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$Al_2(SO_4)_3$ alters the antioxidant mitochondrial metabolism of *Botritys cinerea* and optimizes the production of cellulose and oxidative degrading enzymes

$Al_2(SO_4)_3$ altera o metabolismo mitocondrial antioxidante de *Botritys cinerea* e otimiza a produção de celulose e enzimas de degradação oxidativa

$Al_2(SO_4)_3$ el metabolismo mitocondrial antioxidante de *Botritys cinerea* y optimiza la producción de celulosa y enzimas degradantes oxidativas

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Abstract
The enzymes produced by pathogenic fungi, especially *Botritys cinerea*, deserve specific attention due to the diversity of their applications, mainly in biofuel production, food processing, and the pharmaceutical industry. Thus, this work used $Al_2(SO_4)_3$ as a stressor in order to evaluate if the stress levels caused by the concentrations of 100, 250, 500, and 1000 ppm were sufficient to increase the production of hydrolytic cellulolytic enzymes (FPase, CMCase, Avicelase, β-glucosidase, xylanase) and oxidative (laccase and manganese peroxidase). The study also evaluated the stress levels in previously treated mycelia of *B. cinerea* and whether they corresponded to the different states of mitochondrial respiration. Our study indicates that $Al_2(SO_4)_3$ increased the production of cellulolytic and oxidative enzymes in all concentrations in a dose-dependent manner and that $Al_2(SO_4)_3$ alters the mitochondrial respiratory rate, with lower ATP productions, indicating that less-coupled mitochondria were obtained and that this may be due to the increase of oxidative stress. Thus, it is plausible to suggest the use of $Al_2(SO_4)_3$ in the production of cellulolytic enzymes, which could be used in the hydrolysis stage of second-generation ethanol production processes, as it reduces the time required for enzymatic expression applications in industrial processes.
Keywords: Oxidative stress; Mitochondrial coupling; Catalase; Peroxidase; *Botritys cinerea*.

Resumo
As enzimas produzidas por fungos patogênicos, especialmente *Botritys cinerea*, merecem atenção especial devido à diversidade de suas aplicações, principalmente na produção de biocombustíveis, processamento de alimentos e indústria farmacêutica. Assim, este trabalho utilizou o $Al_2(SO_4)_3$ como estressor a fim de avaliar se os níveis de estresse causados pelas concentrações de 100, 250, 500 e 1000 ppm foram suficientes para aumentar a produção de enzimas celulolíticas hidrolíticas (FPase, CMCase, avicelase, β-glicosidase, xilanase) e oxidativo (lacase e manganês peroxidase). O estudo também avaliou os níveis de estresse em micélios de *B. cinerea* previamente tratados e se eles correspondiam aos diferentes estados da respiração mitocondrial. Nosso estudo indica que $Al_2(SO_4)_3$ aumentou a produção de enzimas celulolíticas e oxidativas em todas as concentrações de forma dose-dependente e que $Al_2(SO_4)_3$

altera a taxa respiratória mitocondrial, com menor produção de ATP, indicando que foram obtidas mitocôndrias menos acopladas e que isso pode ser devido ao aumento do estresse oxidativo. Assim, é plausível sugerir o uso de $Al_2(SO_4)_3$ na produção de enzimas celulolíticas, poderia ser utilizado na etapa de hidrólise de processos de produção de etanol de segunda geração, pois reduz o tempo necessário para aplicações de expressão enzimática em processos industriais.
Palavras-chave: Estresse oxidativo; Acoplamento mitocondrial; Catalase; Peroxidase; *Botritys cinerea*.

Resumen
Las enzimas producidas por hongos patógenos, especialmente *Botritys cinerea*, merecen especial atención debido a la diversidad de sus aplicaciones, principalmente en la producción de biocombustibles, procesamiento de alimentos y la indústria farmacéutica. Así, este trabajo utilizó $Al_2(SO_4)_3$ como estresor para evaluar si los niveles de estrés provocados por las concentraciones de 100, 250, 500 y 1000 ppm eran suficientes para incrementar la producción de enzimas celulolíticas hidrolíticas (FPasa, CMCasa, Avicelasa, β-glucosidasa, xilanasa) y oxidativos (lacasa y manganeso peroxidasa). El estudio también evaluó los niveles de estrés en micelios de *B. cinerea* previamente tratados y si se correspondían con los diferentes estados de respiración mitocondrial. Nuestro estudio indica que el $Al_2(SO_4)_3$ aumentó la producción de enzimas celulolíticas y oxidativas en todas las concentraciones de manera dosis-dependiente y que el $Al_2(SO_4)_3$ altera la frecuencia respiratoria mitocondrial, con menores producciones de ATP, lo que indica que se obtuvieron mitocondrias menos acopladas y que esto puede deberse al aumento del estrés oxidativo. Por lo tanto, es plausible sugerir el uso de $Al_2(SO_4)_3$ en la producción de enzimas celulolíticas, que podrían ser utilizadas en la etapa de hidrólisis de los procesos de producción de etanol de segunda generación, ya que reduce el tiempo requerido para las aplicaciones de expresión enzimática en procesos industriales.
Palabras clave: Estrés oxidativo; Acoplamiento mitocondrial; Catalasa; Peroxidasa; *Botritys cinerea*.

1. Introduction

The hydrolytic and oxidative enzymes produced by fungi have been highlighted due to the diversity of their applications, such as paper recycling, cotton processing, biofuel production, food processing, the applications in the pharmaceutical industry, and their use as effluent biodegradants (Kamidai et al., 2005; Acharya et al., 2012; Pradeep & Narasimha, 2011). These enzymes are inducible and synthesized by a wide variety of microorganisms, including fungi and bacteria (Abdel-Fatah et al., 2012).

The use of enzymes produced by fungi decomposing lignocellulosic materials has attracted much attention recently. The use of xylanases and laccases in the processes and applications of cellulases for the enzymatic hydrolysis of cellulose in the production of second-generation ethanol from sugarcane bagasse is considered an advance in the understanding of the biodegradation of lignocellulosics. This also allows us to explore and test countless technological applications. The development of these applications occurs simultaneously with the generation of knowledge based on the understanding of the biodegradation mechanisms that fungi develop on lignocellulosic materials (Aguiar & Ferraz, 2011).

Stressful fungal organisms tend to develop adaptive responses to the stressor since eukaryotic cells respond and adapt to external stimuli mainly through signal transduction pathways (Alonso-Monge et al., 2009; Hillmann et al., 2015). Used as chemical stressors, heavy metal ions play essential roles in various physiological processes. In trace amounts, several of these ions are required for the metabolism, growth, and development of organisms. However, problems arise when cells are confronted with an excess of these ions, which leads to cellular damage (Anahid et al., 2011)

The toxicity of heavy metals comprises the inactivation of biomolecules blocking essential functional groups or the displacement of essential metal ions (Malekpouri et al., 2012). In addition, the autoxidation of active redox heavy metals and the production of reactive oxygen species (ROS) cause cell damage and may compromise the functioning of the whole organism (Bai et al., 2003).

The main manifestation of aluminum toxicity is the inhibition of the growth of the exposed organisms, leading to metabolic changes essential for the survival of the individual (Azmat et al., 2015). Therefore, important enzymatic processes can be affected by this, leading to the production of reactive oxygen species, which are usually measured by the increase in antioxidant enzymes (Souza et al., 2016, Zandi & Schnug, 2022).

Due to their wide biotechnological applicability, it is highly desirable to explore processes capable of increasing the

production of cellulolytic enzymes. *B. cinerea* fungus is already known to produce cellulolytic enzymes (Elmer & Reglinski, 2006). The objective of this paper is to associate the potential of *B. cinerea* with processes that optimize the production of cellulolytic enzymes by using $Al_2(SO_4)_3$ as a stressor to verify whether it can increase the production of cellulolytic enzymes in *B. cinerea*.

2. Methodology

2.1 The Cultivation of *B. cinerea*

The *B. cinerea* isolate was obtained from the Forest Fungus Collection of Embrapa Forests. Initially isolated from *Pinus taeda* seedlings, it was submitted to the sequencing of the ITS regions of the ribosomal DNA for identification at the species level by comparison with sequences deposited in GenBank. It was possible to identify the pathogen *Botrytis cinerea* [*Botryotinia fuckeliana* (de Bary) Whetzel] deposited under the code KJ476441.

Initially, *B. cinerea* was harvested and cultivated in nine-cm diameter Petri dishes containing agar-BDA (KASVI®) potato dextrose medium and maintained in a BOD (Biological Organisms Development) chamber at 22°C in the dark (Alfenas & Mafia, 2007) until the complete production of hyphae, mycelia, and conidia.

After growth, the culture was carried out in a liquid broth of potato dextrose (potato-dextrose extract, 39 g, and ultra-purified water q.s.p. 1000 mL) to evaluate the growth of fungal biomass, and consequently, the evaluation of oxidative stress. In this assay, a 200 mL aliquot of the broth was placed in glass vials with plastic caps with a capacity of 500 mL each. $Al_2(SO_4)_3$ was then added at concentrations of 100, 250, 500, and 1000 ppm and control. The vials were autoclaved for 1 h at 120°C. The inoculation of the flasks was done by transferring two 5-mm diameter mycelial agar disks of the fungus into each flask for both static and dynamic methods since in this assay, the fungi were cultivated under a shaker, shaking with a rotation of 100 rpm at a temperature of 20°C and grown without shaking in a BOD chamber at 20°C in the dark for 10 days. After this period, the biomass was separated from the broth by vacuum filtration, and the biomass was used to estimate the yield (mg) and changes in the antioxidant defense system (CAT, SOD, POD, PAL, and PHOL) respiratory. The broth was then reserved for the analysis of the hydrolytic and oxidative enzymes.

2.2 Enzymatic extracts preparation of *B. cinerea*

The biomass from the BD broth culture, with the different concentrations of aluminum sulphate, was frozen in liquid nitrogen and stored at −4ºC until the analysis. About 2 mg of fungal mass of *B. cinerea* were placed in approximately 10 mL of cold extraction medium consisting of 0.4 M mannitol, Tris-HCl 50 mM (pH 7.2), EDTA 1.0 mM, $MgCl_2$ 1.0%, 0.1% cysteine (m/v), and fatty acid-free bovine serum albumin (BSA) at 0.5% (w/v) (Bracht et al., 2003).

2.3 Measurement of oxidative stress

The concentration of proteins in the enzymatic extracts of *B. cinerea* was determined by comparison with a standard curve of bovine serum albumin (BSA), and the activity was determined by spectrophotometry at 594 nm (Bradford, 1976).

The Catalase activity was measured in a medium containing 67 mM potassium phosphate buffer (pH 7.0), H_2O_2 (10 mM) and 0.1-0.4 mg protein of the enzyme extract. H_2O_2 consumption was monitored at 240 nm (ε, 0,036 $mM^{-1}cm^{-1}$) (Aebi, 1984).

Superoxide dismutase activity (SOD) was measured according to Giannopolitis & Ries (1977). The medium contained 50 mM potassium phosphate buffer (pH 7.8) and 6.5 mM L-methionine, 150 µM NBT (nitroblue tetrazolium), 4 µM riboflavin, and 0.02-0.1 mg protein from the enzyme extract. The reaction was initiated by the activation of a light (20 W), which illuminated the medium for 20 minutes at 30°C. A unit of SOD (U) activity was defined as the amount of enzyme

required to cause a 50% inhibition of the photoreduction rate (NBT) at 560 nm, and the results were expressed as SOD units. mg protein^{-1}.

The activity of the guaiacol peroxidase (POD) enzyme was measured in a medium containing 25 mM potassium phosphate buffer (pH 6.8), H_2O_2 (10 mM), guaiacol (2.6 mM), and 0.1-0.4 mg of protein from the enzyme extract. The formation of Tetraguaicol (ε, 25,5 mM^{-1} cm^{-1}) was monitored at 470 nm (Putter, 1974).

The polyphenol oxidase activity was determined by the conversion of catechol to quinone (Duangmal & Apenten, 1999). The substrate used contained 20 mM catechol dissolved in 100 mM sodium phosphate buffer (pH 6.8), to which mg of protein from the enzyme extract was added. The reaction occurred at 30°C and the absorbances were read at 420 nm (ε, 2,47 mM^{-1} cm^{-1}).

The phenylalanine ammonia-lyase (PAL) activity was measured by adding 500 µL of the extract to a mixture containing 2 mL of 0.1 M sodium borate buffer (pH 8.8) and 1 mL of 20 mM L-phenylalanine. The reaction mixture was incubated in a water bath at 30°C for one hour. In the control samples, the extract was replaced with 1 mL of sodium borate buffer. The reaction was terminated after the addition of 0.1 mL of HCl 6 N, and the absorbance of the trans-cinnamic acid derivatives was measured in a spectrophotometer at 290 nm and the molar extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker, 1965).

2.4 Preparation of mitochondrial suspension to evaluate the changes in respiratory activity

The fungal biomasses were subsequently homogenized and then filtered through several layers of gauze and centrifuged at 1000 rpm for 10 min, with the pH adjusted to 7.2 with KOH 1.0 N solution. The supernatant was centrifuged at 20,000 rpm for 10 min. The mitochondrial sediment was suspended in 2 mL of a medium containing 0.3 M mannitol, 1.0 mM EDTA, 20 mM HEPES (pH 7.2), and 0.2% (w/v) bovine serum albumin (BSA) free of fatty acids. The washed mitochondria were subsequently purified on a Percoll ™ gradient, according to the procedure described by Jackson et al. (1979) and Bracht et al. (2003). The resulting mitochondrial pellet containing approximately 1.3–2.4 mg mitochondrial protein was suspended in a final volume of 1.5 mL of a medium containing 0.3 M mannitol, 1.0 mM EDTA, 20 mM HEPES (pH 7.2), and 0.2% (w/v) fatty acid-free BSA. All operations were performed at −4°C.

2.5 Measurement of the respiratory activity

Mycelium and conidiospores from the BD broth culture submitted to the different treatments were weighed and placed immediately in the Clark electrode container, containing 2 mL of nutrient solution (pH 5.8) composed of $Ca(NO_3)_2$ mM, KNO_3 2 mM, 27 µM $FeCl_3$, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$, and 20 µM NaH_2PO_4 (Larkin, 1987). The rates of oxygen uptake were calculated from polarographic records assuming an initial dissolved oxygen concentration of 240 µM at 25 °C, according to Estabrook (1967), Pergo and Ishii-Iwamoto (2011) and Ishii-Iwamoto et al. (2012). To estimate the contributions to the total O_2 uptake (respiration sensitive to KCN), 270 mM potassium cyanide (KCN) was added to the reaction medium used for the measurement of respiratory activity. Oxygen uptake was monitored, and the oxygen consumption rates were calculated from polarographic records assuming an initial dissolved oxygen concentration of 240 mM at 25°C (Estabrook, 1967).

2.6 Spectrophotometric assay of mitochondrial complexes

To detect the activity of the mitochondrial complexes, mitochondrial particles were used for the NADH-Q reductase (NQR, complex) analysis, that is, the suspension of mitochondrial particles, 1 mL of buffer containing 35 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM NaN3, 2 µg/mL antimycin A, and 60 µM CoQ10, at pH 7.2. After incubation at 25°C for 5 min, the reaction was

started by the addition of 0.13 mM NADH. The decrease in absorbance at 340 nm was recorded at 25°C for 1 min. The extinction coefficient of NADH was 6,81 mM^{-1} cm^{-1} (Chance & Willium, 1955; Pecci et al., 1994).

Then, to evaluate the integrity of mitochondria isolated from the enzymatic extract of the fungal mass of the treatments, the respiratory controls (CR) were determined after successive ADP pulses in energized mitochondria with $mmol.L^{-1}$ malate and 10 $mmol.L^{-1}$ glutamate.

The relative consumption (CR) was obtained by the numerical relationship between the rate of oxygen consumption of the mitochondria in state III (maximum speed, in the presence of ADP, P_i e $MgCl_2$) on the rate IV state (resting after phosphorylation).

2.7 Measurement of the activity of hydrolytic and oxidative extracellular enzymes

The enzymatic activities of total cellulase (FPase), endoglucanase (CMCase), and exoglucanase (Avicellase) were evaluated according to the methodology proposed by Miller (1959), Ghose (1987), and Dashtban et al. (2010). The quantification of the reducing sugars content was tested by the 3,5 dinitrosalicylic acid (DNS) method and the substrates used were Filter paper, CMC, and Avicel, respectively.

The FPase activity was determined by homogenizing 500 µL of the sample, 500 µL of pH 4.8 citrate buffer, and one strip of filter paper (1x6 cm). The solution was held at 50°C for 1 h, then the reaction was stopped in an ice bath. The quantification of the reducing sugar content was performed by the DNS method. 500 µL of DNS was added, and the samples were placed in a boiling bath for 5 min, then cooled and spectrophotometric read at 540 nm.

The CMCase activity was determined by homogenizing 250 µL of the sample and 250 µL of the 2% CMC solution in sodium acetate buffer. The solution was held at 50°C for 1 h and then the reaction was stopped in a cold-water bath. The quantification of the liberated reducing sugar content was assayed by the DNS method; 500 µL of DNS was added, the samples were placed in a boiling bath for 5 min, and then cooled and spectrophotometric read at 540 nm.

The Avicelase activity was determined by homogenizing 250 µL of the sample and 500 µL of the 1% Avicel solution in sodium acetate buffer. The solution was held at 50°C for 1 h under constant stirring and then the reaction was stopped in a cold-water bath. The solution was then centrifuged at 12,000 rpm for 10 min at 4°C. For the quantification of the reducing sugar content, 28 µL of the supernatant was homogenized with 700 µL of DNS and the samples were boiled for 5 min and spectrophotometric read at 540 nm.

The enzymatic activity for xylanase was determined according to Damiano et al. (2003), in which a reaction mixture containing 0.1 mL of the crude enzyme solution and 0.9 mL of 0.5% Birchwood-Sigma xylan in 0.1 M acetate buffer at p`H 5.0 was incubated for 10 min at 60°C. The released reducing sugar was quantified by the DNS method (Miller, 1959), adding 1 mL of the DNS reagent to the reaction mixture. Such a mixture was incubated in a water bath at 100°C for 5 minutes, then cooled, and 10 mL of distilled water was added to it. After homogenization, a spectrophotometer reading was determined at 540 nm. A unit of xylanase activity was defined as the amount of enzyme capable of releasing 1 µmol of xylose per minute, under the reaction conditions, using a standard xylose curve.

The β-glucosidase activity was performed by the method of Coston & Loomis (1969), adding 100 µL of the 15 mM pPNPG (pNP-β-D-glucopyranoside - Sigma®) reagent, prepared in 50mM sodium citrate buffer pH 4.8 and 100 µL of the culture supernatant. The reaction was incubated for 1 hour at 50°C, then 1 mL of 0.5 M sodium carbonate was added to stop the reaction, and the reading was performed at 410 nm.

The standard curve for the FPase, CMCase, Avicelase, and β-glucosidase tests was established using glucose at concentrations of 1–20 µmol/mL. The standard curve for the test was established using glucose at concentrations of 0.15 to 1.5 mg/mL.

The manganese peroxidase activity was measured at 610 (Ɛ610= 4460M^{-1} cm^{-1}) using the methodology described by Kuwahara et al. (1984). The reaction mixture (1 mL) contained culture medium (500 µL), phenol red (100 µL), 250 mM sodium lactate (100 µL), 0.5% bovine albumin (200 µl), 2mM manganese sulfate (50mL), and 2mM hydrogen peroxide (50mL), prepared in 20 mM sodium succinate buffer, pH 4.0. Reactions occurred at 30°C for 5 minutes and were stopped by the addition of NaOH 2 N (40 µL).

The laccase activity was also measured spectrophotometrically, as oxidation of oxyanidine at 525 nm (Ɛ 525= 65,000M^{-1} cm^{-1}). The reaction mixture contained in 1 mL 0.5 M citrate-phosphate buffer pH 5.0 (200 µL); 1 mM o-dianisidine solution (100 µL); culture medium (600 µL); and 1 mM hydrogen peroxide (100 µL). All analyses used a boiled culture medium as a control (Kuwahara et al., 1984).

2.8 Statistical analysis

All assays were performed in triplicate. For each test, the data were submitted to the analysis of variance, and when the effects of the treatments were significant (p <0.05) in relation to the control, the means were compared by the Dunnet test. All results were analyzed considering the level of significance α = 5%.

3. Results

The $Al_2(SO_4)_3$ interfered in the final biomass production in *B. cinerea*, and the absence of agitation also contributed to lower production of *B. cinerea*, dependent on the increase of $Al_2(SO_4)_3$ (Table 1). In addition, the concentration of 1000 ppm in the culture with agitation caused a reduction in the biomass production by 72.9%; however, a lower biomass reduction was observed in the cultivation without agitation (76.5% in relation to the control) (Table 1).

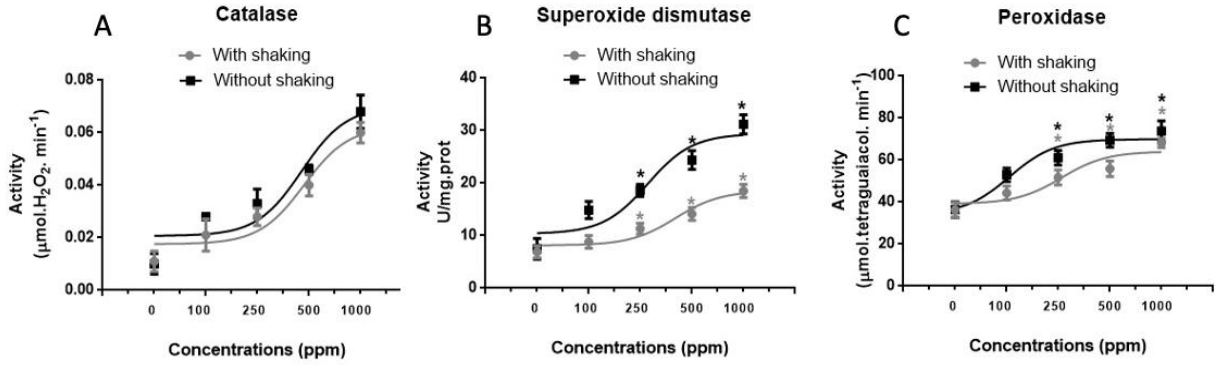
Table 1 - Production of fungal biomass from *Botrytis cinerea* from cultivation with $Al_2(SO_4)_3$, with and without shaker.

Biomass Production (mg)		
Concentration ppm	**With Shaking**	**Without Shaking**
1000	13,50 ± 1,12^b	5,00 ± 1,17^b
500	21,20 ± 1,17^b	8,00 ± 1,30^b
250	24,60 ± 1,90^b	8,70 ± 1,50^b
100	33,30 ± 1,13^b	12,00 ± 1,23^b
Control (0)	49,80 ± 1,20^a	21,30 ± 1,19^a

NOTE: Results expressed as mean ± standard deviation *Means followed by the same letter do not differ according to Dunnett test (p < 0.05). Source: Authors.

The reduction of biomass in *B. cinerea* may be related to the increase of enzymes that act in the antioxidant defense system, resulting from H_2O_2 formation and in response to treatments with varying concentrations of $Al_2(SO_4)_3$. The ability to detect environmental stimuli, including stress, and activate signal transduction to modulate appropriate acute and adaptive responses is crucial for fungal survival. The lowest activity values of CAT, SOD, and POD were detected for the control groups that did not receive $Al_2(SO_4)_3$ (Figure 1).

Figure 1 - The effect of different concentrations of $Al_2(SO_4)_3$ on the activity of antioxidant enzymes.

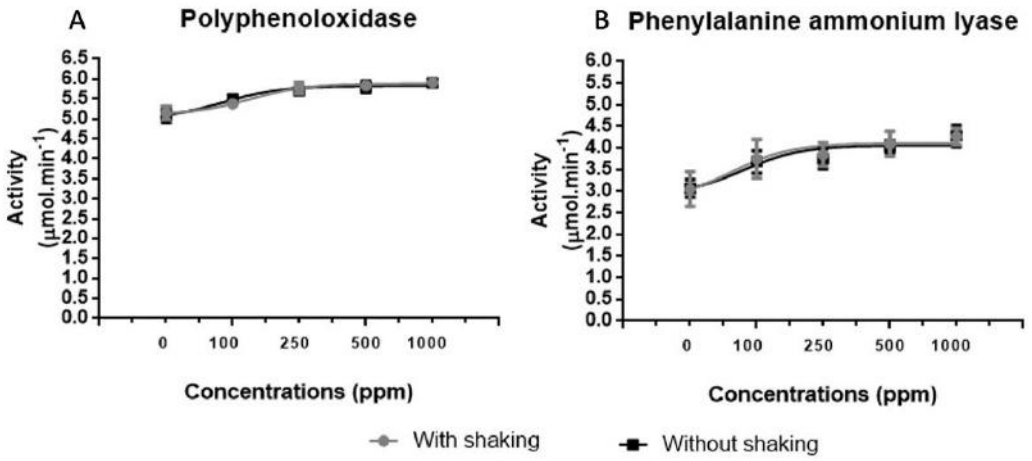


Source: Authors.

The addition of the 100-ppm concentration did not significantly alter the levels of the antioxidant enzymes tested when compared to the control group, and the increase of the concentration to 250 ppm increased the activities of CAT, SOD, and POD and reached the values of 0.060 µmol. H_2O_2.min^{-1}; 18.5 U/mg; and 68.1 µmol.tetraguaiacol.min^{-1} for the concentration of 1000 ppm. The pattern of changes in CAT, SOD, and POD activities was similar in the cultivated samples without agitation and did not differ for the concentration of 100 ppm in relation to the control. The increase of these enzymes was higher in the concentration of 1000 ppm, with values of 0.068 µmol.H_2O_2.min^{-1}; 31.2 U/mg; and 73.8 µmol.tetraguaiacol.min^{-1} (Figures 1A, B, and C).

As shown in Figures 2 A and B, polyphenol oxidase and PAL activity were not expressive for *B. cinerea* grown with and without agitation over the experimental growth period. However, a small increase as a function of the concentration of $Al_2(SO_4)_3$ is noted for polyphenol oxidase (Figure 2A) and phenylalanine ammonium lyase (Figure 2B).

Figure 2 - The effect of different concentrations of $Al_2(SO_4)_3$ on the activity of antioxidant enzymes.

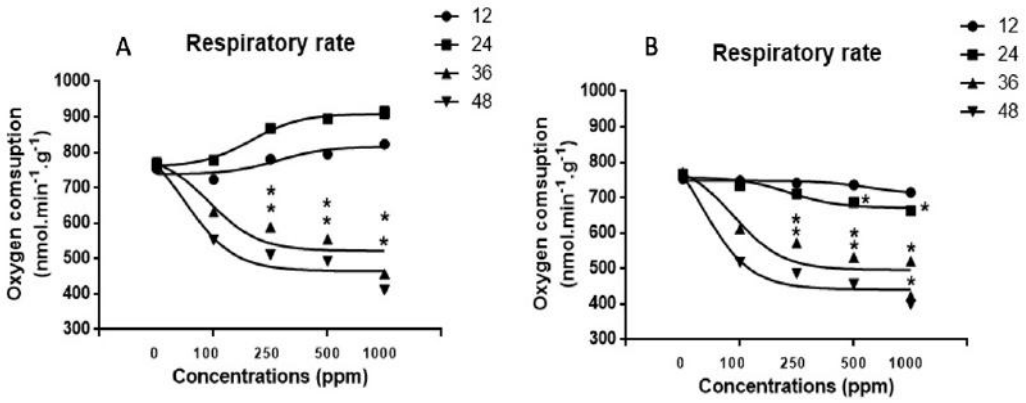


Source: Authors.

The parameters of mitochondrial respiratory rate (Figure 3) were altered in mycelia treated with different concentrations of $Al_2(SO4)_3$, and the presence agitation (Figure 3A) or without agitation (Figure 3B) was a determining factor for decreasing total respiration in treated mitochondria.

Concentrations, except for 100 and 250 ppm, inhibited total respiratory activity. Compared to the control (untreated mitochondrial preparations), respiration increased about 4 to 5 times in the presence of $Al_2(SO_4)_3$. The decrease in these values is associated with an increase in the concentration of $Al_2(SO_4)_3$, indicating the production of less coupled mitochondria.

Figure 3 - The effect of different $Al_2(SO_4)_3$ concentrations on the respiratory activity of mycelium from *B. cinerea.*

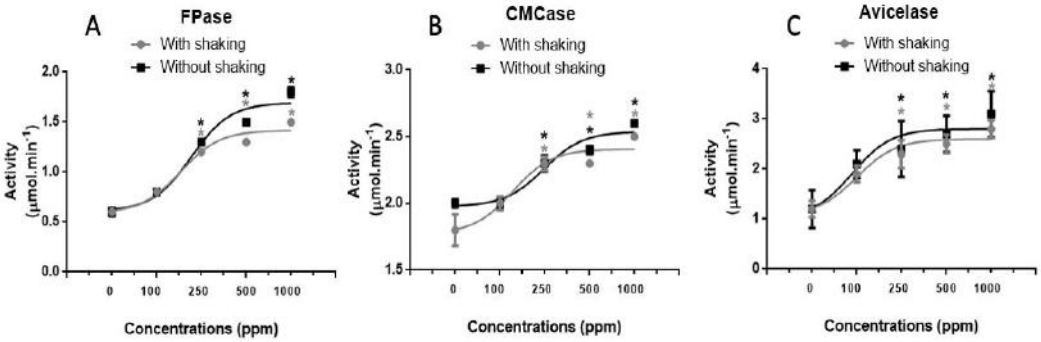


Source: Authors.

The induction of stress in *B. cinerea* also caused an increase in the levels of cellulose-degrading enzymes. For the FPase, CMCase, and Avicelase levels, the concentration of 100 ppm did not significantly increase the enzyme levels relative to the control (Figure 4). From the concentration of 250 ppm, a stimulus of the production of these enzymes is observed, and its maximum value is observed for the concentration of 1000 ppm. The cultivated biomass without agitation was shown to be more efficient in the production of these enzymes for *B. cinerea* (Figures 4A, B, C).

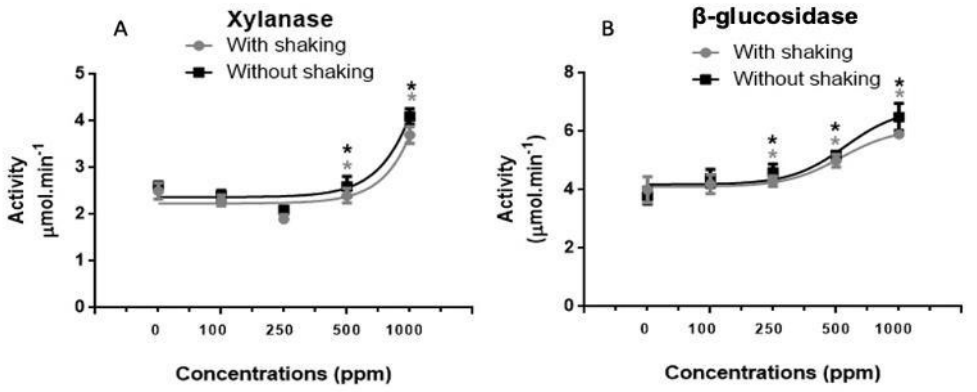
Only the 1000 ppm concentration altered the xylanase production (Figure 5A), and the β-glucosidase production behavior was like FPase, CMCase, and Avicelase enzymes (Figure 5B).

Figure 4 - The effect of different $Al_2(SO_4)_3$ concentrations on enzyme activity.



Source: Authors.

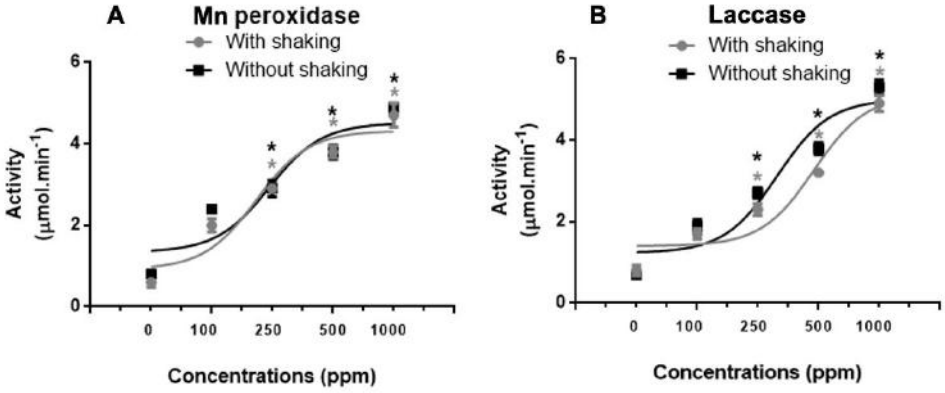
Figure 5 - The effect of different $Al_2(SO_4)_3$ concentrations on enzyme activity.



Source: Authors.

The dose-dependent production is also observed for the oxidative enzymes manganese-peroxidase and laccase, indicating that these activities are related to the increase of $Al_2(SO_4)_3$ concentrations in *B. cinerea* cultivation, with higher productivity for the cultivation without shaking (Figures 6 A, B).

Figure 6 - the effect of different $Al_2(SO_4)_3$ concentrations on enzyme activity.



Source: Authors.

4. Discussion

These results demonstrate that the mitochondrial respiratory activity in mycelium of *B. cinerea* increased due to the metabolic activity, as well as the mobilization of reserved energy, which along with the limitation of oxygen are factors that contributed to the increase in stress levels, and consequently, to the production of cellulose degrading enzymes. In addition, these results probably represent a decrease in the demand for ATP. A pattern of changes like respiratory activity and activity of antioxidant enzymes has been observed in three other fungal species: *Candida albicans* (Butler et al., 2009), *Sacharomices* cerevisiae (Nikolaou et al., 2009), and *Aspergillus flavus* (Fountain et al., 2016).

The occurrence of the enzymatic activities of SOD, CAT, and POD in mycelia treated with $Al_2(SO_4)_3$, together with respiration insensitive to KCN, suggests that the production of reactive oxygen species occurs concomitantly with mitochondrial respiration, which occurs at the beginning of the division of hyphae. KCN-insensitive respiration is responsible for oxygen that is converted to superoxide and H_2O_2 (Puntarullo et al., 1988) or used by alternative mitochondrial oxidase (AOX) and other cytosolic oxidases that may be membrane bound, including lipoxygenases. Although the relative contribution of each process to respiration insensitive to KCN cannot be deduced from our data, all these processes have been previously demonstrated to be stimulated under conditions of oxidative stress (Maheshwari & Navaraj, 2008; Wu et al., 2009).

In mycelia grown at different concentrations with shaking, the patterns of change in the activities of the antioxidant enzymes are like the results obtained in the culture without agitation, where the concentrations increase the responses, reflecting an additional increase in ROS production. The differences that reflect a lower activity in the enzymes from mycelia cultivated with agitation probably occurred due to the greater availability of oxygen compared to mycelia obtained without agitation. According to the observations of Breitenbach et al. (2015), there is an increase in the concentration of free radicals in the mycelium as a direct consequence of the oxygen in the surrounding atmosphere, which occurs at the beginning of hyphae development. When compared to the enzyme activity, SOD and catalase are probably the major antioxidant enzymes involved in the elimination of ROS in *B. cinerea* during their growth. The species also contain accessory enzymes that neutralize H_2O_2, such as peroxidase. The isozyme forms of peroxidases exist in large numbers and are implicated in a wide range of physiological processes (Montibus et al., 2015). Peroxidases bind to cell wall polymers by ionic or covalent interactions to participate in lignin biosynthesis and cell reticulum formation (Brown et al., 2007). Guaiacol peroxidase has also been specifically used as a stress indicator (Almagro et al., 2009). Regardless of the primary mechanisms of $Al_2(SO_4)_3$ to induce

oxidative stress, our data suggest that during development and initial growth, when enzymatic activity increases and neutralizes the detrimental ROS, oxidative effects are still severe, which is observed by the increase in respiratory rates, leaving *B. cinerea* more vulnerable to cellular dysfunctions and cell death.

It is reasonable to suggest that $Al_2(SO_4)_3$ has a high potential to act as an inducer to produce cellulose and oxidative degrading enzymes, either in agitated or non-agitated systems. However, it should be emphasized that in both conditions, the secondary metabolism is activated, also resulting in the production of oxidative phenolic enzymes. Ferreira et al. (2010) state that stress may be one of the factors responsible for the synthesis of oxidative enzymes, explaining why the levels are higher in culture without agitation.

5. Conclusion

Considering what was observed in this study, the time required for the occurrence of an enzymatic expression in this system allows its application in an industrial process, since oxidative stress can be caused by the addition of exogenous (simulated) chemical stressors or under hypoxic conditions. The interactions of the different means of response demonstrate that fungi adapt as a result of metabolic changes due to stress factors. Thus, this study demonstrates the application of $Al_2(SO_4)_3$ as an influencing agent in the production of cellulolytic enzymes involved in the development of processes related to the production of second-generation ethanol, as well as its use in other biotechnological resources. As a result of the analyzed data, studies are suggested that focus on optimizing this resource as a potential opportunity for the industry.

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