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ARTICLE

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

Muthusamy Senthil Kumar [a,*], **Subhash Chandra Nandi** [b]

[a] *Department of Biotechnology, St. Xavier's College, Burdwan 713103, India*
[b] *Department of Botany, Burdwan Raj College, Aftab House, Burdwan 713104, India*

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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 α-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.

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,

Abbreviations: *A. longifolia*, *Asteracantha longifolia*; NAA, α-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 (M. Senthil Kumar).

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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 wildly 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% $HgCl_2$ 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^{-2} 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^{-2} s^{-1} 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^{th}, 30^{th} and 45^{th} 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 $1/4^{th}$ 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