Vol.56, n.6: pp. 904-910, November-December 2013
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

# Cytokinin Dehydrogenase Activity in Primary Roots and Characterization of Primary Metabolites from Leaves and Rootlets of *Ricinus communis*

**Maria Elizabeth da Costa Marques[1], Beatriz Tupinambá Freitas[2], Tarciana Oliveira Guedes[3] and Roberta Pereira Miranda Fernandes[1,2*]**

*[1]Programa de Pós-graduação em Biotecnologia de Recursos Naturais; Universidade Federal de Sergipe; SE – Brasil. [2] Departamento de Fisiologia Universidade Federal de Sergipe; SE – Brasil. [3]Programa de Pós-Graduação em Bioprospecção Molecular; Universidade Regional do Cariri; CE - Brasil*

## ABSTRACT

*The aim of this study was to determine the activity of cytokinin dehydrogenase (CKX) and to measure other biochemical components in the primary leaves and radicles of castor seedlings (BRS Energia) in the initial phase of growth. The crude protein extract obtained after a 1-h extraction from the root tissues of seedlings showed no detectable CKX enzymatic activity when incubated with the substrate isopentenyl adenine for 1 h. However, after precipitation with ammonium sulfate at 70% saturation, the pellet showed CKX activity. The peroxidase enzyme activity was higher in the leaves than in the radicles. The total and reducing sugar content was 1.5 times higher in the leaves than in the radicles. The amino acid and protein contents were 6.4 and 9.2 times higher in the leaves than in the radicles, respectively.*

**Key words:** castor plant, cytokinin dehydrogenase, biochemical characterization, peroxidase

## INTRODUCTION

The castor plant (*Ricinus communis* L.) belongs to the Euphorbiaceae family and has medicinal properties (Kumari et al. 2008). The castor seed has a high oil content (48-50%), and its oil has industrial applications (Carneiro 2003). The cultivation of castor is very promising in many regions of Brazil. Currently, India is the largest producer of castor oil, responsible for 60% of the world's total production, followed by China and Brazil. This plant is distributed over several regions of the world and is commercially grown due to its hardy and heliophilic properties and tolerance to drought periods (Cartaxo et al. 2004). These properties of castor plant allow good adaptation to adverse climatic and soil conditions, fast growth and high production. Given its importance, more detailed surveys of the various aspects of its development and physiology are necessary. The life cycle of higher plants consists of several specific events that involve morphological and physiological changes. These changes are coordinated by the plant growth regulators, which are molecules that operate at low concentrations throughout the plant and bind to specific receptor proteins (Frébort et al. 2011).

Cytokinins (CK) are plant hormones and have a major role in the growth and development of the plants (Zalewski et al. 2010). The control of biologically active cytokinins occurs through a combination of biosynthesis, interconversion

*Author for correspondence: robertafernandes@ufs.br

between different forms, transient inactivation by conjugation (particularly glycosylation) and catabolic reactions, which result in a complete loss of its biological activity (Sakakibara 2006). The cytokinin levels in various tissues are regulated primarily through the action of an enzyme, called cytokinin dehydrogenase (CKX) (EC 1.5.99.12), which preferentially cleaves the isoprenoid cytokinins (Galuszka et al. 2007). It has been reported that the regulation of CKX activity depends directly on its concentration and/or compartmentalization in the cells (Motyka et al. 2003), which may vary in different tissues, or stages of development. The distinct expression profile and subcellular localization of several genes suggest that specialized functions are adapted to certain organs (Schumülling et al. 2003; Werner and Schumülling 2009).

The peroxidases are oxidoreductases (EC 1.11.1.7) that are produced by a variety of plants and microorganisms. These enzymes catalyze a variety of reactions, including the oxidation of various organic and inorganic compounds in the presence of peroxides, such as hydrogen peroxide. Peroxidase activity in the plants is related to the regulation of growth and differentiation processes, such as the inhibition of growth by regulating the cell wall lignification; morphogenic changes in response to physical, chemical and biological stress; wound healing; pathogen defense; the regulation of cell elongation; and other characteristics (Kao 2003; Nieves et al. 2003; Piza et al. 2003; Campos et al. 2004; Maciel 2007; Freitas et al. 2008). Other biochemical components considered are the contents of total and reducing sugars, amino acids and soluble proteins. The same biochemical components are used to identify the metabolic differences occurring in the plants subjected to various treatments. In addition, several studies have sought to correlate metabolic differences with the stages of plant development. Therefore, biochemical markers may help in the early identification of morphogenic processes in plants during the cellular differentiation, growth and multiplication (Piza et al. 2003). These biochemical parameters in the early development of the castor plant offer the prospect of more detailed surveys of the various aspects of its development and physiology. This information may result in a better understanding of this plant's physiology. The aim of this study was to measure the CKX activity in radicles, a site for local synthesis of cytokinins, in castor seedlings at early stages of development, a phase of high CKX enzymatic activity, particularly during the plant growth and differentiation.

## MATERIALS AND METHODS

### Germination

Broken, shriveled or very small seeds were discarded. Subsequently, the seeds were treated with 2% sodium hypochlorite for 5 min, rinsed and immersed in water for 10 h, placed in 72-well polystyrene trays using vermiculite as substrate and watered daily in a photoperiod of 12 h of daylight and 12 h of darkness. The seedlings were collected during the stage IV of development. According to Guedes (2010), this stage is reached when the first pair of primary leaves is completely expanded and the radicle is greater than five centimeters. This stage was reached in approximately 10-12 days after germination, and the radicle and primary leaves were collected. The rootlet samples were used for determining the cytokinin dehydrogenase activity, thereby enabling the expression of this enzyme during early plant development to be verified. Seedlings were grown for 11-12 days under the same conditions for collecting the samples of primary leaves and rootlets for the analysis of biochemical components. Peroxidase activity and the content of total and reducing sugars, protein, and total amino acid were measured.

### Protein extraction

The roots of *R. communis* (7.5 g) were homogenized in 5.0 mL of 0.5 M Tris-HCl (pH 7.4) at 4°C, using a previously cooled pestle and mortar. To determine the best extraction time and optimum activity of cytokinin dehydrogenase, the obtained homogenate was constantly agitated for 1 to 2 h at 4°C. The obtained crude extract was then centrifuged at 3000 xg for 10 min , and the supernatant was used to determine CKX activity. In order to concentrate the proteins, ammonium sulfate, was slowly added to the supernatant until 70% saturation at 0°C; subsequently, the solution was incubated at 4°C for 1h. The proteins were then collected as a precipitate by centrifugation at 3000 xg, re-suspended in a minimum volume of 0.1 M Tris-HCl (pH 7.5) and then stored at 4°C. The total protein content was estimated by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as the standard.

**Cytokinin dehydrogenase assay**

The cytokinin dehydrogenase activity was determined by the Libreros-Minotta and Tripton (1995) method. The reaction mixture (final volume 0.450 mL), containing the protein extract, isopentenyladenine (iP) as the substrate, and cuprum as the electron acceptor (500 mm $CuCl_2$) in 0.2 M imidazole buffer (pH 6.5), was incubated at 37°C for 1, 2 and 3 h. The reaction was stopped by adding 300 µL of trichloroacetic acid and the reaction mixture was centrifuged. After centrifugation, 200 µL of 4-aminophenol (solution in 6% trichloroacetic acid) was added to the supernatant. After 10 min, the absorbance was read at 352 nm to detect the formation of the Schiff base. The enzymatic assays were performed using 0-70% of the crude extract resuspended in 0.1 M Tris-HCl (pH 7.5). All the assays were performed in triplicate. One unit of CKX activity was defined as the amount of enzyme that catalyzed the formation of 1.0 mg of 3-methyl-2-butenal $h^{-1}$ $mL^{-1}$.

**Biochemical Analysis**

**Determination of Total Protein Content**

The concentration of total protein was determined using the Bradford method (Bradford 1976). To obtain the protein extract of the leaf tissues and root tissues, 1.0 g of leaves and 1.0 g of rootlets in 10 mL of 0.2 M sodium phosphate buffer (pH 7.5) were used. Then, each sample was homogenized and centrifuged at 3000 xg for 10 min. Subsequently, the supernatant of each sample was removed and used for analysis. The analysis used 100 µL of crude extract of leaf tissue diluted 20 x with 2.5 mL of Bradford's reagent. For the protein content determination of roots, 100 µL of crude extract was diluted with 2.5 mL of the Bradford reagent. The absorbance was read at 595 nm. Protein quantification was performed using a standard curve of bovine serum albumin (BSA) (0-500 $\mu g.mL^{-1}$).

**Determination of Peroxidase Enzyme Activity**

The peroxidase activity was measured using the method described by Reuveni and co-workers (1995). Briefly, the activity was determined using 20 µg of total protein crude extract from the leaves, 1 mm guaiacol and 25 mm citrate-phosphate buffer (pH 5.4) in a reaction mixture with a final volume of 1.0 mL. The reaction was initiated by the addition of 1.0 µL of 30% hydrogen peroxide. The same test was repeated for the root tissues. Absorbance readings were obtained at 10 second intervals for 1 min at 475 nm at 25ºC. The enzyme activity was expressed as $\Delta A\ min^{-1}\ g^{-1}$.

**Determination of Total Sugar Content**

The total sugar content was estimated according to the method described by Dubois and co-workers (1956). The sugar concentration was determined using a glucose standard curve (0-50 µg). The analysis was performed with 10 µL of leaf crude extract in 490 µL of distilled water. For the rootlets, 20 µL of crude extract was used in 480 µL of distilled water. To both samples, 500 µL of 5% phenol and 2.5 mL of concentrated sulfuric acid were added. The absorbance was read at 490 nm.

**Determination of Reducing Sugar Content**

The content of reducing sugars was quantified according to the DNS method (Miller 1959). The sugar concentrations were determined using a glucose standard curve (0-0.6 mM). The assay was performed with 400 µL of leaf crude extract, and the volume was adjusted to 1.5 mL using the DNS reagent. The reaction mixture was boiled for 5 min and then cooled to room temperature; subsequently, the volume was adjusted to 10 mL with distilled water. The same procedure was followed for the root tissues with the reaction mixture containing 500 µL of root crude extract and 1000 µL of DNS reagent. The reagents were boiled for 5 min and cooled to room temperature, and the volume was adjusted to 10 mL with distilled water. The absorbance was read at 540 nm.

**Determination of Amino Acid Content**

The amino acid content was measured using the method described by Bates and co-workers (1973). The amino acid concentrations were determined using a proline standard curve (0–40 µg). To obtain the leaf extract, 500 mg of leaves were soaked in 10 mL of 3% sulfosalicylic acid. To obtain the radicle extract, 2.5 g of root tissue was soaked in 10 mL of 3% sulfosalicylic acid. Then, each extract was centrifuged at 3000 *g* for 10 min, and the supernatants were used for the analysis. The reaction mixtures, containing 2.0 mL of sample, 2.0 mL of ninhydrin acid and 2.0 mL of acetic acid, were incubated in a boiling water bath for one h before cooling in an ice bath. The absorbance was read at 520 nm.

## RESULTS AND DISCUSSION

The crude extract obtained from the seedlings of *R. communis* showed no detectable activity of cytokinin dehydrogenase. The enzyme appeared to be in very small concentration, which was diluted in the crude extract, hindering the detection of enzymatic activity. This was countered by salting out precipitation. The activity of CKX was observed after $(NH_4)_2SO_4$ precipitation at 70% saturation, which yielded a higher protein concentration. The enzyme showed the highest activity ($1.5 \times 10^{-4}$ $U.mL^{-1}$) at 37°C, after extraction for 1 h in 0.5 M Tris-HCl (pH 7.4). The extraction for 2 h showed a drop in the protein content of the extract, perhaps through the action of some active proteases released during the extraction. Valdés and co-workers (2007) also found CKX enzymatic activity using as assay for the cleavage of the side chains of isopentenyl adenine (iP) and isopentenyl ribosideo (iPr) in *Pinus sylvestris* seeds and seedlings and found that the activity was dependent on the plant tissue. Extracts from the seeds of *Pinus sylvestris* showed a specific activity of CKX that was 85% higher using iPR as a substrate, independent of the electron acceptor. However, for the seedling extracts, using iPR as the substrate gave an activity 55% higher than that observed with iP as the substrate. In this work, the enzyme levels in the seedling radicle protein extract were very low. The expression of CKX is dependent on the environmental conditions and also in the plant developmental stage. In the stage of the plants used here, the growth of the plant was no longer dependent on the high concentrations of CK, which should result in higher activity of CKX enzyme. These results indicated that other factors could be affecting the expression or activity of CKX. Vysotskaya and co-workers (2010) found a 70% increase in the expression of CKX in the shoots of wheat seedlings the first day after transfer to ion-deficient Hoagland-Arnon medium (HA). They also evaluated the excision of four of the five primary roots and observed a decrease in the levels of CKX during the first 60 min, followed by a 65% increase in its expression compared with the control. These results showed that the root tissue was a site for the local synthesis of CKX. Dobra and co-workers (2010) studied the hormonal changes in response to heat, drought stress and the combination of these factors and concluded that CKX was involved in stress response because cytokinin dehydrogenase activity was strongly stimulated in the leaves and roots of stressed plants and coincided with the accentuated negative regulation of bioactive cytokinins.

### Peroxidase Activity

The peroxidase activity varied greatly between the leaves and the root tissues. The peroxidase activity in primary leaves was nine-fold higher than that in the rootlets (Fig. 1).

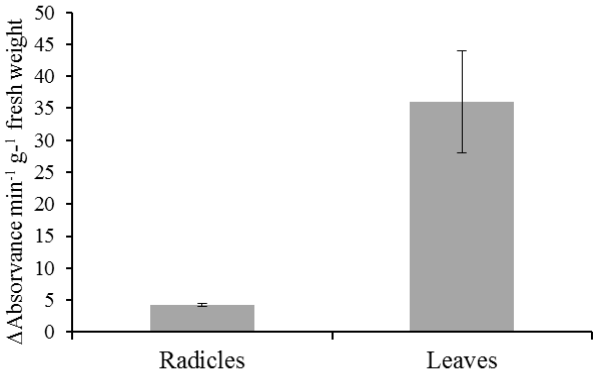


**Figure 1 -** Peroxidase activity in the radicles and leaves from seedlings of *Ricinus communis* with 11-12 days after germination in polystyrene trays using vermiculite as substrate under a photoperiod of 12 h of daylight and 12 h of darkness.

The variations in the peroxidase activity in different plant organs, developmental stages and environmental conditions are well documented in the literature in several plants but not in *Ricinus communis*. However, in this plant, a higher peroxidase activity in male flower, when compared to the females ones has already been shown (Jaiswal et al. 1985). Interestingly in other plants, the opposite mechanism is present; a higher peroxidase activity is found in female plants and it is probably related to differentiation (Jaiswal et al. 1985). Arican and co-workers (2008) observed that the peroxidase enzymatic activity was higher in the calli than in the shoots of *Abies equi-Trojani*. Santos and co-workers (2011) also found higher peroxidase activity in the callus of patchoulli than in the plants grown in a greenhouse and *in vitro*. Piza and co-workers (2003) observed a high peroxidase activity in the roots of pineapple plants cultivated in *vitro* under high salt stress. In addition, peroxidase is associated with oxidative stress conditions, acting in plant defense against reactive oxygen species and promoting the

synthesis of lignin and cell wall strengthening (Gaspar et al. 2002). In this work, the stage at which the plants were collected, as well as the environmental changes, could be responsible for the high peroxidase activity found in these organs, particularly in the leaves.

**Determination of the Total Protein, Amino Acid, Soluble Sugar and Reducing Sugar Contents**

In general, the germination of seeds starts with the absorption of water, and simultaneously, the hydrolysis of macromolecules occurs for embryo nutrition (Sampaio 1998). With a castor seed, after the consumption of carbohydrates through cellular respiration, the oil content begins to decrease on the third day after germination due to the process of oil hydrolysis (Sevast'yanova 1986). In the present studies, the total protein concentration varied between the primary leaves and radicles of seedlings (Table 1). The protein content found in the leaves was approximately 9.2 times higher than that of the radicles. Figueiredo and co-workers (2009) analyzed the biochemical parameters in long pepper subjected to climatic changes during its development and found similar results, as the total protein levels in the leaves were much higher than those observed in the roots. In contrast, Beltrão and co-workers (2003) examined the effects of water stress in the castor plant, variety BRS 188 Paraguaçú, in the initial phase of its growth and did not find any difference in the soluble protein contents in different plant tissues.

**Table 1** - Total soluble sugar, reducing sugar, total amino acid and protein contents in leaves and radicles in *Ricinus communis* with 11-12 days after germination.

| | **Primary Leaves** | **Radicles** |
|---|---|---|
| Total soluble sugars ($\mu g.mg^{-1}$) | 23.70 ± 2.30 | 15.3±0.94 |
| Reducing sugars (mm) | 0.02 ± 0.00 | 0.01±0.00 |
| Total Proteins ($\mu g.\mu L^{-1}$) | 3.04 ± 0.03 | 0.33±0.03 |
| Total amino acids ($\mu g.mg^{-1}$) | 0.14 ± 0.00 | 0.02±0.00 |

In this study, the leaves also showed a higher amino acid content than the radicles. The concentration of these molecules in the leaves was approximately 6.4 times higher than in the roots. The values found in this work were much lower than those observed by Costa and co-workers (2008). They found a total amino acid concentration of 0.698 $\mu g.mg^{-1}$ in the fresh leaves of patchouli grown in the field. Santos and co-workers (2011) also found a higher amino acid content in patchouli cultured *in vitro*. In the present study, the leaves of seedlings BRS Energia showed a concentration of soluble sugars 1.5 times higher than that in the rootlets. Figueiredo and co-workers (2009) examined long pepper under climatic variations and found that the roots and leaves showed a high concentration of soluble sugars three months after the germination. In studies by Beltrão and co-workers (2003), the soluble sugar levels in the roots of castor BRS 188 Paraguaçú were increased when juvenile plants were subjected to flooding conditions compared with water-deficiency stress. In our studies, the reducing sugar content in the leaves was 1.5 times higher than that in the roots, values of 23.7 and 15.3 $\mu g.mg^{-1}$ were found for the leaves and radicles, respectively. Santos and co-workers (2011) found that the levels of reducing sugars in patchouli callus were highest compared to those cultivated in a greenhouse and *in vitro*. Thus, the observed differences in the levels of total sugars, reducing sugars and total amino acids between the leaves and radicles of castor seedlings could be attributed to the dual role of early seedlings first bodies, which provided storage and enable the photo-assimilation of macromolecules for the growth and maintenance of seedlings at 11-12 days after the germination under a natural photoperiod of 12 h of light and 12 h of darkness. Santos et al. (2011) stated that the differences reported for different types of crops could be a result of the different metabolic requirements of these plants. Cordeiro (2007) stated that the processes that regulated the metabolism of the leaves determined the amount of molecules allocated to storage, maintenance and distribution. In summary, the present results showed that the leaves of castor seedlings BRS Energia had higher levels of amino acids, total and reducing sugars and proteins, as well as higher peroxidase activity than the roots. It could be possible that this was due to the necessity of protein synthesis for tissue maintenance and defense of the plant. When the seedling reached 12 days after germination, it already had a pair of primary leaves; thus, it was possible that the seedling might begin to synthesize the reserve substances through photosynthesis. Unlike primary metabolites, which are required for central metabolism, specialized compounds are often biosynthesized in response to

environmental cues or as a consequence of growth and development (Weng et al. 2012.)

## CONCLUSION

*Ricinus communis* seedlings expanding its pair of primary leaves showed CKX activity when subjected to ammonium sulfate precipitation at 70% saturation. The biochemical composition and peroxidase activity were higher in the leaves than in radicles during this stage of development with a natural photoperiod (12 h of light and 12 h of darkness). This was the first report of this enzyme activity in castor plants and could guide further studies of purification and characterization of this enzyme in *Ricinus communis*.

## ACKNOWLEDGMENTS

We thank the Experimental Station of Embrapa Algodão at Missão Velha, CE, for supplying the castor seeds and CAPES for first author Master´s Scholarhip.

## REFERENCES

Arican E, Albayralk G, Gozukirmizi N. Calli cultures from *Abies equi-trojani* (Aschers et Sinten) and changes in antioxidant defense system enzymes. *J Environ Biol*. 2008; 29:841-844.

Bates LS, Waldern RP, Teare ID. Rapid determination of free proline for water stress studies. *Plant Soil*. 1973; 39:205-207.

Beltrão NEM, Souza JG, Santos JW, Costa FX, Lucena AMA, Queiroz UC. Modificações na bioquímica da planta da mamoneira, cultivar BRS 188 paraguaçu, submetida ao estresse hídrico (deficiência e excesso). *Rev Bras Ol Fibros*. 2003; 7:653-658.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248-254.

Campos AD, Ferreira AG, Hampe MMV, Antunes IF, Brancão N, Silveira EP, et al. Atividade de peroxidase e polifenoloxidase na resistência do feijão à antracnose. *Pes Agropec Bras.* 2004; 39:637-643.

Carneiro RAFA. Produção do biodiesel na Bahia. *Revista Conjuntura e Planejamento*. 2003; 112:35-43.

Cartaxo WV, Beltrão NEM, Silva ORRF, Severino LS, Suassuna ND, Soares JJ. O cultivo da mamona no semi-árido brasileiro. Embrapa Algodão. 2004. 20 p. (Circular técnica, 77).

Cordeiro YEM. Aspectos biofísicos e bioquímicos de plantas jovens de mogno brasileiro (*Swietenia macrophylla*/ King) sob dois regimes hídricos [Dissertação-Mestrado em Agronomia]. Belém, Brasil: Universidade Federal Rural da Amazônia; 2007.

Costa AG. Desenvolvimento vegetativo, rendimento e composição do óleo essencial de Patchouli após adubação nitrogenada [Dissertação-Mestrado]. Paraná, Brasil: Universidade Federal do Paraná; 2008.

Dobra J, Motyka V, Dobrev P, Malbeck J, Prasil IT, Haisel D, et al. Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. *J Plant Physiol*. 2010; 167:1360-1370.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for Determination of sugars and related substances. *AnaL Chem*. 1956; 28:350-356.

Figueiredo FJC, Rocha Neto OG, Souza CMA, Silva ESA. Parâmetros bioquímicos de plantas de Pimenta Longa (*Piper hispidinervum* C.D.C.) em diferentes condições de cultivo no município de Igarapé-Açu, PA. *Rev Ciênc Agrar*. 2009; 51:171-189.

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P. Evolution of cytoquinin biosynthesis and degradation. *J Exp Bot*. 2011; 62:2431-2452.

Freitas AA, Francelim MF, Hirata GF, Clemente E, Schmidt FL. Atividades das enzimas peroxidase (POD) e polifenoloxidase (PPO) nas uvas das cultivares benitaka e rubi e em seus sucos e geléias. *Ciênc Tecnol Aliment*. 2008; 28:172-177.

Galuszka P, Popelková H, Werner T, Frébortová J, Pospísilová H, Mik V, et al. Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. *J Plant Growth Regul*. 2007; 26:255-267.

Gaspar T, Franck T, Bisbis B, Kevers C, Jouve L, Hausman JF, et al. Concepts in plant stress physiology. Application to plant tissue cultures. *Plant Grown Regul*. 2002; 37:263-285.

Guedes TO. Isolamento Parcial de uma Citoquinina Oxidase Purificada de Plântulas de *Ricinus Communis* L, Cultivar Brs Energia. [Dissertação de Mestrado em Bioprospecção Molecular]. Crato, Brasil: Universidade Regional do Cariri; 2010.

Hamid M, Ur-Rehman K. Potential applications of peroxidase. *Food Chem*. 2009; 115:1177-1186.

Jaiswal VS, Kumar A, Lal M. Role of endogenous phytohormones and some macromolecules in regulation of sex differentiation in flowering plants. *Proc Indian Acad Sci*. 1985; 95:453-459.

Kao CH. Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice leaves. *Plant Growth Regul*. 2003; 39:83-89.

Kumari KG, Ganesan M, Jayabalan N. Somatic organogenesis and plant regeneration in *Ricinus communis*. *Biol Plant*. 2008; 52:17-25.

Libreros-Minotta CA, Tripton PA. A colorimetric assay for cytokinin oxidase. *Anal Biochem*. 1995; 231:339-341.

Maciel HPF, Gouvea CMCP, Pastore GM. Extração e caracterização parcial de peroxidase de folhas de *Copaifera langsdorffii* Desf. *Ciênc Tecnol Aliment*. 2007; 26:221-225.

Miller GL. Use of dinitosalicylic acid reagent for the determination of reducing sugar. *Anal Chem*. 1959; 31:426-428.

Motyka V, Vaková R, Apková V, Petrásek J, Kamínek M, Schumülling T. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Plant Physiol.* 2003; 17:11-21.

Nieves N, Segura-Nieto M, Blanco MA, Sánchez M, Gonzáles A, Gonzáles JL et al. Biochemical characterization of embryogenic and non-embryogenic caloses of sugarcane. *In vitro Cell Dev Biol Plant*. 2003; 39:343-345.

Piza IMT, Lima GPP, Brasil OG. Atividade de peroxidase e níveis de proteínas em plantas de abacaxizeiro micropropagadas em meio salino. *R Bras Agrociência.* 2003; 9:361-366.

Reuveni R. Biochemical markers for disease resistance. In: Sing, R. P. Sing, U. S. *Molecular Methods in Plant Pathology*. CRC Press; 1995.

Sakakibara H. Cytokinins: activity, biosynthesis, and translocation. *Ann Rev Plant Biol*. 2006; 57:431-449.

Sampaio ES. Fisiologia Vegetal: teoria e experimentos. Ponta Grossa. UEPG, 1998; 190p.

Santos AV, Arrigoni-Blank MF, Blank AF, Diniz LEC, Fernandes RPM. Biochemical profile of callus cultures of *Pogostemon cablin* (Blanco) Benth. *Plant Cell Tiss Org*. 2011; 107:35-43.

Schumülling T, Werner T, Riefler M, Krupková E, Bartrina-Manns I. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *J Plant Res*. 2003; 116:241-252.

Sevast'yanova LB. Germination of seeds. In: Moshkin, V.A. (ed.). Castor. New Delhi: *Amerind*. 1986; 34-35.

Valdés AE, Galuszka P, Fernández B, Centeno ML, Frébort I. Developmental stage as a possible factor affecting cytokinin content and cytokinin dehydrogenase activity in *Pinus sylvestris*. *Biol Plantarum*. 2007; 51:193-197.

Vysotskaya LB, Aval'baev AM, Yuldashev RA, Shakirova FM, Veselov SY, Kudoyarova GR. Regulation of Cytokinin oxidase activity as a factor affecting the content of cytokinins. *J Plant Physyol*. 2010; 57:494-500.

Weng J, Ryan NP, Joseph PN. The Rise of Chemodiversity in Plants. *Science*. 2012; 336:1667-1670.

Werner T, Schumülling T. Cytokinin action in plant development. *Plant Biol.* 2009; 12:1-12.

Zalewski W, Galuszka P, Gasparis S, Orczyc W, Nadolska O. **S**ilencing of the *HvCKX1* gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. *J Exp Bot*. 2010; 61:1839-1851.

Received: November 23, 2012;
Accepted: August 20, 2013.