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Full Length Research Paper

An efficient plant regeneration protocol for *Achyranthes aspera* L.

[1]Monokesh Kumer Sen†, *[1,2]Md. Mehedi Hassan†, [3]Shamima Nasrin, [1]M. Abu Hena Mostofa Jamal, [1]A. N. M. Mamun-Or-Rashid, [1,4]Nirupam Biswas

[1]Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia-7003, Bangladesh
[2]Department of Microbiology and Genetics and Institute for Medical Science, Chonbuk National University Medical School, Jeonju 561-712, South Korea
[3]Scientific Officer, Plant Biotechnology Division, National Institute of Biotechnology, Savar, Dhaka, Bangladesh
[4]Department of Nutrition and Food Sciences, University of Huddersfield, United Kingdom

Abstract

***Achyranthes aspera* L. (Apang locally) belongs to the family Amaranthaceae, is a highly significant medicinal herb found almost everywhere in Bangladesh. Mass collection of the plant from natural habitats has led to its depletion. An attempt was taken to establish an efficient protocol for its mass propagation from field-grown plants (node). Diverse concentrations of phytohormones in single or in various combinations were used to obtain desirable plant shoot and root regeneration. Experimentally, the best shoot induction was observed on half strength MS medium supplemented with BAP 3.0mg/L. *In vitro* raised shoots were allowed to root on different strengths of MS medium fortified with IAA, IBA and NAA at different concentrations. Concerted efforts of 2.0mg/L IBA and 0.5mg/L NAA were enabled to induce 100% root initiation with higher root number and longest shoot (17.0±0.75cm) on full strength MS medium. Afterwards, regenerated shoots with well developed roots were successfully subjected to hardening process and were acclimatized. The survived plantlets showed 86.67% survival frequency without any morphological abnormalities. This protocol might be applied for large scale commercial cultivation of *Achyranthes aspera* L..**

Keywords: *Achyranthes aspera* L., Mass propagation, Phytohormones, MS medium, shoot and root induction, acclimatization.

INTRODUCTION

In Bangladesh, about 500 plant species have been identified as medicinal plant because of their therapeutic properties (Ghani, 2000). One of the many plants which are being evaluated for their therapeutic efficacies is *Achyranthes aspera* (Amaranthaceae family), which is a common plant found as an invasive alien species in northern Bangladesh as weed (Akter and Zuberi, 2009). The plant is a popular folk remedy in traditional system of medicine throughout the tropical Asian and African countries. Researches have carried out by using different parts of the plant to treat leprosy, asthma, fistula, piles, arthritis, wound, renal and cardiac dropsy, kidney stone, diabetes, toothache etc. (Bhandari, 1990; Dwivedi et al.,2008). Phytochemical investigations revealed the presence of sterols, alkaloids, saponins, sapogenins, cardiac glycosides, ecdysterone etc from different parts of the plant (Dey, 2011). Exciting features of this plant is motivating researchers for conducting their investigation on this plant (Kayani, 2008; Gnanaraj et al., 2012).

Despite the development of large number of clinically effective compounds, indigenous phytotherapy is still practiced in many rural areas of developing countries. Worldwide about 85% of all medications for primary health care are derived from natural sources (Abbasia et al.,2010). The increasing demands for medicinal plants will definitely reduce the sustainable supply of medicinal

*Corresponding Author's E-mail: mehedibt07@gmail.com
† These authors have equal contribution

plants in the future. Moreover, plant secondary products are often produced only in small quantities in most of the plant species. Besides, plants are endangered by a combination of factors such as over-collecting, unsustainable agriculture practices, urbanization, no proper regulation on management and conservation. Environmental pollution, climate change and accumulation of heavy metal also affect the continuous supply of fresh plant material. Therefore, biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants (Rao and Ravishankar, 2002).

Micropropagation is an alternative to the conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication (Kaur et al.,1998). It has been viewed as an important technology for enhancing the capability of selected elite high yielding varieties, so as to boost production and productivity. *A. aspera* grows as weeds in agricultural and waste lands. Most of the people collect this plant from any place without considering whether they are grown in polluted or unpolluted areas. The plants that were collected from polluted site were found to contain heavy metal or toxic components such as mercury. As this plant is often utilized by human and in order to maintain sustainable supply of healthy and quality plants for human consumption, *in vitro* propagation technique should be used for growth. The aims of this study are (i) to establish the optimum condition for the enormous propagation of *A. aspera.* (ii) to develop an approach for large scale production of plantlets. (iii) to acclimatize regenerated plantlets.

Throughout the investigation nodal explants of *Achyranthes aspera* L. were cultured in different strength of MS medium with various concentration and combinations of phytohormones for regeneration of shoots and roots. The best shoot induction was observed on half strength MS medium supplemented with BAP 3.0mg/L. 2.0mg/L IBA and 0.5mg/L NAA were enabled to induce 100% root initiation with higher root number and longest shoot (17.0±0.75cm) on full strength MS medium. To our knowledge for the first time an effort was made to develop a protocol for micropopagation of *Achyranthes aspera* L. in different strength of MS medium with various combinations of phytohormones for shoot and root regeneration.

MATERIALS AND MEHODS

Explants and nutrition medium for micropropagation

The experiment was conducted at Plant Biotechnology Division (PBD) of National Institute of Biotechnology (NIB),Dhaka, Bangladesh, during 2011-2012 with the objective to micropropagate Apang efficiently under *in vitro* condition and acclimatize regenerated plantlets. The nodes (explants), those were healthy, free of symptoms of disease, and pest problems collected from the experimental plot of PBD. Explants were inoculated on MS medium (Murashige and Skoog, 1962) after sterilization and inoculated in test tube or jar containing 3% sucrose and gelled with 0.8% agar supplemented with diverse concentrations phytohormones. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20min at 121^0C for 15lbs pressure.

Surface Sterilization

For the surface sterilization, the explants first fresh and juvenile nodal segments were collected and washed thoroughly in running tap water for 30 minutes to remove iota of dist. After that they were again washed with liquid detergent (Vim or Trix) and fungicides for few min with vigorous shaking and repeated washing steps were carried out. After Alcohol dip, explants were surface sterilized with freshly prepared 0.1% aqueous solution of Mercuric chloride for few minutes and washed for 3-4 times with sterile water to remove any traces of Mercuric chloride.

Culture incubation

The test tubes, jars, culture vessels and conical flasks containing explants were incubated in a temperature controlled growth chamber or growth room. The temperature of the growth chamber or growth room was maintained of 25 ± 2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16 hrs day light and 8 hrs dark.

Transplantation of regenerated plantlets

The regenerated plantlets after developing sufficient root system were deemed ready to transfer in soil. Immediately after washing they were transferred to small earthen pots containing soil and compost in 1:1 ratio. The plantlets with pots were covered with polythene bags to check sudden desiccation. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment. The plantlets were adapted under controlled environment of Green house. By this time the plantlets become established in to the soil and ready for field transformation.

Data recording and analysis

The percentage of initiation, days for initiation, length and number of shoots and roots, and regeneration

Table 1. Effects of different concentrations and combinations of phytohormones in different strengths of MS medium for shoot induction and proliferation

Hormone	Concentration	Sample	DSI	NER	NS	NL	SL	RN
BAP+MS	0.5	Q_1	8-9	40	2.33±0.47	8	1.3±0.08	0
	5.0	Q_2	4-5	80	4.33±1.7	16	1.5±0.26	0
	7.0	Q_3	10-12	30	2.33±0.47	14	0.73±0.04	0
BAP+1/2**MS**	0.5	S_1	6	60	3.33±1.89	15.7	1.97±0.12	0
	3.0	S_2	3-4	100	4.33±1.7	15.3	2.76±0.47	0
	7.0	S_3	5-6	40	2±0	8	1.2±0.11	0
BAP+1/3MS	0.5	G_1	5-6	50	2.33±0.47	10	2±0.4	0
	4.0	G_2	4-5	50	2.33±0.47	12	1.6±0.5	0
	7.0	G_3	3-4	80	2.33±0.47	14	0.8±0.1	0
Kin+MS	0.5	K_1	18-19	20	1.33±0.94	6	0.41±0.07	0
	3.5	K_2	15-16	40	0.67±0.47	2.3	0.29±0.01	0
	7.0	K_3	11-12	40	1.67±1.25	6	0.9±0	0
Kin+1/2**MS**	2.0	P_1	12-14	20	1±0.82	4	1.0±0.09	0
	5.0	P_2	6-7	30	3.67±1.25	15.7	1.47±0.42	0
	7.0	P_3	3-4	50	3.67±1.25	16	1.77±0.09	0
Kin+1/3MS	0.5	O_1	12-14	20	1.33±0.94	4	0.44±0.04	0
	4.0	O_2	9-10	30	2.67±0.94	16	2.07±0.17	0
	7.0	O_3	3-4	50	2.33±0.47	14	1.52±0.06	0
BAP+Kin+MS	1.0+0.2	M_1	4-5	80	4±1.63	12	1.46±0.05	0
	1.0+0.5	M_2	5-6	70	5±2.16	16	1.3±0.2	0
	7.0+0.5	M_3	7-8	30	3±0.82	10	0.6±0.1	0
BAP+Kin+1/2**MS**	3.0+0.2	A_1	3-4	100	4±1.63	16	1.44±0.12	0
	3.0+0.5	A_2	5-6	90	3±2.33	6	2.03±0.31	0
	7.0+0.5	A_3	9-10	40	2±0	4	2.9±0.16	0
BAP+NAA+MS	1.0+0.2	H_1	4-5	90	4±1.63	16	1.46±0.04	5.8±0.37
	3.0+0.2	H_2	4-5	80	2.67±0.47	8	1.2±0.08	2.67±0.7
	5.0+0.2	H_3	5-6	80	3±0.82	10	0.86±0.05	2±0
BAP+IAA+MS	3.0+1.0	BA_1	7-8	40	2±0	4	0.8±0.02	0
	3.0+2.0	BA_2	6-7	60	3±0.82	10	1.13±0.9	0
	3.0+3.0	BA_3	5-6	80	2±0	4	0.65±0.04	2.67±0.5
IAA+Kin+MS	3.0+1.0	IK_1	5-6	60	3.33±0.94	12	1.9±0.47	0
	3.0+2.0	IK_2	8-9	30	2±0	4	0.9±0	0
	3.0+3.0	IK_3	9-10	30	2±0	6	1.16±0.20	0
BAP+IAA+Kin+MS	1.0+0.5+0.5	BIK_1	7-8	60	3.33±0.94	10	0.9±0.06	0
	2.0+0.5+0.5	BIK_2	7-8	50	2.67±0.94	8	1.31±0	0
	3.0+0.5+0.5	BIK_3	5-6	80	4±1.63	18	2.01±0.06	0
	4.0+0.5+0.5	BIK_4	8-9	40	2.67±0.94	8	1.5±0	1.33±0.1
BAP+IAA+Kin+1/2**MS**	1.0+0.5+0.5	BK_1	5-6	60	2.67±0.94	10	1.27±0.12	0
	2.0+0.5+0.5	BK_2	5-6	60	2.67±0.94	8	1.3±0	0
	3.0+0.5+0.5	BK_3	3-4	90	3.33±1.9	14	1.77±0.12	2.67±0.5

*Concentration of hormone (mg/L), DSI: Days for shoots initiation, NER: Number of explants regenerated (%), NS: Number of shoots (mean ±SD), NL: Number of leaves (Average), SL: Shoots length (cm, mean ±SD), RN: Root number (mean ± SD), 10 explants inoculated for per sample.

percentage during acclimatization of plantlets were recorded and the data was subjected to standard deviation using computer software.

RESULTS AND DISCUSSION

Shoot initiation time

Half strength of MS medium showed best result to initiate shootlets from nodal explants and it took only 3-4 days (S_1, S_2, G_3, P_3, BK_3 and A_1 samples). BAP took less duration of time than Kin for initiation of shootlets. Kin took 18-19 days at 0.5mg/L of concentration whereas at the same concentration BAP took 8-9 days. Similar type of late initiation of shootlets by using Kin was also examined by Shaheenuzzaman et al.,(2011). The use of BAP with different concentration and combination with Kin, NAA or IAA didn't show significant change in the duration of time for shootlets initiation and the average range was between 7-10 days (Table 1).

Percentage of shoot regeneration

Among different treatments, shoot regeneration percentage varied significantly along with the concentration of cytokinin. BAP at the concentration of

Table 2. Effects of different concentrations and combinations of IBA, NAA and IAA in different strengths of MS medium for rooting of shoots.

Hormone	Concentration	Sample	SR	DRI	NRM	RL	NL	SL
NAA+MS	3.5	A_1	100	7-8	11.3±4.1	3.1±0.6	9.3	6.23±0.04
	4.0	A_2	100	4-5	21.3±5.7	3.17±0.17	14	8±0.14
NAA+1/2MS	1.0	N_1	100	5-6	14.3±3.4	7.6±0.08	8	4.1±0.9
	2.5	N_2	100	7-8	22.7±2.5	3.4±2.1	12	6.0±1.9
NAA+1/3MS	0.5	NA_1	60	17	3.3±2.8	1.0±0.1	8.3	2.1±1.1
	4.0	NA_2	100	10	8.7±5.3	1.3±0.9	8	4.0
IBA+MS	3.5	I_1	100	6-7	12.7±4.1	11.7±1.2	14	8.5.46
	4.0	I_2	100	5-6	26.7±6.7	12.7±2.1	14	10.0
IBA+ 1/2MS	2.5	B_1	100	4-5	20.3±10.3	6.7±0.34	10	6.1±0.01
	4.0	B_2	100	6-7	26.0±10.2	9.1±0.33	16	11±0.17
IBA+1/3MS	0.5	IB_1	100	6-7	12.3±4.9	1.0±0.1	8	5.0±0.32
	2.0	IB_2	100	6-7	11.0±3.7	1.1±0.1	6	4.1±0.34
IAA+MS	2.0	X_1	100	7-8	16.0±7.7	1.3±0.1	10	4.1±1.29
	3.0	X_2	100	5	21.7±6.3	4.0±0.27	10	6.4±1.69
IAA+1/2MS	0.5	T_1	100	5	9.0±5.3	1.6±0.1	6	2.0±1.17
	2.0	T_2	100	5-6	5.6±2.7	1.5±0.1	6	3.1±1.9
IAA+1/3MS	0.5	Y_1	100	6-7	9.3±3.3	1.2±0.1	8	5.0±2.1
	2.0	Y_2	100	9-10	6.6±4.2	0.7±0.25	6	2.9±0.1
NAA+IBA+MS	3.0+3.0	IA_1	80	10-11	7.0±4.08	3.0±2.1	10	8.0±0.33
	4.0+4.0	IA_2	100	5-6	21.7±2.2	1.5±0.1	14	9.0±0.47
IBA+NAA+1/3MS	2.0+0.5	IA_3	100	4-5	25.0± 3.4	8.7±0.9	12	17.0±0.75
	0.5+2.0	IA_4	100	7-8	8.0±2.3	10.7±0.5	10	9.0±1.30
NAA+IBA+ 1/2MS	0.5+0.5	L_1	100	7	14.0±4.2	1.3±0.1	8	4.0±0.33
	6.0+6.0	L_2	60	15	3.3±2.5	0.3±0.1	4	2.0±0.1
NAA+IAA+MS	2.0+2.0	U_1	100	9-10	4.5±5.3	1.0	6	5.6±1.22
	3.0+3.0	U_2	100	6-7	17.3±10.2	1.4±0.2	8	6.0±2.2
IBA+IAA+MS	0.5+0.5	E_1	100	6	19.3±7.7	3.06±0.2	6	4.0±1.1
	2.0+2.0	E_2	100	8-9	8.3±6.4	2.6±0.2	4	3.4±1.3
IAA+IBA+NAA+MS	0.5+0.5+0.5	Z_1	100	10-11	13.3±1.2	3.5±0.18	12	3.0±2.2
	0.75+0.75+0.75	Z_2	70	9-10	11.2±0.3	3.0±0.12	10	4.0±0.30
	1.0+1.0+1.0	Z_3	100	7-8	28.3±10.3	3.6±0.33	14	7.0±0.7
IAA+IBA+NAA+1/2MS	0.5+0.5+0.5	J_1	100	6-7	27.0±2.2	1.0±0.1	6	3.2±0.32
	2.0+2.0+2.0	J_2	100	8-9	5.3±2.9	0.9±0.1	4.3	3.0±0.1

*Concentration of hormone (mg/L), SR: Shoots rooted (%), DRI: Days for root induction, NRM: Number of roots per micro-cutting (mean ±SD), RL: Root length (cm) (mean ±SD), NL: Number of leaves, SL: Shoot length (cm, mean ±SD).

3.0mg/L on half strength MS medium gave highest shoot regeneration (100%).3.0mg/L of BAP showed highest frequency nearly 80% in earlier report (Gnanaraj et al.,2012). Comparatively less shoot regeneration frequency was recorded with Kin among all strength of MS medium application which is in line with the findings of Karim et al.,(2003) and Shabbir et al.,(2012). Kin proved less effective for shoot regeneration and gave comparatively lower regeneration percentages than BAP. But BAP combination with Kin at concentration of 3.0 and 0.2mg/L were fortified in 1/2MS medium, also showed 100% shoot regeneration efficiency. When the concentration of BAP higher than its optimum concentration on both full and half strength MS exhibited negative effect on regeneration frequency. The use of BAP with IAA, Kin with IAA or application of the combined effects of these three on either full or half strength of MS medium played no significant effect on shoot regeneration.

Number of regenerated shoots

Results showed that the number of regenerated shoots per explant ranged between 0.67±0.47 to 5±2.16, depending on the strength of MS medium and concentration and combination of growth regulators (BAP, Kin, NAA and IAA). The number of shoots per explant and leaves were high in BAP induced medium than Kin (Table 1, 2) as shown by Sasikumar et al.,(2009) and Geetha et al.,(1998). But the combination of BAP and Kin gave maximum shootlets (M_2 sample). Other combination of hormones didn't give significantly different results.

Length of regenerated shoots

Significant difference for shoot length was noticed for different concentrations of BAP and Kin in full strength of

Table 3. Survival rate of plantlets of *A. aspera* at different stages of hardening

Sample	Stage of transplantation	Number of plants transplanted	Percentage of survival
T1	Poly House (1st stage)	20	85.00
T2	Shade House (2nd stage)	17	88.23
T3	Field (3nd stage)	15	86.67

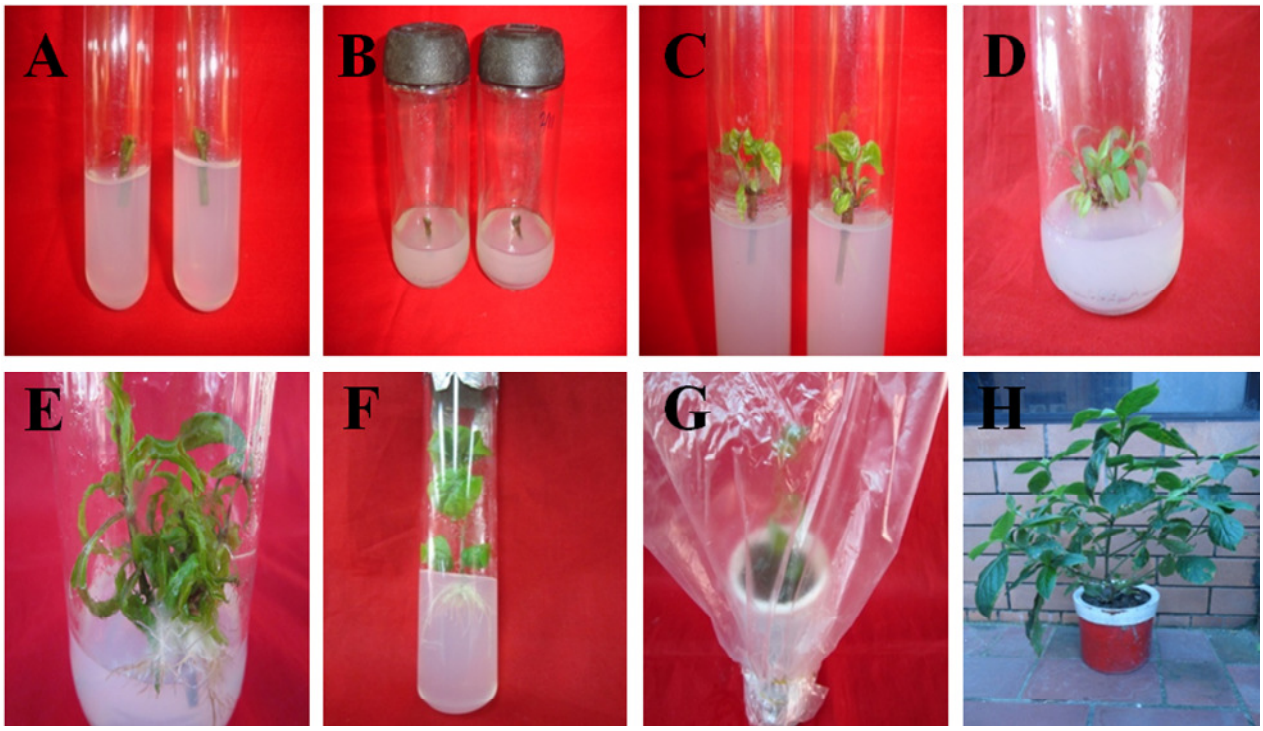

Figure . Different stages of plantlets development from node of *A. aspera.* A. Inoculation of nodal explants in test tube. B. Inoculation of nodal explants in jar. C. Initiation and proliferation of shootlets in test tube. D. Initiation and proliferation of shootlets in jar. E. Root initiation in shooting hormone. F. Root initiation and development. G. Poly bag stage H. Vigorously growing young plant of *A. aspera.*

MS medium but showed almost same results in the 1/3 of MS (Table 1). One third strength MS medium fortified with 7.0 and 0.5mg/L BAP and Kin showed maximum shoots length (2.9±0.16cm); and the lowest shoots length was observed with Kin at concentration of 1.5mg/L in full strength MS.

Rooting in shooting

Magnificently, BAP and Kin were ineffective in root induction. But when these were applied with IAA or NAA (commonly used in root induction) showed root induction irregularly (Table1).

Rooting percentage

Significant differences were not observed among treatments in rooting percentages and it varied from 60 to 100% with the varying concentrations of Auxins (Table 2). Auxin is an essential growth regulator required for the process of root formation. In the present study IBA was found more effective rooting hormone comprising 100% rooting efficiency compare with NAA and other hormones used here for this experiment. Similar results were well documented by earlier reports (Velayutham et al.,2006; Rehman et al.,2003). The efficiency of IAA was also documented in this experiment with high initiation power as mentioned before by Husen and Pal (2001). Mixture of IBA and IAA also had shown 100% rooting frequency. Combined effects of these two were further recorded by Sasikumar et al.,(2009).

Number of regenerated roots

Table 2 shows that exogenous application of NAA, IBA and IAA had a positive effect on root number. Root number varied significantly among different treatments. IAA+IBA+NAA on full strength MS at Z_3 gave maximum root number of 28.3±10.3 and NAA+1/3MS at NA_1 (6.0mg/L) gave 2.3±2.1 roots, which was significantly low as compared to all the concentrations of rooting

hormones applied. Celenza et al.,(1995 and Ujjwala, (2007) found that IBA, IAA and NAA promote root initiation, elongate root length and increase root number.

Root length

Among various treatments, a significant difference in root length was recorded. IBA on full strength MS medium at I_2 (4.0mg/L) gave the longest roots of 12.7±2.1cm whereas NAA+IBA+1/2MS at L_2 gave root length of 0.3±0.1cm, which was significantly lower as compared to all the concentrations of hormones applied (Table 2). Root length at concentration 1.0mg/L of IBA in both full strength and half strength of MS were more than previous report on this plant by Gnanaraj et al.,(2012).

Shoot length

Among the different concentration and combination of hormones studied, the highest shoot length was recorded at IA_3 and it was 17.0±0.75cm and NAA+IBA+1/2MS at L_2 gave 2.0±0.1 shoots, which was significantly lower as compared to all the concentrations applied (Table 2). Rooting hormones increases sugar availability at the site of primordium development and that is responsible for higher shootlets length which previously reported by Metaxas et al.,(2004).

Acclimatization of Regenerated Plantlets

The plantlets developed from different *in vitro* culture were successfully established in soil. In present investigation, the survivals of acclimatized young plants were 86.67% (Table 3).

CONCLUSIONS

Here, we have shown the decent protocol for large scale propagation of *Achyranthes aspera* L. using nodal explants. The survivals of acclimatized young plants were 86.67%. For shoot regeneration the suitable media was half strength MS medium supplemented with BAP 3. For root regeneration the medium composition was 2.0mg/L of IBA and 0.5mg/L of NAA in full strength of MS media. This protocol could be applied for commercial large scale cultivation of *A. aspera*.

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