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In vitro germination, callus induction and phenolic compounds contents from *Pyrostegia venusta* (Ker Gawl.) Miers

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ABSTRACT. A protocol for the *in vitro* germination and callus induction in *Pyrostegia venusta*, a medicinal plant species from the Brazilian savannah (*Cerrado*) is provided. The establishment of cultures of *P. venusta* was originally obtained from seeds germinated *in vitro* and induced callus directly from leaf explants of seedlings. Seeds were germinated on MS and WPM media containing 100 and 50% of salt concentration, supplemented with 30 g L^{-1} sucrose. Callus induction consisted of the inoculation of leaf segments on MS medium plus 2,4-D or BAP in the presence or absence of light. The germination percentage averaged 85% and the aerial parts and roots of seedlings obtained in WPM with 50 and 100% of salt concentration showed elevated contents of total phenolic compounds and flavonoids compared to those obtained on MS medium. Calli induced with lower concentrations of 2,4-D had higher fresh and dry weight values. All treatments resulted in obtaining calli with contents of total phenolic compounds and flavonoids at or above the initial explant, highlighting treatments containing 9.05 μM 2,4-D and 8.88 μM BAP in the presence and absence of light, and 17.75 μM BAP in the absence of light.

Keywords: seeds, auxin, cytokinin, medicinal plant, flavonoids.

Germinação *in vitro*, indução de calos e teores de compostos fenólicos em *Pyrostegia venusta* (Ker Gawl.) Miers

RESUMO. É apresentado um protocolo para a germinação *in vitro* e indução de calos em *Pyrostegia venusta*, uma espécie medicinal do Cerrado. O estabelecimento de culturas foi obtido a partir de sementes germinadas *in vitro* e a indução de calos diretamente de explantes foliares das plântulas obtidas. Sementes foram germinadas em meios MS e WPM contendo 100 e 50% da concentração de sais, suplementado com 30 g L^{-1} de sacarose. A calogênese consistiu na inoculação de segmentos foliares em meio MS acrescido de 2,4-D ou BAP, na presença ou ausência de luz. A percentagem de germinação foi 85%, em média. Partes aéreas e raízes de plântulas obtidas em meio WPM com 50 e 100% da concentração de sais, apresentaram elevados teores de compostos fenólicos totais e flavonoides, em relação àquelas obtidas em meio MS. Calos induzidos com menores concentrações de 2,4-D apresentaram maiores valores de matéria fresca e seca. Todos os tratamentos proporcionaram a obtenção de calos com teores de compostos fenólicos totais e flavonoides iguais ou superiores ao explante inicial, com destaque para os tratamentos com 9,05 μM de 2,4-D e 8,88 μM de BAP, na presença e ausência de luz e 17,75 μM de BAP, na ausência de luz.

Palavras-chave: sementes, auxina, citocinina, planta medicinal, flavonoides.

Introduction

The 'cipó-de-são-joão', *Pyrostegia venusta* (Ker Gawl.) Miers (Bignoniaceae), is a woody vine commonly found in savanna and forest formations of the Brazilian Cerrado, especially in the 'cerradão' and forests edges, fields, along the coast and roadsides (MAGALHÃES et al., 2010; ROSSATTO; KOLB, 2010). It is important as an ornamental species due to its abundant production of orange-colored flowers. From the medical point of view, the aerial parts are used in infusions and decoctions as a tonic and antidiarrheal (SCALON et al., 2008; VELOSO et al., 2010), while the roots are important in the treatment of uterine infections and genital tract, jaundice and erysipelas (CARDOZO et al., 2009; VELOSO et al., 2010). These therapeutic properties are associated with substances of a phenolic nature, mainly flavonoids, found in the leaves and stems, and allantoin in the roots (MAGALHÃES et al., 2010; VELOSO et al., 2010). According to Rossatto and Kolb (2011), *P. venusta* regenerates in nature through seasonally produced seeds that occur between July and November. The germination rate is higher than 80% and occurs within a wide temperature range and absence of photoblastism. This fact allows the species to germinate in open and shaded areas,

favoring its distribution by different 'cerrado' physiognomies. However, the establishment of seedlings is hampered by unfavorable environmental conditions and by pathogen and herbivore attacks (ALMEIDA et al., 1998).

In vitro cultures may be used as an important tool for the characterization, use and preservation of the species, whereas *in vitro* seed germination is an alternative to obtain aseptic explants for further studies. Thus, different culture media, such as MS medium developed by Murashige and Skoog (1962) and WPM medium, manufactured by Lloyd and Mc Cown (1980), may be used for this purpose. MS medium provides a successful growth of cells and tissues due to the high concentration of ammonium, nitrate and potassium. However, this medium has a low quantity of phosphate, which, according to many researchers, is insufficient to sustain growth of various species. On the other hand, WPM medium, developed for shoots culture of woody plants, has a 25% concentration of ammonium, nitrate, sulfate and potassium ions and twice of phostate ions concentration, when compared to MS medium. In fact, higher potassium concentration and a high level of sulfate ions are widely used in the micropropagation of woody plants like shrubs and trees (GEORGE et al., 2008).

Among the *in vitro* culture techniques, callus culture is distinguished as an economically viable alternative that may be used for the preservation of the species and for obtaining bioactive plant metabolites. Calli are tissues that provide partial differentiation, consisting of a mass of irregular cells that multiply widely in response to chemical or physical injuries and have the ability to differentiate into tissues and organs (SMITH, 2012). In general, plant growth regulators are important factors that affect cell growth, differentiation and formation of metabolites in plant cells and tissues. Auxins and cytokinins are the most commonly used classes of plant growth regulators *in vitro* cultures. The use of auxin in the culture medium is related to the formation of adventitious roots and calogenic tissues, while cytokinins cause cell division and growth of shoots. The interaction of auxins and cytokinins is also widely used for the induction and maintenance of calli (MUSTAFA et al., 2011).

The seasonal spread of *P. venusta* and its medicinal potential make the species a good object for biotechnological studies, which are uncommon for Bignoniaceae species and practically do not exist for *P. venusta*. The exception is the study by Loredo-Carillo et al. (2013) who described the effect of osmotic and hydric stress on the content of flavonoids and sterols in *P. venusta* calli established from leaf explants of three-month-old plants grown in a greenhouse. Current study establishes a protocol for obtaining seedlings of *P. venusta* from seeds germinated *in vitro*, induce callus from leaf segments and assess the total phenolic compounds and flavonoids contents in the seedlings and calli produced.

Material and methods

Plant material

Pyrostegia venusta (Ker. Gawl.) Miers (Bignoniaceae) seeds were collected in the Cerrado region located in Divinópolis in the center-western region of the state of Minas Gerais, Brazil (21°11'36.97"S and 44°55'59.07"W) between September and October 2011. Fertile samples were collected and the vouchers were identified by Andreia Fonseca Silva of Herbarium PAMG (PAMG 56307) at the Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG). The seeds were treated for 5 minutes with Captan (1 g kg^{-1} of seeds), following Cengiz et al. (2007), and stored in cold chamber until use (ROSSATTO; KOLB, 2010).

In vitro germination

Seeds were disinfected with NaOCl (25% commercial sodium hypochlorite) for 5 minutes, washed three times in distilled water and autoclaved. The seeds were soaked for 2 hours according to preliminary tests and inoculated into tubes containing 10 mL of media MS (MURASHIGE; SKOOG, 1962) and WPM (LLOYD; Mc COWN, 1980), with 100% and 50% of salt concentration, supplemented with 30 g L^{-1} sucrose, solidified with 7 g L^{-1} agar and pH adjusted to 5.7 ± 0.1 before autoclaving. Seeds were transferred to growth room at 27 ± 1°C, 16 hours of photoperiod and photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}.

The percentages of seeds germinated, oxidation and contamination of the media and of the seeds were observed at 15-day intervals. The physiological criteria, considering all germinated seeds with radicle protrusion, were employed to analyze germination. After 45 days, fresh and dry total and fractional weights of seedlings formed and their total phenols and flavonoids contents were evaluated. Dry weight was determined from the aerial parts and roots dried from the seedlings, in a buffer with forced ventilation at 40°C until constant weight. A completely randomized design was used, with five replicates per treatment, with each replicate consisting of 10 tubes and each tube containing one seed.

Callus induction

The leaf segments from 45-day-old seedlings cultured *in vitro* were placed in a complete basal medium MS with 30 g L^{-1} sucrose plus 2,4-D (0; 4.52; 9.05; 18.10 μM) and BAP (0; 4.44; 8.88; 17.75 μM) to induce callus formation, and solidified with 7 g L^{-1} agar. Further, pH was adjusted to 5.8 ± 0.1 with NaOH 0.1 N after adding plant growth regulators. Medium was sterilized at 120°C (1.37 × 105 Pa) for 20 minutes. The explants were transferred to a growth chamber and kept at 27 ± 1°C in the presence of light (16:8h light / dark regime, with a light intensity of 40 μmol m^{-2} s^{-1}) and absence of light (CASTRO et al., 2009). After 60 days of inoculation, callus induction (%), color, consistency, fresh and dry weights of calli, total phenolic compounds and flavonoids contents were evaluated. The completely randomized experimental design comprised different concentrations of 2,4-D and BAP in the presence and absence of light, with 20 replicates composed of one test tube. Each tube contained an explant, totaling 320 plots.

Preparation of hydromethanolic extracts

Approximately 200 mg of dried callus samples were extracted with 10 mL methanol:water (1:1) solution, under cold maceration, with constant stirring for 4 hours in a shaker device, following Castro et al. (2009). The extract was filtered and the final volume was completed to 10 mL with methanol:water (1:1).

Determination of total phenolic compounds contents

Phenolic compounds were quantified with 100 μL of hydromethanolic extract, following AOAC (1995) procedure. Total phenolic compounds content was calculated by a calibration curve with 100 mg L^{-1} tannic acid solution as standard. Determinations were performed in triplicate and the result was expressed in micrograms of tannic acid equivalents per milligram of dry matter (μg TAEq mg^{-1} DW).

Determination of total flavonoid contents

Total flavonoid assay was performed according to Woisky and Salatino (1998) using 300 μL of hydromethanolic extract. The flavonoid content was calculated by a calibration curve with 100 μg mL^{-1} rutin in a methanol solution of 2% aluminum chloride as standard. Determinations were performed in triplicate and the result was expressed in microgram of rutin equivalents per milligram of dry matter (μg REq mg^{-1} DW).

Results and discussion

In vitro germination

There was no evidence of blackening on the seeds or in the culture medium, which is indicative of oxidative processes, common in the propagation of woody species. The percentage of contamination was less than 2% and showed the efficiency of the disinfection process adopted. According to Donini et al. (2005), rates up to 10% contamination are acceptable in terms of plant tissue culture.

Germination began on the 9th day after inoculation in all media tested and the development of the first leaf pair was observed on the 23rd day of culture (data not shown). Similar pattern was observed by Rossatto and Kolb (2010) in the evaluation of the *in vivo* germination and post-seminal development of *P. venusta*, reporting the onset of germination from the 7th and 8th days and the emergence of the first pair of leaves from the 22nd day from the start of the experiment. In addition, the same authors reported a germination percentage that varied between 88% and 95% at temperatures between 25 and 35°C. In the present study, the germination percentage averaged 85% and the rate of seedling survival reached 100%, without significant variations between culture media and concentrations of salts tested ($p > 0.05$). These results indicated that the difference in the availability of water to the seeds, due to different salt concentrations in the culture media, did not interfere with the process of soaking the seeds, essential for triggering fundamental metabolic processes to the beginning of germination. Other studies show that this behavior is variable for different species of the 'cerrado'. Nery et al. (2008) studied the *in vitro* establishment of embryos of 'ipê amarelo' (*Tabebuia serratifolia* - Bignoniaceae) and Soares et al. (2009) evaluated the germination of 'mangabeira' (*Hancornia speciosa* - Apocynaceae) under similar experimental conditions, and registered a similar behavior for both species, regardless of the culture media employed.

Table 1 shows that the seedlings had higher biomass of aerial parts when compared to the roots, regardless of the treatment employed; and the total and partitioned fresh and dry weight did not vary among the different culture media and concentrations of salts tested ($p > 0.05$). This fact indicated that *P. venusta* seedlings are not demanding from the nutritional point of view to early development. Furthermore, it was observed that low concentration of nitrate in the WPM medium as the sole N source was sufficient to promote good seedling growth when compared to the nitrate, nitrite and ammonium in the MS medium available in larger concentrations.

Table 1. *In vitro* germination of *Pyrostegia venusta* seeds, fresh and dry weight, total phenolic compounds and flavonoids amount in seedlings after 45 days of culture.

Culture Media	Germination (%)	Fresh Weight (mg)		Dry Weight (mg)		Phenolic Compounds (μg TAE mg^{-1} DW)		Flavonoids (μg RE mg^{-1} DW)	
		Aerial Part	Root	Aerial Part	Root	Aerial Part	Root	Aerial Part	Root
MS 50%	82 a	81 a	30 a	17 a	5 a	7.20 b	7.50 c	3.80 a	2.30 a
MS 100%	86 a	75 a	27 a	16 a	3 a	6.40 c	5.50 d	3.60 a	2.00 c
WPM 50%	86 a	85 a	43 a	16 a	6 a	8.50 a	8.90 b	3.50 a	2.30 a
WPM 100%	84 a	69 a	31 a	14 a	5 a	7.20 b	11.20 a	3.70 a	2.00 c

Means in each column followed by the same letter are not significantly different at $p \leq 0.05$ by the Scott-Knott test.

Studies by Ramage and Williams (2002) indicated that higher concentrations of phosphorus in the WPM medium may also be associated with seedling growth. This behavior, however, may vary from species to species, in the case mainly woody species of 'cerrado' as observed in 'barbatimão' (*Stryphnodendron adstringens* - Fabaceae) and 'murici-cascudo' (*Byrsonima verbascifolia* - Malpighiaceae), where the use of MS and WPM complete media provided higher accumulation of total dry weight (CASTRO et al., 2005; 2007).

According to Gago et al. (2011), the culture media must be optimized to meet the nutritional needs required for tissue *in vitro*. As a rule, it is made from variations in the concentration of mineral nutrients, organic substances and plant growth regulators. In some cases, changing the mineral composition may promote greater benefits (KANASHIRO et al., 2009). Ávila et al. (1998) reported that the initiation and growth of roots and shoots in *Syzygium alternifolium* (Myrtaceae) were higher when the concentration of N in the medium was reduced by half, a fact attributed to the greater efficiency in the use of carbon. According to Walch-Liu et al. (2005), low but continuous supply of N initially promotes the formation and growth of root, and induces the synthesis of cytokinins that are important for the expansion of the leaf area.

The phytochemical analysis showed that the highest total phenolic contents were observed in roots of seedlings grown in media WPM 100%, or rather, 11.20 μg TAE mg^{-1} DW. Rubio-Wilhelmi et al. (2012) reported that increased levels of phenolic contents in the presence of lower amount of nitrogen compounds may be attributed to the increase in the phenylalanine ammonia-lyase key enzyme in the metabolism of phenylpropanoids activity. On the other hand, the aerial parts of seedlings showed the highest contents of flavonoids (3.65 μg TAE mg^{-1} DW, on average). They are 42% higher than those observed in the roots, regardless of media and salt concentration tested ($p < 0.05$). This greater accumulation in the aerial part may be related to the presence of flavonoids associated with membranes of chloroplasts (AGATI et al., 2007) which act as powerful antioxidants, preserving membrane integrity during procedures that promote cell dehydration and, consequently, preventing oxidative damage (AGATI et al., 2012).

The importance of nutrients in the culture medium for the growth and development of seedlings has been well described. Unlike micronutrients, which basically have a functional role, the macronutrients are structurally and functionally important by forming a complex system that ensures the plant´s healthy functioning (KANASHIRO et al., 2009). According to Maldaner et al. (2006), nitrogen is the main inorganic nutrient of any culture medium, regardless of the type and purpose of culture, as part of numerous organic structures, composing the nucleotides and forming the nucleic acids, as well as amino acids that constitute proteins, still present in the chlorophyll molecule itself. In shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum,* the amount and source of N influenced the growth, the chemical metabolism, production and accumulation of secondary metabolites (COSTE et al., 2011). The results of this study are interesting from a physiological point of view. They indicated the use of the aerial part of *P. venusta* as a possible source of explants for callus induction and the production of cells with a higher accumulation of flavonoids.

Callus induction

Callus induction started 7 days after inoculation in media containing 2,4-D or BAP in the presence and absence of light. There was no callus formation in the absence of plant growth regulators. The highest callus induction (95%) was found in media supplemented with 4.44 and 17.75 μM BAP in the presence of light and 9.05 μM 2,4-D in the absence of light, whereas the lowest induction (60%) occurred in media with 9.05 μM 2,4-D in the presence of light (Table 2). Results demonstrate the differential effect of the type and the concentration of growth regulator used and light. They also highlight the importance of auxin and cytokinin for the establishment of callus cultures of *P. venusta*. According to Su et al. (2011), auxins and cytokinins.

Table 2. Callus induction, biomass production, total phenolic compounds and flavonoids in callus induced with different concentrations of 2,4-D and BAP in the presence and absence of light after 60 days of culture.

Presence of Light							
Plant Growth Regulators	Callus induction (%)	Fresh Weight (mg)	Dry Weight (mg)	Phenolic Compounds (μg TAE mg^{-1}DW)	Flavonoids (μg RE mg^{-1}DW)	Consistency	Color
4.52 μM 2.4-D	85 b	1406 a	110 a	8.81 b	3.03 c	Compact	Brown/Green
9.05 μM 2.4-D	60 c	570 b	5 b	9.61 a	3.77 a	Compact	Brown/Green
18.10 μM 2.4-D	80 b	660 b	5 b	7.11 d	2.79 c	Compact	Green
4.44 μM BAP	95 a	240 c	3 c	7.66 c	3.35 b	Compact	Green
8.88 μM BAP	85 b	60 d	1 c	9.44 a	3.78 a	Compact	Green
17.75 μM BAP	95 a	160 c	3 c	7.27 c	3.35 b	Compact	Green
Initial Explant	-	-	-	5.78 e	2.98 c	-	-
Absence of Light							
Plant Growth Regulators	Callus induction (%)	Fresh Weight (mg)	Dry Weight (mg)	Phenolic Compounds (μg TAE mg^{-1}DW)	Flavonoids (μg RE mg^{-1}DW)	Consistency	Color
4.52 μM 2.4-D	85 b	1080 a	9 a	6.63 b	2.99 c	Friable	Yellow
9.05 μM 2.4-D	95 a	1460 a	11 a	7.37 a	3.49 b	Friable	Yellow
18.10 μM 2.4-D	85 b	620 b	5 b	7.04 b	2.78 c	Friable	Yellow
4.44 μM BAP	75 c	110 c	2 c	5.33 c	2.88 c	Compact	Yellow
8.88 μM BAP	75 c	80 c	1 c	7.31 a	3.37 b	Compact	Yellow
17.75 μM BAP	85 b	50 c	1 c	7.99 a	4.65 a	Compact	Yellow
Initial Explant	-	-	-	5.78 c	2.98 c	-	-

Means in each column followed by the same letter are not significantly different at $p \leq 0.05$ by the Scott-Knott test.

control cell division in undifferentiated cells even though interaction efficiency depends on the species and plant tissue. Callus color varied between green, yellow, and brown. In the presence of light, calli were predominantly green and had compact consistency, whereas yellow friable calli were found only in the absence of light in 2,4-D (Table 2).

Therefore, the presence of light was not a limiting factor for inducing callus in *P. venusta*. However, light provided the formation of green callus indicating the presence of chloroplasts, whose synthesis is directly influenced by light (FUKUDA et al., 2008). Mustafa et al. (2011) reported that friable calli are mostly used for the cultivation of cells in suspension and for the study of metabolites production because the cells rapidly divide and disperse in culture medium. Yellow friable calli are also considered organogenic and, according to Arunyanart and Chaitrayagun (2005), translucent yellowish parts indicate their embryogenic potential. Some studies have demonstrated the possibility of using auxins to obtain friable calli in different plant species as *Ocimum basilicum* (GOPI; PONMURUMGAN, 2006) and *Uncaria guianensis* (PEREIRA et al, 2007).

Calli showed higher values of fresh and dry weight in media supplemented with 2,4-D and smaller values with BAP in the presence and absence of light (Table 2). However, only the medium supplemented with 9.05 μM 2,4-D in the absence of light was effective in promoting the establishment of 95% friable callus with higher fresh and dry weights. The results suggest the possibility of obtaining *P. venusta*'s calli with higher weight employing low concentrations of 2,4-D in the presence and absence of light, which would resemble those obtained by Loredo-Carillo et al. (2013) also for *P. venusta*. The auxins initiate cell division and control the processes of growth and cell elongation because they induce the transcription of messenger RNA, molecules capable of encoding proteins important for growth (GEORGE et al., 2008). However, the initial explant cell division and growth of calli are sometimes inhibited by the presence of light.

In general, all callus produce significant amounts of phenolic compounds and flavonoids, regardless of the presence or absence of light, or of type and concentration of regulators ($p < 0.05$) (Table 2). In all treatments, calli showed phenolic compounds and flavonoids contents higher than or equal to those found in the initial explant. In the presence of light, the calli induced in 9.05 μM 2,4-D and 8.88 μM BAP had higher total phenolic and flavonoids contents, and the production was stimulated 1.7 and 1.3 times, respectively, when compared to the initial explant. In the absence of light, the increases were smaller for phenolic compounds, but the flavonoids production was stimulated 1.6 times on 17.75 μM BAP medium. These results indicate that *in vitro* techniques both promote and increase the production of phenolic compounds in callus of *P. venusta*. The *in vitro* production of secondary compounds in medicinal plants is possible due to variation of culture conditions, including changes in types and concentrations of plant growth regulators (CASTRO et al., 2009; PALACIO et al., 2011). Results show the potential of *P. venusta*'s callus in producing high amounts of phenolic compounds and flavonoids *in vitro* and disagree with some reports in the literature, which suggest that the formation of specialized tissues is a

prerequisite for the production of secondary metabolites, especially phenolic compounds (PALACIO et al., 2011). However, the treatments cited produce compact calli which are not interesting for the production of these metabolites in cell suspensions (MUSTAFA et al., 2011). The use of friable callus is recommended for the establishment of *P. venusta's* cell suspension from media supplemented with 9.05 μM of 2,4-D, which also exhibits a good weight and yield of total phenolic compounds and flavonoids.

Phenolic compounds and flavonoids contents have an inverse relationship with cell growth given by calli weight (Table 2), especially in media containing BAP, where higher total phenolic compounds and flavonoids contents were observed in calli with lower weight. The relationship between cell growth and the accumulation of secondary metabolites has been reported in the literature, albeit still not well understood (PALACIO et al., 2011; LOREDO-CARILLO et al., 2013). Loredo-Carrillo et al. (2013) describe that decrease in growth may be related to the use of energy from sucrose in the culture medium for the synthesis of secondary metabolites.

Current studies confirm the biotechnological potential of *P. venusta* and agree with Loredo-Carrillo et al. (2013) who reported the *in vitro* flavonoids production in *P. venusta*'s calli in the presence of light and the elicitation of cultures with 2.5% polyethylene glycol. Nevertheless, current results showed that even in the absence of light and without elicitation, it is possible to obtain cultures with high phenolic compounds and flavonoids contents as reported by these authors, employing leaves of *P. venusta*'s seedlings as initial explants.

Conclusion

Results demonstrate the potential of *P. venusta*'s calli as an alternative source of bioactive phenolic compounds *in vitro* and encourage additional agronomic and biochemical studies for the future development of new protocols to obtain *in vitro* cell cultures, especially cell suspensions culture, with high concentrations of bioactive phenolic compounds from a biotechnological approach.

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