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Vegetative and enzymatic analysis of the initial stages of *Coffea arabica* L. grown from seeds treated with humic substances

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Arabica coffee is a perennial crop usually propagated by seeds, which must be healthy and able to form normal plants. The objective of this study was to analyze the vegetative and enzymatic reactions in the initial stages of Arabica coffee seedlings treated with different humic substances and at different concentrations. A germination test was conducted with Arabica coffee seeds of the Topazio MG 1190 cultivar using humic substance doses of 0, 5, 10, 25, 50, 50 and 50 mg.dm^{-3}. The percent germination was evaluated at 15 days; the percent germination and root length were measured at 30 days; and the seedlings were removed at 45 days and stored at -80°C for subsequent evaluation of the enzymatic activity of catalase, superoxide dismutase, alcohol dehydrogenase, esterase, and H^+-ATPase. It is concluded that germination did not differ with the use of humic substances, and root length was greater at the 0 mg.dm^{-3} dose. Although H^+-ATPase responded positively to the application of humic substances, the catalase, superoxide dismutase, alcohol dehydrogenase, and esterase activities were also better at the lowest dose. Humic substances do not present agronomic benefits at the seedling stage for Arabica coffee and are not recommended as a possible seed treatment under these conditions.

Key words: Fulvic acid, humic acid, coffee, seedling formation.

INTRODUCTION

The formation of disease-free coffee seedlings capable of resisting transplanting is essential to obtain a homogeneous stand with high productivity and productive longevity. Several factors can determine the development, quality, and costs of seedling production, including the type of substrate used in seedling cultivation (Oliveira and Miglioranza, 2015). The benefits provided by the addition of organic matter to the soil increasingly demonstrate the importance of its organic acid constituents, specifically, humic acid and fulvic acid. The literature has shown that the influence of these acids on physical, chemical, and biological properties has led to

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better plant development, including of the shoot and root system.

Several experiments using humic substances in agricultural production have shown their positive effects on plant development. The results obtained are variable and depend on, in addition to the tested species, the humic substances used, their concentration, degree of purification of the material, and the conditions under which the experiments were conducted.

In addition to the effects they have on the soil, humic substances also act on plant physiology (Varanini and Pinton, 2001). However, their functions in plant metabolism are related to their structural characteristics, since humic acid has high molecular weight, whereas fulvic acid has low molecular weight (Berbara and García, 2014). In addition, their functions may also vary according to the organic material from which they originated and the time it took for them to undergo transformation (Berbara and García, 2014).

The effect of humic substances on the coffee crop has been little studied, but such studies are increasing due to the growing demand for products produced more sustainably. Thus, humic substances can be great allies when increasing productivity in organic crops.

Therefore, the objective of this study was to analyze the vegetative and enzymatic reactions in the initial stages of cultivated Arabica coffee treated with different humic substances at different concentrations.

MATERIALS AND METHODS

The experiment was conducted at the Laboratory of Seed Analysis, Seed Sector, Department of Agriculture, Federal University of Lavras (Universidade Federal de Lavras), in the municipality of Lavras, Minas Gerais, Brazil.

Coffee seeds of the Topazio MG 1190 cultivar were used. The parchment was removed from each seed. Three sheets of filter paper were used per replicate, with 25 seeds in each replicate. Each treatment had four replicates. For germination, the seeds were placed between two sheets of paper, wrapped into rolls and then placed vertically in a germinator at 30°C (Brasil, 2009)

Humic and fulvic acid (HA and HF, respectively) was extracted from leonardite through the use of a 0.5 mol L^{-1} KOH solution, and, in sequence, purified, following the method recommended by the International Humic Substances Society (Swift, 1996). The humic substances were placed on the paper via water, at a concentration of 0, 5, 10, 25 and 50 $mg.dm^{-3}$ of fulvic acid (FA) and humic acid (HA). The papers were weighed, and the amount of water to be added to the paper was 3x its weight.

Three evaluations were performed, the first at 15 days after sowing (DAS), when the germinated seeds were counted and the paper replaced. The second evaluation was performed at 30 DAS, and in addition to the germinated seeds being counted, the root length (cm) was measured, with 10 seedlings per replicate being established in a plot. The last evaluation was performed at 45 DAS, when the seedlings were removed to quantify the enzymatic activity of catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), esterase (EST), and H^+-ATPase.

For the CAT analysis, a reaction medium composed of 200 mM potassium phosphate (pH 7.0), water, and 250 mM H_2O_2 was incubated at 30°C, and the hydrogen peroxide consumption was monitored (Havir and Mchale, 1987). CAT activity was evaluated by the decrease in absorbance at 240 nm, with a reading, which recorded the decrease in absorbances every 15 s, being carried out for 3 min.

For SOD, a reaction medium composed of 100 mM potassium phosphate buffer (pH 7.8), 70 mM methionine, 10 µM EDTA, water, 1 mM nitrotetrazolium blue (NBT), and 0.2 mM riboflavin was used. A cuvette containing the reaction medium and the sample was illuminated for 7 min, whereas for the control, the same reaction medium without the sample was illuminated, and the blank was kept in the dark. SOD activity was assessed by the ability of the enzyme to inhibit (NBT) photoreduction. The readings were carried out at 560 nm, with one unit of SOD corresponding to the amount of the enzyme capable of inhibiting NBT photoreduction by 50% under the experimental conditions (Giannopolitis et al., 1977).

For ADH, leaves were macerated in the presence of polyvinylpyrrolidone (PVP) and liquid nitrogen. Next, 100-mg samples were placed into microtubes for enzyme analysis. A total of 250 µL of extraction buffer (0.2 M Tris HCl, pH 8.0) and 0.1% β-mercaptophenol were then added. The mixture was left to rest overnight and was then centrifuged at 14,000 rpm for 30 min at 4°C. From the supernatant, a 60-µL aliquot was removed and applied to a 7.5% (separator gel) and 4.5% (concentrator gel) polyacrylamide gel. The electrophoretic run was performed at 150 V for 6 h. After the run, the gels were developed according to the method described by Alfenas (2006).

For the EST, protein extraction was performed by adding 320 µL of extraction buffer (0.2 M Tris) to 100 mg of seed powder, vortexing the solution, and then leaving it in the refrigerator for 1 h. Samples were centrifuged at 16,000 rpm at 4°C for 60 min, and 60 µL of the supernatant was applied to the polyacrylamide gels. The gel/electrode buffer system used was Tris-glycine pH 8.9. Electrophoresis was performed at 110 V for 5 h, and the gels were developed for EST, according to method described by Alfenas (2006). The interpretation of the results was based on the visual analysis of the electrophoresis gels, taking into consideration the intensity of each of the electrophoretic bands in the evaluated system.

For H^+-ATPase analysis, the macerated samples were homogenized in 20 mL of ice-cold extraction medium containing 250 mmol L^{-1} sucrose, 10% glycerol (w:v), 40 kDa 0.5% polyvinylpyrrolidone (PVP), 2 mmol L^{-1} EDTA, 0.2% bovine serum albumin (BSA) (w:v), and 0.1 mol L^{-1} Tris [tris (hydroxymethyl) aminomethane]-HCl, pH 7.5. Immediately before use, 150 mmol L^{-1} KCl, 2 mmol L^{-1} dithiothreitol (DTT), 1 mmol L^{-1} benzamidine hydrochloride, and 1 mmol L^{-1} p-methylphenyl sulfonyl fluoride (PMSF) were added. The entire mixture was prepared at a controlled temperature between 2 and 4°C. The pH of the extraction buffer was monitored during the procedure, remaining in the range of 7.5 to 8.0. After maceration of the root material, the resulting homogenate was subjected to centrifugation at 1,500 x*g* for 10 min at 2°C for removal of residues, unbroken cells, and nuclei. The supernatant was collected and subjected to a new centrifugation at 10,000 x*g* for 15 min at 2°C for separation of the mitochondrial fraction. The new supernatant was then resuspended at 100,000 x*g* for 40 min at 2°C. The precipitate from the new centrifugation, consisting of the microsomal fraction, was resolubilized in 2 mL of buffer solution [resuspension medium: 15% glycerol (v:v), 1 mmol L^{-1} DTT, 1 mmol L^{-1} PMSF, 10 mmol L^{-1} Tris-HCl pH 7.5]. The total protein concentration contained in the preparation was measured by the method described by Bradford (1976).

H^+-ATPase activity was determined colorimetrically, according to the method described by Fiske and Subbarrow (1925). All reactions were initiated with the addition of vesicles/organelles (30 µg mL^{-1} membrane protein) isolated by fractionation and stopped by the addition of 5% trichloroacetic acid (w:v; ice-cold) to the medium. The proton gradient was measured as described by De Michelis and Spanswich (1986), with the modifications proposed by Façanha and De Meis (1998), while monitoring the fluorescence decrease

rate (ΔFmax min^{-1}) of the metachromatic fluorescence probe, 9-amino-6-chloro-2-methoxyacridine (ACMA), which was excited with a beam of 415 nm wavelength, and with the emission captured at 485 nm using a spectrofluorimeter.

The reaction began with the addition of the protein (present in the isolated vesicles) and was stopped by TCA (trichloroacetic acid) addition at a final concentration of 10% (v:v). Development of the hydrolyzed inorganic phosphate was promoted by the addition of 0.5 mL of a mixture containing 2% ammonium molybdate in 2% H_2SO_4 + 1% ascorbic acid after 10 min, and the spectrophotometric reading was performed at a 790-nm wavelength. The composition of the reaction medium was 10 mmol L^{-1} MOPS [3-(N-morpholino)-propanesulfonic acid]-Tris pH 6.5, 3 mmol L^{-1} $MgCl_2$, 100 mmol L^{-1} KCl, 1 mmol L^{-1} ATP and 30 µg mL^{-1} protein. The specific activity of P-type plasma membrane H^+-ATPase was determined by the percentage of activity sensitive to the classical inhibitor of P-ATPases, sodium orthovanadate (0.2 mmol L^{-1}; Na_3VO_4) (DE MICHELIS and SPANSWICK, 1986). That is, the hydrolytic activity of the H^+-ATPase was measured at 25°C, with and without the inhibitor, with the difference between the two activities being the action of the P-type H^+-ATPase.

A completely randomized design with 4 replicates of 25 seeds per plot was used. Analysis of variance was performed for all evaluated variables, and when significant, and if qualitative, the data were subjected to the Scott-Knott test at 5%, and if quantitative, to regression analysis. The SISVAR software was used for the analyses (Ferreira, 2014).

RESULTS AND DISCUSSION

Germination at 15 and 30 DAS was not significant for those factors evaluated according to the F test at the 5% probability level. This finding was expected, since auxins have no direct effects on seed germination. This result corroborates the study of Vendruscolo (2014), who worked with sorghum and observed that doses of humic substances had no effect on seed germination or initial seedling growth. However, this result disagreed with findings on other species that showed responses to the application of humic substances, such as tomato, for which Silva Filho and Silva (2002) demonstrated that humic substances increased germination percentage and speed.

For the variable root length, a significant effect was observed for the different doses but not for the different organic acids or for the interaction of these two factors. Evaluations of the oxidative enzymes showed that all enzyme activity differed significantly according to the acids and doses applied. These evaluations also showed a significant response to the interaction between acids and doses.

H^+-ATPase and the other enzymes showed significant differences by the F test at the 5% probability level for the doses and humic substance types used and for the interaction between doses and types.

The activity of the H^+-ATPase increased with the increasing doses of humic substances applied, and the same was observed when the humic substances were analyzed separately; that is, when humic acid was used, enzymatic activity increased considerably with increasing doses. However, for fulvic acid, this increase is only observed up to a dose of approximately 40 $mg.dm^{-3}$, and thereafter, a slight decrease is seen (Figure 1).

H^+-ATPase is more popularly known as a proton pump and is the main enzyme responsible for nutrient absorption by the roots. It generates an electrochemical gradient that induces the passage of nutrients through the root cell membrane. Canellas et al. (2009) showed that humic acid increased the H^+-ATPase activity in the corn root cells. Jindo et al. (2012) also reported higher H^+-ATPase activity in corn when treated with humic acid.

Although H^+-ATPase has been shown to increase nutrient absorption activity in the roots, in the present study, increased enzymatic activity had no effect on root length (Figure 2). The proton pump stimulates cell wall expansion, promoting cell elongation, and consequently, it increases the root length (Canellas et al., 2004).

Figure 2 shows the complex behavior of the root length curve with increasing dose; nevertheless, the greatest root length was observed at a dose of 0 $mg.dm^{-3}$. The beneficial effects of humic substances on the root system have been observed in tomato, wheat, corn (Canellas et al., 2009; Jindo et al., 2012), and lettuce (Borcioni et al., 2016). However, no satisfactory results were obtained in the Arabica coffee cultivar Topazio MG1190. Root length is an important factor because, the longer the root is, the greater the volume of soil exploited, with several benefits, including better water absorption at greater depths.

The effects of humic substances on plant physiology include the promotion of root growth (Canellas et al., 2015). The activity of plasma membrane H^+-ATPase in root cells is induced by humic substances isolated from agricultural compost in the same way that exogenous auxin induces growth (Dobbs et al., 2010).

Aguiar et al. (2013) studied the bioactivity of humic acids isolated from vermicompost at different maturation stages over a period of 120 days of composting. After 60 days of vermicomposting, the humic acids presented the promotion of lateral root emission, acidification of the growth medium, and proton pump induction, reducing the predicted time by 50%.

The mean catalase, superoxide dismutase, and alcohol dehydrogenase activities were higher when fulvic acid was used, whereas for esterase, the highest mean was obtained when humic acid was applied (Table 1). After the seed is formed, it undergoes some harmful effects due to several factors, one of which is caused by reactive oxygen species (ROS), which are a consequence of the malfunction of some metabolic pathways, as well as of the normal functioning of others (Wang et al. al., 2012). ROS include hydrogen peroxide (H_2O_2), the superoxide anion radical (O^{2-}), and the hydroxyl radical (OH^-), which are capable of oxidizing cell components (Wang et al., 2012). Organisms are capable of producing antioxidant enzymes to eliminate these ROS. Such enzymes include catalase, superoxide dismutase, alcohol dehydrogenase, and esterase.

Increased oxidative enzyme activity is an indication of environmental stress. In this experiment, no specific type

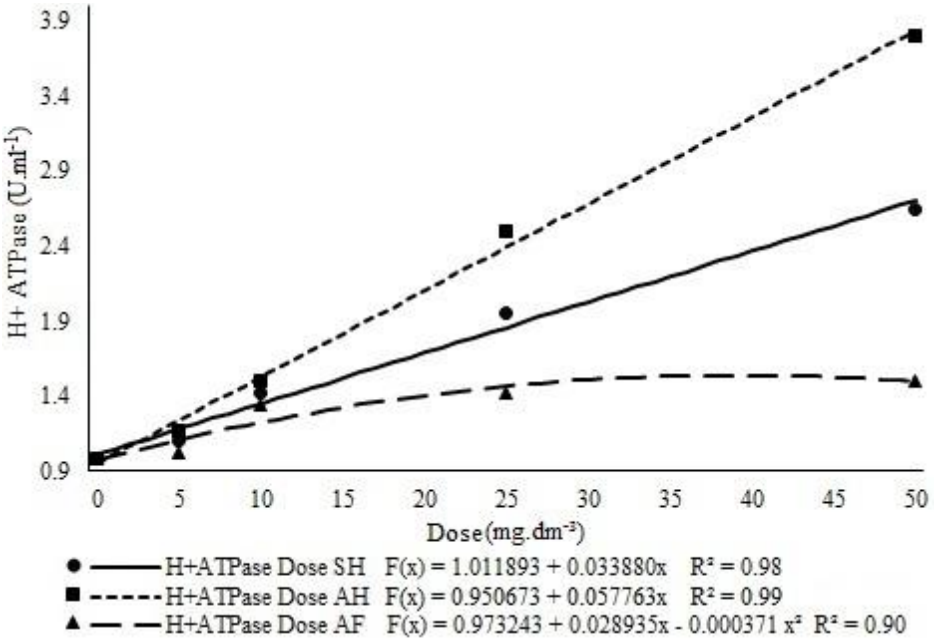

Figure 1. H^+-ATPase activity (U/ml) as a function of the dose of humic substances and of the interaction between the doses and acid types (humic acid (HA) and fulvic acid (FA)).

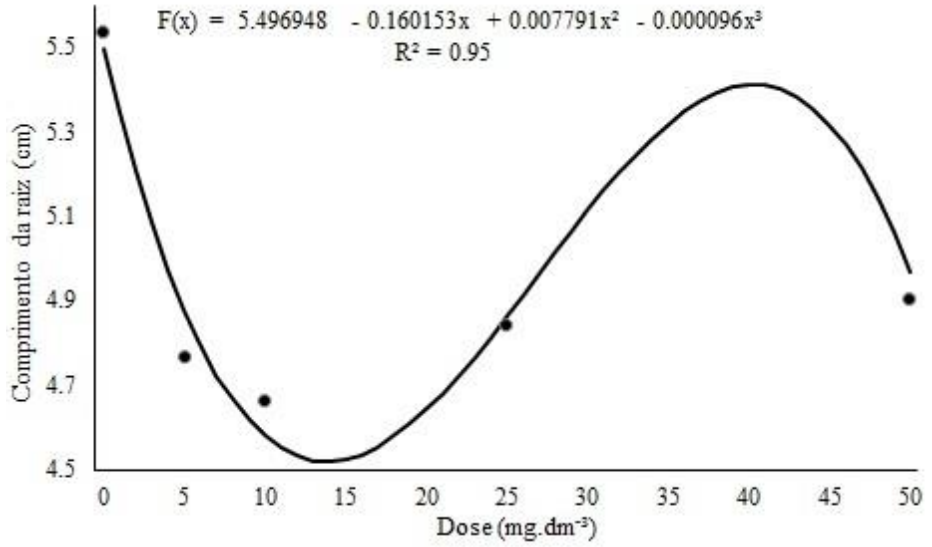

Figure 2. Root length according to the doses of humic substances.

Table 1. Mean enzymatic activity of catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), and esterase (EST) according to the type of humic substance (humic acid (HA) and fulvic acid (FA)).

Acid	Mean enzymatic activity (U.mg^{-1})			
	CAT	SOD	ADH	EST
HA	2.845^{b}	8.894^{b}	5.561^{b}	4.151^{a}
FA	3.535^{a}	11.051^{a}	6.909^{a}	2.796^{b}

Means followed by the same letter in the column did not differ, Scott-Knott test, $p<0.05$.

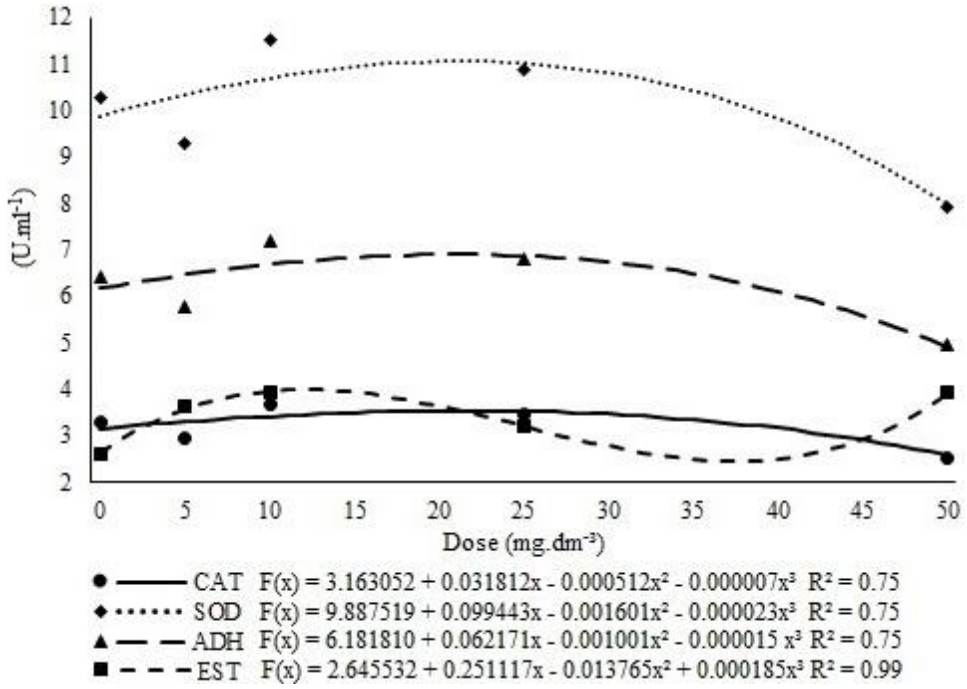

Figure 3. Enzymatic activity of catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), and esterase (EST) according to the doses of humic substances.

of stress was applied to the seeds. The germinator controls the light and temperature, and the humidity was also controlled, so the observed differences in the enzymatic activities are possibly related to the humic substances added to the medium, since that was the only change.

Enzyme activity related to oxidative stress varied with increasing doses of humic substances, but the lowest enzymatic activity rates were observed at the extreme doses (0 and 50 mg.dm^{-3}), except for esterase, which presented different behavior with increasing doses (Figure 3).

All doses used are relatively small when compared to some fertilizers commonly used in agriculture, and even so, considerable changes occurred in the enzymatic reactions. This may be because the humic substances exert a biostimulating effect on plants, which in small doses, is capable of positively or negatively affecting plant metabolism and development (Biostimulant Coalition, 2018).

The interaction of the acid types within each dose of the humic substances was significant for the antioxidant enzymes. At the dose of 5 mg.dm^{-3}, only esterase presented significant differences for the acids, presenting higher mean activity in the treatment with humic acid. At the doses of 10 and 25 mg.dm^{-3}, catalase, superoxide dismutase, and alcohol dehydrogenase showed higher mean activity with fulvic acid, and esterase, with humic acid. At the dose of 50 mg.dm^{-3}, all evaluated enzymes presented higher mean activity in treatments with fulvic acid (Table 2).

This study showed that when enzyme activity was lowest, the coffee seedlings were under the least amount of stress. Catalase, superoxide dismutase, and alcohol dehydrogenase presented curves with similar behavior, with the lowest enzymatic activities observed at the extreme doses of fulvic acid (0 and 50 mg.dm^{-3}), whereas esterase had the lowest activity at the dose of 35 mg.dm^{-3} (Figure 4).

Fulvic acid has a higher molecular weight than humic acid, which facilitates its passage through the plasma membrane of root cells, so it can move within the plant and interact or even influence plant physiology and metabolism (Anjum et al., 2011). In the present experiment conducted with the Arabica coffee cultivar Topazio MG1190, this interaction was not very evident.

The enzymatic activity of catalase, superoxide dismutase, and alcohol hydrogenase decreased with increasing doses of humic acid, with the lowest activity observed at 50 mg.dm^{-3} (Figure 5). This finding may be indicative of lessening of stress with increasing doses of humic acid, since enzymes, after combating the causes of stress, can be deactivated until needed in response to future stress.

When treated with humic acid, lettuce showed a considerable increase in superoxide dismutase activity (Haghighi, 2011). Sweet potato showed an increase in catalase activity, also when treated with humic acid, which functioned as aid in combating ROS (Chen et al., 2017). A study on evergreen azaleas showed the

Table 2. Mean enzymatic activity (U/ml) of catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), and esterase (EST) within each dose of humic or fulvic acid.

Acid	5 mg.dm^{-3}			
	CAT	**SOD**	**ADH**	**EST**
HA	2.940^{a}	9.190^{a}	5.746^{a}	3.767^{a}
FA	2.990^{a}	9.347^{a}	5.844^{a}	3.517^{b}
	10 mg.dm^{-3}			
	CAT	SOD	ADH	EST
HA	3.087^{b}	9.649^{b}	6.033^{b}	5.384^{a}
FA	4.273^{a}	13.358^{a}	8.352^{a}	2.461^{b}
	25 mg.dm^{-3}			
	CAT	SOD	ADH	EST
HA	2.830^{b}	8.846^{b}	5.531^{b}	5.204^{a}
FA	4.130^{a}	12.910^{a}	8.072^{a}	1.243^{b}
	50 mg.dm^{-3}			
	CAT	SOD	ADH	EST
HA	2.083^{b}	6.512^{b}	4.072^{b}	3.776^{b}
FA	2.007^{a}	9.367^{a}	5.857^{a}	4.141^{a}

Means followed by the same letter in the column did not differ, Scott-Knott test, p<0.05.

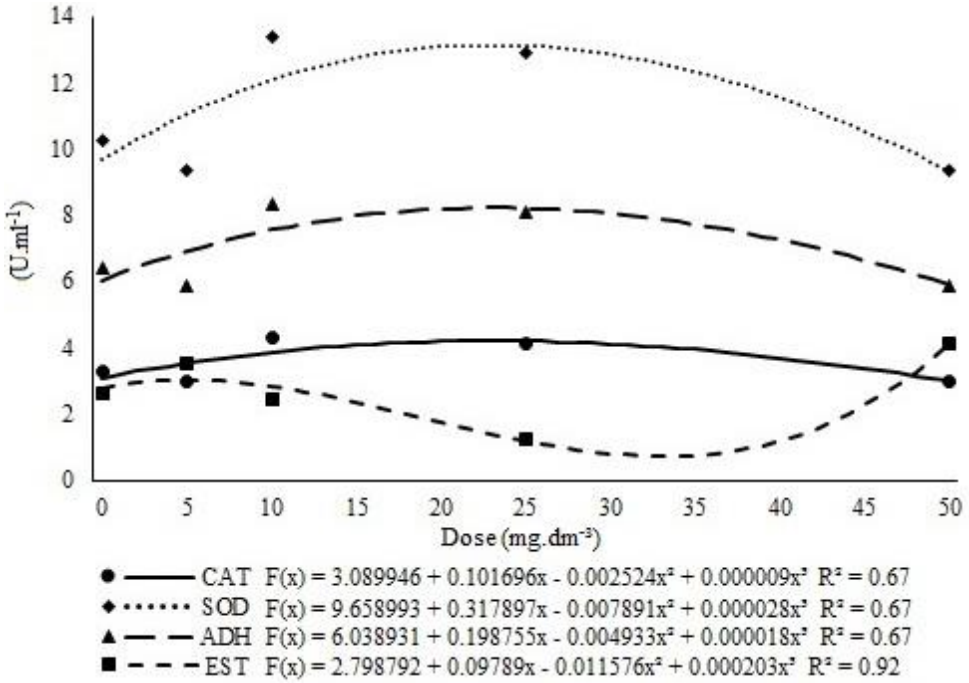

Figure 4. Enzymatic activity of catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), and esterase (EST) according to the doses of fulvic acid.

influence of humic acid on some enzymes, including catalase and superoxide dismutase, since humic acid applications led to greater enzymatic activity (Elmongy et al., 2018).

Applications of humic acids lessened the negative effects of salinity on the vegetative growth and flowering of *Chrysanthemum indicum* in the greenhouse (Mazhar et al., 2012).

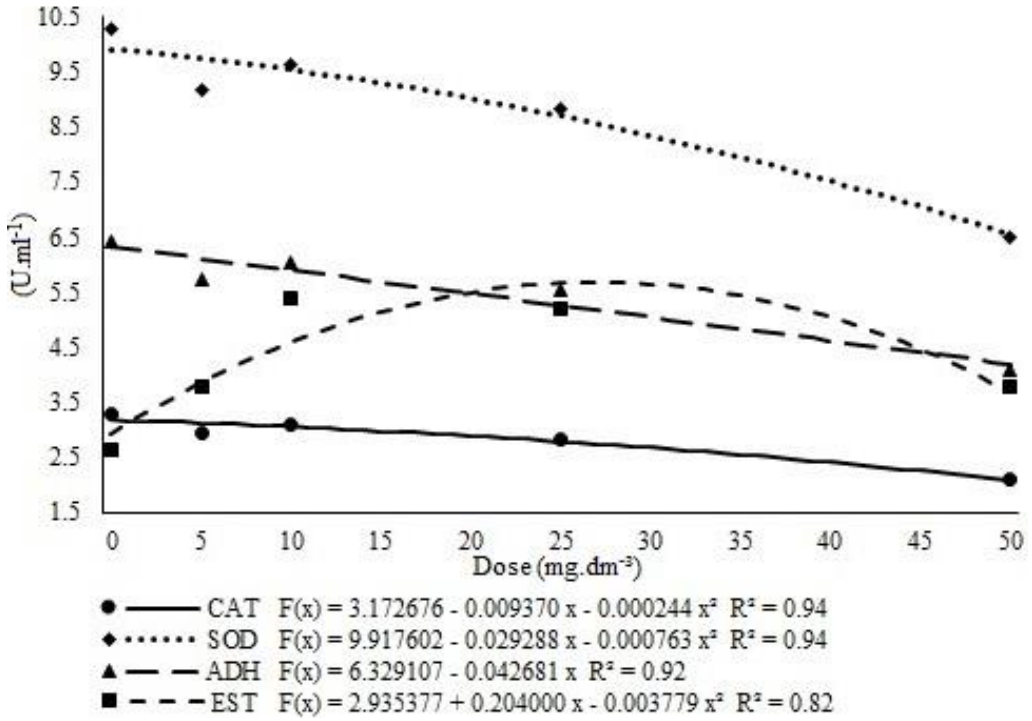

Figure 5. Activity of the enzymes catalase (CAT), superoxide dismutase (SOD), alcohol dehydrogenase (ADH), and esterase (EST) according to the doses of humic acid.

In addition to salt stress, another abiotic factor that harms plants is low tolerance to drought. Humic acid extracted from vermicompost was evaluated in a study on the effects of water deficit on the growth and physiology of rice seedlings (García et al., 2012). Water stress symptoms started to appear at 10 days after germination together with the promotion of seedling growth induced by humic acid. Under water stress conditions, seedlings treated with humic acid had greater root dry weight compared to controls. At 25 days after germination under water stress, the levels of chlorophyll, carotenoids, proteins, and carbohydrates were higher in the treated plants than in the controls, indicating that the photosynthetic capacity of the plants stressed by water deficit was increased by the humic acid. In an experiment with corn, applications of fulvic acid under drought conditions increased the yield (Anjum et al., 2011).

The enzyme esterase had its lowest activity at the dose of 0 $mg.dm^{-3}$ of humic acid, and its activity increased with increasing doses up to 30 $mg.dm^{-3}$ and dropped thereafter (Figure 5). The good functioning of esterase is vitally important because plasma membrane lipid peroxidation is one of the worst deterioration episodes for cells, and esterase is involved in lipid metabolism and ester hydrolysis, helping the membrane undergo stress and remain unharmed (Basavarajappa et al., 1991). Humic acid caused a considerable increase in esterase activity, showing that the least amount of stress was present at the lowest dose.

Conclusion

Humic substances do not interfere in the enzymatic activities of the germination of coffee seeds, nor does it affect vegetative growth of the seedlings

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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