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Induction and Phytochemical Analyses in Stryphno*dendron adstringens* (Mart.) Coville Calli

ABSTRACT

Stryphnodendron adstringens is a medicinal plant considered as endangered species due to the destructive methods used for tannins extraction. The objective of this study was to induce calli development from nodal segments of *Stryphnodendron adstringens* (Mart.) Coville and to evaluate the total phenol and tannin contents of the induced calli. Results indicated that calli induced in the presence of 0.5 and 2.0 mg L^{-1} picloran or picloran associated with 0.1 mg L^{-1} kinetin, showed higher fresh matter values (0.120 g and 0.116 g, respectively). The highest total phenol contents were obtained in calli grown in the absence of growth regulators (9.58%) and in the presence of 0.1 mg L^{-1} kinetin (9.23 %). Highest yields of total tannins was observed in calli induced in the absence of growth regulators (2.36%) and in the presence of 2.0 mg L^{-1} picloran (1.71%).

Key words: Kinetin, Fabaceae, Picloran

Indução e Análises Fitoquímicas em Calos de Barbatimão (*Stryphnodendron adstringens* (Mart.) Coville)

RESUMO

Stryphnodendron adstringens é uma planta medicinal considerada uma espécie ameaçada de extinção devido aos métodos destrutivos usados para a extração dos taninos. O objetivo deste estudo foi desenvolver calos a partir de segmentos nodais de *Stryphnodendron adstringens* (Mart.) Coville e avaliar os teores de fenol total e de taninos dos calos induzidos. Os resultados indicaram que calos obtidos na presença de 0,5 e 2,0 mg L^{-1} de picloram ou picloram associado com 0,1 mg L^{-1} de cinetina apresentaram os maiores valores de matéria fresca (0,120g e 0,116 g, respectivamente). Os maiores teores de fenol total foram verificados em calos cultivados na ausência de regulador de crescimento (9,58%) e na presença de 0,1mg L^{-1} de cinetina (9,23%). Maior produção de taninos totais foi observada em calos induzidos na ausência de reguladores de crescimento (2,36%) e na presença de 2,0mg L^{-1} de picloram (1,71%).

Palavras-chave: Cinetina, Fabaceae, Picloram

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INTRODUCTION

The biotechnology could be described as a method for enhancing the formation and accumulation of desirable natural products, with possible product modification in medicinal plants. Micropropagation, cell and hairy root culture, as well as gene technology, are all important techniques for plant propagation, but mostly used to improve the production and yield of desirable natural products (Julsing et al., 2007). Recently, cell cultures and cell suspensions have been frequently employed to produce phytotherapics with high amounts of active principles (Castro el at., 2009).

Barbatimão (*Stryphnodendron adstringens*) is a native species from the Cerrado, with tannins in the stem bark. Tannins are secondary compounds of phenolic nature, in general, polyphenols of high molecular weight and variable chemical structure. They have an anti-septic, antimicrobial, anti-hemorrhagic, antidiarrheal, healing, and anti-inflammatory effect. Among the species used for tannin extraction, *Stryphnodendron adstringens*, with its tannin-rich bark containing up to 30 % total tannins, is being considered as endangered specie, due to the destructive methods used for tannins extraction. This uncontrolled harvesting operation is depleting the natural populations of the species, calling for new production methods of these compounds, that would make the destruction of these plants unnecessary (Pinto & Bertolucci, 2002; Castro et al., 2009).

The conditions *in vitro* for the enhanced production of secondary products, which include alkaloids, terpenoids, steroids and phenolics, can be regulated in a number of ways. For example, manipulation of secondary product formation is possible by varying the nutrient composition of the growth medium, light, temperature and pH, and by the use of elicitors, permeabilisation and two-stage systems (Collin, 2001).

In view of the sparse information on protocols of *in vitro* production of secondary metabolites in *Stryphnodendron adstringens*, the objective of this study was to evaluate the effect of different picloran and kinetin concentrations on callogenesis in nodal segments and the production of the total phenol and tannins.

MATERIAL AND METHODS

Effect of picloran and kinetin on callus induction in nodal segments

Mature fruits of *Stryphnodendron adstringens* were collected in the region of the "cerrado", in Ijaci municipality, south of Minas Gerais, Brazil. The plantlets were obtained by *in vitro* germination and used as explant source. Nodal segments of 1.0 cm length were inoculated in test tubes containing MS culture medium (Murashige & Skoog, 1962), supplemented with different concentrations of picloran (0.0; 0.5; 1.0 and 2.0 mg L^{-1}) and kinetin (0.0 and 0.1 mg L^{-1}) and 3% sucrose. The medium was solidified with 0.6 % agar and the pH adjusted to 5.8 before autoclaving. Incubation was performed in the dark, at 25 ± 2^0 C.

The callus fresh matter in the different treatments was evaluated 60 days after inoculation.

Evaluation of total phenol and tannin contents of in vitro induced calli

Extract preparation

Samples with variable weights of lyophilized calli and the initial explant (nodal segments derived from plantlets germinated *in vitro*) were ground in 10 mL of a methanol:water (1:1) mixture. Then, the material was macerated for four hours at room temperature, and shaken for 30 minutes in a magnetic stirrer. The extract was then filtered into a 10 mL volumetric flask and the volume completed with the methanol:water (1:1) mixture.

a) Total phenols

An aliquot of 300 mL of the extract was used to determine the total phenol contents by the Folin-Dennis method, in compliance with the norms of the Association of Official Analytical Chemists (AOAC) (1970). Determinations were performed in triplicate and the results expressed in percentage of tannic acid, per gram of lyophilized callus.

b) Total tannins

The total tannin contents were determined by the Radial Diffusion Method, according to Hagerman (1987). A volume of 2 mL of the extract, previously prepared for the determination of the total phenols, was concentrated at 50°C and later dissolved with 0.2 mL of a methanol:water (1:1) mixture. An aliquot of 15 mL was used as sample. All determinations were performed in duplicate and the results expressed in percentage of tannin per gram of lyophilized callus.

Experimental design and statistical analyses

The experimental design was completely randomized, with 15 replications per treatment. Each replication consisted of one test tube, with one explant. The data were analyzed using software SISVAR® and the means compared by the Scott-Knott test, at 5% probability.

RESULTS AND DISCUSSION

Effect of picloran and kinetin on callus induction in nodal segments

Callogenesis was observed during the second week of cultivation in all treatments. The values of callus fresh matter are shown in Table 1

Picloran-supplemented media (0.5 and 2.0 mg L^{-1}) and media with picloran (0.5 and 1.0 mg L^{-1}) associated with 0.1 mg L^{-1} kinetin induced callus formation with higher fresh matter values, compared to the other treatments. Fresh matter values of calli induced in media with high picloran concentration (2.0 mg L^{-1}) associated with kinetin (0.1 mg L^{-1}) or with intermediate picloran concentration (1.0 mg L^{-1}) as well as those

Table 1. Calli fresh matter of *Stryphnodendron adstringens* of nodal segments in the presence of picloran and kinetin

Tabela 1. *Matéria fresco dos calos de barbatimão (Stryphnodendron adstringens) dos segmentos nodais, na presença de picloran e kinetin*

Treatment	Calli fresh matter (g)
Picloran (0.5 mg L^{-1})	0.120 a
Picloran (2.0 mg L^{-1})	0.116 a
Picloran (0.5 mg L^{-1}) + Kinetin (0.1 mg L^{-1})	0.095 a
Picloran (1.0 mg L^{-1}) + Kinetin (0.1 mg L^{-1})	0.088 a
Picloran (2.0 mg L^{-1}) + Kinetin (0.1 mg L^{-1})	0.077 b
Picloran (1.0 mg L^{-1})	0.076 b
Kinetin (0.1 mg L^{-1})	0.055 b
Control	0.033 b

Means followed by the same letter did not differ from each other at 5% significance by the Scott-Knott test

supplemented with only 0.1 mg L^{-1} kinetin were similar to the ones obtained without growth regulators ($p<0.05$).

Although treatments 0.5 and 2.0 mg L^{-1} picloran, 0.5 mg L^{-1} picloran + 0.1 mg L^{-1} kinetin and 1.0 mg L^{-1} picloran + 0.1 mg L^{-1} kinetin induced calli with statistically similar fresh matter values ($p<0.05$), the most economically and most viable medium for callus production with higher fresh matter values was the MS supplemented with picloran at 0.5 mg L^{-1}. Studies conducted by Hagen et al. (1990) also evidenced the efficiency of 2.4 mg L^{-1} picloran for callus induction in potato.

The auxins-cytokinins interaction was co-responsible for the callus induction and increased callus fresh matter values. In *Rudgea jasminoides*, Stella & Braga (2002) verified that MS media, supplemented with 0.48 mg L^{-1} kinetin and with the same picloran concentration, increased callus occurrence.

Kaur & Kothari (2004) tested 2,4-D and picloran, alone or in combination with kinetin, for callus induction and regeneration in *Paspalum scrobiculatum* L. and obtained similar results demonstrating the superiority of picloran over 2,4-D. On the other hand, Flores et al. (1998) testing different 2,4-D and picloran concentrations for callogenesis in two strawberry cultivars (Konvoy-Barkta and Chandler) observed callus formation only in the presence of auxins. Both auxins had the same effect on callus growth with high callus induction being observed in the presence of 2.3 and 2.5 mg L^{-1} of 2,4-D and picloran, respectively, decreasing at higher concentrations.

Evaluation of total phenol and tannin contents of in vitro induced calli

The calli obtained in the different treatments showed varied contents of phenols and tannins. All calli contained total phenols, independent of the type and concentration of the callus-inducing growth regulators. After inoculation, high total phenol contents decreased considerably (Figure 1). It was further observed that calli induced without growth regulators and with 0.1 mg L^{-1} kinetin presented the highest total phenol contents (9.58% and 9.23%, respectively). However, low contents (3.01%) were observed in calli induced with 0.5 mg L^{-1} picloran, which were 68 % lower, when compared to those observed in the control and in the presence of 0.1 mg L^{-1} kinetin.

The reduction in the total phenol concentrations in calli induced by the association of picloran with kinetin does not discard the possibility of the concomitant use of these two regulators, but rather suggests the need of further studies using other concentrations and combinations of them.

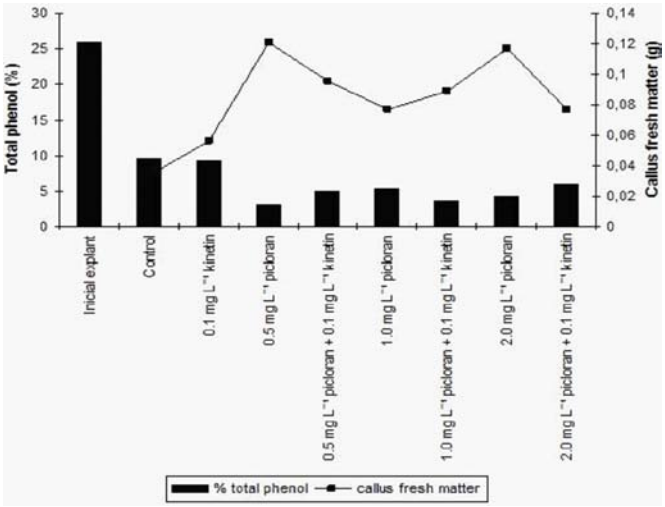

Figure 1. Total phenol average contents obtained in *Stryphnodendron adstringens* calli and callus fresh matter, cultured in the presence of picloran and kinetin

Figura 1. *Conteúdos totais médios de fenol obtidos dos calos e matéria fresca dos calos de barbatimão cultivados na presença de picloran e kinetin*

Only the initial explant, the control treatment and calli induced in the presence of 2.0 mg L^{-1} picloran produced considerable levels of total tannins (4.94%, 2.36% and 1.71%, respectively) (Figure 2). Total tannins were not detected in the other treatments. Calli induced in the absence of growth regulators presented, in average, 27.5% more tannins than those cultivated in the presence of 2.0 mg L^{-1} picloran.

According to Castro et al. (2009), the bark of an adult plant of *Stryphnodendron adstringens* contains 20 to 30% total tannins, which are far higher values than the ones found in nod segments.

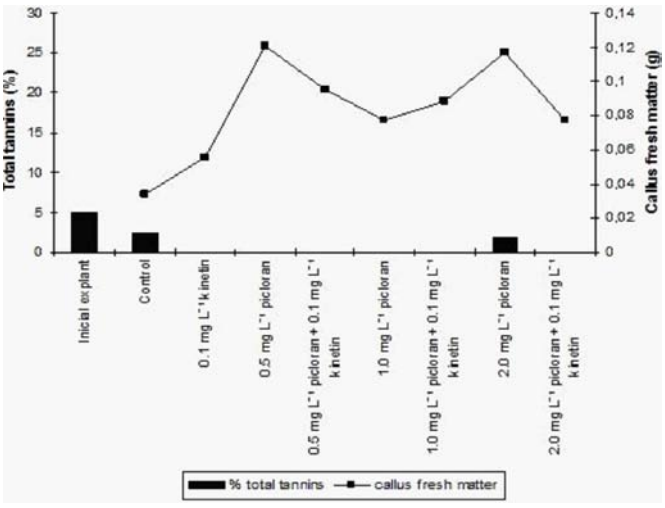

Figure 2. Total tannins average contents obtained in *Stryphnodendron adstringens* calli and callus fresh matter, cultivated in the presence of picloran and kinetin

Figura 2. *Valores médios de taninos totais obtidos de calos e matéria fresca dos calos cultivados na presença de picloran e kinetin*

The high total phenol and tannin contents detected in the initial explants are most likely due to the higher degree of differentiation of these cells, leading to a more regular and constant primary metabolic activity that favors the pathways of secondary biosynthesis.

A simultaneous evaluation of the callus fresh matter production and total phenol and tannin contents in the different treatments (Figures 1 and 2) demonstrated that the calli with the highest fresh matter contents produced the lowest phenol contents and no total tannins, with exception of the 2.0 mg L^{-1} picloran treatment. These results suggested a greater investment of the cells in the calli biomass production and the lower availability of precursors for the production of total phenols and tannins, or may ever reflect the lower degree of differentiation of tissue with high mitotic activity.

Similar observations were reported by Bahorum et al. (1994), whom evaluated the polyphenol production of *Crataegus monogyna* calli and verified an intense synthesis of total phenols in the initial period of calli production (from the fourth to the sixth day), associated to low biomass production.

In *Heliconia chartacea* Lane ex Barreiros cv. Sexy Pink and Sexy Scarlet, the calli formation frequency corresponded to 100% of the inoculated explants in presence of IAA associated with the 2.4-D (Ulisses et al., 2007), but were calli embryogenics. Castro et al. (2009) tested the effect of 2,4-D and BAP to induce callogenesis and total phenols and tannin production in *Stryphnodendron adstringens* and verified that calli grown in medium with 1.0 mg L^{-1} of 2,4-D presented an average content of 1% total phenols, and detected no total tannins at any of the tested 2,4-D concentrations. The highest total phenol contents (mean of 1.3%) were found in calli grown in media with 1.0 and 4.0 mg L^{-1} BAP, while no independent total tannin production was verified in the BAP treatments.

Hence, more studies are needed to adjust the protocol for *Stryphnodendron adstringens* in order to optimize the total phenol and tannin production *in vitro*.

CONCLUSIONS

Picloran, alone or in combination with 0.1 mg L^{-1} of kinetin induced callus formation in *Stryphnodendron adstringens*;

The presence of picloran and kinetin did not improve phenol and tannin production in *Stryphnodendron adstringens*;

Calli with higher fresh matter values presented lower total phenol contents and absence or low tannin concentrations.

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