








A METHOD FOR EVALUATING ERGOSTEROL CONTENT IN WOOD-DECAY FUNGI

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ABSTRACT – Ergosterol is responsible for important functions in the fungal plasma membrane. The influence of fungitoxic agents on membrane ergosterol content is one of the most important mechanisms of antifungal action and its knowledge allows the generation of products that associate active compounds of different mechanisms, consequently improving the effectiveness of wood preservatives. Therefore, this study optimized a method for quantifying ergosterol in wood-decay fungi. The white-rot species selected were *Ganoderma applanatum* and *Trametes versicolor*, while the brown-rot were *Gloeophyllum trabeum* and *Lentinus lepideus*. Mycelial discs of each species were transferred to Petri dishes containing a cellophane-covered potato-dextrose-agar medium. Mycelia of each fungus were collected, weighed, and transferred to test tubes with 5 mL of 25% alcoholic potassium hydroxide. The tubes were vortexed for 5 min, subjected to ultrasound for 5 min, incubated at 85 °C for 4 h, followed by the addition of 2 mL of sterile distilled water and 5 mL of *n*-heptane and subsequent ultrasound shaking for 2 min. The *n*-heptane layer was analyzed by UV spectrophotometry between 230 and 300 nm. The blank sample only contained *n*-heptane. The mycelia wet weight of the fungi ranged from 0.061 to 0.296 g. Ergosterol content was 0.007% for *Lentinus lepideus* and 0.004% for the other species. The absorbance was higher than the ones observed in the blank for all samples. The adapted method was efficient for ergosterol extraction.

Keywords: Sterol; Mycelia; Extraction.

UM MÉTODO PARA AVALIAR O CONTEÚDO DE ERGOSTEROL EM FUNGOS APODRECEDORES DA MADEIRA

RESUMO – O ergosterol é responsável por importantes funções na membrana plasmática dos fungos. A influência de agentes fungitóxicos no conteúdo de ergosterol na membrana é um dos mecanismos mais importantes de ação antifúngica e seu conhecimento permite a geração de produtos que associam compostos ativos de diferentes mecanismos, consequentemente, melhorando a eficácia dos conservantes de madeira. Portanto, este estudo otimizou um método para quantificar ergosterol em fungos apodrecedores da madeira. As espécies de podridão-branca selecionadas foram *Ganoderma applanatum* e *Trametes versicolor*, enquanto que as de podridão-parda foram *Gloeophyllum trabeum* e *Lentinus lepideus*. Discos miceliais de cada espécie foram transferidos para placas de Petri contendo meio batata-dextrose-ágar coberto por celofane. O micélio de cada fungo foi coletado, pesado e transferido para tubos de ensaio com 5 mL de hidróxido de potássio



alcoólico 25%. Os tubos foram agitados em vortex por 5 min, submetidos a ultrassom por 5 min, incubados a 85 °C por 4 h, seguido da adição de 2 mL de água destilada estéril e 5 mL de *n*-heptano e subsequente agitação em ultrassom por 2 min. A camada de *n*-heptano foi analisada por espectrofotometria no UV entre 230 e 300 nm. O branco continha apenas *n*-heptano. A massa micelial úmida dos fungos variou de 0,061 a 0,296 g. O teor de ergosterol foi de 0,007% para *Lentinus lepideus* e 0,004% para as demais espécies. A absorvância foi maior que a observada no branco para todas as amostras. O método adaptado foi eficiente para extração de ergosterol.

Palavras-Chave: Esteróide; Micélio; Extração.

1. INTRODUCTION

Different biological agents can attack wood-based products, including wood-decay fungi (Stangerlin et al., 2013). These organisms can cause serious damage to wooden structures, which may result in considerable economic and resource losses (Cheng et al., 2008). These fungi belong to the class Basidiomycetes and are classified as white and brown rots (Oliveira et al., 2005). The white-rot group is characterized by the capacity to degrade lignin, hemicellulose, and cellulose, while the brown-rot fungi are characterized by the degradation of wood polysaccharides (Martínez et al., 2005).

To circumvent the losses caused by wood-decay fungi on wood products, preservatives are usually used (Mendes et al., 2014; Vivian et al., 2015). However, some preservatives have high toxicity, therefore, research is necessary to obtain alternative fungitoxic agents that are effective in combating wood rot (Brand et al., 2006; Xie et al., 2017).

Ergosterol is the major sterol in the fungal membrane (Hu et al., 2017) and responsible for important growth functions (Mota et al., 2012). Among these functions are its contribution to membrane fluidity and integrity in addition to supporting the normal operation of membrane-bound enzymes (Lupetti et al., 2002). Ergosterol is produced by wood-decay fungi, both in white-rot and brown-rot fungi (Presley and Schilling, 2017; Xie et al., 2017).

The effect of a product on ergosterol content in plasma membrane is a known mechanism of antifungal action (Tian et al., 2012; Kedia et al., 2014; Avançaço et al., 2017). Determining a method for ergosterol evaluation is necessary in order to confirm the mechanism of antifungal action of antifungal agents in wood-decay fungi. Knowledge of fungitoxic compound mechanisms allows the generation of products that associate active compounds of different mechanisms that are more effective as wood preservatives.

Considering that ergosterol content in wood-decay fungi vary between white-rot and brown-rot ones (Niemenmaa et al., 2008), the possibility that the evaluation of this sterol can be used to study mechanism of antifungal action, and that the extraction methods proposed by Arthington-Skaggs et al. (1999) and Tian et al. (2012) were not effective for this group of fungi, the present study optimized a method for ergosterol evaluation in wood-rotting fungi using the white-rot fungi *Ganoderma applanatum* and *Trametes versicolor* and the brown-rot fungi *Gloeophyllum trabeum* and *Lentinus lepideus* as models.

2. MATERIAL AND METHODS

2.1 Fungal growth in PDA with and without cellophane membrane

Both the white-rot fungi (*G. applanatum* and *T. versicolor*) and brown-rot fungi (*G. trabeum* and *L. lepideus*) were provided by the Forest Products Laboratory (*Laboratório de Produtos Florestais*) situated at Universidade de Brasília, DF, Brazil. The fungal strains were deposited at the Herbarium of the Biology Department, UFSM, Brazil. *Ganoderma applanatum* (SMDB 17,748), *T. versicolor* (SMDB 17,749), *G. trabeum* (SMDB 17,751), and *L. lepideus* (SMDB 17,750) were initially cultivated in 9-cm Petri dishes containing potato-dextrose-agar (PDA). Two treatments were used: PDA with and without a cellophane membrane (Bento et al., 2014) in order to confirm whether the membrane influences mycelial growth. Mycelial discs (11 mm of diameter) of each fungus species were transferred (in three repetitions) to the Petri dishes which were then sealed with parafilm and kept in a Biochemical Oxygen Demand (BOD) chamber at 25 °C (± 1) at light/dark cycle of 12/12 h. The first evaluation was done on the 7th day after the beginning of the experiment through two measurements diametrically opposite to each other. The test was considered finished when the fungal growth of each

dish completely covered the medium (Badawy and Abdelgaleil, 2014). Data normality and homogeneity of variances were confirmed, one-way ANOVA performed, and the averages were compared by Tukey's test using SigmaPlot 11.0 software.

2.2 Ergosterol content analysis in the plasma membrane of the wood-decay fungi

The white-rot fungi *G. applanatum* and *T. versicolor* and the brown-rot *G. trabeum* and *L. lepideus* were first cultivated in Petri dishes containing PDA, for fifteen days, in a BOD chamber at 25 °C (± 1) at a light/dark cycle of 12/12 h. Then, mycelial discs (11-mm diameter) of each fungal species were transferred to Petri dishes containing cellophane membrane-covered PDA (Bento et al., 2014). Afterwards, the dishes were sealed and moved to a BOD chamber in which they remained for 5 days. All treatments were done with three repetitions.

An adaptation of the method by Arthington-Skaggs et al. (1999) and Tian et al. (2012) was developed for mycelial ergosterol extraction. After the incubation period, mycelia from each wood-decay fungus were harvested and their wet weight determined. Fungal mycelia along with 5 ml of 25% alcoholic potassium hydroxide were transferred to test tubes and vortex mixed for 5 min (Velp Scientifica, Vortex Mixer, Wizard X). The mixed samples were transferred to ultrasound (Unique Ultrasonic Clean, model Ultra Cleaner 1450A with heating) and remained there for 5 min. After ultrasound treatment, the test tubes were incubated at 85 °C for 4 h. Then, 2 ml of sterile distilled water and 5 ml of *n*-heptane were added to the test tubes, which remained in an ultrasound bath for 2 min. The solution layers of each fungus were separated into funnels for 1 h at room temperature. The *n*-heptane layer of each tube was collected and analyzed by UV spectrophotometry (Biospectro sp-220) between 230 and 300 nm. The absorbance at 282 nm characterized the presence of ergosterol in *n*-heptane layer, while the reading obtained at 230 and 282 nm corresponded to the late sterol

intermediate 24(28) dehydroergosterol (Tian et al., 2012). Blank samples containing only *n*-heptane were also analyzed by UV spectrophotometry at the same wavelengths.

The estimation of ergosterol amount was done based on the fungal mycelia wet weight (g) and absorbance values obtained by considering the formulas adapted from Tian et al. (2012): %24(28) dehydroergosterol = (A230/518)/mycelia wet weight; % ergosterol + %24(28) dehydroergosterol = (A282/290)/mycelia wet weight. For the described formulas, 290 and 518 were considered the E (%/cm) values for crystalline ergosterol and 24(28) dehydroergosterol. Graphics and analysis were obtained using Microsoft Office Excel and SigmaPlot 11.0.

3. RESULTS

After 15 days, all Petri dishes containing PDA with and without cellophane membrane were completely covered by wood-decay fungi. No significant differences were found in mycelial growth in PDA among *G. applanatum*, *T. versicolor*, *G. trabeum*, and *L. lepideus* with and without the cellophane membrane (Table 1).

The procedure tested in this study obtained mycelial wet weights of 0.079 ± 0.0225 , 0.296 ± 0.0267 , 0.079 ± 0.0225 , and 0.061 ± 0.0044 g for *G. applanatum*, *T. versicolor*, *G. trabeum*, and *L. lepideus*, respectively (Figure 1). For the first three species, ergosterol content was $0.004 \pm 0.0005\%$. Ergosterol content for *L. lepideus* was $0.007 \pm 0.0013\%$ (Figure 1).

In order to demonstrate the efficiency of the ergosterol extraction method, the results are presented in two different manners: the percentage of ergosterol obtained for each fungus and the UV spectrophotometric profiles of this sterol for each species (Figure 2). For all samples, the absorbance values between 230 and 300 nm wavelengths were higher than the blank, indicating the presence of ergosterol.

Table 1 – *In vitro* mycelial growth (mm) of *Ganoderma applanatum*, *Trametes versicolor*, *Gloeophyllum trabeum*, and *Lentinus lepideus* with and without cellophane membrane (N = 3).

Tabela 1 – Crescimento micelial (mm) *in vitro* de *Ganoderma applanatum*, *Trametes versicolor*, *Gloeophyllum trabeum* e *Lentinus lepideus* com e sem membrana de celofane (N = 3).

Cellophane membrane	<i>Ganoderma applanatum</i>	<i>Trametes versicolor</i>	<i>Gloeophyllum trabeum</i>	<i>Lentinus lepideus</i>
With	52.2 \pm 2.3	44.9 \pm 1.0	25.3 \pm 0.6	21.6 \pm 1.3
Without	54.4 \pm 2.1	38.2 \pm 2.4	27.3 \pm 0.8	16.6 \pm 1.6

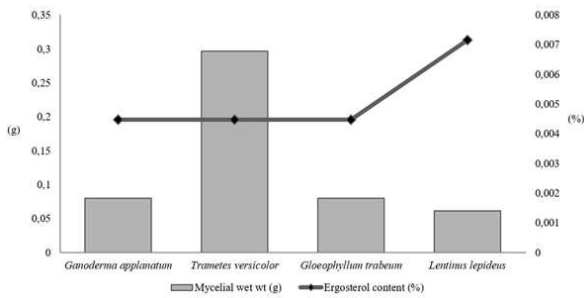


Figure 1 – Mycelial wet weight (primary axis) and ergosterol content (secondary axis) of different species of wood-decay fungi (N = 3).

Figura 1 – Massa micelial úmida (eixo primário) e conteúdo de ergosterol (eixo secundário) de diferentes espécies de fungos apodrecedores da madeira (N = 3).

4. DISCUSSION

The cellophane membrane was first used to determine growth of wood-decay fungi in a study by Bento et al. (2014) in order to obtain a nutrient medium free of mycelium. The aforementioned authors determined the activity of antioxidant enzymes of filamentous fungi in the presence of plant extracts and only evaluated the white-rot species *Trametes villosa* and *Pycnoporus sanguineus*. In some previously described methods, mycelia were washed in sterile distilled water after removing the culture medium (Arthington-Skaggs et al., 1999; Tian et al., 2012; Kedia et al., 2014). The use of cellophane membranes make the removal of the fungal mycelia possible without the need for washing.

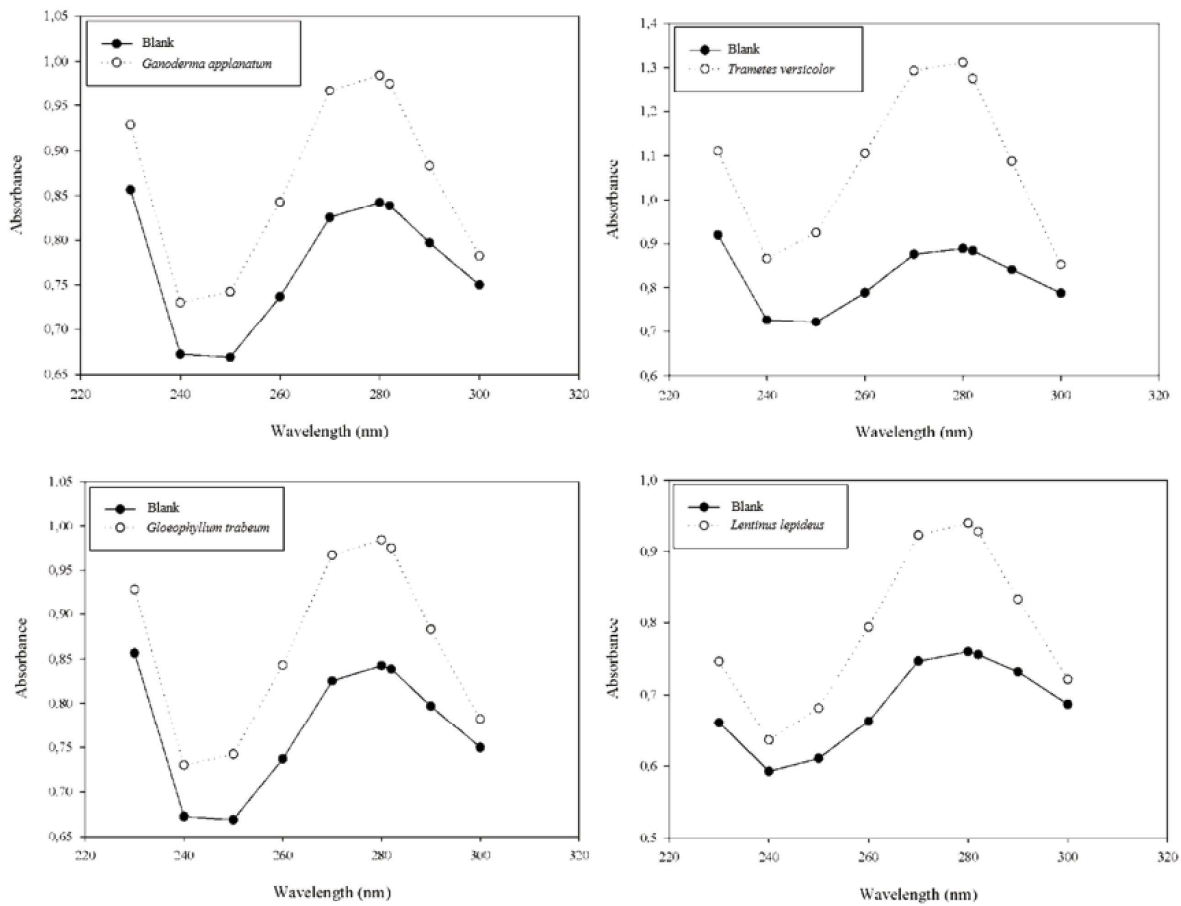


Figure 2 – UV spectrophotometric profile of ergosterol extracted from different wood-decay fungal species (N = 3).

Figura 2 – Perfil espectrofotométrico no UV do ergosterol extraído de diferentes espécies de fungos apodrecedores da madeira (N = 3).

This alternative prevents the washing water from influencing mycelial wet weight.

The percentages of ergosterol vary depending on the fungal species (Barajas-Aceves et al., 2002). The readings of absorbance values between 230 and 300 nm wavelengths were previously used as an indication of ergosterol presence in different studies (Breivik and Owades, 1957; Tian et al., 2012; Kedia et al., 2014). Several studies on ergosterol in wood-decay fungi are found in the literature (Gao et al., 1993; Barajas-Aceves et al., 2002; Eikenes et al., 2005; Niemenmaa et al., 2008; Chedgy et al., 2009; Song et al., 2012), although no information was reported regarding the extraction procedure used in this study. Nevertheless, a similar method was already used for extracting ergosterol from yeasts, such as *Saccharomyces cerevisiae* (Breivik and Owades, 1957), *Candida albicans* (Arthington-Skaggs et al., 1999), and the filamentous fungus *Aspergillus flavus* (Tian et al., 2012; Kedia et al., 2014). Since the previously described methods were not effective for ergosterol extraction of white and brown-rot fungi, the standardization of a new method was necessary.

In addition to the use of cellophane membrane, other steps were added and changes applied to the previously described methods (Tian et al., 2012; Kedia et al., 2014) in order to extract ergosterol from the selected species. After adding 25% alcoholic potassium hydroxide to the test tubes containing mycelia, the literature recommends a vortex mix for 2 min (Tian et al., 2012; Kedia et al., 2014). In our method, the time of vortexing was extended to 5 min and a period of 5 min in ultrasound was added. After the addition of 2 ml sterile distilled water and 5 ml *n*-heptane to the test tubes, the previous methods described a vortex mix for 2 min (Tian et al., 2012; Kedia et al., 2014). In present study, we opted to transfer the test tubes to ultrasound for 2 min.

5. CONCLUSION

Our study describes a method to quantify ergosterol in the plasma membrane of wood-decay fungi. The use of cellophane membrane did not interfere in the mycelial growth of the white-rot species *G. applanatum* and *T. versicolor*, or the brown-rot species *G. trabeum* and *L. lepideus*. The adaptation and additional steps added to the methods already described in the literature were efficient for ergosterol extraction of wood-rot fungi and their quantitative evaluation.

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