Induction and determination of total phenols of callus of barbatimão

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ABSTRACT

Barbatimão [Stryphnodendron adstringens (Mart.) Coville], is a tree with predominant occurrence in the Cerrado regions in the Brazilian States. Its bark is rich in tannins which is used in folk medicine due to its astringent function. In recent years, the indiscriminate exploitation of barbatimão tree led to a decrease in bark production of this medicinal plant. The aim of this study was to induce calluses from cotedyledon segments and determine the weight of fresh and dry matter and the levels of phenols the induced calluses. For callus induction, cotedledon segments were inoculated in a medium with different combinations of growth regulators: 2.4-D × TDZ (0, 0.5, 1.0 and 2.0 mg L⁻¹) and kinetin × picloran (0; 0.5, 1.0 and 2.0 mg L⁻¹). Cultures were incubated in the dark and at a temperature of 27 ± 2 º C. At 60 days of subculture the fresh and dry weight and the levels of total phenols were evaluated. The best results obtained for the calluses fresh weight with the combination (2,4-D × TDZ) were: 0.5 mg L⁻¹ of 2,4-D associated with 0.5, 1.0 and 2.0 mg L⁻¹ concentrations of TDZ. For dry matter values the best combination was: 0.5 and 2.0 mg L⁻¹ TDZ in the absence of 2,4-D. For the (Kinetin × Picloram) combination, the best results for callus induction were: 2 mg L⁻¹ of kinetin and 0.5 mg L⁻¹ of Piclorom, with an average of 0.2 g of fresh weight and 0.02 g of dry weight. The results indicated that higher levels of total phenols were found in calluses induced on a medium supplemented with 1.0 mg L⁻¹ of 2,4-D; 2.0 mg L⁻¹ of kinetin and 1.0 mg L⁻¹ of Piclorom. A negative effect for TDZ regarding the total phenol levels when combined with 2,4-D was noted. The tests performed in this study, where different growth regulators were used for barbatimão callus induction in order to increase phenol concentrations, did not present satisfactory results. Although a low concentration of phenolic compounds was found, it is important to note that the culture medium may be optimized for large scale in vitro production of this compound.

INTRODUCTION

The technique of plant tissue culture enables the induction and proliferation of undifferentiated masses of cells, known as calluses or cellular suspensions (Paz et al., 2006). The in vitro cultures are used as an alternative to agricultural processes for the production of interesting medicinal compounds. In this context, calluses may be stored in long term in order to maintain the specific characteristics of the tissues that may be lost during the normal in vitro maintenance, such as production of secondary metabolites which have pharmaceutical importance (Estrada-Zúñiga et al., 2009; kim; wyslouzil; Weathers, 2002). The callus culture, however, is an essential tool, not only to establish an efficient protocol for valuable metabolites production but, also to evaluate parameters of cellular growth (Khiet et al., 2006).

Various wild medicinal plants from Brazil are threatened, mainly because they are used as extracts or infusions by the population. The barbatimão [Stryphnodendron adstringens (Mart.) Coville] is among those species because, from it, we can extract around 20% to 30% of tannins (Lima; Martins; Souza Júnior, 1998). A way to minimize this problem is using plant tissue cultures, which provide an alternative procedure for aseptic, controlled and automated extraction of tannins from cells, tissues and plant organs, resulting in large-scale
cultivation. Because callus cultures may guarantee homogeneity of production and fast growing, they describe one of the best systems for production of secondary metabolites (Estrada-Zúñiga et al., 2009). Therefore, through this study, we aimed to induce calluses with potential for production of phenolic compounds.

**MATERIALS AND METHODS**

**Plant specimen:**
Ripe fruits of *S. adstringen* were harvested, yet closed, from plants of wild population located in the Ijací city, southern region of Minas Gerais, Brazil. Subsequently, seeds were manually extracted and, those which showed physical integrity were selected and transferred for glass flask and stored in the refrigerator at 4°C for 30 days. Before inoculation, all seeds were scarified into 95% sulphuric acid for 60 minutes and, then, washed in water (Martins; Nakagawa, 2008). Working in a laminar flow hood, these seeds were sterilized in 70% alcohol for around one minute and, then, transferred to a disinfectant solution of 2% sodium hypochlorite, during five minutes. Then, seeds were washed through three rinses into sterile distilled water to remove excessive disinfectant, and placed on dishes containing MS medium (Murashige; Skoog, 1962), added 10 g.l-1 of sucrose and 7 g.l-1 of agar. After inoculation, seeds were maintained in a growing room, under constant 36 μmol m-2.s-1 photons irradiation, for 16 hours photoperiod at 25±2°C. After 30 days, dicotyledon leaves were used as explant sources.

**Induction of callogenesis:**
In the first experiment, small square tissue sections of cotyledon leaves, around 0.25 cm2, obtained from seedlings *in vitro* were inoculated in the test tube containing MS medium supplemented with 0, 0.5, 1.0 and 2.0 mg.l-1 of acid 2.4 -dichlorophenoxyacetic (2.4-D) and 0, 0.5, 1.0 and 2.0 mg.l-1 of Thidiazuron (TDZ), in all possible combinations, and 3% of sucrose. The medium was solidified adding 0.7% of agar, and the pH was adjusted to 5.8 before being placed in an autoclave at 121°C for 20 minutes. Cultures were incubated in the dark at 27±2°C, and the callus sub-cultivation was carried out after 60 days from the first inoculation.

The second experiment was performed as outlined in the first, but were used as supplement of the culture media, quantities of Picloram and Cinetin, instead. The assessment of the weight of fresh and dry materials, and content of total phenols in all two experiments were assessed after 120 days of cultivation of calluses of *barbatimão*.

**Determination of total phenol:**
Analyzes were carried out in triplicate. Determination of the content of total phenols from the samples of the ethanolic extract of the calluses was performed through spectroscopy in the visible region using the Folin-Ciocalteu method (Singleton et al., 1999). The ethanolic extract (100 mg) was dissolved in methanol and qualitatively transferred to a 100 ml volumetric flask, and the final volume was completed with methanol. An aliquot of 7.5 ml from this solution was transferred to a 50 ml volumetric flask and, then also adjusted adding methanol. An aliquot of 100μl from this last solution was shaken with 500μl of the reagent Folin-Ciocalteu and 6μl of distilled water for 1 minute, after which was added 2.0 ml of 15% Na2CO3 and re-shaken for 30 seconds. The final solution was adjusted to 10 ml volume adding distilled water. After 2 hours, the samples absorbance was measured at 750 nm using glass cuvettes, using methanol and all reagents except the extract, as the blank test. The content of total phenols (TP) was determined by interpolating the absorbance of the samples against a calibration curve designed with standards of Gallic Acid (10 to 350 μg.ml-1), expressed as mg of GAE (Gallic Acid Equivalent) per gram of the extract.

**The curve of calluses growing:**
The calluses formed on the culture medium supplemented with 1.0 mg.l-1 of 2.4-D, in the first experiment, and with 2.0 mg.l-1 of Cinetin and 1.0 mg.l-1 of Picloram, in the second experiment, showed high concentrations of total phenols. We used calluses from the concentration of 1.0 mg.l-1 of 2.4-D, which were more similar to friable appearance when compared with calluses of the other treatments (Figure 1). Seedlins obtained *in vitro* were used as explant sources. Small square tissue sections of cotyledon leaves, around 0.25 cm2, were inoculated in the test tubes containing MS medium, 3% of sucrose, 0.7% of agar, and 1.0 m.l-1 of 2.4-D. The explants were incubated in a growing room in the dark at 27±2°C. Fresh mass of the inoculated material was weighted once a week from the day of inoculation (time zero) to the 63rd day of cultivation, then for designing the calluses growing curve. The experiment was performed in completely randomized design with 12 replicates, each consisted in a test tube and each tube containing one explant. One-way analysis of variance (ANOVA) was conducted to determine significance of differences and means were compared by Tukey test at 5% significance level using SISVAR statistical software (Ferreira, 2011).
Fig. 1: Appearance of calluses induced in medium supplemented with: (A) 1.0 mg.l-1 of 2.4-D; and (B) 2.0 mg.l-1 of Cinetin and 1.0 mg.l-1 of Picloram.

**Results:**

**Induction of callogenesis:**

The callogenesis in the cotyledon explants, in the first experiment, was found only with presence of growth regulators. Culture media with no growth regulators, and supplemented with 0.5 mg.l-1 of 2.4-D, associated to concentration of TDZ (0.5, 1.0 and 2.0 mg.l-1) induced formation of calluses with high weight of fresh mass. This also happened to culture media containing 1.0 mg.l-1 of 2.4-D associated to 0.5 mg.l-1 of TDZ and 2.0 mg.l-1 of 2.4-D associated to 1.0 mg.l-1 of TDZ (Figure 2).

Among treatments, was found different characteristics in relation to dry mass weight. The highest values were obtained when the culture media were supplemented with 0.5 and 2.0 mg.l-1 of TDZ and no growth regulator (2.4-D). The calluses only induced on culture media containing 2.4-D resulted in the lowest dry weight. Associating this regulator with TDZ, also showed reduction of the calluses dry weight, especially when the concentration of auxin was increased (Figure 2).

![Graph A](image1.png)

*Fig. 2: Averages of fresh mass (A) and dry mass (B) of calluses of barbatimão, induced from cotyledon leaves using association among different concentrations of 2.4-D and TDZ.*

However, by using only Cinetin or Picloram in the second experiment (Figure 3), even in the inoculated leaf sections with no of these growth regulators, there was no calluses formation in the explants, regardless the
concentrations of these supplements. The highest dry weight was found in the treatment containing 2.0 mg.l⁻¹ of Citenin and 0.5, 1.0 and 2.0 mg.l⁻¹ of Picloram (0.02g), what made them statistically different from all other treatments. Therefore, we can found an increase in the callogenesis with the increment of the Cinetin concentration. But, when the Picloram concentration was high, the calluses formation was low.

**Fig. 3:** Averages of fresh mass (A) and dry mass (B) of calluses of barbatimão, induced from cotyledon leaves using association among different concentrations of Cinetin and Picloram.

**Determination of total phenols:**

Analyzes of data from the first experiment showed that all calluses contained total phenols, regardless the concentration or association of the growth regulators. High content of total phenols were found in calluses induced on culture media supplemented with 1.0 mg.l⁻¹ of 2,4-D. We also found that calluses induced on culture media supplemented with 1.0 and 2.0 mg.l⁻¹ of 2,4-D contained low content of dry weight and high content of phenols in relation to other treatments (Figure 4).

**Fig. 4:** Averages of dry weight and total phenols of calluses of barbatimão, induced from cotyledon leaves using association among different concentrations of 2,4-D and TDZ.
By analyzing data from the second experiment (Figure 5) we realize that there was the presence of total phenols in all treatments that induced calluses. The highest content of phenols was found in the calluses induced on culture media containing 2.0 mg.l-1 of Cinetin and 1.0 mg.l-1 of Picloram. However, by comparing contents of total phenols with the calluses dry weight, we found that calluses induced on culture media containing 0.5 and 1.0 mg.l-1 of Cinetin associated to 0.5, 1.0 or 2.0 mg.l-1 of Picloram showed an increment of content of total phenols as the Picloram concentration increases.

Therefore, culture media with 2.0 mg.l-1 of Cinetin associated to 1.0 mg.l-1 of Picloram provided higher content of phenols than when associated to 0.5 or 2.0 mg.l-1 of Picloram. We also found that calluses with similar dry weight or less than those from other treatments showed high increment of total phenols.

**Fig. 5:** Averages of dry weight and total phenols of calluses of barbatimão, induced from cotyledon leaves using association among different concentrations of Cinetin and Picloram.

**The calluses growth curve:**

The calluses growth suggested a curve sigmoidal-type with distinct five phases, namely, lag, log, linear, deceleration and stationary (Figure 6).

**Fig. 6:** Growth curve of calluses formed from sections of cotyledon leaves of barbatimão inoculated on MS culture medium supplemented with 1.0mg.l\(^{-1}\) of 2,4-D during 63 days of cultivation.
**Discussion:**

Castro *et al.* (2009) found low fresh weight of calluses induced in the dark from leaf sections of *S. adstringens* using different concentration of 2,4-D. Nogueira *et al.* (2007), working with material obtained from murici-pequeno (*Byrsonima intermedia*), found that the association of 1.0 mg.l-1 of 2,4-D with 0.5, 1.0 and 2.0 mg.l-1 of TDZ caused reduction of calluses induction, as high with the increment of TDZ concentration. These quotations show that the calluses induction is affected by the hormonal balance between auxin and cytokine.

These data are according to results of Damião Filho (1995), from which most of woody plant species require high concentration of growth regulators on the culture media, greater than 1.0mg.l-1, for induction of calluses formation in their explants. Nicioli *et al.* (2010), found similar results in relation to fresh weight for calluses induced from nodal sections on culture media supplemented with association of 0.5, 1.0 or 2.0 mg.l-1 of Picloram with 0.1mg.l-1 of Cinetin.

However, we found here that cotyledon leaf sections of barbatimão require the association of Cinetin and Picloram in the supplementation of culture media for callus induction. This positive effect was also found in the experiments for callogenesis of Rudgea jasminoides, on which were necessary 0.48 mg.l-1 of both two plant growth regulators for calluses induction (Stella; Braga, 2002). Kaur e Kothari (2004) also tested the effect of Cinetin and Picloram in the induction of calluses of Paspalum scrobiculatum. They found that association among concentrations of these two growth regulators showed high fresh mass of the calluses.

Auxins are required for calluses formation because they are responsible for the beginning of cellular division and control of growth and cellular elongation processes (Taiz; Zeiger, 2009). The cytokinins are also required for plant cellular division, with positive results in the calluses induction (Pasqual, 2001), confirming that the callogenesis observed in this study probably resulted from ensemble action of these growth regulators.

Besides, calluses formed from cotyledon leaves using culture medium with different concentration of 2,4-D and TDZ, contained different contents of phenols. Castro *et al.* (2009), found the presence of total phenols during induction of callogenesis in leaf sections using different concentrations of 2,4-D. They also found that the increment of concentration of 2,4-D resulted in high dry weight and increase of total phenols. In fact, the association of 2,4-D and TDZ provided here, high calluses dry weight, but low content of phenols; what shows that TDZ affects negatively the content of total phenols, so that is not a great growth regulator for this plant specie.

The Picloram, in this study, was indispensable for the increase of content of phenols when associated to Cinetin on calluses induced from cotyledon leaves of the barbatimão. Nicioli *et al.* (2010), during callogenesis induction from nodal sections of the barbatimão, they used culture media with different association of Cinetin and Picloram or separated. Then, they found that 1.0 mg.l-1 of Cinetin promoted greater contents of total phenols than the association of these two growth regulators and only the Picloram. This result is according to what we found here in relation to association of dry weight of the calluses and concentration of total phenols, because they also found higher concentration of total phenols in calluses with low weight.

Therefore, experiments performed here using different growth regulators in the induction of callogenesis of barbatimão, aiming to increase the concentration of total phenols, did not show satisfactory results. This is because a study with barbatimão, performed by Macedo *et al.* (2007), described contractions of total phenols around 13.96%, 11.47% and 2.35%, respectively for samples obtained from leaves, bark and stem. Then, although we described low concentration of phenolic compounds, it is important to emphasize that culture media may be optimized for large-scale production of total phenols, since was a in vitro process while in conventional way they are extracted from the bark of plants *in vivo*.

The lag phase, on which cells prepare themselves for division, occurred from the first to the 21st day after inoculation, presenting growth around 81%. The log phase or exponential growth phase ranged from the 21st to the 28th day of cultivation, with 41% of growth. According to Serra, Paiva and Paiva (2000). Between the 42nd and the 49th day after inoculation occurred the growth deceleration, presenting only 14% of growth. The beginning of this phase is the ideal moment to calluses transplantation for a new culture medium, due to the reduction of nutrients, drying of agar and accumulation of toxic substances (Smith, 1992). From the 49th day to the 63rd day of incubation, was characterized the stabilization phase, where there are a greater number of cells with weak capacity for division.

Nogueira *et al.* (2007), by analyzing the growth curve of leaf explants calluses of murici-pequeno induced on MS culture medium supplemented with 1.0 mg.l-1 of 2,4-D, also found that the calluses growth followed a sigmoidal-type curve. But another researches suggested that the calluses transplantation for a new culture medium should be between the 60th and the 80th day after inoculation. However, the period of deceleration of calluses growth of barbatimão here was between the 42nd and the 49th day after inoculation, and this is the best moment for the calluses transplantation.
Therefore, the sigmoidal-type distribution curve of growth of ingazeiro calluses was also described in Stein et al., (2010) when leaf sections were inoculated on culture media containing supplement of 2.4-D and, the stationary phase began from the 70th day after inoculation.

Conclusions:
According to results, we concluded that association of different concentrations of auxin and cytokine promotes the formation of calluses in cotyledon leaves of barbatimão. The high concentration of total phenols is found in calluses formed only with supplementation of culture medium with 1.0 mg.l-1 of 2.4-D or the association of 2.0 mg.l-1 of Cinetin and 1.0 mg.l-1 of Picloram. Therefore, the transference of calluses induced adding 1.0 mg.l-1 of 2.4-D on the culture medium should be performed at 49th day of cultivation.

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