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Field culture of micropropagated *Passiflora caerulea* L. histological and chemical studies

[Cultivo a campo de *Passiflora caerulea* L. micropropagada: estudios histológicos y químicos]

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Abstract

In Argentinean popular medicine, *Passiflora caerulea* L. (Passifloraceae) is used mainly as sedative. The objective of this work was to put in culture *P. caerulea* plants obtained by micropropagation as a tool for the propagation and culture at a commercial scale of selected plants according to the qualitative identification of their chemical constituents, as well as to know the origin of their regeneration. Leaves were cultured in Murashige and Skoog (MS) medium, with 1 mg/l of 6-benzylamino-purine (BAP). The material was processed for the histological study, which showed that regeneration is produced by direct organogenesis. Being a climbing species, the field culture of micropropagated plants was carried out on a vertical espalier. The chemical study was carried out through a Thin-Layer Chromatography (TLC); the fingerprint obtained showed that there is no difference in the secondary metabolites among the plant from which the explants were extracted (mother plant) and those obtained by in vitro culture.

Key words: *chemistry, field culture, histology, in vitro culture, Passiflora caerulea.*

Resumen

En la medicina popular de Argentina, *Passiflora caerulea* L. (Passifloraceae) es utilizada principalmente como sedante. El objetivo del presente trabajo fue poner en cultivo plantas de *P. caerulea* obtenidas por micropropagación, como herramienta para propagar y cultivar a escala comercial plantas seleccionadas de acuerdo a la identificación cualitativa de sus componentes químicos, como así también conocer el origen de su regeneración. Las hojas fueron cultivadas en medio de cultivo de Murashige and Skoog (MS), con 1 mg/l de 6-bencil amino purina (BAP). Para el estudio histológico el material se procesó y se concluyó que la regeneración se produce por organogénesis directa. La puesta a campo de las plantas micropropagadas, por ser una especie trepadora, se realizó sobre espalderas de tipo vertical. El estudio químico se realizó a través de una Cromatografía en capa fina (CCF); se obtuvo un fingerprint, que demostró que no hay diferencias entre los metabolitos secundarios de la planta de la cual se extrajeron los explantos (planta madre) y los obtenidos por cultivo *in vitro*.

Palabras clave: *cultivo a campo, cultivo in vitro, histología, Passiflora caerulea, química.*

INTRODUCTION

The use of aromatic and medicinal plants goes back to the dawn of our civilization, with testimonies dated from the year 2000 a.C. (McHoy & Westland, 1994). Their economic importance also dates from that time (Garland, 1979). In recent years, in most developed countries, a growing interest in the study, research, consumption, and production of medicinal and aromatic plants developed together with a wide and growing field of application in pharmaceutical, food and cosmetic industries (Muñoz, 1993).

Seeds of the native medicinal species were collected in its natural habitat. This has caused serious problems in relation to its preservation, since such species have been disappearing in areas where they were abundant until not many years ago. This resource depredation is caused because the exploitation is not carried out properly and there is not an efficient and methodic control of such depredation (Martínez *et al*., 2000).

Cultures of these native herbs in Argentina may offer a series of advantages, which would allow getting an uniform high quality product, and avoiding the excessive pressure on the natural resource (López, 1996). The propagation, either through conventional techniques (cuttings, shoots, division of plants), or through tissue culture, is a crucial and fundamental stage to achieve the domestication of species. For native plants, there are no fundamental multiplication experiences, and in most cases the domestication of cultures is still to be achieved in order to obtain a culture with constant characteristics (Ocampo, 1944).

This research team began to work from explants in the domestication of native species with pharmaceutical interest. First works started with *Passiflora caerulea* L. ("mburucujá" or "pasionaria"), Passifloraceae family, a native medicinal species, valuable in the pharmaceutical industry for its aerial parts, leaves, flowers, and fruits. It is used as sedative, since pharmacological studies have determined that chrysin flavonoid would be the main responsible for the anxiolytic effect, with a sedative action similar to diazepan, but ten times lower and with no myorelaxant effect (Medina *et al*, 1990; Speroni *et al*, 1996).

This plant was very important for American natives, since its fruits had an important economical value as exchange money with other villages. In the XIX century it was recommended as non-narcotic sedative and digestive. This use was popularized in Europe, more precisely during the First World War, as a treatment for the post-war anguish. In 1937, it was registered in the French Pharmacopoeia and in the following years it was adopted by most pharmacopoeias of the Old Continent (Alonso, 2004).

The objective of this work was to put in culture *P. caerulea* plants obtained by micropropagation, as a tool for the propagation and culture at a commercial scale of selected plants, according to the qualitative identification of their chemical constituents, as well as to know the origin of its regeneration.

MATERIALS AND METHODS

Vegetal material

Ripe fruits were collected in February, 2001, in the city of Rosario (Argentina). Seeds were removed manually from the fruit, washed with running water to eliminate the aril, and subsequently dried in the shade. *P. caerulea* seedlings were obtained from germinated seeds in asepsis conditions (Severin *et al.*, 2003 a, b); they were kept *in vitro* as explants donors (mother plants). Taxonomic identification of the vegetal material was carried out in the Vegetal Biological Area, Faculty of Biochemical and Pharmaceutical Sciences (National University of Rosario, Argentina). Voucher specimens, with original seeds, are kept in the U.N.R. Herbarium.

Explants obtainment and culture medium

The micropropagation was achieved using, as explants, foliar segments extracted from the mother plants and cultivated in Murashige and Skoog (1962) medium with 1 mg/l BAP (6-benzylamino-purine) (Gattuso *et al.*, 2003).

Acclimatization

Shoots obtained after rooting in medium (MS) without hormones were transplanted to containers with a mixture of humus-rich soil and perlite of volcanic lava, covered with transparent polyethylene bags to prevent plant dehydration. They were kept 20 days in a growing chamber with temperature of 25 ± 2 °C, during a 16 h photoperiod and irradiance of 60 $\mu mol.m^{-2}.s^{-1}$. Then, they were put in a greenhouse.

Histological studies

In vitro behavior of explants was evaluated through visual observations and histological studies. For this purpose, the material was obtained at 0, 6, 10, 15, 19, 22, 28, 43, and 48 days of culture, processed using conventional techniques for its soaking in paraffin, cut at 10 μm, colored with Safranin-Fast Green (Strittmatter, 1979), and mounted with Canada balsam. Photomicrographs were taken with a Zeiss Axiolab microscope furnished with a MC 80 photographic equipment.

Chemical studies

Aerial parts from the mother plant and from *in vitro* culture were used for the chemical study. Two methods for the extraction of flavonoids were tested (Farmacopeia Brasileira 3º Ediçao, 1977; Wagner *et al.*, 2001). The dichloromethanic extraction was selected.

Preparation of the extracts

Aerial parts (stems and leaves) were dried in the shade at room temperature. 200 mg of dried material was ground and extracted for maceration with 10 ml of dichloromethane and 200 mg with 10 ml of methanol during 20 min by means of constant agitation; each of them was filtered and then evaporated up to approximately 1 ml in a bain-marie at 60 ºC. 30 µl of each sample was cultured by dichloromethane extraction and 10 µl for the methanol extraction. Stationary phase: silica gel G F_{254} (Merck) plates of with aluminum base. Mobile phases: for the methanolic extraction, the mobile phase was EtOAc/formic acid/acetic acid/H_2O (100:26:11:10), and for the dichloromethane extraction, the mobile phase was Toluene/EtOAc

(7:3). Detection: plates were developed under UV (Chromatoview) at 365 nm, with sulfuric anisaldehyde and solution at 1% of 2-aminoethyl diphenylborinate in methanol (NPR). The extract chromatogram with methyl alcohol was revealed with NPR, observed at visible light and that obtained with dichloromethane was detected with sulfuric anisaldehyde and observed at visible light.

Field culture

Wild plants and micropropagated plants already acclimatized in a greenhouse were transferred to the field. Since the passion flower is a climbing species, a conduction system, which allowed a proper growth, was provided. Vertical espaliers or hard-wood fence posts were built in terminals, and rods of 1.8 m height and less diameter were built for middle clearances. Four rows of steel wire were arranged with a 40 cm separation from each other along the espalier, and crossing the middle rods with a final fastening in fence posts or turnbuckles through fence turnstiles. The orientation of the espalier was carried out in the East-West sense of direction to allow a better use of sunlight.

The presence of forest curtains in the lot allowed tempering the effect of dominating winds and, at the same time, prioritized the light effect for the orientation of espaliers. The conduction was carried out tying the plants to the structure; then, the tendrils held by themselves on to the structure. Basal branches or "*chupones*" were removed to allow the developing of superior branches. Branches that surpassed the height of conduction wires in 15-30 cm were sprouted (cut of apical bud) to allow the developing of lateral sprouts (secondary guides). Plants were watered in a regular basis and fertilized with triple 15 (N:P:K) with total doses of 350 g per plant during the first 14 months of growth. Weeds were manually removed and the plague control was not required during the growth cycle.

For the material dehydration, a drying tunnel composed by a chamber measuring 8.1 m length, 2.33 m width and 2.01 m height was arranged and built with walls and roofs insulated with expanded polystyrene of 10 cm width. Internal and external walls were built with a 1 mm thick aluminum plate. Within this chamber, four bodies of six flat trays were placed on top of each other, measuring 2.16 m length and 2.02 m width, and built with a tube structure. The floor, where the material was arranged for dehydration, was built with middle shade of 60%. In each apex, an insert was applied so that the trays could stack without the need of additional structure.

RESULTS AND DISCUSSION

A successful *in vitro* culture establishment was obtained from leaves, and the formation of various shoots was observed in each explant (Fig.1, A). They principally originated in the edges and in the area of leave veins, without previous callus formation, unlike the information reported by Otahola (2000), who in his work, obtained callus in leaf-derived explants. After 50 days of culture, 70 % of the explants regenerated shoots, which formed roots in MS medium without hormones. Appezzato da Gloria *et al*. (1999) who obtained rhyzogenesis in presence of (ANA) working with *P. edulis* f. *flavicarpa*. Two months after the implantation, the plants obtained were ready to initiate the acclimatizing phase. With this protocol, complete plants could be obtained after 60 days of culture (Fig.1, B).

The histological analysis showed that at day zero, the foliar laminas showed a dorsiventral structure, with a layer of palisade cells and 4-5 layers of spongy parenchyma cells (Fig.2, A). Up to the tenth day after culture, no changes were observed in explants. From that day, a proliferation tissue appeared which originated from the parenchyma close to the veins (Fig.2, B). After 19 days, all the mesophyl parenchyma was found dedifferentiated, and after 22 days, buds became visible, which originated from the proliferation tissue mass without previous callus formation (Fig.2, C). 43 days after culture, stems and leaves were completely differentiated, and roots started to appear (Fig.2, D). Finally, after 48 days, a great number of shoots could be observed in different developing stages. The histological analysis showed that the organogenesis is direct, as proposed by Appezzato da Gloria *et al*. (1999).

The acclimatizing and the field culture of micropropagated plants were successful and such plants showed a higher qualitative development than the wild plants (Fig.3). Due to the vigorous growth of the plants obtained by *in vitro* culture, various pruning of basal shoots were required, as well as of the branches which grew above the conduction steel wire. This material, together with other cuts carried out in different sectors of the espalier, was

Fig. 1: A-B: A, shoot proliferation from *P. caerulea* leaves after 48 days of culture; **B**, plants after 3 months.

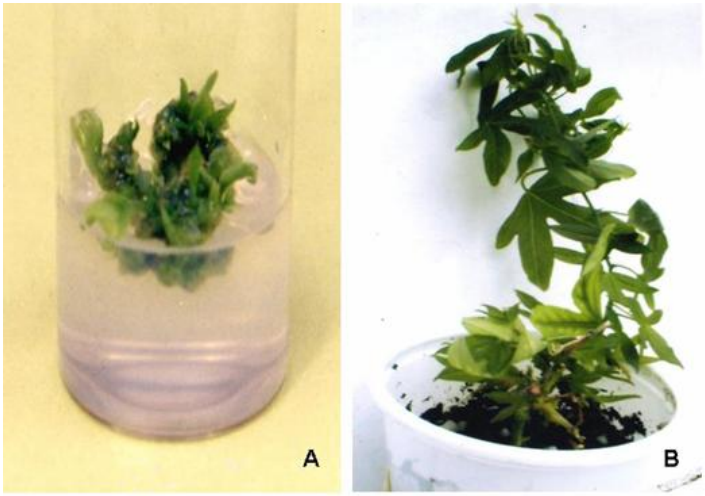


Fig. 2: A-D: histology of *in vitro* regulation of *P. caerulea*; **A,** leaf lamina cross section, day 0; **B,** vascular bundle region, generalized proliferation of spongy parenchyma after 10 days of culture; **C,** tissue growth after 22 days of culture, visualization of stem; **D,** 48 days of culture, visualization in long section of root and shoot primordia. **e,** explant; **l,** leaf; **n,** secondary vascular bundle; **p,** spongy parenchyma; **pe,** palisade parenchyma; **r,** root; **s,** stem; **st,** shoot primordia. Scale bar: **A-B**: 25 µm; **C**: 250 µm; **D**: 150 µm.

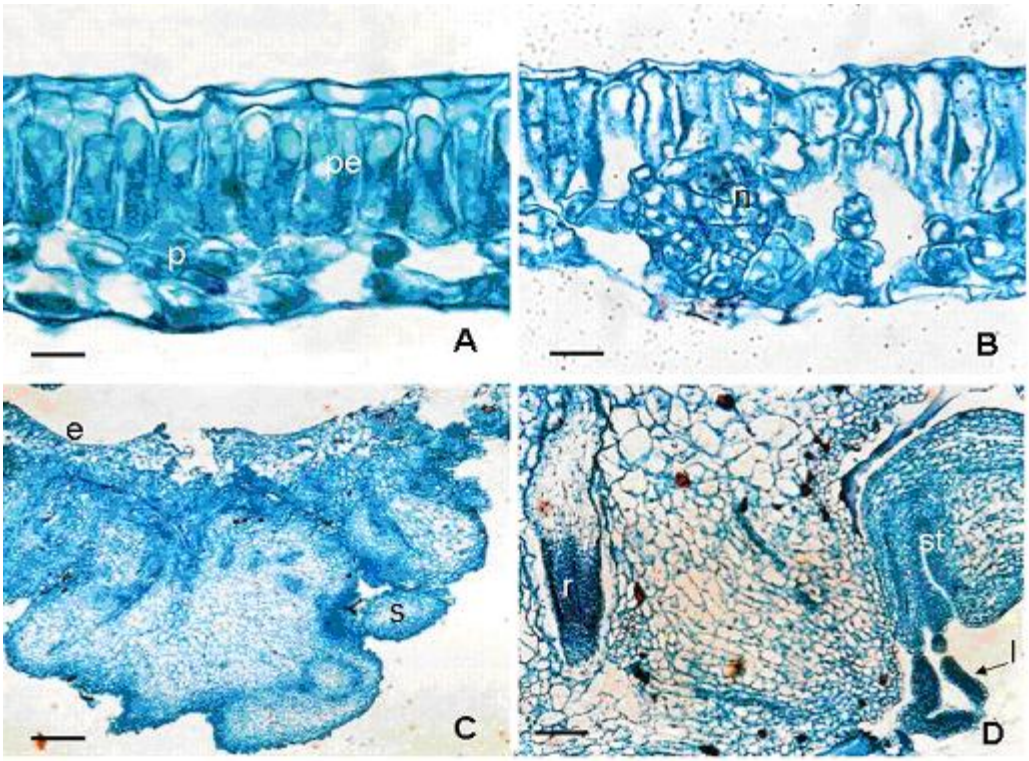
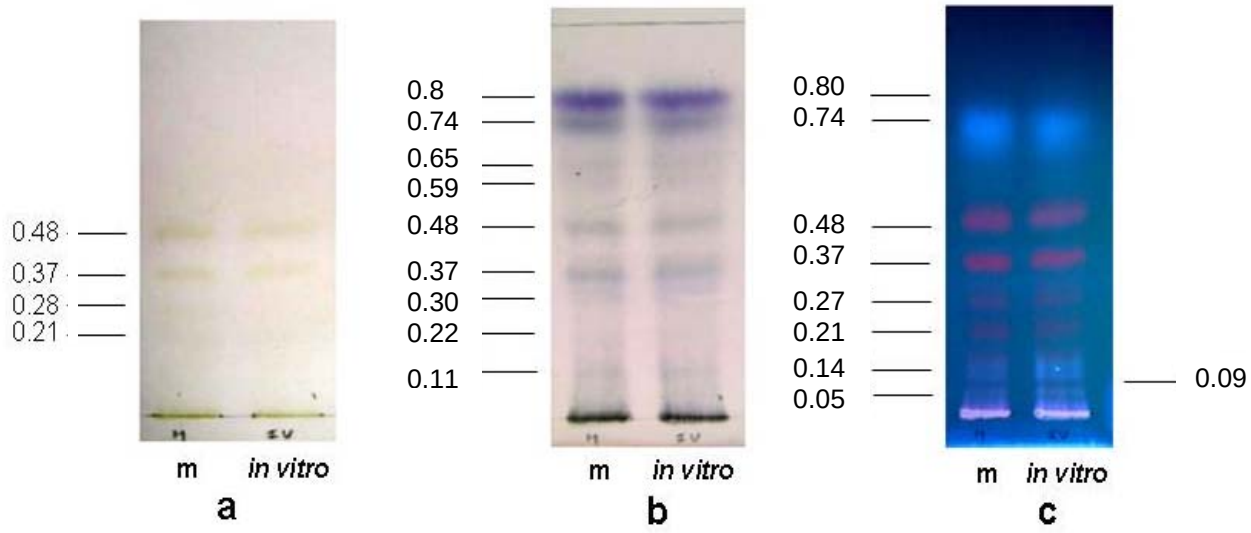


Fig. 3: regenerated plant of *P. caerulea* in field.

Fig. 4: chromatogram corresponding to mother plant (m) and to that derived from *in vitro* culture: **a,** at visible light; **b,** chromatogram developed with sulfuric anisaldehyde; **c,** chromatogram observed at UV at 365 nm.



dehydrated in order to be subjected to the chemical studies.

To obtain dehydrated quality herbs, an artificial drying was required, which avoids the loss of quality,the development of microorganisms, and the chemical alterations of components. According to Page and Stearn (1992), Muñoz (1993), Venkutonis (1997), and Paakkonen *et al.* (1999), the optimal temperatures for leaf and flower dehydration should range between 25 and 40 °C; therefore, drying was carried out in tunnels specially designed where the temperature control and the uniform air distribution were possible (Lara *et al.*, 2001).

Quality parameters of the material obtained from the qualitative point of view, such as color, aroma, and uniformity, proved to be optimal. The obtained chromatogram allowed the visualization of four well-defined bands in the visible light, and nine bands when it was detected in sulfuric anisaldehyde. Extraction protocols were adjusted to work with explants of foliar lamina. The results are shown in the following figures:

Chromatogram corresponding to the mother plant (m) and to that derived from *in vitro* culture.

1. Chromatogram corresponding at visible light (Fig. 4 a), where four well defined bands were observed with Rf of 0.21 (yellow); 0.28 (yellow); 0.37 and 0.48 (green).

2. Chromatogram detected with sulfuric anisaldehyde (Fig. 4 b), where nine bands can be distinguished with Rf of 0.11; 0.22; and 0.30 (gray); 0.37 and 0.48 (green); 0.59; 0.65 and 0.74 (gray) and 0.8 (violet).

3. Chromatogram detected at UV at 356 nm (Fig. 4 c) allows visualizing new bands with characteristic colors and values with Rf of 0.05-0.09 and 0.14 (light blue); 0.21; 0.27; 0.37, and 0.48 (orange), and 0.74 and 0.80 (light blue).

CONCLUSION

This work describes the procedure for the micropropagation and field culture of *P. caerulea* plants. From the chemical study, similar chromatograms were obtained for plants derived from the mother plant, as well as from those obtained from tissue culture; thus, the secondary metabolites are similar. Therefore, selected plants can be produced at a great scale and can be cultivated in field.

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