BIOLOGIA PLANTARUM 46 (2): 181-185, 2003

Micropropagation of *Crataeva nurvala*

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Abstract

A simple protocol for mass multiplication of *Crataeva nurvala*, a medicinal tree, from seedling-derived explants is described. Six different types of explants (cotyledonary nodes, epicotyl nodes, hypocotyl segments, first pair of leaves, cotyledons, and root segments) developed shoots on Murashige and Skoog's (MS) basal medium or the same supplemented with different concentrations of 6-benzylaminopurine (BAP). Among the explants tested for caulogenic potential, only the epicotyl and cotyledonary nodal explants developed shoots on MS basal medium, while on BAP (0 - 2.0 mg dm^{-3}) adjuvated media all the explants exhibited caulogenesis. The optimum concentration of BAP varied for these explants. The shoots could be rooted on half strength MS with 0.02 mg dm^{-3} α-naphthalene acetic acid to get plants, which have been transferred to soil. The explants from *in vitro* regenerated shoots also possessed a similar caulogenic potential.

Additional key words: caulogenesis, growth regulators, seedling-derived explants.

Introduction

The long generation cycles and allogamous nature of ligneous species pose major limitations in conventional methods of breeding. *In vitro* techniques not only allow mass multiplication and propagation under pathogen-free conditions, but also override the dependence on season for availability of the plant material. In addition, under *in vitro* conditions, genetic manipulation of the plant material by employing techniques such as genetic engineering, parasexual hybridization, *etc.*, is also facilitated. To fulfil these objectives, a well-standardized tissue culture protocol is a prerequisite. Keeping this in mind, standardization of a protocol for *in vitro* multiplication of a medicinal tree, *Crataeva nurvala*, belonging to the family *Capparidaceae*, was attempted.

Crataeva nurvala is a medium-sized deciduous tree, with trifoliate leaves. The extract of its bark is traditionally used for treating urinary tract diseases. The natural propagation of this tree is hampered because of poor seed germination and heavy infestation by insects.

There are earlier reports on regeneration of *Crataeva nurvala* from the explants derived from mature trees. Inamdar *et al.* (1990) reported somatic embryogenesis in callus cultures derived from shoot apices of *Crataeva nurvala*. Sharma and Padhya (1996) obtained shoots from the axillary buds of *in vitro* cultured nodal explants. However, there has been no report of regeneration of plants from juvenile explants. The present investigation is the first report on recurrent production of multiple shoots using juvenile explants of *Crataeva nurvala*.

Materials and methods

Fruits of *Crataeva nurvala* were collected in August, each year during a period of three years (1998 - 2000). Seeds were depulped using 5 M HCl followed by repeated washing in tap water. Seeds were then air-dried and stored.

Seeds were treated with concentrated H_2SO_4 for two

Received 28 November 2001, *accepted* 16 May 2002.

Abbreviations: BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; MS - Murashige and Skoog's medium; NAA - α-naphthalene acetic acid.

Acknowledgements: The financial assistance in the form of a research project from the University Grants Commission, New Delhi is gratefully acknowledged.

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hours (for softening the seed coat and for removing the adhering pulp). After thorough washing in tap water, seeds were treated with 1 % (m/v) *Hilzim* (carbendezine) for 10 min. Subsequently, seeds were washed in 5 - 6 drops of Tween 20 in 25 cm^3 of distilled water for 5 min and thereafter, with 0.2 % (m/v) mercuric chloride for 10 min. After rinsing with sterile distilled water four to five times, seeds were left overnight in water for imbibition. These were inoculated on Knop's medium supplemented with 1 % (m/v) sucrose and gelled with 0.8 % (m/v) agar. After 4 - 5 weeks of inoculation, seedlings attaining height of 5 - 6 cm were used as source of various explants. Cotyledonary nodes, epicotyl nodes, first pair of leaves, hypocotyl segments, root segments and cotyledons were inoculated on Murashige and Skoog's medium (1962), gelled with 0.8 % (m/v) agar and supplemented with 3 % sucrose. The media were adjuvated with different concentrations of BAP (0 - 2 mg dm^{-3}). For rooting, 2.5 - 4.0 cm long *in vitro* regenerated shoots were excised and placed on half-strength MS medium gelled with 0.8 % agar and supplemented with NAA (0.02 mg dm^{-3}) and 2 % sucrose. After excision of differentiated shoots, the residual explants were sub-cultured, on fresh shoot induction medium. Nodal explants from the *in vitro* regenerated shoots were also cultured on MS media supplemented with BAP (0.1 - 2.0 mg dm^{-3}).

Before autoclaving at 103.36 kPa at 121 °C for 15 min, pH of the media was adjusted to 5.8 with 1 M NaOH or 1 M HCl. The cultures were incubated at 25 ± 2 °C, under continuous light of 17.76 μmol m^{-2} s^{-1} provided by cool daylight fluorescent tubes. For hardening, 6- or 7-week-old plantlets were transferred to small disposable cups containing autoclaved garden soil. These were maintained under culture room conditions described above. However, to maintain high humidity, pots were covered initially with transparent polythene sheets and were irrigated with twenty times diluted inorganic salt solution of MS medium for initial 2 - 3 weeks, and thereafter, with tap water. The *in vitro* hardened plants were then transferred to garden pots and subsequently, to the field.

The experiments described here were repeated at least once and each time 24 replicates per treatment were raised. The data were subjected to statistical analysis employing χ^2 test ($P = 0.05$).

Results and discussion

The seeds germinated after 1 - 2 weeks of inoculation and seedlings (Fig. 1*A*) attained a height of 5 - 6 cm after 4 - 5 weeks. On MS basal medium, among the different explants cultured, only epicotyl nodes and cotyledonary nodes developed shoots (one shoot per axillary bud; Table 1). However, on all concentrations of BAP supplemented media, all these explants developed shoots (Fig. 1*B-D*). The degree of caulogenic response varied among the explants and was affected by concentration of BAP. The optimum concentration of BAP, for different explants was either 0.5 or 1.0 mg dm^{-3}. Differentiation of shoots was either direct or *via* a callus phase. Likewise, in *Cardiospermum halicacabum*, both direct and indirect caulogenesis was reported from various seedling-derived explants cultured on BAP supplemented MS medium (Babber *et al.* 2001). The requirement of cytokinins in *in vitro* caulogenesis in *Crataeva nurvala* was also noted by Sharma and Padhya (1996). They observed regeneration of healthy shoots from the nodal explants excised from a mature tree and cultured on media adjuvated with BAP and kinetin, used in combination. When used individually, only fragile shoots developed, which could not be transferred to soil. However, in the present study, normal healthy shoots were regenerated on media supplemented with BAP alone. Moreover, in their study, shoots developed from incipient shoot buds, which pre-existed in the nodal explants. While in the present study, shoots differentiated even from the explants, such as roots, hypocotyl segments, cotyledons, which do not have shoot buds, in response to BAP stimulus.

Hypocotyl segments, first pair of leaves and cotyledonary explants were found to be the most responsive on medium adjuvated with 0.5 mg dm^{-3} BAP. On this medium, hypocotyl and cotyledonary explants developed, maximum number of shoots (Fig. 1*B-C*; Table 1). The leaf explants developed maximum number of shoots on 1.0 mg dm^{-3} BAP adjuvated medium (Table 1). Subbaiah and Minocha (1990) also reported multiple shoot regeneration in hypocotyl explants of *Eucalyptus tereticornis* on medium supplemented with 0.5 mg dm^{-3} BAP. Similarly, direct organogenesis from hypocotyl segments of *Tamarindus indica* has been observed on BAP adjuvated MS medium (Jaiwal *et al.* 1998). However, elongation of shoots regenerated from these three explants of *Crataeva*, was the best on medium adjuvated with 1.0 mg dm^{-3} BAP.

Among all the responding explants, cotyledonary nodes developed maximum number of shoots (an average of 6.2 ± 2.6 per explant; Table 1). Likewise, cotyledonary nodes were the most responsive, in terms of the percentage of responding explants, followed by epicotyl node and root segments. The response in terms of percentage of responding explants, of the cotyledonary nodes, was cent percent even on the medium supplemented with the lowest concentrations of BAP (0.1 mg dm^{-3}). However, maximum number of shoots per explant developed on the medium adjuvated with 0.5 mg dm^{-3} BAP and the same supported optimum elongation of the shoots. High caulogenic potential of

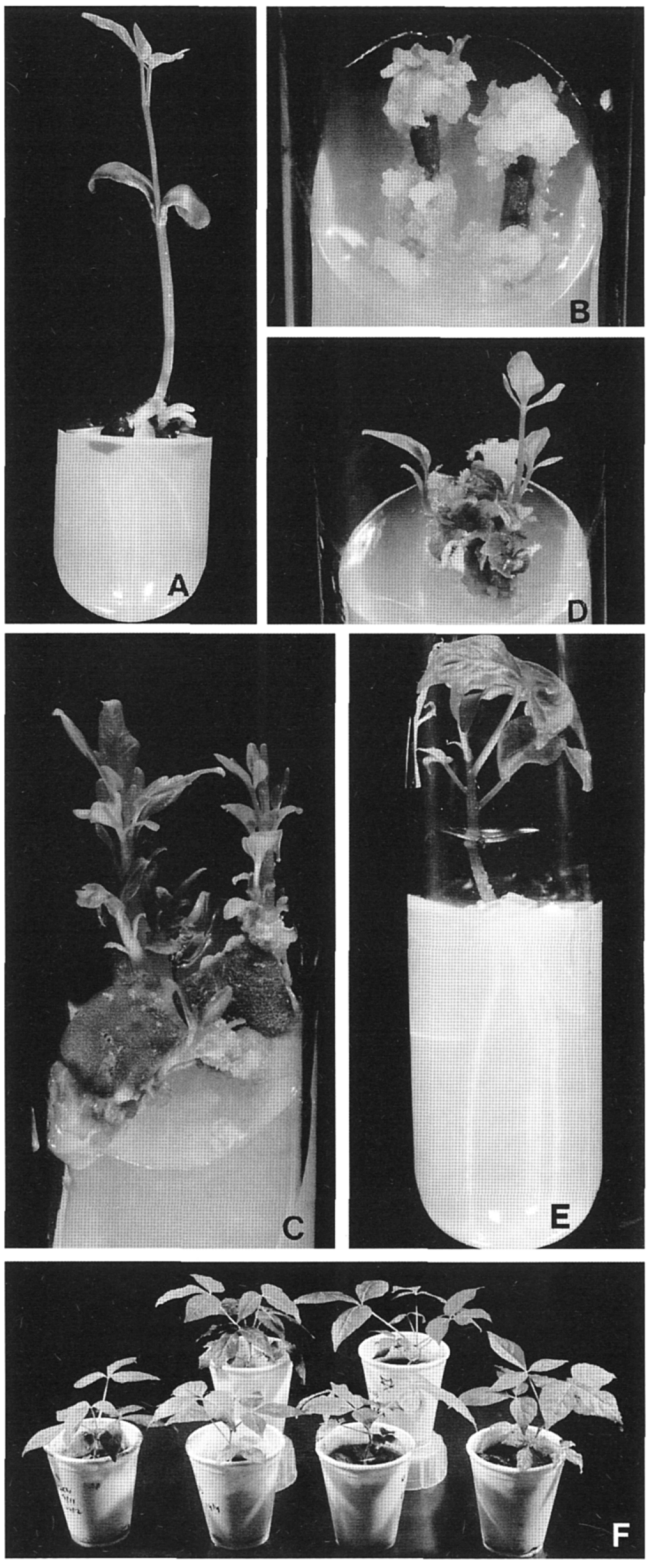

Fig. 1. *A* - four-week-old *in vitro* raised seedling; *B* - development of calli and shoot buds from hypocotyl segments after 5 weeks of culture on MS medium supple-mented with 0.5 mg dm^{-3}; *C* - development of shoots from cotyledonary explants, after 6 weeks of culture on MS medium supplemented with 0.5 mg dm^{-3} BAP; *D* - development of callus and shoots from root segments after 5 weeks of culture on MS medium supple-mented with 0.1 mg dm^{-3} BAP; *E* - a rooted shoot on 1/2 MS medium supplemented with 0.02 mg dm^{-3} NAA, after 4 weeks of transfer; *F* - plants transferred to paper cups containing garden soil for acclimatization.

Table 1. Effect of different concentrations of BAP on the *in vitro* caulogenic response of different types of explants of *Crataeva nurvala*. Means ± SE. Values followed by the same letter(s) in each column are not significantly different ($P = 0.05$).

Explant type	BAP [mg dm^{-3}]	Explants forming shoots [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
Epicotyl nodes	0	35.0^{b}	1.0 ± 0^{e}	0.53 ± 0.30^{e}
	0.1	100.0^{a}	3.4 ± 2.0^{c}	1.26 ± 1.04^{d}
	0.5	85.0^{a}	4.0 ± 1.22^{a}	1.32 ± 1.00^{b}
	1.0	83.3^{a}	3.7 ± 1.39^{b}	1.34 ± 0.87^{a}
	2.0	65.0^{a}	2.1 ± 0.86^{d}	1.31 ± 1.12^{c}
Cotyledonary nodes	0	63.2^{a}	1.2 ± 0.39^{e}	0.8 ± 0.40^{e}
	0.1	100^{a}	3.3 ± 0.66^{c}	1.35 ± 0.85^{d}
	0.5	100^{a}	6.2 ± 2.60^{a}	1.72 ± 0.96^{a}
	1.0	100^{a}	3.9 ± 2.03^{b}	1.48 ± 0.97^{b}
	2.0	100^{a}	3.2 ± 1.36^{d}	1.45 ± 1.09^{c}
Hypocotyls	0	0^{c}	0^{e}	0^{e}
	0.1	65.20^{b}	3.46 ± 1.30^{b}	1.29 ± 0.78^{d}
	0.5	91.66^{a}	3.59 ± 2.12^{a}	1.30 ± 0.63^{b}
	1.0	80.95^{a}	3.06 ± 1.06^{c}	1.58 ± 0.87^{a}
	2.0	70.0^{a}	2.29 ± 1.07^{d}	1.03 ± 0.77^{c}
Cotyledons	0	0^{d}	0^{d}	0^{d}
	0.1	0^{d}	0^{d}	0^{d}
	0.5	68.18^{a}	4.07 ± 2.02^{a}	1.06 ± 0.72^{c}
	1.0	63.64^{b}	3.50 ± 0.94^{b}	1.60 ± 0.72^{a}
	2.0	52.17^{c}	1.50 ± 0.67^{c}	1.24 ± 0.67^{b}
First pair of leaves	0	0^{e}	0^{e}	0^{e}
	0.1	54.17^{d}	3.46 ± 1.27^{c}	1.07 ± 0.59^{c}
	0.5	76.19^{a}	3.13 ± 1.36^{b}	1.40 ± 0.81^{b}
	1.0	65.20^{b}	3.53 ± 1.80^{a}	1.52 ± 0.85^{a}
	2.0	59.09^{c}	2.31 ± 1.18^{d}	0.98 ± 0.65^{d}
Root segments	0	0^{c}	0^{e}	0^{e}
	0.1	59.1^{b}	2.85 ± 1.07^{d}	0.74 ± 0.60^{d}
	0.5	80.0^{a}	3.88 ± 2.31^{c}	1.16 ± 0.91^{c}
	1.0	91.3^{a}	4.45 ± 1.57^{a}	1.33 ± 0.71^{a}
	2.0	87.5^{a}	3.88 ± 1.36^{b}	1.32 ± 0.61^{d}
Nodes from *in vitro* regenerated shoots	0	0^{b}	0^{e}	0^{e}
	0.1	100^{a}	2.00 ± 0.85^{d}	0.76 ± 0.57^{b}
	0.5	98.68^{a}	3.19 ± 1.32^{b}	1.04 ± 0.93^{a}
	1.0	89.66^{a}	3.88 ± 2.10^{a}	0.66 ± 0.73^{c}
	2.0	85.71^{a}	3.00 ± 1.68^{c}	0.45 ± 0.25^{d}

cotyledonary nodal explants was also observed in *Boswellia serrata* by Purohit *et al.* (1995). They obtained an average of 6 - 8 shoots per explant on (0.5 - 1.0 mg dm^{-3}) BAP adjuvated MS medium.

Epicotyl nodal explants were found to be the most responsive on 0.1 mg dm^{-3} BAP adjuvated medium (Table 1). A similar observation was made by Manzanera and Pardos (1990) for juvenile explants of *Quercus*, where 0.1 mg dm^{-3} BAP was found to be optimum for inducing response. Maximum number of shoots per explant were regenerated on 0.5 mg dm^{-3} BAP supplemented medium and the same was optimum for shoot elongation.

There have been reports on regeneration from root explants from *Acacia albida* (Ahee and Duhoux 1994), *Citrus* spp. (Burger and Hackett 1986) and *Citrus aurantifolia* (Bhat *et al.* 1992). The optimum requirement of hormones varies from species to species. Few of the genera required both cytokinin and auxin for bud induction. However, root segments from *Crataeva*

seedling required only BAP for shoot bud induction. The percentage of responding explants as well as the number of shoots per responding explant were maximum on medium supplemented with 0.1 mg dm^{-3} BAP (Fig. 1*D*, Table 1).

After excision of differentiated shoots the residual explants were transferred to fresh medium containing BAP (0 - 2.0 mg dm^{-3}), where they differentiated more shoots. Likewise, nodal explants excised from *in vitro* differentiated shoots cultured on MS medium supplemented with BAP (0.1 - 2.0 mg dm^{-3}) also exhibited caulogenic response. The percentage of responding explants was maximum at 0.1 mg dm^{-3} BAP. However, maximum number of shoots per explant were formed on medium supplemented with 1.0 mg dm^{-3} BAP and on medium with 0.5 mg dm^{-3} BAP best elongation of shoots were observed (Table 1).

In the present study, the shoots differentiated from all the explants, grew normally on the same medium and therefore, no separate medium was required for their elongation, unlike reported by Sharma and Padhya (1996). They had reported that shoots, regenerated from nodal explants, derived from mature trees of *Crataeva*, failed to elongate on shoot induction medium. Therefore, these had to be transferred to medium adjuvated with GA_3 and thiamine hydrochloride.

In vitro regenerated shoots (2.5 - 4.0 cm long), developed adventitious roots when transferred to half strength MS medium supplemented with 0.02 mg dm^{-3} NAA (Fig. 1*E*), where 41.65 % of these rooted after 3 - 4 weeks of transfer. The rooted shoots were transferred to paper cups containing garden soil (Fig. 1*F*) and are now transferred to the field with a survival rate of 26 %.

To conclude, the present study reports *in vitro* production of plants of *Crataeva nurvala*, from six types of seedling-derived explants (including root segments) via a caulogenic phase. The ease with which shoots developed from all the explants including roots is indicative of a high caulogenic potential of the species.

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