

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

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

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 colchicine solution 0.001 and 0.01% P/V, in 1% DMSO. Segments submerged in water and in DMSO 1% aqueous solution were used as controls. DNA contents from recovered plants was measured and characterized and their phenotype described. Two tetraploid plants originated from independent events were detected. These plants showed significant differences in size and colour both in leaves and flowers compared to untreated controls.

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 restorer 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. 2005). These improved ornamental products could be either included into a breeding program or transferred to potential users together with the technology developed.

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 Ali INTA-JICA.

MATERIALS AND METHODS

Tissue culture

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Table 1. Nodal segments response of *B. monnieri* toward different BAP concentrations.

| Treatment | BAP (mg/L) | | | | |
|-----------|------------------------------|------------------------------|-------------------------------|-----------------------------------|---------|
| | 0.0 | 0.10 | 0.25 | 0.50 | 1.00 |
| Response | (s) 7,25 ^T ± 0,39 | (s) 8,55 ^T ± 0,38 | (s) 18,37 ^J ± 0,38 | (c) (s) 17,94 ^J ± 0,38 | (c) (v) |

Letters in brackets indicates the main response for each treatment. (s): shoot. (c): callu. (v): vitrified tissue. Average and SD of the shoots number per explant of two repetitions n = 20 each one, 95% of confidence. T and J indicate no differences between the means observed.

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 cytokinin concentrations. The MS medium used for the culture was supplemented with 20 g/L sucrose, 7 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°C ± 2°C.

After one subculture three or four centimetres long rooted plantlets were transferred to an 8.0 cm diameter pot containing turf, perlite and vermiculite 3:1:1 (V/V) (Escandón et al. 2003) and kept inside a humidity chamber. The nylon bags used to make the humidity chamber were perforated once per day 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 containing 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 0.25 mg/L BAP. Culture conditions as well as temperature an photoperiod were the same than in the tissue culture experiments (16 hrs light photoperiod under an irradiance of 3,000 lux, and an average temperature of 23°C ± 2°C).

The ploidy level was determined using the flow cytometer (Partec, CA), following commercial indications: approximately 0.5 cm² of leaf tissue were chopped with a sharp blade submerged in 0.5 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 HR B, Partec, CA. (De Schepper et al. 2001; Sari et al. 1999). The different flow cytometer parameters were adjusted with untreated material to secure well defined and reproducible readings. The nuclear DNA of 150 colchicine treated plants was used in these determinations.

For the phenotype analysis of the recovered treated plants, the diameter of ten flowers and ten stems and the size of the ten leaves were gauged. The stem diameter was measured at the third leaves pair node. Also, the third leaves pair was chosen to measure the ratio length/ wide to establish the size of the leaves. 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 the software Statistica 2.0.

RESULTS AND DISCUSSION

Tissue culture



Figure 1. a) Development of lateral buds, *de novo* morphogenesis from the bottom of the explant and from leaves edge. b) Growth capacity difference between a tetraploid plant (centre) and their control.

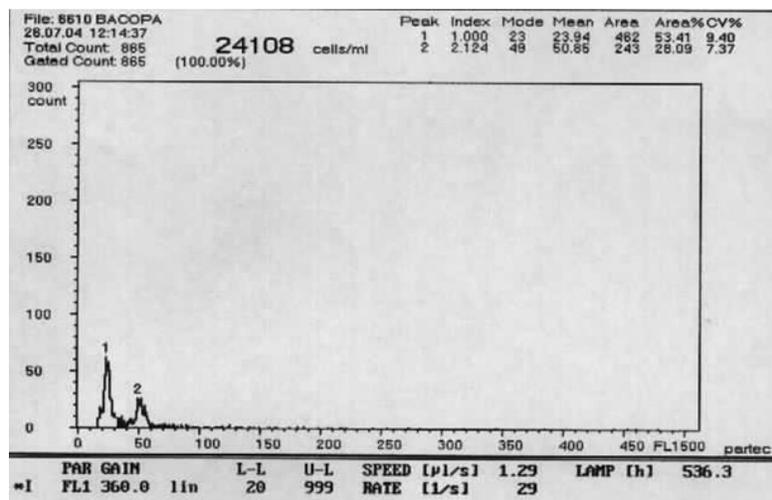


Figure 2. Profiles obtained by flow cytometry of a mixture of a diploid plant and a putative tetraploid one. The peak indicated as "1" corresponds to the control without treatment, and the peak indicated as "2" corresponds to a plant treated with a colchicine dose of 0.001% 48 hrs.

Disinfection experiment showed an efficiency of 80%. *B. monnieri* did not showed difficulties to grow *in vitro* conditions. Neither browning nor oxidation processes were detected at the amount of ethanol 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 and 0.5 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. Although callus production was important in the treatment with 0.5 mg/L BAP, no vitrification was detected in this treatment. Neither callus formation nor vitrification process were found in the 0.25; 0.1 and 0.0 mg/L BAP treatments, and except for the 1.0 mg/L BAP treatment, in all the others the novo shoots developed from the bottom of the explant

Table 2. Relative sizes and SD between Ali INTA-JICA and their control. The datas are the average of 10 determinations. 1) mm²; 2) mm; 3) internodal number within 5 cm of stem measured from the apex.

| Organ | Control | Ali INTA-JICA |
|------------------------------|--------------|---------------|
| Leaf area ¹ | 30.81 ± 6.13 | 79.30 ± 11.72 |
| Flower diameter ² | 9.4 ± 1.5 | 15.1 ± 1.28 |
| Stem diameter ² | 0.86 ± 0.09 | 1.76 ± 0.24 |
| Internodal N° | 4.0 ± 0.8 | 7.6 ± 0.67 |

and at the edge of the leaves (Figure 1a). All recovered shoots rooted spontaneously. No problem was detected for the acclimatization step, all plants transferred to pots were successfully rusticated. These results indicate that for an efficient *in vitro* 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 fine-tuning of *in vitro* micropropagation is required as the first step. The tissue culture experiment in this study 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 they 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 specie. Not enough data are available to explain adequately the difference between the result presented here and those reported by Tiwari et al. (2001).

***In vitro* plant polyploidization**

Complete, viable plants of *B. monnieri* were obtained from *in vitro* growing conditions after the colchicine treatment. Multiplication rate was similar for both, treated and control plants. This result is in agreement with those reported previously by Escandón et al. (2005) with *Scoparia montevidiensis*, another *Schrophulareaceae* species working with the same colchicine doses (0.001 and 0.01%, 24 and 48 hrs). The only difference observed under *in vitro* conditions between the control and the colchicine treated plants was growing capacity: treated plants grew less compared to the controls (Figure 1b).

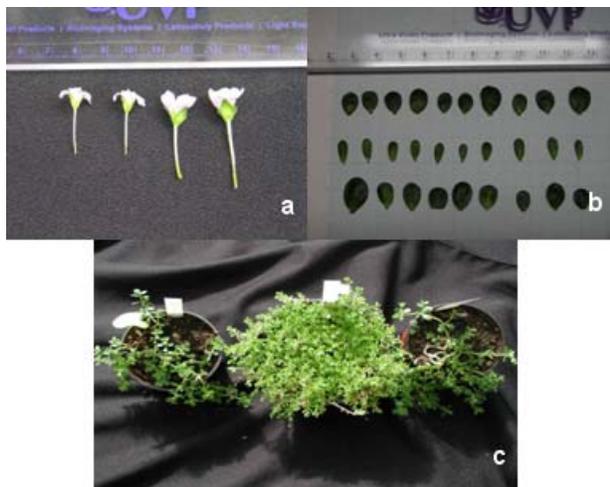


Figure 3. Relative sizes of:
 a) Flowers.
 b) Leaves.
 c) Entire plants (diploids arrowed).

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 tissues. Peak “1”, with a mean relative DNA content of 23.94, corresponds to the control cells, whereas 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 the

Table 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 the controls. Differences in colour, size and firmness are apparent in panel “b” in which tetraploid (arrowed) and diploid flowers are shown in detail. Enlargement of organs (flowers and leaves), intensification of colours, harder and more robust plants, thicker and more rigid foliage, an discernible increase in the tolerance to different stresses, and the resistance to diseases and pests (Petit and Callaway, 2000) are 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 breeders benefit from it for the domestication of certain genotypes (Van Tuyl and Lim, 2003). Actually, this strategy was extensively used during the last 30 years in many species such as banana (Baziran and Ariffin, 2002), grapes (Notsuka et al. 2000), blueberry (Lyrene and Perry, 1982), potato (Hermsen et al. 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), *Rhododendron* (Eeckaut et al. 2002; Väinölä, 2000), *Cyclamen* (Takamura and Miyajima, 1996; Ishizaka and Uematsu, 1994).

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

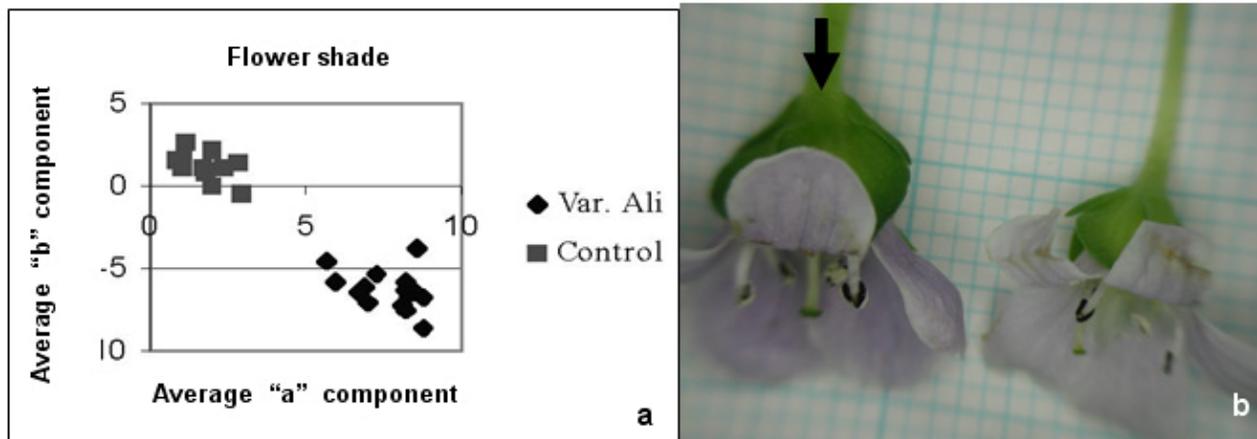


Figure 4. a) Distribution of the colour shade of: the tetraploid and the diploids flowers, where “a” and “b” are parameters measured by the Minolta CR 321 colorimeter. b) The row indicates the tetraploid flower; the differences in colour, size and firmness between them are evident.

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