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POLYAMINES AS SALINITY BIOCHEMICAL MARKER IN CALLUS OF *Eucalyptus urograndis*

POLIAMINAS COMO MARCADORES BIOQUÍMICOS DE SALINIDADE EM CALOS DE *Eucalyptus urograndis*

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ABSTRACT

Biochemical markers have been used for the analysis of plant cells submitted to several types of stress, among them salinity. This work aimed at analyzing the effect of saline stress in callus of *Eucalyptus urograndis* on polyamine contents. Explants (hypocotyls) obtained from seeds were inoculated in callus inductive medium, submitted to different levels of NaCl and analyzed at 10, 20 and 30 days after the inoculation. The free polyamines were extracted, isolated and quantified using TLC (Thin-Layer Chromatography). Putrescine content was higher and a fall in the spermidine content was observed in callus submitted to salinity condition. The results showed that polyamine accumulation is related to NaCl exposure in callus of *Eucalyptus urograndis*. The decrease in spermine content could be used as a biochemical marker for *Eucalyptus* callus subjected to salinity.

Key words: micropropagation, salt stress, polyamines, *Eucalyptus.*

RESUMO

Marcadores bioquímicos têm sido usados na análise de plantas submetidas a vários tipos de estresse, entre eles a salinidade. Este trabalho teve como objetivo analisar o estresse salino em calos de *Eucalyptus urograndis* em relação ao conteúdo de poliaminas. Explantes (hipocótilos) obtidos de sementes foram inoculados em meio indutivo de calos e foram submetidos a diferentes níveis de NaCl e analisados aos 10, 20 e 30 dias de inoculação. As poliaminas livres foram extraídas, isoladas e quantificadas usando TLC (Cromatografia de Camada Delgada). O conteúdo de putrescina foi maior, enquanto que o conteúdo de espermidina apresentou decréscimo, em calos submetidos a condições salinas. Os resultados mostram que o acúmulo de putrescina está relacionado com a exposição a NaCl em calos de *Eucalyptus urograndis*. A diminuição do conteúdo de espermina pode ser usada como marcador bioquímico de calos de *Eucalyptus* sujeitos à salinidade.

Palavras-chave: micropropagação, estresse salino, poliaminas, *Eucalyptus.*

INTRODUCTION

Plants frequently encounter stresses, external conditions that adversely affect growth, development, or productivity. Stresses trigger a wide range of responses, from altered gene expression and cellular metabolism to changes in growth rates and crop yields. Little is known about how plants recognize stresses. Salinity is a complex environmental constraint that presents two main components: an osmotic component due to the decrease in the external osmotic potential of the soil solution, and an ionic component linked to the accumulation of ions which become toxic at high concentrations (mainly Na^+ and Cl^-) and to a stress-induced decrease in the content of essential elements, such as potassium and calcium (Lefrèvre *et al*., 2001).

Water or saline stress results in a wide variety of physiological and biochemical changes in plants, such as turgidity loss, wilting and metabolism changes (Bray *et al*., 2000). External stress can either result in an increase or decrease in cellular polyamines, depending upon the type of stress, the plant species and the time of stress application (Reggiani *et al*., 1993), and according to Aziz *et al*. (1999) osmotic stress induces

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putrescine accumulation.

Spermidine, spermine and the diamine putrescine play an important role in the study of plant biochemistry and physiology; they are involved in important biological processes e.g. membrane fluidity, DNA, RNA and protein stabilization. Polyamines have an outstanding role as modulators of several biological processes such as enzyme activation and ionic balance maintenance in growth and development regulation. They can have hormonal activity and can act on cell division. Due to such functional versatility, studies of polyamines represent an interesting area of modern plant biology research (Bouchereau *et al*., 1999).

Several reports show that polyamine accumulation occurs during stress, and this might be for tissues protection against many types of injuries. Polyamines have also been suggested to participate in the plasticity of nitrogen metabolism, and possibly, survival of higher plants subjected to environmental stresses (Aziz *et al*., 1998).

Important studies on *Eucalyptus* have been performed, aiming at the selection of elite trees adapted to certain environment stresses, such as salinity. Salinization is affecting millions of hectares of land around the world mainly due clearing of forests and shrub lands for agriculture and also due to excessive irrigation (Niknam and McComb, 2000). To select tree genotypes tolerant to salinity, an obvious method would be cloning trees surviving and growing in saline areas. Tolerant species may have mechanisms of compartmentalization of ions in vacuoles, or deposition into bark, ray cells, vessel element, walls and lumens, or older senescent leaves. *Eucalyptus grandis* tolerates low to medium levels of soil salinity through exclusion mechanisms, but once these break down tissues, they are very sensitive to salt. Sun and Dickinson (1993) found *Eucalyptus grandis* to be the most tolerant among 16 *Eucalyptus* studied, in experiments that 50, 100, 150 and 200 mmols NaCl.

Thus, the objective of the present work was to study the effect of salinity on polyamine contents in callus of *Eucalyptus urograndis*.

MATERIAL AND METHOD

Seeds of *Eucalyptus urograndis* obtained through open pollination were used. For disinfection seeds were washed in 2% Tween 20 solution for five minutes, washed in deionized water several times, immersed in commercial solution of sodium hypochlorite (2.5% of active chlorine) for thirty minutes, and washed in sterilized deionized water. Then, they were germinated in Murashige and Skoog (1962) medium. Thirty days later, the hypocotyls were removed and inoculated in nutrient medium as proposed by Gonçalves (1980) supplemented with NAA – naphthalene acetic acid (0.2 mg L^{-1}) and BAP benzylaminopurine (1.0 mg L^{-1}) for callus formation. The medium was gelled with 6 g L^{-1} agar and pH was adjusted to 5.8 before autoclaving. This medium was used as control (without NaCl).

Callus were exposed to 50 and 100 mmol L^{-1} NaCl, and the harvests for the free polyamine analysis were made in 10-day-intervals, in a period of 30 days. The entire callus were grown at 23 ± 2 ^{o}C under 16 h day photoperiod, 1000 lux fluorescent light.

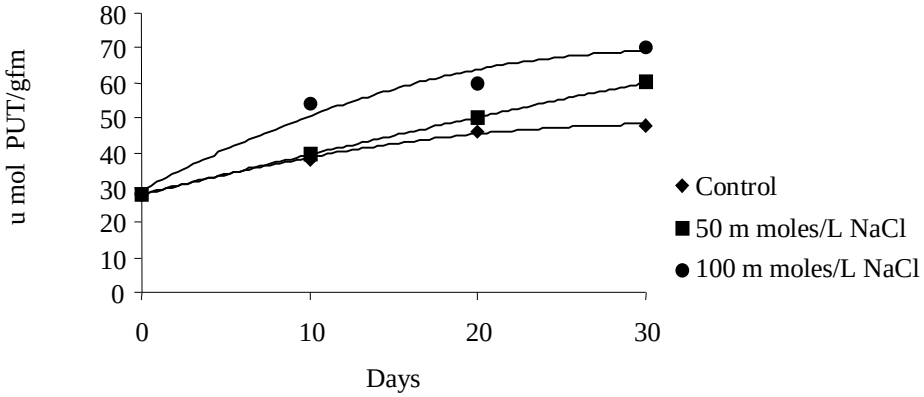
Polyamines were extracted, isolated and quantified according to Flores and Galston (1982). Polyamines were extracted by homogenization with 5% (v/v) cold $HClO_4$. The homogenates were kept on ice 30 minutes before centrifugation at 12.500 g for 20 minutes. After centrifugation the supernatant fraction containing free polyamines was dansylated using dansyl chloride and separated by silica gel (60G) thin-layer chromatography (TLC) (flexible plates – Merck-Germany) using cloroform-triethylamine (20:1, v/v) as the developing solvent. The dansylates amines were quantified using a VDS (Video Densitometer Scanner - Pharmacia) with program Image Pro-IPW.

The experimental design was a completely randomized with a factorial (3 x 3) for salt doses and time with four replications. The regression analyses were run separately for the treatments. All statistical calculations were done with the statistical package ORIGIN 6.0 (Microcal Software, Inc.).

RESULTS AND DISCUSSION

Callus growth in 100 mmol L^{-1} of NaCl was low when compared to the control. The maximum growth was observed in 20 days old callus in all the treatments. In spite of lower growth of cells in saline medium, the tissues survived well up to 20 days in the respective medium. Gangopadhyay *et al*. (1997) reported that cultured cells and/or callus of adapted glycophytes grew slower in medium containing NaCl than cells not adapted to salinity growing without stress.

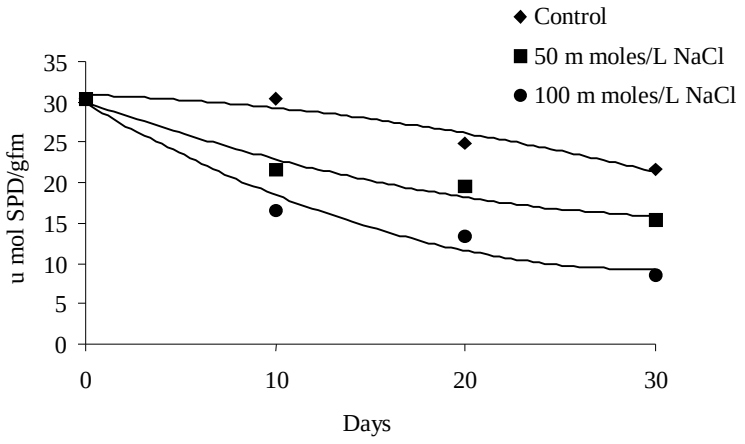
The levels of polyamines putrescine (Put) (Figure 1), spermidine (Spd) (Figure 2), and spermine (Spm) (Figure 3), showed changes during growth of the *Eucalyptus urograndis* callus when maintained in different levels of NaCl. Under saline conditions, the Put contents increased progressively with increasing NaCl concentration, while Spd and Spm levels decreased especially when the medium was supplemented with 100 mmols L^{-1} of NaCl.



Control: $y = -0,0202x^2 + 1,2855x + 27,78$; $R^2 = 0,9965$; 50 m moles/L NaCl: $y = -0,0036\ x^2 + 1,1768x + 28,078$; $R^2 = 0,9997$; 100 m moles/L NaCl: $y = -0,0404\ x^2 + 2,5375x + 29,235$; $R^2 = 0,9683$.

FIGURE 1: Effect of NaCl on the putrescine (Put) levels in callus of *Eucalyptus urograndis*, in 10, 20 and 30 days.

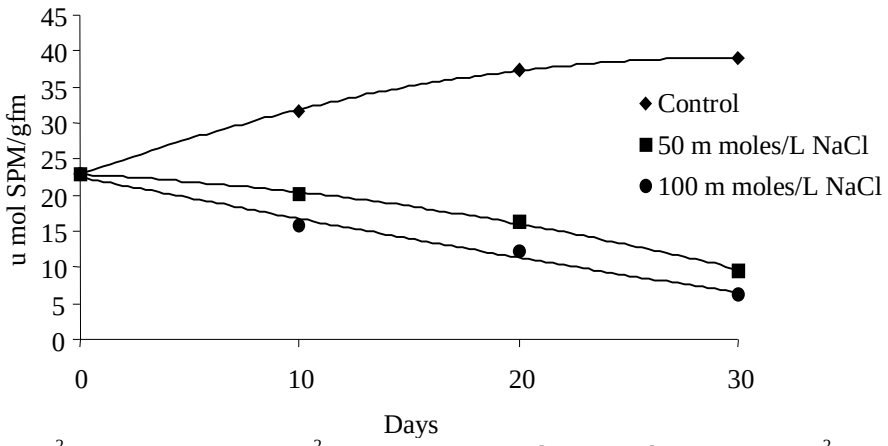
FIGURA 1: Efeito de NaCl nos níveis de putrescina (Put) em calos de *Eucalyptus urograndis*, em 10, 20 e 30 dias.



Control: $y = -0,008x^2 - 0,0783x + 30,853$; $R^2 = 0,9502$; 50 m moles/L NaCl: $y = -0,012\ x^2 - 0,8336x + 30,054$; $R^2 = 0,9701$; 100 m moles/L NaCl: $y = -0,0224\ x^2 - 1,3638x + 29,867$; $R^2 = 0,9718$.

FIGURE 2: Effect of NaCl on the spermidine (Spd) levels in callus of *Eucalyptus urograndis*, in 10, 20 and 30 days.

FIGURA 2: Efeito de NaCl nos níveis de espermidina (Spd) em calos de *Eucalyptus urograndis*, em 10, 20 e 30 dias.



Control: $y = -0,0183x^2 + 1,0888x + 22,838$; $R^2 = 0,9997$; 50 m moles/L NaCl: $y = -0,0096\ x^2 - 0,1467x + 22,82$; $R^2 = 0,999$; 100 m moles/L NaCl: $y = 0,0024\ x^2 - 0,607x + 22,557$; $R^2 = 0,9848$.

FIGURE 3: Effect of NaCl on the spermine (Spm) levels in callus of *Eucalyptus urograndis*, in 10, 20 and 30 days.

FIGURA 3: Efeito de NaCl nos níveis de espermina (Spm) em calos de *Eucalyptus urograndis*, em 10, 20 e 30 dias.

External stress can either result in an increase or decrease in cellular polyamines, depending upon the type of stress, the plant species and the time of stress application (Bray *et al*., 2000). Polyamines have been proposed to participate in the plasticity metabolism, and possibly survival, of higher plants subjected to environmental stress (Aziz *et al*., 1998).

The effect of NaCl has been attributed to changes in osmotic potential resulting from reducing water content and specific toxic effects caused by accumulation of sodium and chloride ions. LI and Chen (2000) reported that in wheat, the level of S-adenosymethionine decarboxilase (SAMDC) transcript was detected earlier in salt–stressed seedlings than in drought-treated seedlings. Several lines of experimental evidence indicate that the ionic component of salt stress may trigger polyamine accumulation independently of any osmotic component, even after short-term exposure (Lefèvre *et al*., 2001).

After 10 days in culture a tendency to accumulate Put in the presence of 100 mmols L^{-1} NaCl was observed. The lowest Put values were detected in control callus. Spd content in callus, in general, decreased during period of culture; however, in the control the highest level was detected. A fall in the Spm content was observed in 50 and 100 mmols L^{-1} NaCl. Moreover, this polyamine content in the control was the highest throughout the study.

These results suggest that the salinity responses monitored at the polyamine level involve not only a rise in Put biosynthesis, but also a stimulation of Spd and Spm oxidation and/or inhibition of enzymes responsible for their synthesis (Aziz *et al*., 1999). Put accumulation was probably a consequence of two factors, 1) inhibition of Spd and Spm synthesis, and 2) de novo Put synthesis (Willadino *et al*., 1996). Under moderate salinity conditions, putrescine accumulation has an adaptive function, whereas in high salinity conditions, the excess of diamine can be toxic by causing an increase in hydrogen peroxide concentration and free radicals from its oxidation (Di Tomaso *et al*., 1989; Willandino *et al*., 1996). NaCl has been already shown to stimulate the activity of PAO (polyamine oxidases) in tomatos (Aziz *et al*., 1999).

The observed increase in Put content may be related to its regulatory effect in maintaining the structural and functional integrity of membranes (Ali, 2000). Polyamines could interact with membranes by binding to negatively charged phospholipids and protecting them from viability loss caused by salt (Aziz *et al*., 1999). The results of Borrell *et al.* (1997) suggest that inhibition of lipid peroxidation may be one the mecanisms responsible for the anti-senescence of the polyamines. This contention is supported by the observation of Riedell (1987) that polyamines inhibit Na^+ influx in excised maize (*Zea mays*) roots. However, the tolerance or sensitivity to NaCl in different cultivars of the same species seems to be

associated not only to the capacity of accumulating putrescine, but also of keeping polyamine metabolism active (Galston and Kaur-Sawhney, 1990).

Increase in Put levels have been observed in salt sensitive rice (*Oryza sativa)* cultivars, moreover the tolerant cultivars showed a small increase in Put and high increase in Spd and Spm (Krishnamurthy, 1991). Shevyaokova *et al*. (1985) suggested that an increase in putrescine content might be a protective or osmorregulator factor during the adaptation of *Nicotina silvestris* cells in medium with NaCl stress.

It is possible that Spd and Spm are able to protect membranes during salt stress, but generally they do not accumulate in this stage. However Spm showed accumulation in callus under control conditions, the opposite being observed on NaCl treatments; this would suggest that callus of *Eucalyptus urograndis* are sensitive to salt stress. This polyamine may be used, as biochemical marker of salinity damage with putrescine, on callus of *Eucalyptus urograndis*, but comparative studies are required to fully establish this possibility. Velikova *et al*., (2000) showed that after stress with acid rain, polyamines are involved in plant responses like protective properties and the more pronounced protective effect of Spm in comparison with Spd could be accounted for by its longer chain and greater number of positive charges with allows more important neutralizing and membrane stabilizing ability.

CONCLUSION

The results showed that polyamine accumulation was related to NaCl exposure in callus of *Eucalyptus urograndis*. The Spm could be of importance as a biochemical marker for the analyses of salinity stress in this system.

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