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Endogenous and exogenous polyamines in the organogenesis in *Curcuma longa* L.

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ABSTRACT

The present work evaluated the development of different *Curcuma longa* L. explants (leaves basis, root tips and ancillary buds from rhizome) stimulated by exogenous polyamines, combined with naphtalenacetic acid (NAA) or with 6-benzyl-aminopurine (BAP), to produce callus and its subsequent differentiation. The explants, isolated from field plants, were previously subjected to a basic cleaning method and were inoculated onto Murashige and Skoog culture medium (MS) [Murashige, T.S., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15, 473–497] supplemented with NAA (2.0 mg L^{-1}). Buds were subjected to different treatments, with or without 5.0 and 10.0 mmol L^{-1} exogenous polyamines (mixture of putrescine:-spermine:spermidine, 1:1:1) combined with NAA. The calluses obtained were transferred into the same medium, supplemented with the mixture of polyamines combined with BAP, in order to induce plant differentiation. For *C. longa*, buds were the most efficient explants for callus induction ($p < 0.05$). The application of exogenous polyamines (5.0 and 10.0 mmol L^{-1}) produced the most developed callus, with numerous roots. The medium supplemented with 10 mmol L^{-1} polyamine mixture, combined with BAP, induced good regeneration, producing vigorous plants and excellent shoot formation.Polyamines addition promoted the formation of callus, roots and leaves, representing an important factor in the determination of indirect organogenesis in *C. longa* L., and putrescine content may be considered a valuable marker of the differentiation process in this specie, as well as the enzyme peroxidase.

1. Introduction

Turmeric (*Curcuma longa* L.) is a plant native of India, belonging to the ginger family Zingiberaceae and it grows almost spontaneously in Brazil. It is widely used in cosmetics, gravy flavourings, aromas, seasonings, food coloring and cloth dyeing industry. In Brazil, researchers' attention on turmeric is increasing because of the possibility to use it as a substitute of synthetic dyes and for its antimicrobial and antioxidant activity (Maia et al., 2004).

Plant morphogenesis, *in vitro*, is related with that of genus *Curcuma* (Prathanturarug et al., 2005; Cousins et al., 2007). The propagation of *C. longa* occurs by vegetative growth from rhizome segments containing one or two buds (Prathanturarug et al., 2005) or by plant multiplication. These practices lead to the reduction of genetic variability, limit natural selection and the genetic improvement of the specie.

In vitro culture techniques for indirect embriogenesis and especially organogenesis can be an useful instrument in the reducing of genetic variability. Furthermore, it can promote the propagation of diseases caused by the use of rhizomes as propagation unit (Grattapaglia and Machado, 1998).

Polyamines (PAs) belong to a class of aliphatic amines, which is found in all organisms and it is, probably, involved in a great number of biological processes, including vegetative growth, regulation of DNA duplication, transcription of genes, cellular division, development of organs, ripening of fruits, leaves senescence and response to environmental changes (Lima et al., 2003). Some literature works proposed the use of exogenous polyamines, during the process leading to plants and calluses formation *in vitro* (Takeda et al., 2002; Mogor et al., 2007; Debiasi et al., 2007) and their endogenous concentration has been related with several organo-genetic processes (Francisco et al., 2008).

Peroxidase (POD, EC 1.11.1.7) is an enzyme related to organogenesis. Tian et al. (2003) showed alterations of peroxidase activity during the differentiation and formation of buds in the cultivation of strawberry *in vitro*. Tang and Newton (2005) associated the activity of peroxidase with shoot formation in *Pinus strobus* and they observed that the development steps can be associated to the alterations in the enzyme activity. Furthermore, literature showed that peroxide radicals and hydrogen peroxide

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should be formed during organogenesis, with the concomitant increase of the activity of antioxidant enzymes, such as peroxidase (Tian et al., 2003). According to these authors, during the differentiation process of strawberry callus, a decrease of peroxidase activity occurred during initial phases of organogenesis, followed by its increase in the final phase.

The aim of the present work is to evaluate the potential use of turmeric buds (obtained from rhizomes) for the production of calluses and the subsequent regeneration of plants, under the action of exogenous polyamines (putrescine, spermidine and spermine), and the possible relationship among endogenous polyamine content, the peroxidase activity and the organogenesis of these plants.

2. Materials and methods

Vegetal material used during the present work was collected from plants of *C. longa* L. (turmeric) coming from the field. Plant rhizomes were collected at approximately 180 days, in good physiological and healthy status. Subsequently, the material was subjected to a disinfection process, constituted of washings in abundant water containing a surfactant, under stirring, followed by immersion in 20% sodium hypochlorite solution (obtained with commercial deionized sterile water containing 2.5% active chlorine) for 25 min.

Rhizomes were rinsed three times in deionized sterile water in an aseptic room, then buds were isolated. These buds were subjected to a second disinfection by immersion in 70% (v/v) ethanol for 1 min, then in a solution of 2% (v/v) Tween-20 for 5 min, in a 1% (v/v) sodium hypochlorite solution for 10 min and finally, rinsed three times in deionized sterile water.

After the disinfection, the epidermis was retreated and the buds were transferred into test tubes containing previously determined nutritional medium. For induction of the calluses in buds isolated from rhizomes, the basic medium was the MS (Murashige and Skoog, 1962), supplemented with 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} thiamine, 0.5 mg L^{-1} piridoxine, 2 mg L^{-1} glycine, 30 g L^{-1} sucrose and, as growth regulators, 10.7 μM naphtalen-acetic acid (NAA), and 10 mM polyamines (putrescine, spermidine and spermine). Medium pH was adjusted to 5.8 and 8 g L^{-1} agar was added for gelification (MS + 10.7 μM NAA + 10.0 mM PAs).

During callus induction phase, treatment n. 1 (T_1, MS, control), no addition of polyamines occurred. Other treatments [T_2 (MS + 10.7 μM NAA + 5.0 mM PAs) and T_3 (MS + 10.7 μM NAA + 10.0 mM PAs)] received different doses of polyamines (putrescine, spermidine and spermine). Polyamine mixture (putrescine, spermidine and spermine, in the proportion 1:1:1) was added to the medium after autoclaving, before cold sterilization, at two concentrations: 5.0 and 10.0 mM, using a disposable sterile syringe filter (diam. 26 mm) by Corning (NY, USA), with a porosity of 0.2 μm, adapted on a disposable sterile syringe.

After polyamine addition, the medium was distributed in sterile culture flasks (20 mL/flask).

In order to induce callus formation and to reduce tissue oxidation, explants were put in a dark room at 25 ± 2 °C, for 60 days. During the process of organogenesis, calluses were disposed in growing chamber maintained at 25 ± 2 °C, and under a light intensity of 40 μL μm m^{-2} s^{-1} following a photoperiod of 16/8 h (light/dark). After callus development from rhizomes (60 days), materials were transferred in the MS culture medium (similar to that used to obtain callus), containing 8.8 μM 6-benzylaminopurine (BAP) and polyamine mixture at concentration 5 and 10 mM, and were left for other 90 days, under illumination (16 h light, at 40 μL μm m^{-2} s^{-1}, and 8 h dark period), changing the medium every 30 days in order to promote plant regeneration.

During this phase, treatments were: $T_{1.1}$ (MS + 8.8 μM BAP), $T_{2.2}$ (MS + 8.8 μM BAP + 5.0 mM polyamine mixture) and $T_{3.3}$ (MS + 8.8 μM BAP + 10.0 mM polyamine mixture). Samples ($n = 10$) were collected for each treatment for polyamine dosage and peroxidase activity determination.

The determination of polyamine content (putrescine, spermidine and spermine) was carried out according to Lima et al. (2006) after 150 days culture that is 60 days in the medium for callus induction and 90 days for the organogenesis induction. Analysis of polyamine content was carried out in regenerated plant (roots and leaves) and callus.

Peroxidase activity, expressed as μmol H_2O_2 reduced g^{-1} wet weight min^{-1}, was determined after 150 days culture according to Lima et al. (1999).

Experimental data were subjected to variance analysis (F Test) and the averages were compared by the Tukey test ($p < 0.05$). Variance analysis was accomplished using the least squares method, by ANOVA procedure (SAS, 1996).

3. Results and discussion

Rhizome buds gave rise to the growth of calluses, confirming results reported in the literature, being these explants the most used in micropropagation because of the presence of tissues with the highest meristematic activity (Prathanturarug et al., 2003). After 30 days, when the formation of calluses occurred within all the treatments, it was observed that the material inoculated in the MSO medium, containing NAA, without polyamines (T_1), presented the lowest development of callus and no formation of roots. Generally, auxin can induce the formation of callus and inhibit the formation of roots, as reported by Radmann et al. (2002), who described plant response to different auxin molecules and concentrations. This difference can be attributed to the time of observation, because this treatment induced the formation of roots in all the samples at 60 days (Fig. 1). As for other treatments used, as higher was the concentration of exogenous polyamines, as higher the observed development of calluses, suggesting that exogenous polyamines act as growth regulators in *C. longa*, inducing the process of cellular division. The idea that polyamines act as vegetable regulators, promoting the cellular division, is in agreement with the results described by several authors (Kaur-Sawhney and Applewhite, 1993; Teixeira da Silva, 2002; Mógor et al., 2007).

Callus subjected to PA treatment for 30 days presented larger volumes and the formation of roots. In none of the treatments, the

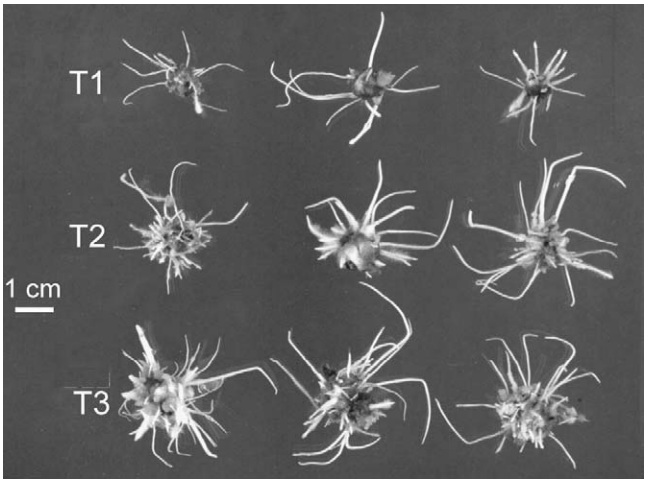

Fig. 1. General aspect of calluses of *Curcuma longa* formed after 60 days with different treatments (T_1 MS + 10.7 μM NAA; T_2 MS + 10.7 μM NAA + 5.0 mM PAs; T_3 MS + 10.7 μM NAA + 10.0 mM PAs).