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Full Length Research Paper

Micropropagation of *Polygonum acre* Kunth var. *aquatile* (Mart.) Meisn, and seasonal variation of tannins in acclimatized plants

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A protocol for *in vitro* clonal propagation of *Polygonum acre* Kunth var. *aquatile* (Mart.) Meisn. is described as an alternative method to standardize raw material for production and experimentation. Disinfected nodal segments were inoculated into Murashige and Skoog basal medium (MS) and initially subcultured in the same kind of medium. They were then transferred to solid MS medium supplemented with 0.26 µM 6-benzyladenine (BA) in combination with either 0.26 - 6.5 µM α-naphthaleneacetic acids (NAA) or 0.26 - 6.5 µM indole-3-acetic acid (IAA). The best treatment for *in vitro* multiplication of *P. acre* contained MS + 0.26 µM BA + 1.3 µM NAA. The plants were successfully acclimatized, and the phenolics and tannins in these plants were quantified. Seasonal changes in condensed tannins were detected, with the highest accumulations in August. Total phenolics did not vary significantly throughout the year. Because the biological activity of *P. acre* has been ascribed to some polyphenols such as tannins, determination of seasonal changes that can improve the harvest of medicinal raw material.

Key words: Medicinal plant, seasonal phytochemical changes, condensed tannins, *Polygonum acre*, tissue culture.

INTRODUCTION

Polygonum acre Kunth var. *aquatile* (Mart.) Meisn. (Polygonaceae), synonym *Polygonum punctatum* Elliot, is a native New World plant known as water-pepper. Medicinal preparations containing aerial parts of this plant are often used in folk medicine to treat circulatory problems such as varices or hemorrhoids. Pharmacological studies of different extracts have shown anti-inflammatory, anti pyretic, anti-diarrheic (Simões et al., 1989; Almeida et al., 1995), and anti-hemorrhagic activity (Teixeira et al., 1989). Aqueous and hydro-alcoholic extracts also reduce the vascular permeability induced by histamine, elicit bradycardia, and lower arterial blood pressure (Simões et al., 1989).

This biological activity has been ascribed to tannins and flavonoids (Simões et al., 1989; Teixeira et al., 1989; Almeida et al., 1995), which are polyphenols, and may play an important role in the prevention of human pathology (Tapiero et al., 2002; Leifert and Abeywardena, 2008). Plants contain many compounds of undetermined biological

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activity, and the quality and quantity of these compounds can vary substantially based on the region where the plant is grown, the season in which it is harvested, and the genotype cultured (Currier et al., 2000; Ma et al., 2003). Tissue-culture techniques such as micropropagation provide means to standardize plant raw material (Rout et al., 2000). Rapid clonal propagation can be attained with micropropagation, and the plants are cultured in controlled conditions. The purpose of the present investigation was to establish a micropropagation protocol as an alternative method for standardizing raw material, contributing to the conservation of germplasm and allowing commercial cultivation of this medicinally important species. This study is also the first report on seasonal changes in total phenolics and tannins in this species.

MATERIALS AND METHODS

Establishment of *in vitro* cultures and treatments with growth regulators

Nodal segments were used as the source of plant material. A voucher specimen of plant (RFA-23928) has been deposited in the Biology Institute Herbarium RFA, Federal University of Rio de Janeiro, Brazil. The nodal segments were first cleaned with tap water and neutral detergent.

The explants were then surface-sterilized in 50% commercial bleach solution (NaClO, 2 - 2.5% active chlorine) for 30 min, rinsed three times for 5 min in distilled water, dipped in 70% ethanol solution for 5 min, and rinsed three times for 10 min in sterile distilled water. The disinfected nodal segments were placed in individual glass tubes (20 x 2.0 cm) containing 15 ml MS basal medium (Murashige and Skoog, 1962) with 3% sucrose added and solidified with 0.7% agar. The explants were maintained under white light illumination (*Sylvania* daylight fluorescent lamps) under 1.6 W m^{-2}, 23 μmol m^{-2} s^{-1} daily photoperiod of 16/8 h light/dark at 25 ± 2°C.

Plantlets regenerated from disinfected explants were subcultured in MS basal medium until enough shoots were available to establish the experiments. The nodal segments were transferred to solid MS medium supplemented with 0.26 μM 6-benzyladenine (BA), in combination with either 0.26-6.5 μM α-naphthaleneacetic acid (NAA) or 0.26 - 6.5 μM indole-3-acetic acid (IAA) (Table 1). The cultures were maintained under the same conditions as described above. The number of shoots per explant (n = 30 x 2), number of axillary buds per explant (n = 30 x 2), shoot length (cm), percentage of rooted explants, and percentage of explants developed callus were recorded after 2 months. Thirty random explants were used per treatment, and each experiment was performed twice. The data were submitted to ANOVA analysis (analysis of variance), and the mean values were compared by Dunn's test, at a 5% significance level.

Acclimatization

Plants cultured for 2 months in MS basal medium without added growth regulators were removed from the culture medium, and their roots were gently washed in tap water and then transferred to a seed pot containing soil conditioner Plantmax (Eucatex). The pots were kept under greenhouse conditions for 1 month before being transferred to outdoor conditions, in the city of Muriaé, Minas Gerais (21° 9' 26.03''S/42° 23' 54.34''W). The plantlets were irrigated daily with filtered UV-sterilized water.

Quantification of total phenolics and tannins

Aerial parts of acclimatized plants were harvested monthly from April to November. The samples were stored freeze-dried at -20°C until analysis. The plant material (500 mg dry weight) was extracted four times with acetone/water (7:3) solution. The extracted volume was filtered and completed to 25 ml with acetone/water (7:3). For the assay of total phenolics (TP), aliquots of 0.5 ml were utilized; for both assays of condensed tannins (CT) and gallotannins (GT), the aliquots utilized contained 1 ml each.

TP were measured by the Folin-Denis method, CT were measured through the proanthocyanidin BuOH-HCL method, and gallotannins at the Rhodanine method (Waterman and Mole, 1994). The percentages of TP, CT, and GT were calculated from the mean content in μg/mg of the dry weight of the sample in relation to TP from tannic acid at 760 nm of optical density in absorbance (OD), CT from Quebracho (*Schinopsis spp.*) at 540 nm OD, and GT from gallic acid at 520 nm OD, respectively. Each assay was performed in triplicate. The data are presented as a mean ± SD. One-way ANOVA was applied to the data, and differences between means were detected using the Tukey test ($P \leq 0.05$ or ***$P \leq 0,001$). All statistical analyses were carried out using GraphPad InStat 3.0 for Windows.

RESULTS

Shoot induction, multiplication, and *in vitro* plant development

The most new shoots per explant were obtained in media containing MS + 0.26 µM BA + 0.26-1.3 µM NAA or MS + 0.26 µM BA + 6.5 µM IAA (Table 1, Figures 1c, d and h). However, the best development of axillary buds was observed in the medium containing MS + 0.26 µM BA + 1.3 µM NAA, which produced 14.6 ± 0.63 buds from per explant (Table 1, Figure 1d). The shoot length was longest (5.0 to 6.29 cm) in MS + 0.26 µM BA + 1.3 - 6.5 µM NAA (Table 1, Figures 1d and e), although these treatments also induced callus development. The calluses were formed in the transition region between root and stem, reaching about 0.8 cm in diameter. Rooting was induced in all treatments, and no statistical difference was found in the percentage of rooting in any treatment (Table 1, Figure 1a - h).

These results show that all the stages of micropropagation, such as multiplication, elongation, and rooting, can be performed within one treatment, making this protocol cost-effective. The developmental responses of plants cultured *in vitro* and treated with growth regulators are shown in Figure 1.

It is clear from the data obtained that the best treatment to promote *in vitro* multiplication of *P. acre* contained MS + 0.26 µM BA + 1.3 µM NAA. The plantlets developed 2.82 ± 0.22 shoots per explant, 14.6 ± 0.63 axillary buds per explant, and 6.29 ± 0.24 cm in height. No subsequent treatment was required for plant rooting, after 8 weeks (60 days) of culture (Table 1).

Subculture of plants cultured in medium containing MS

Table 1. Effect of growth regulators on *in vitro* development of nodal segments of *Polygonum acre* after 60 days.

Treatments (µM)	Number of shoots per explant [a]	Number of axillary buds per explant [a]	Shoot length (cm)	Rooting (%)	Callus development (%)
MS [b]	1.17 ± 0.07	8.63 ± 0.41	4.08 ± 0.19	100	0
0.26 µM BA	1.44 ± 0.13	8.86 ± 0.31	3.90 ± 0.14	100	0
0.26 µM BA + 0.26 µM NAA	2.27 ± 0.17 ***	9.16 ± 0.56	4.02 ± 0.20	95.6	0
0.26 µM BA + 1.3 µM NAA	2.82 ± 0.22 ***	14.6 ± 0.63 ***	6.29 ± 0.24 ***	100	100
0.26 µM BA + 6.5 µM NAA	1.55 ± 0.13	8.90 ± 0.33	5.00 ± 0.19 *	100	100
0.26 µM BA + 0.26 µM IAA	1.62 ± 0.18	7.50 ± 0.36	3.81 ± 0.17	100	0
0.26 µM BA + 1.3 µM IAA	1.75 ± 0.12	9.56 ± 0.65	4.08 ± 0.21	93.7	0
0.26 µM BA + 6.5 µM IAA	2.12 ± 0.18 **	11.0 ± 0.52	4.57 ± 0.15	100	0

[a] Values are mean ± standard deviation. Values in same column followed by asterisks differ statistically from the control treatment (MS medium) at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).
[b] MS - control treatment, medium without growth regulators.

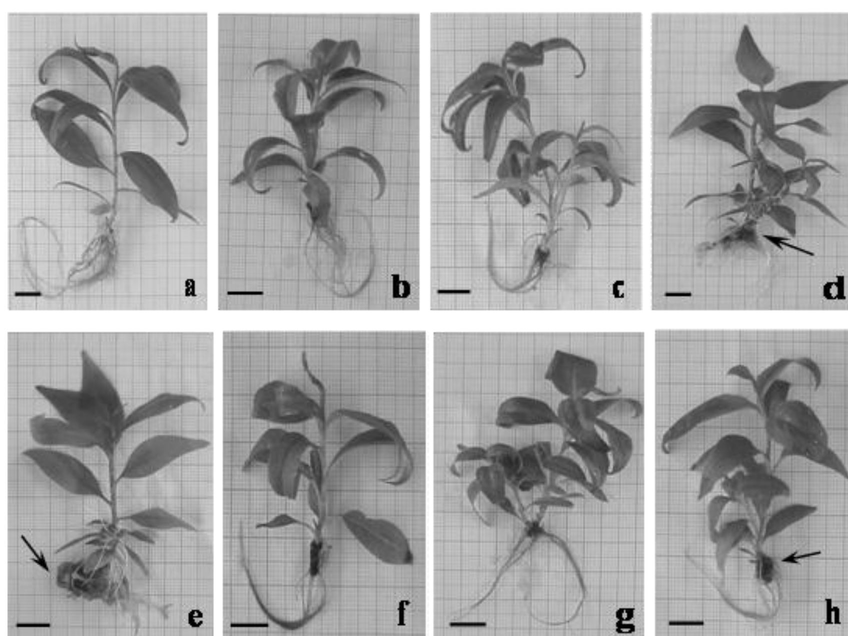

Figure 1. Developmental response of plants treated with growth regulators after 2 months of *in vitro* culture. a) MS medium without growth regulators; b) 0.26 µM BA; c) 0.26 µM BA + 0.26 µM NAA; d) 0.26 µM BA + 1.3 µM NAA; e) 0.26 µM BA + 6.5 µM NAA; f) 0.26 µM BA + 0.26 µM IAA; g) 0.26 µM BA + 1.3 µM IAA; h) 0.26 µM BA + 6.5 µM IAA. Arrows indicate the callus formation. Bars = 1.0 cm.

+ 0.26 µM BA + 1.3 µM NAA to medium without added growth regulators can reduce callus growth, but the yield of the culture can remain higher than the control treatment (MS only). Depending on the purpose of the culture, these plants can be considered appropriate for acclimatization.

Acclimatization

Hardening of *P. acre* in the greenhouse for 1 month was very effective. All the plantlets survived after their transfer into a seed pot, and all of them survived in outdoor conditions (Figure 2). The plants appeared to be morphologically normal and uniform, and were successfully adapted to field conditions, reaching at least 2 m in height after approximately 5 months, and forming well-developed roots. Blooming and seed formation occurred normally.

Only plantlets obtained from MS media were acclimatized (Figure 2a), in order to eliminate the variable growth regulators in subsequent experiments, which were begun

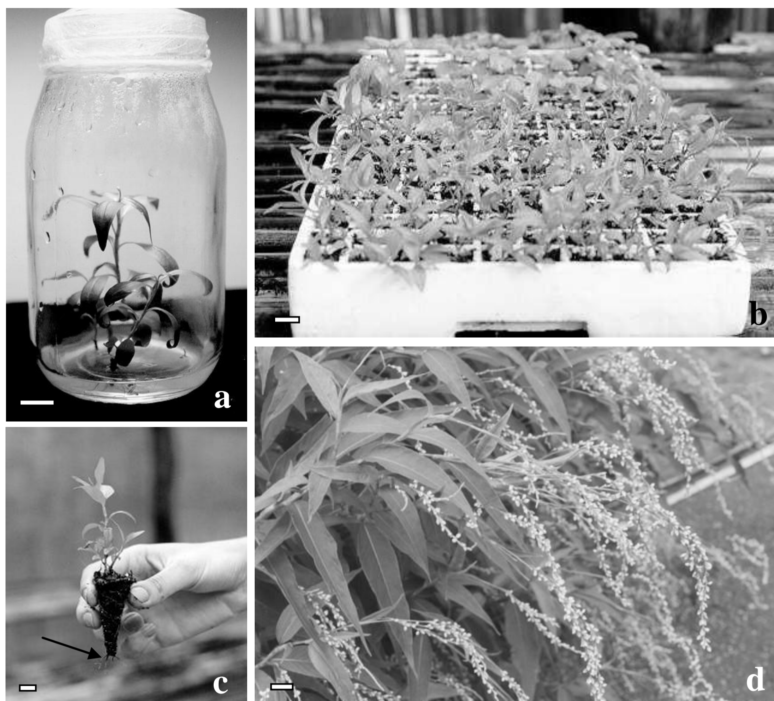

Figure 2. Acclimatization of *Polygonum acre*. a) Multiplication of *P. acre* in MS medium without growth regulators. Plantlets cultured *in vitro* for 2.5 months. Only plantlets obtained from MS media were acclimatized. b) Plants grown in a greenhouse for a month. c) Plant acclimatized for a month, removed from the seed pot and transferred to the field. The arrow indicates well developed roots. d) *P. acre* with 5 months of cultivation in the field. Flowering plants. Bars = 1.0 cm.

4 months after the plants were established in field conditions. The plants cultured in the MS basal medium did not develop calluses, allowing more effective acclimatization.

Seasonal changes in total phenolics and tannins

Concentrations of total phenolics did not vary signifycantly, remaining around 1.57% throughout the period of analysis (Table 2). In contrast, the contents of condensed tannins varied significantly: the amount increased from April to August (highest content detected), and decreased from August to November (Table 2).

In the assay of gallotannins, the absorbance of samples was always very close to the blank. Therefore, gallotannins were considered not to be detected in the samples.

For the analyses of seasonal changes in total phenolics and tannins, the samples were harvested monthly from April to November. All the samples were analyzed, although the results from July and October are not shown in Table 2. Because the collection site is located far from the laboratory, the samples taken in July and October could not be readily transported to the laboratory and lyophilized. Therefore, these samples were dried at the ambient temperature in shade, according to popular tradition. In these non-lyophilized samples, the content of total phenolics and tannins was far below the average, probably as a result of degradation of these metabolites.

Table 2. Seasonal changes in content (%) of total phenolics (TP) and condensed tannins (CT) of *P. acre* in acclimatized plants (mean ± SD):

Month analyzed	TP	CT
April	1.46 + 0.02 c	1.83 + 0.02 c
May	1.57 + 0.05 b c	7.20 + 0.27 d
June	1.59 + 0.02 b	8.93 + 0.42 b
Aug	1.85 + 0.06 a ***	12.32 + 1.02 a ***
Sep	1.5 + 0.06 b c	7.38 + 0.98 b d
Nov	1.56 + 0.02 b c	2.06 + 0.31 e c

Different letters in a column indicate a significant difference ($P \leq 0.05$ or *** = $P \leq 0.001$, Tukey test).

DISCUSSION

Because *P. acre* is used in phytopharmaceuticals, micropropagation represents an alternative to produce more-homogeneous and source-controlled raw material. Plants

of the genus *Polygonum* have been cultured *in vitro* as callus culture or cell culture, generally in studies on the production of secondary metabolites (Banthorpe et al., 1989; Hagendoorn et al., 1994; Kim et al., 1996; Ono et al., 1998; Shim et al., 1998). In a micropropagation protocol established for *Polygonum multiflorum*, several growth regulators were employed to promote *in vitro* development of shoots and roots, including MS + 2.0 mg/l of BA, which induced the development of 4.7 shoots per explant after 6 weeks of culture. 88 - 100% of these plants elongated (3.02 - 4.28 cm) and rooted in MS basal medium supplemented with NAA or IAA (Lin et al., 2003).

The sprouting response and the characteristics of proliferated shoots were affected by the cytokinin-auxin combination (source and concentration). The presence of auxin and cytokinin in the medium often induced new shoots or axillary buds. In general, the composition of the culture medium is manipulated to induce an increase of *in vitro* plant multiplication. The highest multiplication rate is reached when more axillary buds are regenerated from each explant, but the shoot must be well elongated in order to facilitate excision of the buds. In this study, MS + 0.26 µM BA + 1.3 µM NAA was the best treatment to promote *in vitro* multiplication of *P. acre* because it induced shooting, budding, and growth in height (Table 1). No subsequent treatment was required for plant rooting, but the callus formation induced by this medium may interfere with the acclimatization process. An alternative to diminish the impact of callus formation on acclimatization consists in working with the residual effects of the growth regulators, which allows some problems in tissue culture to be overcome (Sato et al., 2006).

Only plantlets obtained from MS media were acclimatized. Plants cultured in MS media did not develop calluses, allowing more effective acclimatization (Figure 2). Moreover, acclimatization of only plantlets from MS media was preferred, to ensure that the results from analysis of seasonal variation of total phenolics and tannins were not related to the growth regulators. In this study, *P. acre* was micropropagated through shoot formation, from nodal segments with one bud. Plantlets obtained from the organized meristem of vegetative buds normally show no visible signs of morphological variations, and are genetically identical (Bajaj et al., 1988). This homogeneity is especially important for micropropagation of medicinal plants.

Plants are dynamic organisms, and therefore it is expected that levels of plant secondary metabolites will vary spatially within a plant, among individuals, and temporally over in the course of a season or in successive years (Brooks and Feeny, 2004). However, changes in levels of plant secondary metabolites interfere with the effectiveness of phytomedicines (Currier et al., 2000; Lee et al., 2005; Gurib-Fakim, 2006). Studies of changes in active compounds as a consequence of biotic and abiotic factors can help to optimize the harvest time of medicinal raw material or to obtain the largest amounts of active compounds (Ma et al., 2003; Abreu and Mazzafera, 2005; Lee et al., 2005). Phenolic compounds are the largest category of natural products, and are the most widely distributed in the plant kingdom. Many phenolic compounds have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antibacterial, antiviral, or anti-inflammatory activity to a greater or lesser extent (Tapiero et al., 2002; Seeram et al., 2006).

The phenolic compounds in *P. acre* have not been studied, in spite of their possible therapeutic properties (Simões et al., 1989; Almeida et al., 1995). Concentrations of condensed tannins varied significantly throughout the period of analysis (Table 2), and the highest content was detected in August (Table 2). The winter season in countries of the Southern Hemisphere includes the months of June, July, August, and September. Many events may contribute to tannin accumulation during the winter. Environmental factors such as water stress, mainly caused by seasonal reduction of precipitation in winter, have been related to tannin accumulation in plant aerial parts (Alonso-Amelot et al., 2007; Wang et al., 2007). In the city of Muriaé where the plants were acclimatized, the winter is dry, and the lowest precipitation occurs in June and July (INMET, 2007). Although the plants were irrigated regularly throughout the growing period, a combination of environmental variations including water stress may explain these results.

Gallotannins were not detected in the samples. Condensed tannins are widely distributed in the plant kingdom, whereas gallotannins have a more restricted occurrence. Gallic-acid metabolites occur within clearly defined taxonomic limits in woody and herbaceous dicotyledons; the ellagitannins are found in the lower Hamamelidae, Dilleniidae, and Rosidae, and have been used as important chemotaxonomic markers for these groups (Okuda, 2005; Haslam, 2007).

Other information generated from the analyses of seasonal changes in total phenolics and tannins was that the post-harvesting method affected the content of total phenolics and tannins. The samples treated according to popular tradition had a much lower content of total phenolics and tannins, in contrast with the lyophilized samples, indicating a probable degradation of these metabolites.

Conclusion

The best treatment for *in vitro* multiplication of *P. acre* contained MS + 0.26 µM BA + 1.3 µM NAA. The acclimatization process, usually a critical point, was very effective both in time and in yield, and no damage was recorded with the procedures adopted. Because the largest accumulation of condensed tannin was observed in August, this is the best period to obtain the raw, tannin- rich

material. Different classes of compounds can vary differently through the year, and therefore the most favorable periods for harvest according to the desired compound and expected biological activity should be determined.

This study aimed to contribute to the knowledge, culture, and use of *P. acre* as a medicinal plant.

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