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Quercetin and indole 3-butyric acid (IBA) as rooting inducers in *Eucalyptus grandis* × *E. urophylla*

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Abstract

Vegetative propagation is the main form of *Eucalyptus* cutting production, but some clones are still difficult to propagate, including rooting. Auxins and co-factors such as flavonoids can improve root development. This study aimed to evaluate the effects of the flavonoid quercetin, either in the presence or absence of auxin, in different rooting stages of *E. grandis* × *E. urophylla*, using biochemical markers. Assessments of stem diameter, height, rooting, number of roots and root length were analyzed and the content of polyamines (PAs) (putrescine, spermidine and spermine), ascorbic acid, indole-3-acetic acid (IAA), quercetin and peroxidase activity were determined in leaves and roots. Indole-3-butyric acid (IBA) induced a higher PA content in roots and leaves mainly after 60 days, indicating that auxin can induce PA production and cell division rate, which is higher at this stage of development. The ascorbic acid content was increased in roots by IBA and quercetin increased the ascorbic acid content in roots and leaves, demonstrating the activation of the antioxidant mechanism of cells. A reduction in peroxidase activity following the addition of auxin and quercetin to roots after 90 days indicates a reduction in oxidative stress. Application of IBA induced the highest IAA content in roots. Both the roots and leaves showed detectable levels of quercetin. All treatments promoted changes in biochemical markers at any time-point, indicating that these substances can reduce oxidative stress and increase cell division. Other *Eucalyptus* clones must be tested to confirm the effect of quercetin and auxin on growth and root development.

Keywords: polyamine, ascorbic acid, peroxidase, indole-3-acetic acid.
Abbreviations: 2,4-D_2,4-dichlorophenoxy acetic acid; NAA_1-naphthalene acetic acid; IAA_indole-3-acetic acid; IBA_indole-3-butyric acid; PA_polyamine; POD_peroxidase; Put_putrescine; Spd_spermidine; Spm_spermine.

Introduction

Vegetative propagation offers some advantages compared to sexual reproduction, including more rapid production and more genetically stable material. The accurate reproduction of individual genetic characteristics, results in a more uniform and higher quality of the product (wood or fiber) (Lelu-Walter et al., 2013). Despite advances achieved in vegetative propagation techniques, some clones are still difficult to propagate, often due to a low rooting rate, which causes production losses. Thus, some substances such as auxins and their co-factors have been studied, to improve rooting.

Auxins contribute to the formation of root architecture and are considered to be components of endogenous development that can be influenced by environmental stimuli (Overvoorde et al., 2010). The best-characterized auxin in plants is IAA; however, synthetic molecules such as 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and indole-3-butyric acid (IBA), can cause similar biochemical, molecular and physiological responses (Rybel et al., 2012). Thus, the use of auxin is a viable alternative to the stimulation of root formation in cuttings.

It is reported that other substances such as flavonoids are associated with rooting. In cuttings of *Eucalyptus gunnii*, levels of isoquercetin (3-O-quercetin-glucoside) and quercetin rhamnoside (3-O-quercetin-rhamnoside) were determined. The two identified flavonoids were present in very low amounts (0.2–0.5 mg 100 g^{-1} fresh weight) in cuttings that were unable to root, and in higher amounts (8 mg 100 g^{-1} fresh weight) in cuttings that could root (Curir et al., 1990), highlighting the potential successful effect of phenolic compounds in *Eucalyptus* on rooting.

Therefore, it is relevant to consider the use of auxins and co-adjuvants, such as flavonoids, to promote rooting in several plant species. Additional compounds that might affect rooting include ascorbic acid, peroxidases (PODs) and polyamines (PAs). The role of ascorbic acid in the regulation of root formation was observed in cuttings of tomato, which showed a large increase in ascorbic acid in the root zone in the first few days of culture (Tyburski et al., 2006).

Many studies have demonstrated that the induction of adventitious rooting is characterized by a strong reduction in POD activity, which increases the initiation phase, and gradually decreases the expression phase (Metaxas et al., 2004; Syros et al., 2004).

A positive correlation between the accumulation of PAs and the initial phase of adventitious rooting was observed in some species, suggesting that PAs can be used as rooting markers (Neves et al., 2002). The levels of agmatine (Agm), spermidine (Spd) and spermine (Spm) are positively related to root development, whereas the content of putrescine (Put) has a neutral or negative effect (Su et al., 2006).

Table 1. Morphological parameters of cuttings of *E. grandis* × *E. urophylla*: height, diameter, survival rate, rooting rate, root length, 30, 60 and 90 days after planting*.

Days	Height (cm)	Diameter (cm)	Survival (%)	Rooting (%)	Root length (cm)
30	7.22 c	0.84 c	89.29 a	89.29 a	5.40 c
60	12.04 b	1.65 b	74.29 b	74.29 b	7.46 b
90	17.08 a	2.03 a	71.25 c	69.46 c	9.53 a

*Data in each column indicated by the same letters are not significantly different by the Tukey's test ($p \leq 0.05$).

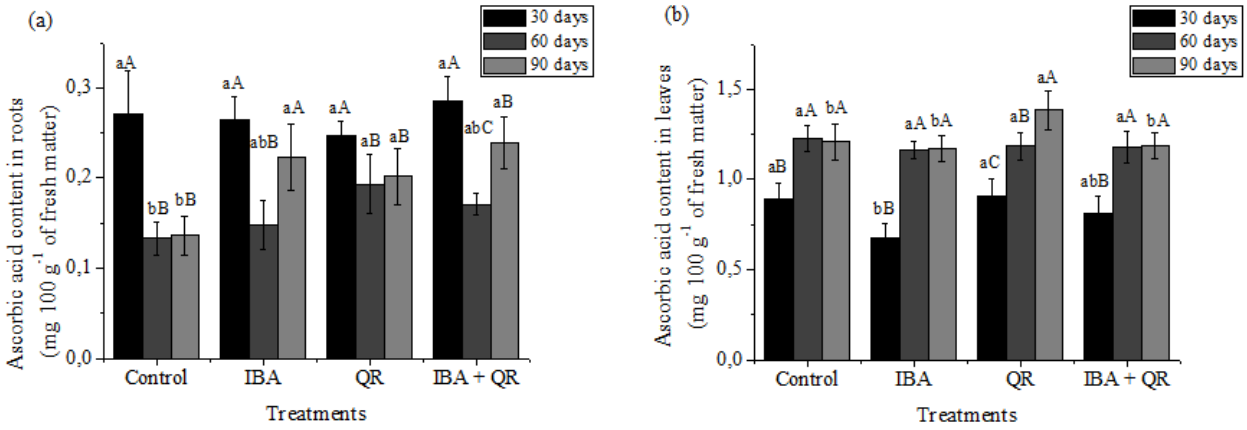

Fig 1. Levels of ascorbic acid (mg 100 g^{-1} fresh matter) in roots (a) and leaves (b) of *E. grandis* × *E. urophylla* following treatment with IBA (1.0 g kg^{-1}), quercetin (0.5 g kg^{-1}) and their combination, 30, 60 and 90 days after planting. Lowercase letters compare means between treatments with IBA and quercetin. Capital letters compare analysis times. Means followed by the same letters do not differ statistically by Tukey's test ($p \leq 0.05$).

This study aimed to verify the effect of quercetin and IBA on the rooting of *Eucalyptus grandis* × *Eucalyptus urophylla* (clone CL1) and to correlate the levels of ascorbic acid, PAs, IAA, quercetin and POD activity with different stages of cutting development.

Results

Effect on height, diameter, survival and rooting

Treatments with auxin and quercetin had no effect on the height, diameter, rooting percentage, mean root length and root number in *Eucalyptus grandis* × *Eucalyptus urophylla* (clone CL1). There was an increase in height, diameter and mean root length over time, due to the natural growth and development of cuttings. A reduction in survival and the rooting percentage occurred between 30 and 60 days (Table 1).

Indole acetic acid and quercetin contents

The highest IAA content in roots occurred following exogenous IBA application (Table 3). Both roots and leaves of *Eucalyptus* cuttings showed detectable levels of quercetin. However, there were no significant differences between treatments. The content of quercetin was higher in leaves than in roots.

Effect on PA content

A difference in the level of PAs (Put, Spd and Spm) in roots and leaves was observed (Table 3). In roots and leaves, all treatments promoted an increase in Put levels after 60 days, compared to after 30 and 90 days. Treatment with IBA alone or in combination with quercetin increased the Spd level in roots and leaves after 60 days and the Spd level in leaves after 90 days. In roots, the Spm level increased following treatment with IBA or quercetin alone after 60 days. The quercetin also increased the Spm level at day 90. The use of IBA combined with quercetin increased the Spm level over time.

Effect on ascorbic acid content

The level of ascorbic acid varied in roots and leaves (Fig. 1), either at several the time-points of analysis or with treatments. In roots (Fig. 1a), the highest level of ascorbic acid occurred after 30 days. However, at this time, the treatments did not affect the ascorbic acid level. Quercetin increased the ascorbic acid level after 60 days, and after 90 days. All treatments enhanced the ascorbic acid level compared to the control. The application of IBA, either alone or in combination with quercetin, increased the ascorbic acid content after 90 days, compared to after 60 days. In leaves (Fig. 1b), the opposite effect was observed, in which the level of ascorbic acid was lower at 30 days than at other time-points (60 and 90 days). The ascorbic acid content was decreased after 30 days following IBA application, but did not differ significantly from the control in other treatments. Quercetin caused an increase in the level of ascorbic acid over time.

Effect on peroxidase activity

The activity of POD was significant for the interaction between time and treatments in roots and leaves (Fig. 2), but in roots was decreased by treatment with IBA alone or in combination with quercetin (Fig. 2a) over time. All treatments reduced POD activity after 90 days. Treatment with quercetin alone increased POD activity after 60 days. The lowest levels of POD in leaves (Fig. 2b) occurred after 90 days. Quercetin increased POD activity after 30 and 60

Table 2. Indole acetic acid and quercetin contents (mg kg^{-1}fresh matter) in leaves and roots of *Eucalyptus grandis* × *Eucalyptus urophylla* following treatment with quercetin (0.0 and 0.5 mg kg^{-1}) and indole-3-butyric acid (0.0and 1.0 g kg^{-1}) during autumn*.

		IAA		Quercetin	
		IBA (g kg^{-1})		(mg kg^{-1})	
	Quercetin (mg kg^{-1})	0	1.0	0	1.0
Roots	0	nd	0.17 ±	0.06 ±	0.19 ± 0.01
	0.5	nd	Nd	0.07 ±	0.28 ±
Leaves	0	nd	0.07 ±	1.73 ± 0.11	0.94 ± 0.05
	0.5	nd	Nd	0.98 ± 0.14	0.74 ± 0.04

*Values are the means of three replicates ± SD. nd – not detected (<LOD and LOQ).

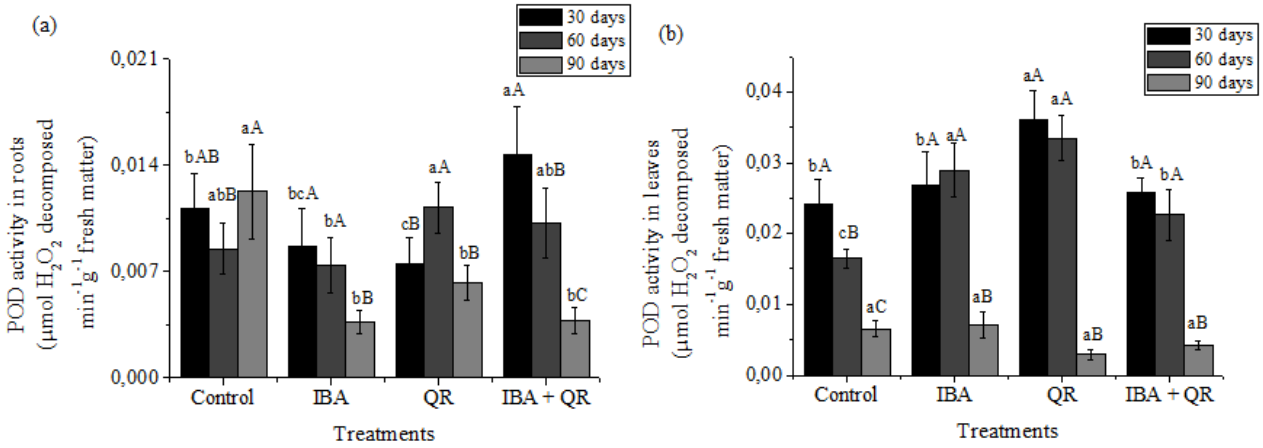

Fig 2. The activity of POD (H_2O_2 decomposed $min^{-1}g^{-1}$ fresh matter) in roots (a) and leaves (b) of *E. grandis*×*E. urophylla* following treatment with IBA (1.0 g kg^{-1}), quercetin (0.5 g kg^{-1}) and their combination, 30, 60 and 90 days after planting. Lowercase letters compare means between treatments with IBA and quercetin. Capital letters compare analysis times. Means followed by the same letters do not differ statistically by Tukey's test ($p \leq 0.05$).

Table 3. Polyamine content (Put, Spd and Spm) in leaves and roots of *Eucalyptus grandis*×*Eucalyptus urophylla*, after 30, 60 and 90 days, in control and the following treatments: IBA (1.0 g kg^{-1}), quercetin (0.5 g kg^{-1}), IBA (1.0 g kg^{-1}) + quercetin (0.5 g kg^{-1}) (µmolg^{-1} fresh matter)*.

Treatment		Control	IBA	Quercetin	IBA + Quercetin
	Days		*Leaves*		
Put	30	2.87 ± 0.19 cC	7.74 ± 0.65 aB	6.19 ± 0.61 bB	2.45 ± 0.64 cB
	60	3.98 ± 0.32 cB	8.90 ± 0.35 aA	7.31 ± 0.29 abA	6.69 ± 0.79 bA
	90	5.57 ± 0.45 aA	5.32 ± 0.32 aC	6.12 ± 0.39 aB	6.12 ± 0.64 aA
Spd	30	10.14 ± 0.10 aB	7.50 ± 0.18 bB	5.06 ± 0.53 cAB	6.31 ± 0.42 bcC
	60	13.68 ± 1.91 bA	14.30 ± 1.33 abA	6.52 ± 0.62 cA	15.92 ± 0.63 aA
	90	5.68 ± 0.05 bC	8.17 ± 0.18 aB	3.78 ± 0.14 cB	8.90 ± 0.62 aB
Spm	30	10.56 ± 1.29 aB	9.84 ± 0.93 aB	6.19 ± 1.06 bC	8.88 ± 0.24 aB
	60	17.25 ± 1.40 aA	14.47 ± 0.59 bA	13.42 ± 0.23 bA	14.40 ± 1.38 bA
	90	8.87 ± 0.53 aB	9.08 ± 0.53 aB	9.21 ± 1.45 aB	6.43 ± 0.17 bC
			Roots		
Put	30	6.69 ± 1.06 bB	6.05 ± 0.45 bB	8.56 ± 0.51 aB	7.71 ± 0.85 abA
	60	8.58 ± 0.62 cA	11.57 ± 0.84 abA	10.63 ± 0.84 abA	8.88 ± 0.86 bcA
	90	6.99 ± 0.60 aAB	5.52 ± 0.61 abB	1.33 ± 0.15 cC	4.39 ± 1.27 bB
Spd	30	5.81 ± 0.40 abA	5.51 ± 0.61 bA	6.07 ± 1.05 abB	6.90 ± 1.14 aA
	60	2.33 ± 0.02 cB	4.66 ± 0.58 aA	2.68 ± 0.60 bcC	3.86 ± 0.53 abB
	90	6.64 ± 0.64 aA	2.95 ± 0.23 bB	7.73 ± 0.12 aA	1.57 ± 0.33 bC
Spm	30	15.79 ± 0.67 aA	3.16 ± 0.46 cB	5.51 ± 0.57 bB	5.09 ± 1.13 bcC
	60	9.41 ± 0.77 cC	12.34 ± 1.11 bA	16.05 ± 1.75 aA	7.01 ± 0.73 dB
	90	13.36 ± 0.58 bB	13.01 ± 0.23 bA	15.45 ± 1.08 aA	13.63 ± 0.16 abA

*Values are means of five replicates ± SD. Lowercase letters compare means between treatments with IBA and quercetin. Capital letters compare analysis times. Means followed by the same letters do not differ statistically by Tukey's test ($p \leq 0.05$).

days compared to the control and other treatments, except for IBA alone after 60 days.

Discussion

Growth parameters

Although the treatments did not affect the development or rooting of *E. grandis* × *E. urophylla*, high levels on rooting, height and diameter were observed in all treatments, including the control (Table 1). The same results were also found in a previous study with the same species (Prado et al., 2014). The period between 60 and 90 days was characterized by changes in environmental conditions of cuttings from shade to full sun. This phase was also characterized by a decrease in irrigation and fertilization, which might explain a higher mortality rate.

Several studies have shown contradictory results in the use of auxin or flavonoids to stimulating rooting. The application of exogenous IBA promoted the rooting of *E. benthamii* Maiden & Cambage × *E.dunnii* Maiden, and increased the rooting percentage and the rate of root formation and adventitious rooting (Brondani et al., 2012). Our results show that there was no effect of exogenous auxin application on rooting, despite an increased concentration of endogenous auxin (Table 3) in leaves and roots. Analyses using LC/MS indicated the presence of indole acetic acid (IAA), a natural auxin in plants, in both leaves and in roots in cuttings treated with IBA alone. Genetic evidence in *Arabidopsi* suggests that the synthetic auxin IBA is converted via peroxisomal β-oxidation into IAA (Zolman et al., 2000), which acts in cell expansion in specific tissues, including in roots and cotyledon cells (Strader et al., 2010) which also occurred in this study.
The results in this study show that exogenous quercetin applied alone or in combination with IBA did not affect rooting, although the endogenous level of this flavonoid was detectable in the leaves and roots of *Eucalyptus* cuttings (Table 3). Quercetin did not induce the formation of IAA, as the IAA concentration was below the detection limit. Generally, phenols are responsible for reducing the decarboxylation of IAA, thereby increasing IAA levels in plants (De Klerk et al., 2011). However, this effect was not observed in the studied clone. In mate herb (*Ilex paraguariensis*), the exogenous application of quercetin resulted in an increased rooting percentage of 17% to 55% (Tarragó et al., 2005). However, there was a reduction in the rooting of wild *Arabidopsis thaliana* when quercetin was applied alone or in combination with IAA. This effect was not observed in a mutant cultivar of the same species (Correa et al., 2012). These results demonstrate that the action of quercetin and auxin in plants can be diverse, and can positively or negatively influence rooting, or have no effect.

Polyamine modifications

The polyamines Spd and Spm have been described as having a positive correlation with root development, whereas Put has a neutral or negative effect (Su et al., 2006). However, this correlation was not confirmed in this study, because there was no significant difference in rooting between treatments (Table 3). Other studies have also reported that these PAs do not influence rooting, as shown for *Withania somnifera* (Sivanandhan et al., 2011) and olive cuttings, where Put was the most effective PA, but Spd and Spm failed to promote rooting (Denaxa et al., 2014). Plant PAs have been associated with morphogenesis, growth, embryogenesis, organ development, leaf senescence and responses to biotic and abiotic stress (Groppa and Benavides, 2008). Thus, the variations in PA levels in this study were probably due to other cellular metabolic changes.

The activity of Put tends to increase activity of growing tissues (Rey et al., 1994), suggesting that all treatments were efficient in causing an increase in cell division in roots and leaves of *Eucalyptus* after 60 days (Table3).

Treatments with IBA enhanced the Put, Spd and Spm content at several experimental time-points. Several studies have shown that treatments with exogenous auxins increased the concentration of endogenous PAs (Jarvis et al., 1985), suggesting that auxins stimulate PA biosynthesis (Kyriakidis, 1983). Polyamines were also shown to be involved in polar auxin transport, the differentiation of vascular tissue and the definition of vein positions (Clay and Nelson, 2005). However, in this study, the increased PA content that resulted from the treatments did not alter the formation of roots or leaves of *Eucalyptus*.

Effect of ascorbic acid

Our results showed that exogenous IBA caused an increase in the ascorbic acid content in roots after 90 days. Exogenous auxins can activate plant antioxidant mechanisms (Pasternak et al., 2005). Thus, in response to auxin, cells might have produced compounds having antioxidant activity, such as ascorbic acid, protecting them against oxidation activated by ascorbic acid production (Cooks and Samman, 1996) (Fig. 1a). Other compounds with an antioxidant function, such as quercetin, are described as having a synergistic action with ascorbic acid to scavenge free radicals (Altunkaya et al., 2009). Our results suggest that the application of exogenous quercetin increased the concentration of ascorbic acid in the leaves and roots of *Eucalyptus* after 60 and 90 days, which might be important for plant cultivation under stress, because ascorbic acid protects cells against the action of free-radical species and acts as a potent antioxidant.

Changes in peroxidase activity

Changes in POD activity and its isoforms have been proposed to be biochemical markers for the successive rooting phases (Syros et al., 2004). In this study, POD activity was analyzed during the root expression phase (Figs. 2a and 2b). Many studies have demonstrated that a gradual reduction in POD activity occurs during the expression phase in adventitious rooting (Metaxas et al., 2004, Syros et al., 2004). Our results show that treatment with IBA caused a gradual reduction in POD activity in roots. This effect was less pronounced in leaves, which might indicate the compound contribution to rooting. Although there was no significant difference in the rooting percentage, root number or mean length of roots, a previous study showed that the exogenous application of IBA also affected POD and IAA-oxidase activities, to maintain high levels of endogenous auxin in mung bean (*Vigna radiata*L.) (Nag et al., 2001).

At the end of the cutting production cycle (day 90), all treatments caused a decrease in the POD activity in roots, which did not differ statistically, leading to a conclusion that treated cuttings are more likely to adapt to the field conditions, due to low stress levels.

Quercetin can act as an antioxidant (Brand-Williams et al., 1995), as well as peroxidases that catalyze the oxidation of many phenolic compounds in the presence of H_2O_2, which eliminates ROS (Gaspar et al., 1999). It has been reported

that the generation of H_2O_2 is a result of the auto-oxidation of quercetin, suggesting that this flavonoid might exert an oxidative function (Canada et al., 1990). Thus, this study suggests that the generation of ROS by quercetin supplementation has been occurred, due to the increase in POD activity after 30 and 60 days, compared to the control. After 90 days, this effect did not occur, probably due to the cessation of the oxidation reactions.

Materials and Methods

Experimental location

The experiment was conducted at the central nursery of Duratex S.A., in the mid-western region of São Paulo state (48º48'W; 22º35'S) at 560 m of altitude. The region is classified as Aw (tropical) according to the Köppen classification, a mean annual rainfall of 1,359.6 mm and a mean annual temperature of 23.3ºC.

Collection of cuttings

Cuttings of *E. grandis* × *E. urophylla* (clone CL1) from 3 to 6 cm length, 1.5 to 2.5 mm in diameter and with one to three leaf pairs were collected in the clonal garden of the company. Half of the leaf area was removed to reduce excessive transpiration. The cuttings were placed in a thermal box and were pulverized in the presence of nutrient solution (water 15 ± 5ºC + Ca + B) to maintain turgidity and reduce oxidation.

Preparation, application of treatments and planting of cuttings

Quercetin and IBA were dissolved in acetone, and were then mixed with $CaSO_4$ to form a smooth paste. The treatments were prepared in the following proportions: control, quercetin (0.5 g kg^{-1}), IBA (1.0 g kg^{-1}) and quercetin (0.5 g kg^{-1}) + IBA (1.0 g kg^{-1}). The bases of the cuttings were dipped in the treatments and planted in a substrate consisting of Canadian peat, rice husk and vermiculite plus 4 kg m^3 of Simple Superphosphate Fertilizer (SSF) (P_2O_5) and slow-release fertilizer, Basacote® Plus (12/8/15) plus micronutrients.

Implementation and experimental procedure

The experiment was implemented in completely randomized 4 × 3 factorial design, with four treatments and three evaluation periods (30, 60 and 90 days) and five replications of 48 plants per experimental unit, totaling 960 plants. After planting, the cuttings were moved to the greenhouse, where they remained for 23 days with temperatures between 25°C and 30°C and a relative humidity above 75%. After this period, cuttings were transferred to shade house for 20 days for acclimatization. At the end of this period, cuttings were moved into full sun for 50 days.

Assessment of rooting and development

From each experimental unit, 28 central plants were evaluated, due to the removal of plants at the border. Periodically, after 30, 60 and 90 days, the diameter and height of the cuttings were measured with a caliper and tape measure, respectively. At each assessment period, a destructive evaluation of five central cuttings from each experimental unit was performed and the rooting percentage and number of roots per cutting was determined, considering those that originated from the base, and the mean length of roots, calculating the mean number of major roots for each replicate.

Biochemical measurements

Leaves and roots were separated and were ground in liquid nitrogen and stored at -80°C for the conservation of physiological and biochemical characteristics.

Auxin and quercetin content

The quantification of quercetin and IAA was performed using HPLC coupled with mass spectrophotometer following the method proposed by Inbaraj et al. (2009) and Nakurte et al. (2012). Samples were injected into a LC-MS/MS (Varian 325-MS). The run time was 9 min, at 50°C with 20 µL injection volume. The column used was the Pursuit XRS model 5u C18 (50 × 2.0 mm). The mobile phases consisted of acidified water (0.1% acetic acid) and acidified acetonitrile (0.1% acetic acid).

Polyamines

Roots and leaves of *Eucalyptus* were homogenized in $HClO_4$ (5%). Dansyl chloride and saturated calcium carbonate were then added to supernatant and this mixture remained for 16 h in the dark. After this period, proline and toluene were added to the solution. The extract was applied to a TLC plate (silica gel G-60). The PAs were separated using chloroform-triethylamine (20:1 v/v). The separation of polyamines was monitored by UV light and the measurement of the polyamines Put, Spd and Spm was performed in a densitometer (Clinscan2, Helena Laboratories, Beaumont, TX) (Flores and Galston, 1982, adapted by Lima et al., 2008).

Ascorbic acid

The ascorbic acid content was determined in leaves and roots according to Terada et al., 1978.

Peroxidase assay

The POD activity was determined in leaves and roots by monitoring the decomposition of H_2O_2 (Lima et al., 1999).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and the means were compared by Tukey's test ($p \leq 0.05$).

Conclusion

Treatments with IBA, quercetin and their combination did not influence the height, diameter, rooting, number of roots, and root length of *E.grandis* × *E. urophylla*. A higher Put content after 60 days might be related to the high growth rate in this period caused by all treatments in both roots and leaves. Exogenous IBA application increased PAs after several time-points, which suggests that auxins might be involved in PA production and regulation. Increases in the ascorbic acid level due to quercetin and IBA treatment indicate the activation of antioxidant mechanisms. All treatments with auxin and quercetin caused a reduction in POD activity in roots at the end of cutting production. The application of these compounds should improve cutting performance under field

conditions, due to oxidative stress reduction and the enhancement of cell division. *Eucalyptus* clones with a low rooting rate should be tested to confirm the effect of the tested compounds on cutting growth and root development.

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