



ARTICLE

# High frequency plant regeneration with histological analysis of organogenic callus from internode explants of *Asteracantha longifolia* Nees



Muthusamy Senthil Kumar <sup>a,\*</sup>, Subhash Chandra Nandi <sup>b</sup>

<sup>a</sup> Department of Biotechnology, St. Xavier's College, Burdwan 713103, India

<sup>b</sup> Department of Botany, Burdwan Raj College, Aftab House, Burdwan 713104, India

Received 17 April 2014; revised 18 September 2014; accepted 23 December 2014  
Available online 21 January 2015

## KEYWORDS

*Asteracantha longifolia*;  
Histological analysis;  
Plant growth regulators;  
Plantlet regeneration;  
Callus proliferation;  
Internode

**Abstract** *Asteracantha longifolia* Nees is an ayurvedic medicinal herb. The internode explants of this plant were used for high frequency plant regeneration on Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs) in different concentrations. Apical meristem and leaf primordium formations were confirmed through microscopic analysis of histological sections of the organogenic callus tissues. The synergistic effect of  $\alpha$ -naphthaleneacetic acid (NAA) 0.5 mg/l with  $N^6$  benzyladenine (BA) 0.25 mg/l increased the percentage of explants response for callus induction while comparing other treatments. Various concentrations of NAA were also found to be best for explants response to callus induction than 2,4-dichlorophenoxyacetic acid (2,4-D). The callus morphology (color and texture) was different according to the growth regulators and their concentrations. The highest percentage of response per culture for shoot bud regeneration was noted for the concentration of NAA 0.5 mg/l with BA 2.0 mg/l, the same concentration effectively increased the number of shoots per culture. Different concentrations of indol-3-butyric acid (IBA) and NAA were used in half strength MS medium for *in vitro* rooting of regenerated shoots. The maximum percentage of shoot response for rooting and the highest number of root formations per shoot were observed on the medium containing 0.5 mg/l of IBA. The survival rate (86.7%) of the regenerated plants was noted after 20 days of transplantation.

© 2015 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

**Abbreviations:** *A. longifolia*, *Asteracantha longifolia*; NAA,  $\alpha$ -naphthaleneacetic acid; BA,  $N^6$  benzyladenine; MS, Murashige and Skoog's nutrient medium; IBA, indole-3-butyric acid; PGRs, plant growth regulators

\* Corresponding author at: Department of Biotechnology, St. Xavier's College, Burdwan 713103, India.

E-mail address: [msen78@gmail.com](mailto:msen78@gmail.com) (M. Senthil Kumar).

Peer review under responsibility of National Research Center, Egypt.

<http://dx.doi.org/10.1016/j.jgeb.2014.12.002>

1687-157X © 2015 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

## 1. Introduction

The wetland medicinal herb *Asteracantha longifolia* Nees (Syn. *Hygrophila auriculata* (Schum) Heine) belongs to the Acanthaceae family. It grows throughout India in stagnant streams, fresh water swamps and ponds. The herb is useful in treating diseases of blood and also shows hepatoprotective and antioxidant activities [13,24]. The whole plant, roots,

seeds, and ashes of the plant are extensively used in the traditional system of medicine for various ailments like rheumatism, inflammation, jaundice, hepatic obstruction, pain, urinary infections, edema and gout. It is classified in the ayurvedic system as seethaveeryam, mathuravipaka and is used for the treatment of premeham (Diabetes), athisaram (Dysentery), etc. [18,8].

The plant is known to possess antitumor [2,15], antidiabetic [17], anthelmintic, antibacterial [20], anti-inflammatory, antipyretic [21], antioxidant [9] and aphrodisiac [5,6] activities. Aqueous extract of aerial parts and roots was reported for its antinociceptive property [23]. The root contains an alkaloid named hygrosterol [26]. The plant has also been used in ayurvedic preparations, such as Lukol, Speman, and Confindo, by the Himalaya Health Care Private Ltd. [27].

In India, the wild medicinal plants are used in large quantities due to increasing demand of raw materials for domestic consumption and export. It is essential to overcome the pharmaceutical demands through *in vitro* conservation of valuable wild medicinal herbs. *A. longifolia* is not in traditional cultivation practice and is collected from wild resources for the preparation of ayurvedic medicines. Therefore, the *in vitro* plant propagation technique is an ideal method for the conservation of the valuable medicinal herb. Though the plantlet regeneration has been achieved from leaf segments of *in vitro* seedlings of *A. longifolia* [19], the present study illustrates *in vitro* callus induction, callus growth and high frequency plant regeneration with histological analysis of organogenic callus from internode explants of wild growing *A. longifolia*.

## 2. Methods

### 2.1. Explants collection and sterilization

The plant twigs of *A. longifolia* with 3–4 nodes were collected from wild grown plants. The internode explants were excised and cleaned with 5% teepol solution for 5 min. followed by keeping the explants in a running tap water for 5–10 min. Explants were surface sterilized by treating with 70% ethanol for 30 s followed by 0.1% HgCl<sub>2</sub> for 6 min., and the explants were rinsed with sterile water at least 3 times after each disinfectant treatment.

### 2.2. Culture medium and condition

The basal medium Murashige and Skoog (MS) [16] used for all treatments was MS medium supplemented with 3% (w/v) sucrose (Himedia) (used as carbon source) and gelled with 0.8% (w/v) agar (Himedia). The pH of the medium was adjusted to 5.8 before autoclaving and the medium was autoclaved at a pressure of 1.06 kg cm<sup>-2</sup> at 120 °C for 15 min. All cultures were maintained at 25 ± 2 °C with 55–60% relative humidity under a 16 h photoperiod with a light intensity of 60 μmol m<sup>-2</sup> s<sup>-1</sup> by white fluorescent light.

### 2.3. Callus induction and morphology

The surface sterilized explants were inoculated on MS medium containing different concentrations of auxins such as NAA and 2,4-D (0.5–3.0 mg/l) and cytokinin: BA (0.25–3.0 mg/l) either alone or in combination for callus induction. Callus

Index (CI) was computed by multiplying percent cultures initiating callus with growth score (G), which was assessed by visual rating (poor = 1, medium = 2, good = 3, and prolific = 4). The mean score was expressed as growth score (G). The percentage of callus induction and morphology such as color and texture was noted after 20 days of inoculation.

### 2.4. Callus proliferation and plantlets regeneration

The callus developed from NAA 2.0 mg/l was subcultured on MS medium containing different concentrations of PGRs with combinations such as auxin (NAA 0.5 and 1.0 mg/l) and cytokinin: (BA 0.25–3.0 mg/l) for their proliferation and plantlets regeneration. Calli derived from all the concentrations were subsequently subcultured in the same medium every 20 days once, for 60 days.

### 2.5. Histology of plant regeneration from callus

The callus at different stages of growth after subculture i.e., at the stages of 15<sup>th</sup>, 30<sup>th</sup> and 45<sup>th</sup> days was selected for histological studies. Thin sections were taken, stained with crystal violet, mounted on glass slides and covered with a cover glass for microscopic observations. Photographs were taken by an Olympus light microscope.

### 2.6. Rooting and acclimatization

*In vitro* regenerated shoots of *A. longifolia* (about 5 cm in height) were carefully separated from clumps of shoots. Shoots were rooted on half strength MS medium containing IBA (0.1–2.0 mg/l) and NAA (0.1–2.0 mg/l). After 15 days, the rooted plantlets were transferred to small pots containing soil and sand (1:1, v/v) and pots were irrigated every day with 1/4<sup>th</sup> strength of MS nutrients for two weeks and they were transplanted to fields. The survival rate of the plants was noted after 20 days of plantation.

### 2.7. Statistical analysis

Morphogenetic response of the culture was closely observed every week and the percentage of callus induction, number of shoots per culture, percentage of shoot response for root formation and number of roots per shoot were recorded. Each treatment factor consisted of 20 replicates and the experiment was repeated three times. A completely randomized design was used in all experiments and analysis of variance and mean separations were carried out using Duncan's multiple range test ( $P < 0.05$ ) using the SPSS (Statistical Package for the Social Sciences) statistics. Values expressed are mean of replicate determinations ± standard error.

## 3. Results and discussion

### 3.1. Callus induction and morphology

The percentage of callus induction in MS medium was observed for the internode explants under the influence of various PGRs. PGRs such as NAA and 2,4-D were used in the medium to induce callus and found to be the best for callus

**Table 1** Effect of NAA and 2,4-D on internode explants of *Asteracantha longifolia* Nees on callus induction and callus morphology.

Growth regulators (mg/l)		Percentage of response	Growth score (G)	Callus index (CI)	Callus morphology	
NAA	2,4-D				Color	Texture
0.5	0.0	81.7 ± 1.7 <sup>cd</sup>	2.6 ± 0.2 <sup>bc</sup>	212.4	White	Compact
1.0	0.0	85.0 ± 2.9 <sup>bcd</sup>	3.5 ± 0.1 <sup>a</sup>	297.5	White	Hard and compact
2.0	0.0	93.3 ± 3.3 <sup>ab</sup>	3.6 ± 0.2 <sup>a</sup>	335.9	Greenish white	Hard and compact
3.0	0.0	80.0 ± 2.9 <sup>d</sup>	2.4 ± 0.3 <sup>c</sup>	192.0	Greenish white	Hard and compact
0.0	0.5	76.7 ± 4.4 <sup>d</sup>	1.6 ± 0.2 <sup>d</sup>	122.7	White	Compact
0.0	1.0	83.3 ± 3.3 <sup>cd</sup>	2.7 ± 0.3 <sup>bc</sup>	224.9	White	Compact
0.0	2.0	90.0 ± 2.9 <sup>abc</sup>	2.9 ± 0.4 <sup>abc</sup>	261.0	White	Friable
0.0	3.0	96.7 ± 1.7 <sup>a</sup>	3.3 ± 0.2 <sup>ab</sup>	319.1	White	Nodular

Observations were made after 20 days of inoculation. Values represent mean ± standard error of 20 replicates per treatment and each experiment was repeated thrice for percentage of explants response.

Mean values within a column followed by different letters are significantly different from each other at  $P < 0.05$  level comparison by Duncan's multiple range test (DMRT).

induction. Different concentrations of growth regulators facilitate the degree of variations in callus induction. The presence of NAA (2.0 mg/l) and 2,4-D (3.0 mg/l) in the culture medium resulted in the highest percentage of callus induction, 93.3 and 96.7 respectively, than other concentrations of NAA and 2,4-D (Table 1). The callus induction plays a vital role in the explants and gives significant variations among the different growth regulators which were studied earlier in *Lithospermum erythrorhizon* and *Cephalotaxus harringtonia* [10,12] and the high concentrations of NAA and 2,4-D produced a high amount of callus in *Phyllanthus urinaria* [4].

Gopi and Vatsala [11] found the maximum callus growth of *Gymnema sylvestre* on the medium containing auxins such as 2,4-D and NAA, and also with BA. Accordingly, the synergistic effect of NAA (0.5 mg/l) and BA (0.25 mg/l) in the present study also slightly increases the percentage of callus induction (98.3 ± 0.3) than the NAA used alone in various concentrations. Moreover, a decrease of calli percentage in proportion to the BA level increase was observed the medium fortified with NAA 2.0 mg/l shows a better callus index (335.9) and callus score (3.6) where as the synergistic effect of NAA (1.0 mg/l) and BA (0.25 mg/l) produces maximum callus index (300.2) and callus score (3.4) as shown in Table 2.

The morphology of the callus varies according to the type and concentrations of growth regulators used in the medium

either alone or in combination. The color of the callus such as white, whitish green, green and greenish white was noted for various concentrations of PGRs. The medium containing lower concentrations of NAA 0.5 and 1.0 mg/l and all concentrations of 2,4-D produced a white colored callus without the combination of BA, but at higher concentrations of NAA 2.0 and 3.0 mg/l a greenish white callus was developed (Table 1).

The synergistic effect of NAA 0.5 mg/l and BA 0.25 mg/l in the medium shows a greenish white callus whereas higher concentrations of BA along with NAA 0.5 mg/l produced a green colored callus. Whitish green and green color calli were developed under the influence of lower concentrations (0.25 and 0.5 mg/l) and higher concentrations (1.0 mg/l and 2.0 mg/l) of BA respectively combined with 1.0 mg/l of NAA (Table 2).

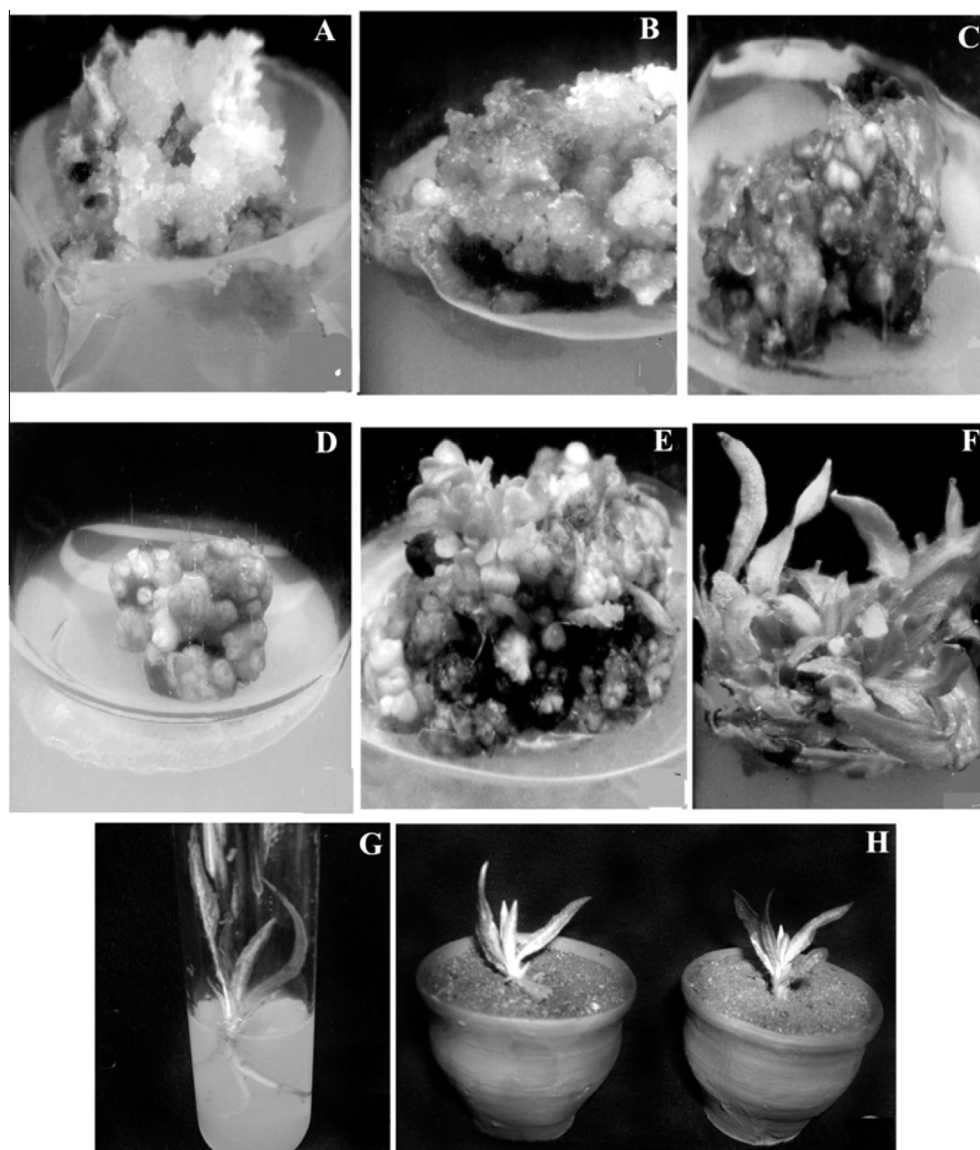
While comparing the texture of the callus it was compact, hard and compact, friable, nodular and compact with regeneration of buds after 20 days of inoculation with different concentrations of growth regulators. The lowest concentration of NAA in the culture medium produced a compact callus, but higher concentrations promote the formation of a hard and compact callus. The medium containing 2,4-D in lower concentration produced a compact callus, when the concentration increased the callus texture was friable and nodular (Table 2). The texture of the callus was compact which was derived from various concentrations of the synergistic effect

**Table 2** The synergistic effect of NAA and BA on internode explants of *Asteracantha longifolia* Nees on callus induction and callus morphology.

Growth Regulators (mg/l)		Percentage of response	Growth score (G)	Callus index (CI)	Callus morphology	
NAA	BA				Color	Texture
0.5	0.25	98.3 ± 0.3 <sup>a</sup>	2.5 ± 0.3 <sup>bcd</sup>	245.8	Greenish white	Compact
0.5	0.50	90.0 ± 5.8 <sup>ab</sup>	3.2 ± 0.3 <sup>ab</sup>	288.0	Green	Compact
0.5	1.0	90.0 ± 2.9 <sup>ab</sup>	3.1 ± 0.3 <sup>ab</sup>	279.0	Green	Compact
0.5	2.0	86.7 ± 4.4 <sup>ab</sup>	2.4 ± 0.2 <sup>bcd</sup>	208.1	Green	Compact (with sprouts)
1.0	0.25	88.3 ± 4.4 <sup>ab</sup>	3.4 ± 0.2 <sup>a</sup>	300.2	Whitish green	Compact
1.0	0.50	85.0 ± 2.9 <sup>ab</sup>	2.7 ± 0.2 <sup>abc</sup>	229.5	Whitish green	Compact
1.0	1.0	83.3 ± 3.3 <sup>b</sup>	2.3 ± 0.3 <sup>cd</sup>	191.6	Green	Compact
1.0	2.0	80.0 ± 5.0 <sup>b</sup>	2.0 ± 0.1 <sup>d</sup>	160.0	Green	Compact

Observations were made after 20 days of inoculation. Values represent mean ± standard error of 20 replicates per treatment and each experiment was repeated thrice for percentage of explants response.

Mean values within a column followed by different letters are significantly different from each other at  $P < 0.05$  level comparison by Duncan's multiple range test (DMRT).



**Figure 1** *In vitro* regeneration of callus from internode explants of *Asteracantha longifolia* Nees on MS medium. (A and B) Callus produced under the influence of NAA and 2,4-D respectively. (C and D) Callus produced under the synergistic effect of NAA with BA, (E and F) plantlet regeneration from callus, (G) *in vitro* rooting (H) Hardening.

of NAA and BA, but the highest concentration of BA 2.0 mg/l with NAA 0.5 mg/l produced a compact callus with numerous regenerating shoot buds (Table 2 and Fig. 1A–E).

### 3.2. Plantlets regeneration from callus

Plantlets regeneration from the callus was highly influenced by the plant growth regulators and their combinations and concentrations. Several studies clearly indicated the requirement of high auxin: low cytokinin ratio for callus induction and low auxin: high cytokinin ratio for shoot induction from the callus. The synergistic effect of NAA and BA in most of the concentrations in the medium showed a better response for callus regeneration. The highest percentage ( $98.3 \pm 2.9$ ) of plantlet regeneration and the maximum number of shoot (42.3 shoots per culture) formations from the callus were found to

be in the medium containing NAA 0.5 mg/l with BA 2.0 mg/l. The higher concentrations of NAA with lower concentrations of BA significantly reduced the percentage of regeneration and the number of shoots in the callus (Table 3 and Fig. 1E and F). Contrasting to this result, Ahmad et al. [1] reported the highest percentage of callus response and the maximum number of shoots per unit callus from leaf explants of *Ruta graveolens* in the media containing higher concentrations of NAA with lower concentrations of BA.

### 3.3. Histological study on plant regeneration from callus

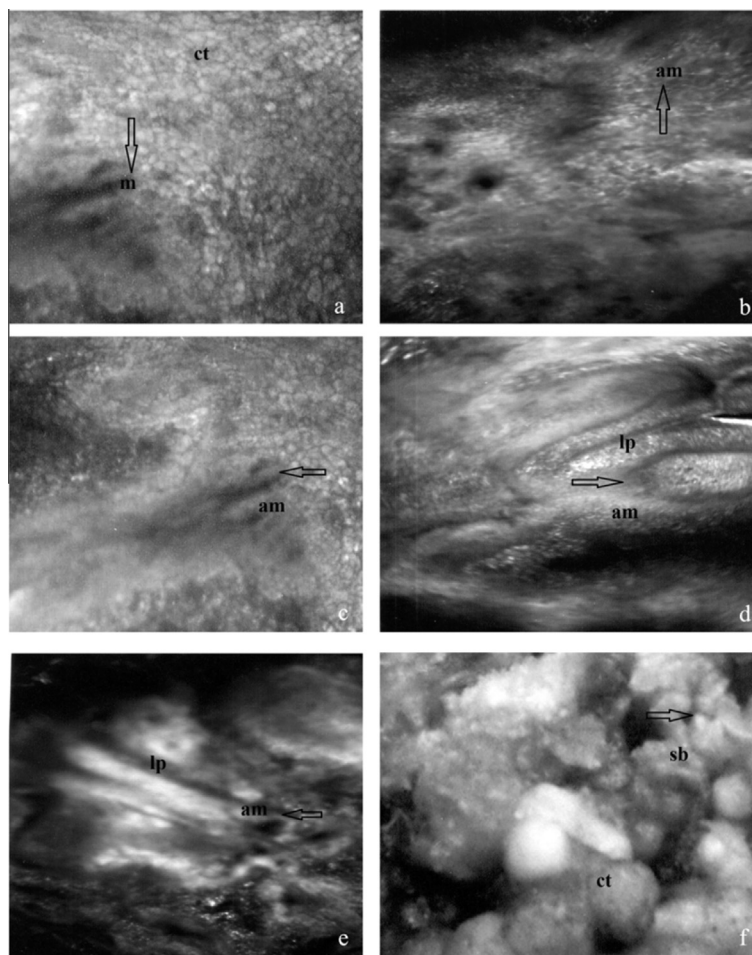
The meristematic regions, which were characterized with small cells with densely stained nuclei and small buds with the tunica corpus organization of a shoot apical meristem and the origin of shoot primordia were observed from internode derived calli

**Table 3** The synergistic effect of growth regulators such as NAA and BA at various concentrations on plantlets regeneration from the internode derived callus of *Asteracantha longifolia* Nees.

Growth regulators (mg/l)		Percentage of response/culture	Number of shoots/culture
NAA	BA		
0.5	0.25	Nil	Nil
0.5	0.50	Nil	Nil
0.5	1.0	95.0 ± 2.9 <sup>ab</sup>	37.7 ± 0.3 <sup>b</sup>
0.5	2.0	98.3 ± 2.9 <sup>a</sup>	42.3 ± 0.4 <sup>a</sup>
1.0	0.25	80.0 ± 2.9 <sup>c</sup>	26.5 ± 0.6 <sup>f</sup>
1.0	0.50	85.0 ± 5.0 <sup>bc</sup>	28.4 ± 0.6 <sup>c</sup>
1.0	1.0	90.0 ± 2.9 <sup>abc</sup>	30.1 ± 0.3 <sup>d</sup>
1.0	2.0	91.7 ± 6.0 <sup>abc</sup>	33.3 ± 0.7 <sup>c</sup>

Observations were made after 60 days (three times subcultured) from the 1st subculture. Values represent mean ± standard error of 20 replicates per treatment and each experiment was repeated thrice for percentage of explants response.

Mean values within a column followed by different letters are significantly different from each other at  $P < 0.05$  level comparison by Duncan's multiple range test (DMRT).



**Figure 2** Light micrographs of longitudinal sections of the callus derived from internodes and a photograph of callus regeneration from *Asteracantha longifolia* Nees (a) The arrow indicates the formation of a shoot meristemoid from the callus tissue (ct) (15<sup>th</sup> day), (b and c) The arrow indicates the development of the apical meristem (am) (15<sup>th</sup> day), (d) Longitudinal section of the callus showing the apical meristem (am) with leaf primordium (lp) (30<sup>th</sup> day), (e) The well developed apical meristem (am) and leaf primordium (lp) (45<sup>th</sup> day), (f) Shoot bud (sb) regeneration from the callus tissue (ct) (45<sup>th</sup> day).

**Table 4** *In vitro* rooting of *Asteracantha longifolia* Nees shoots on half strength MS medium with various concentrations of IBA and NAA.

Growth regulators (mg/l)		Percentage of shoot response	Number of roots/shoot
IBA	NAA		
0.1	0.0	46.7 ± 4.4 <sup>bc</sup>	2.3 ± 0.2 <sup>bc</sup>
0.5	0.0	95.0 ± 2.9 <sup>a</sup>	5.6 ± 0.3 <sup>a</sup>
1.0	0.0	38.3 ± 1.7 <sup>c</sup>	1.5 ± 0.2 <sup>c</sup>
2.0	0.0	Nil	Nil
0.0	0.1	51.7 ± 3.3 <sup>b</sup>	2.3 ± 0.2 <sup>b</sup>
0.0	0.5	56.7 ± 4.4 <sup>b</sup>	2.8 ± 0.3 <sup>b</sup>
0.0	1.0	Nil	Nil
1.0	2.0	Nil	Nil

Observations were made after 20 days of inoculation. Values represent mean ± standard error of 20 replicates per treatment and each experiment was repeated thrice for percentage of explants response.

Mean values within a column followed by different letters are significantly different from each other at  $P < 0.05$  level comparison by Duncan's multiple range test (DMRT).

after 15<sup>th</sup>, 30<sup>th</sup> and 45<sup>th</sup> days of subculture in regeneration medium. Histology of organogenesis from the callus culture of black pepper was reported by Sujatha et al. [25] and the formation of vascular nodules in callus cultures might represent or be associated with an early stage of the development of shoot meristems investigated by Chen and Galston [7]. Cassels [3] also reported that nodules containing xylem elements in the callus of *Pelargonium* developed into shoots when moved to an auxin free medium.

The organogenic potential of a compact and green colored callus was taken for histological studies. Different stages of meristem development were photographed using an Olympus light microscope. The callus developed on the MS medium containing NAA 0.5 mg/l and BA 2.0 mg/l showed many meristematic regions after the 45<sup>th</sup> day of subculture in regeneration medium than other concentration of the same growth regulators. The development of vascular nodules in the callus tissues indicated the formation of shoot meristem. Effect of these growth regulators on plant regeneration was confirmed from histological studies of the callus (Fig. 2A–F).

#### 3.4. *In vitro* rooting of shoots and acclimatization

The regenerated shoots were rooted on half strength MS medium supplemented with different concentrations of IBA and NAA. IBA was found to be superior to NAA for root formation. The maximum percentage (95.0%) of shoot response for root formation was noted for IBA 0.5 mg/l than other concentrations in the medium while the same concentration of IBA produced the highest number (5.6 roots/shoot) of roots (Table 4). The high efficiency *in vitro* root formation under the influence of IBA was supported by some earlier findings in *Heliotropium indicum* [14] and *Vitex negundo* [22]. The *in vitro* rooted plants were transferred to small earthen pots to acclimatize the plants in laboratory condition for two weeks for field transfer. After 20 days of transplantation the survival rate (86.7%) of the plants was noted (Fig. 1G and H). There was no noticeable variation among the plants with respect to morphological and growth characteristics when compared with the mother plants.

#### 4. Conclusions

The standardized protocol for high frequency plantlet regeneration from internode explants of *A. longifolia* with a histological evidence for apical meristem formation from its organogenic callus is highly reproducible and reliable.

#### References

- [1] N. Ahmad, M. Faisal, M. Anis, I.M. Aref, S. Afr. J. Bot. 76 (2010) 597–600.
- [2] S. Ahmed, A. Rahman, M. Mathur, M. Athar, S. Sultana, Food Chem. Toxicol. 39 (1) (2001) 19–28.
- [3] A.C. Cassels, Physiol. Plant. 46 (1979) 159–164.
- [4] E. Catapan, M. Luis, B. Silva, F.N. Moreno, A.M. Viana, Plant Cell Tissue Organ Cult. 70 (2002) 301–309.
- [5] N.S. Chauhan, V. Sharma, V.K. Dixit, Nat. Prod. Res. 14 (2009) 1–9.
- [6] N.S. Chauhan, D.K. Saraf, V.K. Dixit, Eur. J. Integr. Med. 2 (2010) 89–91.
- [7] H.R. Chen, A.W. Galston, Physiol. Plant. 20 (1967) 533–539.
- [8] R.N. Chopra, S.L. Nayar, I.C. Chopra, Council of Scientific and Industrial Research, New Delhi, 1986 (Including the Supplement).
- [9] N. Dasgupta, B. De, Food Chem. 101 (2007) 471–474.
- [10] R.W. Enaksha, N.A. Richard, Plant Cell Rep. 12 (1993) 80–83.
- [11] C. Gopi, T.M. Vatsala, Afr. J. Biotechnol. 5 (12) (2006) 1215–1219.
- [12] H. Inouye, S. Veda, K. Inoue, H. Matsumura, Phytochemistry 18 (1979) 1301–1308.
- [13] K.R. Kirtikar, B.D. Basu, Indian Medicinal Plants, Vol. 3, International Book Distributors, Dehradun, 2005.
- [14] M.S. Kumar, M.V. Rao, Indian J. Biotechnol. 6 (2007) 245–249.
- [15] U.K. Mazumdar, M. Gupta, S. Maiti, D. Mukherjee, Indian J. Exp. Biol. 35 (1997) 473–477.
- [16] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473–497.
- [17] M. Muthulingam, Int. J. Pharm. Biomed. Res. 1 (2) (2010) 28–34.
- [18] K.M. Nadkarni, Indian Materia Medica, Popular Prakashan, Mumbai, India, 1978.
- [19] J. Panigrahi, M. Behera, S. Maharana, R.R. Mishra, Indian J. Exp. Biol. 45 (10) (2007) 911–919.
- [20] A. Patra, S. Jha, P.N. Murthy, V.D. Aher, Res. J. Pharm. and Tech. 1 (2008) 531–532.

- [21] A. Patra, S. Jha, P.N. Murthy, V.D. Aher, P. Chattopadhyay, G. Panigrahi, D. Roy, J. Pharm. Res. 8 (2009) 133–137.
- [22] Y. Sahoo, P.K. Chand, Plant Cell Rep. 18 (1998) 301–307.
- [23] P. Shanmugasundaram, S. Venkataraman, Afr. J. Trad. CAM 2 (2005) 62–69.
- [24] P. Shanmugasundaram, S. Venkataraman, J. Ethnopharmacol. 104 (2006) 124–128.
- [25] R. Sujatha, L.C. Babu, P.A. Nazeem, J. Trop. Agric. 41 (2003) 16–19.
- [26] K.M. Wad kiranis, Indian Materia Medica, Popular Prakashan Private Ltd, Bombay, India, 2002.
- [27] < <http://www.himalayahealthcare.com/herbfinder/hygrophila-auriculata.html> > Accessed 13 July 2013.