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Micropropagation of *Ajuga bracteosa* Wall ex. Benth.-an important medicinal plant growing in Kashmir Himalaya

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ABSTRACT

During the present study an efficient in vitro propagation protocol has been developed viz; callus induction, multiple shoot regeneration and rooting of regenerated shoots from petiole and internodal explants. MS (Murashige and Skoog) medium supplemented with different concentrations and combinations of auxins and cytokinins proved to be best for callus induction in both the explants. Multiple shoot regeneration was achieved after sub-culturing the callus on MS medium fortified with different growth regulators. In petiole explants maximum shoots were regenerated on MS medium containing BAP (3mg/l) in 80% of cultures within 37 days. In internodal explant maximum shoot regeneration were obtained in 100% of cultures within 31 days on MS medium supplemented with BAP (2mg/l) and NAA (5mg/l). Rooting differentiation was best achieved on MS medium fortified with IAA (2mg/l) within 33 days. The in vitro raised plantlets were successfully acclimatized/hardened under green house conditions and successful hardening was obtained within 4 weeks. The hardened plants were successfully transferred to field conditions.

Keywords: Ajuga bracteosa, Callus, multiple shoot regeneration, Petiole, root regeneration.

I INTRODUCTION

Ajuga belongs to the family Lamiaceae. This is one of the largest families among dicotyledons represented by 220 genera and more than 4000 species [1]. *Ajuga bracteosa* is commonly known as "bungle" in English and "Jan-i-adam" in Kashmiri. It is a perennial erect or ascending hairy herb, often prostrate with oblanceolate or sub-spathulate leaves and grows up to 5-50cm tall (Fig. 1). *A.bracteosa* is distributed in subtropical and temperate regions from Kashmir to Bhutan, Pakistan, Afghanistan, China and Malaysia [2, 3]. In India, it abounds in western Himalaya at an altitude of 1300 masl [4]. This plant has also been used traditionally for curing a number of diseases like fever, and a variety of neuro and inflammatory disorders [3]. The herb is in use since

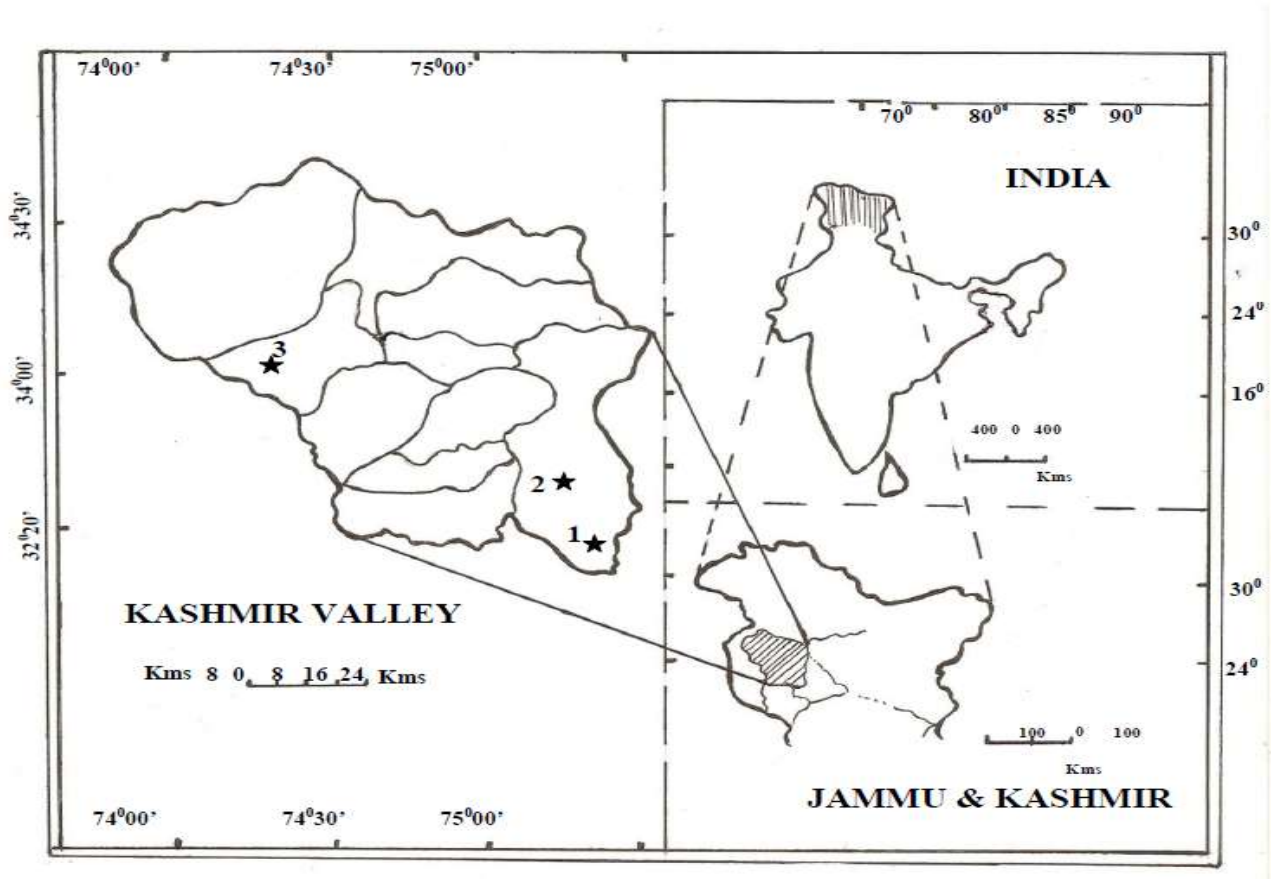
ancient times and recommended in ayurveda for the treatment of rheumatism, gout, palsy and amenorrhea. It is also credited with astringent, febrifugal, stimulant, tonic, and diuretic properties. The juice of the leaves is used as a blood purifier and powdered form for burns and boils [5]. Leaves are employed for application on blisters, venereal and skin diseases, in malarial fever, in eruptions and fissures on skin, in jaundice and leucorrhoea, in diabetes and body swellings [6, 7]. Anti-inflammatory and anti-cancerous properties of *Ajuga bracteosa* have been reported [8]. Investigators have also reported anti-malarial activities [9,10]. It could also be an alternative to Artemisia currently being used as an anti-malarial [11]. *In vitro* evaluation of antihelmentic efficacy of *Ajuga bracteosa* on *Ascaridia galli* (a poultry worm) has also been studied [12]. During the present study *in vitro* propagation of this plant species was carried out because of its tremendous medicinal importance and tough habitat conditions.

Fig.1 *Ajuga bracteosa* Wall ex. Benth (a) Habit of the plant (b) Flower

II Materials and Methods

Ajuga bracteosa was collected from Jawahar Tunnel, Kulam Chinar Anantnag and Ferozpur Nallah at an altitude of 2523, 1734 and 2200 masl respectively (Fig. 2), and transplanted at Kashmir University Botanical Garden (KUBG). Explants of *Ajuga bracteosa* were collected from plants grown at KUBG. Explants were first washed thoroughly under running tap water for 30 minutes in order to remove dust, dirt and other unwanted materials. These were then washed with a detergent solution Labolene 1% v/v containing 2-3 drops of surfactant, Tween 20 (1% v/v). This was followed by washing with tap water to remove the detergent and finally washed 2-3 times with double distilled water under laminar air flow hood. Finally the explants were disinfected with 2 % sodium hypochlorite solution (NaOCl) for 10 minutes. After 10 minutes disinfectant solution was decanted and the surface sterilized explants were washed 5-6 times with double distilled water so as to remove any traces of the sterilant. The sterilized plant material was then aseptically inoculated on to the culture medium in the laminar airflow cabinet. MS medium supplemented with different concentrations and

combinations of 6-benzylaminopurine (BAP), α-Napthalene acetic acid (NAA), Indole-3-acetic acid (IAA) were used for callus induction and shoot regeneration. The cultures were maintained at 25± 2°C, light intensity of 3000 lux and a regular photoperiod of 16 hrs.



1= Jawahar Tunnel, 2= Kulam Chinar Anantnag, 3= Ferozpur Nallah

Fig. 2: Map showing collection sites of *Ajuga bracteosa* Wall ex Benth

III RESULTS

3.1 *In vitro* response from petiole explants

3.1.1 Callus production

Callus was obtained from petiole explants when inoculated on MS medium fortified with auxins and cytokinins in different combinations (Table 1). Among auxin-cytokinin combinations, only BAP + IAA in different concentrations were effective in inducing callus differentiation. When MS medium was supplemented with BAP (2mg/l) + IAA (3mg/l) and BAP (3mg/l) + IAA (2mg/l), compact and green coloured callus was obtained in 51 and 60 days in 100% and 40% cultures respectively (Fig. 3a, 3b). Compact and green coloured callus was

obtained when petiole explants were inoculated on BAP (1mg/l) + IAA (0.5mg/l) containing MS medium in 30% cultures within 65 days (Fig. 3c).

Table No. 1: Effect of different hormones on callus production from petiole explant

Treatments	*Initiation of callus (days)*	*Amount of callus produced*	*% Culture response*
MS Basal	*No response*	_	_
MS+BAP(0.5mg/l)	_	_	*0*
MS+BAP(1mg/l)+IAA(0.5mg/l)	*65*	*Moderate*	*30*
MS+BAP(2mg/l)+IAA(3mg/l)	*51*	*High*	*100*
MS+BAP(3mg/l)+IAA(2mg/l)	*60*	*High*	*40*

(30 replicates per treatment)

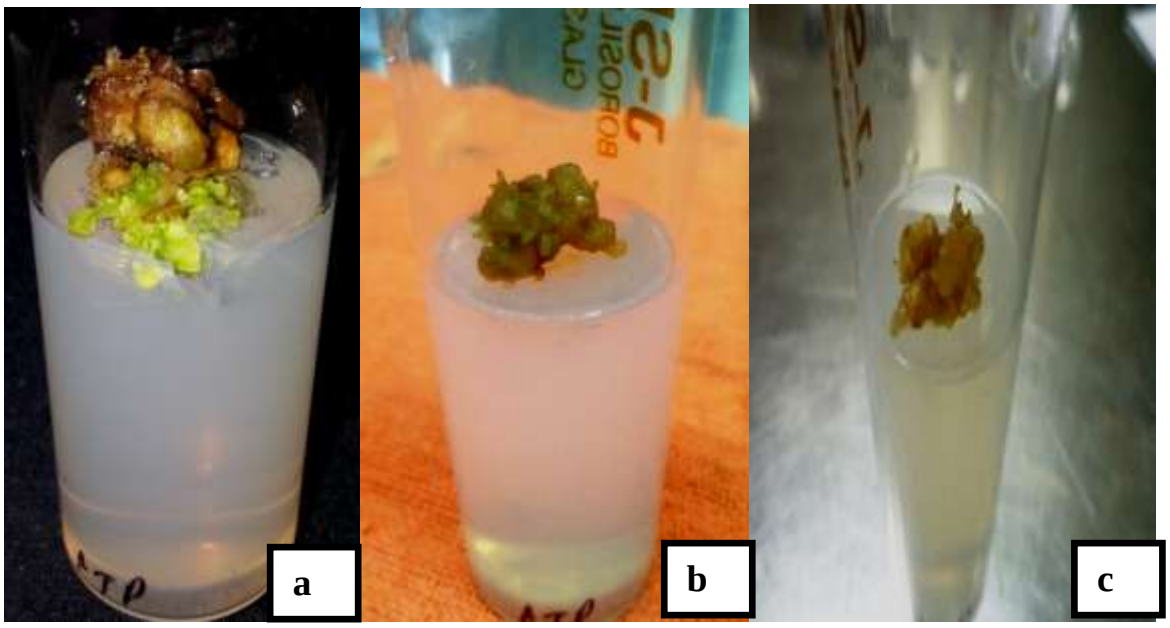


Fig. 3: Callus production from petiole explant on MS medium containing

a) BAP (2.0 mg/l) + IAA (3.0 mg/l) b) BAP (3.0 mg/l) + IAA (2.0 mg/l)

c) BAP (1.0 mg/l) + IAA (0.5 mg/l)

3.1.2 Shoot regeneration

The petiole callus when sub-cultured on MS medium fortified with different growth hormones (Kn, NAA, IBA and 2,4-D) both individually as well as in different combinations did not regenerate shoots (Table 2). The shoots regenerated from the callus initiated on MS medium containing BAP (3mg/l) with (5.9±0.6) mean number of shoots and (2.4±0.26cm) mean shoot length in 80% of cultures within 37 days (Fig. 4a). Shoots also regenerated on MS medium supplemented with BAP (2mg/l) + IAA (2mg/l) with (4.1±0.37) mean number of shoots and (3±0.21cm) shoot length in 60% of cultures within 40 days (Fig. 4b).

Table No.2: Effect of different hormones on multiple shoot formation from petiole derived callus

Treatments	*Average number of shoots±SE*	*Average height of shoots(cm)±SE*	*Mean number of Days*	*% Culture response*
MS Basal	**–**	**–**	**–**	**–**
MS+BAP(2mg/l)+IAA(2mg/l)	***4.1±0.37***	***3±0.21***	***40***	***60***
MS+ BAP(3mg/l)	***5.9±0.6***	***2.4±0.26***	***37***	***80***

(30 replicates per treatment)

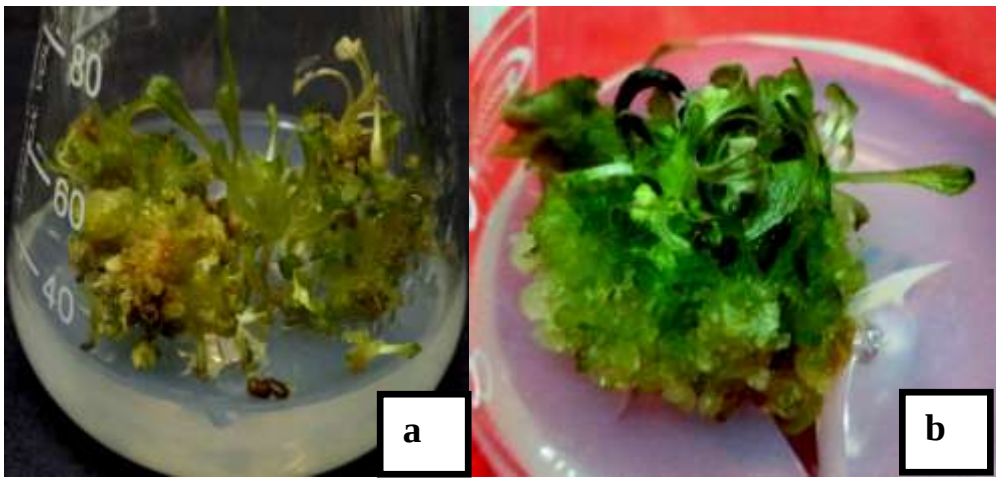


Fig.4: Shoot regeneration from petiole callus on MS medium containing

a) BAP (3.0 mg/l) (b) BAP (2.0mg/l) + IAA (2.0 mg/l)

3.2 *In vitro* response of inter nodal segments

3.2.1 Callus production

Internodal segments when inoculated on MS basal medium showed no response. However, these produced callus on MS medium supplemented with both auxins (NAA) and cytokinins (BAP) individually as well as in different combinations (Table 3). Among cytokinins, BAP at a concentration of 3mg/l was effective in differentiation of compact and green coloured callus in 60% cultures within 37 days (Fig 5a). Among auxins, NAA at a concentration of 2mg/l was effective in inducing callus differentiation in 40% cultures within 37 days (Fig. 5b). The callus produced was compact and greenish in colour. Among auxin-cytokinin combinations, only BAP + NAA in different concentrations were effective in inducing callus differentiation. When MS medium was supplemented with BAP (2mg/l) + NAA (5mg/l) and BAP (2mg/l) + NAA (3mg/l), compact and green colored callus was obtained in 35 and 38 days in 100% and 45% cultures respectively (Fig. 5c, 5d).

Table No. 3: Effect of different hormones on callus production from internodal explant

Treatments	*No. of days taken for callus production*	*Amount of callus produced*	*% Culture response*
MS Basal	***No response***	**–**	**–**
MS+NAA(2mg/l)	***35***	***Moderate***	***40***
MS+BAP(3mg/l)	***37***	***High***	***60***
MS+BAP(2mg/l)+NAA(3mg/l)	***38***	***Moderate***	***45***
MS+BAP(2mg/l)+NAA(5mg/l)	***35***	***High***	***100***

(30 replicates per treatment)

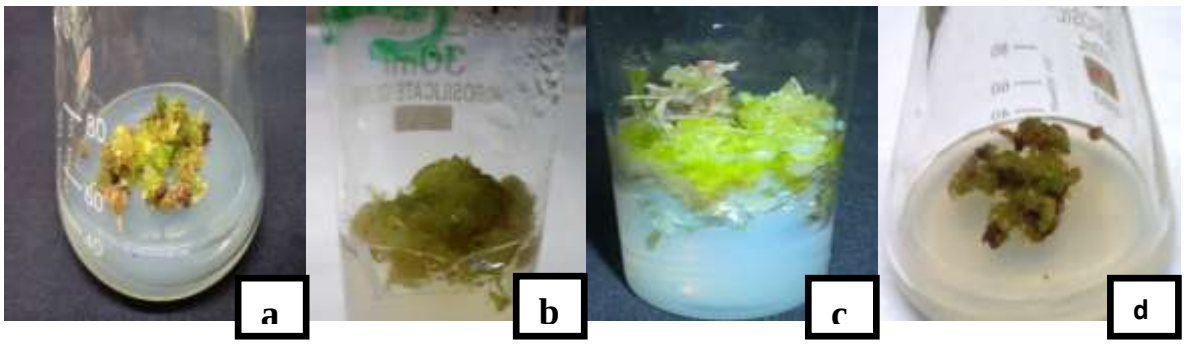


Fig. 5: Callus production from internodal explant on MS medium containing
a) BAP (3.0 mg/l) b) NAA (2.0 mg/l)
c) BAP (2.0 mg/l) + NAA (5.0 mg/l) d) BAP (2.0mg/l) + NAA (3.0 mg/l)

3.2.2 Shoot regeneration

Callus obtained from internodal explants regenerated shoots on MS medium containing BAP and NAA individually and in combinations (Table 4). After sub-culturing the callus, (5.6±0.54) mean number of shoots with mean length of (2.2±0.20cm) were obtained in 100% of cultures within 31 days (Fig. 6a) when MS medium was supplemented with BAP (2mg/l) and NAA (5mg/l). When MS medium was supplemented with BAP (1mg/l) and NAA (2mg/l) (5.8±0.4) mean number of shoots with (1.2±0.05cm) mean length was produced in 70% of cultures within 37 days (Fig. 6b). (8.6±0.54) mean numbers of shoots with (2.22±0.5cm) mean length were produced in 60% cultures within 39 days (Fig. 6c) when MS medium was supplemented with BAP 2mg/l.

Table No.4: Effect of different hormones on multiple shoot formation from internodal derived callus

Treatments	*Average number of shoots±SE*	*Average height of shoots(cm)±SE*	*Mean number of Days*	*% Culture response*
MS Basal	*–*	*–*	*–*	*–*
MS+BAP(2mg/l)	***8.6 ±0.54***	***2.22±0.20***	***39***	***60***
MS+BAP(2mg/l)+NAA(5mg/l)	***5±0.36***	***2.4±0.26***	***31***	***100***
MS+BAP(1mg/l)+NAA(2mg/l)	***5.8±0.4***	***1.2±0.05***	***37***	***70***

(30 replicates per treatment)

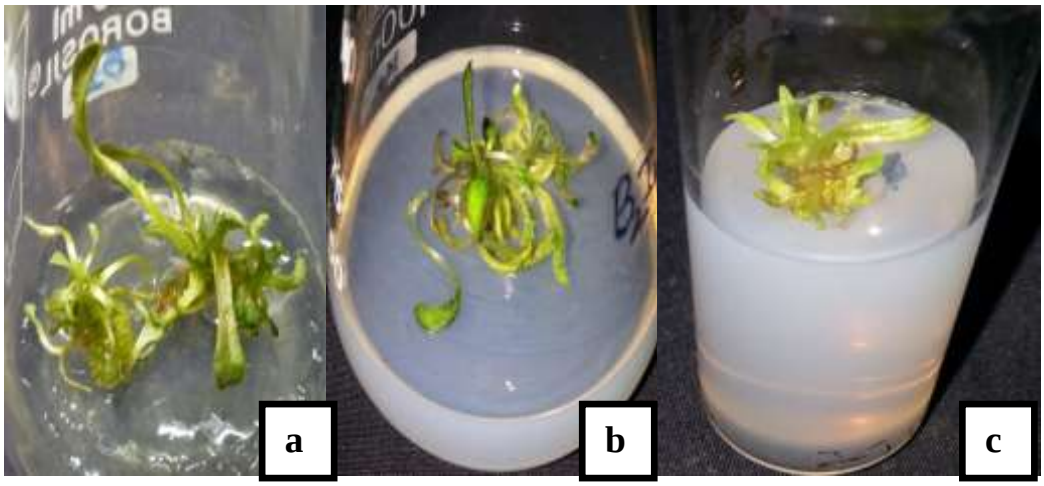


Fig. 6: Shoot regeneration from internodal callus on MS medium containing

a) BAP (2.0mg/l) + NAA (5.0 mg/l) b) BAP (1.0 mg/l) + NAA (2.0 mg/l)

c) BAP (2.0 mg/l)

3.3 Rooting of regenerated shoots

For the induction of roots, the *in vitro* differentiated shoots were excised from shoot clumps and subcultured on both full and half salt strength MS medium supplemented with different concentrations of auxins viz, (IAA, IBA, NAA and 2,4-D) (Table 5). Roots regenerated from the shoots subcultured on full salt strength MS medium supplemented with IBA (0.5-2mg/l), IAA (0.5-2mg/l) and NAA (0.5-2mg/l). The most effective concentration at which maximum root induction was achieved was MS basal medium augmented with IAA (2mg/l). The mean number of roots obtained were (5.9±0.92) in 80% cultures within 33 days (Fig 7).

Table No. 5: Effect of different growth hormones on *in vitro* raised shoots of *Ajuga bracteosa* for root induction

Treatments	*Average no. of roots/explant*	*No. of days taken for root induction*	*%Culture response*
MS Basal	_	_	_
MS+IAA(0.5mg/l)	_	_	_
MS+IAA(1mg/l)	***5.4±0.50***	***39***	***40***
MS+IAA(2mg/l)	***5.4±0.92***	***33***	***80***
MS+IBA(0.5mg/l)	***4±0.31***	***35***	***60***
MS+IBA(1mg/l)	***3.6±0.50***	***38***	***20***
MS+IBA(2mg/l)	_	_	_
MS+NAA(0.5mg/l)	_	_	_
MS+NAA(1mg/l)	***5.2±1.01***	***45***	***30***
MS+NAA(2mg/l)	***3.6±0.50***	***40***	***40***

(30 replicates per treatment)

Fig. 7: Rooting of regenerated shoots on MS medium containing IAA (2.0 mg/l)

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3.4 Hardening

For acclimatization, 35 days old plantlets with well developed shoots as well as roots were taken out of the culture vials. The medium adhering to the basal portion of plantlets was washed off with double distilled water. After washing they were transferred to jiffy pots containing soil and sand in 1:1 ratio and maintained under controlled conditions of temperature (22±4˚C) and relative humidity (60%) in green house (Fig 8a, 8b). The plantlets were watered every alternate day with Hoagland's salt solution. The hardening of the plants was achieved within 4 weeks with 100% survival rate. The hardened plantlets were finally transferred to pots and were maintained under field conditions (Fig. 8c). The plants grew successfully and got established under the natural conditions of growth, where in they also produced flowers (Fig. 8d)

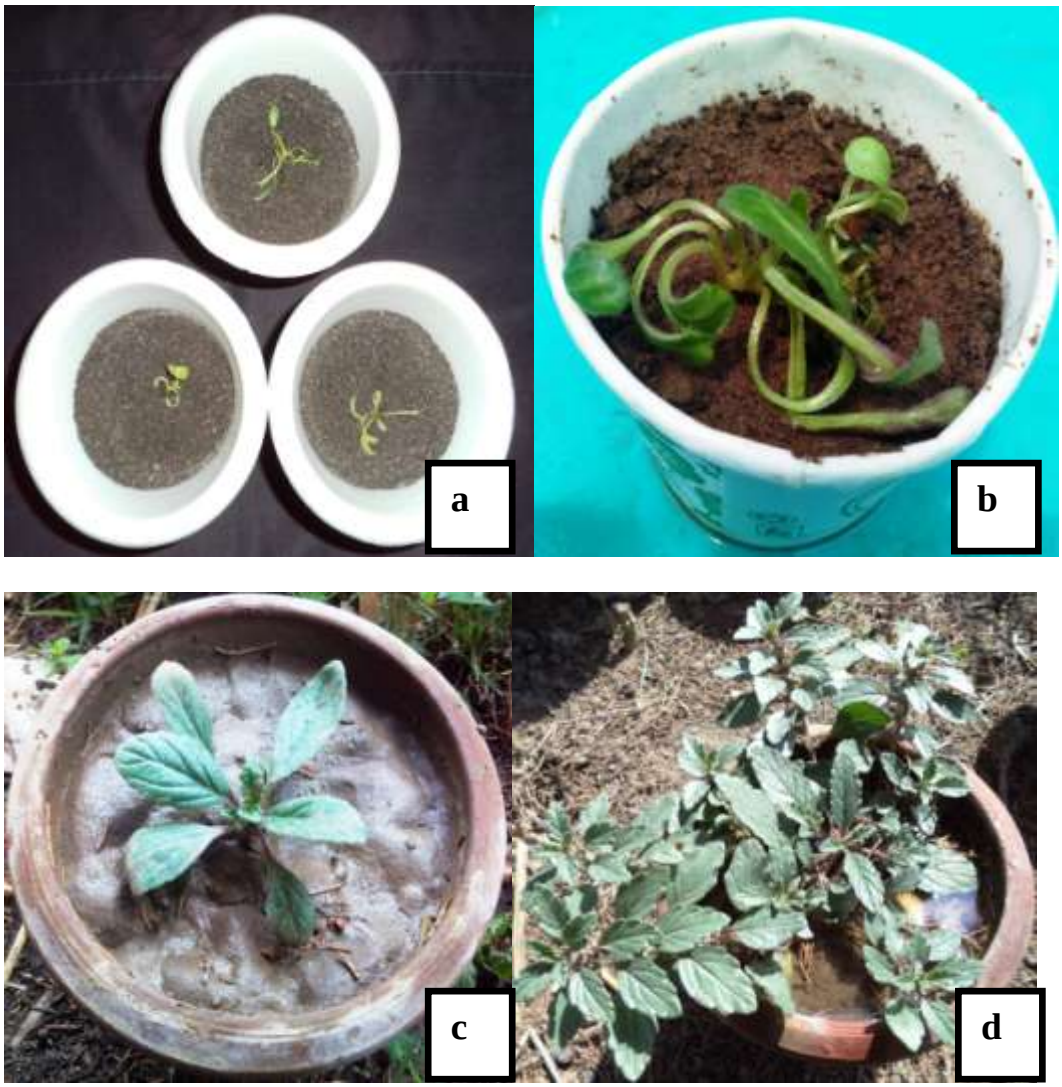

Fig. 10: Acclimatization/Hardening of *in vitro* raised plants

(a and b) in green house (c and d) in field conditions

IV DISCUSSION

During the present study, different plant growth hormones were used either individually or in combinations for callus production and shoot regeneration from petiole and internodal explants of *Ajuga bracteosa*. Petiole explants produced maximum callus in 100% cultures when MS medium was supplemented with BAP (2mg/l) + IAA (3mg/l). The time taken was 51 days. The results obtained were in accordance with Srivastav *et al*. (2013) who obtained callus induction from petiole explants of *Ajuga bracteosa* on MS medium supplemented with BAP and IAA. Petiole callus initiated on MS medium containing BAP (3mg/l) also regenerated shoots within 37 days. From internodal explants (5.6±0.54) mean number of shoots with mean length of (2.2±0.20 cm) was obtained in 100% of cultures within 31 days on MS medium supplemented with BAP (2mg/l) and NAA (5mg/l). The results obtained reveal that for the regeneration of shoots, auxins and cytokinins were required in combinations. Similar results were obtained by Senthil and Kamaraj (2012) for shoot regeneration from inter nodal explants of *Mentha viridis* L. Highest regeneration rate was achieved at BAP (3.5mg/l) after 20 days of culture. Full salt strength MS medium supplemented with IAA (2mg/l) was effective in inducing *in vitro* regeneration of roots in 80% of cultures within 33 days.

V CONCLUSION

A rapid micropropagation protocol was devised for callus induction, multiple shoot and root regeneration from petiole and internodal explants of *Ajuga bracteosa.* During this study, different plant growth regulators were used in order to see their effect on callus induction, shoot and root regeneration. Among all the plant growth regulators BAP proved to be most effective for both callus induction as well as shoot regeneration. However best root regeneration was achieved on full salt strength MS medium augmented with IAA.

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