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DIRECT PLANT REGENERATION FROM MATURE NODAL EXPLANTS OF *ANDROGRAPHIS ECHIOIDES* (L.) NEES – A VALUABLE MEDICINAL PLANT

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ABSTRACT

The present study describes an effective, reproducible and rapid regeneration protocol for *in vitro* shoot development of the important medicinal plant *Andrographis echioides* (L.) Nees. via mature nodal explants inoculation on Murashige and Skoog (MS) medium supplemented with 6-Benzyl amino purine (BAP) 2.0 mg/l combined with α-Naphtalene acetic acid (NAA) 0.5 mg/l on which the highest percentage of shoot bud induction (90±2.88), number of shoot per explant (5±1.00) having a shoot length (2.0±0.57 cm) were produced. This investigation strongly recommends that the perfection of BAP (2.0 mg/l), NAA (0.5 mg/l) in combination with Gibberellic acid (GA_3) 0.5 mg/l on which a maximum percentage of shoot bud response (95±5.00), number of shoots per explants (9±1.00) and shoot length (6.8±1.11 cm) were found in *A. echioides*. MS medium, fortified with Indole-3-acetic acid (IAA) 1.0 mg/l combined with Indole-3-butyric acid (IBA) 0.5 mg/l on which highest percentage of response (90±5.77), number of roots per explants (12±1.15) and root length (6.5±1.26 cm) was observed. The regenerated plantlets were successfully transferred to field conditions with 80% survival rate.

Keywords: Direct regeneration, MS medium, nodal explants, *Andrographis echioides,* Plant growth hormones.

Introduction

Andrographis echioides (L.) Nees is an herbaceous plant belongs to the family Acanthaceae and is commonly called "false waterwillow" and in Tamil "Gopuram Tangi". *A. echioides* is pharmaceutically most known species in the genus *Andrographis* with habitat found in dry land, plain and waste places across the South Asian countries mostly in India, China and Srilanka (Qadrie *et al*., 2009; Mathivanan and Suseem, 2015). The *Andrographis* species are used to cure liver diseases, malarial fever, goiter, influenza, dyspepsia, fertility problems and respiratory related disorder (Chopra *et al*., 1980). *A. echioides* are also containing more number of therapeutic properties to cure cholera, stomach pain, controlling of hair fall, blood cancer, snake bite, scorpion sting and dysentery (Hemalatha and Vadivel, 2010; Padma *et al*., 2012; Purushotham *et al*., 2016). The leaf juice of *A. echioides* is used to cure fever, therefore this plant species is listed in the Indian Materia Medica as a cure for the treatment of ailments (Shen *et al*., 2013).

Even tablets were prepared from leaf extracts in mixture with other ingredients and used as a drug for the treatment of fever (Anandanayaki and Uma, 2014). This plant species was also reported to possess activities of anti-microbial, anti-diabetic, anti-oxidant, anti-pyretic, anti-diuretic anti-ulcer, analgesic, hepatoprotective and anti-inflammatory (Premkumar *et al*., 2010; Rupeshkumar *et al*., 2015; Raja and Jeevanreddy, 2014). Therefore the *A. echioides* has been over exploited by the pharmaceutical industries to prepare various drugs.

Plant tissue culture techniques could be effective method for mass production of such overexploited and important medicinal plants. For an efficient large-scale culture, perpetual explant source that is fast and stable growing is needed for securing the culture materials. There are no previous reports on direct regeneration from nodal explants of *A. echioides*. The present study describes *in vitro* propagation of *A. echioides* could be applied for large scale propagation and also ensure a continuous supply of plants produced in limited time.

Materials and Methods

Plant material and explants preparation

The wild grown healthy *A. echioides* species were collected and nodal explants were selected (Fig. 1-a), washed in running tap water followed by few drops of 10% (v/v) Tween-20 to remove the exterior dust particles including microbes. Then, they were surface sterilized with 0.1% (w/v) mercuric chloride solution ($HgCl_2$) for 1-2 minutes followed by rinsing with sterile double distilled water for 4-6 times. Afterwards sterilized nodal explants were trimmed at both the ends and inoculated on sterilized media.

Medium and culture condition

The surface sterilized nodal explants were inoculated on MS (Murashige and Skoog's, 1962) solid medium fortified with 3% w/v sucrose and 0.8% agar (Himedia, Mumbai,

India). The media were adjusted to pH 5.6 with 0.1 N NaOH or 0.1 N HCl after addition of the plant growth regulators (PGRs) before gelling. The macro, micro nutrients and other chemical of analytical grade procured from Himedia, Mumbai, India. Around 15 or 20 ml of medium was dispensed into test tubes (25 mm x 150 mm, Borosil, India), correspondingly. The medium was sterilized for 20 min at 121 $^{\circ}$C 1.4 × 104 kg m^{-2} pressure. All cultures were incubated at 25±2 $^{\circ}$C under a 16/8 h light/dark regime, under a photon flux density of 50µmol $m^{-2}s^{-1}$ provided by cool white fluorescent light (40 W, Phillips, India). The relative humidity within culture room was maintained with 50-60%. The cultures were subcultured on the fresh medium after 15-20 days intervals.

Effect of growth hormones on shoot and multiple shoot induction

Growth hormones free from MS medium were used as a control in all our experiments. A single nodal segment was vertically inoculated in each culture tube. MS medium containing with different concentrations of two cytokinins like BAP and KIN along with NAA was used to shoot induction and multiple shoot proliferation of nodal explants. All cultures were subcultured to maintain the fresh medium of same composition every four weeks of culture. The maximum percentage of shoot bud response, number of shoots per explants and shoot length were documented after four weeks of culture.

Shoot bud elongation and multiplication

For shoot bud multiplication and elongation, the shoot buds were cultured on a MS medium fortified with different concentration of BAP, NAA and combined with GA_3.

Formation of *in vitro* rooting

For *in vitro* rooting, the well grown or completely proliferated adventitious shoots were transferred to root induction MS medium supplemented with different concentrations of IBA and IAA individual and combination with IAA plus IBA. The percentage of root induction, number of roots per explants and root length after four weeks of inoculation were recorded.

Hardening and acclimatization

The complete *in vitro* grown plantlets were gently taken off from culture tubes and thoroughly rinsed with sterile double distilled water (ddH_2O) to clean all traces of culture medium. Then it was transferred into paper cups filled with garden soil: vermiculite: sand in the ratio of 2:1:1 (w/w). To maintain optimum humidity of 60-70% polyethylene covers were used to close the paper cups. The paper cups were then acclimatized for two to three weeks under 16/8 photoperiod at 25±2^{0}C after removing polybags in an aseptic condition. After 4 weeks plantlets were transferred to glass house. Finally, plantlets were gradually exposed to sunlight and were maintained in the garden.

Statistical analysis

Twenty explants were used for all experiments and were repeated thrice (three replicates) to get optimum result. Statistical Packages for Social Sciences version 16 (SPSS. 16) were used to find out mean values and standard error (IBM Corporation SPSS, North America USA). The one way analysis of variance (ANOVA) by Duncan's multiple range test (DMRT) followed to find out the significance of differences among at $P \leq 0.05$. The results are expressed as mean ± SE of three experiments.

Results and Discussion

Effect of cytokinins and auxin concentration on shoot induction and multiple shoot formation

Nodal explants were cultured for direct shoot regeneration on BAP and KIN alone and combination of BAP+NAA and KIN+NAA along with various growth hormones supplemented MS medium (Table-1; Fig. 1-b). The MS medium without growth hormones served as control. Among two cytokinins (BAP and KIN) were verified, 2.0 mg/l (BAP) was found most suitable for the maximum percentage of shoot bud response, nevertheless the *in vitro* regenerated shoots were smaller in size due to stunted growth. This medium produce highest percentage of shoot response (60±5.00), number of shoots per explants (2±0.57) and shoot length (1.3±0.32 cm) was found on MS medium fortified with 2.5 mg/l BAP (Table 1; Fig. 1-c). But KIN was found to be less effective against the percentage of shoot bud response. The superiority of BAP over KIN has also been described in *Eclipta alba* (Husain and Anis 2006) and *Stevia rebaundiana* (Debnath 2007). Higher level cytokinins concentration decrease the shoot bud induction was observed. The BAP and NAA combinations, highest percentage of multiple shoot regeneration response (90±2.88), number of shoots per explants (5±1.00) and shoot length (2.0±0.57 cm) was observed on MS medium supplemented 2.0 mg/l (BAP) combination with 0.5 mg/l (NAA) (Table-1; Fig. 1-d). Amzad Basha Kolar *et al.*, (2008) reported that MS medium fortified with 6.0 mg/l BAP and 0.5 mg/l NAA produced highest percentage of shoots from nodal explants of *Solanum nigrum.* When increasing the BAP and NAA concentration beyond 2.0 mg/l decreases the regeneration frequency in all parameters. Mahmad *et al*. (2014) demonstrated that the maximum percentage of shoot bud induction produced in *Nelumbo nucifera*. Besides, the synergistic effect of cytokinin and auxin at lower concentration was stated to be optimal for shoot regeneration and higher number of shoot buds formation from nodal segments of *Stevia rebaudiana* by Jain *et al*. (2009). Consequently, our findings exhibited that BAP in combination with low concentration of NAA was observed to be the most effective for shoot bud induction and multiplication. In fact, the presence of cytokinin and auxin was essential to induce shoot bud and multiple shoot production from shoot tip explants of *Prunella Vulgaris* by Rasool *et al*. (2009).

Effect of optimum concentration of GA_3 on shoots elongation and regeneration

In the present investigation microshoots developed on BAP plus NAA containing media failed to elongate when subcultured on the medium. *In vitro* raised stunted growth shoots were inoculated on MS medium containing with BAP (0.5-3.0 mg/l) in combination with optimum concentration of NAA (0.5 mg/l) and GA_3 (0.5 mg/l). Here also control (MS basal medium) has not promoted any form of positive shoot elongation. Shoot elongation in regenerated plantlets is a critical step of culturing *in vitro*, which requires alteration in media composition (Malik and Saxena, 1992; Prakash *et al*., 1994), plant growth regulator substitution (Mohamed *et al*., 1991), change in light conditions etc. A combination of BAP with optimum concentration of NAA plus GA_3 not only

promoted shoot elongation but also enhanced shoot proliferation of even more shoots. The best combination for highest percentage of shoot response (95±5.00), mean number of shoots (9±1.00) and shoot length (6.8±1.11 cm) was recorded on MS medium supplemented with BAP (2.0 mg/l) with optimum concentratin of NAA (0.5 mg/l) and GA_3 (1.0 mg/l) after 4 weeks of culture (Table-2; Fig. 1-e). GA_3 effects shoot bud growth, cell division as well as shoot elongation in many plants reported by George *et al*. (2008). Our observation is in agreement with earlier findings for *Isodon wightii* by Thirugnanasampandan *et al*. (2010). In *Salvia sclarea* subculture of the shoots on the medium fortified with BAP and GA_3 resulted in the shoot elongation and multiple shoot produced by Liu *et al*. (2000).

Effect of IBA and IAA on rooting of regenerated shoots

Regenerated shoots must be relocated to rooting medium which is generally carried out on auxins containing medium. In the current investigation MS medium fortified with various concentrations of growth hormones (0.5-3.0 mg/l) IAA and IBA individually and combinations of IAA plus IBA were tested. *In vitro* raised shoots failed to induce rooting on control medium free from growth hormone in MS medium. Root induction with maximum percentage of root response (90±5.77), mean number of roots per explants (12±1.15) and root length (6.5±1.26 cm) was observed on MS medium supplemented with the combination of IAA (2.0 mg/l) plus IBA (0.5 mg/l), after one month of culture (Table-3; Fig. 1-f&g). Initially the roots were formed as a cluster from the basal cut end and gradually develop into thick and a healthy root was formed. Subsequently rapid *in vitro* shoot elongation was detected after 7 days of root induction. Similarly, Savitikadi *et al*. (2020) reported that MS medium fortified with 1.0 mg/l IAA plus IBA 1.0 mg/l produced maximum percentage of rooting response from leaf explants of *A. echioides*. The mixture of growth hormones like IAA and IBA was also earlier reported in stimulating the highest percentage of rooting in other plant species by Laribi *et al*. (2012). Similarly, Sheik Mohamed *et al*., (2017) demonstrated that the maximum amount of root formation through nodal explants in *Ammannia baccifera* was obtained on MS medium supplemented with 2.5 mg/l IAA and 1.5 mg/l IBA.

Acclimatization

Well grown *in vitro* regenerated shoot and rooted plants at the age of six weeks are gently plucked from the medium and sterilized with double distilled water (ddH_2O) to clean the traces of media were transferred to paper cups filled with garden soil : vermiculite : sand in the ratio of 2:1:1 (w/w). They were then transferred to pots containing as 3:2:1 (w/w) mixture of garden soil, vermiculite and sand for hardening and acclimatization (Fig.1 h). Humidity was maintained by frequent spraying of water and after that, the plantlets were transplanted to the soil. It was recorded that 80% plantlets were survived. Similarly, Siwach and Gill, (2011); Hesami and Daneshvar, (2018) reported the same method of acclimatization in a medicinal plant in *Ficus religiosa*.

Conclusion

The present investigation defined a simple, rapid large scale production and efficient protocol for direct plant regeneration from mature nodal explants of *A. echioides* a medicinally important plant. Maximum percentage of shoot bud induction (90%) devoid of attaining callus was observed in MS medium fortified with 2.0 mg/l BAP+NAA 0.5 mg/l. Our investigation strongly suggest that the superiority of BAP (2.0 mg/l), NAA (0.5 mg/l) in combination with GA_3 (1.0 mg/l), is essential for a shoot induction, multiple shoot bud formation as well as enhanced shoot elongation and also proliferation of even more shoots. MS medium, fortified with IAA 2.0 mg/l combination with IBA 0.5 mg/l was observed to be the best for a highest rooting response as well as the well-developed regenerated plantlets were effectively transferred in soil conditions with 80% survival rate. To the best of our knowledge, this is the first report on high frequency of plant regeneration from mature nodal explants of *Andrographis echioides*.

Table 1: Effect of different concentrations of Cytokinins (BAP, KIN) and combination of Auxin (NAA) on shoot regeneration from mature nodal explants of *Andrographis echioides* after 4 weeks of culture.

Growth regulators (mg/l)			(%) of shoot bud induction (Mean ± SE)	Number of shoots per explants (Mean ± SE)	Shoot length (cm) (Mean±SE)
BAP	KIN	NAA			
Control			00±00	00±00	00±00
0.5			25±5.77^{ghij}	2±0.57^{bcd}	0.7±0.15^{cde}
1.0			40±5.77^{cdefgh}	2±0.00^{bcd}	1.1±0.26^{abcd}
1.5			55±7.63^{cde}	2±1.00^{bcd}	1.5±0.28^{abc}
2.0			75±2.88^{ab}	4±1.00^{ab}	1.8±0.40^{ab}
2.5			60±5.00^{bcd}	2±0.57^{bcd}	1.3±0.32^{abcd}
3.0			35±5.77^{efgh}	2±1.00^{bcd}	0.9±0.20^{bcde}
	0.5		15±5.00^{ijk}	1±0.00^{cd}	0.4±0.05^{de}
	1.0		30±5.00^{fghi}	2±0.57^{bcd}	0.7±0.15^{cde}
	1.5		45±7.63^{cdefg}	2±0.00^{bcd}	0.9±0.26^{bcde}
	2.0		50±5.00^{cdef}	3±1.00^{abc}	1.0±0.28^{bcd}
	2.5		25±7.63^{ghij}	2±0.57^{bcd}	0.6±0.15^{cde}
	3.0		10±0.00^{jk}	1±0.00^{cd}	0.4±0.11^{de}
0.5		0.5	30±5.00^{fghi}	2±0.00^{bcd}	1.2±0.32^{abcd}

1.0		0.5	45±7.63^{cdefg}	2±0.57^{bcd}	1.4±0.50^{abcd}
1.5		0.5	60±7.63^{bcd}	4±1.00^{ab}	1.8±0.41^{ab}
2.0		0.5	90±2.88^{a}	5±1.00^{a}	2.0±0.57^{a}
2.5		0.5	55±7.63^{cde}	2±0.57^{bcd}	1.3±0.30^{abcd}
3.0		0.5	40±5.00^{cdefgh}	2±1.00^{bcd}	1.0±0.00^{bcd}
	0.5	0.5	20±5.00^{hij}	2±0.57^{bcd}	0.7±0.15^{cde}
	1.0	0.5	35±5.77^{efgh}	3±0.57^{abc}	0.9±0.30^{bcde}
	1.5	0.5	50±7.63^{cdef}	3±1.00^{abc}	1.0±0.00^{bcd}
	2.0	0.5	65±7.63^{bc}	5±0.57^{a}	1.0±0.50^{bcd}
	2.5	0.5	45±7.63^{cdefg}	2±0.00^{bcd}	0.7±0.30^{cde}
	3.0	0.5	20±7.63^{hij}	2±1.00^{bcd}	0.4±0.05^{de}

Each values represent mean ± standard error of 20 replicates per treatment in three repeated experiments. P≤0.05 level, using Duncan's multiple range test.

Table 2: Effect of BAP, NAA and GA_3 on multiple shoot induction and elongation from *in vitro* derived nodal explant of *Andrographis echioides.*

Growth regulators (mg/l)			**(%) of response (Mean ± SE)**	**Number of shoots per explants (Mean ± SE)**	**Shoot length (cm) (Mean±SE)**
BAP	NAA	GA_3			
Control			00±00	00±00	00±00
0.5	0.5	1.0	30±5.77^{d}	3±1.52^{c}	2.8±0.95^{b}
1.0	0.5	1.0	55±7.63^{c}	4±0.57^{bc}	3.4±0.50^{b}
1.5	0.5	1.0	70±7.63^{b}	6±0.57^{b}	4.0±0.28^{b}
2.0	0.5	1.0	95±5.00^{a}	9±1.00^{a}	6.8±1.11^{a}
2.5	0.5	1.0	60±7.63^{bc}	5±1.52^{bc}	3.8±0.85^{b}
3.0	0.5	1.0	35±7.63^{d}	3±0.57^{c}	2.4±0.30^{b}

Each values represent mean±standard error of 20 replicates per treatment in three repeated experiments. P≤0.05 level, using Duncan's multiple range test.

Table 3: Effect of IAA and IBA alone, and combination of IAA and IBA auxins in different concentration of growth hormones for root induction from *in vitro* raised shoots of *Andrographis echioides*

Growth regulators (mg/l)			**(%) of root induction (Mean ± SE)**	**Number of roots per explants (Mean ± SE)**	**Root length (cm) (Mean±SE)**
IAA	IBA	IAA+IBA			
Control			00±00	00±00	00±00
0.5			20±2.88^{gh}	2±0.57^{ef}	1.6±0.30^{fg}
1.0			35±7.63^{efg}	2±1.00^{ef}	1.9±0.23^{efg}
1.5			55±2.88^{cd}	4±1.52^{cdef}	2.3±0.58^{defg}
2.0			65±7.63^{bc}	6±1.15^{bcd}	2.8±0.52^{cdef}
2.5			40±2.88^{def}	3±0.00^{def}	2.0±0.57^{defg}
3.0			25±2.88^{fgh}	2±1.00^{ef}	1.2±0.47^{fg}
	0.5		10±2.88^{h}	1±0.00^{f}	0.5±0.15^{g}
	1.0		20±5.00^{gh}	2±0.57^{ef}	0.7±0.20^{fg}
	1.5		35±2.88^{efg}	2±0.57^{ef}	1.0±0.28^{fg}
	2.0		50±2.88^{cde}	3±0.57^{def}	1.8±0.30^{efg}
	2.5		35±7.63^{efg}	3±1.00^{de}	1.5±0.28^{fg}
	3.0		20±5.00^{gh}	2±0.57^{ef}	1.0±0.28^{fg}
		0.5+0.5	40±7.63^{def}	4±1.52^{cdef}	3.8±0.75^{bcde}
		1.0+0.5	55±0.00^{cd}	6±0.57^{bcd}	4.6±0.97^{abc}
		1.5+0.5	75±7.63^{ab}	9±1.52^{ab}	5.2±1.13^{ab}
		2.0+0.5	90±5.77^{a}	12±1.15^{a}	6.5±1.26^{a}
		2.5+0.5	60±7.63^{bc}	7±2.08^{bc}	4.7±1.10^{abc}
		3.0+0.5	35±7.63^{efg}	5±1.15^{cde}	4.0±0.28^{bcd}

Each values represent mean±standard error of 20 replicates per treatment in three repeated experiments. P≤0.05 level, using Duncan's multiple range test.

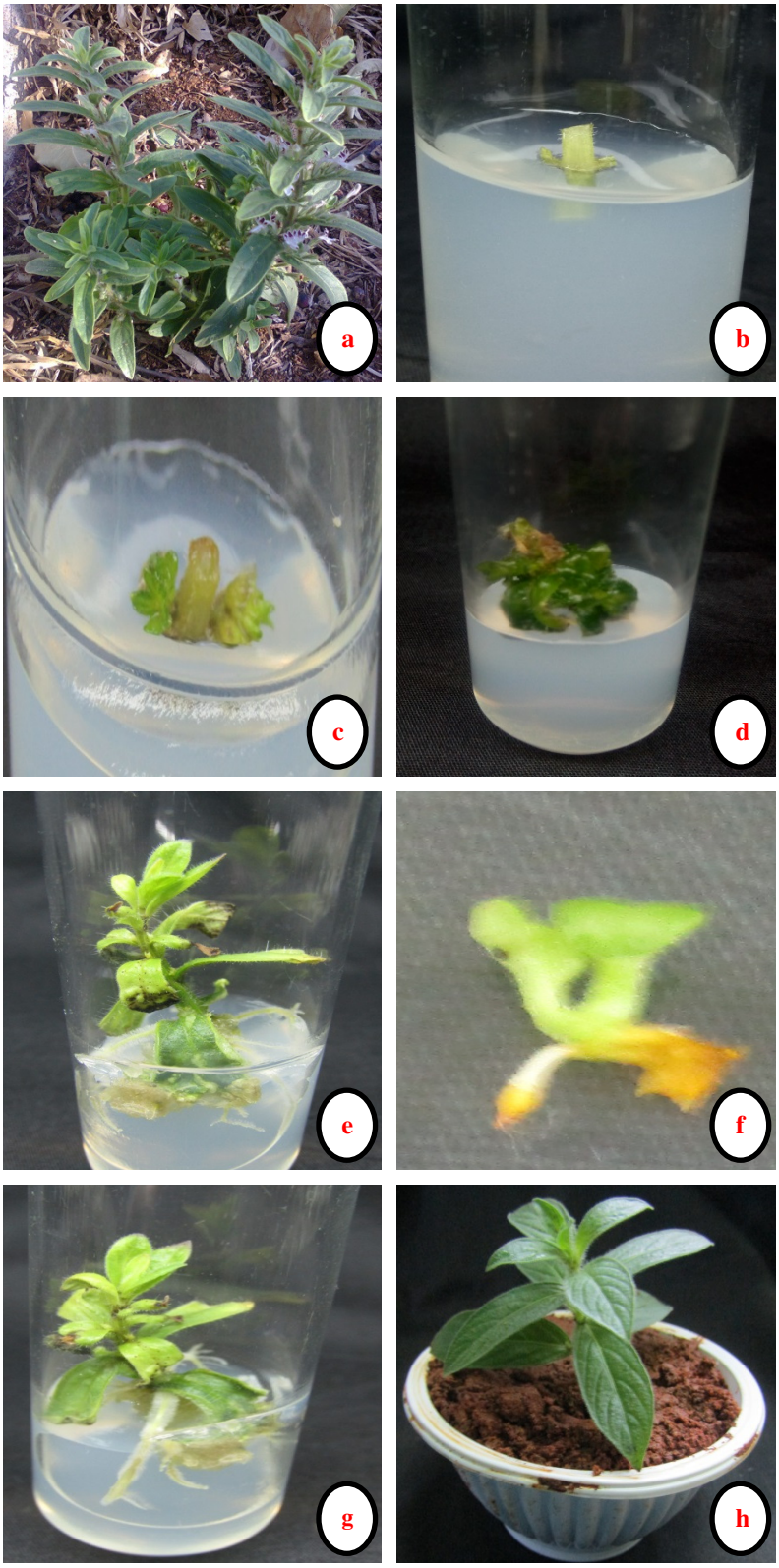

Fig. 1: Direct plant regeneration from nodal explant of *Andrographis echioides*.

a – Habit of *A. echioides* b- Nodal explant c - Shoot induction d - Multiple shoot proliferation
e - Shoot elongation f – Root initiation g – Well developed shoots and roots
h- Acclimatized plants on sterile soil.

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