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Full Length Research Paper

Effect of auxins on adventitious root development from nodal cuttings of *Saraca asoka* (Roxb.) de Wilde and associated biochemical changes

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An attempt was made to induce rooting from nodal cuttings of *Saraca asoca* under controlled conditions and study its biochemical changes during rooting. The nodal cuttings were pretreated with different concentrations of IAA, NAA and IBA and kept in a growth chamber [25±2°C, 16 h photoperiod (55 µmolm^{-2}s^{-1}) with cool, white fluorescent lamps and 65% relative humidity] for 12 h. Among the three auxins used for pretreatment, IBA showed more positive response on rooting as compared to IAA and NAA within 4 weeks of transfer to growing medium under mist condition. Among four concentrations of IBA tested, 500 ppm gave maximum percentage of rooting, number of roots and root length. Therefore, IBA was used further in experiments for biochemical investigation. The adventitious rooting was obtained in three distinct phases that is induction (0 to 10 d), initiation (10 to 20 d) and expression (20 to 30 d). IAA-oxidase activity of IBA-treated cuttings increased slightly as compared to control. The activity was found to decrease during induction and initiation phases and increase during expression phase. The peroxidase activity in IBA-treated cuttings increased up to initiation phase and declined at the expression phase. Polyphenol oxidase activity increased both in IBA-treated and control cuttings during induction and initiation phase but declined slowly during expression phase. Total phenolic content was higher in IBA-treated cuttings, particularly in initiation and expression phases and it also correlated with peroxidase activity. Phenolics might be playing key role for induction of adventitious rooting, and phenolic compounds can be used as rooting enhancer in *S. asoca*, an important medicinal plant.

Key words: Auxins, enzyme activity, nodal cuttings, *Saraca asoca*, propagation.

INTRODUCTION

Saraca asoka (Roxb.) de Wilde (Family Leguminosae) is an important medicinal plant used by uterine tonic. It grows in the central and Eastern Himalays as well as in the West Coast of India. Bark of the plant has medicinal properties. Bark is also used as a uterine tonic and reported to cure biliousness, dyspepsis, dysentery, colic piles and pimples. It contains tannins and glycosides. Alkaloids leucopelargonidin and leucocyanidin have been extracted from the bark. Leaves are known to have blood purifying properties and flowers are used to treat diabetes. Most of the pharmeceutical companies are collecting the bark from the plants growing in the forest. In recent years, many of the plant genetic resources have been lost due to unplanned construction and destruction of natural forest. Conventionally, this plant propagated by seeds and the seed viability is very short. Lepidopterous caterpillars attack the seeds in the early stage of fruit (pod) formation. Commercial exploitation of the cuttings is limited by heavy losses during rooting and hardening procedures. Normal cuttings without treatment of plant growth regulators are also difficult for rooting. Adventitious root formation from the stem cutting is influenced

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by a number of internal and external factors (Davis et al., 1988; Cheniany et al., 2010). Different researchers have recognized different phases of adventitious root formation in plants based on physiology (DeKlerk et al., 1999; Gaspar et al., 1994; Kevers et al., 1997; Kulka, 2008; Cheniany et al., 2010). Auxin has been known to be intimately involved in the process of adventitious root formation (Wiesmann et al., 1988) and the interdependent physiological stages of the rooting process are associated with changes in endogenous auxin concentrations (Gaspar et al., 1997; Satisha et al., 2008). In several cases, high levels of IAA are associated with the promotion of adventitious rooting. In some cases, root development also occurred by decreasing the endogenous level of IAA (Hausman, 1993).

Root initiation has also occurred without any changes in IAA levels in the root occurring region (Nordstrom and Eliasson, 1991). In addition, several studies have emphasized that polyamines and auxins played major roles during the induction of rooting (Davis et al., 1988; Nag et al., 2001). The formation of adventitious roots involves the process of redifferentiation in which predetermined cells switch from their morphogenetic path to act as mother cells for the root primordia (Aeschbacher et al., 1994). Among these changes, the process of lignification in the cell wall, catalyzed by a particular peroxidase, may occur during rooting (Sato et al., 1993). It is well known that adventitious root formation can be stimulated by application of auxins exogenously but the mechanism of this physiological response is still a matter of debate. This plant is going to extinct in near future. There is a need to conserve *S. asoca* for *ex Situ* conservation. The aim of the present study is to establish the rooting efficiency from cuttings of *S. asoca* under mist conditions and to investigate the biochemical changes during rooting process.

MATRIALS AND METHODS

Collection of plant material

Freshly growing twigs (15 cm long) of *S. asoca* were collected from Champagarh reserve forest at Khurda district of Orissa, India and brought to the laboratory in a water cool box. Each twig was further segmented into smaller segments resulting into 3 to 4 node with leaf in each segment. The cuttings were used for rooting experiments.

Pretreatment of cuttings

The cuttings were put in 500 ml glass beakers containing 50 ml MS (Murashige and Skoog, 1962) nutrient solution without organics and sucrose. The basal region of the nodal cuttings was dipped into the solution. The pH of the nutrient solution was maintained at 5.8. Different concentrations of IAA, NAA and IBA (0, 100, 200, 300, 500 and 700 ppm) were used for pretreatment. Nutrient solution without growth regulator was used as control. The pretreatment of cuttings was maintained in a controlled growth chamber (25 ±2°C), 16 h photoperiod (55 $\mu molm^{-2}s^{-1}$) with cool, white fluorescent lamp and 65% relative humidity] for 12 h. After 12 h, the cuttings were planted into rooting medium.

Rooting media and growth conditions

Pre-treated cuttings were planted in the nursery bed containing sterile sand and soil in the ratio of 1:1. Hundred cuttings were planted in each bed under mist condition with 75% relative humidity for 4 weeks. Misting was made at every 30 min interval.

Scoring of data in rooting experiment

Rooting percentage, number of roots per cutting, root length was recorded at every week interval up to 4 weeks. 50 cuttings were used per treatment. The experiment was repeated thrice.

Biochemical investigation

After recording the information from the rooting experiment, an independent experiment was conducted for biochemical studies. For biochemical experiment, 20 cuttings were pretreated with nutrient solution with or without 500 ppm IBA for 12 h and subsequently planted in the rooting medium. The experiment was repeated 3 times. The growth condition was used as earlier. On the basis of the preliminary observation, it was decided to collect samples from 5 to 30 days after pretreatment. The basal part (about 0.5 cm of the rooting zone) of the cuttings was taken after 5, 10, 15, 20, 25 and 30 days of transferring to the medium for biochemical analysis.

Extraction for enzyme assay

1 g of fresh tissue was ground in mortar and pestle with 10 ml of 50 mM potassium phosphate buffer (pH 6.0). The homogenate was centrifuged for 20 min at 20,000 g. After centrifugation, the pellet was discarded and the supernatant was mixed with cold acetone to a final concentration of 70% and centrifuged at 20, 000 g for 15 min. The precipitate was resuspended in the same buffer and centrifuged at 20,000g for 20 min and the supernatant was used for enzyme assay. Protein concentrations were determined according to Bradford (1976) and bovine albumin (BSA) was used as the standard.

Peroxidase assay

The reaction mixture was made by mixing 0.1 ml enzyme extract, 0.01 ml 20 mM Guaiacol, 0.1 ml 50 mM H_2O_2, and 0.76 ml 2.5 mM 3,3-dimethylglutaric acid (3,3-DGA)-NaOH at pH 6.0. Peroxidase activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol at 470 nm after 10 min incubation at 30±0.5°C (Beffa et al., 1990). Each value is the mean of 3 replicates. One unit of peroxidase activity corresponds to a ΔA_{470} of 1.0 for 1 mg of protein in 10 min.

IAA oxidase assay

The reaction mixture was made by mixing the 0.2 ml enzyme extracts, 0.78 ml of 50 mM potassium-phosphate buffer (pH 6.0), 0.01 ml of 5 mM $MnCl_2$, 0.01 ml of 5 mM 2,4-dichlorophenol and

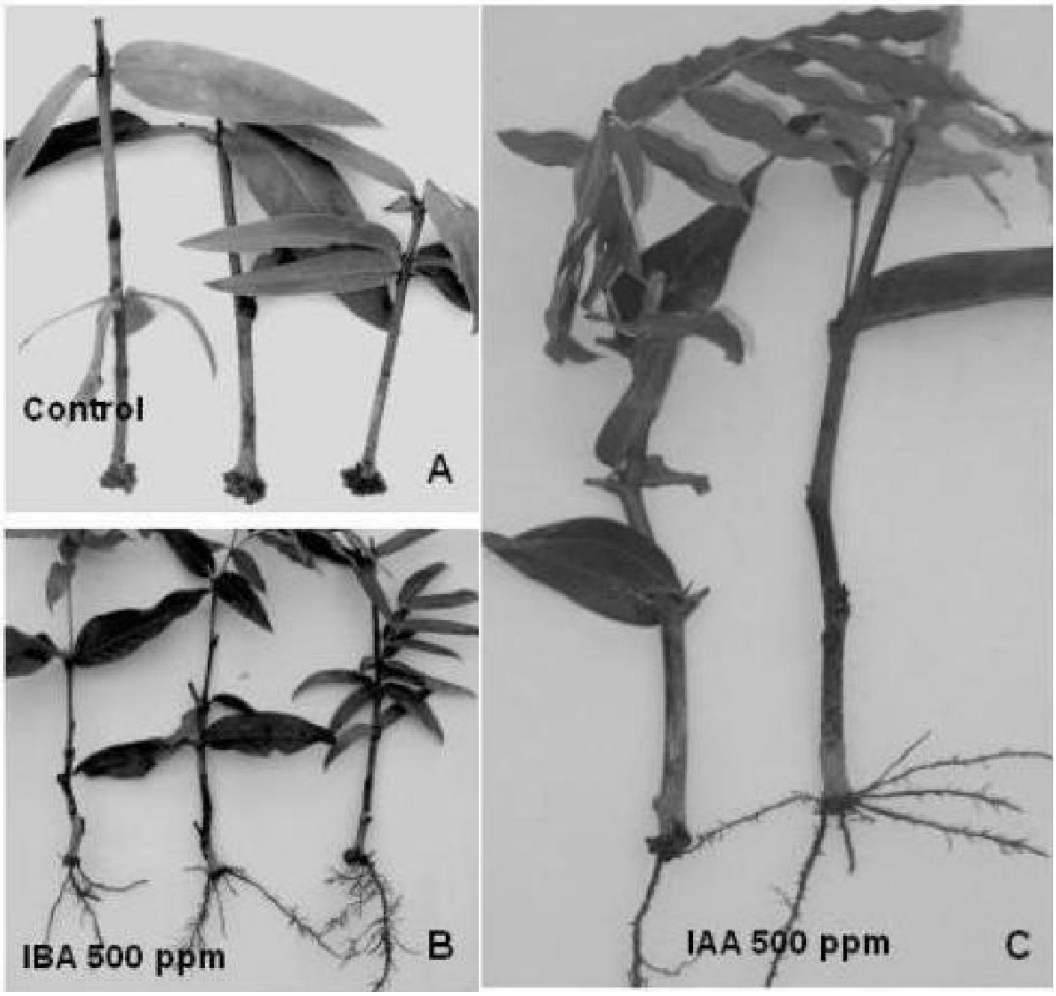

Figure 1. A) Cuttings pretreated with nutrient solution after 3 weeks transferred to growing medium under mist condition. B) Cuttings pretreated with 500 ppm IBA after 4 weeks transferred to potting medium. C) Cuttings pretreatment with 700 ppm IAA after 4 weeks transferred to potting medium.

0.02 ml of 2.5 g/L IAA. Assay was conducted at 25± 0.5°C for 30 min. The Salkowski reagent (2 ml) was then added and the destruction of IAA was determined by measuring the absorbance at 535 after 30 min (Beffa et al., 1990). Each value was the mean of 3 replicates. One unit of IAA oxidase activity is equivalent to a ΔA_{535} of 1.0 for 1 mg of protein in 30 min.

Polyphenol oxidase assay

Polyphenol-oxidase enzyme assay was made by using pyrogallol as the substrate (Kar and Mishra, 1976). The activity was determined in terms of enzyme activity/mg. protein/min. Each value was the mean of 3 replicates.

Total phenol content

Total phenol was extracted and estimated by Folin-Phenol reagent (Bray and Thorpe, 1954) and expressed as mg/gm fresh weight. Each value was the mean of 3 replicates.

RESULTS AND DISCUSSION

Effect of IAA-, IBA- and NAA-pretreatments on rooting

Cuttings treated with nutrient solution without growth regulators did not show any response on root development after 3 weeks on transfer to potting medium (Figure 1A). Root initiation was developed after 20 days of transfer to potting medium in most of the cuttings treated with growth regulators. The nodal cuttings pretreated with IBA showed higher response on rooting than NAA and IAA pretreatments (Table 1). The cuttings treated with 500 ppm IBA showed maximum percentage of rooting and maximum number of roots per cutting (Figure 1B). Among the different concentrations of IAA used for pretreatment of cuttings, the maximum percentage (54.5%) of rooting (Figure 1C) was obtained in cuttings treated with 500 ppm IAA. Among the different concentrations of

Table 1. Effect of different pretreatment of IAA, IBA and NAA on rooting from cuttings of *Saraca asoca* after 4 weeks transferred to potting medium.

Different treatments	% of rooting (mean ± S.E)*	No. of roots/cutting (Mean ± S.E)*	Av. root length (cm) (mean ± S.E)*
Control (H_2O)	0	0	0
IAA 100 ppm	16.4 ± 0.8^{b}	1.2 ± 0.12^{a}	0.38 ± 0.10^{a}
IAA 200 ppm	20.5 ± 0.6^{d}	2.4 ± 0.30^{b}	0.74 ± 0.22^{d}
IAA 300 ppm	38.2 ± 0.9^{g}	3.6 ± 0.71^{d}	0.91 ± 0.25^{e}
IAA 500 ppm	54.5 ± 0.7^{l}	3.1 ± 0.61^{d}	1.01 ± 0.41^{f}
IAA 700 ppm	42.8 ± 0.5^{l}	2.1 ± 0.4^{b}	0.88 ± 0.32^{e}
IBA 100 ppm	24.8 ± 1.0^{e}	2.4 ± 0.34^{b}	0.76 ± 0.12^{d}
IBA 200 ppm	42.7 ± 1.1^{l}	2.1 ± 0.2^{b}	0.89 ± 0.14^{e}
IBA 300 ppm	56.6 ± 1.0^{m}	3.6 ± 0.42^{d}	1.16 ± 0.33^{g}
IBA 500 ppm	76.8 ± 1.4^{n}	4.5 ± 0.53^{e}	1.36 ± 0.52^{n}
IBA 700 ppm	52.4 ± 0.8^{k}	2.4 ± 0.31^{b}	0.45 ± 0.2^{b}
NAA 100 ppm	18.2 ± 0.5^{c}	1.1 ± 0.26^{a}	0.32 ± 0.08^{a}
NAA 200 ppm	30.6 ± 0.4^{f}	2.3 ± 0.32^{b}	0.62 ± 0.11^{c}
NAA 300 ppm	48.2 ± 0.9^{j}	2.7 ± 0.45^{c}	1.01 ± 0.31^{f}
NAA 500 ppm	56.8 ± 0.7^{m}	2.2 ± 0.62^{b}	1.12 ± 0.33^{g}
NAA 700 ppm	40.6 ± 0.8^{h}	1.1 ± 0.21^{a}	0.72 ± 0.08^{d}
IAA 200 + IBA 200 ppm	42.4 ± 1.1^{l}	1.1 ± 0.45^{a}	0.75 ± 0.06^{d}
IAA 200 + IBA 500 ppm	57.2 ± 0.7^{m}	2.1 ± 0.32^{b}	0.86 ± 0.04^{e}
IAA 500 + IBA 500 ppm	15.5 ± 0.6^{a}	1.2 ± 0.25^{a}	0.30 ± 0.02^{a}

*Mean having the same letter in a column were not significantly different by Duncan`s multiple comparison test ($P< 0.05$). 35 cuttings/treatment; repeated twice.

NAA used for pretreatment, the maximum percentage of rooting (56.8%) was observed in cuttings treated with 500 ppm NAA within 4 weeks transfer to potting medium. However, the cuttings pretreated with 500 ppm IBA showed higher percentage of rooting (76.8%) as well as higher number of roots per cutting within 30 days of transfer to the medium. Therefore, this concentration was used further for biochemical studies during rooting process. The formation of adventitious roots from *in vitro* raised shoots of different plant species promoted by different auxins has been reported by various researchers (Sharma et al., 1999; Kulka, 2008). Sharma et al. (1999) achieved root initiation (71.6%) from *in vitro* grown tea micro-shoots on pretreatment with 500 ppm IBA for 30 min.

Subsequently, Gunasekare and Evans (2000) reported that the root initiation from microshoots with a pretreatment of IBA (50 ppm for 3 h) followed by culture of shoots in auxin-free half-strength MS liquid medium with continuous agitation.

Biochemical changes during rooting

Biochemical make up plays a major role during rooting process in plant. The biochemical studies were conducted from 5 to 30 days at every 7 days intervals during the rooting process. Generally, the adventitious rooting occurred in three distinct phases that is induction, initia-tion and expression, as proposed for poplar (Hausman et al., 1997). The results showed that the IAA-oxidase activity of the IBA-treated cuttings decreased during induction (0 to 10 d) and initiation phases (10 to 20 d) and increased during expression phase (20 to 30 d). The IAA-oxidase activity was higher in control treated cuttings as compared to IBA-treated one (Figure 2). The low IAA-oxidase activity during the induction period in IBA-treated cuttings appears to be responsible for better development of adventitious roots with IBA, possibly serving as the source of free auxin (Nag et al., 2001). Kulka (2010) reported that auxin produced by the plantlets induces root development. Hartmann et al. (1993) reported that the adventitious root formation initially occurs in two phases: an auxin-sensitive phase and an auxin-insensitive phase. At the expression phase, the IAA-oxidase activity was high which might be related to low IAA content (Nag et al., 2001). Figure 3 indicates higher peroxidase activity in IBA-treated cuttings as compared to control. The activity of peroxidases in IBA-treated cuttings clearly increased up to the expression phase, whereas in the case of control cuttings it showed stabilization or modest increase during the initiation and expression phases (Figure 3). The increase in peroxidase activity observed in IBA-treated cuttings during induction and initiation phase

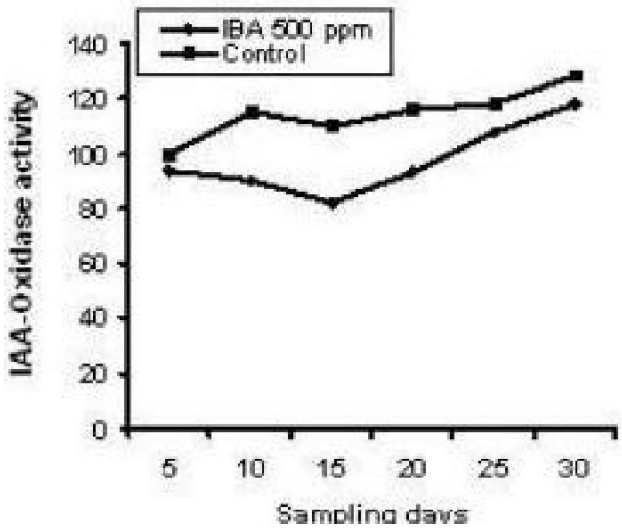

Figure 2. Changes of IAA-oxidase activity in different time period of root development from cuttings of *Saraca asoca* pretreated with or without IBA (500 ppm). One unit of IAA-oxidase activity is equivalent to a ΔA_{535} of 1.0 for 1 mg of protein in 30 min. Values are means ± SE.

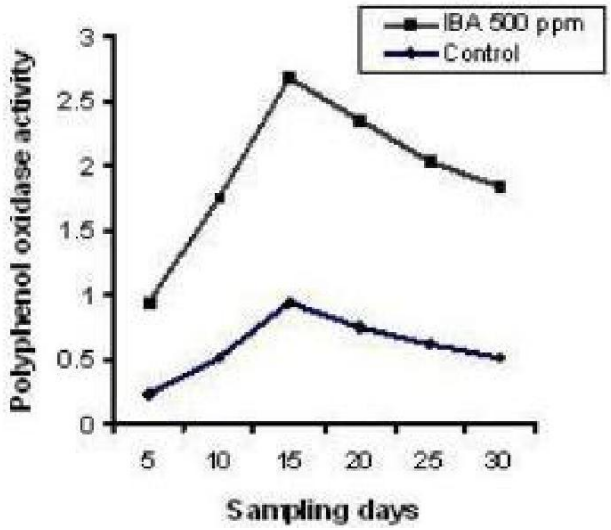

Figure 4. Changes of polyphenoloxidase activity in different time period of root development from cuttings of *Saraca asoca* pretreated with or with out IBA (500 ppm). The activity was determined

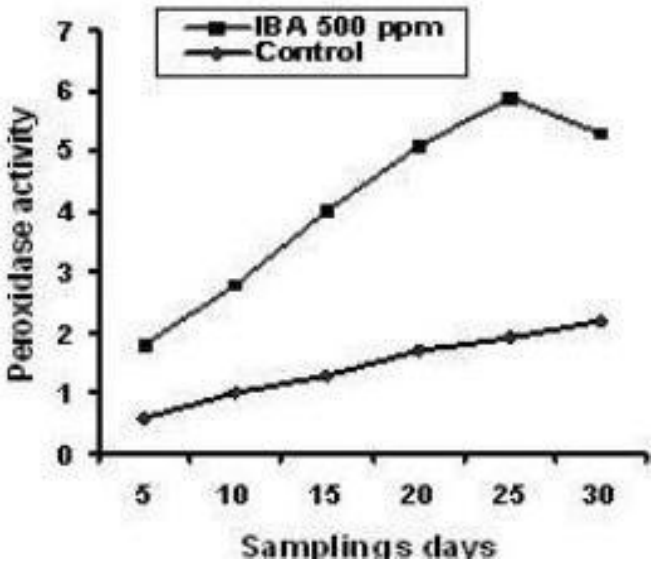

Figure 3. Changes of peroxidase activity in different time period of root development from cuttings of *Saraca asoca* pretreated with or without IBA (500 ppm). One unit of peroxidase activity is equivalent to a ΔA_{470} of 1.0 for 1 mg of protein in 10 min. Values are means ± SE

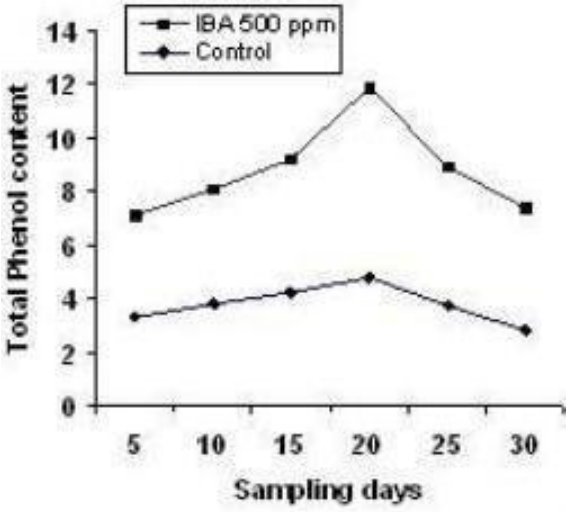

Figure 5. Total phenol content in different time period of root development from cuttings of *Saroca asoca* pretreated with or with out IBA (500 ppm). The phenol content was expressed as mg/gm. fresh weight. Values are means ± SE.

might serve as a good marker for rooting ability in cuttings (Moncousin and Gaspar, 1983). Plant peroxidases are known to be involved in auxin metabolism as well as lignification processes in the cell wall in the presence of phenol, and the present results showed that peroxidase activity increased later than IAA-oxidase activity which indicates that peroxidase activity is more involved in cell wall synthesis at the later phase and obligatory step in root formation (Sato et al., 1993).

An auxin-induced change in peroxidase and IAA-oxidase during the rooting process has also been reported (Mato et al., 1988; Fett-Neto et al., 1992; DeKlerk, 1996).

Polyphenoloxidase activity increased both in IBA-treated and control-treated cuttings during induction and initiation phase but declined slowly during expression phase (Figure 4). The present result also showed that total phenol content was maximum in IBA-treated cuttings, particularly in initiation and expression phases (Figure 5). It might be possible that phenolics play key role for induction of adventitious rooting. Polyphenol oxidase activity has also significant role during the rooting of hardwood cutting as reported (Satisha et al., 2008; Yilmaz et al., 2003). De Klerk et al. (1999) indicated that the wounding related compounds and successive application of auxins leads to higher percentage of rooting. They found that the phenolic compounds act as antioxidants, thereby protect-ting IAA from oxidation and plant tissue from oxidative stress due to wounding. Santos et al. (2009) reported that root induction linked to oxidative stress and that activity of stress induced alternative oxidase (AOX) is importantly involved in adventitious rooting. Cheniany et al. (2010) reported that peroxidase and polyphenol oxidase acti-vities are involved in induction of rooting in *Juglans regia*.

In conclusion, IBA pre-pretreatment helps adventitious root formation from cuttings of *S. asoca*. Among all the biochemical parameters evaluated, the more distinct between IBA-treated and control cuttings were IAA-oxidase (which includes a subgroup of peroxidases, and whose activity was lower in rooting cuttings during the induction phase) and total phenolic content (higher in rooting cuttings during the initiation phase) (Figures 2 and 5). Peroxidase activity was higher in IBA-treated cuttings up to expression phase whereas polyphenol oxidase activity was higher up to initiation phase and thereafter declined (Figures 3 and 4). Phenolic compounds as modifiers of the activities of peroxidase and polyphenol oxidase, as both inhibitors and stimulators, may influence the emergence of roots from cuttings of *S. asoca*. Thus, this investigation will be useful for mass-scale propa-gation and conservation of important medicinal plants; future experiments should examine the validity of these results with recalcitrant and easy-to-root woody germplasm.

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