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Antioxidative enzymes of *Cucumis sativus* seeds are modulated by *Leucaena leucocephala* extracts

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ABSTRACT. This study aimed to analyze oxidative stress produced in cucumber seeds and seedlings when exposed to aqueous extract from dried leaves of leucaena, as well as its effect on the germination behavior, early growth and the antioxidant enzymes activity. It was evaluated the percentage, the speed index, the average time, the frequency and germination synchronization, the root length and shoot, as well as the catalase and peroxidase enzymes activity. There was no significant inhibitory effect of the leaf extracts on the germination percentage. However, there was delay in the seeds germination, as the extract proportion increased. A stimulatory effect of the extract compared to the shoot length was observed, however the root growth was significantly reduced. The catalase activities had a peak at 24 hours after soaking the seeds, however, the activities were reduced in seedlings. The peroxidase activity was low in the seeds and increased in the seedlings at 168 hours after immersion. The results suggest that there was oxidative stress due to allelochemicals present in the leaves extracts from leucaena, verified by germination and initial growth changes, causing alterations in the plants rootlets.

Keywords: allelopathy, reactive oxygen species, antioxidant enzymes.

Enzimas antioxidativas de sementes de *Cucumis sativus* são moduladas por extratos de *Leucaena leucocephala*

RESUMO. Este trabalho objetivou analisar o estresse oxidativo produzido em sementes e plântulas de pepino quando submetidas a extrato aquoso de folhas secas de leucena bem como, seu efeito sobre o comportamento germinativo, crescimento inicial e atividade de enzimas antioxidantes. Foram avaliadas a porcentagem, índice de velocidade, tempo médio, frequência e sincronização da germinação, comprimento de raiz e parte aérea e atividade das enzimas catalase e peroxidase. Não houve efeito inibitório significativo dos extratos sobre a porcentagem de germinação. No entanto, houve atraso na germinação das sementes, à medida que se aumentou a proporção do extrato. Foi observado efeito estimulatório do extrato em relação ao comprimento da parte aérea, porém o crescimento da raiz foi reduzido significativamente. As atividades da catalase tiveram pico às 24 horas de embebição das sementes, tendo sido reduzido nas plântulas. No entanto, a atividade da peroxidase foi baixa nas sementes e teve aumento nas plântulas, às 168 horas após a embebição. Os resultados sugerem que houve estresse oxidativo devido aos aleloquímicos presentes nos extratos foliares de leucena, verificado pelas alterações na germinação e de crescimento inicial, o que causou alterações nas radículas das plântulas.

Palavras-chave: alelopatia, espécies reativas de oxigênio, enzimas antioxidantes.

Introduction

Invasive species can have profound effects on ecosystems (Kimbro et al., 2009), through changes in the structure and habitat quality (Espinola & Julio Junior, 2007), making changes in the diversity and relative abundance of native species and altering the succession dynamics of communities.

In this way, some species are able to change the environment of others via chemicals released mostly into the soil, a phenomenon known as allelopathy (Rice, 1984). According to Rizvi et al. (1992) some invasive plants release allelochemicals in the environment, being in the aqueous phase of the soil or substrate, or by volatilized gaseous substances in the air surrounding soil plants. The secondary metabolites, after being produced and released, can cause direct and indirect effects on other plants.

Direct effects are characterized by alterations on plant metabolism and growth, affecting membranes

and its permeability, hormone concentrations, and enzyme activity (Sunmonu & Van Staden, 2014; Cheng & Cheng, 2015). Gholami, Faravani, and Kashki (2011) report that inhibitory effects on root and shoot elongation can contribute to reduction in cell division, due to damage of cell membrane caused by allelochemicals. The retarded germination and root and shoot length might be influenced by damage of root and shoot cells due to interference of absorption of nutrients and other growth processes caused by allelochemicals found in seed and leaf extracts (Elisante, Tarimo, & Ndakidemi, 2013). Many of these processes being the allelochemicals action results, which can act on the cell degradation processes signaling, through the production and accumulation of reactive oxygen species (ROS), resulting in oxidative stress (Aumonde et al., 2013).

To cope with oxidative stress conditions that are often imposed by the environment, plants have developed ROS removal systems, performed by the enzymatic antioxidant system, highlighting the peroxidase (POD) and catalase (CAT) (Gill & Tuteja, 2010). CAT is an enzyme that promotes H_2O_2 decomposition into H_2O and O_2. It is mainly found in peroxisomes and can also be present in mitochondria and cytoplasm. The Peroxidases (POD) are enzymes belonging to the class of oxidoreductases, and its role is to catalyze the hydrogen peroxide oxidation (H_2O_2) or organic peroxides (Sharma, Jha, Dubey, & Pessarakli, 2012). The peroxidases have been correlated in plants resistant to diseases, ethylene biosynthesis, lignification and suberization, and protection against H_2O_2 and other oxidants (Gomes, Smedbol, Carneiro, Garcia, & Juneau, 2014).

Therefore, this study aimed to evaluate the allelopathic potential of *L. leucocephala* aqueous extract, through the antioxidative enzymes analysis, along with the germination and early growth of the *Cucumis sativus* (cucumber), due to its characteristic bioindicator species, which presents fast and uniform germination, besides expressing phytotoxicity results even in low concentrations.

Material and methods

The study was conducted at the Plant Physiology Laboratory at the *Universidade Estadual do Oeste do Paraná*, Cascavel Campus - PR, from June to September 2014.

The seeds used in the experiment were from *C. Sativus*, long green variety, lot 30129, commercially acquired.

The *L. leucocephala* extract was obtained from leaves, at a senescence stage, observed due to loss of green color in leaves results from chlorophyll degradation, from a permanent preservation area, located between coordinates 25°40'23.03" and 54°39'46.50". The leaves were dried at 40° C in air circulating oven until obtaining its constant weight to avoid the loss of volatile compounds. After drying out, they were crushed in a Willey knife mill and packaged in labeled glass vials, protected from humidity, at ambient temperature and stored for three months until their usage.

For the matrix extract obtention, it as prepared a solution containing 100 g of *L. leucocephala* leaves powder for 1 L of distilled water solution, which rested for 24 hours and was filtered through a fine mesh strainer tissue, resulting in a matrix extract at 10% (w/v).

From this extract, 2.5, 5.0 and 7.5% proportions (w/v) of the extract in distilled water were obtained. In the control treatment only distilled water was used. In order to assess this extract's effect on germination, the early growth and antioxidative enzymes of *C. sativus* seeds and seedlings, the following tests were performed:

Germination test: For the germination tests four repetitions of 20 seeds were performed. The seeds were placed in petri dishes with three sheets of wetted filter paper with distilled water (control) or by the extract different proportions in the proportion of 2.5 times the paper weight, and then put in a growth chamber at 25° C and 12 hours photoperiod (Brasil, 2009). Germination was recorded daily, for seven days, being taken into consideration the germinated seeds with 2 mm of primary root (Hadas, 1976). To analyze the germination, the following parameters were taken: germination percentage (GP%), germination speed index (GSI), as per Maguire (1962), average germination time (AGT) and frequency and synchronization of germination, both as per Labouriau (1983). At the end of the germination test, after seven days, a measure of the germinated seedlings primary root and shoot was made using a ruler. The seedlings measurement results were expressed in centimeters.

For the germination tests, the experimental design was completely randomized (CRD), with five treatments and four replicates with 20 seeds per repetition. The means were compared statistically by Tukey test.

Enzymatic extraction: Five collection points were chosen: seeds at 0, 2, 12 and 24 hours after soaking period and seedlings at 168 hours after the soaking period. 100 mg of vegetal material were homogenized in potassium phosphate buffer, at 0.1 mol L^{-1} and pH 6.8. The homogenate was

centrifuged at 12,000 rpm for 20 minutes at 4° C. The total protein determination was carried out according to Bradford (1976) for the enzyme specific activity calculation purpose.

Peroxidase (POD): The peroxidase activity was measured according with the described by Teisseire & Guy (2000), with the addition of crude enzyme extract, 20 mmol L^{-1} of pyrogallol (1,2,3-benzenetriol), 50 mmol L^{-1} of potassium phosphate buffer, pH 6.5, and hydrogen peroxide (H_2O_2) 5 mmol L^{-1}. The purpurogallin formation was measured by a UV-visible spectrophotometer at 430 nm and its molar extinction coefficient (2.5 mmol L^{-1} cm^{-1}) was used to calculate the specific enzyme activity.

Catalase (CAT): The catalase activity was calculated according to Peixoto, Cambraia, Sant'ana, Mosquim, and Moreira (1999), with the addition of 50 µL of enzyme extract, 0.05 mol L^{-1} of sodium phosphate buffer pH 7.0, and H_2O_2 12.5 mmol L^{-1}. The absorbance reading was performed at 240 nm. The enzyme activity was calculated using the molar extinction coefficient of H_2O_2 (39.4 mmol L^{-1} cm^{-1}).

The enzymatic activities data presented were the average values of assays in duplicate, in which the enzymes behavior were observed. The enzymatic activities were expressed in specific activity (POD - µmol of purpurogallin min^{-1} mg^{-1} of protein; CAT- nmol H_2O_2 min^{-1} mg^{-1} protein).

For the analysis of antioxidative enzymes, there was no adjustment for parametric statistics, in this way, the data were analyzed by descriptive statistics.

Results and discussion

Effect of *Leucaena leucocephala* extract on seeds germination and early seedling development in *C. sativus*

Table 1 shows the results for the *C. sativus* seeds germination. There was no significant difference in the seeds germination percentage, however it was found that, with respect to GSI and AGT variables, all proportions of *L. leucocephala* extract differed from the control, with delay and greater time for this process occurence as the extracts proportions increased, corroborating with Ferreira & Aquila (2000), which state that the allelopathic effect is generally not observed on the germination final percentage, but on the germination speed or another parameter such as the average time of germination.

Bioassays with leaf extracts of exotic species such as *Emilia sonchifolia* (L.) DC. (Oliveira Belinelo, Almeida, Aguilar, & Vieira Filho, 2011) *Lolium multiflorum* Lame and *Brachiaria brizantha* cv. Marandu (Castagnara et al., 2012) also found that extracts delayed the *C. sativus* seeds germination.

Table 1. Percentage, germination speed index (GSI) and average time of germination (AGT) of *Cucumis sativus* L. seeds submitted to aqueous extract of *Leucaena leucocephala* (Lam.) De Wit. Cascavel - PR, 2015.

Treatment % (w/v)	Germination percentage (%)	Germination Speed Index	Average Germination Time (days)
0	88	13.87 a	1.58 b
2.5	86	10.49 b	1.93 ab
5	79	7.68 bc	2.26 a
7.5	85	7.86 bc	2.40 a
10	78	6.95 c	2.45 a
CV (%)	9.76	12.81	11.06

Numbers followed by the same letter do not differ significantly as determined by Tukey test ($p<0.05$)

The germination rate of the *C. sativus* seeds results are illustrated in Figure 1. The graphs show different behavior between control and other *L. leucocephala* extract proportions. It can be seen that in the control (0% w/v) the germination peak occurred on day 1 after the experiment installation, showing a better performance in terms of germination speed and increased process synchronization (U).

As for the *C. sativus* seeds under *L. leucocephala* extract different proportions showed a peak germination 48 hours after the experiment installation (Day 2), with a lower germination synchronization followed by higher AGT and lower GSI (Table 1, Figure 1).

It is worth mentioning that the frequency and synchronization are inversely related parameters, the higher the synchronization value (U), the lower the frequency, resulting in a more uniform germination at a certain point of time (Bufalo et al., 2012).

Therefore, despite the *C. sativus* seeds germination final percentage not being influenced when subjected to *L. leucocephala* extract different proportions, it is possible to observe in a more detailed and joint analysis of other variables, as seen in Table 1 (GSI , AGT, frequency and synchronization) (Figure 1), that in general, the main effects found were delays in *C. sativus* seeds germination in contact with the extracts, demonstrated by the frequency curves, an increase in the germination average time and a reduction of the germination speed index.

Therefore, the plant extract under study can interfere with other species germination process by the allelochemicals presence.

Regarding the seedlings length, the results indicated that there was *L. leucocephala* extract effect on the *C. sativus* growth, as the applied extract proportion increased (Table 2). Regarding the primary root length, all the extract proportions differed from the control, showing a negative effect for this variable. However, the seedlings shoots had growth increase at 2.5; 5 and 7.5% proportions (w/v), however at 10% (w/v) there was no significant difference compared to the control.

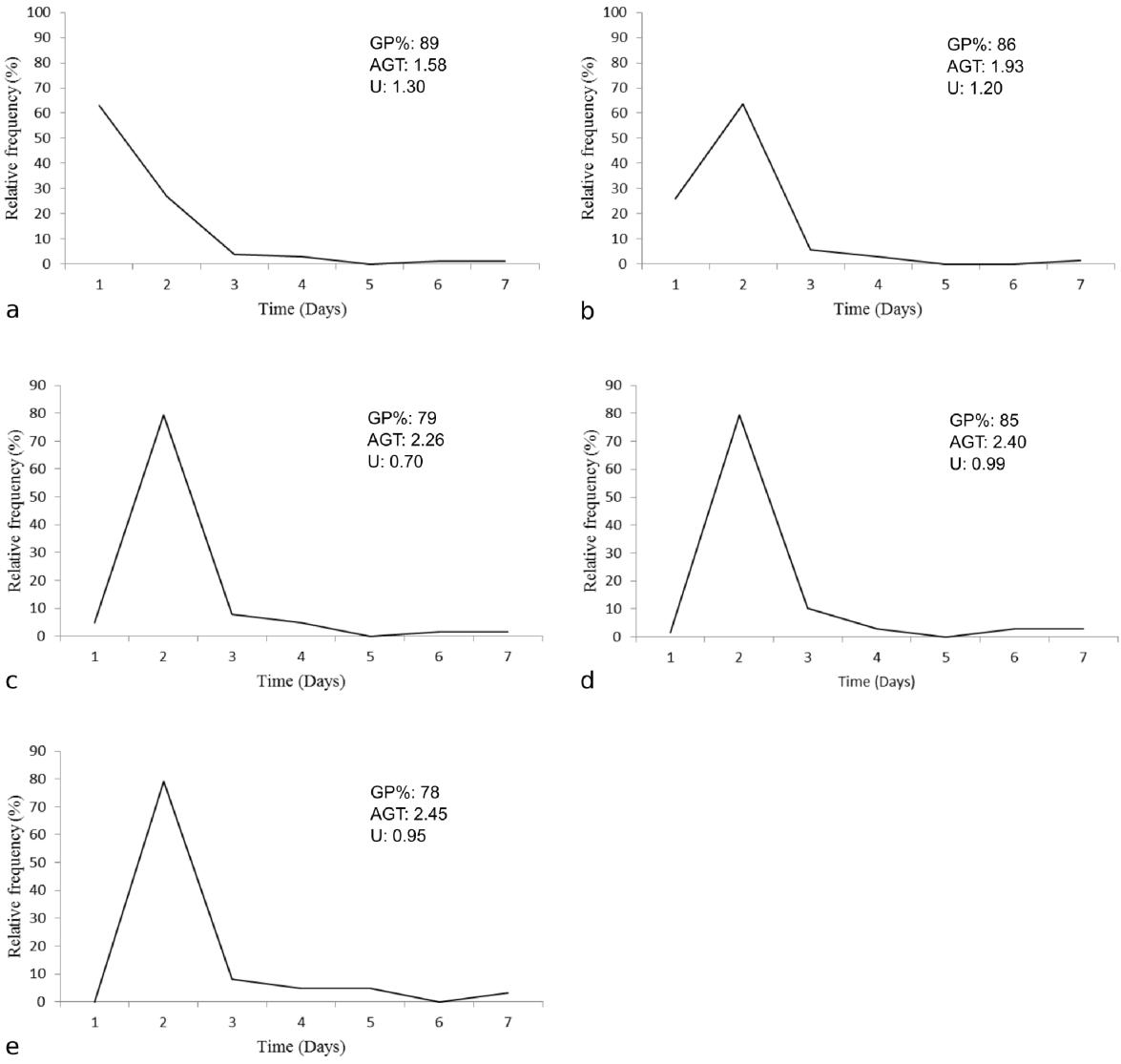

Figure 1. Relative frequency of germination of *Cucumis sativus* L. seeds in seed development. a. extract 0% (w/v); b. extract 2.5% (w/v); c. Extract 5% (w/v); d. Extract 7.5% (w/v); e. Extract 10% (w/v).

Table 2. Primary root length and shoot of *Cucumis sativus* L. seedlings subjected to aqueous extracts of *Leucaena leucocephala* (Lam.) De Wit. Cascavel - PR, 2015.

Treatment % (w/v)	Root length (cm)	Seedlings shoots length (cm)
0	10.11 a	5.76 b
2.5	8.56 b	7.18 a
5	6.79 c	7.33 a
7.5	5.73 c	7.43 a
10	1.84 d	5.54 b
CV(%)	7.26	8.54

Numbers followed by the same letter do not differ significantly as determined by Tukey test ($p<0.05$)

It is observed that the plant extracts usually affect more specifically the root than the shoot growth, as observed in this experiment, mainly due to the roots direct and prolonged contact with the extract (Tuan Noorfatiehah, Nashriyah, Hasbullah, Raja Danial, & Muhamad Azhar, 2011; Aliyu & Mustapha, 2014).

This root length decrease may be due to the interaction between allelochemicals and plant hormones, for studies show that allelopathic compounds tend to inhibit the gibberellins action as well as the acetic indole acid function (AIA), thereby preventing the cycle stages and cell elongation (Rice, 1984; De Klerk, Guan, Huisman, & Marinova, 2011).

According to the *L. leucocephala* phytochemical profile, there are in its composition compounds as mimosine (b-[N-(3-hydroxy-4-oxopyridyl)] - a-aminopropionic acid), and gallic acids, protocatechuic, p-hydroxybenzoic, p-hydroxyphenylacetic, vanillic, ferulic, caffeic and p-coumaric acids (Chou & Kuo, 1986; Aderogba, McGaw, Bezabih, & Abegaz, 2010; Hassan, Tawfik, & Abou-Setta, 2014). These

phenolic compounds can both reduce as increase the indole acetic acid concentration (IAA) in plant tissues, which is a plant hormone of the auxin group, having the cell elongation as its primary function (Rahman, 2013). The *L. leucocephala* allelopathic effects have been attributed to the phenolic compounds presence, such as those mentioned above. The coumaric acid and the hydroxybenzoic acid potentiate the IAA oxidase system, responsible for its inactivation, causing the roots inhibition (Souza Filho & Alves, 2002).

According to Cothren and Oosterhuis (2010), the root growth is inhibited by auxin in proportions promoting the stems and coleoptile elongation, which could explain the shoot growth stimulation in contrast with the root inhibition, as demonstrated in Table 2.

The extracts effect on the enzyme activity

To determine the oxidative stress presence in the *C. sativus* seeds and seedlings submitted to *L. leucocephala* extract, it was analyzed the antioxidant enzymes activity involved in ROS detoxification and balance, during the (2, 12, 24 and 168 hours) immersion periods, as seen in Figure 2.

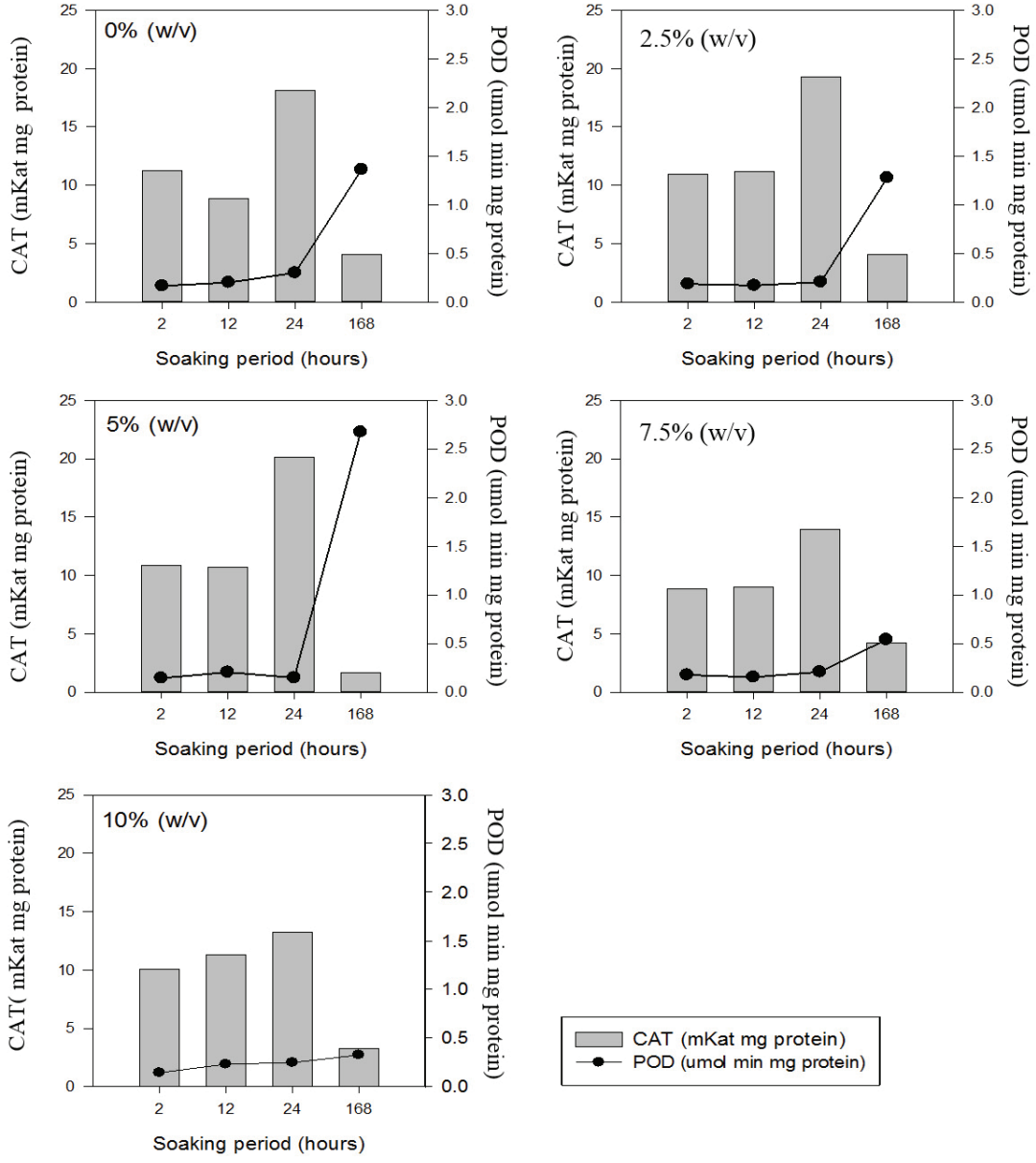

Figure 2. Peroxidase activity (POD) and catalase (CAT) in seeds (2, 12 and 24 hours) and seedlings (168 hours) of *Cucumis sativus* L. submitted to treatments with *Leucaena leucocephala* (Lam.) De Wit extract at different soaking periods

It was found that the CAT activity increased after 24 hours in all the extract proportions, as well as in the control, but decreased after 168 hours soaking, that is, this enzyme activity was low in *C. sativus* seedlings and high in the seeds after 24 hours immersion in all the tested proportions. This catalase high activity, also present in the control (0% extract), leads us to suppose that the oxidative damage arose in response to the stress caused in the natural process of the seeds soaking, that way the catalase activity increase occurred in order to start the process of damage repair, this repair process being efficient, for the seeds showed high rates of germination percentage, the germination average speed and their germination occurring in a short time (Table 1).

The CAT is the enzyme that has a high potential in the H_2O_2 dismutation process into H_2O and O_2, being essential for the ROS detoxification during the toxic radicals production conditions (Garg & Manchanda, 2009). According to Figure 3, the catalase activity was similar in all the extract proportions in the germination first two hours.

After 12 hours of soaking, small changes in the seeds activity were observed, on the extract different proportions. However, at 24 hours a high CAT activity was seen in 0, 2.5 and 5% proportions (w/v) compared to 7.5 and 10% proportions (w/v), for which the enzyme activity decreased. This CAT activity reduction did not reflect in the germination percentage decrease of the seeds submitted to 7.5 and 10% extract (w/v), however may have led to increased time and low germination rate index (Table 1).

According to Umair, Ali, Tareen, Ali, and Tareen (2012) the damage repair caused by the lipid peroxidation occurs during the Phase I of the water procurement by the seeds, mainly through the antioxidant enzymes production. It is at this germination stage that higher activities of enzymes, promoting the reactive oxygen species elimination, are observed, such as CAT and POD. According to Gurgel Jr., Torres, Oliveira, and Nunes (2009) the *C. sativus* seeds have moisture gain mainly after 12 and 26 hours of immersion, which may explain the fact that cucumber seeds soaked in water also showed increase of CAT 24 hours after immersion (Figure 2).

After 168 hours of immersion, an increased activity of this enzyme at 0% (w/v) extract ratio was observed, with a decline at 2.5 and 5% proportions (w/v), increasing again at 7.5 to 10% ratios (w/v), reaching then values similar to the control.

The POD activity was similar in all ratios during the first 24 hours of germination (comprising values between 0.14 and 0.30 umol min mg protein), however, at 168 hours after immersion at 5% extract proportion (w/v), an increase in its activity occured, as shown in Figures 2 and 3. Siddique & Ismail (2013) also observed a POD activity increase in rice seedlings, when submitted to *Fimbristylis miliacea* extract.

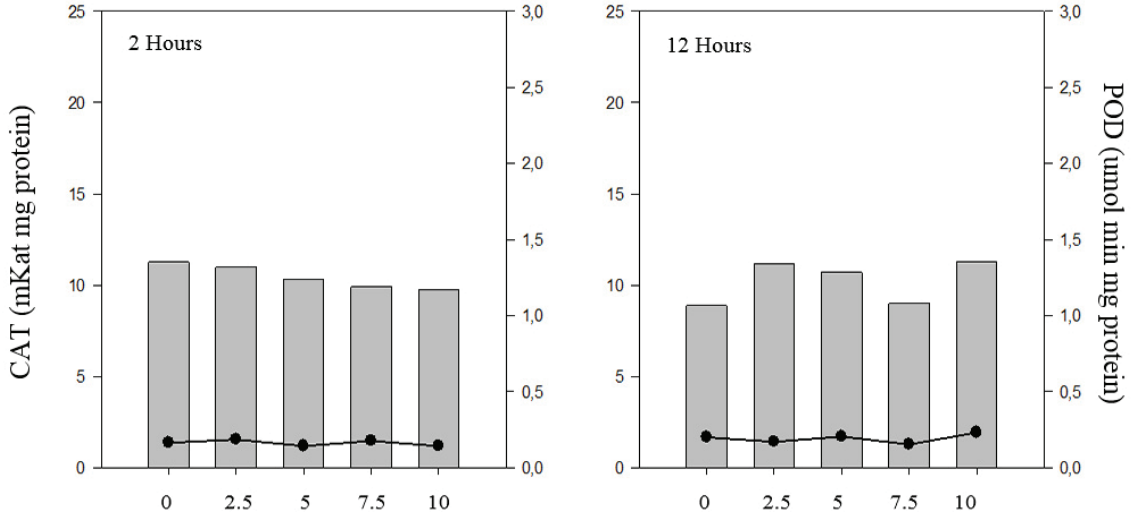

Figure 3. Activity of peroxidase (POD) and catalase (CAT) in seeds and seedlings of *Cucumis sativus* L. submitted to different proportions of *Leucaena leucocephala* (Lam.) de Wit extract at different times of soak.

This enzyme activity was more pronounced in seedlings (exposed to 168 hours immersion) (Figure 3), its activity peak being at 5% proportion (w/v) of the extract, in contrast with the catalase low activity, significantly reducing its activity at 7.5 to 10% proportions (w/v) of the extract. Apel & Hirt (2004) suggest that when changes in the antioxidant enzymes balance happen, there is a compensatory mechanism occurrence in the tissues. For example, when the catalase activity is reduced, other enzymes are generated in large quantities in a compensatory effect, in order to guarantee the oxidative damage repair. However, this was not observed in the *C. sativus* roots development. It is possible that the low POD activity in the extract higher proportions was not sufficient to repair the oxidative damage, causing the seedlings stress, due to possible membrane damage, as observed in the roots length average low values (Table 2).

Therefore, plants subjected to stressful conditions increase the antioxidant enzymes activities in order to prevent damage. Consequently, the energy of the seedling allelochemicals mediated stress is directed to the antioxidants biosynthesis, enabling this way to face adverse environmental conditions. This energy shift may be responsible for reducing the growth of the plants that are conditioned to this stress (Sunaina & Singh, 2014).

Conclusion

The *Leucaena leucocephala* extract has allelopathic potential.

There was a delay in the seeds cucumber germination and a decrease in the radicle length of seedlings due to the oxidative stress facing the allelochemicals present in the extract.

An oxidative stress was induced by the allelochemicals present in the extract.

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