Plant Tissue Cult. & Biotech. **19**(2): 237-245, 2009 (December)

PTC&B

Effect of Bavistin and Adenine Sulphate on *In vitro* Shoot Multiplication of *Picrorhiza scrophulariiflora* Pennell.: An Endangered Medicinal Plant of Indo-China Himalayan Regions

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Abstract

An alternative protocol for *in vitro* propagation of *Picrorhiza scrophulariiflora* is described using bavistin and adenine sulphate. The explants differentiated into multiple shoot buds on MS supplemented with various concentrations of bavistin and adenine sulphate ranging from 0 - 400 mg/l either alone or in combination. Maximum number of multiple shoots were obtained on MS containing the combination of bavistin (100 mg/l) and adenine sulphate (100 mg/l). In this combination as high as 28 shoots per explant was achieved and also vetrification of the cultures were not recorded. This study also demonstrates that the bavistin has stronger cytokinin-like activity than adenine sulphate. For instance, it was observed that bavistin alone in the concentration of 300 mg/l produced as high as 24 shoots per explant, however, adenine sulphate (100 mg/l) could produce a maximum of 18 shoots per explant. Moreover, higher or lower concentration did not improve the shoot multiplication. The microshoots were separated from the multiple shoots and transferred to MS containing various concentrations of auxins. Among them, NAA (1 mg/l) produced as high as 6 roots per explant. The regenerated plantlets were hardened in plastic cups (6 x 8 cm) containing 9 : 1 virgin soil and soil at Kyongnosla nursery and acclimated for four weeks. A 90% survival rate of the plants was recorded after 60 days.

Introduction

The Eastern Himalayas of India comprising North-Eastern states including Darjeeling, Bhutan as well as eastern part of Nepal hills are rich in floral diversity. Many of these plants are endangered, but medicinally useful with high demand in national as well as international markets (Manandhar 1999). One such

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species is Nepali's Kutki (*Picrorhiza scrophulariiflora* Pannell.). It is a small herbaceous plant found in sub-alpine or alpine zones of Sikkim, Nepal and China with an altitude above 3000 m (Hara et al. 1982).

The genus *Picrorhiza* is well characterized chemically for its two species namely, *P. kurroa* and *P. scrophulariiflora.* The rhizomes are used for traditional medicines to treat several ailments. In Nepal, rhizome of this species is also used as a cathartic, in case of dyspepsia, as a purgative, as well as in the treatment of scorpion bites (Anon. 1993). Though the species *P. kurroa* and *P. scrophulariiflora* are rich source of irridoid glycosides such as picroside-I, II, III and kutkoside as major bioactive compounds, *P. scrophulariiflora* contains an additional phenylethanoids glycosides and plantamajoside which are absent in *P. kurroa* (Li et al. 1998). Thus, *P. scrophulariiflora* is not only the substitute but also chemically superior than *P. kurroa* (Smith et al. 2000).

Several reports indicated the need of conservation, sustainable utilization and cultivation of *P. scrophulariiflora* (Ohba and Akiyama 1992, Olsen 1998, Subedi 2000). Due to the high demand in the pharmaceutical industries as well as Tibetan and Chinese medicine, plants are not only heavily uprooted by the local traders but also the natural regeneration is hampered. In addition, intentional fires set by shepherds for grazing cattle and domestication in place ultimately leaded to unsustainable management. Moreover, availability of the plants are decreasing every year in natural habitat (Uprety 2001, Bantawa et al. 2009) because of premature and faulty collection methods (which often does not allow them to set seeds and sprout suckers).

It is noteworthy to mention here that in attempts to sterilize the explants collected from wild condition for *in vitro* culture, the authors found accidentally the positive effect of bavistin and along with adenine sulphate which are reported here.

Materials and Methods

Shoots were used from previously established *in vitro* culture for the present study. Briefly the terminal or single nodes of the rhizomes were cut into small pieces (2 - 4 cm) and were washed with detergent Tween-80 (M/S Himedia Laboratories Pvt. Ltd., India) for 10 min and then again washed under running tap water for 30 min. Subsequently, they were treated with fungicide bavistin (M/S BASF India Pvt. Ltd., India) solution (0.5% w/v) along with another fungicide Tata Master (M/S Tata Chemicals Ltd., India) solution (0.2%) and antibiotics rifampicin (M/S Himedia Laboratories Pvt. Ltd., India) solution (0.5% w/v) for 4 hr. Afterwards, they were taken under laminar hood and treated with 0.1% mercuric chloride (w/v) (M/S Himedia Laboratories Pvt. Ltd., India) for 3 min which were further washed three times with autoclaved sterile water each

with 10 min. The surface sterilized explants were aseptically cut into 2 - 4 cm segment and were carefully inoculated onto WPM (Lloyd and McCown 1980) fortified with Kn (0.5 mg/l) for multiplication. Alternatively the seeds were also germinated under charcoal containing media and germinated shoots were multiplied *in vitro* (Fig. 1A).

For *in vitro* multiplication, Bavistin (M/S BASF India Pvt. Ltd., India) at the concentration of 50, 100, 200, 300 and 400 mg/l and adenine sulphate (M/S Himedia Laboratories Pvt. Ltd., India) 25, 50, 100, 200 and 300 mg/l were added to MS either alone or in various combinations. For root induction, multiple shoots were separated and transferred to MS supplemented with 0.1, 0.5, 1.0, 2.0 mg/l NAA, IBA and IAA separately. All the basal media contained 3% (w/v) sucrose (M/S Himedia Laboratories Pvt. Ltd., India) and 0.8% (w/v) agar (M/S Himedia Laboratories Pvt. Ltd., India). The media were adjusted to pH of 5.8 before sterilization prior to the addition of agar. The media were autoclaved at 15 Ib/sq inch pressure for 18 min.

Established shoots (4 - 5 cm) were cut at the base and these shoots were used as explants. Shoot tips were inoculated vertically on the multiplication media with about 5 mm basal portion embedded in ordinary jam bottle (300 ml) containing 75 ml of medium.

Each culture bottle contains four explants in multiplication experiment. All the cultures were kept on culture rack at 25 ± 1ºC under 12 hr photoperiod at light intensity of 2000 lux from cool fluorescent tube light (M/S Philips India Ltd., India) and 70% relative humidity was maintained.

The rooted explants were directly transferred to potting mixture containing sterile 9 : 1 virgin soil and sand under hot house at Kyungnosla nursery (3758 m), Department of Forest and Wild Life, Government of Sikkim, India. The survival percentage was recorded after 60 days.

The experiments were set up in a randomized block design. Data were analyzed using ANOVA to detect significant differences between means (Sokal and Rohlf 1987). Means differing significantly were compared using DMRT at $p \leq 0.05$ with Statistical software ver. 5.0 (INC StatSoft). Data are presented as the mean ± SE.

Results and Discussion

Bavistin is a systemic fungicide that belongs to benzimidazole family, and the molecular structure of methyl benzamidazole carbamate or carbendazim has some resemblance to Kn, adenine and many other cytokinins based on adenine (Tripathi and Ram 1982). It is also well documented that the bavistin (Tiwari et al. 2006) as well as adenine sulphate promotes shoot multiplication (Shrivastava and Banerjee 2008).

Presence of bavistin in the present study showed a marked improvement in shoot multiplication rate of *P. scrophulariiflora*. Bavistin alone at concentration of 300 mg/l produced average shoot multiplication number of 24 shoots per explants (Fig. 1B) after six weeks of inoculation. This is also in agreement with Tiwari et al. (2006), who observed similar result with *Bacopa monniera*. The average height of shoots was varied in the range of 6.4 to 8.8 cm in MS (Table 1). On the other hand adenine sulphate alone showed the multiplication rate of 3 - 18 shoots per explant in 25 - 100 mg/l, respectively. Higher or lower concentration of adenine sulphate did not improve the multiplication rate. Instead successive vetrification of shoots was resulted in due to the increase of its concentrations (Table 1). In general, the combination of bavistin and adenine sulphate gave significantly higher multiplication rate. The highest shoot multiplication of 28 shoots per explant was recorded in MS supplemented with bavistin (100 mg/l) in combination with adenine sulphate (100 mg/l) (Table 1). In this combination as high as 82.63% culture showed responses to the treatment. Similarly, Ramesh et al. (2006) reported that the addition of adenine sulphate (60 mg/l) along with other growth regulators was the most effective in inducing shoot multiplication. The frequency of vetrification was lowest (0%) in the concentration of 50 - 100 mg/l of bavistin. Nevertheless, vetrification increased significantly at higher concentration of bavistin and was recorded about 49.2% at the concentration of 400 mg/l. The shoot number was decreased when the concentration of bavistin was more than 400 mg/l. The basal callusing was also noted at that concentration. Additionally, at that concentration of bavistin the shoot growth was significantly reduced and the colour of the plantlets becomes pale yellow (Table 1). Note that in *Bacopa monniera* Tiwari et al. (2006) observed similar result, which may be due to the toxic effect of bavistin at higher dose. In case of controlled cultures there were no multiplication, instead elongation of plantlets were noticed. About 80 - 92% of culture responded to the treatment, however adenine sulphate started showing vetrification above 100 mg/l concentration with 10.6, 28.6 and 59.1% at 100, 200 and 300 mg/l, respectively. In contradiction, Burikam et al. (1988) reported the reduction of vetrification in *Carica papaya* culture by the addition of adenine sulphate (1.5 mg/l) in the medium. However, in the present experiment, the higher dose of adenine sulphate significantly reduced the shoot length and the cultures became thin and yellowish green in colour. At the same time 200 - 300 mg/l adenine sulphate showed frequent basal callusing of the explants. Interestingly, the combination of bavistin and adenine sulphate did not manifest any basal callusing at all the tested concentrations but 5 - 19% of the cultures showed vetrification at higher concentration of bavistin.

Table 1. The multiplication of shoots of *P. scrophulariiflora* in different concentrations of bavistin.

Sl. No.	Medium	Conc. of PGR used (mg/l)	No. of shoots/ explants	% of explants showing response	Shoot length (cm)	Vertifi-cation (%)	Remarks
1	MS + B	0	1 ± 00 q	100 a	8.8±0.95 a	0 j	Healthy with dark green shoots
2	MS + B	50	5 ± 0.82 o	82.63 ± 0.18 l	8.5 ± 0.42 ab	0 j	"
3	MS + B	100	12 ± 0.55 k	88.56 ± 0.23 f	7.2 ± 0.88 cde	0 j	"
4	MS + B	200	19 ± 0.55 e	87.86 ± 0.55 fg	7.2 ± 0.62 cd	18.3 ± 0.17 f	Healthy with green shoots
5	MS + B	300	24 ± 1.20 b	91.93 ± 0.5 cd	6.4 ± 0.21 ef	23.6 ± 0.18 d	"
6	MS + B	400*	17 ± 1.02 g	90 ± 0.58 m	3.7 ± 0.31 i	49.2 ± 0.18 b	Stunt growth with yellowish green shoots
7	MS + AS	25	3 ± 0.74 p	84.8 ± 0.5 k	8.6 ± 0.17 ab	0 j	Dark green but thin shoots
8	MS + AS	50	10 ± 0.68 m	86.8 ± 0.26 hi	7.0 ± 0.26 cde	0 j	"
9	MS + AS	100	18 ± 0.63 f	80.1 ± 0.32 m	7.0 ± 0.14 c	10.6 ± 0.18 g	"
10	MS + AS	200*	14 ± 0.55 j	89.93 ± 0.24 e	5.9 ± 0.14 g	28.6 ± 0.2 c	Greenish with thin shoots
11	MS + AS	300*	11 ± 1.20 l	92 ± 0.29 cd	4.5 ± 0.31 h	59.1 ± 0.38 a	Yellowish green, stunt growth
12	MS + B + AS	50 + 25	7.8 ± 0.55 n	85.56 ± 0.23 jk	7.2 ± 1.1 cde	0 j	Healthy with green shoots
13	MS + B + AS	50 + 50	14.5 ± 1.02 ij	88.57 ± 0.1 f	6.7 ± 0.8 def	0 j	"
14	MS + B + AS	50 + 100	18.9 ± 0.76 e	88.62 ± 0.15 f	6.6 ± 0.66 efg	0 j	"
15	MS + B + AS	100 + 25	10.5 ± 0.66 lm	91.34 ± 0.4 e	8.5 ± 0.58 ab	0 j	"
16	MS + B + AS	100 + 50	20.8 ± 1.20 d	86.36 ± 0.48 ij	8.2 ± 0.58 ab	5.6 ± 0.2 i	"
17	MS + B + AS	100 +100	28.6 ± 0.55 a	82.63 ± 0.09 l	8.2 ± 0.92 ab	10.2 ± 0.2 g	"
18	MS + B + AS	200 + 25*	15.6 ± 1.41 h	87.36 ± 0.17 gh	7.6 ± 0.22 bc	6.6 ± 017 h	Greenish with thin shoots
19	MS + B + AS	200 + 50*	22.6 ± 0.62 c	92.47 ± 0.15 bc	7.4 ± 0.52 cd	10.5 ± 0.29 g	"
20	MS + B + AS	200 + 100*	15.2 ± 0.62 hi	93.34 ± 0.29 b	6.5 ± 0.56 b	19.8 ± 0.23 e	"

*– Basal callusing; B– Bavistin; AS– Adenine sulphate. Data (mean ± SE) pooled from three independent experiments; Means followed by the same letter does not differ significantly according to DMRT.

Of the two additives, bavistin appeared to have much stronger cytokinin-like activities than adenine sulphate. This was evident from a promontory effect of bavistin on adventitious shoot bud regeneration. Cytokinin-like physiological functions of carbendazim of bavistin has been well documented by several workers like Davidse (1973), Tripathi et al. (1982), Tripathi and Ram (1982). This further supports the cytokinin-like activities of bavistin.

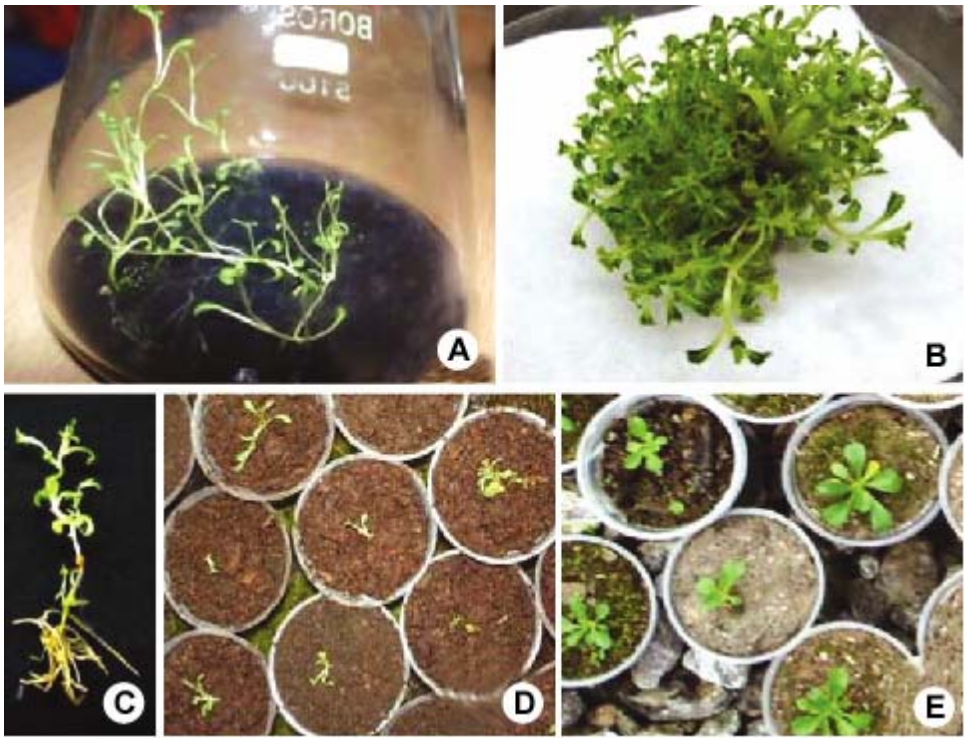

Fig. 1A-E: Micropropagation of *P. scrophularflora*. A. Germination of seed in MS containing 0.1% activated charcoal. B. Shoot multiplication in MS fortified with bavistin and adenine sulphate (100 mg/l each). C. Rooting in MS in combination of NAA (1 mg/l). D. Hardening and (E) Acclimated.

For rooting IAA, IBA and NAA were tested in the concentrations of 0.5, 1.0 and 1.5 mg/l. Among these tested auxins, NAA was found to be the best for root induction (Fig. 1C). The findings are in agreement with those observed in other plant species such as *Adhatoda vasica* (Amin et al. 1997, Azad and Amin 1998), *Caphaelis ipecacunha* (Jha and Jha 1989), *Ocimum sanctum* (Begum et al. 2000). In the concentration of 1.0 mg/l NAA as high as 6 roots per explant was recorded with the maximum length of 5.1 cm long. It has also been noted that though rooting was initiated within 10 to 12 days but maximum rooting was noted only after 18 to 21 days. However, higher concentration of IAA and IBA (1.5 mg/l)

caused profuse basal callusing and 1 mg/l IBA and 1.5 mg/l NAA also resulted in moderate basal callusing (Table 2).

Table 2. The rooting behaviour of *P. scrophulariiflora* in response to different hormonal combinations.

Sl. No.	Auxins	Conc. of auxins	% of rooting	No. of roots/explant	Average length of root	Days emergence of root
1	Control	0	26.0 g	2.3 ± 0.15 e	3.9 ± 0.27 f	18 - 24
2	"	0.5	30.0 f	3.4 ± 0.25 d	4.2 ± 0.11 cd	15 - 18
3	"	1.0	69.0 d	5.1 ± 0.22 b	4.2 ± 0.31 e	15 - 18
4	"	1.5	26.0 **g	3.3 ± 0.18	3.6 ± 0.25 g	12 - 17
5	"	0.5	67.0 e	3.9 ± 0.08 cd	4.3 ± 0.23 c	15 - 18
6	"	1.0	95.0 a	6.2 ± 0.22 a	5.1 ± 0.19 a	10 - 14
7	"	1.5	89.0 *b	5.1 ± 0.36 b	4.6 ± 0.23 b	10 - 14
8	IBA	0.5	66.0 e	3.2 ± 0.33 bcd	3.9 ± 0.09 f	11 - 18
9	"	1.0	79.0 *c	4.5 ± 0.15 bc	3.9 ± 0.17 f	14 - 18
10	"	1.5	70.0 **d	3.3 ± 0.15 d	2.6 ± 0.23 h	14 - 21

Extent of callusing *viz*. *Slight callusing; **Considerable callusing. Data (mean ± SE) pooled from three independent experiments; Means followed by the same letter do not differ significantly according to DMRT.

The rooted shoots were transferred from rooting media to plastic cups (Fig.1D) containing hardening mixture (9 : 1 virgin soil and sand). The hardened plants were acclimated in the hot house at Kyungnosla nursery for four weeks which were later transferred to the field and survival percentage was about 90 after 60 days of transfer (Fig. 1E).

In this study an efficient protocol has been developed using common fungicide: bavistin and a growth regulator: adenine sulphate for mass scale propagation of *P. scrophulariiflora* for sustainable conservation of this endangered species. It is also expected that this protocol may be used for constant supply of the high valued medicinal plants of higher altitude of Eastern Himalayas. This study also demonstrates the novel role of bavistin and adenine sulphate alone or in combination in inducing high frequency shoot buds in *P. scrophulariiflora* cultures. Direct shoot regeneration in *P. scrophulariiflora* cultures further provides the basis of commercial cultivation as well as germplasm conservation.

Acknowledgements

The authors are thankful to Professor Swapan Kumar Ghosh, Dean, Faculty of Horticulture for his kind encouragements and thankful to Department of Biotechnology and Department of Science and Technology, Govt. of India for financial assistance. We are also grateful to Mr. Bijoy Gurung, Department of

Wildlife and Forest, Govt. of Sikkim, India for his help to conduct the survey and Mr. Kamal Das of this laboratory for his assistance.

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