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First Report of *Neousicoccum luteum* as the Causal Agent of Canker and Die-Back of *Cupressus sempervirens*

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Abstract. The Mediterranean cypress (*Cupressus sempervirens*) is a species native to the eastern Mediterranean region, with many uses and properties. The *Botryosphaeriaceae* fungal family and in particular *Neofusicoccum luteum* are known for being both primary pathogens and opportunists, mainly on woody hosts such as conifers. This species was first reported in Portugal in 2012 on several coniferous hosts, including *C. sempervirens*. However, no report of *N. luteum* causing disease in this host has been reported either in Portugal or in any other country. In an attempt to understand the underlying causes of dieback of one *C. sempervirens* tree in the municipality of Aveiro (Portugal), we conducted a multi analytical study based on fungal isolation and identification. Fungal isolations were made from unhealthy plant material, resulting in the *N. luteum* identification. Moreover, Koch's postulates were carried out, leading to the development of lesions at the inoculation spots in the tested plants. Further re-isolation attempts from lesion areas lead to the confirmation of the presence of *N. luteum.* Our results point that *N. luteum* was the causal agent of disease on the sampled tree, marking this as the first report of *N. luteum* causing dieback in *C. sempervirens*. These results can be important in

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future diagnosis of this disease in this host, as well as be the kick-start for prevention regarding the aforementioned fungus.
Key words: *Botryosphaeriaceae*; cypress; disease; Koch's postulates

Primeiro Relato de *Neofusicoccum luteum* como Agente Causal de Cancro e Declínio de *Cupressus sempervirens*

Sumário. O cipreste mediterrâneo (*Cupressus sempervirens*) é uma espécie originária da região mediterrânea oriental, com múltiplas propriedades e fins de utilização. A família de fungos *Botryosphaeriaceae* e em particular o fungo *Neofusicoccum luteum* são conhecidos tanto como patógenos primários como oportunistas, principalmente em hospedeiros lenhosos como coníferas. Esta espécie foi reportada pela primeira vez em Portugal em 2012 em várias coníferas, incluindo *C. sempervirens*. Contudo, até à data não houve qualquer relato de *N. luteum* causar doença neste hospedeiro, seja em Portugal ou qualquer outro país. Numa tentativa de entender as causas do declínio de uma árvore de *C. sempervirens* no município de Aveiro (Portugal), levámos a cabo um estudo multi-analítico com base em isolamento e identificação fúngica. Isolamentos fúngicos foram feitos a partir de material vegetal que apresentava sinais de doença, resultando na identificação de *N. luteum*. Foram verificados os postulados de Koch, uma vez que após inoculação do fungo nas plantas se verificou desenvolvimento de lesões nos locais de inoculação, obtendo-se posteriormente o re-isolamento de *N. luteum* a partir das lesões. Os nossos resultados indicam que *N. luteum* era o agente causal de doença da árvore amostrada, fazendo deste o primeiro relato de *N. luteum* como agente causal de declínio em *C. sempervirens*. Estes resultados poderão ser importantes em diagnósticos futuros de doença neste hospedeiro, bem como ser impulsionadores para prevenção no que toca ao fungo em causa.
Palavras-chave: *Botryosphaeriaceae*; cipreste; doença; postulados de Koch

Premier Rapport de *Neofusicoccum luteum* comme Agent Causal du Chancre et du Dépérissement de *Cupressus sempervirens*

Résumé. Le cyprès méditerranéen (*Cupressus sempervirens*) est une espèce originaire de la région méditerranéenne orientale, aux multiples usages et propriétés. Les champignons de la famille des *Botryosphaeriaceae* et en particulier *Neofusicoccum luteum* sont connus pour être à la fois des pathogènes primaires et des opportunistes, principalement sur des hôtes ligneux tels que les conifères. Cette espèce a été signalée pour la première fois au Portugal en 2012 sur plusieurs hôtes conifères, dont *C. sempervirens*. Cependant, aucun signalement de *N. luteum* provoquant une maladie sur cet hôte n'a été signalé au Portugal ou dans un autre pays. Dans une tentative de comprendre les causes sous-jacentes du déclin d'un arbre *C. sempervirens* dans la municipalité d'Aveiro (Portugal), nous avons mené une étude multi-analytique basée sur l'isolement et l'identification des champignons. Des isolations de champignons ont été réalisées à partir de matériel

végétal insalubre, ce qui a permis l'identification de *N. luteum*. En outre, les postulats de Koch ont été réalisés, ce qui a conduit au développement de lésions aux sites d'inoculation dans les plantes testées. D'autres tentatives de ré-isolation à partir des zones de lésions ont permis de confirmer la présence de *N. luteum*. Nos résultats indiquent que *N. luteum* était l'agent causal de la maladie sur l'arbre échantillonné. Il s'agit du premier signalement de *N. luteum* provoquant le dépérissement de *C. sempervirens*. Ces résultats peuvent être importants pour le diagnostic futur de cette maladie chez cet hôte, ainsi que pour la prévention de la maladie.
Mots-clés: *Botryosphaeriaceae*; cyprès; maladie; postulats de Koch

Introduction

The Mediterranean cypress (*Cupressus sempervirens*) is a tree species native to the eastern Mediterranean region, with human introductions spreading it over Southern Europe, Northern Africa and the Middle East (BAGNOLI *et al*., 2009). It is mostly used as an ornamental tree, even though its essential oils have previously been reported to have antibacterial, antifungal, antiviral, antiparasitic, insecticidal, antioxidant, wound healing, anticancer, estrogenic and anticoagulant effects (AL-SNAFI, 2016). The *Cupressus* genus can host several species of microfungi, many of which can cause cankers and other diseases. *Seiridium cardinale* is among the most aggressive fungi for this host as it causes the cypress canker disease (BONTHOND *et al.,* 2018). It was first reported in Europe in 1944 (GRANITI, 1986), and first recorded in Portugal in 1980 (CAETANO, 1980). This fungus has been reported to cause serious disease in *Cupressus* spp., being particularly severe in *C. sempervirens* in the Mediterranean region (GRANITI, 1998). Symptoms on this host encompass crown reddening, branch dieback and resin exudation (DANTI *et al*., 2009).

The *Botryosphaeriaceae* family is well known for encompassing both primary pathogens and opportunists, mainly on woody hosts (ABDOLLAHZADEH *et al.,* 2009). Among them is *Neofusicoccum luteum*, a species which has been reported as being pathogenic for conifers, such as *Sequoia sempervirens* (branch canker) (AĆIMOVIĆ *et al*., 2017), as well as for broad-leaved trees, such as *Pistacia lentiscus* (LINALDEDDU *et al*., 2016) and *Crataegus mexicana* (ADESEMOYE *et al*., 2013). It was first reported in Portugal in 2012 on several coniferous hosts, as an endophytic fungus in asymptomatic *C. sempervirens* (ALVES *et al*., 2013). *Neofusicoccum luteum* has also been isolated in *Q. robur*, where it, alongside *Neofusicoccum australe*, were associated with canker and dieback (BARRADAS *et al*., 2013). However, no report of *N. luteum* causing disease in *C. sempervirens* has been issued, be it in Portugal or any other country. Another member of the *Neofusicoccum* genus is *Neofusicoccum parvum*, a species which has been reported to cause canker and dieback of *Eucalyptus* (ITURRITXA *et al*., 2011; BARRADAS *et al.* 2016), as one of the most aggressive causal agents of dieback in grapevine (MASSONNET *et al*., 2017), causing decline and cankers of Norfolk Island pine in Australia (GOLZAR and BURGESS, 2011), being one of the species associated with *C. sempervirens* decline in Iran (MOHAMMADI *et al*., 2014) where it was reported as the most virulent and the causer of larger lesions among the several species of fungi identified and, recently, as the second most aggressive species during pathogenicity trials of several *Botryosphaeriaceae* species on *Quercus suber* and

Quercus ilex, having shown similar aggressiveness towards both hosts (MAHAMEDI *et al.*, 2020).

In an attempt to understand the underlying causes of dieback of one *C. sempervirens* ornamental tree from the municipality of Aveiro (Portugal), we conducted a multi analytical study based on fungal isolation morphological and phylogenetic analysis. Our results indicate that *N. luteum* was the causal agent of disease, marking this as the first time this species is described as pathogenic for *C. sempervirens*. These results could prove invaluable in future diagnosis of disease in this host, as well as be the kick-start for prevention regarding the aforementioned fungus on *C. sempervirens* and other conifers.

Materials and methods

Sampling and fungal isolation

One ornamental *C. sempervirens* tree present in the city of Aveiro (Portugal) was reported to show signs of dieback in shoots and branches, alongside lesions and cankers with resin exudates in the trunk. To understand the possible origin of such symptoms, samples of diseased trunk and branches were collected and prepared for macro/microscope observation for fungal structures as well as for isolation on fungal culture media. For fungal isolation, samples were surface disinfected with 1,5% sodium hypochlorite for 2 minutes, followed by two wash cycles with distilled water for 2 minutes each. Disinfected plant material was plated onto Potato Dextrose Agar (PDA) (Difco, USA) and incubated at 25±1ºC for 7 days. Developing fungal cultures were further isolated onto PDA until sufficient mycelium growth for DNA extraction and identification was observed.

Several saprobic fungal isolates were obtained; these were discarded, as they were not the causal agents of plant diseases. Two identical isolates belonging to *Botryosphaeriaceae* were obtained, with one of them being followed and further investigated from this point on. We proceeded with the morphological and molecular identification of this isolate that has been kept in culture for further use. A voucher specimen under the code MUM 21.33 has been deposited in the culture collection of Micoteca da Universidade do Minho (MUM) (Braga, Portugal).

To induce sporulation, cultures were grown on 3.9% w/v water agar medium bearing pieces of sterilized pine needles, and the resulting cultures were then incubated at 25ºC for 30 days before microscope observation.

DNA extraction, PCR amplification, amplicon sequencing and phylogenetic analysis

DNA extraction of the pure culture of MUM 21.33 was conducted with the REDextract-N.Amp PCR ReadyMix kit (Sigma, USA), with some modifications. The fungal mycelium was suspended on 40 µL of extraction solution and incubated at 94ºC for 10 minutes, followed by 60ºC for 13 minutes and 10ºC for 15 minutes. The reaction was stopped with the addition of 40 µL of dilution solution and the resulting mixture centrifuged at 16000g for 5 minutes. The resulting supernatant was transferred to a new tube and the pellet was discarded. Obtained DNA was used as template for two individual PCR reactions targeting the ITS and the β-tubulin regions (*benA*). The reaction mixes used for both procedures consisted of 12.5 µL of JumpStart Taq ReadyMix (Sigma, USA), 9.5 µL of ddH_2O, 1 µL of each primer and 1 µL of DNA template. For the amplification of the ITS region, the primer pair ITS1F/ITS4 (WHITE *et al*., 1990; GARDES and BRUNS, 1993) was used, with the reaction consisting of an initial denaturation step of 5 minutes at 94ºC, followed by 35 cycles of denaturing, annealing and extension steps, for 1 minute each, with the temperatures being 94ºC, 55ºC and 72ºC respectively, and lastly a final extension of 5 minutes at 72ºC. Complementarily, the partial β-tubulin gene (*benA*) was amplified using the primer pair Bt2a/Bt2b (GLASS and DONALDSON, 1995), with the reaction consisting of an initial denaturation step of 2 minutes at 95ºC, followed by 29 cycles of denaturation, annealing and extension steps, for 1 minute each, with the temperatures being 95ºC, 53ºC and 72ºC respectively, and a final extension with 5 minutes at 72ºC. Obtained amplicons were purified with the EXO/SAP Go PCR Purification Kit (GRISP, Portugal), by adding 10 µL of PCR product to 2 µL of RSAP and 2 µL of Exo I. The resulting mixture was then incubated at 37ºC for 5 minutes followed by enzyme inactivation at 80ºC for 10 minutes. Amplicons were then sequenced using an ABI 3730xl DNA Analyzer system (96 capillary instruments) at STABVIDA, Portugal.

Obtained DNA sequences were processed using Geneious® R11.0.02 and deposited in GenBank database with the accession numbers MZ440613 (ITS) and MZ476038 (*benA*). A preliminary molecular analysis was conducted by comparison with sequences available in the NCBI database using the BLAST tool (ALTSCHUL *et al*., 1997). Taking into consideration these results, a further confirmation of the recovered isolate's identity was performed by phylogenetic interference. For this analysis, a simplified dataset consisting of a concatenated matrix of ITS and *benA* individual alignments was constructed, based on the

Neofusicoccum clade IV according to LOPES *et al.* (2016). Each individual matrix was aligned using the online version of MAFFT v.7 (KATOH and STANDLEY, 2013) and manually adjusted using UGENE v.1.26.3 (OKONECHNIKOV *et al.*, 2012). The two resulting alignments were further concatenated using SeaView v.4 (GOUY *et al.*, 2010). Lastly, a maximum likelihood phylogenetic analysis with 1000 bootstrap replicates was conducted in the concatenated alignment using the MEGAX bioinformatics software (KUMAR *et al.*, 2018).

Pathogenicity tests

To verify Koch's postulates, pathogenicity tests were performed in a greenhouse by inoculating eight seedlings of *C. sempervirens* (stem diameters 6 to 8 mm) with the studied isolate. Small strips of bark (10 × 4 mm) were cut from the stems (around 30mm from the base of the stem) and pieces of PDA colonized by the fungus were placed in the wounds and covered with wet sterilized cotton and sealed with Parafilm. Eight seedlings inoculated with sterile PDA served as negative controls. The plants were maintained in the greenhouse and observed every two weeks for nine months. After that, plants were subjected to fungal re-isolation, with the obtained culture being studied by molecular methods as previously described. The resulting sequences were also deposited in GenBank database with the accession numbers MZ440614 (ITS) and MZ476039 (*benA*).

Results and Discussion

Morphological and molecular characterization of the fungus

The isolate was initially identified morphologically as *Neofusicoccum* sp.. Moreover, the isolate developed abundant, white aerial mycelium after 3-4 days on PDA having then gained a dark grey pigment after 7-10 days (Figure 1).

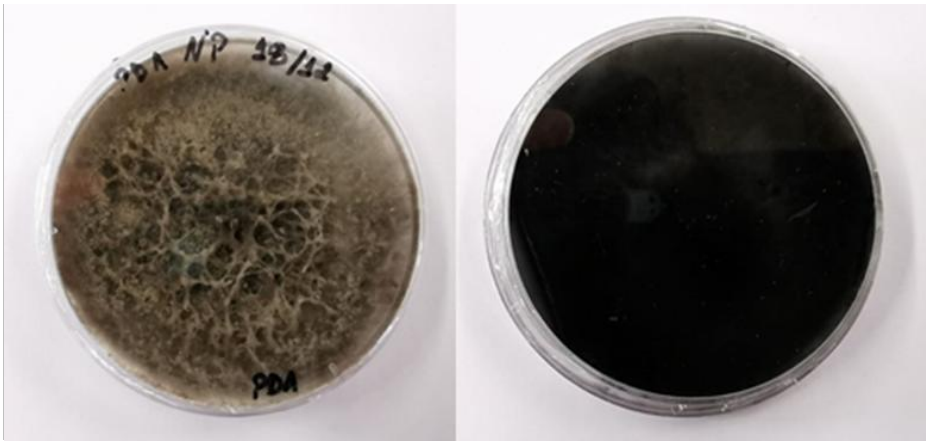

Figure 1 - Isolate obtained from the diseased samples in PDA growth medium after 20 days. Pictured from front and back.

After 30 days, the cultures inoculated on water agar medium with sterile pine needles showed ascomata erumpent through the pine needles surface (Figure 2), which are in line with other descriptions of the species in literature (PHILLIPS *et al.*, 2013).

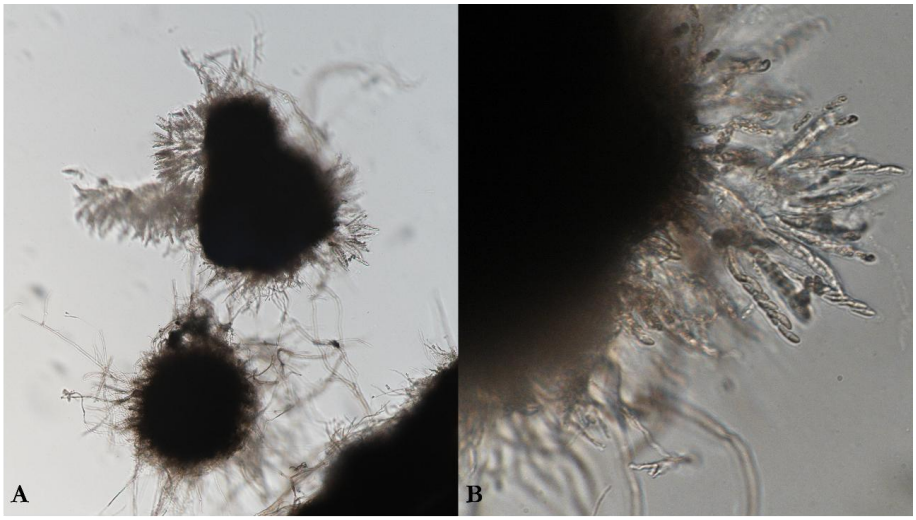

Figure 2 - *Neofusicoccum luteum* disrupted ascomas (A), with exposed asci bearing eight ascospores (B), observed under the microscope with a magnification of 100x and 400x respectively.

As mentioned previously, the obtained sequences from the original isolate and from the one recovered from inoculated plants were handled using NCBI's blastn tool (ALTSCHUL *et al.*, 1997) with the program optimized for highly similar sequences. The results showed that every sequence from the original isolate (sequences MZ440613 and MZ476038) and from the recovered fungal culture (sequences MZ440614 and MZ476039) were identified as *N. luteum*, with sequence MZ440613 showing 100% identity similarity with isolates previously identified as *N. luteum* (GenBank MH183385.1), sequence MZ476038 100% identity similarity (GenBank MH118938.1), sequence MZ440614 100% identity similarity (GenBank MH183385.1) and sequence MZ476039 100% identity similarity (GenBank MH118938.1). The sequences from the original isolate and the one obtained from Koch's postulates were also compared using BLAST's multiple sequence alignment tool. Comparing both ITS sequences (sequences MZ440613 and MZ440614) and comparing both β-tubulin gene sequences (sequences MZ476038 and MZ476039) they showed a 100% identity similarity between them respectively. Moreover, the recovered isolate's identity assessment by phylogenetic interference of the dataset consisting of a concatenated matrix of ITS and *benA* individual alignments, confirmed the identity of the isolate as being *N. luteum* (Figure 3).

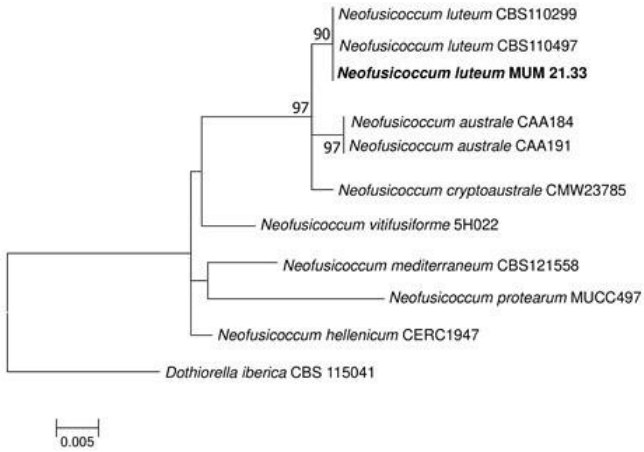

Figure 3 - Maximum likelihood phylogenetic tree obtained from an ITS/*benA* concatenated alignment (887 characters, 517 for ITS and 370 for benA. **MUM 21.33** in the tree is the original isolate. The tree was rooted to *Dothiorella iberica* CBS 115041. The scale bar indicates the number of substitutions per site and the bootstrap values (>75% based on 1000 replicates) are also shown.

Pathogenicity tests

The first symptoms appeared after two months in two inoculated plants, and after nine months only 50% of the plants inoculated present symptoms on the twigs around the lesions (Figure 4). Symptomatic plants did not show severe symptoms or visible necrosis around the wound on the main stem and none of them died. No external symptoms were observed on control plants. The fungal pathogen inoculated was re-isolated from all four symptomatic plants thus confirming Koch's postulates. These results indicate that *N. luteum*, the only pathogen detected on the sampled impaired tree, cause disease in *C. sempervirens*.

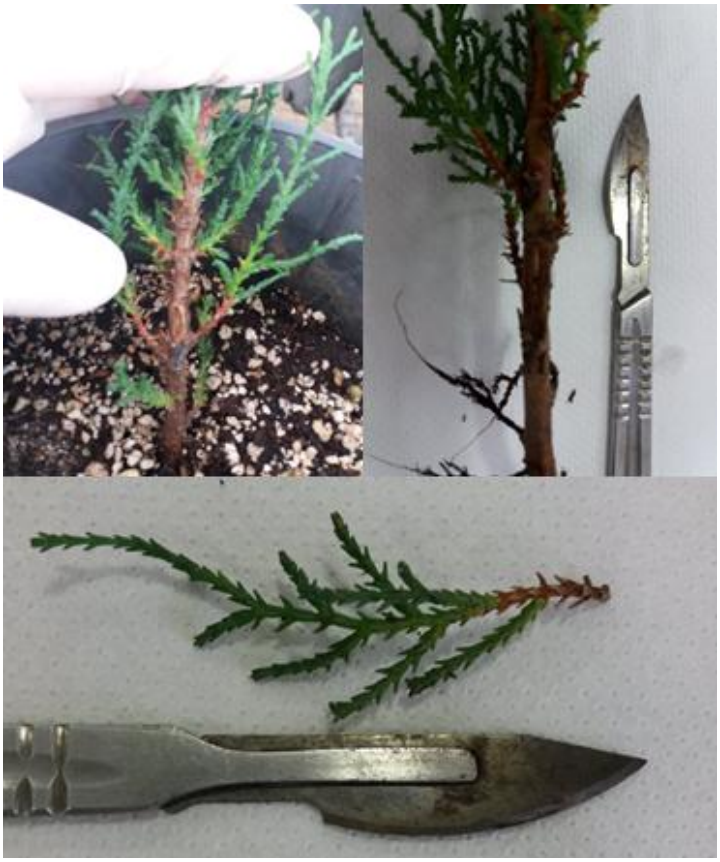

Figure 4 - Lesions in *C. sempervirens* plants after inoculation with *N. luteum* isolate.

These results are new evidence that *N. luteum* can cause disease on *C. sempervirens*. This species has previously been reported on this host (ALVES *et al.*, 2013), however no signs of it causing disease were ever reported on this conifer. As such, this is, as far as the authors are aware, the first time *N. luteum* is reported to cause disease on *C. sempervirens*. Moreover, it is also reported as a potential threat to other hosts, as is the case of eucalyptus (PAVLIC *et al*., 2007) and grapevines (AMPONSAH *et al.,* 2014). Its history as a pathogen for other conifers and other woody hosts, alongside the already known capabilities of other *Neofusicoccum* species causing dieback on this host, make a strong case for *N. luteum* to be considered as a brand-new light pathogen for *C. sempervirens*, which can cause mild lesions and cankers on some occasions.

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