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In vitro multiple shoot regeneration and plant production in *Alysicarpus rugosus* DC. var. *heyneanus* Baker

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A protocol for *in vitro* multiple shoot regeneration and plant production through seedling (shoot tip) culture was established for *Alysicarpus rugosus* DC. var. *heyneanus* Baker. Maximum number of adventitious shoots (14.4) per shoot tip explant were initiated after two subcultures on MS solid medium supplemented with IAA (2.85 μ*M*) plus BAP (2.22 μ*M*) after 4 weeks. Shoot elongation (3.0–3.5 cm) was achieved on MS medium without any hormones. Stunted shoots elongated on half MS medium without growth hormones. Rooting occurred in MS medium containing IAA (1.14 – 2.85 μ*M*) alone or in combination with IBA (0.89 – 2.46 μ*M*) and or NAA (1.07 – 2.69 μ*M*). Maximum rooting was established in MS medium supplemented with IAA (2.85 μ*M*). The plants were acclimatized successfully with 55% survival in pot containing cocoa peat and sand (1:1). After a month, hardened plants were transferred to pots with manure, garden soil and sand (1:2:1) for further growth and finally planted in field.

Keywords: *Alysicarpus rugosus*, Multiple Shoot regeneration
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Forage legumes are major components of grassland agriculture and are of paramount importance for meat/milk production and for soil conservation. *Alysicarpus* Neck, commonly known as Alyce clover or Buffalo clover, is a low spreading annual plant grown for its forage value[1]. It is widely distributed in Maharashtra, India, and is represented by 9 species and 2 varieties[2]. The seeds of these species are hard with long dormancy and need scarification for effective germination[3]. The plant has nutritional potential and contains about 16.40% of crude proteins in its leaves and 8.50% of crude proteins in the stem[4]. The seeds contain more crude protein and crude lipid than other commonly consumed legumes[5]. An extensive work on cytogenetical studies with respect to karyotype analysis, meiotic analysis, DNA contents, breeding behaviour etc. was carried out by John and Thengane[6-8]. The present studies were undertaken to develop a protocol for *in vitro* multiple shoot regeneration and better surviral rate under solid condition.

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Abbreviations:
MS– Murashige and Skoog's basal medium; BAP– 6 Benzyl amino purine; IAA– Indole-3-acetic acid; IBA– Indole-3-butyric acid; NAA– Naphthalene acetic acid; SDW– Sterile distilled water; ANOVA– Analysis of variance; LSD– Least squared difference.

Materials and Methods

Mature pods of *Alysicarpus rugosus* DC. var. *heyneanus* Baker. were collected from Kolhapur, India. The seeds were de-coated manually by hand and soaked in sterile distilled water (SDW) for 10 min. They were then treated with concentrated sulfuric acid for 1min for scarification followed by thorough washing for 3-4 times in SDW. The seeds were allowed to pre-soak overnight in SDW under aseptic conditions and then inoculated into germination medium comprising 1/2 strength plain MS medium[9] without any growth hormones, containing sucrose 3% and gelled with 0.8% of Agar-Agar (Hi-Media India). The *p*H of the medium was adjusted to 5.7-5.8. Three seeds per tube were inoculated with total 100 seeds used. The experiment was repeated 3 times. The cultures were incubated at 25°±2°C temperature, 16/8hr photoperiod with 35 μE^{-2} m^{-2} s^{-1} illumination frequency using cool white fluorescent tubes.

Induction of shoot buds from seedling explants—*In vitro* germinated seedlings of 1.5-2.5 cm length were selected from which shoot tip, cotyledons and hypocotyl were used as the explants. The cotyledonary leaves were removed from shoot tip. The hypocotyl (0.5-0.8 cm), shoot tip and cotyledons were cultured aseptically on semi-solid MS medium supplemented with various concentrations of 2,4-D (2.26–22.62 μ*M*), IAA (2.85–28.54 μ*M*) and BAP (2.22 – 22.19 μ*M*).

For shoot tip 2,4-D (2.26–9.05 μ*M*)/IAA (2.85 μ*M*) alone or in combination with BAP (2.22 μ*M*) was used, while for hypocotyl and cotyledonary explants combination of 2,4-D (2.26 – 22.62 μ*M*) + IAA (5.71– 28.54 μ*M*) + BAP (2.22 – 22.19 μ*M*) was used in the induction medium. For each experiment 20 cultures with 3 replicates were maintained. Observations based on 20-30 days incubations were used for evaluation. Each experiment was repeated three times.

Elongation and rooting of multiple shoots—Multiple shoots especially induced on IAA (2.85 μ*M*) + BAP (2.22 μ*M*) were stunted and therefore transferred to 1/2 MS medium without any hormones for elongation. However, the multiple shoots induced on different combinations of hormone were subcultured on MS medium without hormone for elongation. The elongated shoots (3.0-3.5 cm) were transferred to medium containing IAA (1.14 – 11.42 μ*M*) alone or in combination with IBA (0.89 – 2.46 μ*M*)/NAA (1.07–2.69 μ*M*) for root induction. The rooted shoots were acclimatized in pot containing cocoa peat and sand (1:1) under green house conditions and after a month transferred in pots containing manure, garden soil and sand (1:2:1).

Statistical analysis—The results were analyzed statistically by ANOVA[10]. Treatments were compared at 1% level. All calculations were based on 20 readings for each treatment.

Results and Discussion

The seeds inoculated on 1/2 strength MS medium at 16/8hr photoperiod germinated within 3-4 days (Fig. 1a). Pre-soaking and scarification of the seeds facilitated best germination (76%).

Shoot organogenesis—Adventitious shoot induction was observed only from shoot tip explants (Fig.1b). Cotyledon and hypocotyl explants did not

Table 1—Effect of growth hormones on adventitious shoot induction

Explant	Phytohormone conc. (μ*M*)			% Response	Type of response	Amount of callus	Average No. of shoots after 2 subcultures
	2,4 D	IAA	BAP				
Cotyledon							
	2.26	-	-	82.20 (65.58)	c	+	Nil
	4.52	-	2.22	78.63 (62.47)	c	+++	Nil
	9.05	-	2.22	73.42 (58.97)	c	+++	Nil
	4.52	5.71	2.22	63.28 (52.65)	c	++	Nil
	9.05	11.42	4.44	69.11 (56.23)	c	++	Nil
	13.55	16.12	8.87	50.88 (45.46)	c	+	Nil
	22.62	28.54	13.31	49.64 (44.77)	c	+	Nil
	22.62	28.54	22.19	28.17 (32.01)	c	+	Nil
Shoot tip							
	2.26	-	-	74.30 (59.60)	c,ms,r	+	1.13
	2.26	-	2.22	88.34 (70.28)**	c,ms	+	2.35
	-	2.85	-	91.59 (73.15)**	c,ms,r	-	1.75
	-	2.85	2.22	96.17 (78.83)**	c,ms,r	-	14.42
	4.52	-	2.22	49.36 (44.60)	c,ms	++	0.97
	9.05	-	2.22	41.77 (40.22)	c,ms	+++	0.83
Hypocotyl							
	2.26	-	-	63.53 (52.84)	c	+	Nil
	4.52	-	2.22	81.82 (64.78)	c	++	Nil
	9.05	-	2.22	93.52 (75.31)	c	++	Nil
	4.52	5.71	2.22	56.41 (48.68)	c	++	Nil
	9.05	11.42	4.44	69.78 (56.60)	c	+	Nil
	13.55	16.12	8 87	50.23 (45.11)	c	+	Nil
	22.62	28.54	13.31	43.57 (41.27)	c	+	Nil
	22.62	28.54	22.19	31.64 (34.20)	c	+	Nil

c : Callus induction, ms: Multiple shoot formation, r: Rooting.
+ : Indicates the degree of callusing.
** Significant at 1% level.
Figures in parenthesis are angular transformation values of percentage of response.

produce shoots, but almost all the combinations of 2,4 D (2.26 – 22.62 μ*M*) supplemented with BAP (2.22–22.19 μ*M*) and/IAA (5.71–28.54 μ*M*) produced callusing with rare rhizogenesis.

Small shoot primordia were observed at the base of shoot tip explants after 15 days of inoculation in 74–96% cultures depending on media with different hormone compositions *viz;* 2,4-D (2.26 – 9.05 μ*M*), IAA (2.85 μ*M*) and BAP (2.22 μ*M*) (Table 1). Average number of shoots varied from 0.83 – 14.42 per shoot tip explant. The maximum number was observed in the medium containing IAA (2.85 μ*M*) + BAP (2.22 μ*M*). The shoots were induced from the base of shoot tip without callus induction. Direct shoot regeneration from explants without a callus phase would maintain the genotype fidelity that could be lost with the shoots arising from callus and would be an ideal system for genetic transformation[11]. Medium containing 2,4-D (2.26 – 9.05 μ*M*) alone or in combination with BAP (2.22 μ*M*) also produced multiple shoots in 41–88% cultures. However, from the cut surface of explant profuse callusing was observed. Few explants showed good multiple shoot induction with less amount of callus from the cut surface on the medium containing 2,4 D (2.26 μ*M*) alone and/or supplemented with BAP (2.22 μ*M*). For

statistical analysis, ANOVA was calculated. LSD at 1% level was 8.13 for shoot induction. It was observed that for multiple shoot induction from shoot tip explant, IAA (2.85 µ*M*) alone or in combination with BAP (2.22 µ*M*) and/or 2,4 D (2.26 µ*M*) + BAP (2.22 µ*M*) were significantly superior over 2,4 D alone. All the cultures were subcultured onto the same medium at every 15-day interval. Increase in the shoot induction was observed after two subcultures. Maximum average number of shoots 14.42 per explant were noted on IAA (2.85 µ*M*) + BAP (2.22 µ*M*) after a period of 45 days. (Fig.1c). Even though combination of IAA (2.85 µ*M*) + BAP (2.22 µ*M*) was found best for multiple shoot induction, the shoots produced were stunted. Although BA stimulates multiple shoot formation, it interfered with development and elongation of shoots. Most of the workers have observed the induction of stunted shoots along with some elongated ones when BA was used[12]. Elongation of stunted shoots occurred when such shoots were transferred to ½ strength MS medium without hormones. Shoots induced on 2,4-D (2.26 – 9.05 µ*M*) alone or in combination with BAP (2.22 µ*M*) and on IAA (2.85 µ*M*) alone were transferred to MS basal medium for further growth. Continued incubation on the same medium composition did not promote further multiplication of shoots.

The shoots (3.0-3.5cm) were transferred to rooting medium containing IAA (1.14 – 11.42 µ*M*) alone or in combination with IBA (0.89 – 2.46 µ*M*) and or NAA (1.07 – 2.69 µ*M*), (Table 2). Medium containing IAA (1.14 – 2.85 µ*M*) supplemented with IBA (0.89 – 2.46 µ*M*) and / NAA (1.07 – 2.69 µ*M*) induced rooting (Fig.1d) in 20-25 days. Combination of auxins IAA (1.14 µ*M*) + IBA (0.89 µ*M*) · NAA (1.07 µ*M*) showed 22.70% response. Increase in the concentration of all the auxins further increased rooting up to 55.13%. However, maximum 86.28% rooting was observed on the medium supplemented with IAA (2.85 µ*M*) alone. Higher concentration of IAA (5.71 – 11.42 µ*M*) alone or in combination with IBA (0.89 µ*M*) and NAA (1.07 µ*M*) did not induce rooting, rather the explants (elongated shoots) became yellow and ultimately died. The LSD at 1% level was 4.85. Thus, it was observed that for root induction IAA (2.85 µ*M*) was significantly superior over other auxin combinations used.

Table 2—Rooting of elongated shoot explants

Phytohormone concentration (µ*M*)			% Response	Average No. of roots
IAA	IBA	NAA		
1.14	0.89	1.07	22.70 (28.44)	3.81
2.85	2.46	2.69	55.13 (47.78)	5.93
2.85	-	2.69	30.91 (33.72)	6.00
2.85	-	-	86.28 (68.32)**	5.27
5.71	-	-	Nil	Nil
5.71	0.89	1.07	Nil	Nil
11.42	0.89	1.07	Nil	Nil

** Significant at 1% level.

Figures in parenthesis are angular transformation values of percentage of response.

Well-grown plantlets were transferred to sterilized pot containing mixture of cocoa peat and sand (1:1) under green house conditions at 70% RH and 26°±2°C for acclimatization, where 55% survival was observed. After about one month, the hardened plants were transferred to pots containing manure, garden soil and sand (1:2:1) for further growth and finally planted in field. The planting of micropropagated plants was carried out during the months of May-June just before the onset of the monsoon. Pits ($20cm^3$) were made in the field of botanical garden of botany department, Pune University, which were filled with garden soil and leaf mould (2:1). The plants in field after 3-4 months (Fig.1 e) showed good growth and development. The plants were watered every 3-4 days till onset of monsoon and were once supplemented with urea (IFFCO, India).

The present work is the first report on *in vitro* propagation of *Alysicarpus rugosus* DC. var. *heyneanus* Baker., an unexploited legume with better fodder potential as compared to other forage legumes. The above protocol can thus be used further for plant improvement programmes.

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