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Development of an Efficient *In Vitro* Micropropagation Protocol for Medicinally Important Plant *Achyranthes bidentata* Blume

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ABSTRACT

The current situation of medicinal plants and increasing demand of plant derived drugs suggest an immediate need to conserve our medicinal plant resources. The present study attempted to develop *in vitro* micropropagation protocol for the important medicinal plant *Achyranthes bidentata* Blume. Shoot tip and nodal segment of healthy plants from natural condition were used as explants for direct organogenesis. Varying concentrations of auxins and cytokinins both alone and in combination were used. Nodal segment explants produced best response in terms of shoot induction percentage and maximum number of shoot than shoot tip. Single cytokinins media was far better than combined effect of cytokinins and auxins. 5.0 mgL^{-1} and 4.5 mgL^{-1} of BAP (6-Benzyl amino purine) produced the highest number of shoot which was 10.5 and 10.2, respectively from nodal segment explants whereas the same media produced 9.8 and 9.2 shoot from shoot tip. For root induction and root development, IBA (Indole-3-butyric acid) in full strength MS media was better than IBA in ½ MS (Murashige and Skoog) media. Among different concentrations of full and ½ strength MS media tested, 1.5 mgL^{-1} of IBA in full strength MS media proved to be the best in terms of root induction (97.78 %) and root number (15.8). Almost 80 % of *in vitro* grown shootlets were established and acclimatized in natural soil.

Keywords: *In Vitro*, Micropropagation, *Achyranthes bidentata*, Shoot Tip, Nodal Segment.

1. Introduction

Medicinal plants are the richest source of drugs for traditional medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates etc. [1]. For many years, tissue culture techniques are being applied for germplasm conservation, mass propagation and disease free plant production of medicinal plants. With the help of tissue culture it is possible to produce a huge number of plantlets from single explants within shortest span of time [2]. Micropropagation has been used to conserve the germplasms of many rare and endangered species of medicinal plants [3, 4]. Bangladesh has a great natural resource of medicinal plants and plant based health remedies are in practice here for long since. To harvest proper benefits from medicinal plants measures need to be taken regarding conservation and management of medicinal plants. Pharmaceutical industries can also utilize this technique for drug development approach from medicinal plants and gain economic success.

Achyranthes bidentata is one of the important medicinal plant species of Amaranthaceae family. This species is an erect, perennial herb found in many tropical areas of the Asia and Africa including China, India, Java, Japan [5, 6]. This plant contains a great variety of phytochemicals like alkaloids (achyranthine), rutins, oleanolic acid, caffeic acid, polysaccharides, saponins, terpenoids, triterpenoids, sitosterol, stigmasterol, ecdysterone, rubrosterone, amino acids, etc. most of which have therapeutic values [7-12]. Many of these phytochemicals were identified and isolated and some need more investigation. Different parts of this plant are effectively used for treating a number of ailments including cough, asthma, fever, fistula, skin rash, renal dropsy, diarrhoea, diabetes, tumor, toothache, indigestion, pyorrhea, antifertility, anti-inflammatory, piles, snake bites, immune stimulant [13-21].

In folklore medicine, *A. Bidentata* has been reported to be useful in arthritis, abdominal cramp, and chest pain and as antispasmodic [22-24]. In Bangladesh this medicinal plants are found as wild species in waysides and hilly areas of Chittagong. But attempts regarding *in vitro* tissue culture and conservation of this plant are very rare. Commercial scale mass propagation of this medicinal species has great economic potential. Great demand of these species for medicinal purposes and negligence as wild species may threaten them as extinct in near future. Therefore, *in vitro* tissue culture of this medicinal species was carried out in the present investigation. The major objective was to establish a standard and highly reproducible *in vitro* propagation and regeneration protocol for the medicinally important herb *Achyranthes bidentata* through direct organogenesis. More specifically, investigation was carried out to determine the suitable surface sterilizing conditions, best source of explants, appropriate nutrient media for multiple shoot regeneration and root formation and finally, acclimatization of the *in vitro* raised plantlets in soil conditions.

2. Materials and Methods

Healthy plants of *A. bidentata* Blume were collected from Chittagong University campus. Young and green shoots of *A. bidentata* Blume were harvested and washed with running tap water and rinsed twice with distilled water. Then the explants were surface sterilized with 0.1 % and 0.2 % (w/v) $HgCl_2$ solutions for different duration (2-7 minutes). After rinsing with sterile distilled water for 4-6 times, shoot tips and nodal segments were cut into smaller segments (1-1.5 cm) and used as the explants. The explants were placed vertically on semi-solid basal MS medium [25] supplemented with 3 % sucrose, 0.8 % (w/v) agar (Hi-Media, Mumbai, India). Different concentrations and combinations of 6-benzyl amino purine (BAP), kinetin (Kin), naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D) and indole butyric acid (IBA) for direct regeneration of shoots. For rooting, the *in vitro* raised shootlets were sub-cultured on both full strength and 1/2 strength MS medium supplemented with various concentrations and combinations of auxins (IBA). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ^{0}C for 15 min. The cultures were incubated at (25±2 ^{0}C) under cool fluorescent light of 16 h/day photoperiod). When the plantlets attained 4-8 cm heights with few leaves and well developed root system, they were ready for transplantation or acclimatization into soil. Before transfer into soil, the plantlets were taken out from the controlled environment of growth chamber, unplugged and were kept in room temperatures for 2-4 days to bring them in contact with normal temperature for gradual acclimatization. After 2-4 days of hardening the plantlets were taken out from culture vessel and washed the roots under running tap water to remove the agar and media. Then the plantlets were dipped in a beaker with tap water for 30 minutes. Then the plantlets were ready for transfer to natural or garden soil. The soil was prepared in the ratio of 1:1 (Garden soil: compost) and dried. Then this soil was autoclaved to make microorganism free. Finally some sand was mixed with the compost soil and the plantlets were planted into the soil. These plants were observed on 14th and 28th day.

The data for different parameters were recorded and analyzed using SPSS (Version 16) statistical software by one way ANOVA procedures. Differences among the means were compared following Duncan's Multiple Range Test (DMRT) at 5 % level of significance.

3. Results and Discussions

The complete protocols for *in vitro* tissue culture and plant regeneration of this important medicinal plant using different explants (shoot tip, nodal segment) were established through the present investigation. Variations in culture responses, multiplication and regeneration rates were observed depending upon the plant species, type of explants used and composition of the culture medium. The results obtained are discussed in the light of available literatures under the major headings as mentioned below:

3.1 Surface sterilization techniques of explants for initial establishment of the culture

For surface sterilization, the explants (shoot tip and nodal segment of *A. bidentata* collected from natural habitat) were treated with 0.1 % and 0.2 % (w/v) $HgCl_2$ solutions for different durations (2-7 min). Efficacy of 70 % (v/v) ethanol and savlon (3 % w/v cetrimide, an antiseptic plus detergent, marketed by ACI Bangladesh Ltd.) were also tested. The results of this experiment is presented graphically in Fig.1.

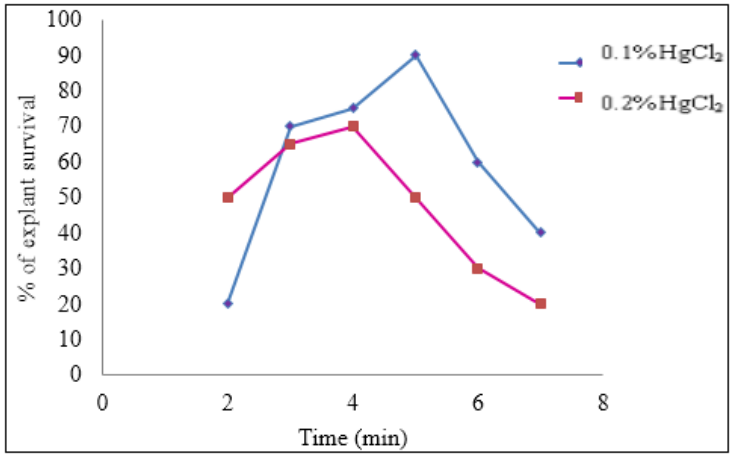

Fig1: Effect of $HgCl_2$ concentration and duration of treatment on survival of contamination free explant of *Achyranthes bidentata*

In case of shoot tip and nodal segment explant, treatment with 0.1 % $HgCl_2$ for 5 minutes in *A. bidentata* was proved to be the best. The percentage of explant survival in 5 min treatment with 0.1 % $HgCl_2$ was 90 % for *A. bidentata*. This result corresponds with some previous findings [26, 27]. The highest degree of contamination was observed in treatment with 0.1 % and 0.2 % $HgCl_2$ for 2 minutes. The lowest percentage of explant survival was obtained in long duration of treatment (6 and 7 min) not due to contamination but due to inhibitory effect of $HgCl_2$ and ultimately tissue killing. However, 0.2 % $HgCl_2$ was showed greater tissue killing effect and hence produced poor results. This tissue killing effect of $HgCl_2$ with long exposure is evident from many previous studies [28, 29, 30]. Treatment with savlon and 70 % ethanol prior to treating with $HgCl_2$ also yielded poor results. Surface sterilization of explants collected from natural habitats as wild species is a prerequisite for successful establishment of *in vitro* culture. Fungal and bacterial contaminations are a common nuisance in plant tissue culture [31]. Concentrations and duration of treatment with sterilizing agents

need to be adjusted for each explants [32].

3.2 Shoot initiation and multiple shooting of *Achyranthes bidentata* using shoot tip and nodal segment explants

For shoot induction and multiple shoot production, shoot tip and nodal segment of *A. bidentata* collected from natural habitat were used as explants. Shoot induction and development of multiple shoot is the main target of micropropagation. In this study, different concentrations of cytokinins (BAP and Kinetin) alone and in combination with lower concentrations of auxins (2, 4-D, NAA, IBA) were used for the purpose of shoot initiation and multiple shooting. BAP and Kinetin concentrations used in this investigation ranged from 1.0-5.0 mgL^{-1}. Low concentrations (only 0.1 and 0.5 mgL^{-1}) of auxins (2,4-D, NAA, IBA) were used in combination with 2.0 and 3.0 mgL^{-1} BAP to examine the combined effect or hormonal interaction on shoot induction and multiple shooting. In every case, primarily induced shoots were sub-cultured in fresh media with same concentration for shoot multiplication and shoot elongation. This was done because after a certain period (16-18 days) the nutrients get exhausted. Cytokinins were reported by many workers to induce shoot by apical dominance and by inhibiting the effects of auxins [33, 34]. Interaction of explant types and shooting media also made significant difference regarding days for shoot initiation, number and length of shoot per culture [35].

In case of *A. bidentata* shoot tip, when single cytokinin containing media was used, 3.5 mgL^{-1} BAP induced the highest percentage of shoot (97.20 %) and the quickest response (8 d) while 5.0 mgL^{-1} BAP produced maximum number of shoots (9.8) followed by 9.2 shoot per explant in 4.5 mgL^{-1} BAP and 3.0 mgL^{-1} Kinetin produced most elongated shoot (5.8 cm) (Table 1).

Table 1: Effect of varying concentration of cytokinins in MS medium for micropropagation of *Achyranthes bidentata* using shoot tip as explants isolated from field grown plant

Treatment	Hormone	Hormone conc. (mgL^{-1})	% of shoot initiation	Days Required	No. shoot /explant	Average length of shoot (cm)
T_1	**MS_0**	0	50.00 d	12.00 a	2.20 i	2.50 i
T_2	**BAP**	1.0	66.66 bc	12.00 a	3.60 g	4.60 cd
T_3		2.0	75.00 b	10.00 abc	4.20 f	4.20 de
T_4		2.5	75.00 b	10.00 abc	4.00 f	3.80 ef
T_5		3.0	83.33 ab	8.00 c	5.80 e	4.00 e
T_6		3.5	97.20 a	8.00 c	7.30 d	3.50 fg
T_7		4.0	83.33 ab	10.00 abc	8.10 c	3.20 gh
T_8		4.5	75.00 b	10.00 abc	9.20 b	3.40 fgh
T_9		5.0	66.66 bc	9.00 bc	9.80 a	3.00 h
T_{10}	**Kinetin**	1.0	66.66 bc	10.00 abc	2.80 h	4.20 de
T_{11}		2.0	83.33 ab	10.00 abc	3.40 g	5.20 b
T_{12}		3.0	75.00 b	12.00 a	3.60 g	5.80 a
T_{13}		4.0	55.63 cd	9.00 bc	2.80 h	4.80 bc
T_{14}		5.0	50.00 d	11.00 ab	3.00 h	4.50Cd

Figures in the same column denoted by the same letter (s) did not differ significantly according to DMRT at $P < 0.05$. [a,b,c,….↓]

For *A. bidentata,* nodal segment cultured on single cytokinin media, 3.5 and 4.5 mgL^{-1} BAP induced the highest percentage of shoot (97.22 %), the highest number of shoots was 10.5 in 5.0 mgL^{-1}BAP followed by 10.2 in 5.0 mgL^{-1} BAP whereas maximum shoot length was observed in 3.0 mgL^{-1} Kinetin (Table 2). Best shoot proliferation in higher BAP concentration was also reported in *Pongamia pinnata* nodal segment[36]. For both shoot tip and nodal segment, MS media without hormone produced lowest number of shoot and minimum length of shoot, minimum frequency of shoot regeneration. All other concentrations of BAP and Kinetin produced varying results which were better than control but worse than those mentioned earlier.

Table 2: Effect of varying concentration of cytokinins in MS medium for micropropagation of *Achyranthes bidentata* using Nodal segment as explants isolated from field grown plant

Treatment	Hormone	Hormone conc.	% of shoot Initiation	Days required	No. shoot /explant	Average length of shoot
T_1	**MS_0**	0	58.33 de	10.00 ab	2.80 h	2.00 k
T_2	**BAP**	1.0	66.66 cde	10.00 ab	3.20 gh	2.50 j
T_3		2.0	66.66 cde	9.00 b	3.50 fg	3.00 hi
T_4		2.5	75.00 bc	8.00 bc	3.80 ef	3.40 gh
T_5		3.0	75.00 bc	7.00 c	6.20 d	4.20 cde
T_6		3.5	97.22 a	10.00 ab	9.25 c	4.00 def
T_7		4.0	83.33 ab	10.00 ab	9.80 b	3.80 efg
T_8		4.5	97.22 a	9.00 b	10.20 ab	3.60 fg
T_9		5.0	75.00 bc	10.00 ab	10.50 a	3.40 gh
T_{10}	**Kinetin**	1.0	58.33 de	9.00 b	3.40 fg	2.80 ij
T_{11}		2.0	75.00 bc	10.00 ab	3.70 fg	4.40 bcd
T_{12}		3.0	83.33 ab	12.00 a	4.20 e	5.10 a
T_{13}		4.0	66.66 cde	8.00 bc	3.20 gh	4.80 ab
T_{14}		5.0	52.78 e	8.00 bc	2.80 h	4.50 bc

Figures in the same column denoted by the same letter (s) did not differ significantly according to DMRT at P <0.05. [a,b,c,….↓]

When combination of cytokinins (BAP) and auxins (2,4-D, NAA, IBA) were used to investigate the combined effect on shoot induction and shoot proliferation of *A. bidentata*, the response greatly varied with that observed in single cytokinins media. For *A. bidentata* shoot tip, 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA was the best in terms of percentage of shoot induction (96.67 %) and number of shoot (5.6) while 3.0 mgL^{-1} BAP + 0.1 mgL^{-1} NAA produced the highest shoot length (4.4 cm) and 5.2 shoot per explant. 3.0 mgL^{-1} BAP + 0.1 mgL^{-1} IBA produced 5.4 shoot per explant and 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} IBA produced better shoot length (4.2 cm) (Table 3).

Table 3: Effect of different concentration of BAP in combination with 2, 4-D, NAA and IBA in MS medium for *in vitro* micropropagation of *Achyranthes bidentata* using shoot tip as explants isolated from field grown plant.

No. of treatment	Hormone	Hormone Conc.	% of shoot initiation	Days required for shoot initiation	No. of shoot/ explant	Length of shoot (cm)	No. of root
T_1	**BAP+ 2,4-D**	2.0+0.1	60.00 cd	10.00 ab	2.20 f	2.70 f	0.00 c
T_2		2.0+0.5	40.00 d	12.00 a	2.50 ef	3.30 e	0.00 c
T_3		3.0+0.1	70.00 bc	12.00 a	2.80 ef	3.70 cde	0.00 c
T_4		3.0+0.5	60.00 cd	9.00 b	3.00 e	3.40 e	0.00 c
T_5	**BAP+NAA**	2.0+0.1	80.00 abc	8.00 b	4.53 cd	3.60 de	0.00 c
T_6		2.0+0.5	90.00 ab	8.00 b	4.83 bcd	4.20 ab	1.00 bc
T_7		3.0+0.1	80.00 abc	10.00 ab	5.20 abc	4.40 a	2.00 ab
T_8		3.0+0.5	96.67 a	9.00 b	5.60 a	4.10 abc	3.00 a
T_9	**BAP+IBA**	2.0+0.1	80.00 abc	9.00 b	4.50 cd	3.90bcd	1.00 bc
T_{10}		2.0+0.5	70.00 bc	8.00 b	4.70 cd	4.10 abc	2.00 ab
T_{11}		3.0+0.1	70.00 bc	8.00 b	5.40 ab	3.30 e	2.00 ab
T_{12}		3.0+0.5	90.00 ab	10.00 ab	4.30 d	4.20 ab	3.00 a

Figures in the same column denoted by the same letter (s) did not differ significantly according to DMRT at P <0.05. [a,b,c,….↓]

For *A. bidentata* nodal segment, the best combination was 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA that induced 90 % shoots, required minimum days, produced highest number of shoot (6.4) and maximum number of root also (3). However, 3.0mgL^{-1} BAP + 0.1 mgL^{-1} NAA was induced highest percentage of shoot (96.67 %) and 2.0mgL^{-1} BAP + 0.5 mgL^{-1} IBA produced highest shoot length (5.0 cm). Number of shoot was also better in shoot number (6.2) (Table 4). In both shoot tip and nodal segment, combination of BAP+2, 4-D reduced shoot induction percentage, number of shoot, shoot length and delayed the response.

Table 4: Effect of different concentration of BAP in combination with 2, 4-D, NAA, IBA in MS medium for *in vitro* micropropagation of *Achyranthes bidentata* using nodal segment as explants isolated from field grown plant.

No. of treatment	Hormone	Hormone Conc.	% of shoot initiation	Days required for shoot initiation	No. of shoot/ explant	Length of shoot (cm)	No. of root
T_1	BAP+ 2,4-D	2.0+0.1	70.00 bcd	11.00 ab	3.20 hi	3.40 def	0.00 c
T_2		2.0+0.5	60.00 cd	10.00 abc	3.40 h	3.10 ef	0.00 c
T_3		3.0+0.1	70.00 bcd	10.00 abc	2.80 i	3.50 de	0.00 c
T_4		3.0+0.5	56.67 d	12.00 a	3.20 hi	3.00 f	0.00 c
T_5	BAP+NAA	2.0+0.1	70.00 bcd	10.00 abc	5.30 de	3.80 d	0.00 c
T_6		2.0+0.5	80.00 abc	10.00 abc	4.90 ef	4.30 c	2.00 ab
T_7		3.0+0.1	96.67 a	8.00 cd	5.80 bc	4.80 ab	2.00 ab
T_8		3.0+0.5	90.00 ab	7.00 d	6.40 a	4.60 abc	3.00 a
T_9	BAP+IBA	2.0+0.1	80.00 abc	9.00 bcd	6.20 ab	4.50 bc	0.00 c
T_{10}		2.0+0.5	90.00 ab	8.00 cd	5.40 cd	5.00 a	1.00 bc
T_{11}		3.0+0.1	70.00 bcd	10.00 abc	4.80 f	4.80 ab	1.00 bc
T_{12}		3.0+0.5	80.00 abc	10.00 abc	4.20 g	4.60 abc	2.00 ab

Figures in the same column denoted by the same letter (s) did not differ significantly according to DMRT at P <0.05. [a,b, c,….↓]

To summarize, *A. bidentata,* nodal segment explant produced the best response in terms of shoot induction percentage and maximum number of shoot than shoot tip. It was also observed that, for *A. bidentata,* single cytokinins media was far better than combined effect of cytokinins and auxins. BAP 5.0 and 4.5mgL^{-1} produced the highest number of shoot (10.5 and 10.2, respectively) from nodal segment whereas the same media produced 9.8 and 9.2 shoot from shoot tip. But combination of BAP with auxins did not yielded promising result. 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA was the best medium for both shoot tip and nodal segment among all the combinations tested. Maximum shoot proliferation on this combined media was 5.6 and 6.4 shoot per explant from shoot tip and nodal segment, respectively. This finding is authenticated by earlier some previous works where BAP alone induced best shoot proliferation [37, 38, 39].

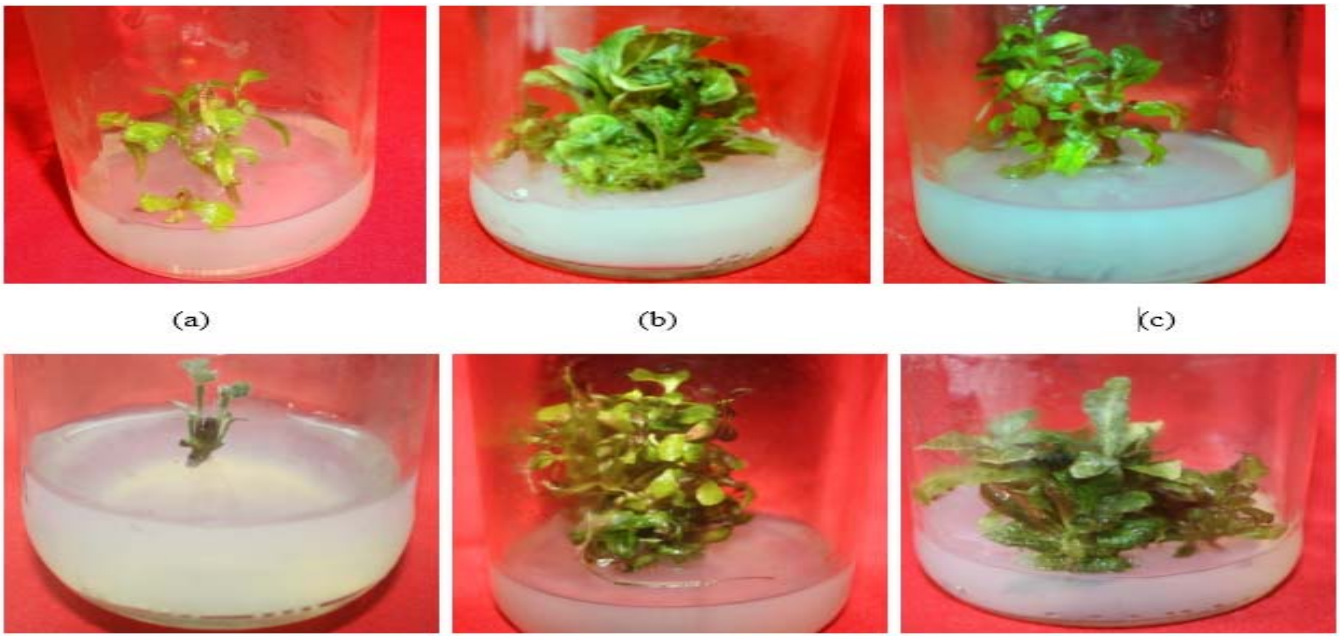

Fig 2: Shoot initiation and multiple shooting of A. bidentata shoot tip and nodal segment in different hormonal concentrations and combinations: (a) shoot initiation from A. bidentata shoot tip; (b) multiple shoot regeneration from shoot tip in BAP 4.5 mgL^{-1}; (c) multiple shooting from shoot tip in 3.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA; (d) shoot initiation from nodal segment; (e) multiple shooting from

nodal segment in BAP 5.0 mgL^{-1} and (f) multiple shoot development from nodal segment in 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} IBA

3.3 Root initiation of *Achyranthes bidentata* using *in vitro* propagated microcuttings of shoot.

In vitro proliferated shoots from shoot tip and nodal segment of *A. bidentata* were then transferred into full and ½ strength of MS medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5, 2.0 mgL^{-1}) for root induction and development of extensive root system. MS media with 0.4 % agar was used for root induction which allowed easy penetration of root in media and made the roots easily visible. MS basal media without hormone was used as control. In this study, *A. bidentata* microcuttings of shoot produced roots in all the concentrations of IBA both in full and half strength MS medium. IBA in full strength MS was better than IBA in ½ MS media. However, the extensive rooting was observed in *A. bidentata*. 1.5 mgL^{-1}IBA in full strength MS media was best as it induced the highest percentage of root (97.78), responded quickly (8 days), produced maximum number of root (15.8) and the highest shoot length (7.2 cm). However, 0.5mgL^{-1} IBA produced the highest root length in both full strength MS (6.4 cm) and ½ MS (5.6 cm). 1.5mgL^{-1} IBA was also best for ½ MS media for root induction percentage (73.33 %) and root number (10.6) (Table 5).

Table 5: Effect of different concentration of IBA (Auxin) in full strength and half strength MS medium for root initiation of the elongated microshoots of *Achyranthes bidentata*

Treatment	Media strength	Hormone conc. (IBA)	% of root initiation	Days for root initiation	No. of shoot/ explant	Average length of shoot	No. of root /explant	Average length of root (cm)
T_1	**MS_0 (control)**	0	66.66 cd	12.00 a	2.50 e	4.80 cd	5.60 f	4.80 de
T_2	**MS**	0.5	80.00 bc	10.00 ab	3.20 cd	5.10 c	7.50 e	6.40 a
T_3		1.0	86.66 ab	12.00 a	3.40 bc	4.60 de	12.60 b	5.80 ab
T_4		1.5	97.78 a	8.00 b	3.70 b	7.20 a	15.80 a	5.20bcd
T_5		2.0	80.00 bc	12.00 a	4.20 a	6.40 b	12.50 b	4.70 de
T_6	**½ MS**	0.5	53.33 d	10.00 ab	2.50 e	3.80 f	4.80 g	5.60 bc
T_7		1.0	66.66 cd	12.00 a	2.80 de	4.20 ef	8.40 d	4.30 e
T_8		1.5	73.33 bc	11.00 a	3.00 cd	4.00 f	10.60 c	4.6 de
T_9		2.0	66.66 cd	10.00 ab	3.20 cd	4.60 de	8.70 d	5.10 cd

Figures in the same column denoted by the same letter (s) did not differ significantly according to DMRT at P <0.05. [a,b,c,….↓]

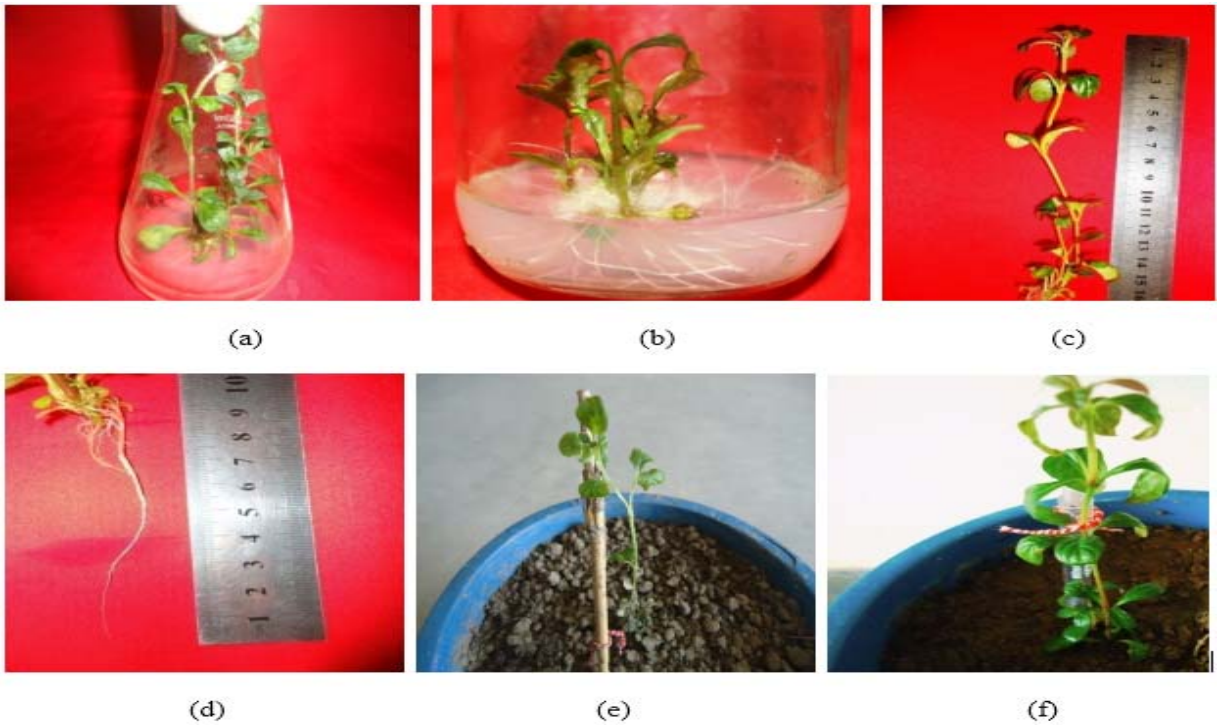

Fig 3: Root induction and acclimatization of in vitro grown plantlets: (a) shoot elongation after 35 days of inoculation; (b) development of extensive root systems in 1.5 mgL^{-1} IBA; (c) measurement of shoot length before acclimatization; (d) measurement of root length before acclimatization; (e) and (f) acclimatization of the *in vitro* grown plant in natural soil.

The shoot development as well as the rooting of *in vitro* raised shootlets is crucial for regeneration of whole plant through tissue culture [40]. Many previous investigation suggested the importance of auxins in root induction and concluded that auxins are the main factor in root initiation of microcuttings of *in vitro* grown shoots [34, 41-43]. The suitable range of auxins for root initiation was 0.1-1.5 mgL^{-1} [30]. The superiority of IBA on root induction was reported by many scientists [44, 45]. The results obtained for rooting was in support with many previous reports who achieved similar or nearly similar rooting response in some other medicinal plant species [46-49].

3.4 Acclimatization and establishment of *in vitro* regenerated plantlets under *ex vitro* condition.

After development of extensive root system, these rooted shoot or plantlets were gradually acclimatized to natural environment and finally planted in specially prepared soil in plastic pot. The soil was prepared in the ratio of 1:1 (Garden soil: compost) and dried. Then this soil was sterilized to prevent contamination. Finally, some sand was mixed with the compost soil and the plantlets were planted into the soil. These plants were observed on 14th and 28th day. Almost 80 % of *in vitro* regenerated plantlets were successfully established in soil. It was observed that those *in vitro* plantlets with root system developed in ½ MS failed to establish in soil. The method employed in this study for acclimatization was the same established by previous studies [50, 51]

4. Conclusion

The regeneration protocol developed in this study is superior to previous results. This could be exploited for commercial scale mass propagation of *A. bidentata* by pharmaceutical industries for drug development. Further studies on tissue culture of these species or other species of this family could be benefitted from this result. Besides, this protocol can be utilized for cryopreservation study or conservation purpose.

5. Acknowledgement

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6. References

1. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999; 86(6):985.
2. Bajaj YPS. Cryopreservation of germplasm of medicinal and aeromartic plants. In Y.P.S Bajaj (ed.), Biotechnology in agriculture and forestry, Cryopreservation of plant germplasm, springer-Verlag, Berlin, 1995; 32:419-434.
3. Fay MF. Conservation of rare and endangered plants using *in vitro* methods. *In vitro* cell and Dev. Biol 1992; 14:28.
4. Mikulik J. Propagation of endangered plant species by tissue culture. Biological 1999; 37:27-33.
5. The wealth of India, "Raw Materials ", Publication and Information Directorate, CSIR, New Delhi, 1, 57(1985).
6. Chopra R.N. "Indigenous Drugs of India" Art Press, Calcutta, 457-462 (1958).
7. Nguyen T, Nikolov S, Nguyen TD. Chemical research of the aerial parts of *Achyranthes bidentata* Blume Tap. Chi Duoc Hoc 1995; 6:17-18.
8. Yip TT. Fung SC, Kong YC. Uterotonic activity of Achyanthes bidentata saponins Asian symp. Med Plants spices 1980; 9:63.
9. Takemoto T, Ogawa S, Nishimoto N, Hirayama H, Taniguchi S. Studies on the Constituents of *Achyranthes radix* VII. The insect mounting substances in *Achyranthes* and *Cyathula genera* "Supplement". Yakugaku Zasshi 1968; 88, 1293-1297.
10. Bishit G, Sandhu H. Chemical constituents and antimicrobial activity of *Achyranthes bidentata*. Indian J Chem Soc 1990; 67:1002-1003.
11. Ratra PS. Alkaloids in two species of *Achyranthes* at different stages of their growth, Curr trends life Sci 1979 4:81-85.
12. Nguyen Van Dan and Doan Thi Nhu, 1989. Medicinal Plants in Vietnam World Health Organization ISBN 92 9061 1014.
13. Chandra K, Pandey H. Collection of plants around agora-Dodital in Uttarkashi district, at U.P., Medicinal value and folklore claim, INT.J. Crude Drug Res 1983; 21:21-28.
14. Kong YC, Hu SY, Lau FK, Che CT. Potential anti-fertility plants from Chinese Medicine, Amer. J Chinese Herbal Med 1976; 4:105-126.
15. Li CP, Chinese herbal Medicine, Health, Education and welfare, (NIH), Washington 1974; 75-732.
16. Kosuge T, Ishida H, Yamazaki K. Studies on active substances in the herbs used for Oketsu, blood coagulation in Chinese medicine, Yakugaku Zasshi 1984; 104:1050-1053.
17. Yu SC, Zhang YZ. Effect of *Achyranthes bidentata* polysaccharides on antitumour activity and immune function of S-180 bearing mice, Zhongguo Zhongyao Zasshi 1995; 17:275-278.
18. Kosuge T, Yokoto M, Sugiyama K. Studies on antitumour activities and antitumour principles of Chinese herbs, Yakugaku Zasshi 1985; 105:791-795.
19. Emmanuel SZ, gnanavendan SG. Antisnake venom botanicals from Ethnomedicine, J Herbs Spices and Med Plants 1994; 2(4):45-100.
20. Vetrichelvan T, Jegadeesan M. Effect of alcoholic extract of *Achyranthes bidentata* Blume on acute and sub acute inflammation. Indian Journal of Pharmacology 2002; 34: 115-118
21. Manandhar NP, 2002. Plants and People of Nepal. Timber Press. Oregon. ISBN 0-88192-527-6.
22. Kirtikar KR, Basu BD. "Indian Med. Plants" Bishen Singh Mahendra Pl Singh, Dehradun 1995; 3:2006-2008.
23. Duke JA, Ayensu ES. "Medicinal Plants of China". Algonac, Michigan 1985; 252-361.
24. Bishit G, Sandhu H, Verma S. Constituents of *Achyranthes bidentata*, Fitoterapia 1993; 64:85.
25. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15:473-497.
26. Das T, Mitra GC. Micropropagation of *Eucalyptus tereticornis* Smith. Plant Cell Tiss Org Cult 1990; 22:95-103.

27. Johnson M, Sonali D, Yasmin N, Babu A. Impacts of cytokinins and auxins on organogenesis and somatic embryogenesis of *Vitex negundo* var. negundo L. and evaluation of genetic fidelity. Indian J Biotechnol 2009; 3(4):236-241.
28. Wesely EG, Johnson MA, Mohanamathi RB, Kavitha MS. *In vitro* clonal propagation of *Achyranthes aspera* L. and *Achyranthes bidentata* Blume using nodal explants. Asian Pacific Journal of Tropical Biomedicine 2012; 5.
29. Johnson M, Usha A, Babu A. Rapid multiplication of *Plumbago auriculata* L.-a medicinally important plant. J Basic Appl Biol 2010; 4(1&2):135-141.
30. Bhojwani SS, Rajdan MK. Plant Tissue Culture: Theory and Practice, Developments in Crop Science. 5: Elsevier Science Pub. Amsterdam. The Netherlands 1983; 502.
31. Anon. Rapid clonal propagation of two varieties of *Chrysanthemum* through tissue culture. M. Sc. Thesis Dept. of Botany Rajshahi University 1988.
32. Druart P, Gruselle R. Plum (*Prunus domestica*) In: Biotechnology in Agriculture and Forestry. Bajaj YPS (Ed). Trees. 1 Springer – Verlag. Berlin 1986; 130-154.
33. Rubinstein B, Nagao MA. *Lateral bud out growth and its control by the apex* **.** The Bot Rev 1976; 42:83-113.
34. Kyte L. Plants From Test Tubes: An introduction to micropropagation (Rev. Ed.), Portland. Oreg. Timber Press 1987; 43-44.
35. Khatun R, Islam SM, Jahan AAM, Hossain TM. Mass propagation of *Azadirachta indica* A. Juss through *in vitro* Growth Seedling culture. Bangladesh J Sci INd Res 1998; 33(1).
36. Sugla T, Purkayastha J, Singh KS, Solleti S. Shoo L. Micropropagation of *Pongamia pinnata* through enhance axillary branching. *In Vitro* Cell Dev. Biol.-Plant 2007; 43(3):409-414.
37. Short KC, Price L, Roberts AV. Micropropagation of roses in; the rose annual. harkness, J (eds.) R. N. R. S. St., Albans, UK 1981; 138-144.
38. Bressan PH, Kinn YJ, Hyndman SE, Hasegawa PM. Bressan RA. Factors affecting *In vitro* propagation of rose. J Ameri soc Hor Sci 1982; 107:927-990.
39. Graifenberg A, Guistiniani L, Papanderoo A. *In vitro* culture of shoot apices of *Rosa multiflora* Thumb and *Rosa indica* Major Agric 1975; (3):119-128.
40. Moss JP, Dutt NRG, Lingamaneni A. Root induction of *in vitro* grown shoots of *Arachis* species and a hybrid. IAN 1988; 4:25-25.
41. Gautheret RJ. Une voie nouvelle in biological vegetable, La culture des tissus vegetaux, Gallimard, Paris 1959.
42. Tizio R, Moyano JC, Morales H. Inhibitor like substances in vine cuttings and their possible relationship to the rooting process. Fyton 1968. 25:123-128.
43. Lee CJL, McGuire JJ, Kitechin T. The relationship between rooting co-factors of easy and difficult to root cuttings of three clones of rhododendron. J Amer Soc Hort Sci 1969; 94:45-48.
44. Jabeen N, Shawl SA, Dar GH, Jan A, Sultan P. Micropropagation of *Inula racemosa* Hook. f.a valuable medicinal plant. Intl J Bot 2007; 3(3):296-301.
45. Islam R. Joarder LO. 1994. Regeneration of *Azadirachta indica* by tissue culture. In Abstract of 18th Bangladesh science Conference. Abstract No.60.P.32.
46. Johnson M, Berhanu A, Mulugeta K, Eyayu M, Manickam VS. Regeneration from callus cultures of Rhinacanthus nasutus L. Kurtz. Ethnomed J Sci Technol 2005; 3(1):17-24.
47. Gadidasu K, Umate P, Aileni M, Kota SR, Kokkirala VR, Kasula K. Micropropagation of a valuable ethnomedicinal plant *Streblus asper* Lour. J Phytol 2011; 3(2):18-23.
48. Gutiérrez IEM, Nepomuceno CF, Ledo CAS, Santana JRF. Micropropagation and acclimatization of *Bauhinia cheilantha* (an important medicinal plant). Afr J Biotechnol 2011; 10(8):1353-1358.
49. Loc NH, Kiet HV. Micropropagation of *Solanum hainanense* Hance. Ann Biol Res 2011; 2(2):394-398.
50. Dhir SK, Oglesby J, Bhagsari AS. Plant regeneration via somatic embryogenesis, and transient gene expression in sweet potato protoplasts. Plant cell Rep 1998; 17:665-669.
51. Roy SK, Roy PK. Raman M, Hossain T. Clonal propagation of *Rauwolfia serpentina* through *In vitro* culture. Acta hort. (ISHS) 1995; 390:141-146.