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RESEAI

A new variety of *Bacopa monnieri* obtained by *in vitro* polyploidiza

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Abbreviations: BAP: 6-benzyl amino purine
DMSO: dimethylsulfoxide
MS: [Murashige and Skoog \(1962\)](#)

Abstract

The commercial value of *Bacopa monnieri*, a widespread herbaceous plant in Argentina can be substantially improved increasing its flower size by chromosome doubling with colchicine. MS supplemented with 0.25 mg/L of 6-benzylaminopurine proved to be an appropriate medium for the *in vitro* multiplication of nodal segments of *B. monnieri*. Polyploidization was achieved submerging nodal segments during 24 or 48 hrs in a solution 0.001 and 0.01% P/V, in 1% DMSO. Segments submerged in water and in an aqueous solution were used as controls. DNA contents from recovered plants was measured and characterized and their phenotype described. Two tetraploid plants originated from polyploidization events were detected. These plants showed significant differences in size and colour between leaves and flowers compared to untreated controls.

Article

B. monnieri, an herbaceous plant commonly found in temperate regions ([Zuloaga and Morrone, 1999](#)), is one of the 12 native species of the genus *Bacopa* (*Scrophulariaceae*) present in Argentina. This species has economical relevance as a medicinal plant ([Tiwari et al. 2001](#)), a pot plant and as an ornamental in aquatic gardens.

Flower size is one of the traits in *B. monnieri* that needs to be improved to increase the ornamental value of the specie. Polyploidy agents such as colchicines and oryzaline, often used to modify the plant shape and to restore fertility, are valuable tools to get bigger flowers. These chemicals were successfully applied in the past in many ornamental crops ([Horn, 2002](#)) to obtain new genotypes with variants in size and/or colour of flowers and leaves ([Notsuka et al. 2000](#)). Chromosome doubling under *in vitro* conditions is, as well, a very interesting tool for exploration and domestication of wild germplasm to be used as ornamentals ([Escandón et al.](#)

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[al. 2005](#)). These improved ornamental products could be either included into a breeding program or transferred to potential users together with the technology de

This study presents the tweaking of both the *in vitro* propagation technique of nodal segments of *B. monnieri* and the *in vitro* treatment of this species with colchicine to obtain the new variety JICA.

Materials and Methods

Tissue culture

Nodal segments of *B. monnieri* were disinfected in 70% ethanol for 30 s and then in a solution containing 25% sodium hypochlorite: 0.01% Tween 80 during 25 min. After that, the segments were rinsed three times with distilled and sterile water.

Following disinfection, nodal segments were cultured in a hormone free MS medium ([Murashige and Skoog, 1962](#)) to grow the explants needed to study the response of *B. monnieri* to different concentrations. The MS medium used for the culture was supplemented with 20 g/L sucrose, 10 g/L agar, and four growth regulator concentrations: 0.0; 0.25; 0.5 and 1.00 mg/L BAP, pH was adjusted to 5.7 with KOH. The explants were grown in a 16 hrs light photoperiod under an irradiance of 3,000 lux and an average temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

After one subculture three or four centimetres long rooted plantlets were transferred to a 10 cm diameter pot containing turf, perlite and vermiculite 3:1:1 (V/V) ([Escandón et al. 2003](#)) and placed in a humidity chamber. The nylon bags used to make the humidity chamber were perforated daily until no condensation was detected inside them. Afterwards, the plants were transferred to greenhouse conditions. Twenty explants were used per treatment and the experiment was repeated twice.

In vitro plant polyploidization

Nodal segments from *in vitro* plant of *B. monnieri* were submerged in 1% DMSO solution for the following doses of colchicine (v/v): 0.0; 0.001 and 0.01 % (24 and 48 hrs). Fifteen untreated nodal segments as well as fifteen segment groups submerged in water or in 1% DMSO (aqueous solution) were used as controls. The culture medium used for the controls was MS supplemented with 1 mg/L BAP. Culture conditions as well as temperature and photoperiod were the same than in the culture experiments (16 hrs light photoperiod under an irradiance of 3,000 lux, and temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$).

The ploidy level was determined using the flow cytometer ([Partec, CA](#)), following the following indications: approximately 0.5 cm² of leaf tissue were chopped with a sharp blade submerged in 1 ml nucleus extraction buffer (HR, high resolution, A solution, [Partec, CA](#)) and then incubated in the same buffer during 1.5 min. After being filtered, the solution was incubated 1 min with [Partec, CA](#). ([De Schepper et al. 2001](#); [Sari et al. 1999](#)). The different flow cytometer parameters were compared with untreated material to secure well defined and reproducible readings. The nuclear DNA content of colchicine treated plants was used in these determinations.

For the phenotype analysis of the recovered treated plants, the diameter of ten flowers and the size of the ten leaves were gauged. The stem diameter was measured at the third node. Also, the third leaves pair was chosen to measure the ratio length/ wide to establish the leaf shape. The colour of fifteen leaves and flower was measured by a Minolta CR 321 colorimeter.

Statistical analysis was performed using ANOVA and Tukey test (95%) supported by Statistica 2.0.

Results and Discussion

Tissue culture

Disinfection experiment showed an efficiency of 80%. *B. monnieri* did not showed difficult *in vitro* conditions. Neither browning nor oxidation processes were detected at the amount and sodium hypochlorite used.

[Table 1](#) shows the results obtained with the nodal segments of *B. monnieri*. Significant differences in the shoots multiplication rate were detected between the treatments containing 0.25 mg/L BAP (18.37 and 17.94 shoots/explant, respectively) and the other treatments. The treatment containing 1.0 mg/L BAP showed callus production and a very important tissue vitrification. Callus production was important in the treatment with 0.5 mg/L BAP, no vitrification was observed in this treatment. Neither callus formation nor vitrification process were found in the 0.25 mg/L BAP treatments, and except for the 1.0 mg/L BAP treatment, in all the others the shoots developed from the bottom of the explant and at the edge of the leaves ([Figure 1a](#)). All the shoots rooted spontaneously. No problem was detected for the acclimatization step. The plants transferred to pots were successfully rusticated. These results indicate that for an efficient micropropagation of *B. monnieri* and in order to avoid undesirable responses, the levels of BAP must be carefully adjusted. In fact, to start with the *in vitro* polyploidization experiments, the *in vitro* micropropagation is required as the first step. The tissue culture experiment showed that *B. monnieri* presented a strict hormonal and nutritional requirement.

Contrasting with the results herein, [Tiwari et al. \(2001\)](#) found, in an experiment in which he compared the effects of different cytokinin concentrations, that for *B. monnieri* BAP concentrations higher than 1.0 mg/L were the adequate for *in vitro* multiplication of this species. Not enough information is available to explain adequately the difference between the result presented here and that by [Tiwari et al. \(2001\)](#).

In vitro plant polyploidization

Complete, viable plants of *B. monnieri* were obtained from *in vitro* growing conditions without colchicine treatment. Multiplication rate was similar for both, treated and control plants. This is in agreement with those reported previously by [Escandón et al. \(2005\)](#) with *Scoparia mollis*, another *Schrophulariaceae* species working with the same colchicine doses (0.001 and 0.002 mg/L for 48 hrs). The only difference observed under *in vitro* conditions between the control and colchicine treated plants was growing capacity: treated plants grew less compared to control ([Figure 1b](#)).

Two tetraploid individuals out of 150 colchicine treated plants were recovered. [Figure 2](#) shows the peak readings obtained by flow cytometry analysis of a mixture of diploid and tetraploid cells. The peak "1", with a mean relative DNA content of 23.94, corresponds to the control cells, whereas the peak "2", with mean relative DNA content of 50.85 corresponds to the tetraploid cells. The cell concentration of the mixture was 24,108 cells/mL and the total populations cells counted was 865.

Leaf area, flower diameter, stem diameter and number of internodes were significantly different in the recovered tetraploid plants compared to the control ([Table 2](#)). [Figure 3](#) shows the differences in size, aspect and shape between flowers (panel "a") and leaves (panel "b") referred to in [Figure 2](#). In panel "c" it can be seen that the different growth capacity observed *in vitro* ([Figure 1b](#)) is maintained *in vivo*.

Colour analysis made both in treated and control plants is summarized in [Figure 4](#). Panel "a" shows the different distributions of colour intensity of the tetraploid plant flowers compared to control. Differences in colour, size and firmness are apparent in panel "b" in which tetraploid (a) and diploid flowers are shown in detail. Enlargement of organs (flowers and leaves), intense colours, hardier and more robust plants, thicker and more rigid foliage, an discernible increase in tolerance to different stresses, and the resistance to diseases and pests ([Petit and Callaway](#)

associated with chromosome doubling, a frequent natural event in ornamental species (Horn, 2002). Thus, chromosome doubling is accepted as a source of evolution of flowering plants, and can benefit from it for the domestication of certain genotypes (Van Tuyl and Lim, 2003). This strategy was extensively used during the last 30 years in many species such as banana (Ariffin, 2002), grapes (Notsuka et al. 2000), blueberry (Lyrene and Perry, 1982), potato (Horn, 1981), and sugarcane (Heinz and Mee, 1970). Under *in vitro* controlled conditions polyploidization was employed in several ornamental crops, such as Alocasia (Thao et al. 2003), Rhoeo (Eeckaut et al. 2002; Väinölä, 2000), Cyclamen (Takamura and Miyajima, 1996; Ishizaka and Hagiwara, 1994).

It was widely demonstrated that the *in vitro* colchicine treatment is a powerful tool for ornamental germplasm (Horn, 2002). In our laboratory interesting results were obtained in different genera. The results herein add to previous studies in the genera *Scoparia* (Escandón, 2005) and *Calibrachoa* (Hagiwara et al. 2002).

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