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BRIEF COMMUNICATION

Synergistic effect of trimethoprim and bavistin for micropropagation of *Bacopa monniera*

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Abstract

A micropropagation protocol for *Bacopa monniera* (L.) Wettst., a medicinally important plant, has been developed. Direct organogenesis without callus formation was induced by culturing node, internode and leaf explants on growth regulator free Murashige and Skoog (MS) medium. MS medium supplemented with an antibiotic trimethoprim (TMP) and a fungicide bavistin (BVN) produced axillary shoots from node and adventitious shoot buds on the surface of all explants. The combination of 200 mg dm^{-3} TMP and 200 mg dm^{-3} BVN induced the optimum frequency of shoot formation as well as shoot number. Presence of both TMP and BVN induced multiple axillary shoot formation from the nodal segments and this ability was maintained for four subcultures.

Additional key words: adventitious shoot bud, axillary shoot.

Bacopa monniera (L.) Wettst. (*Scrophulariaceae*), commonly known as brahmi is well exploited in the traditional medicine (Roodenrys *et al.* 2002). The pharmacological importance of this plant is mainly due to the presence of different types of saponins, *e.g.*, bacosides A, B, C and D (Rastogi *et al.* 1994). Over-exploitation of the natural population and inadequate efforts for its cultivation resulted in marked decline in the population of this species. Hence, it is necessary to develop methods for *in vitro* propagation and conservation of this plant. We have previously reported the induction of multiple shoot regeneration from different explants of *B. monniera* (Tiwari *et al.* 1998, 2001). Recently, a mass *in vitro* propagation system devoid of growth regulators has been developed (Tiwari *et al*. 2006).

Antimicrobial agents (antibiotics and fungicides) are generally added in plant tissue culture media to control microbial contamination. Several of these agents are reported to induce shoot regeneration (Burgos and Alburquerque 2003) and somatic embryo formation (Aoshima 2005). Antibiotics are widely used in the selection media for the recovery of transgenic plants (Bhattacharjee *et al*. 2010, Dibax *et al.* 2010). The objective of this study was to evaluate the efficacy of combined effect antibiotic trimethoprim (TMP) and a fungicide bavistin (BVN) on shoot regeneration from different explants of *B. monniera*.

Shoots of *Bacopa monniera* collected from the campus of Banaras Hindu University, were washed under running tap water for 10 min, followed by soaking in 1 % (v/v) cetrimide solution for 10 min. Shoots were surface sterilized with sodium hypochlorite solution containing 0.8 % (v/v) available chlorine for 10 min and washed six times with sterile distilled water. Segments containing single node were cultured vertically on Murashige and Skoog (1962; MS) basal medium. The shoots produced from the axillary buds of the nodal explants were used as the source of the explants.

Node (0.8 - 1.0 cm), internode (1.0 cm) and leaf (0.6 cm) explants were cultured on MS medium containing TMP (0 - 250 mg dm^{-3}), BVN (0 - 350 mg dm^{-3})

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Abbreviations: BA - 6-benzyladenine; BVN - bavistin; IAA - indole-3-acectic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; TMP - trimethoprim.
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and 3 % (m/v) sucrose. The pH of the medium was adjusted to 5.8 prior to addition of 0.8 % (m/v) agar and autoclaving at 121 °C for 20 min. The cultures were incubated at temperature of 24 ± 2 °C and 16-h photoperiod in culture room with an irradiance of 50 µmol m^{-2} s^{-1} provided by white florescent tubes (*Philips*, Mumbai, India).

TMP and BVN containing MS medium does not support elongation of axillary/adventitious shoot buds. Therefore, axillary shoots/shoot buds were transferred for elongation and rooting to MS medium with indole-3-acetic acid (IAA) and 6-benzyladenine (BA) as previously described (Tiwari *et al*. 1998). Plantlets were removed from the culture vials, washed gently under running tap water, placed in a 250 cm^3 beaker containing tap water for 15 min, and finally transferred to cups filled with sterilized *Soilrite* (*Kel Perlite*, Karnataka, India). To achieve high humidity the cups were covered with polythene bags and irrigated daily with 1 - 2 cm^3 tap water. The plants were maintained in culture room at 24 ± 2° C, 16-h photoperiod and irradiance of 20 µmol m^{-2} s^{-1} provided by the cool white fluorescent tubes for another 2 weeks before the plants were transferred to the field.

Data were analyzed as a factorial design by using the factors as four explants types (A), six concentration of TMP (B) and eight concentration of BVN (C) in randomized complete block design [explants type (A) = 0.01, concentrations of TMP (B) = 0.04, concentrations of BVN (C) = 0.03, A × B = 0.03, A × C = 0.04, B × C = 0.02, A × B × C = 0.06] by using analysis of variance and for means comparison critical difference at 5 % probability level was made according to Gomez and Gomez (1984). Each experiment was replicated three times and each replicate consisted of 20 culture tubes.

MS medium supplemented with TMP and BVN had a marked effect on axillary shoot and adventitious shoot bud differentiation from different explants of *B. monniera* (Table 1). Nodal segments cultured on MS medium containing TMP and BVN showed both axillary shoot and adventitious shoot bud differentiation, while internode and leaf explants support the adventitious shoot bud induction only (Table 1). Analysis of variance revealed the synergistic effect of TMP and BVN for axillary and adventitious shoot bud induction (CD at 5 %; Table 1). It was observed that increasing concentrations of BVN upto 200 mg dm^{-3} added in a media with a constant concentration of TMP gradually improved axillary shoot and adventitious shoot buds formation, but higher concentrations was not promontory for morphogenetic responses. Nodal segments cultured on MS medium without TMP and BVN did not show axillary or adventitious shoot bud formation. Maximum number (50) of axillary shoots was differentiated on MS medium containing 200 mg dm^{-3} TMP and 200 mg dm^{-3} BVN (Fig. 1*A*). The number of nodes per axillary shoot was significantly enhanced and multiple branching of

Fig. 1. Response of *B. monniera* explants cultured on MS medium supplemented with 200 mg dm^{-3} each TMP and BVN: *A* - induction of axillary shoots from nodal segment without callus formation, *B* - adventitious shoot buds induction at cut end of internode explant, *C* - shoot buds induction on entire surface of internode, *D* - shoot buds induction from leaf explant, *E* - rooting of elongated shoot, *F* - acclimatized plantlet transferred into plastic cup containing *Soilrite*.

Table 1. The synergetic effect of TMP and BVN [mg dm^{-3}] on number of axillary shoots or adventitious shoot buds [$explant^{-1}$] induced on different explants of *B. monniera* after four weeks of culture. Values are means ± SE. Each experiment was replicated three times and each replicate consisted of 20 culture tubes.

TMP	BVN	Number of axillary shoots node	Number of adventitious shoot buds node	internode	leaf
0	0	0	0	17.1 ± 0.16	14.0 ± 0.17
50	50	3.0 ± 0.14	1.2 ± 0.13	16.0 ± 0.47	13.2 ± 0.20
	100	5.0 ± 0.15	1.4 ± 0.13	18.2 ± 0.35	14.1 ± 0.22
	150	8.0 ± 0.21	1.8 ± 0.11	20.5 ± 0.65	16.1 ± 0.67
	200	8.0 ± 0.20	2.2 ± 0.15	30.0 ± 0.76	20.2 ± 0.45
	250	8.0 ± 0.19	2.8 ± 0.23	25.1 ± 0.78	18.1 ± 0.53
	300	7.0 ± 0.17	1.0 ± 0.11	20.2 ± 0.57	17.1 ± 0.74
	350	5.0 ± 0.16	1.0 ± 0.13	19.2 ± 0.66	16.2 ± 0.65
100	50	8.0 ± 0.21	3.0 ± 0.19	16.0 ± 0.69	15.1 ± 0.51
	100	10.0 ± 0.18	5.0 ± 0.27	20.2 ± 0.92	19.7 ± 0.45
	150	12.0 ± 0.18	6.0 ± 0.27	22.1 ± 0.98	21.1 ± 0.49
	200	21.0 ± 0.59	6.5 ± 0.24	34.8 ± 0.54	32.2 ± 0.54
	250	20.0 ± 0.66	5.0 ± 0.28	30.1 ± 0.69	27.5 ± 0.26
	300	20.0 ± 0.65	4.0 ± 0.22	32.2 ± 1.20	25.1 ± 0.84
	350	17.0 ± 0.36	2.2 ± 0.14	25.0 ± 1.12	25.1 ± 0.85
150	50	12.0 ± 0.20	4.0 ± 0.24	20.0 ± 0.91	18.5 ± 0.29
	100	13.5 ± 0.20	7.0 ± 0.24	22.0 ± 0.88	18.4 ± 0.28
	150	20.0 ± 0.65	10.0 ± 0.41	26.0 ± 0.64	20.1 ± 0.46
	200	30.0 ± 0.64	14.0 ± 0.55	40.0 ± 1.31	32.5 ± 0.49
	250	28.0 ± 0.45	12.0 ± 0.56	38.4 ± 1.36	30.4 ± 0.78
	300	27.0 ± 0.42	10.0 ± 0.53	35.2 ± 0.66	31.4 ± 1.23
	350	24.0 ± 0.63	9.0 ± 0.35	25.4 ± 0.76	20.1 ± 0.57
200	50	18.0 ± 0.44	3.0 ± 0.30	30.2 ± 1.17	27.1 ± 0.14
	100	21.0 ± 0.65	9.0 ± 0.45	32.4 ± 0.58	26.5 ± 0.42
	150	30.0 ± 0.54	11.0 ± 0.45	40.5 ± 1.74	38.4 ± 1.06
	200	50.0 ± 1.26	30.0 ± 1.52	135.2 ± 3.94	90.4 ± 2.37
	250	40.0 ± 1.34	22.0 ± 1.02	80.0 ± 2.68	85.2 ± 3.08
	300	37.0 ± 1.03	18.0 ± 0.91	90.0 ± 2.54	80.1 ± 2.70
	350	30.0 ± 0.81	10.0 ± 0.46	70.0 ± 1.89	75.2 ± 3.19
250	50	15.0 ± 0.48	4.0 ± 0.32	25.0 ± 0.81	22.1 ± 0.64
	100	18.0 ± 0.43	6.0 ± 0.26	22.0 ± 0.84	24.2 ± 0.64
	150	18.8 ± 0.35	7.0 ± 0.27	22.1 ± 0.64	25.1 ± 0.65
	200	20.0 ± 0.65	8.0 ± 0.42	98.4 ± 2.62	85.5 ± 3.07
	250	17.0 ± 0.25	4.0 ± 0.20	80.2 ± 2.65	76.3 ± 3.13
	300	12.0 ± 0.40	5.0 ± 0.28	35.2 ± 0.60	40.4 ± 1.33
	350	10.0 ± 0.33	3.0 ± 0.22	30.1 ± 0.86	38.1 ± 0.66

shoots was observed when nodes were cultured on MS medium containing both TMP and BVN. The ability of node to form multiple branching was consistently maintained for four subcultures.

Adventitious shoot buds were induced from all explants (node, internode and leaf) of *B. monniera* cultured on MS media supplemented with TMV and BVN. Internode was the most responsive explant, producing the highest number of adventitious shoot buds (135.2; Fig. 1*B*,*C*) followed by leaf (90.4; Fig. 1*D*) and node (30.0). The shoot buds always originated at a small distance from the cut ends of internode and later developed on entire surface while in nodal segment they developed near nodal ring. Interestingly, the shoot buds induced by TMP and BVN were callus free.

Fungicides (imazalil/imidazol) supplemented media promote shoot regeneration in *Spathiphyllum floribundum* and members of *Araceae* (Werbrouck and Debergh 1995, 1996). Imazalil supplemented media showed improvement in shoot proliferation due to an inhibitory effect on cytokinin degrading enzymes (Werbrouck and Debergh 1995) as well as due to inhibition of gibberelic acid biosynthesis (Werbrouck and Debergh 1997). BVN is a systemic fungicide belongs to benzimidazole family; it has structural similarity with imazalil and imidazol. Whenever it was added in media, it resulted in a significant improvement in axillary shoot induction and multiple branching. This effect of BVN was attributed to its interaction with the general mechanism of cytokinin action (Thomas 1974, Tripathi and Ram 1982). Synergistic effect of some plant growth regulators (thidiazuron and paclobutrazol) on *in vitro* axillary shoot proliferation has been reported in *Aframomum corrorima* (Tefera and Wannakrairoj 2006).

Axillary shoots and shoot buds induced on the optimum TMP and BVN containing MS medium remained stunted and failed to elongate rapidly. Optimum shoot growth in terms of shoot length and number of nodes/shoot was observed on MS medium containing 0.2 mg dm^{-3} IAA and 0.1 mg dm^{-3} BA as previously reported by Tiwari *et al.* (1998). Elongated shoots (4 - 6 cm) showed best rooting (90 %) on MS medium containing 0.5 mg dm^{-3} indole-3-butyric acid (IBA) in terms of number of roots/shoot and its length (Fig. 1*E*). A similar medium was previously reported for optimum rooting of elongated shoots of *B. monniera* (Tiwari *et al.* 1998). The rooted shoots were transferred from culture tubes into plastic cups containing *Soilrite* (Fig. 1*F*) with 90 % survival. The acclimatized plantlets were successfully established in the field. There was no phenotypic variation among the field transferred plants with respect to morphology and growth characteristics.

In conclusion, an efficient regeneration protocol for *B. monniera* has been established in the presence of TMP and BVN. All the plants transferred to field showed a high homogeneity without morphological evidence of somaclonal variation. In spite of this it needs further evaluation of secondary metabolite content of micropropagated plants.

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