ISOLATION AND IDENTIFICATION OF FUNGI WITH POTENTIAL FOR BIOLOGICAL STUMP REMOVAL OF *EUCALYPTUS* SPP.

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This study aimed to identify and evaluate the decay capability of fungi that occur naturally in eucalyptus plantations of three cities in Espírito Santo state, Brazil. Hence, discs from deteriorated stumps were collected and samples isolatied to obtain pure fungal cultures. An experiment using Petri dishes with malt-agar medium and eucalyptus sapwood was performed in order to test the decay of each isolate. The experiment was evaluated by mass loss every four weeks during a total of 20 weeks. Fungi with decay capability were selected and identified via extraction of genomic DNA. Seven isolates were identified: *Penicillium implicatum, Resinicium confertum, Phanerochaete chrysosporium, Cladosporium* sp., *Penicillium* sp., *Trichoderma citrinoviride* and *Acremonium* sp. Isolation and identification of fungi from eucalyptus stumps provided naturally occurring decay fungi that inhabit the eucalyptus plantations that were adapted for this study.

Keywords: Fungi selection, molecular identification, stump decay, wood-decaying fungi

INTRODUCTION

Fungi capable of causing changes in the physical and mechanical properties of wood are found in the phylum of Basidiomycota, Ascomycota and Deuteromycota (Kluczek-Turpeinen et al. 2005, Liers et al. 2011, Riley et al. 2014). The basidiomycetes include mushrooms, plant and animal pathogens, and saprophytic fungi. This phylum has the main decomposers of terrestrial ecosystems (Urairuj et al. 2003).

The wood degrading fungi, Basidiomycetes, vary mainly in relation to their lignocellulolytic capacity, and is classified as white-rot or brown-rot fungi (Tortella et al. 2008). The fungi causing white-rot use hydrolases that gradually degrade cellulose and lignin, and mineralise then to CO_2 and H_2O with peroxidase enzymes, while brown-rot fungi rapidly depolymerise cellulose, and lignin remains as residue (Martínez 2002).

Ascomycetes and Deuteromycetes, capable of decaying wood, are classified as soft-rot fungi which mainly affect the external surface of wood, leaving it darkened and soft, with the presence of transverse cracks when dried (Hatakka &

Hammel 2010). These fungi are problematic on wood and wood-based materials, but also may have potential for beneficial biodegradation, such as biopulping or bioincising (Wu et al. 2005, Franco et al. 2006, Çöpür & Tozluoğlu 2007, Mendonça et al. 2008, Schubert et al. 2011, Thaler et al. 2012). Another beneficial application for processes involving wood is the biological stump removal in forest plantations, whereby xylophagous fungi accelerate the process of deterioration of the stumps that remained after the trees were harvested (Alonso et al. 2007, Andrade et al. 2012, Negrão et al. 2014, Silva et al. 2014).

The process of stump removal in forest plantations is an important step to clean the area for replanting. This process is usually performed mechanically with the use of implements and machines. Mechanical stump removal increases costs, besides causing damages to the soil by increasing the degree of compaction and removal of nutrients during the displacement of the organic blanket which accumulates on the surface (Alonso et al. 2007, Hope 2007).

Due to large areas of *eucalyptus* plantations in Brazil, the improvement of techniques used in this sector has been increasingly researched, always aiming at reducing costs and damage to the environment. As such, biological stump removal using wood-decaying fungi appears as a sustainable and affordable alternative for substituting the process of mechanical stump removal. Thus, the present study aimed to identify and evaluate the deteriorating potential of fungi that occur naturally in eucalyptus plantations located in three municipalities in Espírito Santo, Brazil.

MATERIALS AND METHODS

Collection of material and isolation of fungi

Discs (± 3.0 cm thickness) of deteriorated stumps were collected in three municipalities in Espírito Santo, Brazil: Cachoeiro de Itapemirim (20°46' S, 41°18' W, 100 m), São José do Calçado (20°55' S, 41°37' W, 590 m) and Jerônimo Monteiro (20°51' S, 41°27' W, 800 m). The materials were properly identified and stored in bags of porous paper. For the isolation of fungi, the disks were transformed into samples measuring approximately 1.0 × 1.0 × 3.0 cm (radial × tangential × longitudinal), removed from the transition area which is located between the healthy and decomposed portions.

The indirect isolation of the fungi was carried out in a laminar flow chamber, where the samples were disinfected in sodium hypochlorite solution (2%) for 15 seconds, washed in sterile distilled water, rapidly flamed and aseptically transferred to Petri dishes containing malt:agar (2:1.5%) culture media, on the basis of one sample per plate (Alonso et al. 2007, Alfenas et al. 2016).

Petri dishes were placed in an incubation room (25 ± 2 °C and 65 ± 5% relative humidity) in the absence of photoperiod, until fungi mycelial growth was observed. Isolation was conducted to obtain pure cultures by transferring fungi structures (spores and hyphae) to Petri dishes containing malt agar culture media.

Evaluation of deterioration potential

The objective of this experiment was to test the deterioration potential of each isolate and to select those with the greatest potential for field tests, as presented by Costa et al. (2017). In addition to the isolates from the field, three fungi with recognised deterioration capacity were used as positive controls, i.e, *Trametes versicolor* (Mad 697) (white-rot) and two brown-rot fungi, *Postia placenta* (Mad 698) and *Gloeophyllum trabeum* (Mad 617). In addition, two basidiomycetes that presented satisfactory deterioration capacity in a previous study were also used Silva et al. (2014).

During the assembly of the experiment, five Petri dishes were used containing the respective malt:agar culture media for each fungus mentioned above. Sapwood samples of *Eucalyptus grandis* × *Eucalyptus urophylla* (rotational age six years) with dimensions 1 × 1 × 1 cm (radial × tangential × longitudinal) were used to test the deterioration capacity of the isolates.

The wood samples were oven-dried at 103 ± 2 °C for 48 hours, weighed to obtain the initial dry mass and then autoclaved. After 15 days, the time required for the mycelial growth of the fungus throughout the Petri dish in a laminar flow chamber, the wood samples were rapidly wetted in sterile distilled water and placed in the ratio of 10 samples per Petri dish.

The Petri dishes were incubated (25 ± 2 °C and 65 ± 5% relative humidity) in the absence of photoperiod. The experiment lasted for 20 weeks where evaluation was carried out every four weeks. At the end, the wood samples were removed from the Petri dishes and the mycelium was brushed off, and then oven-dried at 103 ± 2 °C for 48 hours to obtain the final dry mass and mass loss (%) (Figure 1).

Molecular identification of fungi with potential for deterioration

The fungi with potential for wood deterioration were selected and sent for molecular identification in the Laboratory of Seed Pathology and Post-Harvest, Department of Plant Pathology, Federal University of Viçosa, Minas Gerais, Brazil.

The fungi were grown in potato dextrose agar (PDA) and kept in an incubator at 25 ± 2 °C and 65 ± 5% relative humidity for up to four weeks, depending on the growth rate of the isolate, and were subsequently sent for identification.

The mycelium of the isolate was removed with a scalpel and placed in 1.5 mL microtubes. The extraction of genomic DNA was performed using a Wizard® Genomic DNA Purification Kit, as described in Pinho et al. (2012).
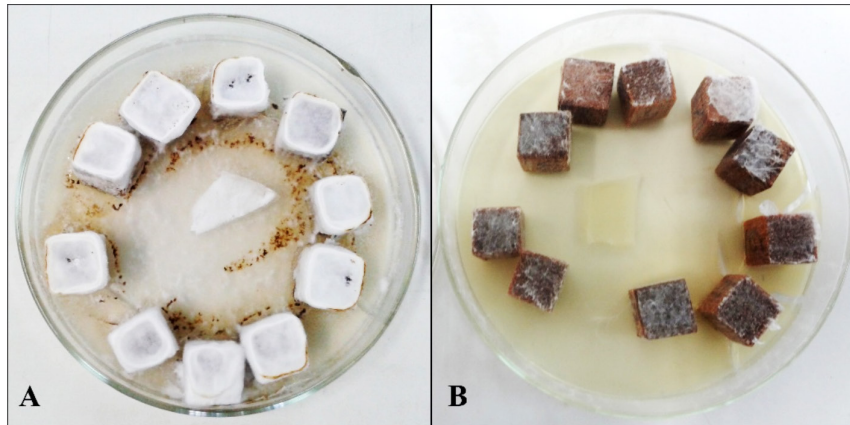
Figure 1   Wood blocks submitted to deterioration by *Acremonium* sp. (A) and *Resinicium confertum* (B) at the end of 16 and 4 weeks, respectively

The primers ITS1 (5'-TCCTCCGCTTATT GATATGC-3') and ITS4 (5'-GGAAGTAAAAGT CGTAACAAGG-3') were used to amplify the internal transcribed spacer (ITS) region of rDNA (White et al. 1990). Polymerase chain reaction (PCR) (amplicon) reactions included the following ingredients for each 25 µl reaction: 12.5 µl of 2X DreamTaq™ PCR Master Mix (MBI Fermentas, Vilnius, Lithuania), 1 µl of 10 µM of each forward and reverse primer synthesised by Invitrogen, 2 µL of genomic DNA (25 ng µl-1), and nuclease-free water to complete the total volume.

The amplifications were performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, initial extension at 72 °C for 30 s and a final extension of 7 min at 72 °C. The amplified products were visualised on a 1% agarose gel to check the product size and purity. The PCR products were purified and sequenced by Macrogen, South Korea.

The nucleotide sequences were edited using the CodonCode Aligner software. All sequences were analysed and the arrangement of the nucleotides at ambiguous positions were corrected by comparison of sense and antisense sequences. The isolates were previously identified by analysis of the ITS sequences in the UNITE database (Nilsson et al. 2014).

Analysis and evaluation of the results

The potential of deterioration of the isolated fungi was evaluated as a function of the mass loss of the wood samples. For this, a completely randomised design was used in a factorial arrangement in which the effect of the isolated fungi and the time of exposure on mass loss (4, 8, 12, 16 and 20 weeks) were analysed.

The Scott-Knott test at 5% significance was used for factors detected as significant by the F test. The mass loss values were transformed into arcsen to meet the requirements of normality and homogeneity of variances.

RESULTS AND DISCUSSION

Isolation and molecular identification of fungi with potential for deterioration

Deteriorated discs of eucalyptus stumps collected from the field resulted in the isolation of 66 pure cultures, of which seven were taxonomically identified as being the ones that caused the greatest mass loss, and were therefore considered to have deterioration potential.

The seven isolates were *Penicillium implicatum and Resinicium confertum* from São José do Calçado, *Phanerochaete chrysosporium* from Jerônimo Monteiro, *Cladosporium* sp., *Penicillium* sp. and *Trichoderma citrinoviride* from Cachoeiro de Itapemirim, and *Acremonium* sp. (Silva et al. 2014), described as Basidiomycete 1.

According to literature, *Penicillium implicatum* does not cause damage to wood. This fungus belongs to the phylum Ascomycota and is an important pathogen associated with the pomegranate fruit (*Punica granatum*), which has irregular patches of brown to caramel on the fruit bark (Labuda et al. 2004, Khokhar et al. 2013, Munhuweyi et al. 2016).

There were no records on word deterioration potential of *Resinicium confertum* fungus, belonging to the phylum Basidiomycota. However, the *Resinicium bicolor* fungus, belonging to the same genus, causes white rot in wood. It is also used in industrial processes, for example in the biofibrillation of wood from *Picea abies* (Bredberg et al. 2002, Job 2002, Toljander et al. 2006, Nakasone 2007, Folman et al. 2008).

Phanerochaete chrysosporium, belonging to the phylum Basidiomycota, causes wood deterioration and is classified as a white rot fungus (Fernandez-Fueyo et al. 2012, Skyba et al. 2016). This potential deteriorator was reported by Koyani and Rajput (2015) with *Azadirachta indica* wood, in which *P. chrysosporium* caused a mass loss of 29% after 120 days of exposure to this fungus.

The fungi of the genus *Cladosporium*, belonging to the phylum Ascomycota, are present in wood during deterioration process (Held et al. 2006, Kirker et al. 2012). The presence of this genus was described in plots of *Pinus radiata* treated with chromium copper arsenate (CCA), where the fungi *Cladosporium oxysporum* and *Cladosporium sphaerospermum* were identified as dominant species (Kim et al. 2007). There are also reports of the presence of *C. cladosporoides* in the deterioration of aged cotton fibers in museums, and the presence of *Cladosporium* sp. in the deterioration of pieces art in closed environments (Pangallo et al. 2009, Kavkler et al. 2015).

Fungi, from the genus *Penicillium,* are often consideredan causative agents in the deterioration of wood. This genus is commonly found in wood during the decomposition stage and internal environments, known as "internal moluds" (Clausen & Yang 2007, Kirker et al. 2012). *Penicillium chrysogenum*, described as a soft rot fungus, is able to penetrate cell walls in less than a month, deteriorating cellulose, but not lignin (Hamed 2013).

The fungus *Trichoderma citrinoviride,* from the phylum Ascomycota, is commonly found in soil and decaying woods (Jeleń et al. 2014). It has the ability to deteriorate wood as it produces the main enzymes of cell wall decay, i.e. cellulases and xylanases (Blaszczyk et al. 2016). The fungus *Trichoderma reesei*, belonging to the same genus of the identified fungus, is classified as a soft rot fungus (Shrestha et al. 2009). In the study by Koyani et al. (2011), the white rot fungus, *Trichoderma harzianum*, caused 43% mass loss in *Azadirachta indica* wood after 120 days of exposure. The genus *Acremonium* has not been reported as a causative agent of wood rot. The *Acremonium cucurbitacearum* is responsible for the root rot and decline of melon vine (*Cucumis melo*), and *Acremonium recifei* is the cause of rapid decline resulting from tree trunk decay of macadamia, (Ko 2009).

Among fungi with wood decay potential, brown- and white-rot fungi (basidiomycetes) provide greater structural damage to the wood cell wall (Ortiz et al. 2013). In the present study, *R. confertum* and *P. chrysosporium* belong to this class of fungi, therefore causing greater wood deterioration.

The other isolates belong to Ascomycota phylum, classified as soft-rot or mould with saprophytic characteristics in most of the aforementioned studies. Although these fungi have low or moderate wood decay capacity, they are capable of causing significant damage under favorable and long-acting conditions. Also, they are often part of the succession process of microorganisms that act together during wood decay process (Oliveira et al. 1986)

Only *Acremonium* sp. is presented as a phytopathogen, however, no citations were found for forest plantations. Isolates obtained from decaying stumps from local eucalyptus plantations minimise the potential for detrimental impact on land reuse for eucalyptus.

Assessment of deterioration potential

Analysis of variance indicated a significant difference between the fungus parameters, time and interaction of fungi x time. A comparison between means of mass loss in fungus x time interaction, it was observed that mass loss in the first month was statistically the same for all fungi, except for *P. placenta* and *T. citrinoviride*, which had lowest means (Table 1).

In the second month, *G. trabeum* caused the highest mean mass loss, differing statistically from the others. Among the isolates obtained in the field, *P. implicatum* and *P. chrysosporium* achieved the second best performance, not statistically differing from *T. versicolor*, which obtained a 50% lower mass loss, compared to *G. trabeum*.

In the third and fourth months, *G. trabeum* and *T. versicolor* caused greater mass loss, differing statistically from others. In the fourth month, *P. placenta* did not differ from the previously

Table 1　　Comparison between the means of mass loss in wood samples as a function of time and fungi tested

Fungus	Mass loss (%)				
	Time (months)				
	1	2	3	4	5
Trametes versicolor	1.72[aD]	4.90[bC]	15.45[aA]	12.13[aB]	10.11[B]
Gloeophyllum trabeum	0.66[aD]	9.87[aB]	12.62[aA]	14.59[aA]	6.57[cC]
Postia placenta	0.01[bD]	2.73[cC]	4.68[cC]	11.79[aB]	17.23[aA]
Penicillium implicatum	1.22[aB]	6.37[bA]	6.31[bA]	7.62[bA]	9.06[bA]
Resinicium confertum	1.41[aC]	3.29[cB]	6.12[bA]	3.70[cB]	6.25[cA]
Acremonium sp.	0.50[aC]	2.84[cB]	5.60[bA]	5.72[bA]	5.48[cA]
Phanerochaete chrysosporium	0.80[aB]	7.07[bA]	0.85[eB]	5.98[bA]	5.02[cA]
Cladosporium sp.	0.93[aC]	4.01[cB]	6.35[bA]	2.80[cB]	3.11[dB]
Penicillium sp.	1.13[aD]	2.58[cC]	3.70[cB]	8.14[bA]	0.94[eD]
T.citrinoviride	0.39[bC]	1.33[dC]	2.49[dB]	1.62[dB]	6.88[cA]
Mean	0.84[C]	4.91[B]	6.17[A]	7.25[A]	6.38[A]

Means followed by the same lowercase (column) or uppercase (line) letter do not differ statistically; Scott-Knott test $p > 0.05$

analysed fungi. Among the fungi that presented the second best performance, *Penicillium* sp. obtained the best mean of mass loss, ie. 44% lower compared to *G. trabeum*.

In the fifth month, *P. placenta* obtained the highest mean of mass loss, differing statistically from others. Among the isolates from field, *P. implicatum* was prominent in relation to the others in the fifth month, not differing statistically from *T. versicolor*.

During the analysis of the mass loss for each fungus as a function of the months, it was observed that, *T. versicolor* and *G. trabeum* showed greatest mass losses in the third month, and a significant decrease in the fifth month. The *P. placenta* gradually increased mass loss a until the fifth month, reaching the largest mass loss among the fungi tested.

Among the isolated fungi in the field, it was observed that *P. implicatum* reached a higher mean of mass loss in the fifth month, however, no significant differences were observed during the second month of attack. A similar result was observed for *P. chrysosporium*, where the greatest loss occurred at the second month.

Among the other isolates tested, the greatest mass loss was reached after the third month of exposure, and some cases of decrease in subsequent months. Since the samples were mounted on different Petri dishes for each month, variations in samples and fungal attacks may have occurred. In all the fungi tested, it was observed that there was a significant increase in the mass loss until the third month, not differing statistically compared to the subsequent ones.

Monteiro and Freitas (1997) and Andrade et al. (2012) tested fungi deterioration capacity in a similar way to the one performed in this study, with Petri dishes and culture medium, finding potential fungi. Results from Monteiro and Freitas (1997) showed that, after eight weeks of exposure to *G. trabeum*, mass losses of 3.94, 4.24, 3.40, 3.82 and 24.91% was observed for corresponding wood of *Astronium* sp., *Anadenanthera* sp., *Hymenaea* sp., *Aspidosperma polyneuron* and *Araucaria Angustifolia*, respectively.

Andrade et al. (2012) isolated *Polyporus guianensis*, *Pycnoporus sanguineus* and *Ganoderma applanatum* from eucalyptus stumps in the field, which caused deterioration in *Eucalyptus grandis* wood, 0.03, 29.0 and 34.0%, respectively, after eight weeks of exposure.

CONCLUSIONS

Isolation of fungi from eucalyptus stumps showed the genera and fungal species with deteriorating potential that inhabit eucalyptus plantations in the regions studied.

Based on the study and in literature, the method used to evaluate the deterioration capacity of isolated fungi allowed a faster and

satisfactory selection of fungi with potential for deterioration, thus allowing the selected fungi to be tested by using other methodologies and even field experiments in order to know the realistic capacity of wood deterioration.

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