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Micropropagation of an important medicinal herb *Eclipta alba* (L.) Hassk.

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Abstract

Nodal segments from naturally grown *Eclipta alba* (L.) Hassk.were used as explants for organogenesis. Multiple shoots were obtained from the explants cultured on MS medium supplemented with various concentrations of BAP and Kn alone or in combination with NAA and IAA. Maximum number of multiple shoots (18.40±0.67) were induced on MS medium supplemented with 1.0 mg/l BAP and 0.1mg/NAA. *In vitro* raised shoots were cultured onto half and full strength MS medium supplemented with different concentration of IBA, IAA and NAA. The best root induction medium was found to be half strength MS containing 0.1 mg/l IBA where 96% shoots rooted. Regenerated plantlets grew normally without showing any morphological variation and flowered after 45 days of transplantation.

Key words: *Eclipta alba*, nodal explant, Micropropagation, *In vitro*, acclimatization

INTRODUCTION

Eclipta alba (L.) Hassk. is a small branched herb with white flower heads belonging to the family Asteraceae. This species grows commonly in moist places as a weed. In Bangladesh this plant grows in rainy season in paddy field and all over the country. Commercial exploitation and elimination of natural habits consequent of urbanization has led to gradual extinction of several medicinal plants (Hassan *et. al.*, 2008). Its natural growth is discreet and on in limited period and specific land types. Due to its inconsistent natural growth it is difficult to harvest as well as preserve seed for large or commercial cultivation. Micropopagation techniques could solve these problems.

Eclipta alba (L.) Hassk. contains wide range of active principles which include coumestans, alkaloids, flavonoids, glycosides, polyacetylenes, tri-terpenoids. The leaves, roots & areal parts are reported to contain a wide range of active principles having medicinal values (Jaglan *et al.*, 2013). The polypeptide isolated from this plant yield cystine, glutamic acid, phenyl alanine, tyrosine & methionine on hydrolysis. Nicotine and nicotinic acid are reported to occur in this plant (Jadhav *et al.*, 2009).

It is an active ingredient of many herbal formulations prescribed for liver ailments and shows effects on liver cell generation. It is used as a tonic and diuretic in hepatic and spleen enlargement (Jaglan *et al.*, 2013). The main active principles of *E. alba* are wedelolactone and demethylwedelolactone, both of which possess anti hepatotoxic

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activity (Wagner *et al.*, 1986 and Franca *et al.*, 1995). It is also used in catarrhal jaundice and for skin diseases. It is commonly used in viral hepatitis to promote bile flow and protect the parenchyma and popularly used to enhance memory and learning (Banji, *et al.*, 2007). *Eclipta alba* is a source of coumestan-type compounds used in phytopharmaceutical formulations of medicines prescribed for treatment of cirrhosis of the liver and infectious hepatitis (Wagner *et al.*, 1986).

Externally it is used for inflammation, minor cuts and burns and the fresh leaf-juice is considered very effective in stopping bleeding. Leaf juice mixed with honey is also used for children with upper respiratory infections and also used in eye and ear infections (Jaglan *et al.*, 2013).

The alcoholic extract of the plant has shown antiviral activity against Ranikhet disease virus (Dalal *et al.*, 2010). The fresh juice of leaves is used for increasing appetite, improving digestion (Lans, 2007) and as a mild bowel regulator. The plant is traditionally used in hair oil for healthy black and long hair (Roy *et al.*, 2008). The plant has a reputation as an anti-aging agent in Ayurveda (Thakur & Mengi, 2005).

In recent years, the harvest of medicinal plants on a mass scale from their natural habitats is leading to a depletion of plant resources. The biodiversity of our country is decreased for over population and civilization. There has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Ajith Kumar & Seeni, 1998, Prakash *et al.*1999). Therefore, it is important to develop an efficient micropropagation technique for *Eclipta alba* for rapid multiplication. In Bangladesh there have been a few reports on the establishment of micropropagation protocol for *Eclipta alba* (Hasan *et al*., 2008). In Bangladesh Hasan *et al*. (2008) developed a protocol for mass clonal propagation of this important medicinal harb using apical and axillary buds with survival rate 80% at outdoor condition. The present study aims at developing a simple, rapid, low-cost effective and high frequency regeneration protocol from nodal explants with higher survival rate so as to give rise to true-to-type clones for potential application in large- scale propagation.

MATERIALS AND METHODS

The experiments were carried out in the Plant Biotechnology Division of National Institute of Biotechnology, Bangladesh. *Eclipta alba* was collected from the National Institute of Biotechnology campus. Nodal segments were used for this purpose. The nodal segments (1.0 – 1.5 cm) were excised and thoroughly washed under running tap water for 30 minutes. Then they were treated with mild detergent followed by rinsing several times with autoclaved distilled water. The explants were dipped in 0.5% antifungal bavistin for 10 mins and again washed 4-5 times with sterilized distilled water. Further sterilization was done in laminar air flow cabinet under aseptic conditions. The explants were dipped in 70% (v/v) ethanol for 1.0 minute and then washed three times with sterilized distilled water. For surface sterilization, the explants were dipped in 0.1% aqueous solution (w/v)

of $HgCl_2$ for 2-3 minutes. Finally they were washed in sterilized distilled water for 3-4 times, till the sterilents were removed completely. The nodal segments were then trimmed/cut at both ends prior to inoculation on culture media. The explants were cultured on MS (Murashige and Skoog, 1962) medium with 3% sucrose and supplemented with different concentrations of cytokinins BAP and Kn (0.5-3.0 mg/l) alone or in combination with auxins NAA and IAA (0.1- 0.5 mg/l). The pH of the medium was adjusted to 5.8 by adding 1N NaOH/ 1N HCl before the addition of agar. The medium was gelled with 0.8% agar and autoclaved at 121° C for 20 minutes.
After inoculation, all the cultures were incubated at 28±2º C temperature and 16/8 (light/dark) photoperiod with 3000 lux intensity illumination provided by cool-white fluorescent tubes. After three-four weeks the explants were subcultured at a regular interval of 10-15 days.

For root induction 3-4 cm long shoots were cut and transferred to half and full strength MS medium fortified with auxins IAA, IBA and NAA (0.1-0.3 mg/l) singly. The rooted plantlets were taken out from the culture vessels and rinsed with sterilized distilled water to remove all trace of medium attached to the roots. After washing, plantlets were transplanted in small plastic pots containing autoclaved garden soil and compost (1:1). In order to maintain a high humidity the pots were then covered with transparent polythene bags and acclimatized in the growth room temperature (28±2º C) for 3 weeks. After 2-3 weeks, these pots were uncovered and then they were exposed to partial and then complete direct sun light. Finally, these hardened plantlets were transferred to the natural condition for their further growth and development.

RESULTS AND DISCUSSION

In the present study nodal segments were used as explants. There are a lot of reports on nodal segments used as explants in other medicinal plants (Biswas *et at*., 2009, Hassan & Roy 2004, Sultana & Handique, 2004, Jain *et al*., 2003, Chandramu *et al*., 2003). Nodal segments were inoculated on MS medium supplemented with different concentrations of BAP (0.5-3.0 mg/l) and Kn (0.5-3.0 mg/l) separately or in combinations with low concentrations of NAA (0.1-0.5 mg/l) and IAA (0.1-0.5 mg/l). Results of these experiments were presented in Table 1 & 2. In case of BAP the maximum number of shoots were obtained in MS medium supplemented with 1.0 mg/l BAP. In this concentration 76.66% explants responded for shoot induction and the maximum number (8.4 ± 0.62) of shoots per explants, highest shoot length (4.23± 0.26) as well as maximum number (6.80±0.51) of leaves per shoots were recorded (Table 1). This result was not similar with earlier report (Sharma *et al.* 2013) where 15-17 shoots/explants in 1.0 mg/l BAP were induced. Gawda & Paratkar (2004) reported 10-12 shoots per explant in 4.44 μ*M* BA. Dhaka & Kothari (2005) also reported a highly efficient and reproducible protocol for *E. alba* through nodal explant on MS+1.0 mg/BAP. In the present investigation the initiation of multiple shoot formation was obtained within six to ten days and BAP showed better response to multiple shoot formation as compared to Kn. This result corroborates with the results obtained by Baskaran & Jayabalan (2003). But the length of shoot and internode, thickness of stem and leaf size is higher in Kn than BAP.

The maximum number (6.50±0.5) of shoots per explants with an average length (4.05±0.20) of shoot and average number of leaves (7.70±0.40) were found in 1.0 mg/l Kn. In the present study the number of shoots were decreased and showed bushy structure with the increase of cytokinins (BAP and Kn) which is similar with the results of Hu & Wang (1983).

Table 1. Effects of BAP and Kn singly on multiple shoot regeneration from nodal segments of *E. alba*

Growth regulators (mg/l)		Explants inducing shoots (%)	Number of shoots/explants	Shoot length (cm)	Number of leaves/shoots
BAP	Kn	Mean	Mean ± SE	Mean ± SE	Mean ± SE
0.5		66.66	4.40 ± 0.34	3.86 ± 0.21	6.30 ± 0.30
1.0		76.66	8.40 ± 0.62	4.23± 0.26	6.80 ± 0.51
1.5		70.00	5.20 ± 0.49	4.09 ± 0.26	6.50 ± 0.31
2.0		65.00	5.60 ± 0.48	4.00 ± 0.27	6.20 ± 0.36
2.5		60.00	5.10 ± 0.53	3.13 ± 0.13	6.00 ± 0.26
3.0		60.00	4.70 ± 0.42	3.24 ± 0.11	6.30 ± 0.30
	0.5	45	5.80 ± 0.42	3.26 ± 0.15	6.50 ± 0.34
	1.0	68.66	6.50 ± 0.50	4.05 ± 0.20	7.70 ± 0.40
	1.5	66.66	4.70 ± 0.45	3.71 ± 0.23	7.20 ± 0.49
	2.0	60	4.50 ± 0.34	3.50 ± 0.24	6.80 ± 0.36
	2.5	55	4.10 ± 0.28	3.98 ± 0.21	6.30 ± 0.30
	3.0	50	4.00 ± 0.37	3.45 ± 0.22	6.20 ± 0.33

Results are mean ± SE of 15 replications.
Data recorded after eight weeks of culture.

Table 2. Effects of different concentrations and combinations of BAP, NAA and IAA on shoot induction

Growth regulators (mg/l)			Explants inducing shoots (%)	Number of shoots/explants	Shoot length (cm)	Number of leaves/shoots
BAP	NAA	IAA	Mean	Mean ± SE	Mean ± SE	Mean ± SE
1.0	0.1		90	18.40 ± 0.67	4.50 ± 0.31	8.50 ± 0.37
1.0	0.2		83.33	12.5 ± 0.96	4.09 ± 0.27	8.10 ± 0.41
1.0	0.3		75	8.10 ± 0.48	4.00 ± 0.27	7.20 ± 0.29
1.0	0.5		70	7.70 ± 0.50	3.99 ± 0.16	7.20 ± 0.33
1.0		0.1	73.33	9.50 ± 0.70	4.09 ± 0.22	8.10 ± 0.41
1.0		0.2	66.66	7.10 ± 0.43	3.97 ± 0.16	7.20 ± 0.36
1.0		0.3	60.00	6.50 ± 0.34	3.90 ± 0.23	7.10 ± 0.28
1.0		0.5	60	5.20 ± 0.42	3.74 ± 0.17	6.80 ± 0.33

Results are mean ± SE of 15 replications.
Data recorded after eight weeks of culture.

Multiple shoot regeneration from *E. alba* nodal explants cultured on MS media supplemented with various concentrations and combinations of BAP, NAA and IAA were studied. The effect of BAP in combination with an auxin has been reported and in most of the cases BAP and NAA were used for the induction of multiple shoots of various medicinal plants (Sudha *et al.*, 1998, Huang *et al.*, 2000, Chen *et al.*, 2001, Biswas *et at*.,

2009). The percentage of responded explants, number of shoots per explants, number of leaves, length of shoots and proliferation of shoots differed with the medium constituents. In the present study, among the four different media used the nodal explants in MS medium supplemented with 1.0 mg/l BAP and 0.1mg/l NAA grew vigorously. The explants (nodal segments) started in producing shoots within one week in MS medium supplemented with BAP and NAA (Plate 1). The highest percentage of responsive explants (90%) was examined in MS+1.0 mg/l BAP+0.1mg/l NAA. The maximum number (18.40±0.67) of shoots per explants, shoot length (4.50± 0.31) and average number (8.50±0.37) of leaves were found in this medium. Sharma *et al* (2013) also reported that MS medium supplemented with 1.0 mg/l BAP and 0.1mg/l NAA was most effective for shoot induction in *E. alba*. In the present study it was observed that explants with small shoots were subcultured in the same medium the number of multiple shoots were increased but the shoot length, leaves size and chlorophyll content were decreased. Repeated subculture in the same medium at 8-10 day intervals resulted profuse shoot multiplication (Plate 2). For shoot elongation and proliferation, the regenerated multiple shoots were transferred into MS medium supplemented with 1.0 mg/l BAP (Plate 3 & 4). These shoots were further multiplied and maintained in same media (MS+1.0mg/l BAP) for further shoot multiplication. Almost similar results were also reported by Dhaka and Kothari (2005) and Gawda & Paratkar (2004). Combination of BAP with IAA was not found suitable for multiple shoot induction. This is identical with the results obtained by Hassan *et al.*, 2008 using apical and axillary bud. The maximum number (9.50±0.70) of shoot induction was found in MS +1.0 mg/l BAP + 0.1 mg/l IAA.

Table 3. Effects of IBA, IAA and NAA on *in vitro* root induction in regenerated shoots of *Eclipta alba* on half and full strength MS

MS strength	Growth regulators (mg/l)			Rooted shoots (%)	Days required for rooting	No. of roots/ shoot	Length of roots (cm)
	IBA	IAA	NAA				
½ MS	0.1			96	15-16	8.70 ± 0.42	4.64±0.23
½ MS	0.2			90	18-20	7.10 ± 0.43	3.96 ± 0.17
½ MS	0.3			70	20-22	6.60 ± 0.45	3.31 ± 0.16
½ MS		0.1		84	20	8.60 ± 0.45	3.99 ± 0.28
½ MS		0.2		78	17-18	7.70 ± 0.37	3.51 ± 0.18
½ MS		0.3		65	21-22	6.60 ± 0.37	3.55 ± 0.17
½ MS			0.1	55	19-20	7.00 ± 0.26	4.12 ± 0.26
½ MS			0.2	42	22-23	6.20 ± 0.25	3.93 ± 0.18
½ MS			0.3	45	22-123	6.00 ± 0.30	3.29 ± 0.16
MS	0.1			90	16-17	8.50 ± 0.34	4.53 ± 0.26
MS	0.2			88	22	7.50 ± 0.34	4.08 ± 0.19
MS	0.3			65	21	5.90 ± 0.38	3.80 ± 0.23
MS		0.1		66	19-20	8.20 ± 0.47	4.67 ± 0.30
MS		0.2		60	16-17	5.50 ± 0.34	4.05 ± 0.25
MS		0.3		55	18-20	6.80 ± 0.47	4.09 ± 0.25
MS			0.1	50	24	6.00 ± 0.42	4.15 ± 0.21
MS			0.2	45	23	6.20 ± 0.33	3.92 ± 0.25
MS			0.3	40	25-26	5.30 ± 0.30	3.78 ± 0.22

Results are mean ± SE of 15 replications.
Data recorded after four weeks of culture.

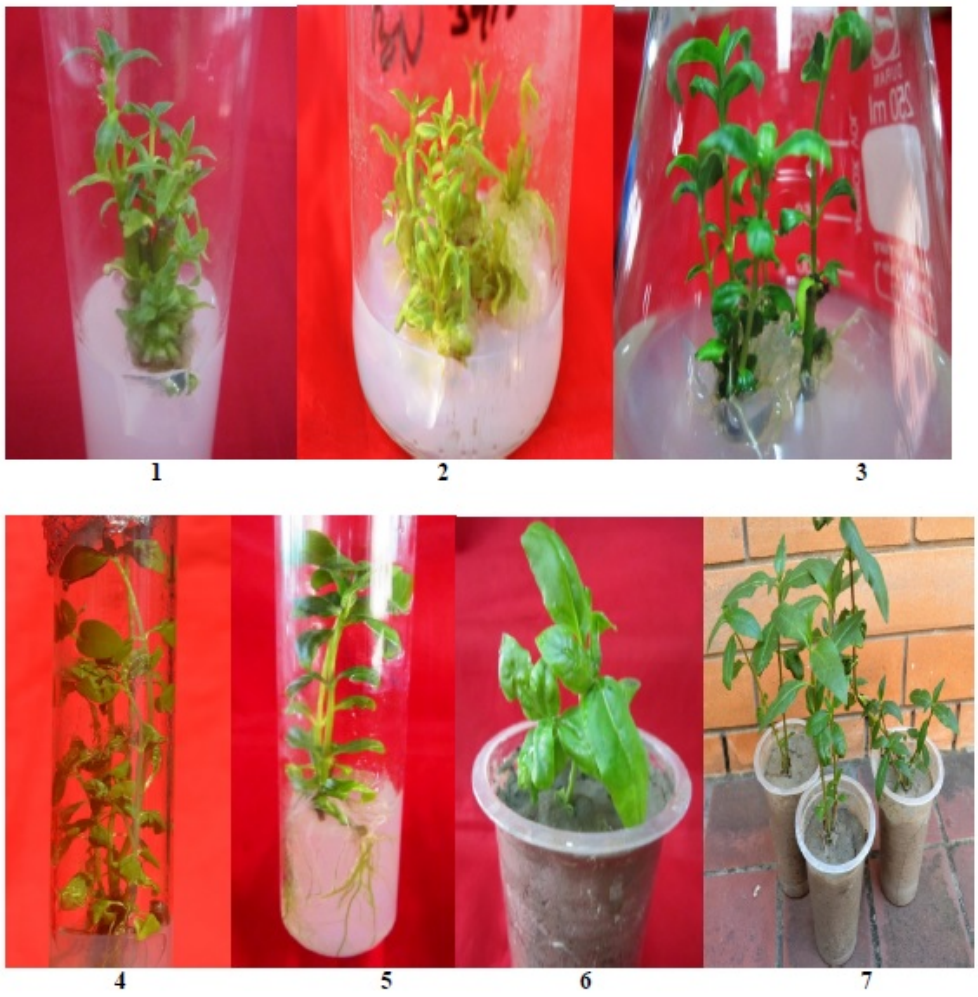

Plate 1-7. *In vitro* regeneration of *Eclipta alba* (L.) Hassk. from nodal segment explants. 1. Induction of multiple shoots on MS + 1.0 mg/l BAP + 0.1 mg/l NAA after three weeks of culture; 2. Development and multiplication of shoots in same medium. 3 & 4. Elongation and proliferation of *in vitro* shoots on MS + 1.0 mg/l BAP; 5. Rooting of *in vitro* regenerated shoots on half MS + 0.1 mg/l IBA; 6. Acclimatization of *in vitro* grown plantlets in small plastic pots. 7. Fully acclimatized regenerated plants in open air after five weeks of transplantation

The regenerated multiple shoots, obtained from MS + 1.0 mg/l BAP and 0.1 mg/l NAA, when subcultured on MS + 1.0 mg/l BAP for elongation and proliferation produced rooting simultaneously. But those were found to be inadequate for transplantation and therefore adequate root induction is necessary.

For induction of roots regenerated healthy shoots of 3-4 cm length were excised and cultured on half and full strength of MS medium supplemented with different concentrations (0.1- 0.3 mg/l) of IBA, IAA (0.1- 0.3 mg/l) and NAA (0.1- 0.3 mg/l) singly. The results of these experiments are presented in Table 3. Use of auxins singly or in combination for rooting in *E. alba* were also reported by different authors (Hassan *et al.*, 2008, Baskaran & Jayabalan, 2003, Sharma *et al.*, 2013). Among the different auxins (IBA, IAA and NAA) the best root induction and development was found in half strength MS medium containing 0.1 mg/l IBA. The highest percentage (96%) of root formation, maximum number of roots (8.70±0.42) and highest length (4.64±0.23) of roots were recorded in this medium within three weeks of inoculation (Plate 5). Several earlier researchers (Khalekuzzaman *et al.*, 2008, Biwas *et al*., 2009, Hassan *et al*., 2010, Das *et al.*, 2008, Karthikeyan *et al*., 2009) reported root induction in different medicinal plants by using IBA in MS and modified MS. In the present study it was observed that IAA and NAA were less effective for root induction in *E. alba*.

After sufficient development of roots the plantlets were transferred into small plastic pots containing soil and compost (Plate 6). Following proper acclimatization, plantlets were transferred to open air and 95% plants survived (Plate 7). 300 plantlets were transferred into experimental field and among them 90% plants survived. The *in vitro* regenerated plants grew vigorously in field condition and they did not show any morphological variation. These plantlets flowered within 45 days after transplantation and set seeds. Sharma *et al*. (2013), Hasan *et al*. (2008), Ragavendran *et al*. (2014), and Borthakur *et al*. (2000) reported 35%, 80%, 70%, and 50% survival rate respectively. The *in vitro* regeneration protocol developed here is cost effective, rapid and reproducible. This protocol can be applied for large scale clonal propagation and conservation of this elite medicinal plant species.

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