BIOLOGIA PLANTARUM 37 (3): 335-340, 1995

In vitro propagation of *Boswellia serrata* Roxb.

S.D. PUROHIT, K. TAK and G. KUKDA

Plant Biotechnology Laboratory, Department of Botany, M.L. Sukhadia University, PB 100, Udaipur-313 001, India

Abstract

An *in vitro* procedure for large scale multiplication of *Boswellia serrata* Roxb. has been developed using cotyledonary node segments. In average 4 shoots per node were obtained on Murashige and Skoog's (MS) medium containing 0.5 mg dm^{-3} 6-benzylaminopurine (BAP) and 0.05 mg dm^{-3} napthaleneacetic acid (NAA) within 22 d. By repeated subculture technique 90 - 100 shoots per node could be obtained after 88 d of initial culture. Shoots could be rooted on MS medium containing 1/4 salts, 1 % saccharose, and a combination of 0.5 mg dm^{-3} indole-3-butyric acid (IBA) and 0.25 mg dm^{-3} indole-3-acetic acid (IAA). Addition of antioxidants like polyvinylpyrrolidone (PVP - 50 mg dm^{-3}) and ascorbic acid (100 mg dm^{-3}) in both multiplication and rooting media prevented browning of cultures. Approximately 80 % of shoots rooted within 8 - 10 d. Rooted plantlets were kept for 15 d in culture bottles containing Soilrite™ irrigated with a nutrient solution containing 1/4 MS salts and finally transferred to pots containing soil : Soilrite™ (1:1) mixture with 70 % transplantation success.

Key words: afforestation, benzylaminopurine, indole-3-acetic acid, indole-3-butyric acid, kinetin, napthaleneacetic acid

Introduction

Boswellia serrata Roxb. (*Burseraceae*) valued for oleo-gum-resin and cheap quality paper pulp once formed a major forest type of Aravallis in Rajasthan has now been listed as a threatened plant (Sharma 1983). Indiscriminate removal, difficulty in vegetative propagation and poor seed germination (10 - 20 %) account for its depleted population. Plant tissue culture biotechnology offers an opportunity for

Received 20 September 1994, *accepted* 23 March 1995.

Abbreviations: BAP - 6-benzylaminopurine, IAA - indole-3-acetic acid, IBA - indole-3-butyric acid, Kn - kinetin, MS - Murashige and Skoog's (1962) medium, NAA - napthaleneacetic acid, PVP - polyvinylpyrrolidone

Acknowledgements: Authors are thankful to Head, Department of Botany, M.L.Sukhadia University, Udaipur for providing facilities and Department of Biotechnology, Govt. of India, New Delhi for providing grant No. BT/AB/03/006/90 dated 14 March 1991.

rapid multiplication of desired tree species where the conventional methods have limitations (Aitken-Christie and Connett 1992, Mascarenhas and Muralidharan 1989).

The present paper reports a successful procedure for micropropagation of *B. serrata* using cotyledonary node segments.

Materials and methods

The seeds of *B. serrata* collected from superior identified trees were germinated on water or MS (Murashige and Skoog 1962) medium solidified with agar after surface sterilization with 0.2 % mercuric chloride for 10 min and washed thoroughly with autoclaved distilled water for 4 - 5 times. Cotyledonary and epicotyledonary nodes obtained from one month old *in vitro* grown seedlings were cultured on MS medium containing varying concentrations and combinations of cytokinins (0.1 - 5.0 mg dm^{-3}) and auxins (0.01 - 0.3 mg dm^{-3}) for shoot proliferation and multiplication. Repeated subculture of proliferated shoots with/without mother explant was done every 22 d on the same medium. During every subculture clusters of shoots were separated into small groups of 3 - 4 shoots each and transferred onto medium for further elongation and multiplication. Elongated shoots (3 - 4 cm) were excised carefully and transferred for rooting on media containing naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) individually or in combination. Salt concentration of MS medium was varied (full and 1/4 strength) and saccharose concentration was also reduced from 3 % to 1 % in the rooting medium. Antioxidants like PVP (50 mg dm^{-3}) and ascorbic acid (100 mg dm^{-3}) were added to both shoot proliferation and rooting media. pH of the medium was adjusted to 5.8 prior to autoclaving at 1.06 kg m^{-2} s^{-1} for 15 min. All the cultures were incubated under a 16-h photoperiod (cool-white fluorescent tubes, irradiance 45 µmol m^{-2} s^{-1}), relative humidity of 60 % and temperature of 28 ± 2 °C. Each treatment was repeated thrice. Rooted plants after 22 d were transferred directly into soil and also to culture bottles containing Soilrite™ (*Karnataka Explosives Ltd.*, Bangalore, India) irrigated with nutrient solution containing 1/4 MS salts without saccharose and growth regulators. These bottles with caps were kept in culture room for next 15 d. Their caps were removed gradually. Finally, the plants were transferred to pots containing soil:Soilrite™ mixture (1:1) and kept under polyethylene tent where 70 - 80 % relative humidity was maintained by spray misting.

Results and discussion

Origin and type of explant: Seedlings grown on MS medium were sturdy as compared to water-agar and provided better explants suitable for shoot induction and multiplication. Cotyledonary nodes were better explants than epicotyledonary nodes for shoot induction. Explant from sexually mature plant is generally preferred

as starting tissue for obtaining genotypically uniform plants *in vitro* but when the aim of multiplication is to produce a large number of propagules for revegetation of degraded lands, the genotypic uniformity of the regenerants can be compromised, specially, where the seed germination is a problem (Joshi 1991). In present investigation, cotyledonary nodes have been used for large scale multiplication of *B. serrata* as has been done in *Anogeissus pendula* and *A. latifolia* (Joshi 1991).

Shoot induction and multiplication: Cotyledonary nodes inoculated on MS medium without growth regulator produced no shoots. Incorporation of kinetin at lower concentrations (0.1 - 1.0 mg dm^{-3}) induced 2 shoots per node with a maximum length of 1.5 cm in 22 d after inoculation (Table 1). The shoots so produced, developed

Table 1. Effect of cytokinins added to MS medium on number of shoots per node and shoot length (mean ± SD) in cotyledonary nodes of *B. serrata* (subculturing done after 22 d)

Cytokinin [mg dm^{-3}] BAP	Kn	Number of shoots/node 22 d	Shoot length [cm] 22 d	Number of shoots/node 45 d	Shoot length [cm] 45 d	Callus
0.0	-	-	-	-	-	+
0.1	-	3.0 ± 0.82	0.6 ± 0.03	8.0 ± 0.91	1.0 ± 0.21	+
0.5	-	6.0 ± 0.44	0.8 ± 0.07	15.0 ± 1.34	1.5 ± 0.33	+
1.0	-	8.0 ± 0.52	0.5 ± 0.06	12.0 ± 1.06	1.0 ± 0.07	++
1.5	-	3.0 ± 0.63	0.5 ± 0.03	5.0 ± 0.63	0.7 ± 0.03	+++
3.0	-	2.0 ± 0.21	0.3 ± 0.01	4.0 ± 0.54	0.4 ± 0.08	+++
5.0	-	-	-	-	-	+++
-	0.1	2.0 ± 0.15	1.5 ± 0.20	4.0 ± 0.61	1.8 ± 0.84	++
-	0.5	2.0 ± 0.11	1.0 ± 0.51	5.0 ± 0.69	2.0 ± 0.36	++
-	1.0	2.0 ± 0.44	1.0 ± 0.36	3.0 ± 0.44	1.5 ± 0.16	+++
-	1.5	1.0 ± 0.89	0.7 ± 0.07	2.0 ± 0.36	1.0 ± 0.11	+++
-	3.0	1.0 ± 0.16	0.6 ± 0.06	1.0 ± 0.11	1.0 ± 0.12	++++
-	5.0	-	-	-	-	++++
0.5	0.5	2.2 ± 0.61	1.5 ± 0.11	2.6 ± 0.48	3.5 ± 0.76	+
1.0	0.5	2.3 ± 0.44	1.2 ± 0.12	2.6 ± 0.36	2.1 ± 0.31	+
1.5	0.5	2.7 ± 0.89	1.0 ± 0.21	2.9 ± 0.51	2.0 ± 0.38	++
0.5	1.0	2.0 ± 0.54	1.7 ± 0.28	2.5 ± 0.63	1.9 ± 0.27	++
1.0	1.0	2.0 ± 0.43	1.5 ± 0.31	2.3 ± 0.76	1.8 ± 0.16	++
1.5	1.0	1.0 ± 0.11	0.9 ± 0.07	2.1 ± 0.38	1.0 ± 0.34	++
0.5	1.5	2.0 ± 0.16	1.4 ± 0.18	4.2 ± 0.86	1.7 ± 0.16	+++
1.0	1.5	2.0 ± 0.17	1.5 ± 0.21	2.0 ± 0.71	1.7 ± 0.30	+++
1.5	1.5	1.0 ± 0.15	1.0 ± 0.11	2.0 ± 0.66	1.5 ± 0.84	+++

more broad dark reddish green leaves. Increased concentrations of kinetin (5.0 mg dm^{-3}) could not enhance shoot multiplication. BAP when used individually produced 6 - 8 shoots per node at lower concentration (0.5 - 1.0 mg dm^{-3}). A maximum of 15 shoots per node (1.5 cm length) could be produced on MS medium containing 0.5 mg dm^{-3} BAP in 44 d of inoculation (with one subculture of 22 d). Higher concentrations of BAP produced lesser number of shoots with different

degree of callusing. To improve shoot elongation, BAP was combined with kinetin in different concentrations. On combined cytokinins, average shoot length increased upto 3.5 cm on 0.5 mg dm^{-3} each of cytokinins but there was no improvement in number of shoots/node. Little to moderate callusing was always observed with such shoots. Incorporation of auxins (IAA and NAA) in the concentration range of 0.01 - 0.3 mg dm^{-3} in combination with BAP (0.5 mg dm^{-3}) promoted shoot elongation (Table 2).

There was slight reduction in number of shoots/node with this combination. In average 4 shoots per node were obtained on MS medium containing 0.5 mg dm^{-3}

Table 2. Effect of auxins combined with BAP (0.5 mg dm^{-3}) added to MS medium on number of shoots per node and shoot length (mean ± SD) in cotyledonary nodes of *B. serrata* (subculturing done after 22 d).

Auxins [mg dm^{-3}]		Number of shoots/node	Shoot length [cm]	Number of shoots/node	Shoot length [cm]	Callus
IAA	NAA	22 d	22 d	45 d	45 d	
0.01	-	3.6 ± 0.84	2.5 ± 0.41	10.3 ± 0.94	3.1 ± 0.46	+
0.05	-	4.1 ± 0.91	2.7 ± 0.36	11.9 ± 1.01	3.5 ± 0.87	+
0.1	-	2.1 ± 0.61	2.0 ± 0.64	11.3 ± 1.06	2.9 ± 0.34	++
0.2	-	2.0 ± 0.28	1.9 ± 0.32	11.1 ± 1.64	2.8 ± 0.64	++
0.3	-	1.8 ± 0.16	1.5 ± 0.36	11.0 ± 1.21	2.0 ± 0.44	+++
-	0.01	3.0 ± 0.86	1.8 ± 0.64	11.7 ± 1.41	2.9 ± 0.71	+
-	0.05	4.3 ± 0.92	1.9 ± 0.71	13.1 ± 2.01	3.9 ± 0.94	+
-	0.1	2.3 ± 0.16	2.0 ± 0.62	11.2 ± 1.74	2.5 ± 0.81	++
-	0.2	2.0 ± 0.12	1.0 ± 0.13	10.3 ± 0.96	2.0 ± 0.24	++
-	0.3	2.0 ± 0.15	0.7 ± 0.08	10.5 ± 0.99	1.2 ± 0.11	+++

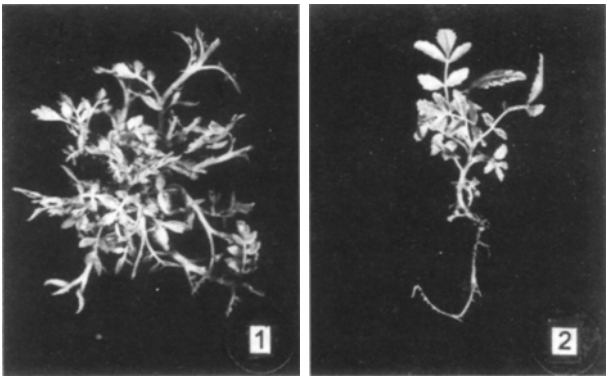

Fig. 1. Multiplication of shoots on MS medium containing BAP (0.5 mg dm^{-3}), NAA (0.05 mg dm^{-3}) and antioxidants (PVP 50 mg dm^{-3}, ascorbic acid 100 mg dm^{-3}) after 45 d.

Fig. 2. Rooting in regenerated shoots on MS medium containing 1/4 salts, 1.0 % saccharose, 0.5 mg dm^{-3} IBA, 0.25 mg dm^{-3} IAA and antioxidants.

BAP and 0.05 mg dm^{-3} NAA in 22 d of initial culture. NAA was considered a better auxin as compared to IAA to help elongation of shoots upto mean length of 3.9 cm. It was concluded that MS medium containing 0.5 mg dm^{-3} BAP and 0.05 mg dm^{-3} NAA was best combination on which 13 shoots (length 3.9 cm) could be produced per node (Fig. 1). In all, 90 - 100 shoots were obtained in 88 d (involving 3 subcultures) on above medium. Addition of NAA in shoot multiplication medium was necessary to help rooting at a later stage. Callusing during every subculture caused browning affecting the growth and health of cultures adversely. This problem could be solved to some extent by incorporation of antioxidants like PVP (50 mg dm^{-3}) and ascorbic acid (100 mg dm^{-3}) in the medium. The superiority of BAP over kinetin has been shown in *Maytenus emarginata* (Rathore *et al.* 1992) and *Tecomella undulata* (Rathore *et al.* 1991). In present case also the BAP was more effective cytokinin than kinetin. Addition of lower concentration of auxin (0.05 mg dm^{-3} NAA) with BAP has been found useful for elongation of shoots as has been reported in *Tecomella undulata* and *Maytenus emarginata*. Beneficial effects of antioxidants have been reported by various workers (McComb and Newton 1981, Hu and Wang 1983). In our case also PVP (50 mg dm^{-3}) and ascorbic acid (100 mg dm^{-3}) proved useful for healthy growth of cultures.

Rooting of shoots: Of three auxins (NAA, IAA, IBA) only IAA and IBA in the concentration range of 0.5 - 2.0 mg dm^{-3} induced rooting in the shoots inoculated on MS medium. NAA could not induce rooting on any of its concentrations and caused callusing only. 50 % of shoots could be rooted within 30 - 35 d on MS medium containing 0.5 mg dm^{-3} IAA. Maximum (40 %) rooting response was observed with 1.0 mg dm^{-3} IBA in the rooting medium. The rooting could be further improved by combining IAA and IBA in the rooting medium (Table 3) and 80 % rooting was

Table 3. Effect of IAA and IBA alone or in combinations added to 1/4 MS medium with 0.8 % agar and 1.0 % saccharose on rooting (mean ± SD) of regenerated shoots of *B. serrata*.

IBA [mg dm^{-3}]	IAA [mg dm^{-3}]	Rooting [%]	Number of roots/shoot	Root length [cm]	Callus
0.0	0.0	-	-	-	+
0.5	-	50.0	2.5 ± 0.38	1.8 ± 0.64	+
1.0	-	40.0	1.0 ± 0.40	1.5 ± 0.56	++
2.0	-	40.0	1.0 ± 0.34	1.5 ± 0.48	++
-	0.5	40.0	1.5 ± 0.16	1.2 ± 0.21	++
-	1.0	30.0	1.5 ± 0.24	1.0 ± 0.16	++
-	2.0	20.0	1.0 ± 0.13	1.0 ± 0.12	+++
0.5	0.1	60.0	1.0 ± 0.12	2.7 ± 0.44	++
0.5	0.25	80.0	2.8 ± 0.51	4.0 ± 0.51	++
0.5	0.5	60.0	1.5 ± 0.37	2.2 ± 0.32	+++

observed in shoots inoculated on MS medium containing 0.5 mg dm^{-3} IBA and 0.25 mg dm^{-3} IAA (Fig. 2). To obtain such response it was also necessary to reduce the salt concentration of MS medium to 1/4 and saccharose to 1.0 %. Such modifications helped in early induction of roots (8 - 10 d) with least or trace of callus. Antioxidants like PVP (50 mg dm^{-3}) and ascorbic acid (100 mg dm^{-3}) were also incorporated in the rooting medium. The strength of inorganic and organic salts of MS medium played significant role in the rooting behaviour of shoots *in vitro*. Beneficial effect of low salt concentration on rooting has been reported (Joshi 1991). In *B. serrata* low salt strength (1/4) of MS and 1.0 % sucrose helped in callus free rooting.

Hardening and acclimatization: Direct transfer of plantlets from culture medium to pots showed high rate of mortality. Plants obtained from culture bottle showed 70 % survival at pot level. Hardening is most critical factor for achieving success in pot transfer of the regenerated plantlets. We have observed that gradual shifting of the plants from medium to the culture bottles with low nutrients, without saccharose allowed stress, compelling plants to become partially autotrophic. This step proved useful in achieving more success in hardening. Using the optimum conditions described in this paper, a large number of propagules can be obtained in a short period.

References

Aitken-Christie, J., Connett, M.: Micropropagation of forest trees. - In: Kurata, K., Kozai, T. (ed.): Transplant Production Systems. Pp. 163-194. Kluwer Academic Publishers, Dordrecht - Boston - London 1992.

Hu, C.Y., Wang, P.J. Meristem, shoot tip and bud cultures. - In: Evans, D.A., Sharp, R.W., Ammirato, P.V., Yamada, Y. (ed.): Handbook of Plant Cell Culture. Vol. 1. Pp. 177-227. Macmillan, New York 1983.

Joshi, R.: *In vitro* studies on forest plants of Rajasthan. - Ph.D. Thesis, University of Jodhpur, Jodhpur 1991.

Mascarenhas, A.F., Muralidharan, E.M.: Tissue culture of forest trees in India. - Curr. Sci. **58**: 606-613, 1989.

McComb, J.A., Newton, S.: Propagation of Kangaroo Paws using tissue culture. - J. hort. Sci. **56**: 181-183, 1981.

Murashige, T., Skoog, F. : A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Rathore, T.S., Singh, R.P., Shekhawat, N.S.: Clonal propagation of desert teak (*Tecomella undulata*) through tissue culture. - Plant Sci. **79**: 217-222, 1991.

Rathore, T.S., Deora, N.S., Shekhawat, N.S.: Cloning of *Maytenus emarginata* (Willd.) Ding Hou-a tree of the Indian Desert, through tissue culture. - Plant Cell Rep. **11**: 449-451, 1992.

Sharma, S.: A census of rare and endemic flora of South-East Rajasthan. - In: Jain, S.K., Rao, R.R. (ed.): Threatened Plants of India. Pp. 630-670. Botanical Survey of India, Howrah 1983.