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Micro-propagation of *Astercantha longifolia* (L.) Nees – An Ethno-medicinal Herb

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Abstract

An efficient rapid protocol for plant regeneration and micropropagation from leaf explants of *Asteracantha longifolia* (L.) was reported. The multiple shoots obtained from leaf explants cultured on MS medium supplemented with different concentration of Thidiazuron (TDZ), N^6- Benzyladenine (BA) and α-Napthalene acetic acid (NAA). The highest number of shoots per explant (158.4±0.75) was obtained on MS medium fortified with 0.5 mg/l TDZ. Maximum numbers of roots were developed from healthy shoots when cultured on half-strength MS medium with 0.1mg/l NAA. The rooted were hardened and transferred to soil with 80% of survival rate. The continuous production of *A. longifolia* regenerated plants could be used as a possible micropropagation system.

Keywords: *Asteracantha longifolia*, TDZ, Micropropagation, Plant regeneration.

Introduction

Asteracantha longifolia (L.) Nees (Family- Acanthaceae) is a medicinal herb of pharmaceutical significance. The plant parts including leaves, inflorescences, seeds, roots and ashes have been extensively used in preparation of herbal medicine for

various ailments including diuretic, jaundice, diopsy, rheumatism, hepatic obstructions and dissolution of gallstones, kidney stones, liver dysfunction and diseases of urino-genital tracts[1,2,3]. The plant extract are possessed with antitumor[4,5], hypoglycaemic[6], antibacterial[7,8], hepatoprotective[9,10,11,12], anti-nociceptive[13], hematopoietic[14,15], anitipyretic[16] and antioxidant properties[11,17]. The seeds were also act as enhancer of spermatogenesis[18]. The plant parts have been used in several ayurvedic preparations- Lukol, Speman, Confindo, Tentex Royal etc. by Himalaya Health Care Pvt. Ltd. (www. Himalayahealthcare.com), Breastone by Vedic Biolabs Pvt. Ltd. (www.vedicbiolabs.com), Biogest and Rasanagugul by Trihealth Care, Kerala Ayurveda Pharmacy division (www.oilbath.com) and Microlactin by Victoria Health Care Ltd. (www.victoriahealth.com), Cardiraksh & Arjit Liniment (oil& spray) by Capro Labs Exports India Pvt. Ltd. (www.caprolabs.com), Goutnil-Natural by Rajavel Diabetes centre (www.diabetesayuevedic.com).

Astercantha longifolia is a marshy, hispid herb, growing in the bank of fresh or stagnant water ditches and swampy grounds, mixed with marshy grasses and sedges. The requirement of *A. longifolia* is presently obtained from the natural populations. This extensive utilization has a positive threat for the plant, to the extent of being extinct. Commensurate with this, the intervention of *in vitro* culture for accelerating clonal multiplication of this important drug yielding plant through micropropagation and their conservation are warranted in the right earnest. Only few reports were available on *in vitro* organogenesis of *A. longifolia*[19,20,21].

Therefore the present study has been initiated to standardize a simple and rapid protocol for *in vitro* micropropagation of this important medicinal herb.

Materials and Methods

Plant materials and explant preparation

The plants of *A. longifolia* were collected from paddy fields of Narayanapatna, Orissa and maintained in the experimental garden of the Division of Biotechnology, MITS, Rayagada, Orissa, India. Leaves washed with 5 % (v/v) Teepol (E-Merck, India) solution for 2 min were surface sterilized with 70% alcohol for 45 sec followed by 3min of soaking in 0.1 % (w/v) $HgCl_2$ (E-Merck, India). The sterilized leaves were washed thoroughly with sterilized double distilled water (ddH_2O) and used as explants.

Culture medium and conditions

The culture medium was that of Murashige and Skoog (1962)[22] MS with 100 mg/l Meso-inositol (Hi-media, India) and 3% (w/v) Sucrose (Qualigen, India). The medium was augmented with different concentrations and combinations of N^6-Benzyladenine (BA), α-Napthalene acetic acid (NAA) and TDZ (Hi-media, India). The pH of the medium was adjusted to 5.8 prior to gelling it with 0.8% Agar-agar (bacteriological grade, Hi-media, India). All the media containing culture vessels were autoclaved at 104 kpa and 121^{o}C for 20 min. One explant (~25mm^2) was implanted in each tube and cultures were incubated at 25±1^{o}C, 60-70% relative

humidity and 16 hr photoperiod of 35 μ $Em^{-2}s^{-1}$ irradiance level provided by cool white fluorescent tubes (Philips, India).

Effect of phytohormones for multiple shoot induction and shoot elongation

The experiment was designed to study the effect of different concentration and combination of BA, NAA and TDZ on shoot bud regeneration and multiple shoot induction. After eight week of culture, the numbers of shoot buds or shoot initials per explant were counted. Each treatment was replicated five times using five explants for each treatment.

Rooting of the elongated shoots and acclimatization

Elongated shoots with 5-6 leaves (>3 cm long) were excised *in-vitro* and were transferred to half-strength MS medium containing 0.1 to 0.5 mg/l of NAA. Cultures were incubated as described previously. Plantlets with well-developed roots were transferred to plastic cups containing autoclaved sand and soil (1:1) and maintained in same environmental condition for one week. They were watered regularly with $1/10^{th}$ strength MS liquid medium. Subsequently, they were transferred to earthen pots containing coarse sand, compost and garden soil and kept in shade for two weeks before transferring to the experimental garden.

Observation and statistical analysis

Visual observations were made every week and data on explant response, number of shoot buds/ shoots per explants and numbers of roots per shoot were recorded at the end of 8^{th} week. Each phytohormone treatment consists of five replicated tubes and was repeated thrice, and the standard error was calculated. Data on shoot bud regeneration, multiple shoot production and rooting were statistically analyzed using a completely randomized block design and means were evaluated at p=0.05 level of significance using Duncan's multiple range test[23]. For this SPSS V 8.0.1 software used with parameters - one way ANOVA and homogeneity of variance.

Result and Discussion

A simple and effective protocol has been developed for plant regeneration and micropropagation of *A. longifolia*. Surface sterilized leaf explants (20-25 mm^2) were used for *in-vitro* studies. The implanted leaf segments, enlarged and produced protuberances from the cut ends within 7 to 10 days of culture (Fig. 1a) on semi solid MS medium supplemented with BA (0.5 to 2.5 mg/l) and TDZ (0.01 mg/l to 0.5mg/l). Initially these protuberances were concentrated at the cut surface near the midrib of the proximal end but subsequently developed from the distal end and also from the abaxial surface in contact with the medium. By the end of 5^{th} week, these protruded structures develop into shoot buds without intervening callus phase and 80±2% of them were flanked by green leaves (Fig.1b). By the end of 8^{th} week well developed shoots (Fig.1c) were obtained on the same medium. MS medium supplemented with BA (2mg/l) and NAA (0.5mg/l) produced 91.6±0.75 number of shoots/plankton. Similar type of observation was reported by Panigrahi *et al.* (2006)[19], Mishra *et al.*

(2006)[20] and Panigrahi *et al.* (2007)[21] for the plant taking node, internode & leaves as explants. But maximum number of healthy shoots/plankton (158.4±0.75) was obtained in MS medium with 0.5 mg/l TDZ (Fig.1d) (Table 1). A lot of reports have been published earlier by taking TDZ as growth regulator for micropropagation.

Table 1: Effect of different Plant growth hormones on multiple shoot bud regeneration from leaf explants of *A. longifolia* after 8th week.

Media Code	Conc. of plant growth regulators (mg/l)			No. of shoot buds ± SEM
	BA	NAA	TDZ	
A_1	Control			00±0.0[a]
A_2	0.5	0.0	0.0	38.4±0.98[f]
A_3	1.0	0.0	0.0	41.6±0.98[g]
A_4	1.5	0.0	0.0	43.2±0.94[gh]
A_5	2.0	0.0	0.0	13.6±0.73[c]
A_6	2.5	0.0	0.0	14.4±0.60[c]
A_7	0.5	0.2	0.0	14.4±0.60[c]
A_8	1.0	0.2	0.0	13.2±0.73[c]
A_9	1.5	0.2	0.0	38.4±0.98[f]
A_{10}	2.0	0.2	0.0	68.8±0.49[i]
A_{11}	2.5	0.2	0.0	18.8±1.2[d]
A_{12}	0.5	0.5	0.0	41.6±0.98[g]
A_{13}	1.0	0.5	0.0	72.8±0.8[j]
A_{14}	1.5	0.5	0.0	76.4±0.4[k]
A_{15}	2.0	0.5	0.0	91.6±0.75[m]
A_{16}	2.5	0.5	0.0	41.6±0.98[g]
A_{17}	0.5	1.0	0.0	28.8±0.49[e]
A_{18}	1.0	1.0	0.0	26.8±0.49[e]
A_{19}	1.5	1.0	0.0	41.6±0.98[g]
A_{20}	2.0	1.0	0.0	10.2±0.73[b]
A_{21}	2.5	1.0	0.0	Callus 00±0.0[a]
A_{22}	0.0	0.0	0.05	45.2±0.49[h]
A_{23}	0.0	0.0	0.1	74.4±0.98[jk]
A_{24}	0.0	0.0	0.2	82.4±0.98[l]
A_{25}	0.0	0.0	0.3	118.4±0.98[n]
A_{26}	0.0	0.0	0.5	158.4±0.75[o]

*Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's multiple range test (SPSS V 8.0)

Leave explants of *Rehmmania glutinosa* when cultured in MS medium supplemented different concentration of TDZ produce multiple shoots. The highest

number of shoots per explant (2.1) and shoot growth (1.2 cm) was obtained on MS medium containing 1 mg/l TDZ[24]. A regeneration protocol using thidiazuron (TDZ) with a high frequency in *Astragalus cicer* was developed by Basalma *et al.* (2008)[25]. For this, hypocotyl and cotyledon explants were cultured on MS medium supplemented with different concentrations of TDZ. The highest frequency of shoot regeneration (53.3%) was achieved from hypocotyl segments through an initial callus growth stage on MS medium containing 0.25 mg/l TDZ. The shoots were cultured on the different strength (1/1, 3/4, 1/2 and 1/4) of basal MS medium containing different concentrations of NAA. High rooting (100%) and survival (100%) were achieved using half strength MS medium supplemented with 0.25 and 0.50 mg/l NAA. Observation shows that TDZ along produce multiple shoot from nodal explants of *Psoralea corylifolia*. Proliferation of shoots was achieved on MS medium supplemented with 0.5, 1, 2, 3, 4 and 5 μM TDZ. The maximum numbers (13.6 ± 1.4) of shoots per explant were obtained from nodal segment cultured on 2 μM TDZ for 4 weeks[26]. When the hypocotyl explants of same plant was cultured on L2 medium supplemented with 2μM BA, 4μM TDZ and 50mg/l bavistin produced 89.5±1.18 number of shoots per explant[27]. Similar types of observations were seen by using TDZ in combination with different growth regulator for *Hydrastis canadensis* L.[28] and *Kigelia pinnata* L.[29].

Elongated and well developed shoots (>3 cm long) were excised from the shoot clumps and cultured on half-strength MS supplemented with 0.1 to 0.5 $mgl^{-1}$of NAA. The maximum numbers of roots were produced in MS medium supplemented with 0.1 mg/l NAA (Table 2, Fig. 1e). After four weeks, the rooted plantlets were transferred into plastic pots containing autoclaved sand and soil mixture (1:1) and were maintained in the culture room for two weeks (Fig. 1f), and then transferred to shade and then to field conditions. The survival rate is 80±4%. The regenerated plantlets did not show any variation in morphology or growth characteristics when compared with the respective donor parents.

Table 2: Influence of auxins on rooting of *in-vitro* derived shoots of *A. longifolia* after 3 weeks*.

Media code	Plant growth regulator conc. (mg/l)	*No. of roots developed from the shoots (Mean ± S.E.)*
	NAA	
R_1	Control	*10.8±0.58* ^{a}
R_2	0.1	*32.4±1.96* ^{d}
R_3	0.2	*24.8±1.16* ^{c}
R_4	0.5	*20.4±0.24* ^{b}

*Means within a column having the same letter are not statistically significant (p=0.05) according to Duncan's Multiple range test (SPSS V 8.0.1)

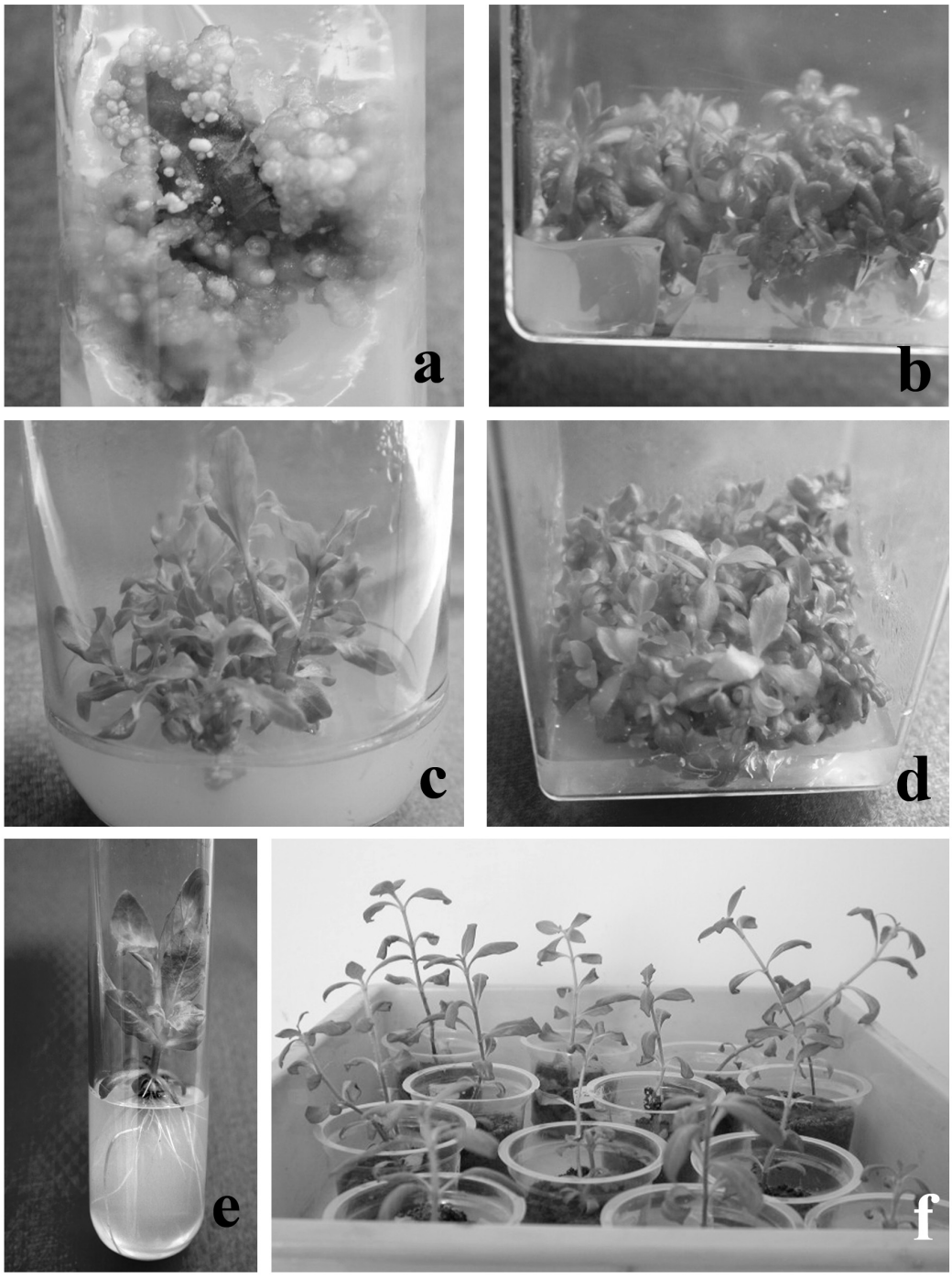

Figure 1 (a-f): Plant regeneration in *Astercantha longifolia* (a) Protuberances at the cut end of leaf explant implanted on MS+ 0.5 mg/l TDZ, (b) Multiple shoot buds after 5th week on MS + TDZ (0.5mg/l), (c) Well developed multiple shoots in MS + BA 2.0 mg/l + NAA 0.5mg/l after 8th week, (d) Highest number of multiple shoots in MS + TDZ 0.5 mg/l after 8th week, (e) Rooting of shoot in half-strength MS medium + NAA 0.1mg/l, (f) A set of hardened plantlets in plastic cups.

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