Micropropagation of *Ajuga bracteosa*, a medicinal herb

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Abstract For conservation and genetic transformation, a successful in vitro micropropagation protocol for *Ajuga bracteosa*, a medicinal herb has been established for the first time. MS medium supplemented with IAA (2 mg/L) and BA (5 mg/L) induced 100 % shoot regeneration with an average of 41.4 shoots of 8.4 cm per culture. Excised in vitro shoots when transferred to MS + IBA (0.5 mg/L) produced 20 roots/shoot of 20.2 cm average length in 100 % cultures. Of the three explants, leaf, petiole and root, leaf displayed quickest response followed by petiole while root was the slowest. Hardening of plantlets was achieved with 82 % survival. The hardened plants were maintained in pots with garden soil under controlled (Temp. 25±2 °C) conditions. RAPD exhibited genetic fidelity with 100 % monomorphism in regenerants.

Keywords *Ajuga bracteosa* · Micropropagation · Medicinal plant · RAPD

Introduction

In the absence of a credible programme for cultivation of medicinal plants, 85 % of the herbs are still collected from

the wild (Hamayun et al. 2006). The unplanned practice of collection is leading to rapid depletion of valuable raw material. As a consequence, there is an inevitable need to ensure their availability in sufficient quantities. The in vitro micropropagation offer potential alternative for availability of raw material throughout the year. The collection of *Ajuga* from the wild by unskilled hands like digging out the whole herb results in the loss of the propagules. Therefore, for conservation of *Ajuga bracteosa* a reliable and efficient micropropagation protocol is needed. In the present study, in vitro micropropagation of *Ajuga bracteosa* has been successfully achieved.

Ajuga bracteosa (Lamiaceae), commonly called ‘Nilkanthi’ in Sanskrit and ‘Jaan-e-Adam’ in Urdu is a hairy herb found in temperate regions, distributed from Kashmir to Nepal at an altitude of 2,000 m. It is a diffusely branched perennial evergreen herb (Fig. 1) that remains compressed to the ground. The yellowish white flowers are crowded in axillary whorls. The herb is in demand for the treatment of gout, rheumatism, palsy and amenorrhoea (Anonymous 1985). Locally the leaves help in curing headache, pimples, measles, stomach acidity, burns, boils, jaundice, hypertension and sore throat (Hamayun et al. 2006) and as blood purifier. Singh et al. (2006) have reported expectorant, anti-inflammatory and anti-cancerous properties of *A. bracteosa*. Most importantly, the leaves are used in the treatment of fevers as a substitute for quinine (<http://www.pfaf.org/database/plants.php?Ajuga+bracteosa>). Investigators report that the herb possesses anti-malarial activities (Njoroge and Bussmann 2006; Kuria et al. 2001, 2002). The two isolates, ajugarin-1 and ergosterol-5,8-endoperoxide are effective against chloroquine sensitive *Plasmodium falciparum* (Kuria et al. 2002). It could be an alternative to *Artemisia*.

The limited distribution of this herb coupled with tough habitat, valuable medicinal importance and the consequent ruthless exploitation, may lead *Ajuga bracteosa* to become endangered. In fact, there has thus far been no micropropagation study on *Ajuga bracteosa*. Ours is the first report of a protocol for in vitro regeneration of *A. bracteosa*.

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Fig. 1 *Ajuga bracteosa*: mother plant

Materials and methods

Micropropagation

Ajuga bracteosa was collected from hills of ‘Sanasar’ and ‘Panchari’ district Udhampur, Jammu and Kashmir and introduced during winter months (5–15 °C) in the Herbal Garden at Hamdard University, New Delhi. The plants were irrigated when required to keep the soil moist and maintained under shade by covering with green nylon net. After washing under running tap water for 30 min, leaf and petiole explants were treated with 0.2 % cetrimide for 5 min, streptomycin sulfate (0.25 %) 30 min, bavistin (0.5 %) 30 min, 0.1 % mercuric chloride 3 min and finally with 70 % alcohol for ½ min. Before inoculation, the explants were rinsed thoroughly with sterile water. The explants were cut to 0.75–1 cm with sterile blade and inoculated on MS medium (Murashige and Skoog 1962) with 3 % sucrose and

Table 1 *Ajuga bracteosa*: Interactive effect of IAA + BA on leaf explants after 6 weeks

Medium MS + (in mg/L)		Percent shoot regeneration	Average number of shoots/culture	Average height of shoots (cm)	Percent rooting	Average number of roots/culture	Average length of roots (cm)
IAA	BA	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$
0	0	—	—	—	—	—	—
0.2	0.2	86.10±2.40	13.4±0.46	1.8±0.23	—	—	—
0.2	0.5	87.5±5.00	26.4±0.36	3.4±0.39	—	—	—
0.2	1	91.66±5.00	33.4±0.36	3.7±0.27	—	—	—
0.2	2	91.66±4.16	33.8±0.60	6±0.32	—	—	—
0.2	5	91.66±4.31	8.6±0.46	3.8±0.23	—	—	—
0.5	0.2	94.44±2.78	16±0.50	2.8±0.23	16.67±2.40	2±0.57	1.5±0.14
0.5	0.5	100±0	26±0.40	3.3±0.34	23.61±2.77	4.2±0.34	1.4±0.17
0.5	1	100±0	33.8±0.22	5.2±0.39	—	—	—
0.5	2	100±0	33.4±0.44	3.1±0.41	—	—	—
0.5	5	100±0	6.8±0.34	1.4±0.17	—	—	—
1	0.2	94.44±1.39	13.2±0.44	6.8±0.23	—	—	—
1	0.5	100±0	24±0.40	6.8±0.40	41.66±6.36	4.2±0.34	4.6±0.30
1	1	100±0	17±0.70	5.3±0.44	23.61±1.39	9.2±0.53	1.6±0.26
1	2	100±0	19.8±0.88	5.8±0.37	—	—	—
1	5	100±0	16.2±0.34	7.9±0.30	27.78±1.39	3.2±0.44	2.7±0.18
2	0.2	94.44±2.78	5.4±0.61	1.3±0.11	—	—	—
2	0.5	93.05±3.57	8.8±0.34	2.6±0.39	—	—	—
2	1	100±0	13±0.5	4±0.24	37.5±2.40	4.2±0.18	4.4±0.36
2	2	100±0	26.0±0.22	5.4±0.17	31.94±1.38	4.6±0.22	5.3±0.56
2	5	100±0	41.4±0.44	8.4±0.14	—	—	—
5	0.2	90.27±7.73	9.6±0.22	1.9±0.26	—	—	—
5	0.5	90.27±8.33	12.2±0.18	2.2±0.30	38.89±1.39	4.4±0.22	3.5±0.40
5	1	90.27±9.10	20.2±0.18	2.8±0.30	50±3.57	3±0.5	3.7±0.18
5	2	90.27±5.00	12.0±0.40	2±0.24	22.22±1.39	3.2±0.44	2±0.24
5	5	93.05±3.67	9±0.40	2.7±0.38	—	—	—

Percent response was best on IAA 0.5 mg/L + BA 0.5, 1, 2 and 5 mg/L. IAA 2 mg/L and BA 5 mg/L induced maximum number of shoots

Fig. 2 *Ajuga bracteosa*:
a Callus initiation
 [MS + IAA(2 mg/L) + BA
 (5 mg/L)], **b** Eight-week-old
 cultures [MS + IAA(2 mg/L) +
 BA(5 mg/L)], **c** Rooting [MS +
 IBA(0.5 mg/L)], **d** Plantlet in
 MS (basal) medium and **e**
 Hardened plants in garden soil

supplemented with various concentrations of plant growth regulators (IAA, NAA, IBA, BA, KN, GA₃; Sigma-Aldrich, USA) either alone or in combinations. The pH of the medium was adjusted to 5.7, gelled with 0.63 % agar and autoclaved at 121 °C (1.06 kg/cm²) for 20 min. The cultures were maintained in a culture room illuminated by cool, white fluorescent tubes of 36 Watts (Philips, India) at 25 ± 2 °C with 55 ± 5 % relative humidity. All the experiments were repeated thrice with 24 replicates. Regenerated shoots of 3–5 cm were transferred to MS medium supplemented with IBA for rooting. The rooted plantlets were transferred to basal medium (MS medium without supplements) and after 4 weeks to ½ strength MS medium. The plants were shifted to plastic pots containing a mixture of autoclaved

soilrite and soil (1:1) and covered with self-sealing transparent polyethylene bags to maintain humidity. The plants were watered with ½ strength MS salts for 2 weeks and later by sterile distilled water. After 4 weeks, the polyethylene bags were removed and the hardened plants were finally shifted to pots with garden soil.

RAPD analysis

To determine the clonal fidelity of the regenerants RAPD analysis was carried out. The DNA of the mother plant and ten randomly selected in vitro regenerants of *Ajuga bracteosa* were isolated by modified Doyle and Doyle (1990) method. The DNA samples were then analyzed using

Table 2 *Ajuga bracteosa*: Effect of IAA + KN interaction on leaf explants after 6 weeks

Medium MS + (in mg/L)		Percent shoot regeneration	Average number of shoots/culture	Average height of shoots (cm)	Percent rooting	Average number of roots/culture	Average length of roots (cm)
IAA	KN	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$	$\bar{X} \pm \text{S.E.}$
0	0	—	—	—	—	—	—
0.5	0.5	68.05±1.38	4.4±0.25	0.54±0.08	70.83±2.40	4.6±0.15	1.1±0.06
0.5	1	79.17±2.40	8±0.35	0.4±0.04	72.27±2.72	7±0.20	0.7±0.07
0.5	2	84.72±1.39	8.6±0.15	0.75±0.07	65.28±7.73	9.6±0.25	0.7±0.07
0.5	5	87.5±0	10.4±0.25	1±0.10	56.94±3.67	10.4±0.25	0.6±0.06
1	0.5	79.17±2.40	8.6±0.25	0.48±0.09	80.55±3.67	9.2±0.12	0.8±0.07
1	1	77.78±1.39	10.4±0.15	0.55±0.03	69.44±11.36	11.8±0.24	0.9±0.03
1	2	84.72±1.39	17.6±0.25	1.1±0.12	48.55±1.39	3.6±0.15	0.85±0.06
1	5	86.11±1.39	13±0.20	0.58±0.11	51.39±5.00	3.4±0.32	0.85±0.25
2	0.5	68.05±2.77	3.6±0.32	0.65±0.06	87.5±2.40	11.6±0.15	5.4±0.15
2	1	73.61±1.39	9±0.20	1±0.17	94.44±2.77	21±0.28	8.1±0.15
2	2	93.05±3.67	18±0.28	1.2±0.07	72.22±5.00	4.4±0.15	0.75±0.07
2	5	100±0	32±0.20	1.3±0.07	50±2.40	3.4±0.25	0.7±0.25
5	0.5	34.72±5.90	3.4±0.32	0.6±0.03	95.83±2.40	9.6±0.15	0.7±0.07
5	1	31.94±3.57	7±0.20	0.6±0.06	94.44±3.67	17.4±0.15	1.08±0.06
5	2	79.16±4.31	12.2±0.24	0.36±0.05	94.44±3.67	18.8±0.12	1.1±0.06
5	5	73.61±1.39	7.2±0.24	0.7±0.07	87.5±2.40	8.8±0.12	1.24±0.12

Kinetin did not prove as effective as BA. The maximum percent regeneration and number of shoots was on IAA 2 mg/L + Kn 5 mg/L

random decamer primers- G-01 (CTG CTC TGC CC), G-02 (GGG TGA CGC AG), G-03 (TTG GGG GAC TC), G-05 (AGG TCA CCT CA), G-06 (GGA TGA CCG CC), G-07 (GAA AGC TGC GG), G-08 (TGT CGC CGT CA), G-09 (GCC GCG TCT TG), G-10 (TGG ACC TGC TG) and G-14 (GCC CCG ATG CT). For PCR, each reaction mixture

Fig. 3 *Ajuga bracteosa*: comparison of height of shoots on MS + IAA + BA and MS + IAA + KN from leaf explant after 6 weeks

Fig. 4 *Ajuga bracteosa*: percent rooting from in vitro shoots after 6 weeks

prepared in 0.2 ml PCR tubes (Tarsons, India) consisted of 1X Reaction buffer (10 mM Tris–HCl, pH 8.3 and 50 mM KCl), 1.67 mM MgCl₂, 1 U *Taq* DNA polymerase; 0.67 mM dNTP, 0.67 μM decamer primer and 50 ng genomic DNA template. The amplification carried out in a thermocycler (Eppendorf) was as follows: one cycle at 94 °C for 4 min, 36 °C for 2 min and 72 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 36 °C for 1 min and elongation at 72 °C for 2 min. The 35th cycle was followed by primer extension step at 72 °C for 10 min and then held at 8 °C until electrophoresis. Amplified DNA fragments (15 μl) along with 3 μl gel loading dye and *EcoRI* and *HindIII* digested λ DNA as molecular size marker were electrophoresed on 1 % agarose gel, detected by ethidium bromide and photographed under ultraviolet light using the Gel Documentation (UVP, UK).

Results

A reproducible 4 month duration micropropagation protocol has been established for *Ajuga bracteosa*. None of the explants responded on basal medium. On MS medium supplemented with auxin and cytokinin, although all the explants produced callus and shoots, response was fastest in leaf and slowest in roots. Both direct and indirect regeneration was

observed in leaves. Of the combinations tried, MS + IAA + BA proved best for callusing as well as shoot regeneration and MS + IBA for rooting.

Response on MS + IAA + BA medium

After 4 weeks, compact green regenerating callus was induced from leaf explants in all the cultures (Table 1). Fastest callusing was observed in 100 % cultures after just 10 days of inoculation on MS + IAA (2 mg/L) + BA (5 mg/L). The callus developed multiple shoots that elongated within 8 weeks (Fig. 2b). Out of 25 combinations of MS + IAA + BA, the best was MS + IAA (2 mg/L) + BA (5 mg/L) inducing 41.4 average number of shoots per culture with 8.4 cm average height. At IAA (0.2 mg/L) no rooting was recorded in the shoots. On MS + IAA (5 mg/L) + BA shoots showed improved rooting (Table 1).

Response on MS + IAA + KN medium

The interactive effect of IAA and KN on leaf explants is presented in Table 2. After 6 weeks shoot regeneration in 100 % cultures with 32 shoots per explant was recorded on MS + IAA (2 mg/L) + KN (5 mg/L). Rooting was also observed in 95.83 % cultures on MS + IAA (5 mg/L) + KN (0.5 mg/L). A comparison in response on MS + IAA + BA and MS + IAA + KN is shown in Fig. 3. 50 % of the cultures

Table 3 *Ajuga bracteosa*: Rooting response of in vitro raised shoots

Medium MS + IBA (in mg/L)	Percentage $\bar{X} \pm \text{S.E.}$	Average number of roots/culture $\bar{X} \pm \text{S.E.}$	Average length of roots (cm) $\bar{X} \pm \text{S.E.}$
After 4 weeks			
0	—	—	—
0.1	66.67±2.40	5.4±0.15	1.4±0.12
0.2	75±1.96	7.6±0.25	1.5±0.14
0.5	98.61±1.39	16.4±0.25	7.8±0.21
1	100±0	11.2±0.24	3.4±0.25
2	100±0	10.2±0.12	1.5±0.20
3	87.5±2.40	8.2±0.37	2.5±0.10
4	70.83±2.40	5.8±0.12	1±0
5	69.44±2.77	3.2±0.12	1.3±0.07
7	29.17±2.40	1.4±0.15	0.9±0.06
10	—	—	—
After 6 weeks			
0	—	—	—
0.1	68.05±1.38	8±0.40	4.6±0.26
0.2	83.34±2.39	11.4±0.36	5.8±0.23
0.5	100±0	20±0.28	20.2±0.30
1	100±0	14.2±0.34	15.6±0.33
2	100±0	13.6±0.79	12.6±0.56
3	94.44±1.38	10.4±0.22	7.9±0.30
4	83.33±2.40	8±0.28	5.5±0.28
5	81.94±1.38	4.4±0.46	3.6±0.22
7	29.17±2.40	3±0.28	1.3±0.18
10	—	—	—

Rooting in maximum percent of cultures was obtained on IBA 1 and 2 mg/L

showed vitrification on MS + IAA + KN but recovered when transferred to MS + IAA + BA.

Rooting

In vitro differentiated shoots showed poor rooting but MS medium supplemented with IBA promoted thick and healthy roots. With increasing concentration of IBA the rooting percentage reached to 100 on MS + IBA (0.5, 1 and 2 mg/L); beyond IBA (2 mg/L) the percentage declined. At IBA (10 mg/L) all the explants turned brown and succumbed (Fig. 4). MS + IBA (0.5 mg/L) induced maximum average number (20) as well as length of roots (20.2 cm) after 6 weeks (Table 3).

Hardening and transplantation

The plantlets (shoots obtained on MS + IAA + BA and rooted on MS + IBA) were transplanted for 4 weeks to MS basal medium without any supplement (Fig. 2d). Sequential

Fig. 5 *Ajuga bracteosa*: analysis of clonal fidelity of in vitro raised regenerants through RAPD fingerprints with Primer G-5. Lanes- **L**: molecular weight marker (HindIII and EcoRI digested DNA), **M**: DNA from Mother Plant, **1–10**: DNA from regenerants

hardening was achieved by transferring the plantlets to $\frac{1}{2}$ strength MS medium for 2 weeks and then to $\frac{1}{4}$ strength for another 2 weeks. The plantlets were then transferred to plastic pots containing a mixture of autoclaved soil and soilrite (1:1). 82 % of the plantlets survived. The plants were finally transferred to earthen pots containing garden soil (Fig. 2e).

Clonal fidelity of in vitro regenerants

RAPD analysis confirmed the true to type nature of the in vitro raised regenerants of *A. bracteosa*. Genomic DNA of mother plant and ten randomly selected in vitro raised regenerants were isolated and amplified with 10 decamer primers. Of which 8 produced clear easily scorable amplification profiles (Fig. 5).

Discussion

The effect of explant, combination and concentration of phytohormones on the in vitro shoot regenerative potential of *Ajuga bracteosa* a herb, hitherto unstudied was tried. Young leaves responded better as compared to older leaves. Establishment of cultures through young explants has also been reported by Veltcheva and Svetleva (2005). Low IAA

and high BA was favourable for shoot regeneration while high IAA and low BA favoured rooting. Superiority of BA in shoot regeneration has been widely reported (Akbas et al. 2011; Dohling et al. 2012; Faisal et al. 2012). Effectiveness of IAA + BA in shoot regeneration has been recorded in other medicinal plants including *Zehneria scabra* (Anand and Jeyachandran 2004), *Ocimum gratissimum* (Gopi et al. 2006), *Withania somnifera* (Sharada et al. 2007), and others. Contrary to our results, Purohit et al. (1995) and Pande et al. (2002) have recorded superiority of IAA + KN in *Ammi majus* and *Lepidium sativum*, respectively. NAA has been widely used for regeneration (Zhang et al. 2006; Torabi-Giglou and Hajieghrari 2008; Khawar et al. 2008) but for *Ajuga bracteosa*, NAA did not prove favourable.

MS + IBA supported the production of well differentiated roots. 0.5 mg/L IBA induced best response. Stimulatory effect of IBA in rooting has been amply demonstrated for other medicinal plants including, *Ammi majus* (Purohit et al. 1995), *Eclipta alba* (Husain and Anis 2006), *Withania somnifera* (Sharada et al. 2007), etc. The ultimate success of a micropropagation protocol depends upon the large scale survival of plants after in vivo transfer (Hussey 1986). Full grown plantlets of *Ajuga* were sequentially hardened by transferring them to MS basal medium, then to ½ strength and ¼ strength and finally to pots. 82 % plantlets survived.

Somaclonal variations, have been detected in DNA fingerprint analysis of *Ocimum americanum* (Rady and Nazif 2005) and *Musa paradisica* (El-DougDoug et al. 2007). Genetic stability of in vitro regenerated plants of *Ajuga bracteosa* were assessed by RAPD. 100 % monomorphism was observed. The pattern of monomorphic bands in *Drosera binata* (Kawaik and Lojkowska 2004), *Phoenix dactylifera* (Saker et al. 2006), *Dioscorea bulbifera* (Dixit et al. 2003; Narula et al. 2007) and *Dioscorea rotundata* (Mandal et al. 2008) are in concurrence with our findings.

With the help of successful in vitro regeneration system developed by us *Ajuga bracteosa* can be made available for scientific experimentations throughout the year.

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