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Full Length Research Paper

Establishment of an efficient protocol for micropropagation of stem explants of *Tylophora indica*, an important medicinal plant

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An efficient reproducible protocol has been developed for *in vitro* propagation of endangered medicinal plant (*Tylophora indica*). The concentration of plant growth regulators and explant types exhibited discrete roles in the efficiency of plant regeneration. Stem explants cultured on 8.8 µM 6-benzylaminopurine (BAP) resulted in formation of nodular meristemoids, which developed into green leafy shoots 6 to 8 weeks after culturing on the same medium. 29.4 µM α-naphthalene acetic acid (NAA) and 4.65 µM kinetin (Kn) induced green solid callus within 7 to 8 days of culturing. Callus thus formed, revealed the presence of cells of variable shapes and sizes together with xylogenesis. Calli when subcultured on MS medium supplemented with 8.8 µM BAP developed adventitious shoots, however, initially, few shoots were formed but the number increased further on subsequent subculturing. Microshoots thus formed, were cultured on indole 3-butyric acid (IBA) and half strength basal MS media for induction of roots. Regenerated plantlets with healthy shoots and roots were acclimatized in moist cotton followed by their hardening in soil : vermicompost potting mixture with 90% survival rate.

Key words: *Tylophora indica*, organogenesis, 6-benzylaminopurine, α-naphthalene acetic acid, indole 3-butyric acid.

INTRODUCTION

Tylophora indica (Asclepiadaceae) is an important medicinal plant found in the sub-Himalayan tract from Uttar Pradesh to Meghalaya and in the central and peninsular India. This plant has been traditionally used as a folk remedy for the treatment of bronchial asthma, bronchitis, rheumatism, inflammation, allergies and dermatitis (Shivpuri et al., 1968; Gore et al., 1980; Exoticnatural, 2005). Apart from this, it also seems to be a good remedy in traditional medicine as anti-psoriasis, seborrheic, anaphylactic, leucopenia and as an inhibitor of the Schultz-Dale reaction (Sarma and Misra, 1995). The leaves and roots are used medicinally (Bhavan, 1992) and are known to have laxative, expectorant, diaphoretic and purgative properties. Major constituent in this plant are alkaloids (Rao et al., 1971) such as tylophorine and tylophorenine which are responsible for a strong anti-inflammatory action (Gopalakrishnan et al., 1979; Faisal and Anis, 2005). Threat of plant propagation is critical to meet the pharmaceutical demand for alkaloids present in the plant, due to low seed viability and germination rate (Thomas and Philips, 2005). In addition, the destruction caused by harvesting the roots as a source of drug has threatened the survival of the plant. Thus, large scale demand necessitates rapid multiplication of the endangered plant, *T. indica* (Faisal and Anis, 2007) within a short period of time through tissue culture.

In vitro direct shoot induction is an easy way for rapid plant propagation as reported in a number of plants like *Murraya koeningii* (Rout, 2005) and *Euphorbia nivulia* (Martin et al., 2005). This investigation was aimed to develop rapid micropropagation protocol through direct and indirect o rganogenesis from stem explants of *T.*

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Abbreviations: BAP, 6-Benzylaminopurine; **IBA,** indole 3-butyric acid; **Kn,** kinetin; **NAA,** α- naphthalene acetic acid.

indica to meet the growing demands of pharmaceutical industry.

MATERIALS AND METHODS

Explants were collected from mature and healthy field grown plants of *T. indica* grown in Thapar University campus, Patiala and were surface sterilized using different sterilizing agents. Stem explants were washed under running tap water for 30 min followed by washing in 0.1% (v/v) liquid detergent (Teepol) for 5 min and was subsequently washed with tap water. The explants were then treated with bevistin (0.1% w/v) for 10 to 12 min, followed by repeated washing with water. Finally, the explants were surface sterilized with 0.1% solution of mercuric chloride for 2 to 4 min followed by 3 to 4 washing in sterile distilled water. Fresh cuts were given to the segments after sterilization to remove dead portions and explants of 5 to 6 mm in length were cut.

The excised explants were cultured on MS (Murashige and Skoog, 1962) medium augmented with various plant growth regulators, 2% sucrose and 0.8 to 1% agar with pH adjusted to 5.8 before the addition of agar. Culture tubes containing medium were autoclaved at 121°C for 15 lbs/inch^2 for 15 min. All the inoculated cultures were incubated in growth room in controlled conditions at a temperature of 25 ± 2°C, 16 h light/8 h dark photoperiod and continuous illumination was provided by cool white fluorescent tubes at 3500 lux.

Organogenesis from stem explants

De novo adventitious shoot formation was observed when stem explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of 6-benzylaminopurine (BAP) (4.4 to 17.6 µM). Formation of nodular meristemoids was initiated from the cut ends and they covered the entire surface of the explant. Eventually, these developed into green leafy shoots on further subculturing on the same medium. Formation of callus was obtained on different concentrations of α-naphthalene acetic acid (NAA) (7.35 to 29.4 µM) and Kn (4.65 to 18.6 µM) supplemented media. Differentiation of shoots occurred when callus was transferred to BAP (4.4 to 17.6 µM) supplemented media. Initially, fewer shoots were formed but the number increased further on subsequent subculturing. Rhizogenesis was also observed in callus when cultured on different concentrations of indole 3-butyric acid (IBA) (4.92 to 19.68 µM). Microscopic analysis of callus was carried out to reveal the nature and presence of different types of cells. Microshoots thus formed were rooted on IBA and half strength basal MS medium for root initiation. In order to acclimatize, rooted microshoots were removed from the medium using forceps to avoid any mechanical damage to the plantlets and were transferred to culture bottles containing moist cotton and kept in growth room. Plantlets were further transferred to polybags containing sterile potting mixture of soil : vermicompost (1:1) covered with perforated plastic bags and were again kept inside the growth room. The hardened plantlets in plastic bags were then transferred to the green house before transferring them to full sunlight outdoor.

RESULTS

De novo adventitious shoot formation

Stem explants were cultured on variously supplemented MS medium for *de novo* adventitious shoot formation directly from the explant. Differentiation of nodular meristemoids from cut ends was observed within 7 to 8 days of culturing on 8.8 µM BAP supplemented medium (Figure 1a). Eventually, these nodular meristemoids differentiated into green leafy shoots after 6 to 8 weeks of culturing in about 90% of the cultures (Figure 1b). Initially, fewer shoots were formed, but the number further increased to 40 to 50 per flask on subsequent subculturing (Figure 1c). Repeated subculturing accelerated the formation and proliferation of shoots in large numbers without any further decline.

Callus induction and differentiation

Stem explants were inoculated on MS medium with different combinations of auxins and cytokinins for induction of callus. However, the best callus growth took place on NAA (29.4 µM) and K (4.65 µM) supplemented media (Figure 1d). Initiation of callus from cut ends was observed after 7 to 8 days of culturing in about 85% of the cultures and within 4 weeks, the entire segment turned into a mass of green solid compact callus (Figure 1e). Callus when subcultured on same medium, proliferated further and showed sustained growth. The stem callus formed was solid and hard and was heterogeneous, comprising cells of different shapes and sizes. Differentiation of shoots was observed when callus was transferred to MS supplemented with BAP (8.8 µM). Initially, 4 to 5 shoots were formed (Figure 1f), but the number increased further on subsequent subculturing. Rhizogenesis from callus was also observed when cultured on MS supplemented with different concentrations of IBA. Roots were formed on IBA (9.84 µM) after 2 weeks of culturing (Figure 1g). Initially, fewer roots were formed, but with further proliferation of callus, more roots appeared randomly in about 80% culture. These roots were long, white or greenish in color.

Xylogenesis

Two to three weeks old stem callus revealed differentiation of tracheids which occurred singly or in groups forming nodules and possessed sclariform thickenings on their walls (Figure 1h).

Rooting of microshoots

Regenerated shoots were carefully rescued from the jars and were inoculated upright in the MS medium with or without hormones. Shoots were inoculated on half strength basal MS medium and MS medium supplemented with different concentrations of IBA for root initiation. Among the various growth regulators tested, the best root initiation however occurred on half strength MS medium where 2 to 3 healthy roots emerged in 90% of

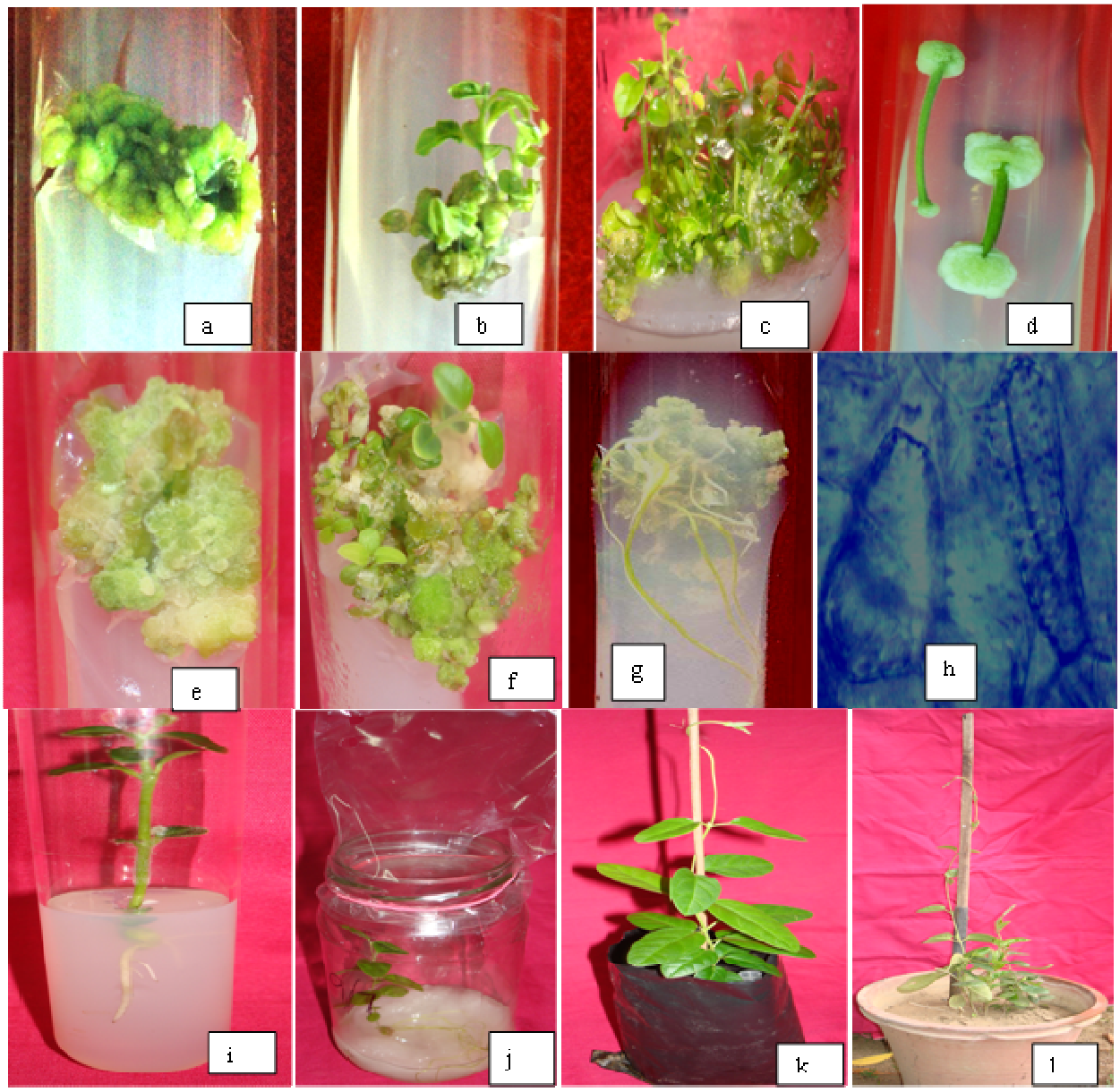

Figure 1. a) Formation of nodular meristemoids on MS medium supplemented with 8.8 µM BAP after 7 to 8 days of culturing; b) differentiation of green leafy shoots from nodular meristemoids on subsequent subculturing; c) further proliferation of shoots (40 to 50 per explant) on same medium; d) formation of callus from leaf explant on NAA (29.4 µM) and K (4.65 µM) supplemented media after 7 to 8 days of culturing; e) formation of green solid hard callus within 4 weeks of culturing; f) differentiation of 4 to 5 shoots from callus when transferred to MS supplemented with BAP (8.8 µM); g) rhizogenesis from stem callus on IBA (9.84 µM) supplemented medium after 2 weeks of culturing; h) section showing tracheids in 2 to 3 weeks old callus having sclariform thickenings on their walls; i) formation of roots on ½ strength MS medium after 7 to 8 days after culturing; j) plantlets formed transferred to moist cotton for acclimatization; k) transfer of plantlets to soil : vermicompost potting mixture; l) an 8 months old well acclimatized plant in the soil.

the cultures after 7 to 8 days of culturing (Figure 1i). The roots were long, thin and bear profuse root hairs.

Acclimatization of rooted microshoots

For acclimatization, plantlets were rescued safely from culture medium, washed under tap water to remove traces of agar sticking to them and were initially transferred to the culture bottles containing moist cotton covered with perforated plastic covers and were kept for a period of 15 days under growth room conditions (Figure 1j). The plantlets were then transferred to plastic cups containing potting mixture of soil : vermicompost (1:1).

The cups were covered with perforated plastic bags to maintain internal humidity and aeration and were kept inside the growth room for another 15 days (Figure 1k). The hardened plantlets in plastics were then transferred to the green house for another 2 weeks before transferring them to full sunlight outdoor. By this time, plants had become sturdy, developed an efficient root system, formed new leaves and became phytosynthetically active. The plants were subsequently transplanted to earthen pots containing same potting mixture and were transferred to open field conditions (Figure 1l). The acclimatized plants showed 90% survival rate with well developed shoot and root systems and large number of secondary and tertiary branches, and all the plants thrived very well in field conditions.

DISCUSSION

A regenerative organogenesis protocol has been developed from stem explants of *T. indica.* The present material has a great organogenic potential as it exhibited a high efficiency of shoot bud formation and plant regeneration from various vegetative parts taken from mature field grown plants. *De novo* adventitious shoot formation was observed on 8.8 µM BAP where formation of 40 to 50 shoots/flask was observed after 6 to 8 weeks of culturing. The result is in accordance with different reports from the literature where cytokinins have been regarded as one of the most important factors affecting the response of shoot proliferation (Lane, 1979; Stolz, 1979; Bhojwani, 1980; Garland and Stolz, 1981). A wide range of cytokinins like kinetin, BAP, 2-iP and zeatin have been employed in shoot proliferation (Bhojwani and Razdan, 1982). Similar results are also reported for direct shoot bud formation from root explants of *T. indica* on MS supplemented with different concentrations of BAP (10.72 to 26.80 µM) (Chaudhuri et al., 2004).

Indirect organogenesis through intervening callus formation has been achieved on NAA (29.4 µM) and K (4.65 µM) supplemented medium. Callus thus formed was hard, solid and heterogeneous in nature. Callus when transferred to BAP supplemented medium resulted in the formation of multiple adventitious shoots. Plant regeneration through indirect shoot organogenesis was achieved from leaf explants by some workers as leaf segments are more regenerative than internodal and petiole explants (Verma et al., 2010). Induction of adventitious shoots from leaf callus was obtained on MS supplemented with Kn (5 µM) (Faisal and Anis, 2003) and on MS with thidiazuron (8 µM) (Thomas and Philip, 2005). The spectrum of induced differentiation from calli was wide and included xylogenesis, rhizogenesis and caulogenesis from calli formed from different explants. Rhizogenesis occurred when callus was transferred to IBA (9.84 µM) supplemented medium. Similar report for root induction from callus was observed in *Aconitum balfourii* when it was cultured on different concentrations of IBA (Pandey et al., 2002). Induction and development of roots at the bases of *in vitro* grown shoots is an essential and indispensable step to establish tissue culture derived plantlets in the soil. Shoots thus formed were excised and subjected to rooting on MS medium supplemented with auxins. Half strength basal MS media was best suited for the induction of roots from microshoots. After the complete plantlet formation, the regenerants were hardened in soil : vermicompost potting mixture with 90% regeneration rate.

In conclusion, an efficient reproducible mass propagation protocol was developed from stem explants of mature field grown plant of *T. indica*, an important threatened plant.

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