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Shoot Organogenesis and Plantlet Regeneration from Stem Explants of *Cleome rosea* Vahl (Capparaceae)

Claudia Simões*, Alessandra S. Santos, Norma Albarello, Solange Faria Lua Figueiredo

Departamento de Biologia Vegetal/Laboratório de Biotecnologia de Plantas (LABPLAN) – Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro (UERJ), Rua São Francisco Xavier, 524, PHLC, s/509, Maracanã, RJ, CEP 20550-013, Brazil

Abstract

The medicinal value of the genus *Cleome* justifies biotechnological studies of *Cleome rosea*, a Brazilian annual species from sandy coastal ecosystems (*restinga*), which have been submitted to an intense process of antropogenic degradation. In the present work, was analyzed the influence of cytokinins, 6-benzyladenine (BA) and 6-furfurylaminopurine (kinetin) added to the Murashige and Skoog medium (MS), on the proliferation capacity of explants from the stem axis (hypocotyl, node and internode) for a period of five monthly subcultures (150 days). Regardless of the explant sources, plantlet regeneration by direct and indirect organogenesis was observed. The largest number of shoots proliferated through direct organogenesis was obtained on medium with 4.4 μM BA. Also, the highest proliferation capacity through indirect organogenesis was found on medium with 4.4 μM BA + 4.6 μM kinetin. The presence of kinetin alone was not effective for multiplication of the species. Elongation and rooting were obtained when shoots were transferred onto growth regulator-free medium, and acclimatization rates from 70% to 81% were achieved depending on explant sources used. Plants were then successfully established in soil and showed normal phenotypes.

Key words: Cytokinin, medicinal plant, micropropagation

Introduction

The genus *Cleome* has a tropical and subtropical distribution (Brummitt 1992). Several species have been objects of phytochemical and pharmacological studies with promising medicinal applications (Di Stasi et al. 1994; Selloum et al. 1995, 1997; Nicola et al. 1996; Nagaya et al. 1997; Hashem and Wahba 2000). *Cleome rosea* is a herbaceous annual species, a native of Brazil, and with ornamental value, due to its pink and attractive inflorescences. It is found in sandy coastal ecosystems (*restinga*), which have been submitted to an intense process of antropogenic degradation, that has been committing the survival of the species. This can be verified by the fact that populations previously registered are not found now in many of these areas.

Only two works were reported about biotechnological researches with the genus *Cleome* (Nassem and Jha 1994, 1997). The present work is the first about *in vitro* propagation with the species *C. rosea*. The medicinal potential of the species is being investigated, having already been obtained positive results with methanolic crude extracts related to anti-inflammatory and antioxidant activities (Simões C, unpublished results). The unique phytochemical study with the species is related to the isolation and identification of a glucosinolate, the glucocaparine, in aqueous ethanol crude extract of its seeds (Ahmed et al. 1972). The objective of this study was to establish a protocol of *in vitro* propagation for mass production of *C. rosea* in a short period of time.

Materials and Methods

Bud induction and shoot proliferation

Two month-old plants, developed from nursery-grown

* Corresponding author, E-mail: csimoes04@yahoo.com.br
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seeds, were used as a source of explants. Plants were washed with 1% triclosan detergent (Fisiohex II), rinsed in running tap water, followed by removal of roots. Aerial part was disinfested by immersion in 0.5% NaOCl plus 0.05% Tween 80 (v/v) for 10 min, under agitation, and then rinsed three times with sterile distilled water (5 min each), under agitation. Explants (1 cm) were excised from the stem axis, from sections corresponding to the hypocotyl and the epicotyl, separated in node (with 1-2 lateral buds) and internode. The basal medium consisted of MS mineral salts and vitamins (Murashige and Skoog 1962), 30 g/L sucrose and three concentrations of BA (1.1, 2.2, 4.4 μM) and kinetin (1.2, 2.3, 4.6 μM) used alone or in combination. The pH was adjusted to 5.8 before gelling with 8 g/L agar (Merck) and autoclaved at 121℃ for 15 min. Five explants were inoculated in flasks (60 x 80 mm) containing 30 mL of the culture medium. Four replicates were used for each treatment, with a total of 20 explants per treatment. The flasks were closed with polypropylene caps and incubated in a growth chamber at 26 ± 1℃, under a 16-h photoperiod provided by cool daylight fluorescent and Grolux tubes (45 μmol/m^{2}s). The regeneration frequency for direct and/or indirect organogenesis (percentage of explants with shoot proliferation), the mean number of shoots per explant and the length of shoots were evaluated. In cultures from node explants, the adventitious and axillary shoots were considered together as direct organogenesis. At each subculture, explants without bud induction or with shoots <0.5 cm in length were recultured under the same conditions. Shoots (≥ 0.5 cm) were excised and subcultured onto fresh medium monthly. Each experiment was evaluated for five subcultures (150 days).

Shoot elongation and rooting

After five subcultures, 45 shoots multiplied from each type of explant mainly by direct organogenesis (2 to 2.5 cm) on medium supplemented with 4.4 μM BA were transferred to flasks (110 x 50 mm) containing 30 mL of growth regulators-free MS medium. Three shoots were inoculated per flask and closed with polypropylene caps. The cultures were maintained for 25 days under the same physical conditions, mentioned above, and shoot elongation and rooting percentage were measured.

Acclimatization

Forty elongated and rooted plantlets (>4.0 cm aerial part), derived from cultures of each explant type, were selected for acclimatization. The plantlets were washed to remove adhering medium and transferred to plastic pots (75 x 70 mm) containing a mixture of soil and sand (1:1). Each pot was covered with a transparent plastic cloche (200 mm in height). The pots were incubated under environmental conditions (25℃ - 30℃) and 12-h photoperiod, for four weeks. After the first week, the cloches were perforated (five 4 mm diameter perforations) in order to reduce the relative humidity in the pots and removed at four weeks from planting. The acclimatization process was evaluated by the percentage of healthy plants after three months of the beginning of the process.

Statistical Analysis

Data was analysed using an experimental design of randomized blocks. Each block was represented by one repetition. Four repetitions of five explants were used for the evaluation of the regeneration frequency and the mean number of shoots. Fifteen repetitions of three shoots were considered to evaluate the rate of elongation and rooting. Four repetitions of ten plantlets were used to evaluate the acclimatization process.

Data was analysed using one-way analysis of variance (ANOVA) and the means were compared by Tukey test at the 5% level of significance. The statistical program used was MSTATC.

Results and Discussion

Bud induction and shoot proliferation

On all media tested the development of axillary buds in node explants was observed after seven days in culture. Adventitious buds were visible by direct and/or indirect organogenesis between two and three weeks from hypocotyl, node and internode explants, mainly on media supplemented with BA alone or in combination with kinetin. The direct-shoots developed on the apical region of the explants, while the indirect-shoots originated from callus formed at the basal region (Figure 1A). The callus showed internally compact consistence and friable surface with beige coloration.

In previous studies with other species of the genus *Cleome*, when auxins were used in association with cytokininsonly only indirect organogenesis was achieved (Nassen & Jha 1994; 1997). However, when cytokinins were used alone, was observed direct-shoots (Nassen and Jha 1997). Based on these reports, in the present work, we used only cytokinins for shoot-bud induction and multiplication.

Media with BA were more effective on shoot multiplication, with the highest values of regeneration frequency by direct and indirect organogenesis (Table 1). The statistical analysis

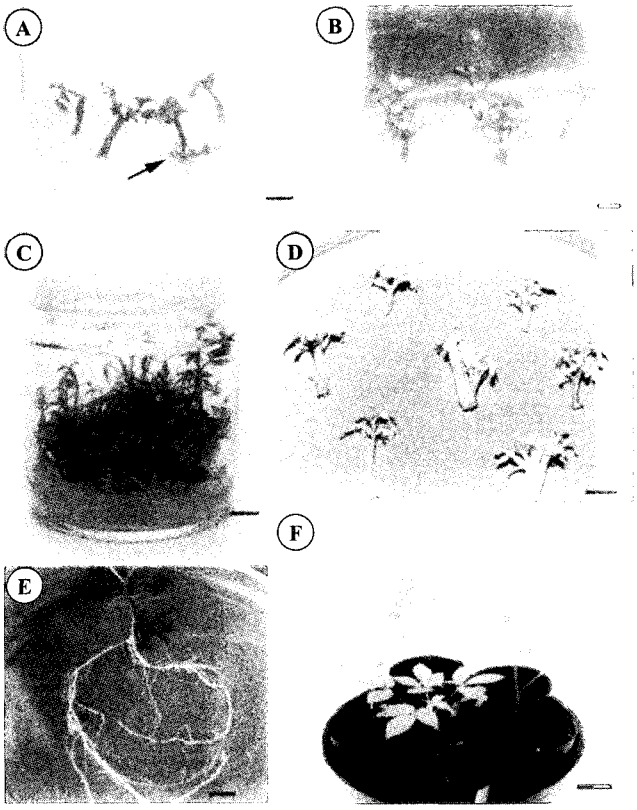

Figure 1. *In vitro* propagation of *Cleome rosea* from hypocotyl explants cultured in medium with 4.4 μM BA. Shoot development by direct and indirect (arrow) organogenesis after six days in culture (A); Proliferated shoots cultivated during seven days (B) and during 30 days (C), after four subcultures; Fasciated shoots (D); *In vitro* rooting after 30 days on growth regulators-free medium (E); Three month-old acclimatized plants (F).
Scales: A - 0.69 cm; B - 0.52 cm; C - 0.87 cm; D - 1.01 cm; E - 0.96 cm; F - 3.56 cm

of shoot proliferation by direct organogenesis showed a significant efficiency of media supplemented with BA alone and in association with kinetin.

The addition of 4.4 μM BA and 4.4 μM BA + 4.6 μM kinetin, resulted in a significant increase in the regeneration frequency by indirect organogenesis in all cultures. However, in internode explants, medium supplemented with 2.2 μM BA has also shown a similar response (Table 1).

When media were supplemented with kinetin alone, in spite of an efficient proliferation capacity not being observed, the regeneration frequency by direct organogenesis prevailed on the indirect organogenesis (Table 1). The induction of small roots and subsequent growth of the stem axis was observed in most of the regenerated shoots after the first subculture. Hussey (1986) reported that the use of cytokinins often inhibits rhizogenesis. However, as well as in *C. rosea*, in cultures of *Alpinia galanga* (Borthakur et al. 1998) and *Eclipta alba* (França et al. 1995) rhizogenesis was observed when shoots were cultured on media containing only kinetin.

Regardless of the origin of the explant, a progressive increase in the mean number of shoots through direct organogenesis was verified when BA was used alone (Table 2 and Figure 1B, 1C). The supplementation of 4.4 μM BA provided the best statistical results, however, in cultures from internode explants the association of 2.2 μM BA and 2.3 μM kinetin also achieved a similar statistical value.

Through indirect organogenesis, the highest number of shoots was found on media supplemented with the highest concentration of BA alone and in association with kinetin in all cultures, without significant differences among them (Table 2). It should be considered that, in cultures from

Table 1. Effect of BA and KIN on regeneration frequency of *Cleome rosea*, by direct and indirect organogenesis, considering five subcultures.

BA / KIN (μM)	Regeneration frequency (%)					
	Hypocotyl explants		Internode explants		Node explants	
	DO	IO	DO	IO	DO	IO
0.0 / 0.0	9 g	3 d	9 d	2 d	14 e	1 c
1.1 / 0.0	89 ab	61 b	92 ab	66 b	87 bc	54b
2.2 / 0.0	97 a	27 c	97 ab	81 ab	96 ab	51 b
4.4 / 0.0	97 a	83 a	98 ab	83 ab	95 ab	96 a
0.0 / 1.2	27 f	9 d	45 c	2 d	40 d	3 c
0.0 / 2.3	55 de	7 d	49 c	5 d	38 d	1 c
0.0 / 4.6	37 ef	8 d	35 c	1 d	57 d	3 c
1.1 / 1.2	81 bc	59 b	96 ab	38 c	92 abc	65 b
2.2 / 2.3	88 ab	64 b	99 a	41 c	99 a	61 b
4.4 / 4.6	70 cd	86 a	91 b	86 a	78 c	95 a

DO - direct organogenesis; IO - indirect organogenesis
Same letters on each column are not significantly different by Tukey test at 5%.

node explants, the values reached on these media by indirect organogenesis were superior to the ones obtained by direct organogenesis. A low shoot multiplication on media supplemented only with kinetin was also reported by Hu and Wang (1983) in species from different families. Such fact was also verified in cultures from *C. rosea* and might have been due to a differentiated number of protein receptors with likeness for BA or kinetin. Another hypothesis could be the enzymatic degradation of kinetin to the enzyme cytokinin oxidase or other enzymes, as mentioned by Forsyth and Van Staden (1987).

Shoot proliferation was observed on growth regulators-free medium only during the first month in culture, however with low rates. Probably, the regeneration found during this period had been due to the presence of endogenous hormones from the donor plant. The subcultivated shoots just re-generated roots. The adventitious radicular system was constituted by numerous roots with 5.4 ± 1.8 cm of length and light green coloration in the proximal region near the shoots and white in the opposite extremity, with well developed hair root zones.

While the higher proliferation capacity was verified during the fourth or fifth subcultures on media with BA or with BA + kinetin (data not shown), this response was verified mainly during the first month in culture on media only with kinetin.

The propagated shoots did not achieve a long length. In a general way, they presented a mean size between 1.0 to 3.0 cm during the subcultures. This small growth of shoots was also observed by Tyagi and Kothari (2001) for *Capparis decidua* proliferated on media with BA. According to Brassard et al. (1996), although the cytokinins promote bud induction and shoot proliferation, they can inhibit elongation.

A phenomenon observed during the experiments was the formation of fasciated shoots after the third subculture on media with BA, alone or in association with kinetin. They presented a wide stem and although slightly compressed flat, exhibited ondulations resembling the fusion of stem axis of several shoots (Figure 1D). Their number was directly related to the increase of the cytokinin concentration and time in culture. The same morphological aspect had been observed on cultures of *Capparis spinosa* on media with cytokinin (Safrazbekyan et al. 1990). The authors suggested that the phenomenon was associated with the development of dormant axillary meristems and could be prevented with the alternation of subculturing on cytokinin-enriched and hormone-free media. Srivastava and Glock (1987) observed fasciated shoots in cultures of *Betula pendula* on medium supplemented with p-fluorophenylalanine (FPA), a phenylalanine analog. The phenomenon of fasciated shoots has been reported by Nieuwkerk et al. (1986), Yusnita et al. (1990), Preece et al. (1991) and Huetteman and Preece (1993) in cultures maintained on media with thidiazuron (TDZ), a urea derivative with potent cytokinin-like response. According to Geneve (1990), in many cases, fasciation came about through spontaneous mutations.

Another aspect observed during the experiments was the change in shoot coloration independent of explant sources. The stem and sometimes the leaves of some proliferated shoots took on a violaceous coloration in all media tested.

Table 2. Effect of BA and KIN on the mean number of shoots per explant of *Cleome rosea*, by direct and indirect organogenesis, considering five subcultures.

BA / KIN (μM)	Number of shoots/explants					
	Hypocotyl explants		Internode explants		Node explants	
	DO	IO	DO	IO	DO	IO
0.0 / 0.0	0.60±1.34 e	0.54±1.20 cd	0.84±1.84 d	0.44±0.98 de	0.32±0.52 f	0.06±0.13 c
1.1 / 0.0	2.51±0.60 c	1.69±0,27 b	2.52±0.47 c	2.51±0.53 bc	2.45±0.45 cd	1.66±0.33 b
2.2 / 0.0	3.61±0.64 b	1.19±0.77 bcd	3.82±1.17 b	3.48±0.97 b	2.70±0.74 bc	1.63±0.35 b
4.4 / 0.0	6.57±1.19 a	4.81±1.74 a	4.84±0.98 a	3.57±0.93 ab	4.89±0.89 a	7.00±1.48 a
0.0 / 1.2	0.54±0.29 e	0.37±0.21 d	0.81±0.63 d	0.10±0.15 e	0.75±0.53 ef	0.17±0.23 c
0.0 / 2.3	0.90±0.33 d	0.20±.014 d	1.11±0.68 d	0.17±0.15 e	0.87±0.55 ef	0.09±0.20 c
0.0 / 4.6	1.22±0.25 de	0.73±0.20 bcd	0.97±0.38 d	0.11±0.10 e	1.33±0.70 e	0.23±0.26 c
1.1 / 1.2	1.64±0.50 c	1.88±0.61 b	2.15±0.33 c	1.08±0.34 de	2.03±0.35 d	1.80±0.57 b
2.2 / 2.3	2.52±0.62 c	1.69±0.93 bc	4.19±0.68 ab	1.43±0.68 cd	3.20±0.68 b	1.93±1.05 b
4.4 / 4.6	3.46±0.50 b	6.01±1.20 a	3.78±0.94 b	4.65±1.38 a	3.09±1.51 bc	7.33±1.68 a

DO - direct organogenesis; IO - indirect organogenesis
Data represent mean ± standard deviation
Same letters on each column are not significantly different by Tukey test at 5%.

This phenomenon was observed in the first week after the beginning of the experiments and of each subculture, tending to disappear gradually through time until the next subculture. Fragments of such organs took on a bluish coloration when submitted to an atmosphere saturated with hydroxide of ammonia vapour and became red with hydrochloric acid vapour. This alteration in the coloration on account of pH is an indicative of the presence of anthocyanic compounds (Hopkins 1995). The synthesis of these compounds might have been stimulated by the mechanical and hydric stress suffered during the manipulation of the shoots in each subculture as also reported by Chalker-Scott (1999).

Shoot elongation and rooting

In the present work, the transfer of the shoots proliferated on medium with 4.4 μM BA to growth regulators-free medium stimulated shoot elongation, observed only after the development and/or establishment of the roots. After 25 days of culture, an increase in stem axis growth up to 2-fold of length was found, without statistical differences with regard to the type of explants used. Shoot elongation of *Cleome viscosa* (Nassem and Jha 1994) and *Cleome gynandra* (Nassem and Jha 1997) was also found on medium without plant growth regulators.

The choice of the growth regulators-free medium to rooting was based on the results verified on medium M1, when the development of roots in the propagated shoots was observed. Rooting was verified within three-five days, reaching the roots a length of 6.5$\pm$2.1 cm after 25 days in culture. High rooting percentages of regenerated shoots (91 to 94%) were achieved without statistical differences among the explant sources. The process was successfully induced in *in vitro* conditions (Figure 1E).

Different from what was observed for *C. rosea*, the supplementation with auxin was necessary for root regeneration in other species of Capparaceae: in *Capparis spinosa* AIA was used (Rodriguez et al. 1990), IBA for *Cleome viscosa* (Nassem and Jha 1994), *Cleome gynandra* (Nassem and Jha 1997) and *Capparis decidua* (Deora and Shekhawat 1995) and ANA for *Crataeva nurvala* (Sharma and Padhya 1996).

Acclimatization

After three months of the transfer to *ex vitro* conditions, acclimatization rates of 81% were found for plants obtained from hypocotyl, 74% from node and 70% from internode cultures, without statistical difference among them. Acclimatization was reached with sucess (Figure 1F), as the methodology used prevented the plants from being submitted to an abrupt change of atmospheric humidity. The efficient capacity of acclimatization of *C. rosea* was also observed in many cultures of herbaceous species (Grattapaglia and Machado 1998). The fact that these species present a great capacity to differentiate organs that guarantee the fixation (roots) and the gaseous changes (leaves), facilitates the acclimatization process.

The *in vitro*-regenerated plants were phenotypically normal and were transferred to *in situ* conditions completing the biological cycle after 8 months with flowering and frutification.

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