera of the **Botryosphaeriaceae** were recovered from symptomatic citrus trees, namely: *L. mediterranea*, *D. seriata*, *D. mutila*, *Doth. viticola* and *L. mitidjana*. The latter is introduced here, as new species. To our knowledge, except for *D. seriata* and *L. mediterranea*, this is the first report of *D. mutila*, *Doth. viticola* and *L. mitidjana*, causing branch canker disease on citrus and any crop, in Algeria. The **Botryosphaeriaceae** species were recovered from more than half of the trees sampled and were found in all the prospected orchards.

*Lasiodiplodia* was the most commonly isolated genus that was found in six of the ten surveyed orchards. This fact is consistent with previous studies, which showed that *Lasiodiplodia* species have the ability to target a wide variety of plants, distributed worldwide [40-42]. In fact, *Lasiodiplodia* species do not only occur as latent endophytes in asymptomatic plants but are also associated with different symptoms occurring on a variety of hosts including stem-end rot, fruit rot, decline, canker and dieback [41, 43-45]. In this study, *L. mediterranea* and *L. mitidjana* sp. nov. were the most frequently encountered species. *L. mediterranea* has been reported as the causal agent of canker and dieback of grapevine, holm oak as well as citrus, indicating its capability to target different hosts [28]. The latter findings lead Andolfi et al. [46] to isolate and characterize the main secondary metabolites produced by *L. mediterranea*, as well as to evaluate its phytotoxic and antifungal activities. According to the former authors [28], *L. mediterranea* has been found only on V-shaped necrotic sectors of grapevine while, it has been isolated from all the lesion types of citrus trees in this study. *L. mitidjana* sp. nov. was found in the five surveyed orchards. Isolates of this species were present predominantly in the wedge-shaped necrosis.

*Dothiorella viticola* was isolated at low frequency compared to *Lasiodiplodia* species found in this study. Interestingly, it was detected in six sampling sites. This species was first described as *Spencermartinsia viticola* by Phillips et al. [47]. It was obtained for the first time from *Vitis vinifera* in Spain. Recently, Yang et al. [38] regarded *Spencermartinsia* a synonym of *Dothiorella* and thus transferred the epithet *viticola* to *Dothiorella* as *Doth. viticola*. This taxonomic change was supported by a multi-gene phylogeny that included *Spencermartinsia* in *Dothiorella* genus [38, 48]. *Dothiorella viticola* has been reported from a wide range of woody hosts, including citrus trees [16, 21]. Recently, it has been also described as the causal agent of gummosis on citrus trees, in Tunisia [20]. According to Phillips et al. [18] and Dissanayake et al. [49], this species is known from China, Chile, USA, Spain, France, Australia, South Africa and Tunisia. Therefore, this study constitutes the first record of *Doth. viticola* in Algeria, which thus expands its known geographical range.

Two species of **Diplodia** genus, *D. seriata* and *D. mutila* were isolated from the surveyed orchards. **Diplodia** species are well known to cause damage on several economically important species and causing numerous disease symptoms including blight, dieback, rot diseases and canker [22, 28, 50-54]. In this study, *D. seriata* was frequently recovered from the sampling sites, which matches the findings of previous studies indicating the cosmopolitan nature of this species. This latter species is commonly reported as a pathogen on a large number of hosts and has been reported from hundreds of plant species [18, 49, 50]. *Diplodia mutila*, the second **Diplodia** species isolated in this study, was less frequently found in the prospected orchards. Moreover, to our knowledge, this is the first report of this species.
in our country. In addition to Algeria, the USA is the only other country in which both \(D.\) seriata and \(D.\) mutila have been associated with citrus dieback [21]. These species have been found on apples in the USA [55, 56], Chile [57], France [58], Germany [59], Uruguay [60] and South Africa [61]; as well as in pear trees [60, 61], plum [62], peach and apricot [60, 63] and walnut [51].

All the Botryosphaeriaceae species of this study caused necrosis on the citrus shoots, with differences in the lengths of the lesions. These differences were observed between the species and also among isolates of the same species. Thus, the results suggest that Diplodia seriata and \(L.\) mediterranea could be considered as being the most aggressive, since they produced the longest lesion. However, \(D.\) seriata was significantly different compared to the rest of the isolates, which is consistent with previous studies that showed significant impact of \(D.\) seriata on several hosts, across the globe [28, 64-66].

For \(L.\) mediterranea, our results are in accordance with a previous study, which highlighted its aggressiveness in artificial inoculation experiments [28]. The least aggressive species of this case were \(D.\) mutila and Doth. viticola, producing the smallest lesion size. Nevertheless, these findings contradict previous study in which \(D.\) mutila was found to be the most aggressive based on lesion length [60]. According to Linaldeddu et al. [28] and Chakusary et al. [50], these differences in aggressiveness maybe due to several factors including genetic variability of isolates, age, type of host tissue, differences in susceptibility as well as inoculation methods and experimental conditions. In this case, extensive sampling from citrus as well as other hosts are required to further emphasise the findings and draw a final solid conclusion.

Overall, almost all the Botryosphaeriaceae species we identified have previously been detected on citrus trees with the exception of \(L.\) mitidjana, which was described for the first time associated with citrus dieback. Given the major impact of the Botryosphaeriaceae species isolated on declining trees, worldwide, it is important to emphasize the urgent need to implement prevention techniques and management strategies in order to minimize the incidence of these pathogens and to prevent their spread to new orchards. For a better understanding of citrus dieback, it is necessary to set up larger surveys that include all citrus production areas. These surveys would assess, more accurately, the impact of the trunk diseases pathogens and eventually identify the factors that influence the dieback. This will be set in order to identify a number of practices to prevent their development.

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Supplementary table S1. Details of strains included in the phylogenetic and/or morphological analyses.

| Species          | Culture collection number(s)¹ | Substrate                  | Country       | Collector(s)                         | GenBank accession numbers | GenBank accession numbers |
|------------------|--------------------------------|----------------------------|---------------|--------------------------------------|---------------------------|--------------------------|
| *L. avicenniae*  | CMW 41467                      | *Avicennia*, asymptomatic branches | South Africa  | J. A. Osorio & J. Roux              | KP860835                  | KP860680                 |
| *L. avicenniae*  | LAS199                         | *Avicennia*, asymptomatic branches | South Africa  | J. A. Osorio & J. Roux              | KU587957                  | KU587947                 |
| *L. brasiliense* | CMM 4015, ex-type              | *Mangifera*, stems          | Brazil        | M. W. Marques                       | JX464063                  | JX464049                 |
| *L. brasiliense* | CMM 4469                       | *Anacardium*                | Brazil        | -                                    | KT325574                  | KT325580                 |
| *L. bruguierae*  | CMW 41470                      | *Bruguiera*, asymptomatic branches | South Africa  | J. A. Osorio & J. Roux              | KP860833                  | KP860678                 |
| *L. bruguierae*  | CMW 42480                      | *Bruguiera*, asymptomatic branches | South Africa  | J. A. Osorio & J. Roux              | KP860832                  | KP860677                 |
| *L. caatinguensis* | CMM 1325                      | *Citrus*                   | Brazil        | I. B. L. Coutinho & J. S. Lima       | KT154760                  | KT008006                 |
| *L. caatinguensis* | IBL 381                       | *Spondias*                | Brazil        | J. S. Lima & J. E. Cardoso           | KT154757                  | KT154751                 |
| *L. chinensis*   | CGMCC 3.18061                  | Unknown, branch            | China         | W. He & Z. P. Dou                   | KX499889                  | KX499927                 |
| *L. chinensis*   | CGMCC 3.18044                  | *Vaccinium*, branch         | China         | J. H. Zhao                          | KX499875                  | KX499913                 |
| *L. cinnamomi*   | CFCC 51997                     | *Cinnamomum*, branch       | China         | N. Jiang                            | MG866028                  | MH236799                 |
| *L. cinnamomi*   | CFCC 51998                     | *Cinnamomum*, branch       | China         | N. Jiang                            | MG866029                  | MH236800                 |
| L. citricola     | CBS 124706 | Citrus sp., twigs | Iran | A. Shekari | GU945353 | GU945339 |
|-----------------|------------|-------------------|------|------------|----------|----------|
| L. citricola    | CBS 124707, ex-type | Citrus, twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945354 | GU945340 |
| L. crassipora   | CMW 13488 | Eucalyptus, wood | Venezuela | S. Mohali | DQ103552 | DQ103559 |
| L. crassipora   | CBS 118741, ex-type | Santalum | Australia | T. I. Burgess & B. Dell | DQ103550 | EU673303 |
| L. euphorbicola | CMW 33350 | Adansonia | Botswana | - | KU887149 | KU887026 |
| L. euphorbicola | CMW 36231 | Adansonia | Zimbabwe | - | KU887187 | KU887063 |
| L. exigua       | BL 184 | Retama, branch canker | Tunisia | B. T. Linaldeddu | KJ638318 | KJ638337 |
| L. exigua       | CBS 137785, ex-type | Retama, branch canker | Tunisia | B. T. Linaldeddu | KJ638317 | KJ638336 |
| L. gilanensis   | CBS 124704, ex-type | Citrus, fallen twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945351 | GU945342 |
| L. gilanensis   | CBS 124705 | Citrus sp., fallen twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945352 | GU945341 |
| L. gonubiensis  | CMW 14077, ex-type | Syzygium | South Africa | D. Pavlic | AY639595 | DQ103566 |
| L. gonubiensis  | CMW 14078, ex-paratype | Syzygium | South Africa | D. Pavlic | AY639594 | DQ103567 |
| L. gravistriata | CMM 4564 | Anacardium, stems | Brazil | M. S. B. Netto | KT250949 | KT250950 |
| L. gravistriata | CMM 4565 | Anacardium, stems | Brazil | M. S. B. Netto | KT250947 | KT266812 |
| L. hormozganensis | CBS 124708 | Mangifera, twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945356 | GU945344 |
| **L. hormozganensis** | CBS 124709, ex-type | *Olea*, twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945355 | GU945343 |
|-----------------------|---------------------|---------------|------|---------------------|---------|---------|
| **L. hyalina** | CGMCC 3.17975 | *Acacia*, cankered stems | China | Y. Zhang & Y. P. Zhou | KX499879 | KX499917 |
| **L. hyalina** | CGMCC 3.18383 | unidentified woody plant, cankered branches | China | Z. P. Dou & Z. C. Liu | KY767661 | KY751302 |
| **L. iraniensis** | IRAN 1520C, ex-type | *Salvadora*, twigs | Iran | J. Abdollahzadeh & A. Javadi | GU945346 | GU945334 |
| **L. iraniensis** | IRAN 1502C | *Juglans*, twigs | Iran | A. Javadi | GU945347 | GU945335 |
| **L. laeliocattleyae** | CBS 167.28 | *Laeliocattleya*, leaves | Italy | C. Sibilia | KU507487 | KU507454 |
| **L. laeliocattleyae** | CBS 130992 | *Mangifera*, leaves | Egypt | A. M. Ismail | JN814397 | JN814424 |
| **L. lignicola** | CBS 134112 | Wood | Thailand | - | JX646797 | KU887003 |
| **L. macrospora** | CMM 3833, ex-type | *Jatropha*, collar and root rot | Brazil | A. R. Machado & O. L. Pereira | KF234557 | KF226718 |
| **L. mahajangana** | CMW 27801, ex-type | *Terminalia*, healthy branches | Madagascar | J. Roux | FJ900595 | FJ900641 |
| **L. mahajangana** | CMW 27820 | *Terminalia*, healthy branches | Madagascar | J. Roux | FJ900597 | FJ900643 |
| **L. margaritacea** | CBS 122519, ex-type | *Adansonia*, dying twigs | Australia | T. I. Burgess & M. J. Wingfield | EU144050 | EU144065 |
| **L. margaritacea** | CBS 122065 | *Adansonia gibbosa*, dying twigs | Australia | T. I. Burgess | EU144051 | EU144066 |
| **L. mediterranea** | CBS 137783, ex-type | *Quercus*, branch canker | Italy | B. T. Linaldedita | KJ638312 | KJ638331 |
| **L. mediterranea** | CBS 137784 | *Vitis*, brown stripe under the bark | Italy | S. Serra | KJ170150 | KJ170151 |
| **L. missouriana** | CBS 128311, ex-type | Wedge-shape canker of grapevine cv. Catawba (complex hybrid of North America *Vitis* species) | USA | K. Striegler & G. M. Leavitt | HQ288225 | HQ288267 |
|---------------------|---------------------|---------------------------------------------------------------------------------|------|-----------------------------|-------------|-------------|
| **L. missouriana** | CBS 128312          | Wedge-shape canker of grapevine cv. Catawba (complex hybrid of North America *Vitis* species) | USA | K. Striegler & G. M. Leavitt | HQ288226 | HQ288268 |
| **L. parva**       | CBS 456.78, ex-type | Cassava-field soil                                                              | Colombia | O. Rangel           | EF622083 | EF622063 |
| **L. parva**       | CBS 494.78          | Cassava-field soil                                                              | Colombia | O. Rangel           | EF622084 | EF622064 |
| **L. plurivora**   | STE-U 5803, ex-type | *Prunus*, wood canker                                                          | South Africa | U. Damm              | EF445362 | EF445395 |
| **L. plurivora**   | STE-U 4583          | *Vitis*, symptomatic                                                            | South Africa | F. Halleen           | AY343482 | EF445396 |
| **L. pontae**      | CMM 1277            | *Spondias*, necrotic canker                                                    | Brazil | J.S. Lima & F.C.O. Freire | KT151794 | KT151791 |
| **L. pseudotheobromae** | CBS 116459, ex-type | *Gmelina*, twigs                                                                | Costa Rica | J. Carranza- Velazquez | EF622077 | EF622057 |
| **L. pseudotheobromae** | CGMCC 3.18047       | *Pteridium*, twigs                                                              | China | -                  | KX499876 | KX499914 |
| **L. pyriformis**  | CBS 121770, ex-type | *Pinus*, fruiting structures                                                   | Namibia | F. J. J. van der Walt & J. Roux | EU101307 | EU101352 |
| **L. pyriformis**  | CBS 121771          | *Pinus*, fruiting structures                                                   | Namibia | F. J. J. van der Walt & J. Roux | EU101308 | EU101353 |
| **L. rubropurpurea** | WAC 12535, ex-type | *Eucalyptus*, canker                                                            | Australia | T. I. Burgess         | DQ103553 | EU673304 |
| **L. rubropurpurea** | WAC 12536 | *Eucalyptus*, canker | Australia | T. I. Burgess | DQ103554 | DQ103572 |
|---------------------|------------|---------------------|-----------|--------------|----------|----------|
| **L. sterculiae**   | CBS 342.78, ex-type | *Sterculia* | Germany | S. Bruhn | KX464140 | KX464634 |
| **L. subglobosa**   | CMM 3872, ex-type | *Jatropha*, collar and root rot | Brazil | A. R. Machado & O. L. Pereira | KF234558 | KF226721 |
| **L. subglobosa**   | CMM 4046 | *Jatropha* | Brazil | A. R. Machado & O. L. Pereira | KF234560 | KF226723 |
| **L. thailandica** | CGMCC 3.18382 | *Podocarpus*, cankered branch | China | D. Zhipeng & L. Zuchen | KY767662 | KY751303 |
| **L. thailandica** | CGMCC 3.18384 | *Albizia*, cankered branch | China | Z.P. Dou & Z.C. Liu | KY767663 | KY751304 |
| **L. theobromae**   | CBS 164.96, ex-neotype | Fruit along coral reef coast | Papua New Guinea | A. Aptroot | AY640255 | AY640258 |
| **L. theobromae**   | CBS 111530 | *Leucospermum* | USA | J. E. Taylor | EF622074 | EF622054 |
| **L. venezuelensis** | WAC 12539, ex-type | *Acacia*, wood | Venezuela | S. Mohali | DQ103547 | EU673305 |
| **L. venezuelensis** | WAC 12540 | *Acacia*, wood | Venezuela | S. Mohali | DQ103548 | DQ103569 |
| **L. viticola**     | CBS 128313, ex-type | Wedge-shape canker of grapevine cv. Vignoles (complex hybrid of North America *Vitis* species) | USA | R. D. Cartwright & W. D. Gubler | HQ288227 | HQ288269 |
| **L. viticola**     | UCD 2604MO | *Vitis* | USA | J. R. Urbez-Torres | HQ288228 | HQ288270 |
| **L. vitis**        | CBS 124060, ex-type | *Vitis*, wood fragment | Italy, Sicily | S. Burruano | KX464148 | KX464642 |
| **D seriata**       | CBS 112555 | *Vitis*, dead stems | Portugal | A. J. L. Phillips | AY259094 | AY573220 |
| **D mutila** | CBS 112553 | **Vitis** | Portugal | A. Alves | AY259093 | AY573219 |
|-------------|------------|-----------|----------|----------|----------|----------|

1 BL: Personal number of B.T. Linaldeddu; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Beijing, China; CMM: Culture Collection of Phytopathogenic Fungi “Prof. Maria Menezes”, Universidade Federal Rural de Pernambuco, Recife, Brazil; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; WAC: Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia.
Supplementary figure S1.

Maximum Parsimony phylogenetic tree resulting from the analysis of the combined ITS and tef1-α sequence data from *Lasiodiplodia* species. The tree was rooted to *Diplodia mutila* and *Diplodia seriata*. 
**Lasiodiplodia mitidjana** sp. nov. and other *Botryosphaeriaceae* species causing branch canker and dieback of *Citrus sinensis* in Algeria

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**Abstract**

Several *Botryosphaeriaceae* species are known to occur worldwide, causing dieback, canker and fruit rot on various hosts. Surveys conducted in ten commercial citrus orchards in the northern region of Algeria revealed five species of *Botryosphaeriaceae* belonging to three genera associated with diseased trees. Morphological and cultural characteristics as well as phylogenetic analyses of the internal transcribed spacer (ITS) region and the translation elongation factor 1-alpha (tef1-α) identified *Diplodia mutila*, *Diplodia seriata*, *Dothiorella viticola*, *Lasiodiplodia mediterranea* and a novel species which is here described as *Lasiodiplodia mitidjana* sp. nov.. Of these, *L. mitidjana* (14.1% of the samples) and *L. mediterranea* (13% of the samples) were the most widespread and abundant species.

Pathogenicity tests revealed that *L. mediterranea* and *D. seriata* were the most aggressive species on citrus shoots. This study highlights the importance of *Botryosphaeriaceae* species as agents of canker and dieback of citrus trees in Algeria.

**Key words:** Citrus cultivation, trunk diseases, fungi, identification, taxonomy, pathogenicity.
Introduction

Citrus cultivation is one of the major contributors to Algerian wealth and is part of the traditional agriculture of the country. Many types of citrus are grown in Algeria, including oranges (48 400 ha), clementine (10 817 ha), mandarins (2 347 ha), lemons (4 409 ha) and grapefruits (83 ha) [1]. Despite the high adaptation capacity of citrus trees to different climates [2], a number of unfavourable factors has led to a decrease of the total citrus yield in Algeria. Among these factors, ageing trees, droughts, inappropriate cultural practices and the effects of various pests and pathogens are the most important [2, 3]. Citrus diseases are numerous and diverse, and are caused by phytopathogenic agents belonging to viruses, viroids, phytoplasmas, bacteria, and fungi [2]. Some pathogens cause very serious diseases, predisposing to, and inciting dieback, while others are less serious [2-5].

Recently, trunk diseases have become a growing threat in both, old and newly established orchards of citrus, worldwide. Symptoms include leaves that become yellow and fall early, shoots and twigs die, increasing the risk of citrus decay as the damage expands to the trunk [2, 6-8]. To date, among the fungi that impact citrus, Diaporthe species are well known for causing stem-end rot and melanose of fruits, young leaf and shoot gummosis and blight of perennial branches and trunks, in Greece, Italy, Malta, Portugal, Spain, China, Korea, New Zealand, and the USA [8-11]. Fusarium and Neocosmospora have also been reported causing canker and dieback diseases of citrus, in Tunisia, Greece, Italy and Spain [12-14]. The Diatrypaceae are other canker and dieback pathogens impacting citrus orchards [15]. Several Eutypella spp. have been reported from Citrus sp. In southern California desert, three distinct species of Eutypella are found associated with citrus branch canker, namely: Eutypella citricola, E. microtheca and a Eutypella sp. [15-17].

In addition to the above fungal pathogens that compromise citrus crops, several Botryosphaeriaceae species are known to colonize citrus trees. The Botryosphaeriaceae family is recognized as an important and widely distributed plant pathogen, which impacts on a variety of economically important hosts. It comprises 24 genera encompassing 222 species, living as endophytes, saprobes, or plant pathogens [18, 19]. Recent studies carried out in California, Italy and Tunisia have highlighted the Botryosphaeriaceae as the most prevalent fungi that cause cankers, vascular necrosis and dieback of citrus trees [7, 15, 16, 20]. Adesemoye et al. [21] recovered various Botryosphaeriaceae species from necrotic tissues of citrus branch canker and rootstock, including, Diplodia seriata, D. mutila, Dothiorella viticola, D. iberica, Lasiodiplodia parva, Neofusicoccum australe, N. luteum, N. mediterraneum, N. parvum and Neoscytalidium dimidiatum. In Iran, Abdollahzadeh et al. [22], described Lasiodiplodia citricola from citrus trees showing symptoms of branch dieback.

In Algeria, members of the Botryosphaeriaceae have been reported to cause diseases on Vitis vinifera [23-25], Quercus suber [26] and Cupressus macrocarpa [27]. Linaldeddu et al. [28] isolated and described Lasiodiplodia mediterranea from a cankered branch of Citrus sinensis trees in northern Algeria. However, the impact of Botryosphaeriaceae species on citrus trees has not been studied in detail. Therefore, the aim of this study was to investigate
and determine the incidence of the *Botryosphaeriaceae* species associated with branch canker and dieback in the major citrus-growing region of Algeria.

**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies. This study did not involve endangered or protected species.

**Field survey and sampling**

Surveys were conducted in ten commercial orchards in the northern region of Algeria. Specifically, in the Mitidja plain at the base of the Tell Atlas Mountains. The sampling was done in four municipalities; namely Oued El Aleug (4 orchards), Chiffa (2 orchards), Boufarik (2 orchards) and the coastal town, Sidi Fredj, located within the territory of the Staoueli municipality, situated by the Mediterranean Sea (2 orchards). The field diagnosis and sampling were performed between April 2013 and March 2015. Samples were collected from the orchards with permission of landowners. Trunks and branches showing symptoms such as dead shoots, defoliation, cankers, wood necrosis, and dieback were collected, randomly. A total of 80 symptomatic sweet orange (*Citrus sinensis*) trees were sampled (Table 1).

**Table 1. Citrus orchards surveyed and number of samples collected.**

| Locality     | GPS coordinates | Orchards | Area (ha) | Number of trees sampled | Number of samples processed |
|--------------|-----------------|----------|-----------|-------------------------|----------------------------|
| Oued El Alleug | 36°33'21"N 2°47'22"E | a       | 18        | 5                       | 9                          |
|              |                 | b       | 16        | 5                       | 6                          |
|              |                 | c       | 28        | 5                       | 7                          |
|              |                 | d       | 6.8       | 5                       | 6                          |
| Chiffa       | 36°27'44"N 2°44'27"E | a       | 25        | 10                      | 13                         |
|              |                 | b       | 18        | 10                      | 10                         |
| Boufarik     | 36°34'31"N 2°54'46"E | a       | 43        | 10                      | 10                         |
|              |                 | b       | 27        | 10                      | 11                         |
| Staoueli     | 36°45'12"N 2°53'17"E | a       | 32        | 10                      | 10                         |
|              |                 | b       | 15        | 10                      | 10                         |
Fungal isolation and morphological characterization

In the laboratory, all samples were processed by peeling the outer bark surface with a sterilized scalpel. Longitudinal and transversal cuts were made to reveal the type and localization of the internal necrosis. From each lesion detected, ten pieces of wood, approx. 5 mm², were cut from the margins between necrotic and healthy tissues. These pieces were submerged in 4 % sodium hypochlorite for 15 min, washed thrice with sterile distilled water, dried with sterilized filter paper and placed onto the surface of potato dextrose agar (PDA, Difco Laboratories). Plates were incubated at 25 °C until growth was detected. The mycelium emerging from wood pieces were transferred onto fresh PDA plates and incubated under the same conditions.

Preliminary identifications to genus and tentative species level were based on colony and conidial morphology (colony colour, colony growth pattern, conidial size, shape, colour, striation, septation, conidiogenous cells, and presence of paraphyses) according to Phillips et al. [18]. Isolates that lacked pycnidia production on PDA were placed on autoclaved pine needles in ¼ strength PDA within 2–3 weeks, incubated at 25 °C under mixed near-UV and cool-white fluorescent light in a 12 h light 12 h dark regime for 2–6 weeks, to enhance fruiting body production. Conidiogenous layer and conidia were mounted in 100 % lactic acid and observed with a Nikon 80i light microscope.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 7 days old axenic cultures, grown on PDA at 25 °C, following Santos and Phillips [29]. PCR reactions were carried out with Taq DNA polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania). PCR reaction mixtures were prepared as previously described by Alves et al. [30], with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. The ITS region plus D1/D2 domain of the LSU was amplified with the primer pair ITS1 [31] and NL4 [32]. The amplification conditions were initial denaturation of 5 min at 95 °C, followed by 29 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1.5 min at 72 °C, and a final extension of 10 min at 72 °C. Part of the translation elongation factor 1 alpha gene (tef1-α) was amplified with primers EF1-688F and EF1-1251R [33]. The amplification conditions were: initial denaturation of 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, 1½ min at 72 °C, and a final extension period of 10 min at 72 °C. ITS and tef1-α regions were sequenced in both directions by STAB Vida Lda (Portugal), using the Sanger method.

The nucleotide sequences were read and edited with BioEdit Alignment Editor V.7.0.9.0 [34]. Newly generated sequences were deposited in GenBank (Table 2). Homological sequences of the newly sequenced ones were retrieved from the GenBank using the Basic Local Alignment Search Tool (BLAST) [35].
Table 2. *Botryosphaeriaceae* species included in this study

| Species               | Isolate number | Host/Substrate | Origin                  | Collector                | GenBank accession numbers |
|-----------------------|----------------|----------------|-------------------------|--------------------------|---------------------------|
| *Lasiodiplodia*       | ALG77          | Citrus/wood canker | Algeria, Boufarik       | Akila Berraf-Tebbal      | MN104094, MN159093         |
| *mediterranea*        | ALG76          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104095, MN159094         |
| *mediterranea*        | ALG40          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104096, MN159095         |
| *mediterranea*        | ALG78          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104097, MN159096         |
| *mediterranea*        | ALG41          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104098, MN159097         |
| *mediterranea*        | ALG36          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104099, MN159098         |
| *mediterranea*        | ALG80          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104100, MN159099         |
| *mediterranea*        | ALG73          | Citrus/wood canker | Algeria, Boufarik       | Akila Berraf-Tebbal      | MN104101, MN159100         |
| *mediterranea*        | ALG74          | Citrus/wood canker | Algeria, Boufarik       | Akila Berraf-Tebbal      | MN104102, MN159101         |
| *mediterranea*        | ALG75          | Citrus/wood canker | Algeria, Boufarik       | Akila Berraf-Tebbal      | MN104103, MN159102         |
| *mediterranea*        | CBS 124060     | *Vitis*, wood fragment | Italy, Sicily          | S. Burruano              | KX464148, MN938928         |
| *mitidjana*           | ALG81          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104104, MN159103         |
| *mitidjana*           | ALG44          | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal      | MN104105, MN159104         |
| L. mitidjana  | ALG39 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104106 | MN159105 |
| L. mitidjana  | ALG42 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104107 | MN159106 |
| L. mitidjana  | ALG38 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104108 | MN159107 |
| L. mitidjana  | ALG43 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104109 | MN159108 |
| L. mitidjana  | ALG37 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104110 | MN159109 |
| L. mitidjana  | ALG34 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104111 | MN159110 |
| L. mitidjana  | ALG82 | Citrus/wood canker | Algeria, Oued El Alleug | Akila Berraf-Tebbal | MN104112 | MN159111 |
| L. mitidjana  | ALG72 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104113 | MN159112 |
| L. mitidjana  | ALG71 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104114 | MN159113 |
| L. mitidjana  | ALG70 = MUM 19.90 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104115 | MN159114 |
| L. mitidjana  | ALG69 | Citrus/wood canker | Algeria, Boufarik | Akila Berraf-Tebbal | MN104116 | MN159115 |
| Diplodia seriata | ALG93 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104117 | MN159116 |
| D. seriata    | ALG94 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104118 | MN159117 |
| D. seriata    | ALG98 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104119 | MN159118 |
| D. seriata    | ALG91 | Citrus/wood canker | Algeria, Chiffa | Akila Berraf-Tebbal | MN104120 | MN159119 |
| D. seriata    | ALG92 | Citrus/wood canker | Algeria, Staoueli | Akila Berraf-Tebbal | MN104121 | MN159120 |
| Species              | Code  | Location                  | Authors                  | Accession Numbers |
|---------------------|-------|---------------------------|--------------------------|-------------------|
| D. seriata          | ALG90 | Citrus/wood canker        | Algeria, Chiffa          | MN104122 MN159121 |
| D. seriata          | ALG89 | Citrus/wood canker        | Algeria, Chiffa          | MN104123 MN159122 |
| D. seriata          | ALG96 | Citrus/wood canker        | Algeria, Staoueli        | MN104124 MN159123 |
| D. seriata          | ALG95 | Citrus/wood canker        | Algeria, Staoueli        | MN104125 MN159124 |
| D. seriata          | ALG97 | Citrus/wood canker        | Algeria, Staoueli        | MN104126 MN159125 |
| D. mutila           | ALG99 | Citrus/wood canker        | Algeria, Chiffa          | MN104127 MN159126 |
| D. mutila           | ALG103| Citrus/wood canker        | Algeria, Chiffa          | MN104128 MN159127 |
| D. mutila           | ALG100| Citrus/wood canker        | Algeria, Chiffa          | MN104129 MN159128 |
| D. mutila           | ALG102| Citrus/wood canker        | Algeria, Chiffa          | MN104130 MN159129 |
| D. mutila           | ALG101| Citrus/wood canker        | Algeria, Chiffa          | MN104131 MN159130 |
| Dothiorella viticola| ALG83 | Citrus/wood canker        | Algeria, Staoueli        | MN104087 MN159086 |
| Doth. viticola      | ALG35 | Citrus/wood canker        | Algeria, Oued El Alleug  | MN104088 MN159187 |
| Doth. viticola      | ALG84 | Citrus/wood canker        | Algeria, Chiffa          | MN104089 MN159188 |
| Doth. viticola      | ALG85 | Citrus/wood canker        | Algeria, Staoueli        | MN104090 MN159189 |
| Doth. viticola      | ALG86 | Citrus/wood canker        | Algeria, Oued El Alleug  | MN104091 MN159190 |
| Doth. viticola      | ALG87 | Citrus/wood canker        | Algeria, Chiffa          | MN104092 MN159191 |
| Species         | Accession | Description         | Location       | Researcher     | Accession1 | Accession2 |
|-----------------|-----------|---------------------|----------------|----------------|------------|------------|
| *Doth. viticola* | ALG88     | Citrus/wood canker  | Algeria, Chiffa| Akila Berraf-Tebbal | MN104093   | MN159192   |
**Phylogenetic analysis**

Sequences of all *Lasiodiplodia* species known from culture were retrieved from GenBank (S1 Table) and aligned with sequences of the isolates obtained in this study. Alignments were done with ClustalX v. 1.83 [36] using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Alignments were checked and manual adjustments made if necessary using BioEdit v. 7.2.5 [34]. Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were performed using MEGAX [37]. The best fitting DNA evolution model was determined also by MEGAX. ML analysis was performed on a Neighbour-Joining starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference. MP analysis was done using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The robustness of the trees (ML and MP) was evaluated by 1000 bootstrap replications.

**Pathogenicity test**

The ability of isolates to cause cankers was assessed *in vivo* on detached shoots collected from symptomless citrus trees. From each phylogenetically resolved species, two representative isolates were selected. Pathogenicity of each selected strain was tested on 1-year-old shoots of *Citrus sinensis*. The shoots with 25 mm in diameter were cut into equal length (25 cm long). They were then surface disinfected with 70% ethanol and wounded on an intermediate internode, with a scalpel. From each strain, a 5 mm diameter mycelial plug taken from a 5-day old colony growing on PDA was placed into the wound. Negative controls were inoculated with fresh, non-colonized, PDA plugs. Subsequently, the cuttings were wrapped with wet sterile cotton and sealed with Parafilm® to prevent the desiccation of the agar plug. The shoots were immediately transplanted into pots containing sterilized water as a growth substrate. They were incubated at the ambient room temperature, under daily photoperiod. The water of the container was changed twice a week. There were 10 replicates per isolate, and the same number of cuttings was used as controls. One month after inoculation, lengths of lesions produced by each strain were measured. In an attempt to recover the inoculated fungi and complete Koch’s postulates, necrotic tissue from the margin of the lesions was taken and placed onto PDA.

**Statistical analyses**

Data of lesion lengths caused by the fungal isolates belonging to the different species was subjected to one-way ANOVA (analysis of variance) with $P \leq 0.05$. Prior to analysis data were checked for normality, then, significance of differences between means was determined by
Turkey's honestly significant difference (HSD) test. Statistical analyses were performed on
the software R v. 3.5.1 and a significance level of 0.05 was used.

**Nomenclature**

The electronic version of this article in Portable Document Format (PDF) in a work with an
ISSN or ISBN will represent a published work according to the International Code of
Nomenclature for algae, fungi, and plants, and hence the new names contained in the
electronic publication of a PLOS article are effectively published under that Code from the
electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from
where they will be made available to the Global Names Index. The unique MycoBank
number can be resolved and the associated information viewed through any standard web
browser by appending the MycoBank number contained in this publication to the prefix
http://www.mycobank.org/MB/. The online version of this work is archived and available
from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES
WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS
etc)].

**Results**

**Disease symptoms**

Citrus dieback was detected in all the orchards and regions investigated, with different
degrees of intensity. Various external symptoms, including partial or complete dieback of the
tree, branch and shoot cankers, abnormal growth of epicormic shoots; defoliation and leaf
chlorosis were observed. Moreover, in certain orchards, bark cracking of the trunk and the
branches was also noticeable (Fig 1).

The analysis of the 80 symptomatic sweet orange (*Citrus sinensis*) trees sampled to carry
out the isolations, revealed the existence of 92 necrotic lesions in the trunks and the branches.
They belonged to four types of wood alteration, including: wedge-shaped necrosis (WSN),
that was the most prevalent lesion (n=30) of the total samples collected. The brown central
necrosis (BCN) (n=26) was the second most prevalent lesion, followed by the black spots in
the xylem (BS) (n=24) and yellow soft wood rot (YSW) (n=12).

**Fig 1.** Citrus tree with dieback symptoms (a), bark cracking of the trunk and gummosis
(b), main internal symptoms of sectioned branches and trunks (c–f).

**Fungal isolation and identification**
Isolation carried out from ninety-two samples yielded a total of forty-seven fungal colonies belonging to Botryosphaeriaceae. On the basis of morphological characteristics, it was possible to distinguish three morphological groups according to colour and shape of conidia. Twenty-five isolates with brown sub-globose and striate conidia were grouped as Lasiodiplodia-like fungi. Fifteen isolates with brown oblong to ovoid conidia were considered as Dothiorella-like fungi. A further seven isolates with brown, ovoid thick walled and 1-septate conidia were considered as Dothiorella-like fungi. The identification of the isolates was confirmed by analysis of ITS and tef1-α sequences, which distinguished five separate species. The BLAST searches in GenBank showed 99–100% identity with reference sequences of representative isolates including that of the ex-type. The identified species were: Diplodia seriata (10 isolates), Diplodia mutila (5 isolates), Dothiorella viticola (7 isolates), Lasiodiplodia mediterranea (10 isolates) and a Lasiodiplodia sp. (14 isolates) that could not be assigned to any of the currently known species.

Phylogenetic analysis

Phylogenetic analysis was performed using ITS and tef1-α sequences. Fragments of approximately 500 and 300 bases were determined for ITS and tef1-α regions, respectively. The ML and the MP trees are presented in figure 2 and figure S1, respectively. The combined ITS and tef1-α dataset of Lasiodiplodia consisted of 23 isolates aligned with sequences of 69 isolates retrieved from GenBank, representing a selection of all known Lasiodiplodia and 2 outgroup taxa (Diplodia seriata CBS 112555 and Diplodia mutila CBS 112553). In the ML phylogenetic tree (figure 2), the isolates obtained in this study grouped in two clades. The first clade comprised 10 isolates, which clustered together, with the ex-type strain of Lasiodiplodia mediterranea (CBS 137783) and the ex-type strain of Lasiodiplodia vitis (CBS 124060) (Table S1), forming a single monophyletic group. The second group contained 14 isolates, which formed a distinct clade, with a high bootstrap support (ML/MP = 80/94), was considered to represent a distinct species, which is described here as Lasiodiplodia mitidjana sp. nov. (Fig 2).

Fig 2. Maximum likelihood tree generated from the combined analysis of ITS and tef1-α sequence data. ML/MP bootstrap values are given at the nodes. Support values less than 50% are omitted or indicated with ‘–’. The tree was rooted to Diplodia mutila and Diplodia seriata.

Taxonomy

Lasiodiplodia mitidjana A. Alves, A.E. Mahamedi & A. Berraf-Tebbal sp. nov. (Fig 3) [urn:lsid:mycobank.org:names: MB 832823] Algeria, Mitidja, isolated from a branch canker of Citrus sinensis, June 2015, Akila Berraf-Tebbal, HOLOTYPE AVE-F-7, a dried culture sporulating on pine needles twigs deposited in the Herbarium Universitatis Aveirensis
(AVE), culture ex-holotype MUM 19.90 (=ALG70). Other isolates examined are listed in Table 2.

**Etymology**: named after Mitidja where the fungus was discovered.

**Sexual state**: Not seen. Asexual state: Conidiomata stromatic, pycnidial, produced on pine needles on ¼ strength PDA within 2–3 wks, dark brown to black, covered with dense mycelium, superficial or immersed in the host becoming erumpent when mature, mostly uniloculate, solitary, globose, thick-walled. Paraphyses hyaline, cylindrical, thin-walled, initially aseptate, becoming septate when mature, rounded at apex. Conidiogenous cells holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, sometimes slightly swollen at the base. Conidia subovoid to ellipsoid-ovoid, apex rounded, occasionally tapering to truncate base, widest in middle to upper third, thick-walled, with granular content, initially hyaline and aseptate, remaining so for a long time, becoming dark brown and 1-septate, with longitudinal striations, \((22.6–27.7(−31.9) \times (13.5–16.7(−19.6)) \mu m, \text{95 \% confidence limits} = 27.3–28 \times 16.5–16.9 \mu m)\) (av. of 125 conidia ± SD = 27.7 ± 1.9 × 16.7 ± 1.1 μm, L/W ratio = 1.7).

**Cultural characteristics**: Colonies on PDA with moderate to dense aerial mycelium, initially white to smoke-grey, turning greenish grey on the surface and reverse, becoming dark slate blue with age.

**Cardinal temperatures for growth**: Minimum <10 °C, maximum < 40 °C and optimum 25 – 35 °C, covering the medium surface (90 mm) before 7 days at 25 °C in the dark.

**Habitat**: Twigs and branches of *Citrus sinensis*.

**Known geographic distribution**: Algeria.

**Notes**: Phylogenetically it is very closely related to *L. citricola* being distinguished by three bp in the *tef1*-α locus. Conidia tend to be larger than those of *L. citricola*, 95 % confidence limits = 24.1–24.9 × 15–15.7 μm (av. ± S.D. = 24.5 ± 0.2 × 15.4 ± 1.8 μm) and have a lower L/W ratio = 1.6.

*Lasiodiplodia mediterranea* Linaldeddu, Deidda & Berraf-Tebbal **sp. nov.** (Linaldeddu et al. 2015. Fungal diversity 71:207)

**MycoBank**: MB 808356

**Synonym**: *Lasiodiplodia vitis* Yang & Crous, **sp. nov.** (Yang et al. 2017. Fungal Biology 121)

**MycoBank**: MB817635

**Notes**: Yang et al. [38] described *L. vitis* as a novel species, clearly distinct from the species recognised on *Vitis vinifera* in Italy. However, in their study Yang et al. [38] did not include any representative of *L. mediterranea* which was described by Linaldeddu et al. [28] from
several hosts, including *V. vinifera* in Italy. However, we have shown that *L. vitis* is phylogenetically indistinguishable from *L. mediterranea*. Their ITS sequences are 100% identical and the *L. vitis* tef1-α sequence deposited in GenBank differs from the tef1-α of *L. mediterranea* in 2 nt positions (1 missing G and a C instead of a T in the EF-986R primer binding region). We re-sequenced the tef1-α region of *L. vitis* CBS124060 (Table 2) using primers EF1-688F and EF1-1251R [33] which span a larger region than primers EF1-728F and EF-986R used by Yang et al. [38] and verified that these 2 nt are not real. There is no missing G in the *L. vitis* sequence and the C instead of a T is an artefact in *L. mediterranea* sequence introduced by the EF-986R primer sequence. Thus, the tef1-α region of *L. vitis* CBS124060 is 100% identical to the tef1-α sequence of *L. mediterranea*.

**Fig 3. Lasiodiplodia mitidjana.** (a,b). Pycnidia formed on pine needles. (c). Conidiogenous layer with conidia developing on conidiogenous cells. (d). Conidia developing on conidiogenous cells and paraphyses. (e,f,i). Hyaline aseptate conidia. (g,h). Hyaline aseptate brown 1-septate conidia in two focal planes showing the striations on the inner surface of the wall. (j). Aseptate conidia, one becoming brown. (k,l). Brown 1-septate conidia in two focal planes to show the striations in the inner surface of the wall. Scale bars: e = 20 μm, c,d,f–j = 10 μm; k–l = 5 μm.

**Pathogenicity test**

All the Botryosphaeriaceae isolates tested in the pathogenicity test were pathogenic to the citrus shoots. On the wood tissue under the bark, black to brown lesions developed, upward and downward from the inoculation point, within 30 days. The control plants did not develop any symptoms. Lesion lengths varied between the species and among the isolates of each species tested, with a significant difference (F=10.874; P < 0.001) (Table 3).

The most aggressive isolates were ALG91 (*D. seriata*) and ALG36 (*L. mediterranea*), which produced the longest lesions (5.49±2.65 cm and 4.39±1.31 cm, respectively) with a statistically significant difference recorded between ALG91 and the rest of the species, except for *L. mediterranea*. No significant difference in lesions size was observed between the isolates ALG40 (*L. mediterranea*) and ALG39 (*L. mitidjana*), which presented intermediate lesion lengths (3.83±0.97 and 3.88±1.24 cm, respectively). However, the smallest lesion size was produced by *Doth. viticola* ALG84 with 2.1±0.67 cm and both *D. mutila* isolates ALG102 (2.04±0.54) and ALG103 (2.05±0.4 cm). *D. seriata* was the only species that showed significant difference in lesion length between its two isolates (Table 3).

Koch’s postulates were confirmed by a successful re-isolation of all tested fungal species from the necrotic tissues (Table 3).

**Table 3.** Mean lesion lengths (cm) caused by *Doth. viticola, D. mutila, D. seriata, L. mediterranea* and *L. mitidjana* species implicated in citrus dieback in northern Algeria.
30 days after inoculation of detached green branches with mycelium-colonized agar plugs.

| Species       | Isolate | Mean lesion length (cm) ± SD |
|---------------|---------|-------------------------------|
| D. seriata    | ALG91   | 5.49±2.65 a                   |
| D. seriata    | ALG98   | 2.35±0.55 cd                  |
| L. mediterranea | ALG36  | 4.39±1.31 ab                  |
| L. mediterranea | ALG40  | 3.83±0.97 abc                 |
| Doth. viticola | ALG86   | 2.82±0.86 bcd                |
| Doth. viticola | ALG84   | 2.1±0.67 d                    |
| D. mutila     | ALG102  | 2.04±0.54 d                   |
| D. mutila     | ALG103  | 2.03±0.29 d                   |
| L. mitidjana  | ALG39   | 3.88±1.24 abc                 |
| L. mitidjana  | ALG34   | 2.2±0.67 cd                   |

The same letter after numbers refers to the isolates that do not differ significantly according to Turkey’s HSD test at $P \leq 0.05$.

**Distribution of Botryosphaeriaceae species**

Overall, the *Botryosphaeriaceae* species occurred in 42 of the 80 citrus trees showing canker and dieback symptoms. Five distinct *Botryosphaeriaceae* species were obtained in this study. Each species was found with its respective frequency, as follow: *L. citricola* (14.1%), *L. mediterranea* (13%) and *D. seriata* (10.9%), *Doth. viticola* (7.6%) and *D. mutila* (5.4%).

At least, two different species were found in each orchard. *L. mediterranea* and *Doth. viticola* were found in six of the ten surveyed orchards. They were followed by *L. mitidjana*, recorded from five orchards of two municipalities. *D. seriata* was found in four sampling sites; whereas, *D. mutila* was recovered from only two orchards of the same municipality.

**Discussion**

This study aimed to evaluate and characterize the diversity of the *Botryosphaeriaceae* species associated with dieback of *Citrus sinensis*. It represents the first survey and preliminary investigation of these species in the main citrus orchards in northern Algeria.

Citrus canker and dieback were detected in all regions surveyed. Several external symptoms, including partial or complete dieback of the tree, branch and shoot cankers were observed. Over time, the disease can increase and seriously affected trees can become barren and eventually, die. Similar situation has been described in several citrus orchards, worldwide [7, 15, 20, 21, 39]. According to some authors, abiotic factors, including drought, severe sunburn or freezing predispose the trees to xylem dysfunction, leading to these diseases [2, 3, 39].
In this study, five species belonging to three different genera of the *Botryosphaeriaceae* were recovered from symptomatic citrus trees, namely: *L. mediterranea*, *D. seriata*, *D. mutila*, *Doth. viticola* and *L. mitidjana*. The latter is introduced here, as a new species. To our knowledge, except for *D. seriata* and *L. mediterranea*, this is the first report of *D. mutila*, *Doth. viticola* and *L. mitidjana*, causing branch canker disease on citrus and any crop, in Algeria. The *Botryosphaeriaceae* species were recovered from more than half of the trees sampled and were found in all the prospected orchards.

*Lasiodiplodia* was the most commonly isolated genus that was found in six of the ten surveyed orchards. This fact is consistent with previous studies, which showed that *Lasiodiplodia* species have the ability to target a wide variety of plants, distributed worldwide [40-42]. In fact, *Lasiodiplodia* species do not only occur as latent endophytes in asymptomatic plants but are also associated with different symptoms occurring on a variety of hosts including stem-end rot, fruit rot, decline, canker and dieback [41, 43-45]. In this study, *L. mediterranea* and *L. mitidjana* sp. nov. were the most frequently encountered species. *L. mediterranea* has been reported as the causal agent of canker and dieback of grapevine, holm oak as well as citrus, indicating its capability to target different hosts [28].

The latter findings lead Andolfi et al. [46] to isolate and characterize the main secondary metabolites produced by *L. mediterranea*, as well as to evaluate its phytotoxic and antifungal activities. According to the former authors [28], *L. mediterranea* has been found only on V-shaped necrotic sectors of grapevine while, it has been isolated from all the lesion types of citrus trees in this study. *L. mitidjana* sp. nov. was found in the five surveyed orchards. Isolates of this species were present predominantly in the wedge-shaped necrosis.

*Dothiorella viticola* was isolated at low frequency compared to *Lasiodiplodia* species found in this study. Interestingly, it was detected in six sampling sites. This species was first described as *Spencermartinsia viticola* by Phillips et al. [47]. It was obtained for the first time from *Vitis vinifera* in Spain. Recently, Yang et al. [38] regarded *Spencermartinsia* a synonym of *Dothiorella* and thus transferred the epithet *viticola* to *Dothiorella* as *Doth. viticola*. This taxonomic change was supported by a multi-gene phylogeny that included *Spencermartinsia* in *Dothiorella* genus [38, 48]. *Dothiorella viticola* has been reported from a wide range of woody hosts, including citrus trees [16, 21]. Recently, it has been also described as the causal agent of gummosis on citrus trees, in Tunisia [20]. According to Phillips et al. [18] and Dissanayake et al. [49], this species is known from China, Chile, USA, Spain, France, Australia, South Africa and Tunisia. Therefore, this study constitutes the first record of *Doth. viticola* in Algeria, which thus expands its known geographical range.

Two species of *Diplodia* genus, *D. seriata* and *D. mutila* were isolated from the surveyed orchards. *Diplodia* species are well known to cause damage on several economically important species and causing numerous disease symptoms including blight, dieback, rot diseases and canker [22, 28, 50-54]. In this study, *D. seriata* was frequently recovered from the sampling sites, which matches the findings of previous studies indicating the cosmopolitan nature of this species. This latter species is commonly reported as a pathogen on a large number of hosts and has been reported from hundreds of plant species [18, 49, 50]. *Diplodia mutila*, the second *Diplodia* species isolated in this study, was less frequently found in the prospected orchards. Moreover, to our knowledge, this is the first report of this species
in our country. In addition to Algeria, the USA is the only other country in which both *D. seriata* and *D. mutila* have been associated with citrus dieback [21]. These species have been found on apples in the USA [55, 56], Chile [57], France [58], Germany [59], Uruguay [60] and South Africa [61]; as well as in pear trees [60, 61], plum [62], peach and apricot [60, 63] and walnut [51].

All the *Botryosphaeriaceae* species of this study caused necrosis on the citrus shoots, with differences in the lengths of the lesions. These differences were observed between the species and also among isolates of the same species. Thus, the results suggest that *Diplodia seriata* and *L. mediterranea* could be considered as being the most aggressive, since they produced the longest lesion. However, *D. seriata* was significantly different compared to the rest of the isolates, which is consistent with previous studies that showed significant impact of *D. seriata* on several hosts, across the globe [28, 64-66].

For *L. mediterranea*, our results are in accordance with a previous study, which highlighted its aggressiveness in artificial inoculation experiments [28]. The least aggressive species of this case were *D. mutila* and *Doth. viticola*, producing the smallest lesion size. Nevertheless, these findings contradict previous study in which *D. mutila* was found to be the most aggressive based on lesion length [60]. According to Linaldeddu et al. [28] and Chakusary et al. [50], these differences in aggressiveness maybe due to several factors including genetic variability of isolates, age, type of host tissue, differences in susceptibility as well as inoculation methods and experimental conditions. In this case, extensive sampling from citrus as well as other hosts are required to further emphasise the findings and draw a final solid conclusion.

Overall, almost all the *Botryosphaeriaceae* species we identified have previously been detected on citrus trees with the exception of *L. mitidjana*, which was described for the first time associated with citrus dieback. Given the major impact of the *Botryosphaeriaceae* species isolated on declining trees, worldwide, it is important to emphasize the urgent need to implement prevention techniques and management strategies in order to minimize the incidence of these pathogens and to prevent their spread to new orchards. For a better understanding of citrus dieback, it is necessary to set up larger surveys that include all citrus production areas. These surveys would assess, more accurately, the impact of the trunk diseases pathogens and eventually identify the factors that influence the dieback. This will be set in order to identify a number of practices to prevent their development.

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**Supplementary table S1.** Details of strains included in the phylogenetic and/or morphological analyses.

| Species          | Culture collection number(s)¹ | Substrate              | Country     | Collector(s)                  | GenBank accession numbers |
|------------------|-------------------------------|------------------------|-------------|-------------------------------|----------------------------|
| *L. avicenniae*   | CMW 41467                     | *Avicennia*, asymptomatic branches | South Africa | J. A. Osorio & J. Roux       | KP860680                   |
|                  |                               |                        |             |                               |                            |
| *L. avicenniae*   | LAS199                        | *Avicennia*, asymptomatic branches | South Africa | J. A. Osorio & J. Roux       | KU587947                   |
| *L. brasiliense*  | CMM 4015, ex-type             | *Mangifera*, stems     | Brazil      | M. W. Marques                | JX464049                   |
| *L. brasiliense*  | CMM 4469                      | *Anacardium*           | Brazil      |                               | KT325574                   |
|                  |                               |                        |             |                               |                            |
| *L. bruguiera*   | CMW 41470                     | *Bruguiera*, asymptomatic branches | South Africa | J. A. Osorio & J. Roux       | KP860678                   |
| *L. bruguiera*   | CMW 42480                     | *Bruguiera*, asymptomatic branches | South Africa | J. A. Osorio & J. Roux       | KP860677                   |
| *L. caatinguensis* | CMM 1325                     | *Citrus*               | Brazil      | I. B. L. Coutinho & J. S. Lima | KT008006                  |
|                  |                               |                        |             |                               |                            |
| *L. caatinguensis* | IBL 381                      | *Spondias*             | Brazil      | J. S. Lima & J. E. Cardoso   | KT154751                   |
| *L. chinensis*   | CGMCC 3.18061                 | *Unknown*, branch      | China       | W. He & Z. P. Dou            | KX499927                   |
|                  |                               |                        |             |                               |                            |
| *L. chinensis*   | CGMCC 3.18044                 | *Vaccinium*, branch    | China       | J. H. Zhao                   | KX499913                   |
| *L. cinnamomi*   | CFCC 51997                    | *Cinnamomum*, branch   | China       | N. Jiang                     | MH236799                   |
| *L. cinnamomi*   | CFCC 51998                    | *Cinnamomum*, branch   | China       | N. Jiang                     | MH236800                   |

¹: *=* type strain
| L. citricola       | CBS 124706 | Citrus sp., twigs | Iran     | A. Shekari   | GU945353 | GU945339 |
|-------------------|------------|-------------------|----------|--------------|----------|----------|
| L. citricola      | CBS 124707, ex-type | Citrus, twigs | Iran     | J. Abdollahzadeh & A. Javadi | GU945354 | GU945340 |
| L. crassispora    | CMW 13488  | Eucalyptus, wood | Venezuela | S. Mohali     | DQ103552 | DQ103559 |
| L. crassispora    | CBS 118741, ex-type | Santalum | Australia | T. I. Burgess & B. Dell | DQ103550 | EU673303 |
| L. euphorbicola   | CMW 33350  | Adansonia         | Botswana |                           | KU887149 | KU887026 |
| L. euphorbicola   | CMW 36231  | Adansonia         | Zimbabwe |                           | KU887187 | KU887063 |
| L. exigua         | BL 184     | Retama, branch canker | Tunisia | B. T. Linaldeddu | KJ638318 | KJ638337 |
| L. exigua         | CBS 137785, ex-type | Retama, branch canker | Tunisia | B. T. Linaldeddu | KJ638317 | KJ638336 |
| L. gilanensis     | CBS 124704, ex-type | Citrus, fallen twigs | Iran     | J. Abdollahzadeh & A. Javadi | GU945351 | GU945342 |
| L. gilanensis     | CBS 124705  | Citrus sp., fallen twigs | Iran     | J. Abdollahzadeh & A. Javadi | GU945352 | GU945341 |
| L. gonubiensis    | CMW 14077, ex-type | Syzygium | South Africa | D. Pavlic     | AY639595 | DQ103566 |
| L. gonubiensis    | CMW 14078, ex-paratype | Syzygium | South Africa | D. Pavlic     | AY639594 | DQ103567 |
| L. gravistriata   | CMM 4564  | Anacardium, stems | Brazil   | M. S. B. Netto | KT250949 | KT250950 |
| L. gravistriata   | CMM 4565  | Anacardium, stems | Brazil   | M. S. B. Netto | KT250947 | KT266812 |
| L. hormozganensis | CBS 124708 | Mangifera, twigs | Iran     | J. Abdollahzadeh & A. Javadi | GU945356 | GU945344 |
| Species               | Accession          | Host/Locus                        | Location | Authors                          | Accession1 | Accession2 |
|-----------------------|--------------------|-----------------------------------|----------|----------------------------------|------------|------------|
| L. hormozganensis     | CBS 124709, ex-type | *Olea*, twigs                     | Iran     | J. Abdollahzadeh & A. Javadi    | GU945355   | GU945343   |
| L. hyalina            | CGMCC 3.17975      | *Acacia*, cankered stems          | China    | Y. Zhang & Y. P. Zhou            | KX499879   | KX499917   |
| L. hyalina            | CGMCC 3.18383      | unidentified woody plant, cankered branches | China    | Z. P. Dou & Z. C. Liu            | KY767661   | KY751302   |
| L. iraniensis         | IRAN 1520C, ex-type | *Salvadora*, twigs                | Iran     | J. Abdollahzadeh & A. Javadi    | GU945346   | GU945334   |
| L. iraniensis         | IRAN 1502C         | *Juglans*, twigs                  | Iran     | A. Javadi                       | GU945347   | GU945335   |
| L. laeliocattleyae    | CBS 167.28         | *Laeliocattleya*, leaves          | Italy    | C. Sibilia                      | KU507487   | KU507454   |
| L. laeliocattleyae    | CBS 130992         | *Mangifera*, leaves               | Egypt    | A. M. Ismail                    | JN814397   | JN814424   |
| L. lignicola          | CBS 134112         | Wood                              | Thailand |                                 | JX646797   | KU887003   |
| L. macrospora         | CMM 3833, ex-type  | *Jatropha*, collar and root rot   | Brazil   | A. R. Machado & O. L. Pereira   | KF234557   | KF226718   |
| L. mahajangana        | CMW 27801, ex-type | *Terminalia*, healthy branches    | Madagascar | J. Roux                           | FJ900595   | FJ900641   |
| L. mahajangana        | CMW 27820          | *Terminalia*, healthy branches    | Madagascar | J. Roux                           | FJ900597   | FJ900643   |
| L. margaritacea       | CBS 122519, ex-type | *Adansonia*, dying twigs          | Australia | T. I. Burgess & M. J. Wingfield | EU144050   | EU144065   |
| L. margaritacea       | CBS 122065         | *Adansonia gibbosa*, dying twigs  | Australia | T. I. Burgess                    | EU144051   | EU144066   |
| L. mediterranea       | CBS 137783, ex-type | *Quercus*, branch canker          | Italy    | B. T. Linaldeddu                 | KJ638312   | KJ638331   |
| L. mediterranea       | CBS 137784         | *Vitis*, brown stripe under the bark | Italy    | S. Serra                        | KJ170150   | KJ170151   |
| **L. missouriana** | CBS 128311, ex-type | Wedge-shape canker of grapevine cv. Catawba (complex hybrid of North America *Vitis* species) | USA | K. Striegler & G. M. Leavitt | HQ288225 | HQ288267 |
|-------------------|---------------------|-------------------------------------------------|-----|----------------------------|----------|---------|
| **L. missouriana** | CBS 128312          | Wedge-shape canker of grapevine cv. Catawba (complex hybrid of North America *Vitis* species) | USA | K. Striegler & G. M. Leavitt | HQ288226 | HQ288268 |
| **L. parva**      | CBS 456.78, ex-type | Cassava-field soil                               | Colombia | O. Rangel | EF622083 | EF622063 |
| **L. parva**      | CBS 494.78          | Cassava-field soil                               | Colombia | O. Rangel | EF622084 | EF622064 |
| **L. plurivora**  | STE-U 5803, ex-type | *Prunus*, wood canker                           | South Africa | U. Damm | EF445362 | EF445395 |
| **L. plurivora**  | STE-U 4583          | *Vitis*, symptomatic                             | South Africa | F. Halleen | AY343482 | EF445396 |
| **L. pontae**     | CMM 1277            | *Spondias*, necrotic canker                       | Brazil   | J.S. Lima & F.C.O. Freire | KT151794 | KT151791 |
| **L. pseudotheobromae** | CBS 116459, ex-type | *Gmelina*, twigs                                 | Costa Rica | J. Carranza- Velazquez | EF622077 | EF622057 |
| **L. pseudotheobromae** | CGMCC 3.18047      | *Pteridium*, twigs                               | China    |                     | KX499876 | KX499914 |
| **L. pyriformis** | CBS 121770, ex-type | *Pinus*, fruiting structures                     | Namibia  | F. J. J. van der Walt & J. Roux | EU101307 | EU101352 |
| **L. pyriformis** | CBS 121771          | *Pinus*, fruiting structures                     | Namibia  | F. J. J. van der Walt & J. Roux | EU101308 | EU101353 |
| **L. rubropurpurea** | WAC 12535, ex-type  | *Eucalyptus*, canker                             | Australia | T. I. Burgess | DQ103553 | EU673304 |
| Species                    | Collection | Affected Plant | Location    | Authors                          | GenBank Accession Numbers |
|---------------------------|------------|----------------|-------------|----------------------------------|---------------------------|
| *L. rubropurpurea*        | WAC 12536  | *Eucalyptus*    | Australia   | T. I. Burgess                    | DQ103554, DQ103572       |
| *L. sterculiae*           | CBS 342.78, ex-type | *Sterculia*     | Germany     | S. Bruhn                        | KX464140, KX464634       |
| *L. subgloboasa*          | CMM 3872, ex-type | *Jatropha*, collar and root rot | Brazil      | A. R. Machado & O. L. Pereira | KF234558, KF226721       |
| *L. subgloboasa*          | CMM 4046   | *Jatropha*      | Brazil      | A. R. Machado & O. L. Pereira   | KF234560, KF226723       |
| *L. thailandica*          | CGMCC 3.18382 | *Podocarpus*, cankered branch | China       | D. Zhipeng & L. Zuchen          | KY767662, KY751303       |
| *L. subgloboasa*          | CMM 4046   | *Jatropha*      | Brazil      | A. R. Machado & O. L. Pereira   | KF234560, KF226723       |
| *L. theobromae*           | CBS 164.96, ex-neotype | Fruit along coral reef coast | Papua New Guinea | A. Aptroot                      | AY640255, AY640258       |
| *L. theobromae*           | CBS 111530 | *Leucospermum*  | USA         | J. E. Taylor                     | EF622074, EF622054       |
| *L. venezuelensis*        | WAC 12539, ex-type | *Acacia*, wood | Venezuela   | S. Mohali                       | DQ103547, EU673305       |
| *L. venezuelensis*        | WAC 12540  | *Acacia*, wood  | Venezuela   | S. Mohali                       | DQ103548, DQ103569       |
| *L. viticola*             | CBS 128313, ex-type | wedge-shape canker of grapevine cv. Vignoles (complex hybrid of North America *Vitis* species) | USA | R. D. Cartwright & W. D. Gubler | HQ288227, HQ288269       |
| *L. viticola*             | UCD 2604MO | *Vitis*         | USA         | J. R. Urbez-Torres              | HQ288228, HQ288270       |
| *L. vitis*                | CBS 124060, ex-type | *Vitis*, wood fragment | Italy, Sicily | S. Burruano                      | KX464148, KX464642       |
| *D. seriata*              | CBS 112555 | *Vitis*, dead stems | Portugal    | A. J. L. Phillips               | AY259094, AY573220       |
| Species   | Collection | Country | Author | Accession Numbers |
|-----------|------------|---------|--------|------------------|
| *D. mutila* | CBS 112553 | Portugal | A. Alves | AY259093, AY573219 |

BL: Personal number of B.T. Linaldeddu; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Beijing, China; CMM: Culture Collection of Phytopathogenic Fungi “Prof. Maria Menezes”, Universidade Federal Rural de Pernambuco, Recife, Brazil; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; WAC: Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia.
Supplementary figure S1.

Maximum Parsimony phylogenetic tree resulting from the analysis of the combined ITS and tef1-α sequence data from *Lasiodiplodia* species. The tree was rooted to *Diplodia mutila* and *Diplodia seriata*. 
Dear Dr. Katharina B Budde,

We would like to thank you for considering our manuscript PONE-D-19-29810, entitled "Lasiodiplodia mitidjana sp. nov. and other Botryosphaeriaceae species causing branch canker and dieback of Citrus sinensis in Algeria", for publication in PLOS ONE Journal.

We would like to thank you for the helpful comments and constructive observations. We have made all the suggested revisions. A detailed point-by-point response is reported here below and all new changes have been highlighted in yellow in the revised version of our manuscript.

All authors read and approved the submitted new version of the manuscript.

Best regards,

Dr. AKILA BERRAF-TEBBAL

Reviewer Comments to Author:

Answers to the reviewer 1

General comments:
Akila Berraf-Tebbal and collaborators present in this manuscript the results of a survey on Botryosphaeriaceae diversity and pathogenicity affecting symptomatic sweet orange (Citrus sinensis) in Algeria. Botryosphaeriaceae species were identified in every orchard tested (n=10) and a total of five different species have been isolated from symptomatic samples, with frequencies ranging from 5.4% to 14.1% of the samples. One species appears to form a distinct monophyletic group - not described before - and is claimed by the authors to be a new species. The pathogenicity of two representative isolates for every species isolated (n=5) was tested experimentally on Citrus shoot and Koch’s postulate was verified for all of them. Differences in pathogenicity was observed between isolates.
Overall, the manuscript is well written and there is no doubt about the efforts made by the authors to produce this work. The results presented are interesting and alert about the potential spread and threats brought by these pathogens, as mentioned by the authors. My main comments to improve this manuscript is that authors often goes on conclusions that are not completely supported by the results. I suggest therefore to reconsider these conclusions or change the way they are presented to stick more on the facts.

We took into consideration all the comments regarding the conclusions and we made sure to do all the required modifications.
Furthermore, the GenBank accession for the Tef1-α sequences are not available which hamper my reviewing conclusions. This is even more problematic because the genetic difference observed to discriminate the new species are brought by this marker.

The ITS and the tef1-α sequences have been deposited into GenBank; However, the tef1-α sequences are not automatically deposited into GenBank after being accessioned. Each sequence record is individually examined and processed by the GenBank annotation staff to ensure that it is free of errors or problems.

In this sense, a new species (Lasiodiplodia mitidja) is introduced in this study. This introduction is based on a two loci phylogeny, as well as morphological observations. I’m not a taxonomist myself, but are two SNPs (which I could not verified, and that is not illustrated by an alignment neither in the manuscript) and a bootstrap of 80 enough to consider the organism a new species? Concerning the morphological differences, as the authors mentioned, conidia “tend” to be larger and L/W ratio is different but for both measurements, no statistical significance is brought to the observations to confirm the difference. Can it represent a subpopulation of L. citricola? I presume it is not possible to test if those “species” outcross but if we have to be more rigorous, I would recommend to stay more prudent about the “new species” terminology and presented it more as a suggestion, or inform the readers that all the criteria to say it’s a new species are not completely fulfil.

We agree that this may be debatable. In fact, we have discussed this previously within the team. However, it is clearly aligned with the current trend for introduction of novel Lasiodiplodia species.

In the future it may well be proven that in fact it is not a new species different from L. citricola. But taxonomy is dynamic and hence frequently changing. For the moment we would like to introduce the new species. The fact is that eventually it will be described as a new species, if not by our group, then by someone else.

As an example of a case which is similar to ours, here are the nucleotide differences for the following mentioned species: L. chinensis vs L. lignicola vs L. pseudotheobromae. For all 3 species ITS is 100 % identical

As for Tef1:

L. chinensis vs L. lignicola: 1 nucleotide difference

L. chinensis vs L. pseudotheobromae: 3 nucleotides differences

My second concern is the ambiguity made between the types of wood alteration/symptoms observed in Citrus trees and the presence and implication of Botryosphaeriaceae. Botryosphaeriaceae species can be isolated from certain types of alteration and yet not being responsible of these alterations. Knowing the “opportunist” behaviour of these
pathogens, I would not be surprised if they take over the habitat after a disequilibrium was induced into the microbiome of trees following another pathogen attack.

We agree with the reviewer. After considering your other comments on the same part and your suggestion of removing it and given that it is not relevant for the paper we decided to delete it.

The fact that the isolates were able to provoke symptoms experimentally does not necessarily mean they are responsible of the ones observed on the diseased trees, especially since the symptoms observed after artificial inoculation are not correlated to the ones observed on fields.

We agree with the reviewer, however the goal was to test pathogenicity of the isolates and this is the way to do it. Of course we cannot be sure that they will behave the same way in the field but they have the potential to do so.

Similarly, the presence of basidiocarps on heavily symptomatic Citrus is confusing for me, at least the way it is presented. What is the link between the Botryosphaeriaceae and the basidiocarps emergence, which species correspond to this basidiocarp?

We described the health status of the orchards where the sampling has been carried out (branch and shoot cankers, abnormal growth of epicormic shoots; defoliation and leaf chlorosis). Basidiocarps are the fruiting bodies of the decay-causing fungi. Their presence on the trunk means that it is an already rotting trunk and that some ascomycetes (Botryosphaeriaceae, Diatrypaceae...) and Basidiomycetes (Fomitiporia, Phellinus....) have already colonized the trunk.

In a similar fashion, Figure 4 is confusing as my conclusion on this figure is that Botryosphaeriaceae can be isolated from different types of symptoms and not that one species is more isolated from one type of symptoms than another as the authors tend to say. There is no statistic proving so, and a quick interpretation (but false) from a hurried reader would be that such species is responsible of such symptoms. At this point, those results are more detrimental that beneficial to the study. I either recommend to delete this part or erase those ambiguities by a deeper discussion and a clearer result presentation. What do we know about the multifactorial aspects of dieback diseases? Is there only one pathogen involved? I think study conducted on Botryosphaeriaceae and grapevine trunk disease can be related to this case. Furthermore, if this part is conserved, more insights on what is known about the different symptoms that can cause Botryosphaeriaceae could/should be presented in the introduction.

We agree with the reviewer and his comments. After pointing out these remarks we thoughtfully considered them and decided to delete the figure 4 as well as the paragraphs related to it.
Finally, the statistical methods used to test pathogenicity differences is either not well presented or the conclusions are not correct. This part needs to be improve. Have you tested species effect? Isolate effect? What are the p-value attributed to each ANOVA test, which factor has been tested by the ANOVA? Is LSD method (which is not described by the way) the more appropriate in your case?

We took into consideration your valuable comments and we made sure to change this part and we removed all the ambiguities.

Minor Comments:

L30: 14.1% percent of the samples and 13% of the samples
R: Revised as recommended

L31: what is the difference between widespread and abundant?
R: Widespread means that it is found or distributed over a large area. However, abundant means that it is existing or available in large quantities (it could have the same meaning as plentiful)

L42: I would erase (pomelos)
R: Revised as recommended

L43: Despite the high adaptation capacity of citrus trees to different climates (reference is missing)
R: Revised as recommended

L47: Citrus diseases are numerous and diverse, and are caused by phytopathogenic agents belonging to viruses, viroids, phytoplasmas, bacteria, and fungi (reference is missing).
R: Revised as recommended

L57: reference is missing
R: Revised as recommended

L63: colonize or affect?
R: We deleted ‘affect’
This part on Botryosphaeriaceae should be more documented: classification of Botryosphaeriaceae, how many genera, endophytes with symptomless period, etc...

R: Revised as recommended

L82: Surveys were conducted in ten commercial orchards in the northern region of Algeria, specifically, in the Mitidja plain at the base of the Tell Atlas Mountains (Table 1).

Table 1: I would add coordinates of the orchards and years of sampling.

R: Revised as recommended

L94: was the scalpel sterilized?

R: Yes, the scalpel was sterilized. This detail has been added to the manuscript.

L112: In this paragraph, can you add more info about the PCR conditions?

R: Revised as recommended

L120-121: pyrosequencing?

R: the company used Sanger sequencing method.

L123: Newly generated sequences were deposited in GenBank (Table 2): the Tef1-α isn’t accessible.

R: The sequences are available in GenBank.

L124: Sequences for both DNA regions were retrieved in BLAST searches from GenBank [34].

Check the meaning of this sentence. For example: “Homological sequences of the newly sequenced ones were retrieved from the GenBank by Blast.”

R: Revised as recommended

L125: Table 2: Can you add more info on this table, like type of tissue (trunk/branches), type of symptoms (under your classification), Orchard, etc....

R: Revised as recommended

L132: Please specify the request you made, or put the sequence in the supplemental files.
R: Revised as recommended (the sequences are in the supplementary files)

L136: what kind of adjustments?
R: The ITS and tef1-α sequences were initially aligned separately using ClustalX v. 1.83. The alignments were manually optimized by coding the missing sequences as “?”. Ambiguous sequences at the start and the end were deleted and gaps were adjusted in BioEdit.

L153: diameter
R: Revised as recommended

L164: this part need to be more precise/improve. Which threshold to accept the significance of the ANOVA, which factor tested, what LSD means for, which soft did you use?
R: Revised as recommended

Detailed responses:

Which threshold to accept the significance of the ANOVA
R: when the P value is below the threshold (0.05), the difference between the means is considered as significant.

Which factor tested?
R: We tested the lesions produced by each fungal isolate of the different species.

What LSD means for?
R: We changed the statistical test by using Tukey's honestly significant difference (HSD) test.

Which soft did you use?
R: The R v. 3.5.1 statistical software was used to perform the statistical analysis.

L171: nothing is said about the distribution
R: We removed the word ‘distribution’ from the title

L172-173 and L174-175 could be fused for clarity purposes
R: We removed this paragraph as recommended.

L175: the total number of samples... Samples = branches?
R: The samples mean the different necrotic lesions found in the branches and the trunks of the 80 trees.

L176 – 177: this measurement is completely arbitrary and according to me abusive. If one pathogen would have occurred at 80% frequency, the difference between 11%(very frequent) and 4%(infrequent) would be meaningless. My advice, stick to the numbers and do not try to interpreted it in a frequent or infrequent way, that’s too subjective.

R: We removed the paragraph related to the frequency of occurrence, as recommended.

L186: On heavily infected trees, basidiocarps emerged: that’s ambiguous, as said before.
R: We removed the description of the basidiocarps from the photoplate as well as from the text, as recommended.

L189: why wedge-shaped necrosis is not name WSN?
R: Revised as recommended

L191: similar BCN instead of BCN and YSW instead of NCC.
R: Revised as recommended

L196: the “e.” is missing on the picture, but maybe see in it as a sign for not putting this picture...
R: Revised as recommended

L214: Why MP tree is not shown? At least in supplemental file?
R: Revised as recommended (the tree is in supplementary file)

L218: why 23 isolates and not 24 (10+14)
R: The sequence of one isolate was not good enough to use it for the phylogenetic analysis.

L228: The phylogenetic tree of only one method is presented, although the bootstrap values of the two methods are shown. Can we see the tree constructed with the second method in the supplemental files?
R: Revised as recommended

L279: can you have the sequence accessible please?
R: The sequence has been submitted to GenBank. It will be available online after verification of the annotation. We have included the accession number into the table 2.

L311: from what I’ve read, LSD test is not recommended anymore as sensitive to multiple comparison. Furthermore, as mentioned above, this test is not well conducted and presented. A histogram would be better, with a sign for significant difference, either at the isolate level and species level, with threshold use for significance. The 100% of re-isolation frequency for every isolates are not necessary in the table according to me, if you say it in the text.

R: Revised as recommended.

L317: Ambiguous: are you speaking of distribution in the wood? If yes, cut this paragraph in two: Frequency of occurrence / Distribution of Botryosphaeriaceae in the wood.

R: Revised as recommended

L320: I comment already the frequent and very frequent ranking, that’s abusive according to me.

R: We deleted this paragraph.

L323: For each orchard, at least two different species were isolated, average per orchard?

R: In the paragraph L323, we described the widespread of the species in the orchards. It was only to mention their presence on each orchard.

For more details, here is a table containing all the information about species distribution among the surveyed orchards.

| Species/Orchards | Region          |     |     |     |     |     |     |     |     |     | Total |
|------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|
|                  | Oued El Alleug  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10    |       |
| D. seriata       |                 |     |     |     |     | 2   | 1   |     |     | 3   | 4     | 10    |
| D. mutila        |                 |     |     |     |     | 3   | 2   |     |     |     | -     | 5     |
| L. mediterr.     |                 |     | 2   | 2   | 3   | 1   |     | 2   | 2   |     | -     | 12    |
| L. mitidjana     |                 | 0   | 3   | 3   | 3   |     |     | 2   | 2   |     | -     | 13    |
|        | 1 | - | 1 | - | 1 | 2 | - | - | 1 | 1 | 7 |
|--------|---|---|---|---|---|---|---|---|---|---|---|
| Total  | 3 | 5 | 7 | 4 | 6 | 5 | 4 | 4 | 4 | 5 | 47 |

L328: new paragraph or no paragraph at all as mentioned above, I found this part confusing.

R: Revised as recommended (We removed the paragraph).

L370-371: According to these authors, *L. mediterranea* has been found only on V-shaped necrotic sectors of grapevine while it has been isolated from all the lesion types of citrus trees in this study.

R: Revised as recommended

L373: what the results of Andolfi et al. bring to your results?

R: Andolfi et al. (2016) isolated and characterized the main secondary metabolites produced by *L. mediterranea*. They also, evaluated its phytotoxic and antifungal activities. These findings support our results, which show the ability of this fungal species to colonize and cause damages in the wood.

L374-375: maybe if you had isolated only the 4 or 5 isolates that were from brown central necrosis, would you have said that the species was exclusively found in brown central necrosis? This part of the discussion is not really constructive.

R: we removed the paragraph, as well as the figure 4.

L387: More interesting that this, it confirms its wide geographical range.

R: revised as recommended

L402: Wedge shaped lesion?

R: The paragraph is about the pathogenicity trial and the lesions produced by each *Botryosphaeriaceae* species. We did not consider the shape of the lesions, for the pathogenicity test.
L403-405: this part of the discussion goes beyond what your data show, either improve your statistical analysis or moderate the message.

R: Revised as recommended

L408-415: similar, hard to have this kind of discussion with two isolates per strains, with one phenotyping trial.

R: Revised as recommended

L446: References have to be reformatted: species not in italic, capital letter on every first word letter for some references, etc.; some other problem like L508 Philippis is written bizarrely.

R: Revised as recommended

Answers to the reviewer 2

Reviewer #2: This is a nice study of Botryosphaeriaceae which are important plant pathogens, including fruit trees. Algeria is unexplored both from mycological and pathological perspective and it is nice to see collaborations like this resulting in a good piece of work. I agree with authors regarding Lasiodiplodia mediterranea and L.vitis situation, especially because pcr artifacts introduced by primer sequences are unfortunately a common thing these days (my personal experience). I have small suggestions that would improve the paper.

Abstract, line 25-of Botryosphaeriaceae

R: Revised as recommended

Abstract, line 29- Delete which, add Lasiodiplodia mitidjana is described in this paper as a new species

R: Revised as recommended

Abstract, line 62- delete effect
R: Revised as recommended

**Materials**-line 98-dried on sterilized paper (towels, filter paper?)
R: Revised as recommended

**Materials, line 100**-The mycelium emerging from wood pieces was transferred...
R: Revised as recommended

**Materials, line 105**-Isolates that lacked pycnidia production...
R: Revised as recommended

**Materials, line 150**-How did you select representative isolates?
R: We selected two isolates, from each phylogenetically resolved species.

**Materials, line 151**-Shoots? But above you mentioned branches (line 148)
R: Revised as recommended (We have standardized using shoot instead of branch).

**Materials, line 157**-“... well watered and maintained under favorable conditions” What do you mean by this? Were the cuttings in soil or in water? How many times per week did you change the water? Ambient temperature? Light?
R: The inoculated cuttings were wrapped with wet sterile cotton to avoid the desiccation of the agar plug. The shoots were immediately transplanted into pots containing sterilized water as a growth substrate (10 shoots per pot), which were incubated at the ambient room temperature, under daily photoperiod. The water of the container was changed twice a week.

**Results, line 182**-Degrees of intensity? Where are they?
R: The degrees of intensity refer to the different levels of the dieback symptoms observed in the orchards.

**Results, line 186**-Basidiocarps of which species or genera?
R: We did not identify the basidiocarps. We described all the symptoms related to the citrus trees dieback, including the fruiting bodies emerged from the trunks.
Results, line 309-What about control plants?
R: We did not isolate any of the tested species from the negative control.

Results, line 313-Now you mention branches again
R: revised as recommended

Discussion, line 360-and seriously affected trees can become
R: revised as recommended

Discussion, lines 403-404- “However, D. seriata was significantly different compared to the rest of the isolates” But previously you said that both D. seriata and L. mediterranea were most aggressive species (based on lesion lengths). So which species was in fact most aggressive? Also, what about differences in aggressiveness between different isolates of the same species?

So which species was in fact most aggressive?
R: The significant difference was made based on a comparison between all the tested isolates. It was not about the pairwise comparisons that take one isolate and compare it with each of the rest of isolates. D. seriata and L. mediterranea were the most aggressive species when compared to the rest of the species. However, D. seriata was the most aggressive species, considering the length of the lesion for each isolate, separately.

Also, what about differences in aggressiveness between different isolates of the same species?
R: Significant variation in aggressiveness can occur within and among isolates from the same species. This aggressiveness refers to the quantitative variation of pathogenicity on the susceptible host infection efficiency, the latent period, the spore production rate and the infectious period of each strain. These components are closely related to the genetic variability within the strains of the same species.

Discussion, lines 408-412-Was this previous study also about Bot on citrus trees?
R: the study was about Lasiodiplodia species (Botryosphaeriaceae) on grapevine.

Discussion, lines 409 and 411-In line 404 you are talking about aggressiveness. Now about virulence. In line 413 you talk again about aggressiveness. Virulence and aggressiveness don’t mean the same thing. Replace the term virulence with aggressiveness in lines 409 and 411.
Answers to the academic editor

L.273 ..we have shown...
R: Revised as recommended

L. 279 “…these 2 nt are not real.” Are not real is confusing, maybe instead use “were correctly determined”?
R: Revised as recommended

L. 335 “The remaining species…” better mention the species’ name here.
R: Revised as recommended

L. 351 A similar situation has been...
R: Revised as recommended

L. 363 world
R: Revised as recommended

L. 373 compared instead of comparing
R: Revised as recommended

L. 389 “…known for targeting economically important plants…” targeting sounds as if they are selecting the hosts based on the economical value. Maybe better say: …known to cause damage on several economically important species…”
R: Revised as recommended

L. 393 “The later…”, better say “This latter species..” or just “It…”
R: Revised as recommended
I would suggest to add some research perspectives at the end of the discussion. What kind of studies could help to better understand and develop management recommendations against citrus dieback?

R: Revised as recommended