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OXIDATIVE STRESS IN THE DEVELOPMENT OF *Phaseolus vulgaris* (L.) SEEDLINGS SUBMITTED TO THE EXUDATE OF *Cajanus cajan* (L.) MILLSP. SEEDS

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ABSTRACT: The species used to recover damaged areas can release allelopathic compounds that influence the metabolism of neighboring species promoting oxidative stress. The objective was to evaluate if the exudation of *Cajanus cajan* (L.) Millsp. seeds generates oxidative stress during the early development of *Phaseolus vulgaris* L. seedlings from the analysis of the specific activity of the enzymes peroxidase (POD) and catalase (CAT). Pre-germinated seeds of *P. vulgaris* were submitted to T1 treatment: distilled water (control); T2: immersion on phase I exudate (6 hours) and T3: immersion on phase II exudate (20 hours) of *C. cajan* seeds, 4 replicates of 10 seeds each. Six days after the installation, peroxidase and catalase activity and the measurement of the mean and dry mass of root and shoot of *P. vulgaris* were analyzed. We observed that the allelochemicals released by *C. cajan* seeds during the imbibition phases can modify the metabolism of other species during initial growth and development. The model showed that stress was controlled by the specific activity of peroxidase and catalase enzymes that stimulated epicotyl growth of *P. vulgaris* plants.

Keywords: allelopathy, peroxidase, catalase.

ESTRESSE OXIDATIVO NO DESENVOLVIMENTO INICIAL DE PLÂNTULAS DE *PHASEOLUS VULGARIS* (L.) SUBMETIDAS AO EXSUDATO DE SEMENTES DE *CAJANUS CAJAN* (L.) MILLSP.

RESUMO: Espécies utilizadas na recuperação de áreas degradadas podem liberar compostos alelopáticos que influenciam no metabolismo de espécies vizinhas promovendo o estresse oxidativo. O objetivo foi avaliar se o exsudado das sementes de *Cajanus cajan* (L.) Millsp. geram estresse oxidativo durante o desenvolvimento inicial de plântulas de *Phaseolus vulgaris* L. a partir da análise da atividade específica das enzimas peroxidase (POD) e catalase (CAT). As sementes de *P. vulgaris* pré-germinadas, foram submetidas aos tratamentos T1: água destilada (testemunha); T2: exsudado da fase I de embebição (6 horas) e T3: exsudado da fase II (20 horas) de embebição das sementes de *C. cajan*, cada um com 4 repetições de 10 sementes. Seis dias após a instalação foram analisadas a atividade da peroxidase e catalase e a mensuração do comprimento médio e massa seca da raiz e parte aérea de *P. vulgaris*. Observamos que os aleloquímicos liberados pelas sementes de *C. cajan* durante as fases de embebição podem modificar o metabolismo de outras espécies durante o crescimento e desenvolvimento inicial. O modelo revelou que o estresse foi controlado pela atividade específica das enzimas peroxidase e catalase que estimulavam o crescimento do epicótilo de mudas de *P. vulgaris*.

Palavras-chave: alelopatia, peroxidase, catalase.

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INTRODUCTION

The process of allelopathy is characterized by the synthesis and release of compounds that affect the relationship between plants and the biotic and abiotic environment in which they live. The influence may be positive or negative to the growth and development of neighboring species (Souza Filho et al., 2011). Compounds lie in different segments of the plant and during their life cycle they are distributed in different concentrations within the vegetal tissues (Almeida et al., 2008).

Allelopath compounds may be released through leeching, volatizing of tissues, exudation of roots and/or seeds and decomposition of organic matter (Almeida et al., 2008). When allelopath compounds are released by seed exudation, the cell contents, such as sugars, are released during the soaking process and may trigger or inhibit the development of other plants of the same or of other species (Pinã-Rodrigues et al., 2015).

Allelochemicals are selective molecules in their activity and plants´ growth and development may be impaired or enhanced when these compounds occur in the environment. As a rule, compounds have several molecular targets and may influence respiration, photosynthesis, hydric relationships, opening of the stomata, levels of vegetal hormones, availability of minerals, cell division and elongation, structure and permeability of membranes and cell wall. Several of the above-mentioned processes are due to the oxidative stress triggered by the accumulation of reactive oxygen species (ROS) which impairs the growth and development of other plants (Gniazdowska et al., 2014).

ROSs accumulation, mediated by allelochemicals in the environment, may damage vegetal growth and development (Gniazdowska et al., 2014). According to Barbosa et al. (2014), ROS accumulation in vegetal cells occurs with the activation of anti-oxidant systems and is associated to the synthesis of molecules such as glutathione reductase, ascorbate and tocopherol, coupled to enzyme activity such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) which regulate and maintain ROS basal level in the cells without any apparent damage to vegetal growth and development.

Search for alternatives in the recovery of degraded areas (RDA) has underscored the direct seeding of forest species among the methodologies employed. Actually, it is a more economic option than the conventional planting of seedlings, since the former may be done in difficult-to-access areas and on high slopes (Daltro et al., 2013). The seeding system has been adopted in several regions in Brazil and has been underscored by government and private

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institutions (Oliveira 2013 et al., 2015).

The technique consists of mixing species of several native seeds to a substrate. It is highly recommended that seeds of pioneer, secondary and climax species are mixed, with the possibility of biological interaction of exudates of seeds or seedlings from different species through allelopathy. Exotic species, such as *Cajanus cajan* (L.) Millsp. of the Fabaceae family, known as guandu beans, are also mixed to these species (Pietro-Souza and da Silva, 2015). *Cajanus cajan* (L.) Millsp. is native to tropical West Africa and has a three-year life cycle. The shrub, 2 - 3 m high, grows rapidly, with high productivity of phytomass for soil covering, and hinders the establishment of invading species (Lima Filho et al., 2014). The species is greatly capable of fixing nitrogen, with an important role in the fertilization of degraded soil (Pietro-Sousa and Silva, 2015).

Valmorbida (2016) showed that exudate of *C. cajan* seeds has an allelopathic capacity since the germination in *Solanum lycopersicum* L. seeds was enhanced without any oxidative stress at cell level. In other words, the species is capable of achieving ecological restoration in degraded areas since the compounds released by seeds may stimulate the growth of other plants. Exudates should also be evaluated to verify whether seeds also interfere during the early growth of other species. *Phaseolus vulgaris* (L.) was used in current assay as a study model.

Current assay evaluates whether exudate from *Cajanus cajan* seeds produces oxidative stress during the early development of *Phaseolus vulgaris* seedlings by analyzing the specific activity of peroxidase (POD) and catalase (CAT) enzymes.

MATERIALS AND METHODS

Current study was performed at the Laboratory of Vegetal Physiology of the Biology and Health Center (CCBS) of the Universidade Estadual do Oeste do Paraná (UNIOESTE), Campus Cascavel, Cascavel PR Brazil, between March and August 2016.

Seeds of *Cajanus cajan* cv. BRS Mandarim were used as allelochemical donors by the exudation process and seedlings of the bio-indicator *Phaseolus vulgaris* L. cv. IAC Milênio were the receiving species. *Phaseolus vulgaris* underwent the following treatments: T1: distilled water (control); T2: exudate of soaking curve phase I of *C. cajan* seeds (6 hours); T3: exudate from soaking curve phase II of *C. cajan* seeds (20 hours).

Analysis test for oxidative stress throughout early growth comprised the pre-germination of *P. vulgaris* seeds in Germitest paper soaked with 2.5 times their dry weight in

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distilled water, conditioned in BOD germination chambers at 25°C, light/dark photoperiod (BRASIL, 2013). They were monitored daily till primary root averaged 3 cm and then they were submitted to treatments with exudate of *C. cajan* seeds.

As precursors of the exudate, the *C. cajan* seeds germinated in Germitest paper soaked in distilled water at a proportion of 2.5 times their dry weight and maintained in a germination chamber at 25°C within a 12-h light/dark photoperiod for 6 and 20 hours, representing respectively phases I and II of the seeds´ soaking curve, following Valmorbida (2016). When the *C. cajan* seeds attained phases I and II of the soaking curve, they were removed from the Germitest paper and 10 seedlings of *P. vulgaris*, averaging a 3 cm primary root, were placed instead. Assay was performed during six days. On the sixth day after the start of the experiment, collections were undertaken for further analysis of the specific activity of peroxidase (POD) and catalase (CAT) enzymes, mean length and dry mass of the root and of the aerial section of *P. vulgaris* seedlings.

Peroxidase activity (EC 1.11.1.7) was determined following Teisseire and Guy (2000) and reaction occurred by adding 30 µL of enzymatic extract, 500 µL of potassium phosphate buffer 50 mmolL^{-1}, pH 6.5, 250 µL pyrogallol (1,2,3-benzenetriol) 20 mmol L^{-1} and 250 µL hydrogen peroxide (H_2O_2) at 5 mmol $L^{-}1$, totaling 1 mL. Reaction was incubated for five minutes at room temperature (25°C) and purpurogallin was measured by UV-visible spectrophotometer at 430 nm. Specific activity of peroxidase was calculated with the amount of samples´ total soluble protein following Bradfor´s methodology (1976) and the coefficient of molar extinction of purpurogallin (2.5 mmol L^{-1} cm^{-1}). Results were given in µmol of purpurogallin $min^{-1}mg^{-1}$ of protein.

Catalase (EC 1.11.1.6) activity was assessed according to Peixoto et al. (1999). Reaction occurred with 50 µL of enzyme extract plus 950 µL of sodium phosphate buffer 0.05 mol L^{-1}, pH 7.0 and H_2O_2 12.5 mmol L^{-1}, with total volume 1 mL. Reaction was incubated at room temperature (25°C) for 80 seconds and readings were undertaken with visible UV-spectrophotometer at 240 nm absorbance. Enzyme´s specific activity was calculated by the difference in absorbance between 0 and 80 seconds, the amount of total soluble protein of the samples calculated according to Bradford (1976) and the coefficient of molar extinction of H_2O_2 (39.4 mmol $L^{-1}cm^{-1}$). Results were given in nmol of H_2O_2 at min^{-1} mg^{-1} protein.

Ten seedlings were measured by repetition with a digital caliper on the sixth day from the start of the assay to analyze mean length of the root and the aerial segment of *P. vulgaris*

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seedlings. Length of the aerial segment was calculated by measuring the distance between the base and the peak of the epicotyl; root length was measured by the distance between the apical section and the root cap. Results were given in centimeters (cm).

Dry mass of root and the aerial segment of *P. vulgaris* were measured by weighing five seedlings on a four-decimal-points precision scale. The plants were then conditioned in dark envelopes and placed in a forced air buffer at 32°C till constant mass. They were newly weighed to obtain the dry mass of the root and the aerial segment, given in milligrams (mg).

Assay design was totally randomized, with three treatments, each of which composed of four repetitions of ten pre-germinated seeds of *P. vulgaris*. Data on the activity of enzymes peroxidase, catalase, mean length (MLR and MLAS) and dry mass (MSR and MLAS) of root and aerial segment, which complied to normality, underwent analysis of variance (test F). Measurements were compared by Tukey´s test ($p \leq 0.05$), by R statistical program.

RESULTS AND DISCUSSION

The analysis of anti-oxidant enzymes in *P. vulgaris* seedlings after six days from the start of the experiment showed that peroxidase´s specific activity did not change in the roots, whereas catalase´s activity was reduced to 37.62 and 86.50 nmol min^{-1} mg^{-1} in the presence of the exudate of *C. cajan* seeds, respectively at Phases I and II, when compared to control with a specific activity of the enzyme at 240.57 nmol min^{-1} mg^{-1} (Figures 1A and 1B). In spite of the significant reduction of more than a half of catalase activity in *P. vulgaris* roots, mean length and dry matter of the roots were not altered with the exudate of *C. cajan* seeds since the variables were not statistically different from control (Figures 2A and 2B).

When the specific activity of anti-oxidant enzymes of the aerial segment of *P. vulgaris* seedlings was calculated after submitted to treatments (Figures 1A and 1B), catalase´s specific activity failed to occur even in control. However, Phase II exudate of *C. cajans* seeds caused an increase in peroxidase activity in control from 0.00054 umol min mg^{-1} to 0.0021 umol min mg^{-1}. Associated to increase of peroxidase activity, there was a statistically significant increase ($p \leq 0.05$) of 1 cm in the length of the aerial segment of *P. vulgaris* seedlings with Phase I and II exudates of *C. cajan* seeds when compared to control. Dry mass of the seedlings was maintained statistically equal among treatments six days after the start of the experiment (Figures 2A and 2B).

Figure 2 reveals that exudate of *C. cajan* seeds contains compounds that cause oxidative

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stress when in contact with developing *P. vulgaris* seedlings. Oxidative stress, however, decreases when the activities of anti-oxidant enzymes increase, as in the case of peroxidase

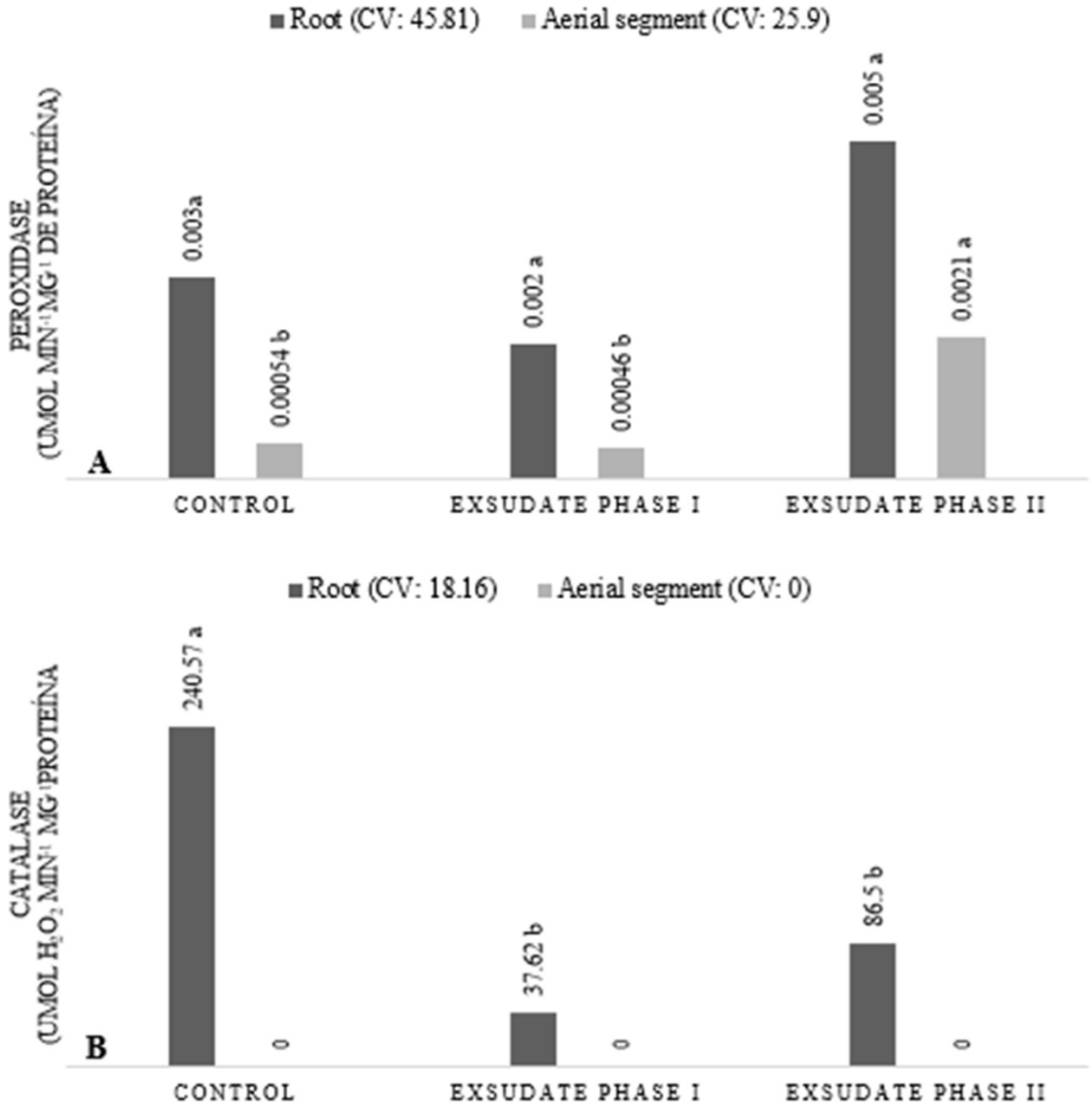

Figure 1: A: specific activity of peroxidase (umol min mg^{-1}) and B: specific activity of catalase (nmol de H_2O_2 consumed min^{-1} mg^{-1} protein) in *Phaseolus vulgaris* (L.) plants, submitted to exudate of *Cajanus cajan* (L.) Millsp. seeds. Means followed by the same letter do not differ by Tukey´s test ($p \leq 0.05$).

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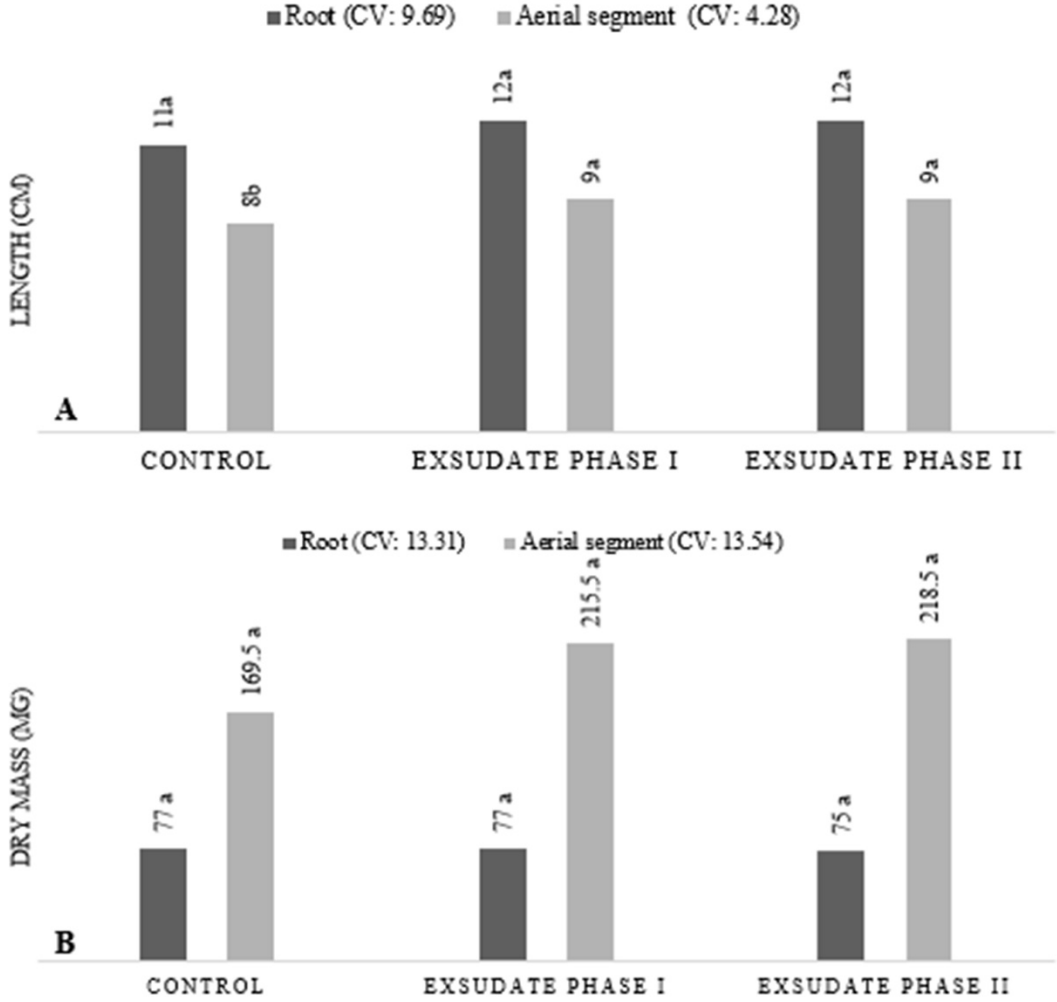

Figure 2: A: mean length (cm) and B: dry mass (mg) of *Phaseolus vulgaris* (L.) seedlings submitted to exudate of *Cajanus cajan* (L.) Millsp. seeds. Means followed by the same letter do not differ by Tukey´s ($p \leq 0.05$).

Current analysis shows that exudate of *C. Cajan* seeds may enhance the growth and development of the aerial segment of other species without jeopardizing the development of the sub-soil system for nutrients (Figures 2A and 2B). The use of the species as green fertilizer in direct seeding technique of forest species may improve the soil´s physical structure and alter the dynamics of ecological relationships in vegetal communities through the release of secondary metabolites (Trezzi et al., 2016).

Valmobida (2016) demonstrated that the exudate of *C. Cajan* seeds predominantly releases secondary metabolites of saponins and pentacycle triterpenes in phase I of the germination process (6 h soaking) and the release of pyrogallic tannins and Phase I compounds during phase II (20 h soaking). Metabolite classes may have caused changes detected in the activity of the enzymes catalase and peroxidase in the root and in the aerial segment of bean

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seedlings (Figures 1 and 2).

Gniazdownska et al. (2015) reported that oxidative stress may be induced by allelochemicals since the compounds underscored the levels of oxygen-reactive species (ORSs) produced in vegetal cells due to metabolic alterations mainly related to the transport chain of respiration and photosynthesis electrons. They are actually the first evidence of allelopathic effect within the germination process and within the early development of the vegetal species.

Arabdopsis thaliana seedlings in contact with catechin, a secondary metabolite, increase ROS levels formed in the root cells. Once formed, the molecules may damage cells or move towards the meristem and the root maturing region of *A. thaliana* to act as a signal for cell elongation (Weir et al., 2004).

The main stress-generated radicals in vegetal cells may be divided into two groups: radical group comprising hydroxyl, superoxide, peroxyl and alkoxyl, and the non-radical group comprising oxygen, hydrogen peroxide and hypochlorous acid. The radical hydrogen peroxide (H_2O_2) is mildly reactive; it has a high life time and passes easily the biological membranes. It is actually one of the motives that warrant its role as regulator of lignification loosening of the cell wall (Gill and Tuteja, 2010). However, so that H_2O_2 regulates the physiological processes in vegetal cells, the control of its levels is required, mainly through the specific activity of peroxidase and catalase enzymes, and thus cell damages are minimized (Barbosa et al., 2014).

The five-fold increase in peroxidase activity in the aerial segment of *P. vulgaris* seedlings submitted to exudates of *C. cajan* seeds (Figure 1A) show that stress was produced by allelochemicals and that the enzyme caused a 1 cm increase in the length of the aerial segment of *P. vulgaris* (Figure 2A). Class III peroxidases (E.C. 1.11.1.7), analyzed in current study, are a set of enzymes in the cell wall of vegetal cells belonging to a multigene family and represented by several isoforms due to two possible catalytic sites they provide. They are enzymes which eliminate free radicals and maintain the levels of hydrogen peroxide (H_2O_2) in the apoplast, while enhancing, by their catalytic or hydroxylic cycle, the architecture of the cell wall, due to the equilibrium between the formation of a flexible or rigid wall which depends on the life cycle phase of the organisms (Passardi et al., 2005).

According to Schopfer (1994), H_2O_2 apolastic levels in eudicotyledons, such as *Glycine Max*, are low within the hypocotyl elongation region. On the other hand, in the case of monocotyledons, Cordoba-Pedregosa et al. (2003) related H_2O_2 levels with lignification and growth of tissues in *Allium cepa* seedlings. Results show that significant changes ($p \leq 5\%$)

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reported in peroxidase´s specific activity of the aerial segment of *P. vulgaris* seedlings in current assay (Figure 1A) indicate the growth dynamics of seedlings when submitted to exudates of *C. cajan* seeds.

The significant increase of peroxidase activity ($p \leq 0.05$) also demonstrates that allelochemicals may have changed the formation of hydrogen peroxide in the epicotyl cells of *P. vulgaris* seedlings with growth enhancement. In fact, the enzymes are necessary to finely adjust H_2O_2 levels and trigger the loosening of the cell wall. The process is caused by peroxidase activity in its hydroxyl cycle which produces the hydroxyl radical causing the break of covalent bonds between the polysaccharides of the cell wall and favoring primary growth (Francoz et al., 2015).

Coupled to significant peroxidase results which indicate its role in the loosening of the cell wall of the epicotyl of *P. vulgaris* seedlings, the authors have verified that in current assay the seedlings respond differently to allelochemicals-mediated stress in the exudate of *C. cajan* seeds during early development. In fact, Figure 1B reveals that catalase was active in the roots but it was not required in the aerial segment, or rather, only peroxidases were active to remove hydrogen peroxide.

The maintenance of peroxidase activity without any statistically significant differences between treatments and control and the reduction of more than half of the catalase activity in *P. vulgaris* roots when submitted to the exudate of *C. Cajan* seeds (Figures 1A and 1B) demonstrate that an oxidative disarrangement of the components of antioxidant system with allelochemicals may occur, as reported by Mahmdi et al. (2010) and, consequently, a reduction of its catalytic activities. Although catalase activity was reduced, the root did not undergo change neither in length nor in dry mass (Figures 2A and 2B). The above evidences no oxidative damage of the cells.

According to Lara-Nunez et al. (2006), catalases are characterized by the great number of reactions they catalyze and their low affinity to H_2O_2. They are efficient tools in the elimination and control of high concentrations of hydrogen peroxide. The enzymes are not adequate for fine adjustments when low concentrations of the radical exist since peroxidases are active in this context. Therefore, *C. cajan* exudate may have decreased H_2O_2 levels in *P. vulgaris* roots. Only peroxidase activity was enough to maintain growth (Figure 2A).

Stimuli mediated by allelochemnicals of the exudate of *C. cajan* seeds were also described in the case of *Solanum lycopersicum* seeds when germinated with exudates of *C.*

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cajan seeds. Valmorbida (2016) reported that tomato seeds were altered during the germination process since mean time was reduced and speed increased. Further, the author registered that early development of *Solanum lycopersicum* seedlings was also stimulated by exudates of *C. cajan* seeds, with more significant effect in the roots than in other segments.

Other experiments also showed that several allelochemical classes may also enhance plant growth. Iqbal and Frey (2012) verified that exudates of *Lepidium sativum* seeds in the early development of *Lactuca sativa* in low concentrations (3 uM) stimulate the hypocotyl when compared to control. The allelopathic effect in this case was attributed to secondary metabolites of the lepidimoide class causing increase of epidermal cells and root circumference of the exudate´s receiving species.

The ecological increase of the epicotyl and hypocotyl axis respectively improves the reception of light and nutrients by vegetal organisms (Larcher, 2000). The accumulation of exudates of *C. cajan* seeds in the soil may trigger the development of the roots and the aerial section of other plants. The development of species competing within the niche and changes within the environment are thus enhanced causing the establishment of new communities.

Results show that, in the direct seeding of forest species, the use of a green fertilizer species may improve the physical and chemical features of soil. It may also be an alternative for the supplementation of growth-regulating molecules (Aumonde et al., 2015) which may be useful for the establishment of a vegetal community in the area that is being recovered.

CONCLUSION

We concluded that allelochemicals released by *C. cajan* seeds during the soaking phases may modify the metabolism of other species during growth and early development. The model under analysis revealed that stress was controlled by the specific activity of peroxidase and catalase enzymes that stimulated the growth of epicotyls of *P. vulgaris* seedlings.

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