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Full Length Research Paper

Biochemical aspects of single-node cuttings of *Ricinodendron heudelotii* (Baill.) in relation with rooting

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Ricinodendron heudelotii **(Njansang) is a valuable multipurpose tree species retained for domestication in Central and Western African regions. To measure the ability of rooting in relation with biochemical changes, basal single-node leafy cuttings were treated with different concentrations of indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA) and cultured in fine sand media under poly-propagator system. The adventitious rooting was obtained in three distinct stages: Induction (0 to 20 days), initiation (20 to 30 days) and expression (30 to 40 days). Rooting response was higher within nodal cuttings pretreated with IBA than those pretreated with NAA. Polyphenoloxidase activity started to increase both in treated and control cutting during the initiation stage of the experiment and decreased after root emergence only in treated cuttings. Indole-3 acetic acid (IAA)-oxidase activity of auxin treated cuttings decreased as compared to the control. The peroxidase activity in IBA-treated cuttings increased slowly at the initiation stages and lightly at the expression stage. Total phenolic content was higher in IBA-treated cutting particularly at the initiation and expression stages. Phenolics and polyphenoloxidase might be playing key role for emergence of adventitious rooting and can be used as rooting enhancer in *R. heudelotii*.**

Key words: Auxins, enzyme activity, nodal cutting, *Ricinodendron heudelotii*, vegetative propagation.

INTRODUCTION

Ricinodendron heudelotii (Baill.) belonging to Euphorbiaceae family is an indigenous tree commonly known as Njansang. It is native to Central Africa and the Golf of Guinea region. It has been classified as one of the top ten species to be domesticated in Centre and West African by International Centre of Research in Agroforestry (ICRAF) in accordance with the priorities setting among candidate species (Franzel et al., 1996). The plant is widely exploited for medicine and food (Tchiegang et al., 2005). Naturally, *R. heudelotii* is dioecious and dispersed by seeds. Due to the genetic variability of the seeds, vegetative propagation is necessary for the domestication of high value individual by large scale cultivation or introduction in agro-forestry system, but the development of suitable vegetative techniques in *R. heudelotii* is limited by the poor capacity for adventitious root formation. Normal cuttings without

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treatment of growth regulators are difficult for rooting. The formation of adventitious root from cuttings is influenced by a number of internal and external factors which include phytohormones (Hausman, 1993; Liu and Reid, 1992). It is well established that auxins play central role in the determination of rooting capacity, which is essential for vegetative propagation. Yet the mechanism of this physiological response is still disputed (Fagaca and Fett-Neto, 2005). Indeed, plants undergo profound changes in their enzyme expression. The formation of adventitious roots involves the process of re-differentiation, in which predetermined cells forms the root primordia.

Indole butyric acid (IBA) is the most widely used auxin because of its high ability to promote root initiation (Rout et al., 1999) and its great stability in comparison to 1-naphtalene acetic acid (NAA) and indole-3-acetic acid (IAA). Positives correlations between IBA and rooting have been reported (Alvarez et al., 1989; Tchoundjeu et al., 2002). Changes in enzyme activities which regulate different biochemical pathways example proteins, carbohydrates, phenolics during the rooting process in cuttings have been investigated (Druege et al., 2000; Kanmegne and Omokolo, 2003).

Changes in peroxidase activities have been proposed as molecular marker for rooting (Beffa et al., 1990). Studies showed that rooting occurs when peroxidase activity increased (Mark et al., 2004) and this activity is similar to those of IAA oxidase. Phenols also play a major role in root process but their effect is under control of the biochemical endogenous level in cuttings. They are the main substrate of peroxidases and their preferential oxidation by peroxidase prevents peroxidase-catalysed oxidation of auxin (Racchi et al., 2001). Polyphenols oxidase in presence of oxygen catalyses oxidation of phenolic compounds and plays a major role in organization and development of root primordial. So, the control of the rooting process by phytohormones and the knowledge of biochemical events associated with root induction and expression are useful, as they will permit the improvement of rooting procedures in Njansang. Furthermore the specific action of auxins or the interaction with other endogenous compounds in rooting of various plant species was reported.

The present investigation was carried out to determine the role of auxin treatment on rooting and evaluate changes of peroxidase, polyphenol oxidase and IAA oxidase activities as well as endogenous phenolic content during root development from cuttings of *R. heudelotii*.

MATERIALS AND METHODS

Study site and stock plant production

This study was conducted from March 2010 to September 2010 in a propagation unit established in the nursery at the Institute of Medical Research and Medicinal Plants Studies in Cameroon (IMPM) at Yaounde in the Centre region of Cameroon, located at latitude 3° 51' N and longitude 11° 25' E., approximately 813 m above sea level. Average rainfall is 1692 mm and is bimodally distributed (Segalen, 1967). Mature seeds of *R. heudelotii* were collected from at least 30 randomly selected trees, located in Yaounde (site described above). Seeds were bulked before sowing at nursery. The resulting seedlings were planted near the nursery and used as cutting source for the present trial.

Preparation of cuttings and propagation system

Basal single-node leafy cuttings used for this trial were harvested from stock plant, at the beginning (April, May 2010) of the rainy season. The base of each cutting was dipped for 1 h in 0, 50, 100, 200 and 300 µg/l IBA and NAA solution. The propagation media were composed of fine sand (FS), treated with a systemic fungicide (Benlate) prior to use. The propagator (1.5 x 0.5 x 1 m) was built with local material and providing an internal irradiance of 20 to 32%. Basal single-node leafy cuttings were inserted into a rooting bed in a randomised complete block design with three replications. Each five treatments (including control) contained 15 cuttings, giving a total of 450 cuttings for the whole experiment (15 cuttings × 05 treatments × 02 phytohormones × 3 replications). Cuttings were assessed every five days interval up to 40 days for survival and rooting rate, number and length of roots.

Subsequently, in a separate experiment, cuttings were treated with the best concentration of each auxin (100 µg/l IBA and 200 µg/l NAA) for biochemical analysis. The experimental unit was made up of 15 cuttings giving a total of 135 (15 cuttings x 3 treatments x 3 replications). The base (about 0.5 cm of the rooting zone) of the IBA and NAA-treated and untreated cuttings (control) of *R. heudelotii* were collected after 10, 15, 20 25, 30, 35, 40 days of transferring to rooting medium for biochemical analysis.

Enzyme preparation and assay

Proteins were extracted by homogenizing 1 g fresh weight of tissues (base of cutting) in mortar with 10 ml of 50 mM potassium phosphate buffer pH 6.0. The homogenate was then centrifuged at 4°C for 30 min at 6000 g and the supernatant was taken off. The pellet was re-suspended in potassium- phosphate buffer and re-centrifuged under the same conditions as before, and the new supernatant was added to the first. Protein concentrations were determined according to the method of Bradford (1976) and, bovine albumin was used as the standard.

Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined by measuring the increase in absorbance at 330 nm according to the study of Van Kammen and Broumer (1964). The reaction mixture incubated at 25°C contains: 2.7 ml of 1/15 M phosphate buffer pH 6.1 and 0.3 ml of 10 mM cathecol. The reaction was initiated by adding 40 ml of enzymatic extract. The enzyme activity was determined according to the change in optical density at 330 nm after 30 s. This activity was expressed on a fresh weight basis, compared to the protein content.

Peroxidase assay

Peroxidase activity was determined according to Thorpe and Gaspar (1978) method by monitoring the formation of guaïacol at 420 nm. Five milliliter (5 ml) of reaction mixture (1V of 0.2% H_2O_2; 2V of 1% guaïacol; 5V of 1/15 M phosphate buffer pH 6) was added to 10 µl of extract. One unit of enzyme activity corresponded to 0.1

is degraded per min, at 420 nm. Peroxidase activity was expressed on a fresh weight basis (unit g^{-1} FW).

IAA oxidase assay

Reaction mixture consisted of 100 mM $MnCl_2$, 50 mM 2,4-dichlorophenol (DCP), 320 mM IAA and 0.5 ml extract in 1/15 M potassium phosphate buffer (pH 6). The measurement of non-oxidized IAA was carried out with Salkowski reagent (2 ml) at 535 nm after 30 min at 25°C and the remaining IAA was calculated (Beffa et al., 1990). IAA oxidase activity was expressed in mg IAA destroyed after 30 min per fresh weight.

Phenols extraction and assay

Phenols were extracted by homogenizing 1 g tissue in 5 ml 80% methanol. The homogenate was then centrifuged for 20 min at 6000 g and the supernatant was collected. The pellet was re-suspended in 3 ml of 80% methanol and re-centrifuged under the same conditions as before and the new supernatant was added to the first. The quantity of phenol was determined as described by Bray and Thorpe (1954) using Folin and Ciocalteu reagent. The reaction mixture contained 100 µl of extract, 2 ml distilled water, 200 µl Folin; 0.5 ml of 20% Na_2CO_3. The reaction mixture was incubated at 40°C for 20 min and absorbance read at 725 nm. Total phenol content was expressed as µg/mg of fresh weight.

RESULTS

Effect of IBA and NAA on root formation

Four concentrations of IBA and NAA (50, 100, 200 and 300 µg/l) were tested and compared to the control (Without phytohormone). The adventitious rooting was obtained in three distinctive stages: Induction, initiation and expression. Roots induction was observed after 10 days of culture but this process was preceded by callus development at the base of cutting (Figure 1A). Root primordium formation (Recognizable stage of development characterized by brown spots of the transverse section at the base of cutting) appeared by the 15th day and roots emerged from the basal part on the 20th day. Meanwhile, nodal cuttings treated with growth regulators showed higher response on rooting than those untreated (Figure 1B). This response was also higher within nodal cuttings pretreated with IBA than those pretreated with NAA (Figure 1C and D). Within 40 days, cuttings treated with different concentrations of IBA showed at 100 µg/l, maximum percentage of survival (80%) and rooting (80%) and maximum number of root per cutting (6.2); while maximum average length of root (8.3 cm) was obtained with 200 µg/l NAA treated cutting (Table 1). In contrast, untreated cuttings showed lower percentage of survival (53.3%) and rooting (50%), lower average number of root per cutting (2) and lower average length of root (1.4 cm) (Table 1). Compare to IBA, NAA is less efficient and the maximum percentage of survival (70%) and rooting (70%), maximum number of root per cutting (2.9) and maximum average length of root (8.3 cm) were obtained with 200 µg/l (Table 1).

Biochemical changes during rooting

The biochemical studies were conducted from 10 to 40 days at every five days intervals during the rooting process. The adventitious rooting occurred in three distinct stages: induction (10 to 20 days), initiation (20 to 30 days) and expression (30 to 40 days). Polyphenols oxidase activity was investigated and compared to rooting ability. PPO activity started to increase both in auxin (IBA and NAA) and control cutting from the early period of growth and reached its maximum level during initiation stage. After that this activity was almost constant in control cutting while in 100 µg/l IBA or 200 µg/l NAA treated cutting, the activity decreased during expression stage of roots. The decrease was more pronounced in IBA treated than NAA treated cutting. Root formation in *R. heudelotii* cuttings was detected on the 20th days of culture (Figure 2).

The effect of auxin (IBA and NAA) was also reflected in peroxidase and IAA oxidase activities during roots formation. In the treated cuttings, peroxidase activity increased at the initiation stage during the first 20 days after the application of the root inducing treatment. After that, it continued to increase up to expression stage. IAA oxidase activity showed an opposite trend with decrease in initiation and expression stages. In the untreated cutting, peroxidase and IAA oxidase activities followed the same variations but at a lower level and one week later (Figures 3 and 4). These results also confirmed that IAA oxidase was not involved in root formation of *R. heudelotii*. In fact, its activity decreases from induction to initiation stage.

The effect of auxin (IBA and NAA) on phenol content of cutting was also investigated. As shown in Figure 5, phenol content significantly increased after auxin treatment particularly in initiation and expression stages and it is also correlated to polyphenol oxidase activity. In the control, phenolics gently increased over the induction stage, reached a peak on day 20 and then decreased.

DISCUSSION

In the present study, effective rooting of *R. heudelotii* cuttings is achieved by the application of the growth regulator IBA. Similar results have been observed in *Eucalyptus saligma* and *Eucalyptus globules* (Fett-Neto et al., 2001) and *Prunus Africana* (Tchoundjeu et al., 2002). In general, auxins played an important role in the process of roots development. But compared to IBA, NAA showed poorer responses in the rooting of *R. heudelotii*. This is consistent with the result reported for *Camelia sinensis* (Rout, 2006). The efficiency of IBA can be explained by the fact that it is less toxic to plant than NAA, and acts as a precursor for endogenous IAA (Han

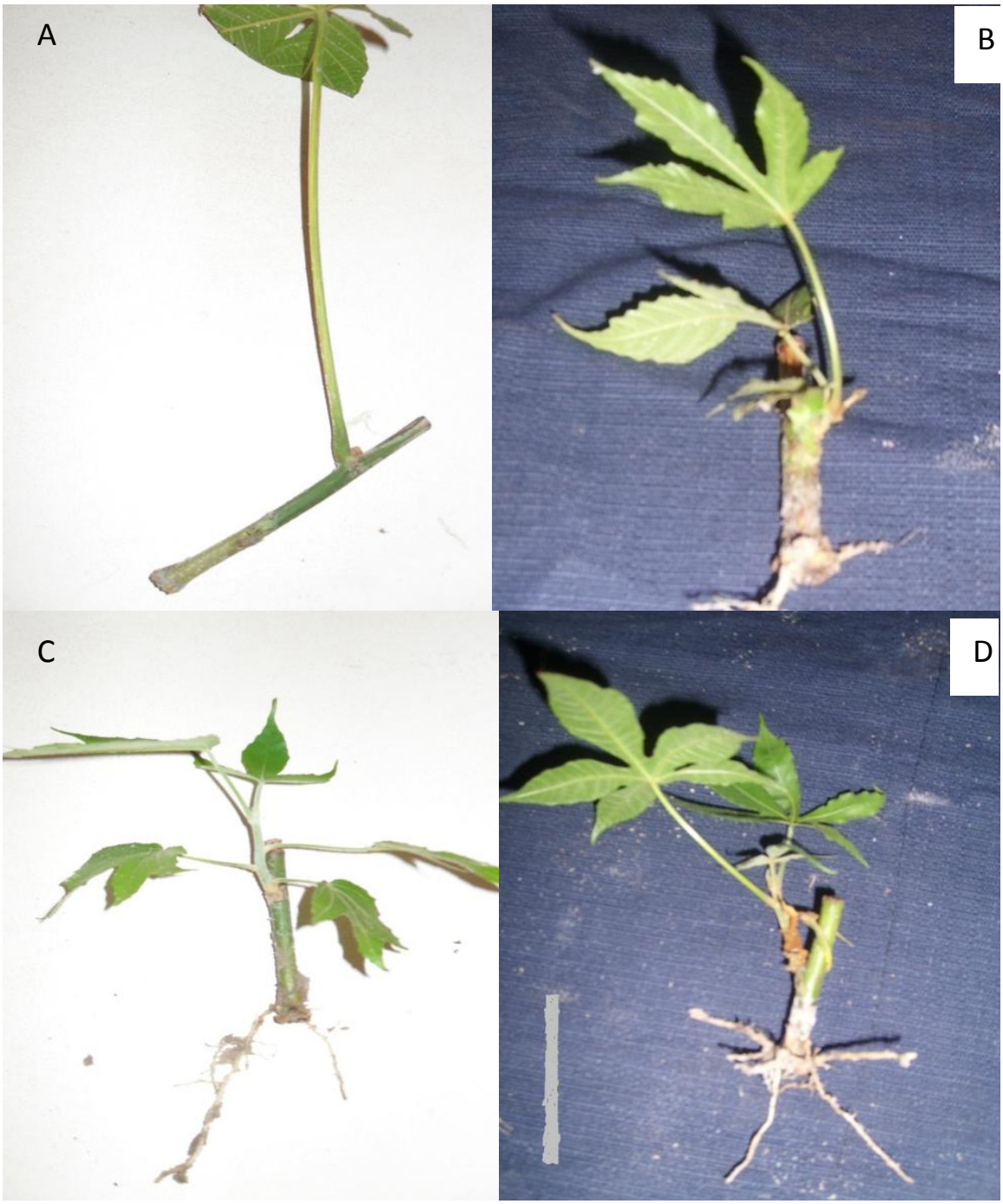

Figure 1. Morphology of *R. heudelotii* cuttings: (A) Single node cutting with a developed callus at the base, (B) Untreated single node cutting showing few leaves and poor root system, (C) Single node cuttings treated with 200 µg/l NAA showing poor root system and (D) Single node cuttings treated with 100 µg/l IBA showing the greatest number of leaves and roots.

et al., 2009).

The formation of adventitious roots from *in vitro* microcuttings of *R. heudelotii* has earlier been reported, but cuttings treated with nutrient solution without growth regulators did not show any response on root development. However our results are contradictory, because rooting occurs in the absence of auxin treatment. This may be partially due to the size and physiological state of cuttings, as well as to the physical or chemical characteristics of the media used. In all

Table 1. Effect of IBA or NAA concentrations on the formation of adventitious roots of *R. heudelotii* cuttings.

Different treatments (µg/l)		Survival rate	Rooting rate	Number of root/cutting	Average root length (cm)
IBA	50	53.3 ± 3.6[a]	50.0 ± 2.1[a]	2.6 ± 0.2[b]	2.4 ± 2.0[a]
	100	80.0 ± 3.0[e]	80.0 ± 3.0[e]	6.2 ± 0.9[d]	7.2 ± 2.6[c]
	200	66.6 ± 2.1[c]	66.6 ± 2.1[c]	4.0 ± 0.6[c]	6.6 ± 1.5[b]
	300	55.0 ± 1.2[a]	55.0 ± 1.2[b]	3.2 ± 0.2[c]	3.1 ± 1.3[a]
NAA	50	55.0 ± 1.9[a]	55.0 ± 1.9[b]	2.1 ± 0.2[b]	6.6 ± 1.7[b]
	100	66.6 ± 2.4[c]	70.0 ± 3.8[d]	2.8 ± 0.3[b]	8.1 ± 2.6[c]
	200	70.0 ± 4.1[d]	70.0 ± 4.1[d]	2.9 ± 0.6 [b]	8.3 ± 3.1[c]
	300	60.0 ± 3.4[b]	56.6 ± 1.8[b]	1.3 ± 0.3[a]	7.1 ± 1.8[b]
Control	0	53.3 ± 3.3[a]	50.0 ± 3.1[a]	2.0 ± 0.1[b]	1.4 ± 2.0 [a]

Means having the same letter in a column were not significantly different by Duncan's multiple comparison test ($p < 0.05$).

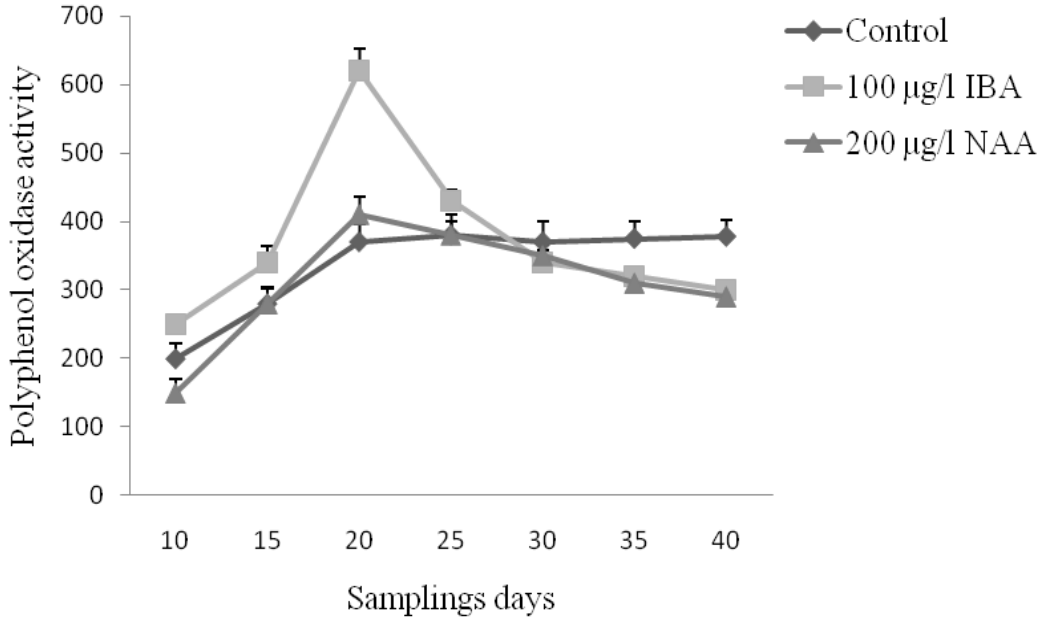

Figure 2. Changes of polyphenoloxidase activity in different time period of root development from nodal cuttings of *R. heudelotii* pretreated with IBA or NAA. Values are means ± SE.

cases, these results emphasized the needs to study biochemical changes during rooting in *R. heudelotii* cuttings.

Biochemical studies showed that there was relationship between PPO activity and the formation of rooting. Many results have been reported about the role of PPO on rooting tissues. Qaddoury and Amssa (2003) showed that PPO is effective for auxin oxidation during root development. Elevated auxin (IBA) concentration is required during the initiation stage but during the expression (root emergence) stage, the phytohormone becomes inhibitory. This pattern has been observed in many plant species. In addition, auxin oxidation by PPO is involved in regulating the synthesis of phenolic precursors needed for lignin biosynthesis during root differentiation (Haissig, 1986). Contrary to PPO, IAA oxidase was not involved in root formation of *R. heudelotii*. These results differed from those of many authors in that the drop of IAA oxidase activity might correspond to the rise in endogenous IAA level (Caboni et al., 1997). This suggests that the reduction of IAA oxidase may be necessary for adventitious root formation. Mato et al. (1985) as in our experiment did not find significant changes of IAA oxidase in treated and untreated cutting. Phenolics analyses suggest that they might play important role for induction of adventitious root formation. These compounds are very heterogeneous group of substances, interacting with intra and interce-

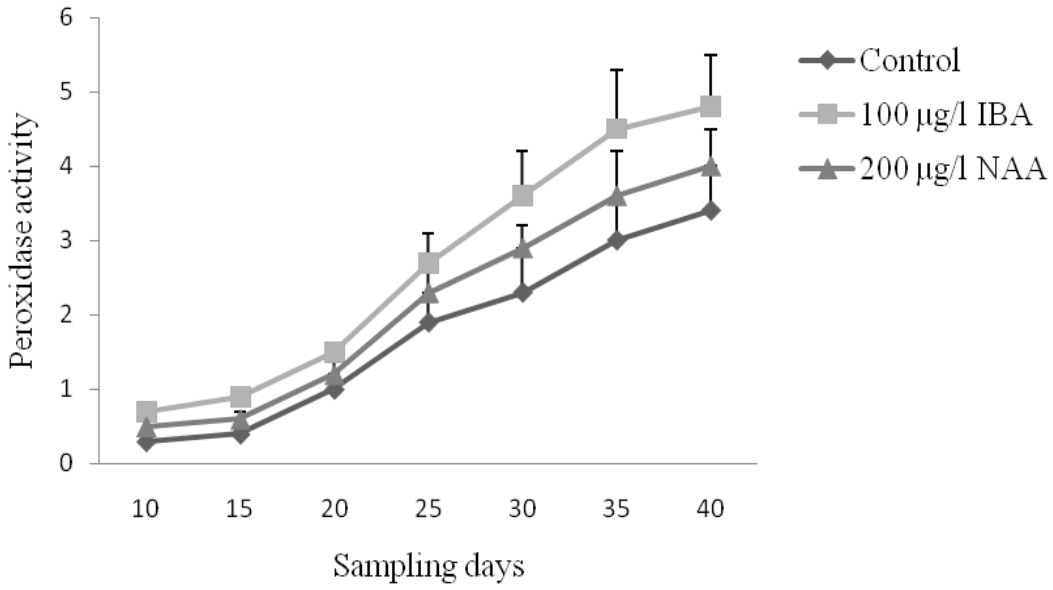

Figure 3. Changes of peroxidase activity in different time period of root development from nodal cuttings of *R. heudelotii* pretreated with IBA or NAA. Values are means ± SE.

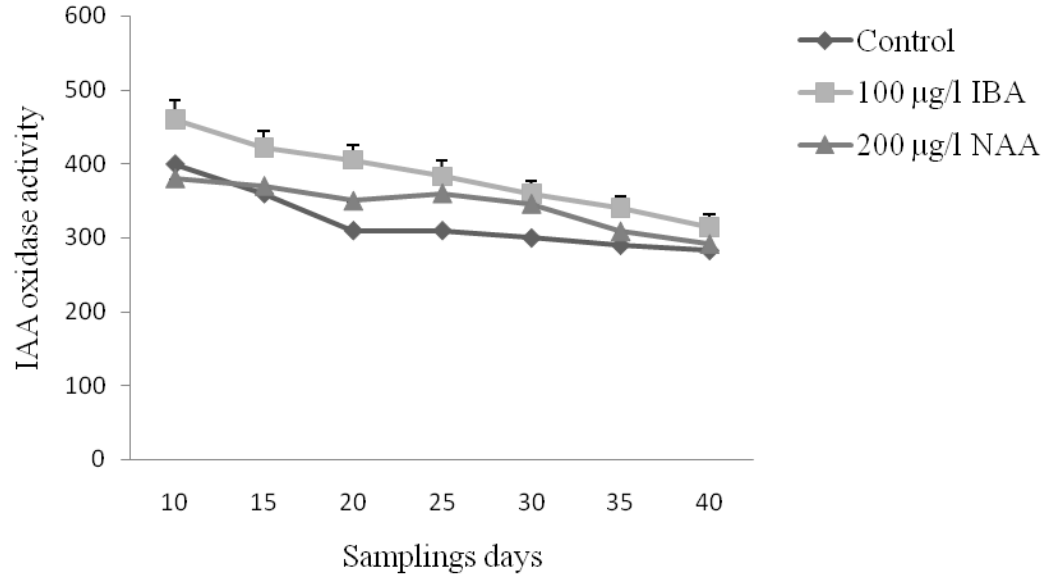

Figure 4. Changes of IAA oxidase activity in different time period of root development from nodal cuttings of *R. heudelotii* pretreated with IBA or NAA. Values are means ± SE.

cellular processes, example, with auxin metabolism (De klerk et al., 1997). The phenolics are used in woody plants to differentiate between juvenile and adult phases and thus serve as a marker for the ability to root formation (Rout, 2006). In this experiment, phenolics which are the main substrate of peroxidase were probably involved in their oxidation which promotes lignifications process due to wounding. In the past, De Klerk et al. (1999) indicated that wounding related compounds and successive application of auxins lead to higher percentage of rooting.

In conclusion, IBA treatment is more favorable for adventitious root formation in single node cuttings of *R. heudelotii*. This rooting process is correlated to changes of endogenous biochemical parameters. Among all these parameters, the more distinct between IBA or NAA-treated and control cutting were total phenolic content (higher in rooting cutting during initiation and expression stage) and polyphenol oxidase (involved in regulating the synthesis of phenolic precursors and whose activity was

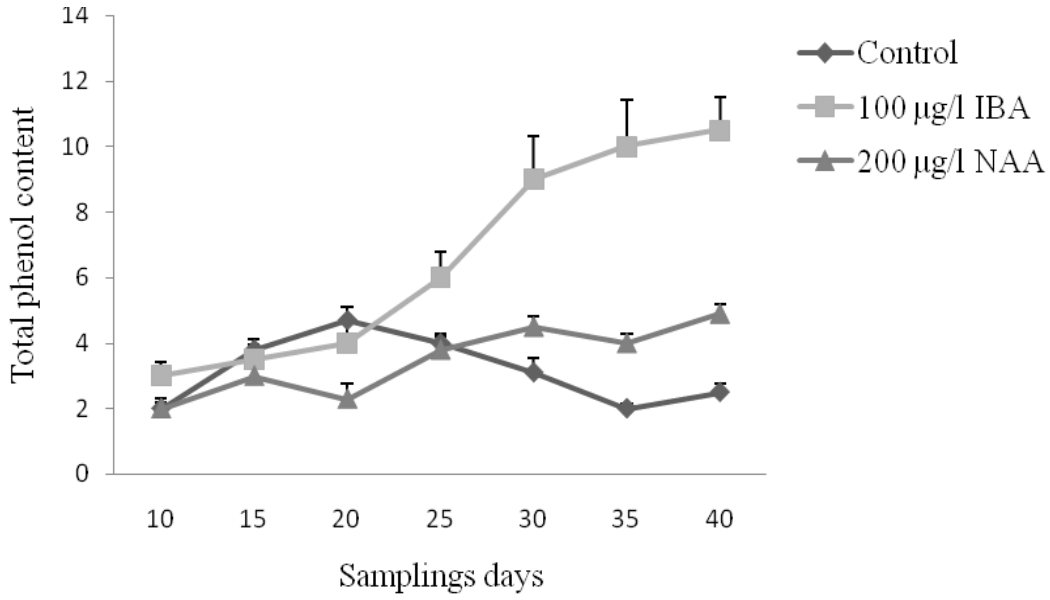

Figure 5. Total phenol content in different time period of root development from nodal cuttings of *R. heudelotii* pretreated with IBA or NAA. Values are means ± SE.

higher in rooting cutting during the initiation phase). Phenolics which are the main substrate of peroxidase were involved in their stimulation and may influence the emergence of roots from nodal cuttings of *R. heudelotii*. Futures studies should examine the validity of these results by better characterizing root ability of various accessions of *R. heudelotii*.

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