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Biochemical Modifications in Seedlings of Cedar Submitted to Salicylic Acid Application

Modificações Bioquímicas em Mudas de Cedro Submetidas à Aplicação de Ácido Salicílico

Maria Eunice Lima Rocha*[a]; Diana Jhulia Palheta de Sousa[b]; Jessica Suellen Silva Teixeira[c]; Marlene de Matos Malavasi[a]; Cândido Ferreira de Oliveira Neto[de]; Ubirajara Contro Malavasi[a]

[a]Universidade Estadual do Oeste do Paraná, Programa de Pós-Graduação Stricto Sensu em Agronomia. PR, Brasil.
[b]Universidade Federal de Viçosa, Programa de Pós-Graduação Stricto Sensu em Fitotecnia. MG, Brasil.
[c]Universidade Federal Rural da Amazônia, Programa de Pós-Graduação Stricto Sensu em Agronomia. PA, Brasil.
[d]Universidade Federal Rural da Amazônia, Programa de Pós-Graduação Stricto Sensu de Ciências Florestais, Produção Animal na Amazônia. AM, Brasil
[e]Rede Bionorte. PA, Brasil.
*E-mail: eunice_agronomia@yahoo.com.br

Abstract

The factors that limit forest production the most are abiotic stresses; soon, when plants are exposed to these conditions there will be secondary metabolites production and the exogenous application of growth regulators can facilitate and accelerate some adaptive responses. Therefore, the research aimed to evaluate biochemical changes associated with nitrogen metabolism in *C.fissilis* seedlings submitted to water deficit with salicylic acid (SA) application. The experiment used 400 seedlings of *C.fissilis* and the experimental design used in the phase prior to the water deficit imposition was completely randomized and composed of 4 doses of SA (0; 100; 200 and 300 mg L^{-1}) with 5 repetitions of 20 seedlings each. SA application was performed weekly for 2 months. At the end of that period, seedlings from each treatment (doses of SA) were randomly selected and submitted to water deficit for 4, 8 and 12 days. The biochemical analyzes performed consisted of relative content of water (RCW), nitrate, free ammonium, total soluble amino acids, proline and glycine-betaine. The dose of 300 mg L^{-1} of SA was considered extremely high because it resulted in a great alteration in seedling metabolism. On the other hand, for most parameters, the dose of 100 mg L^{-1} was efficient in signaling RCW as it reduced water loss and consequently kept seedlings alive longer.

Keywords: Analyzes. Nitrate. Nitrogen Metabolism. Water Deficit.

Resumo

Os fatores que mais limitam a produção florestal são os estresses abióticos, logo, quando as plantas são expostas a essas condições podem sofrer muitas modificações, assim, muitos estudos têm destacado respostas pertinentes com a aplicação exógena de reguladores e alteração metabólica que pode resultar em respostas adaptativa nas plantas. Portanto, a pesquisa teve como objetivo avaliar alterações bioquímicas ligadas ao metabolismo do nitrogênio em mudas de C.fissilis submetidas a déficit hídrico e com aplicação de ácido salicílico (AS). O experimento utilizou 400 mudas de C.fissilis e o delineamento experimental utilizado na fase anterior à imposição de déficit hídrico foi o inteiramente casualizado, composto por 4 doses de AS (0, 100, 200 e 300 mg L^{-1}), com 5 repetições de 20 mudas cada. A aplicação do AS foi realizada semanalmente por 2 meses. Ao final desse período, mudas de cada tratamento (doses de AS) foram selecionadas aleatoriamente e submetidas a déficit hídrico por 4, 8 e 12 dias. As análises bioquímicas realizadas consistiram no conteúdo relativo de água (CRA), nitrato, amônio livre, aminoácidos solúveis totais, prolina e glicina-betaína. A dose de 300 mg L^{-1} de AS pode ser considerada muito alta, pois resultou em uma grande alteração no metabolismo das mudas. Por outro lado, para a maioria dos parâmetros, a dose de 100 mg L^{-1} foi eficiente em sinalizar à condição de déficit hídrico e com isso mantendo o CRA por meio da redução da perda de água e consequentemente mantendo as mudas vivas por mais tempo.

Palavras-chave*: Análises. Nitrato. Metabolismo do Nitrogênio. Déficit Hídrico*

1 Introduction

The use of native woody species has increased in recent years, mainly to generate energy and to reforest landscapes. Therefore, there is a need to produce quality seedlings in large quantities. Recent studies have highlighted the potential of native woody species (ELOY *et al*., 2013) such as cedar.

Cedrela fissilis Vell.belongs to the family Meliaceae and is popularly known as cedar or pink cedar. The species is widely distributed throughout the Brazilian territory, but it is more frequent between the states of Rio Grande do Sul and Minas Gerais. Cedar is found mainly in semi-deciduous and Atlantic rain forests. However, it can develop in areas in the initial and medium stages of regeneration, reaching large numbers in advanced stages of plant succession (JARENKOW; BUDKE, 2009; SAKURAGUI *et al*., 2013).

The most limiting stress to forest production is abiotic. Several studies have been developed to determine the cause and effect arising from such stress (KUMAR *et al*., 2011; FARIDUDDIN *et al*., 2013; FILIPPOU *et al*., 2014). Seedling establishment can be mediated by temperature, solar radiation, nutritional deficiency and water availability (ARAÚJO *et al*., 2018).

Water can be considered one of the most limiting environmental factor as it affects photosynthesis impairing plant production and modifying soil-plant-atmosphere continuum (RODRIGUES *et al*., 2011; TONELLO; TEIXEIRA FILHO, 2012; CUNHA *et al*., 2013).

Situations that expose terrestrial plants to various stresses have advantages and disadvantages, because it can hinder some metabolic activities but on the other hand increase tolerance and consequently induce plant acclimation (BERGAMASCHI; BERGONCI, 2017). When exposed to some adverse condition, terrestrial plants stimulate production of secondary metabolites. Therefore, exogenous application of inductor compounds can facilitate and accelerate some biological responses that promote changes (GONÇALVES *et al*., 2014). Thus, knowing the effects and consequences of applying plant hormones is very important, not only to define criteria for application but also to evaluate doses in order to measure the limit between what can benefit or hinder plant growth and development under adverse environment conditions.

Salicylic acid (SA) is a phenolic phytohormone which performs several functions such as regulation of germination, growth and development, fruits ripening, leaf abscission, transpiration and interference in the water and solutes absorption by roots (RIVAS-SAN; PLASENCIA, 2011; KABIRI *et al*., 2012). In addition, SA is considered very promising as an alternative to induce plant tolerance related to the enzymes and proteins activation associated with protection and signaling (SÁNCHEZ *et al*., 2010; KANG *et al*., 2014).

Among the protection mechanisms, osmotic adjustment is an important one because it activates phyto response to stressful conditions to maintain cell turgor for short periods. This mechanism is activated by the solutes accumulation in the cell vacuole or cytosol such as amino acids, proline and glycine betaine among others, contributing to the maintenance and water balance to preserve the integrity of the substances that act in the primary metabolism of plants and cell membranes (ASHRAF *et al.*, 2011; MARIJUAN; BOSCH, 2013).

Studies have been developed in order to quantify changes resulting from the SA application in seedlings submitted to water deficit, evaluating which characteristics can be modified and which action mechanisms are linked to the primary and secondary plants metabolism (MAZZUCHELLI *et al*., 2014).

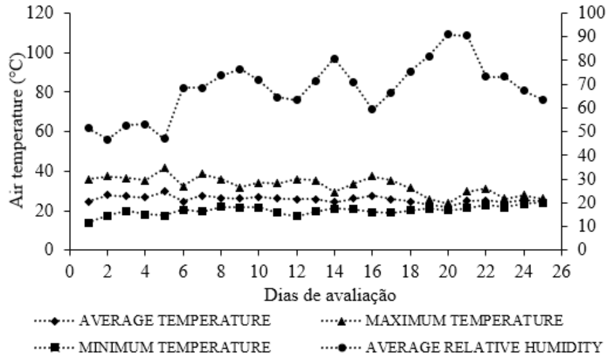
The experiment aimed to quantify biochemical changes associated with nitrogen metabolism in *C.fissilis* seedlings submitted to water deficit after the SA application.

2 Material and Methods

The experiment was conducted under a shade house located at 24°33'24'' S, 54° 05'67'' W and 420 m altitude. According to Instituto Agronômico do Paraná (IAPAR) and Koppen, the region climate receives a Cfa classification, subtropical, maintaining the annual average temperature between 22 and 23 °C, with well-distributed precipitation during the year and hot summers (ALVARES *et al*., 2013; NITSCHE *et al*., 2019).

Air relative humidity and temperature during the water deficit experimental period (Figure 1) were obtained daily with a datalogger (KlimaLogg Smart).

Figure 1 - Maximum, average and minimum air temperature and relative humidity during the experimental period



Source: Research data.

The experiment used four hundred 3-month old C.fissilis seedlings from Instituto Ambiental do Paraná (IAP) which were propagated in plastic plugs of 120 cm^3. Before the treatments imposition, the seedlings underwent a 30-day acclimatization period (in a shade house) during August and September of 2017. During that period, seedlings were fertilized with 3 mL per plug of a nutrient solution (Table 1).

Table 1 - Composition of the nutritive solution used to fertilize *C.fissilis* seedlings

Nutrient Solution					
KH_2PO_4	$MgSO_4$	KNO_3	$Ca(NO_3)_2$ $4H_2O$	Micro Complete	Fe-EDTA
--------------------mL L^{-1}-------------------					
1.0	2.0	5.0	5.0	1.0	1.0

Source: Research data.

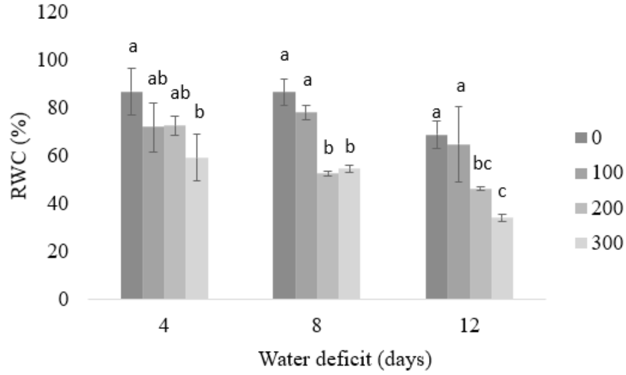
Before water deficit imposition the experiment was conducted as a completely randomized design composed of four treatments with five repetitions of twenty seedlings totaling four hundred seedlings. Treatments consisted of SA solutions such as: (T1) 0 mg L^{-1}; (T2) 100 mg L^{-1}; (T3) 200 mg L^{-1} and (T4) 300 mg L^{-1} applied weekly for 2 months from September 26th to November 14th, 2017. The solutions consisted of SA, deionized water and an adjuvant (Agral®) in the proportion 30 mL to 100 L of water, following the manufacturer's recommendation. The solution was applied by a hand spray between 6:00 and 8:00 AM in order to avoid unfavorable weather conditions.

After the two months, 16 seedlings from each treatment were randomly selected and transplanted to 5-liter pots filled with a mixture of local soil (LATOSOL RED Eutro-ferric with a very clayey texture) and humus in the proportion of 3: 1 and submitted to water deficit for 4, 8 and 12 days. The experimental design was arranged in subdivided plots consisting of 4 doses of salicylic acid (0, 100, 200 and 300 mg L^{-1}), 3 times to stress (E1-4 days of water suspension; E2-8 days of water suspension; E3-12 days of water suspension) and 4 repetitions, resulting in 48 sample units.

Seedling analyses were destructive according to the water deficit periods. Biochemical analyzes were performed at the Laboratory for the Study of Biodiversity in Higher Plants of the Federal Rural University of the Amazon, Pará. Measured variables included relative water content (RWC) (SLAVICK, 1979), nitrate and free ammonium (WEATHERBURN *et al*., 1967), total soluble amino acids (PEOPLES *et al.,* 1989), proline (BATES *et al*., 1973) and glycine-betaine (GRIEVE; GRATTAN, 1983).

Data was subjected to the uniformity and homogeneity tests of the common covariance matrix by GENES program, analysis of variance and chi-square were applied to the parameters that presented restrictions (CRUZ, 1998). Through the data significance, they were tested by the Tukey test, in order to compare the means. The graphs were constructed with MS Excel and the curves were constructed according to the means and their respective standard deviations.

Figure 2 - Relative water content (RWC) in *Cedrela fissilis* seedlings exposed to water stress and treated with salicylic acid



The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data.

3 Results and Discussion

Under water deficit period of 4 days, the dose of 200 mg L^{-1} of SA was efficient in maintaining RWC and consequently maximizing the use of water by the seedling with cell turgor of 72.50%. It should be added that there was no difference ($P > 0.05$) between the control and the other SA treatments (Figure 2).

Naturally, under water deficit, leaf water content is reduced (TAIZ *et al*., 2017). However, looking for alternatives that mitigate that effect has a great value, mainly because it acts by changing the plants' tolerance in adverse conditions due to the expectations of future climate changes.

RWC was reduced in all the experimental periods (4, 8 and 12 days). However, 100 mg L^{-1} of SA helped to maintain the water content after 8 and 12 days of water deficit, resulting in lower decrease when compared to other treatments. Low SA concentrations were efficient to improve water conditions in cedar seedlings but THEY should not be extrapolated to other wood species. The 100 mg L^{-1} dose of SA may have generated a major change in the plant's metabolism, signaling a stress condition and thus maintaining cell turgor, reducing water loss and consequently keeping seedlings alive longer.,

Furthermore, at 12 days of water deficit, seedlings still had a green stem, showing that if seedlings had been rehydrated, they could have possibly recovered and flushed new leaves. After all, the species is deciduous and tends to lose leaves in certain periods in order to reduce water loss and attenuate metabolic activities.

The highest RWC value was quantified in control seedlings subjected to 4 days of water deficit (86.7%) while the lowest was from the 12 days water deficit period with 300 mg L^{-1} of SA (34.1%). High SA doses can intensify the stress effects and foster the modification of essential metabolic activities in plants, reflecting an imbalance in the plant system.

Yuan and Lin. (2008) reported that doses between 0.1 and 0.5 mM of SA can improve tolerance to water deficit. However, that dose can be changed depending on the species, development stage, climate, phytosanitary and nutritional condition (HAYAT *et al*., 2010; MIURA; TADA, 2014).

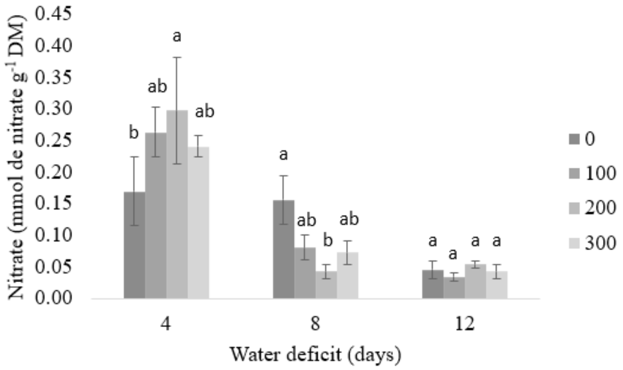
RWC is one of the main determinants for the variation in the content of compatible solutes, that is, the majority of the variations in nitrogen compounds come from fluctuations in water content. In addition, the alteration of those compounds present in cells can serve as an indicator of drought tolerance (SCHONFELD *et al*., 1988).

In *C. fissilis* seedlings, the highest nitrate value from the leaves (Figure 3) was obtained at 4 days of water deficit. The strategy is to signal the plant as to the condition and thus reduce water loss through transpiration. Thus, the plant begins the stomatal closure processes and balance of solutes inside and outside the cells.

It should be noted that osmotic regulation is an immediate measure. Thus, as the deficit condition is intensified, a plant may lose its ability to regulate and then release the solutes in the cellular environment, being lost and not quantified, and may also cause plant intoxication, depending on the content released. Such trend can be interpreted from Figure 3, because after 8 days of water deficit nitrate concentrations was reduced demonstrating that the stress attenuation was not efficient to trigger defensive signaling. In addition to the physiological and biochemical changes, a significant change resulting from foliage yellowing evolving to abscission was noticed.

At 12 days of water deficit there was no difference ($p > 0.05$) among the SA doses, confirming that, under certain circumstances, plants are no longer able to recover turgidity, through osmotic regulation strategy resulting in permanent wilt. Cedar seedlings showed in this experiment great sensitivity to water deficit and high air temperature. Based on those hypotheses, the doses used may have been high and, therefore, presented opposite responses to what was expected, helping to intensify stress in extreme conditions, as those observed at 12 days of water deficit with nitrate (Figure 3).

Figure 3 - Nitrate content in *Cedrela fisilis* seedlings exposed to water stress and treated with salicylic acid



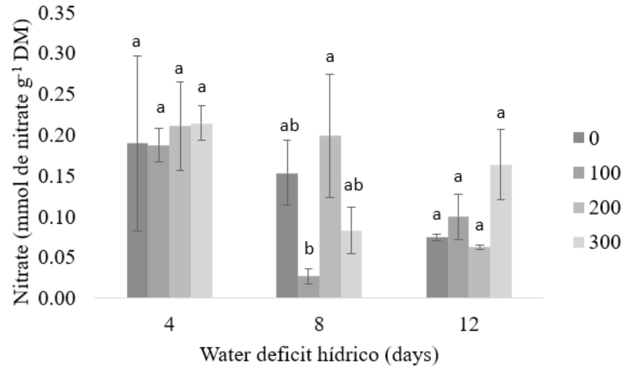
The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data.

Results similar to this study were reported by Teixeira *et al.* (2015), where the reduction in nitrate coincided with the water content reduction of *Morinda citrifolia* L. with a reduction of 82.43 and 83.33% in roots and leaves, respectively for plants under water deficit. The justification for this event is related to the low water flow in the plant and, therefore, reduction or even stagnation in the nutritional transport from leaves to roots. The above noted will trigger numerous blockages in the transport chain especially of nitrate reductase activity that converts nitrogen sources in plants (TAIZ *et al.,* 2017).

The levels of nitrate in roots of *C.fissilis* were reduced (Figure 4) with the water deficit intensification. However, at 8 days the SA dose of 200 mg L^{-1} increased nitrate concentrations in roots, coinciding with the nitrate levels reduction from leaves. The theory of translocation of compatible solutes can explain these changes in concentrations, demonstrating that the roots will be the receiving organ and, consequently, they will be responsible for the storage of those biochemical components for later biological activity, because under water deficit conditions leaves are lost in order to reduce transpiration.

Plant regulators increase as a form of signaling through biotic or abiotic stresses, which may explain the increase in nitrate concentrations under 12 days of water deficit and 300 mg L^{-1} of SA (Figure 4). In addition, SA is a phytohormone which is related to several regulatory functions in plant metabolism, acting both on plant growth and on the amplification and signaling of enzyme protectors with the ability to promote adaptive responses in the plant (KHAN *et al*., 2012; MIURA; TADA, 2014; ASGHER *et al*., 2015).

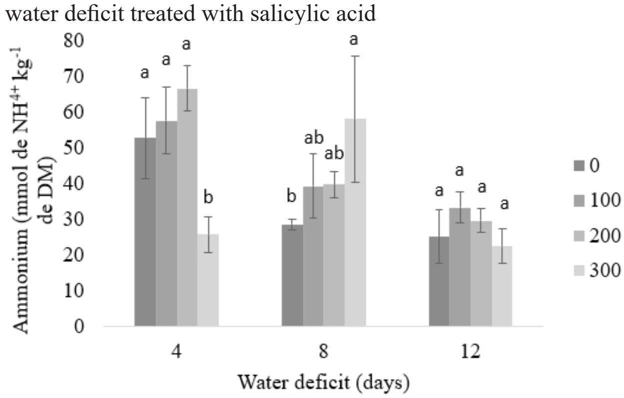
Figure 4 - Nitrate content of *C.fissilis* seedling roots under water deficit treated with salicylic acid



The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data.

Another metabolite that can be altered due to water deficit is ammonium. This, in turn, differs from nitrate by the form of its absorption; while nitrate is assimilated through an active process, that is against an electrochemical gradient, and through a simultaneous input system of H^{+} and NO^{3-} , ammonium is absorbed via passive system. In general, ammonium absorption is a priority, when in equivalent amounts. In cases where ammonium levels are scarce, plants prioritize the NO^{3-} absorption (TAIZ *et al*., 2017). Thus, the ammonium levels in both leaves and roots are much higher than nitrate concentrations, as it can be seen from Figures 3 and 4 compared to Figure 5.

Figure 5 - Ammonium content of *C.fissilis* seedlings subjected to water deficit treated with salicylic acid



The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data. .

In cedar leaves, at 4 days of water deficit the highest averages were measured up to the dose of 200 mg L^{-1} of SA; after that dose the value decreased sharply from 66.5850 to 25.6738 mmol of NH^{4+} kg^{-1} of DM (Figure 5). After 8 days of water deficit, the result was reversed and the highest average was quantified with the maximum dose of SA tested. This is a consequence of the intensification of the stressful condition, demonstrating that in cedar seedlings, SA acted in osmotic signaling, activating specific genes and proteins that triggered the ammonium production and, consequently, helped the water regulation (Figure 5).

Although the increase in ammonium concentrations is advantageous, as it acts on cell protection, osmotic regulation and plant signaling, it can cause some harm because ammonium is a toxic substance that cannot be stored for a long time. This accumulation can impair electronic transport, photosynthesis and respiration, due to the increase in concentrations of that cation. Upon accumulation, an imbalance in the proton gradient and loss of transmembrane selectivity will begin (TAIZ *et al*., 2017).

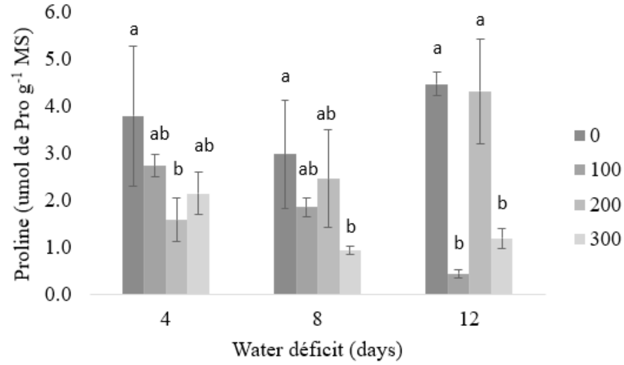
The high ammonium concentrations in seedlings subjected to 4 days of water deficit is a consequence of the first signaling resulting from the lack of water recognition. With the increase of days under water deficit, the plants begin to carry out their primary metabolic activities in a more restricted way and thus, the defense, which is part of secondary metabolism, begins to stand out. However, osmotic regulation works efficiently to some extent. Therefore, at 12 days of water deficit, concentrations were reduced drastically and with that, they did not show any difference in relation to the SA doses (Figure 5).

For free ammonium of *C.fissilis* seedling roots there was no difference (P >0.05) among the treatments and the means obtained ranged from 21.1313; 23.6541 and 37.9720 mmol of NH4 + k g^{-1} of DM at 4, 8 and 12 days of water deficit, respectively. The results indicated that *C.fissilis* seedlings showed no difference (p>0.05) among the treatments for the amino acids concentrations and the means obtained for the respective parts of the plants were 10.66 and 9.21 umol of amino acids g^{-1} of DM in leaves and roots, respectively.

Proline accumulation can be a parameter that defines resistance in plants, as it is considered an osmoprotectant and ensures greater sensitivity to plants. Therefore, proline is one of the most studied amino acids (ASHRAF *et al*., 2011; IQBAL *et al*., 2014; TROVATO *et al*., 2017). In addition, proline has an increased content due to abiotic stresses and studies have highlighted that this content can increase up to 100% under water deficit (CVIKROVÁ *et al*., 2013; FILIPPOU *et al*., 2014).

Cedar is sensitive to water deficit. On the leaves of seedling subjected to 12 days of water deficit, for example, the SA application at 200 mg L^{-1} increased the proline concentration, demonstrating a stress intensification and its correspondent signaling. Plant regulators, as well as phytohormones, are required in small amounts. However, depending on the species, the effect may be inverse to the expected (Figure 6). Similar results were obtained by Gonçalves *et al*. (2015) with *E. grandis* vs. *E. urophylla* submitted to water deficit who reported an increase in proline levels approaching the average of 12.74 µg g^{-1}.

Figure 6 - Proline levels in leaves of *C.fissilis* seedlings exposed to water deficit and salicylic acid



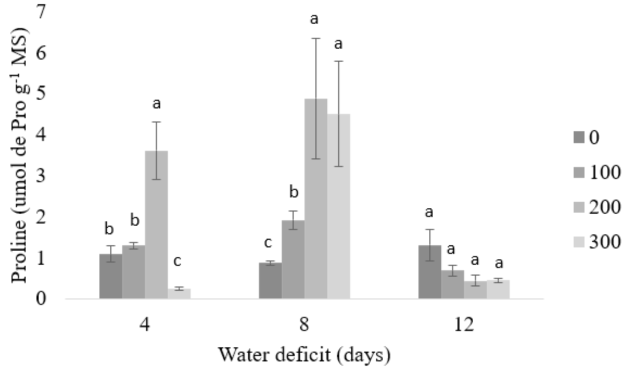
The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data.

Higher proline levels at 12 days of water deficit may result in a rapid increase in order to remedy the adverse situation in which the plants were exposed. Some solutes may show a response after the stimulus, different from what was seen in the previous parameters. The highest proline concentrations were quantified in seedlings submitted to the longest water deficit period (Figure 6).

Moura *et al*. (2016) reported proline in the leaves of *Jatropha curcas* L. varied from 0.31 to 0.38 µmol g-1 MF while at 90 and 120 days under water deficit there was an increase to 0.58 and 0.76 µmol g-1 MF, respectively. The foregoing reinforces the theory that the longer the water deficit the greater increase of proline levels.

In general, plants signalize both roots and leaves depending on the way the stress was imposed or the current plant condition. Thus, in the two plant organs above mentioned, concentrations tended to be inverse. Therefore, the proline levels obtained from roots of seedlings subjected to 8 days of water deficit increased, while in leaves the levels decreased. The hypothesize that translocation of solutes from the leaves to the roots is the cause, because with the intensification of the stressful condition plants begin the process of leaf abscission and consequently if this transfer does not occur the osmotic flags will be lost (Figure 7).

Figure 7 - Proline levels in roots of *C.fissilis* seedlings exposed to water deficit and salicylic acid



The means followed by the same letter do not differ statistically from each other by the Tukey test at the level of 1% probability.
Source: Research data.

This fact was not observed on seedlings submitted at 12 days of water deficit, as the proline concentration was higher on the leaves. This can be explained due to the fact that osmotic adjustment is an efficient activity in the short term. Over the course of the stress period, plants may have entered a period of permanent wilting and with that, the plant strategy became less efficient with the water deficit intensification.

Nascimento *et al*. (2019) published results similar to this study where there was a significant increase in the proline levels after 32 days of water deficit in 7-month-old *Hevea brasiliensis* L. related to the osmotic adjustment between water and osmotic potentials.

In adverse conditions there may be an increase of glycine betaine in order to balance cell water and osmotic potentials cells. Such strategy is to increase the number of solutes in the roots, thus decreasing the water potential and facilitating the water transport in favor of a positive gradient. In theory, transportation should take place from the soil to the roots and to the other parts of the plants, that is, from where there is a greater amount of water (fewer solutes) to the place with less water concentration (more accumulated solutes).

However, glycine-betaine levels from leaves and roots of *C.fissilis* were quantified, SA doses did not result in difference (p>0.05) among treatments with 22.82 and 24.63 mg of glycine betaine g^{-1} DM, respectively. SILVA *et al.* (2012) obtained similar glycine betaine levels from leaves and roots of papaya plants under water deficit for 20 days. Increasing these concentrations is a great alternative for preventing stress, since glycine betaine has great protective potential and is an excellent osmotic adjuster. Additionally, it can protect cell membranes, as well as prevent oxidative stress (ASHRAF; HARIS, 2004).

Nitrogen metabolism can vary depending on the species characteristics and climatic conditions. Thus, it is necessary that new studies be carried out in order to define the threshold between the doses of phytohormones that will be beneficial or harmful, the best time to start application and on which stress conditions the vegetables were imposed. The detailed study of those factors will make it easier to describe the parameters that can improve plant tolerance in nurseries and in the field.

4 Conclusion

Nitrogen metabolism was altered as a result of days of stress and salicylic acid application, moreover, it was observed that the relative water content influenced the variation of compatible solutes, including proline, ammonium and nitrate. The concentration of 100 mg L^{-1} of SA applied on *C.fissilis* seedlings subjected to 8 and 12 days of water deficit maintained water in the cells and could be recommended, since the phytohormones are required in small doses and that amount was enough to signalize the plant defense system and thereby relieves stress and improves the plant's tolerance.

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