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Leaf extracts of *Casearia sylvestris* and *Casearia decandra* affect growth and production of ligninolytic enzymes in wood decay basidiomycetes[1]

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ABSTRACT - (Leaf extracts of *Casearia sylvestris* and *Casearia decandra* affect growth and production of ligninolytic enzymes in wood decay basidiomycetes). White-rot basidiomycetes are able to deteriorate wood products and be pathogenic to living trees, requiring, thus requiring control. The tropical flora is an important source of eco-friendly antifungal compounds; however, the knowledge on how leaf extracts affect the fungal physiology is limited. Therefore, in the present work we investigated the influence of ethanolic leaf extracts of *Casearia sylvestris* and *C. decandra* at 0.1 mg mL^{-1} on the production of ligninolytic enzymes by *Trametes villosa*, *Ganoderma australe* and *Pycnoporus sanguineus*. Overall, the extracts inhibited the mycelial growth and the production of biomass. Additionally, *C. sylvestris* extract reduced the production of manganese peroxidase and laccase; however, the exposure to *C. decandra* extract resulted in variable responses. Therefore, enzymes related to lignin degradation are potential targets to control wood decay fungi by plant bioactive compounds, as their ability to colonize the substrate may be impaired.
Keywords: antimicrobial, biodeterioration, lignin, phytopathogen, white rot

RESUMO - (Extratos foliares de *Casearia sylvestris* e *Casearia decandra* afetam crescimento e produção de enzimas ligninolíticas em basidiomicetos deterioradores de madeira). Basidiomicetos de podridão branca são capazes de deteriorar madeira e atuar como patógenos de árvores. A flora tropical é uma importante fonte de compostos antifúngicos ecologicamente seguros, entretanto, o conhecimento de como extratos vegetais afetam a fisiologia fúngica é limitado. Portanto, neste trabalho foi investigada a influência de extratos etanólicos de folhas de *Casearia sylvestris* e *C. decandra*, na concentração de 0,1 mg mL^{-1}, sobre a produção de enzimas ligninolíticas por *Trametes villosa*, *Ganoderma australe* e *Pycnoporus sanguineus*. De modo geral, os extratos inibiram o crescimento micelial e a produção de biomassa. Além disso, o extrato de *C. sylvestris* reduziu a produção de manganês peroxidase e lacase, porém as respostas ao extrato de *C. decandra* foram variáveis. Deste modo, enzimas envolvidas na degradação da lignina são alvos potenciais para o controle de fungos causadores de podridão em madeira por compostos vegetais bioativos, uma vez que a capacidade desses microrganismos para colonizar o substrato pode ser prejudicada.
Palavras-chave: antimicrobiano, biodeterioração, fitopatógeno, lignina, podridão branca

Introduction

White-rot wood basidiomycetes perform an essential role in nutrient cycling in ecosystems. In addition, these fungi have unique enzymatic system with applications in bioremediation, pulp and paper, textile, and food industries (Elisashvili *et al*. 2010). However, under certain conditions, many fungal species can deteriorate timber products such as railway sleepers, utility poles, and fences, causing significant economic losses. Furthermore, such microorganisms can act as phytopathogens attacking living trees in urban and forest areas (Harsh & Bisht 1997, Luley 2005).

In order to degrade lignin, wood decay fungi produce a complex set of extracellular ligninolytic enzymes including manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2). These enzymes

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are able to degrade oxidatively the lignin shield around the cellulose and hemicellulose components of the plant cell wall (Lundell *et al*. 2010, Levasseur *et al*. 2013). Thus, the action of ligninolytic enzymes provides access to cell wall polysaccharides that subsequently are converted to soluble sugars by hydrolytic enzymes as cellulases and hemicellulases. Therefore, it is hypothesized that compounds able to inhibit the ligninolytic system of fungi may affect negatively colonization and degradation of plant materials as the accessibility to carbon source is impaired.

Traditional methods to control wood decay and phytopathogenic fungi employ synthetic and inorganic compounds that can adversely affect the environment as well as the human health. The increasing concern on the use of chemicals have stimulated the development of alternative methods to control wood deteriorating microorganisms including the use of microbial antagonists and natural compounds as plant extracts and oils (Singh & Singh 2012, Tascioglu *et al*. 2013).

Tropical flora is rich in species that may serve as a source of new antifungal compounds, natural, and environmentally less harmful than chemical biocides. The genus *Casearia* (Salicaceae) has been used in traditional medicine and several pharmacological properties have been attributed to these plants, including antimicrobial activity (Santos *et al*. 2010, Ferreira *et al*. 2011). In previous study, we reported for the first time the antifungal activity of *Casearia* extracts on wood decay fungi. Gas Chromatography Mass Spectrometry (GC/MS) analysis identified a clerodane diterpenoid in leaf extract of *C. sylvestris*, and hydroquinone, β-sitosterol and cinnamic acid in leaf extract of *C. decandra* (Bento *et al*. 2014). Many publications have described the antimicrobial activity of extracts from plant species; however, there are few studies on their mode of action. The knowledge on how plant-derived compounds affect fungal physiology allows to determine vulnerable enzyme systems to be explored as targets and to predict the risk of resistance development.

Trametes villosa, *Ganoderma australe* and *Pycnoporus sanguineus* are among the most important saprophytic white-rot wood basidiomycetes, able to degrade completely all components of lignocellulose. However, these fungi are also capable of causing important economic losses in wood materials and are associated with wood decay in living trees in tropical and subtropical areas (Harsh & Bisht 1997, Luley 2005). Therefore, the present study was aimed at assessing the activity of ethanolic leaf extracts of *C. sylvestris* and *C. decandra* on growth and production of ligninolytic enzymes of the three white-rot wood fungi.

Materials and methods

Leaf extracts - The ethanolic extracts from leaves of *Casearia sylvestris* Swartz (RM17) and *C. decandra* Jacq (M742) were obtained from the plant extract bank located in the Center for Plant Physiology and Biochemistry Research of the Institute of Botany, Sao Paulo, Brazil. The leaf extracts were previously analyzed by GC/MS (Bento *et al*. 2014).

Microorganisms - The basidiomycetes *Trametes villosa* (CCIBT 2628), *Pycnoporus sanguineus* (CCIBT 3732) and *Ganoderma australe* (CCIBT 3801) were obtained from the Algae, Cyanobacteria and Fungi Culture Collection of the Institute of Botany of São Paulo (CCIBt), Brazil.

Antifungal activity - Twenty milliliters of autoclaved Potato Dextrose Agar (PDA) at 45 °C were added in 9 cm diameter sterile plates containing 0.025 g mL^{-1} of sugarcane bagasse powder. After solidification, a 9 cm diameter cellophane membrane was aseptically added on the medium surface. A 2 mm diameter agar plug containing mycelia of each fungus was transferred to the cellophane membrane-covered PDA medium and the plates were kept at 28 ± 2 °C for 4 days. Thereafter, the cellophane membranes containing the fungal colony were aseptically removed and transferred to new plates containing 20 mL of PDA medium supplemented with 0.025 g mL^{-1} of sugarcane bagasse powder and ethanolic extract at a final concentration of 0.1 mg mL^{-1}. In the control plates, the ethanolic extract was replaced by the same volume of ethanol 99.8%. The plates with the fungal colonies were maintained at 28 ± 2 °C and every 4 days, during 16 days, sample plates were collected in order to determine mycelial growth, biomass production (fresh weight), and to perform enzyme extraction. The experiment was carried out in triplicate.

Enzyme extraction - The cellophane membrane containing the fungal colony was removed from the plate and the solid medium used for extracting the enzymes secreted to the medium according to Ballaminut *et al*. (2014), with modifications. The solid medium was homogenized in 50 mM sodium acetate buffer, pH 4.5, in the proportion 1:3 (solid medium: buffer; w/v). The homogenate was kept under constant

agitation at 140 rpm for 1 h at 4 °C and subjected to vacuum filtration. The filtrate was collected and kept at -20 °C for one month prior to the enzyme assays. The protein concentration was determined by the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Enzyme assays - Laccase activity was determined by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (ε = 36000 mol^{-1} cm^{-1}) at 420 nm for 10 min at 25 °C. The reaction mixture contained 250 µL of 50 mM citrate-phosphate buffer, pH 4.0, 50 µL of ultrapure water, 100 µL of 5 mM ABTS, and 600 µL of protein extract. Total peroxidase activity was determined by using the same reagents employed for laccase activity, excepting that ultrapure water was replaced by 50 µL of 2 mM hydrogen peroxide. Total peroxidase activity was given by the difference between the value obtained in the reaction and the laccase activity value (Ballaminut *et al*. 2014).

Manganese peroxidase activity was determined by monitoring the oxidation of phenol red (ε = 4460 mol^{-1} cm^{-1}) at 610 nm for 10 min at 25 °C. The reaction mixture was constituted of 500 µL of protein extract, 300 µL of 200 mM sodium succinate buffer, pH 4.5, containing 100 mM sodium lactate and 0.5 % (w/v) bovine serum albumin, 50 µL of 2 mM manganese sulphate, 100 µL of 0.1 % (w/v) phenol red, and 50 µL of 2 mM hydrogen peroxide (Kuwahara *et al*. 1984).

The enzyme activities were expressed as U mg^{-1} protein, where one unit (U) corresponded to the amount of enzyme that oxidizes 1 µmol of substrate per min under the assay conditions.

Statistical analysis - Analysis of variance (ANOVA) using Tukey's test at $P < 0.05$ was used to examine significant differences between treatments. All results were expressed as mean ± standard deviation (SD).

Results and Discussion

Leaf extract of *C. sylvestris* reduced the mycelial growth of *T. villosa* by 48 % in the first 4 days of exposure; however, after 16 days of treatment the fungus was inhibited only by 15 % when compared to the control (figure 1a). *G. australe* (figure 1b) and *P. sanguineus* (figure 1c) were more susceptible to *C. sylvestris* extract as the mycelial growth was reduced by 76 and 72 %, respectively, after 16 days of exposure.

Overall, the inhibitory effect of *C. decandra* extract was less evident as the mycelial growth of the fungi was not significantly reduced at the end of the experiment. Interestingly, despite having limited effect on the growth rate on plate, *C. decandra* extract altered the morphology of the colonies that produced sparse aerial mycelium (data not shown). Bento *et al*. (2014) reported microscopic changes in hyphal morphology of *T. villosa* and *P. sanguineus* exposed to extracts of *Casearia* spp., including hyphal branching and segmentation, and formation of defective clamp connections. Production of sparse aerial mycelium

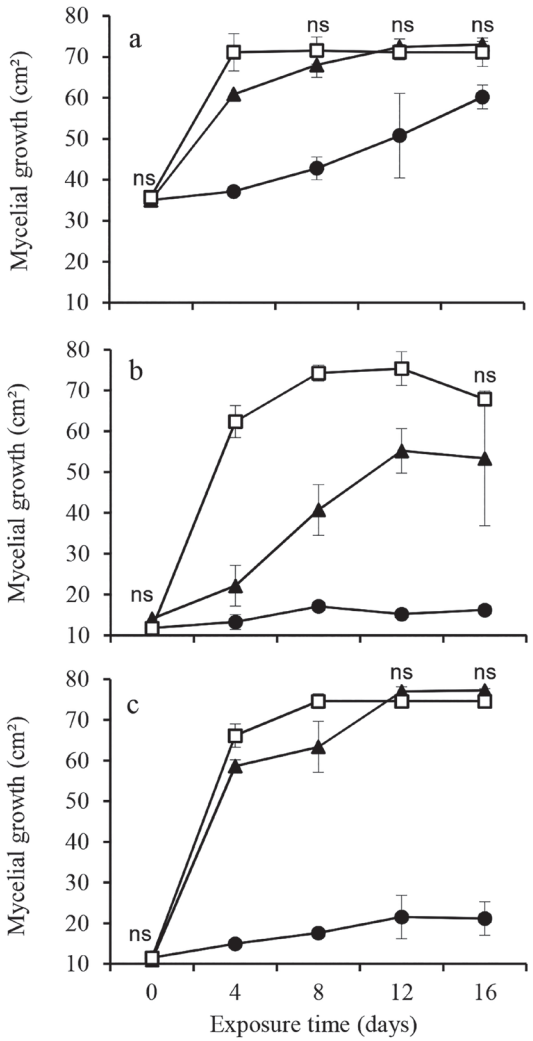

Figure 1. Mycelial growth of *T. villosa* (a), *G. australe* (b) and *P. sanguineus* (c) exposed to leaf extracts of *C. sylvestris* and *C. decandra*. The time 0 indicates the moment when 4 day-old cultures were transferred to PDA medium supplemented with sugarcane bagasse powder at 0.025 g mL^{-1} and leaf extracts at 0.1 mg mL^{-1}. Values are means of three replicates (± SD). ns = non-significant difference from the control at $P < 0.05$, Tukey's test. (—□—) Control; (—●—) C. sylvestris; (—▲—) *C. decandra*.

by the fungi exposed to *Casearia* extracts can result in lower production of biomass. In fact, all fungi presented reduced biomass after 16 days of treatment, approximately 72 % of inhibition for *T. villosa* (figure 2a) and 90 % of inhibition for *G. australe* (figure 2b) and *P. sanguineus* (figure 2c). Therefore, the production of biomass can be considered a better parameter than the mycelial growth to evaluate the antifungal activity of the extracts.

Reports on the antimicrobial activity of *Casearia* extracts are scarce and have been restricted mainly to human pathogens. Extracts from five species of *Casearia* inhibited the human pathogens *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Mosaddik *et al*. 2004). According to the authors, leaf extracts of *Casearia* sp. and *C. grewiifolia* presented the highest antimicrobial activity with minimal inhibitory concentration corresponding to >5 mg mL^{-1}, concentration 50-fold higher than that used in the present study.

Bento *et al*. (2014) identified a clerodane diterpenoid in leaf extract of *C. sylvestris*. Most of the pharmacological properties described for *Casearia* species are attributed to clerodane diterpenoids found mainly in leaves (Ferreira *et al*. 2011). Oberlies *et al*. (2002) isolated from leaves and twigs of *C. sylvestris* three clerodane diterpenoids denominated casearvestrins with cytotoxic activity on tumor cell lines and antifungal activity on *Aspergillus niger*. Bento *et al*. (2014) also identified hydroquinone, β-sitosterol and cinnamic acid in leaf extract of *C. decandra*. Studies have shown the antimicrobial activity of these compounds (Amoroso *et al*. 2009, Sova 2012, Wong *et al*. 2012), including the development of a wood preservative formulation based on cinnamic acid able to protected wood against *T. versicolor* for three months (Kartal *et al*. 2006).

Despite the reduced development of the fungi exposed to *C. sylvestris* extract, it was observed increased levels of extracellular proteins (figure 3). Overall, *C. decandra* extract did not affect the production of extracellular proteins by *T. villosa* and *P. sanguineus*; however, increased production of extracellular proteins by *G. australe* was observed after 8 days of exposure to the leaf extract. Bento *et al*. (2014) reported that exposure of *T. villosa* and *P. sanguineus* to *C. silvestris* and *C. decandra* extracts increased the production of catalase and glutathione reductase, enzymes related to defense against oxidative stress triggered by biotic and abiotic factors. Studies on proteins secreted by fungi exposed to natural compounds are scarce, although it is important to comprehend the physiological effects of these substances on target organisms.

It is essential for white-rot wood decay basidiomycetes to produce extracellular ligninolytic enzymes for complete lignocellulose decomposition. The leaf extracts of *C. sylvestris* and *C. decandra* affected significantly the production of manganese peroxidase and laccase by the three basidiomycetes (figure 4). Overall, the extract of *C. sylvestris* inhibited completely the production of the enzymes by the fungal species. Conversely, the results with *C. decandra* were variable, as its leaf extract inhibited completely the three enzymes in *G. australe*, and

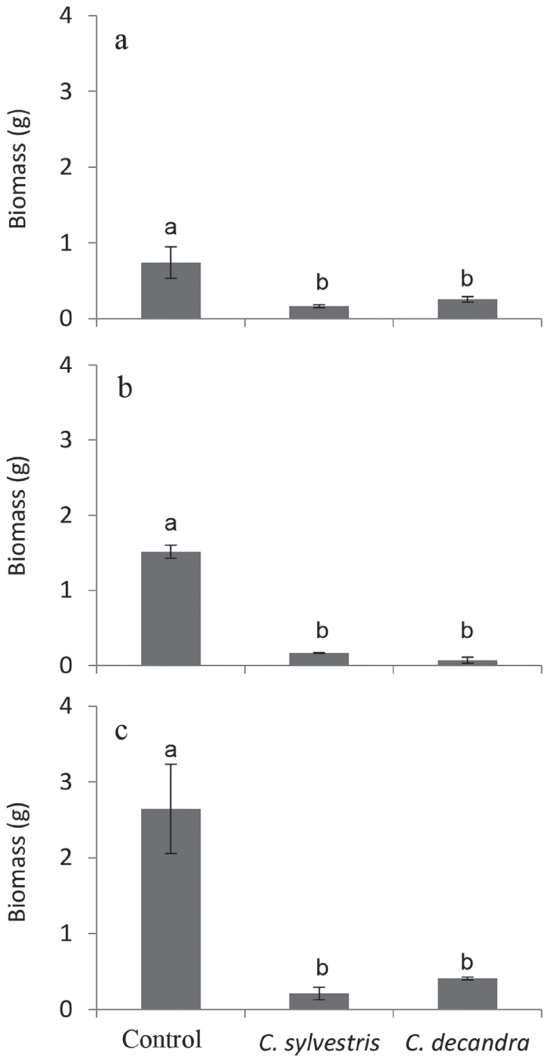

Figure 2. Production of biomass by *T. villosa* (a), *G. australe* (b) and *P. sanguineus* (c) after 16 days of exposure to leaf extracts of *C. sylvestris* and *C. decandra* at 0.1 mg mL^{-1}. Values are means of three replicates (± SD). Bars with the same letters are not significantly different at $P < 0.05$, Tukey's test.

manganese peroxidase in *P. sanguineus*; but, laccase was not significantly affected. *C. decandra* leaf extract inhibited manganese peroxidase and laccase in *T. villosa*; however, at the end of 16 days, the fungus recovered its normal production of manganese peroxidase, and the production of laccase overcame the control treatment by 50 %.

In normal conditions (control treatment), it was not detected production of total peroxidases by *T. villosa* and *P. sanguineus*; however, this enzyme activity was observed in *G. australe* after 12 and 16 days of growth. Furthermore, the three fungi produced manganese peroxidase and laccase; but, *T. villosa* produced higher levels of laccase than *G. australe* and *P. sanguineus*. Moreira-Neto *et al*. (2013) studying 12 basidiomycetes also observed higher ligninolytic potential of *T. villosa* compared with *P. sanguineus*. According to the authors, strains of *T. villosa* produced high levels of manganese peroxidase and laccase whereas *P. sanguineus* produced only low levels of laccase.

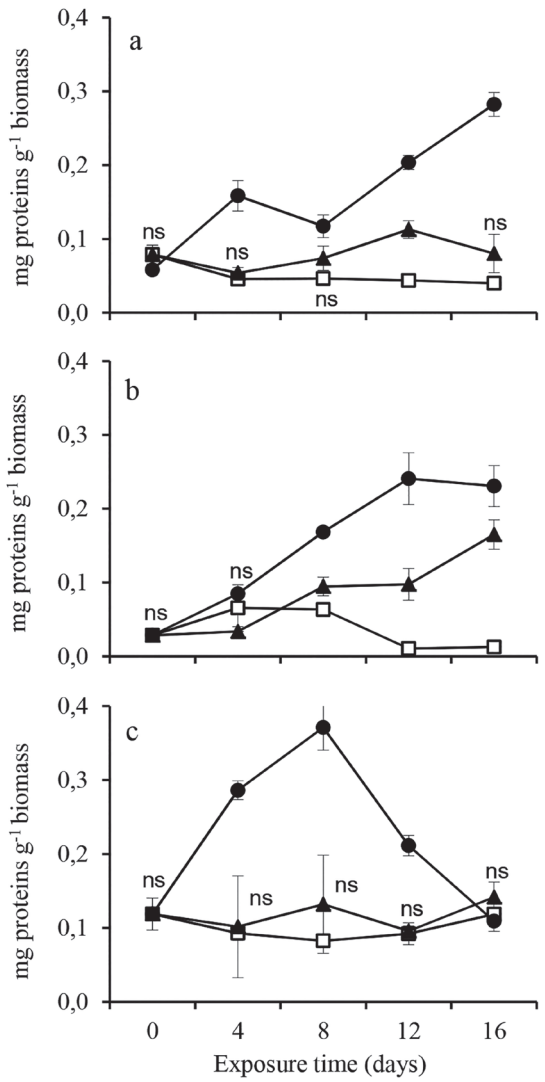

Figure 3. Production of extracellular proteins by *T. villosa* (a), *G. australe* (b) and *P. sanguineus* (c) exposed to leaf extracts of *C. sylvestris* and *C. decandra*. The time 0 indicates the moment when 4 day-old cultures were transferred to PDA medium supplemented with sugarcane bagasse powder at 0.025 g mL^{-1} and leaf extracts at 0.1 mg mL^{-1}. Values are means of three replicates (± SD). ns = non-significant difference from the control at $P < 0.05$, Tukey's test. (—□—) Control; (—●—) C. sylvestris; (—▲—) *C. decandra*.

It was observed increased levels of total peroxidases in *T. villosa* after 16 days of exposure to *C. decandra* extract, whose activity had not been detected in the control treatment. The increased production of total peroxidases in *T. villosa* may function as a defense mechanism against chemical stress caused by the aromatic compounds present in the *C. decandra* extract. Bento *et al*. (2014) observed increased levels of glutathione reductase in *T. villosa* exposed to *C. decandra* extract, indicating an oxidative stress process.

According to Souza *et al*. (2004), induction of laccases by aromatic compounds constitutes a protective response to toxic compounds generated during lignin degradation. Elisashvili *et al*. (2010) also observed increased production of laccase by *T. versicolor* in presence of hydroquinone, a compound present in *C. decandra* extract. Furthermore, it was observed induction of laccase in *G. lucidum* exposed to pyrogallol and 2,6-dimethoxyphenol. Terrón *et al*. (2004) reported that aromatic compounds, particularly, p-coumaric acid and guaiacol, increased the production of laccase by Trametes sp. I-62. The authors also observed that even structurally close-related compounds have different effects on the expression of three laccase isozyme genes. White-rot wood basidiomycetes display a wide diversity of responses to exposure to aromatic compounds. Induction or repression of ligninolytic enzyme encoding genes depends on fungal physiological, genetic, or ecological peculiarities (Myasoedova *et al*. 2008).

Therefore, fungal inhibition by the *C. sylvestris* leaf extract was accompanied by reduced production of ligninolytic enzymes; consequently, the ability of the basidiomycetes to colonize lignocellulose-based substrates may be impaired. In contrast, the inhibition by the *C. decandra* extract was not correlated to reduced enzyme production. Thus, further studies are necessary as the mechanisms involved in the inhibition of the fungi by natural compounds are probably

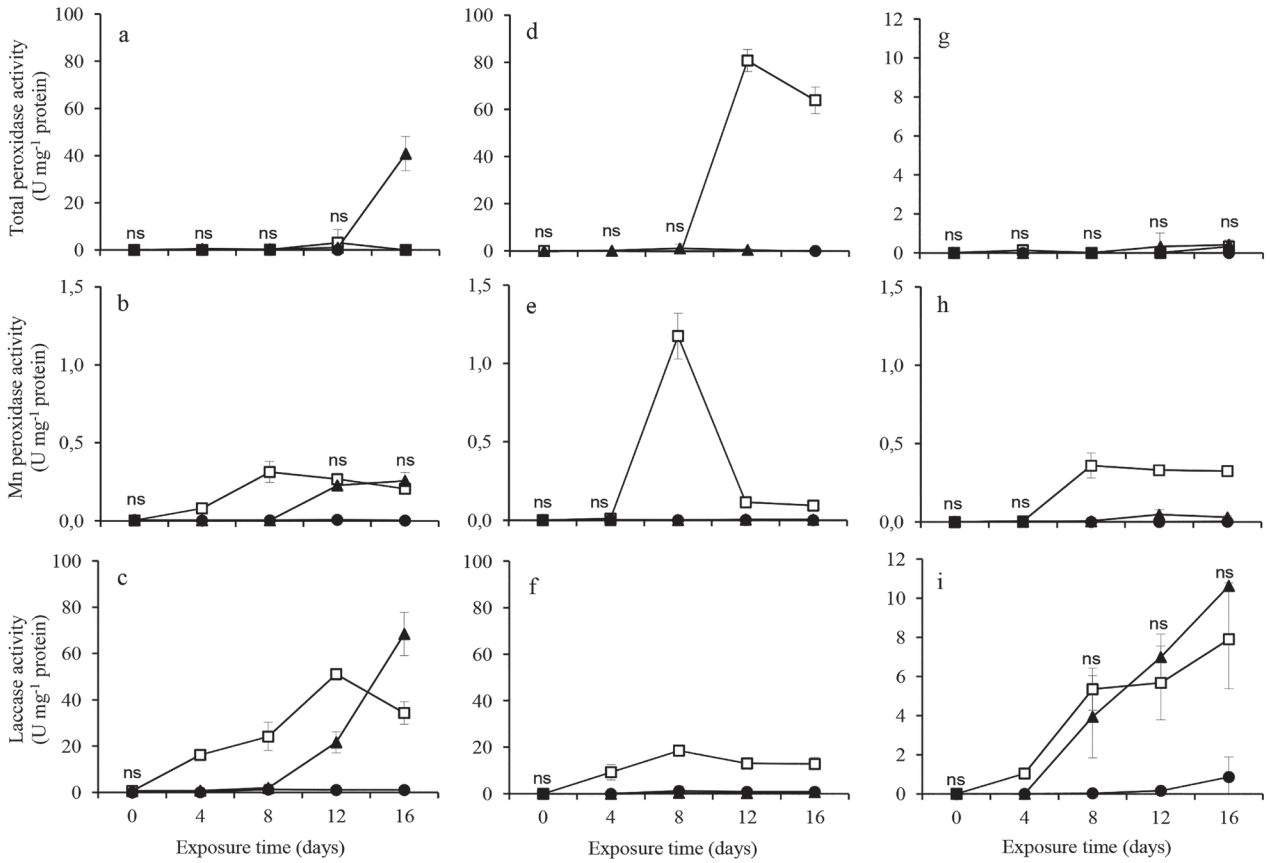

Figure 4. Total peroxidase, manganese peroxidase and laccase activities in *T. villosa* (a, b and c), *G. australe* (d, e and f) and *P. sanguineus* (g, h and i), respectively, exposed to leaf extracts of *C. sylvestris* and *C. decandra*. The time 0 indicates the moment when 4 day-old cultures were transferred to PDA medium supplemented with sugarcane bagasse powder at 0.025 g mL^{-1} and leaf extracts at 0.1 mg mL^{-1}. Values are means of three replicates (± SD). ns = non-significant difference from the control at $P < 0.05$, Tukey's test. (—□—) Control; (—●—) C. sylvestris; (—▲—) *C. decandra*.

complex and multifactorial. The present work provides useful information for the development of eco-friendly formulations based on *Casearia* extracts to preserve wood materials and to protect urban and forest trees against these wood decay fungi.

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