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Original Article

IN VITRO PLANT REGENERATION OF *THEVETIA NERIIFOLIA,* JUSS FROM INTERNODE EXPLANTS *VIA* INDIRECT ORGANOGENESIS

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ABSTRACT

Objective: Present study was carried out to develop an efficient *in vitro* protocol for micro propagation of plantlets *via* indirect organogenesis from internode explants of *Thevetia neriifolia*, a medicinal plant with immense biological potentialities.

Methods: For callus induction, internodes from 4 weeks old seedlings were cultured in Murashige and Skoog (MS) medium supplemented with varying concentrations (0.5-2.0mg/L^{-1}) of Auxins (2,4-D, IBA) and Cytokinins (KIN, BA).

Results: Creamy white callus obtained initially was gradually transformed into the light green meristematic callus during subsequent subculturing in most of the combinations. The best organogenic response was achieved with a combination of IBA+BA (0.5+1.0mg/L^{-1}) with 2.722±0.752 shoots per explant. Shoot initiation was induced/enhanced by transferring the non meristematic IBA+KIN calluses to the above IBA+BA medium. However, better response for maximum shoot proliferation (6.091±1.231) was achieved when cytokinin BA (1.0mg/ L^{-1}) was supplied individually, with 100% efficacy. Good roots were produced in IBA (1.0mg/L^{-1}) medium and the rooted plantlets were transferred to paper cups and acclimatized (72%) subsequently in green houses.

Conclusion: This rapid and successful protocol for micro-propagation definitely facilitates to promote the mass cultivation of this potent medicinal plant.

Keywords: Callus, Micropropagation, Organogenesis, Regeneration, *Thevetia neriifolia.*

INTRODUCTION

Thevetia neriifolia, Juss commonly known as 'Yellow oleander' is one of the most important medicinal shrubs in the family Apocynaceae. Plants are distributed in the tropical and subtropical regions of the world, and they are conventionally propagated by seeds. Various plant parts are reported to have diuretic, cardiotonic, emetic, and purgative properties, hence, traditionally they are used to cure cardiac disorders, arthritis, rheumatism, hemorrhoids, acne, constipation, nausea etc. [1-2].

However, the popularity of this plant is particularly due to the presence of therapeutically well indexed cardiac glycosides as the chief secondary metabolite. Since enormous curative potentialities dwell in this plant, the need for conservation and its *in vitro* propagation deserves prime importance. Studies have been conducted earlier in the establishment of callus cultures [3] and plantlet regeneration from young leaves of this plant [4-5]. Similarly, calli and regenerated shoot lets were developed from immature seeds, leaf, stem and roots of *Thevetia sps* growing in Egypt [6]. Development of *in vitro* procedures will help to establish tissues for germ plasm conservation, rapid propagation and for secondary metabolite production to meet the vastly increasing demand of therapeutic and other industries. Therefore, the present work was designed to develop a simple and efficient protocol for the *in vitro* propagation of this plant *via* indirect organogenesis from healthy juvenile internode explants to benefit conservation and regeneration of this important taxon.

MATERIALS AND METHODS

Source of explants

Mature seeds fallen from yellow flowered plants were collected from various natural habitats of Trichur Dist, Kerala. They were raised into seedlings in polyethylene trays in normal surroundings; and young, healthy seedlings of 4-5 weeks old were used as the source of explants.

Nutrient media and inoculation

Whole plant above the first node was excised from the seedling, thoroughly washed under running tap water for 20 minutes and cleansed with detergent Tween 20 followed by fungicide bavistin. After several rinses with distilled water, explants were surface sterilized with 0.1% Mercuric chloride (w/v). All adhering sterilant was removed by repeated washing (4-5 times) with double distilled water. Tender parts below 1.5 cm from the apical shoot were used as internode and leaf explants, whereas stem apex and intervening nodes of the same seedling were used to study their regeneration potential. Explants of suitable sizes from nodes, shoot tips and internodes (0.6-0.8 cm) were made ready under Laminar Air Flow Chamber and inoculated into MS medium [7] fortified with various plant growth regulators, 3% sucrose (w/v) as carbon source and 0.75% agar (w/v) as solidifying agent. The pH of the medium was adjusted to 5.8±0.02 with the help of 0.1N NaOH or 0.1N HCl. About 5 ml medium was poured into suitable culture tubes and sterilized at 121°C for 20 min. Explants from node and stem apex were placed vertically in the medium, whereas those from the leaves and internodes were inoculated horizontally on agar slants. After inoculation, all cultures were maintained at 26±2°C for a photoperiod of 16 h under cool white light and relative humidity. Observations were done daily and recorded weekly; and the data are presented as Mean ± SD.

Plant growth regulators

Response of explants was studied in different concentrations (0.5-2.0mg/L^{-1}) and combinations of auxins 2,4-D (2,4-Dichloro phenoxyacetic acid) and IBA (Indole 3-Butyric Acid), and cytokinins KIN (Kinetin), BA (6-Benzyl Adenine) supplemented with basal MS medium. First subculturing of all proliferated calluses was done in the same medium after 5 weeks of inoculation, followed by a change to another 5-8 weeks in IBA+BA (0.5+1.0mg/L^{-1}) fortified medium. A final transfer of all calluses into BA medium (1.0mg/L^{-1}) was done for shoot elongation and further maturation.

Rooting and acclimatization

Fully matured microshoots were excised from the mother callus and transferred to various concentrations of rooting media (IBA-0.1, 0.5 and 1.0mg/L^{-1}) for root initiation. Regenerated shoots with the well developed rooting system were taken out of culture tubes, washed

carefully to remove the traces of agar, and placed in autoclaved sand: soil mixture (1:2). A minute quantity (0.1%) of activated charcoal was also added to the soil mix. Subsequently, cultures were covered with moistened polyethylene bags to maintain humidity for 15 days during acclimatization.

RESULTS AND DISCUSSION

Explants of various origins (leaves, shoot apex, node and internode segments) were collected from 4 weeks old seedlings. All explants responded quickly to MS medium supplied with various concentrations and combinations of plant growth regulators. Shoot apex having a lively terminal bud initiated its ongoing growth after 6-8 days of inoculation. Similarly, dormant axillary buds became active after 10 days and they showed similar behavior to terminal bud in growth habit. Callus induction became visible from the wounded ends of all explants within 10 days of inoculation and proliferated rapidly into an appreciable amount of creamy white callus. Depending upon concentrations and combinations of growth regulators, the response of explant and nature of callus varied considerably. Appearance of light green colored clumps of meristematic cells on the internode callus surface was considered as a sign of morphogenic response in the shoot initiation process, but there was no sign of such tissues on calluses in vertically placed samples. Hence the study was primarily concentrated on internode explants. Without any growth regulators, the explants failed to initiate cell division in MS medium even after 6 weeks of inoculation.

Effect of 2, 4-D+KIN combinations

Within ten days of inoculation, both cut ends of internode segments initiated callus formation (fig. 1. a), when both hormones were supplied simultaneously. During first subculturing, the proliferated creamy white friable callus developed groups of embryogenic cells on the callus surface as green colored clumps (fig. 1. b-c), when low concentrations of 2,4-D (0.5-1.0mg/L^{-1}) were supplied with KIN (1.0mg/L^{-1}). Similar calli with numerous unorganized structures were reported by Kumar [4] when leaf discs were used as explants. The frequency of these clusters was less when compared to leaf explants (unpublished data), which are the forerunners of somatic embryos. When the concentration of both hormones was increased slightly (2.0mg/L^{-1}), the explant responded slowly and a reduction in callus biomass was noticed and it failed to differentiate into embryogenic cells. Thus, as reported by Sharma and Kumar [8], a lower 2,4-D+KIN combination (<1.0 mg/L^{-1}) stimulated somatic embryo formation. But, higher frequency of callus induction and proliferation was reported in vertically placed nodal segments as compared to inter-nodal segments and shoot apices, on MS medium supplemented with various concentrations of 2,4-D and Kinetin [9]. From this observation, it is understood that the orientation of explant plays a key role in callus production and subsequent morphogenesis even if the same hormonal combinations were provided.

Effect of IBA+KIN combinations

When auxin IBA was supplied with KIN, according to the concentration of growth hormones, morphological responses varied widely (Table1). KIN (1.0mg/L^{-1}) was active with low concentrations of IBA (0.5mg/L^{-1}) and produced more shoot initials (1.333±0.500) during first subculture (fig. 1. d) than the vice versa composition (0.400±0.548). However, an increase in IBA (2.0mg/ L^{-1}) and decrease in KIN (1.0mg/L^{-1}) initiated root formation directly from the internode explant without the involvement of compact callus (fig. 1. e).

These rooted explants strengthened further with good root cap and numerous root hairs upon first subculturing in the same medium, under the influence of IBA. When an equal amount of IBA+KIN (1.0mg/L^{-1}) was supplied, KIN was more active on internode explants and produced shoot primordium during first subculture. But, for nodal and shoot tip explants which possess developing axillary and terminal buds, instead of KIN, IBA showed its efficacy and produced roots from the compact mother callus. The callus induction, shoot initiation and regeneration potential vary with the developmental stages of explants and depend on the concentration and type of growth regulators supplied in the medium [10].

Effect of IBA+BA combinations

Compared to the above observations, this combination also produced good creamy white callus initially at both cut ends of internode segments, which later converted to light green meristematic calli. Lower concentration of IBA (0.5-1.0mg/L^{-1}) and (a-b)

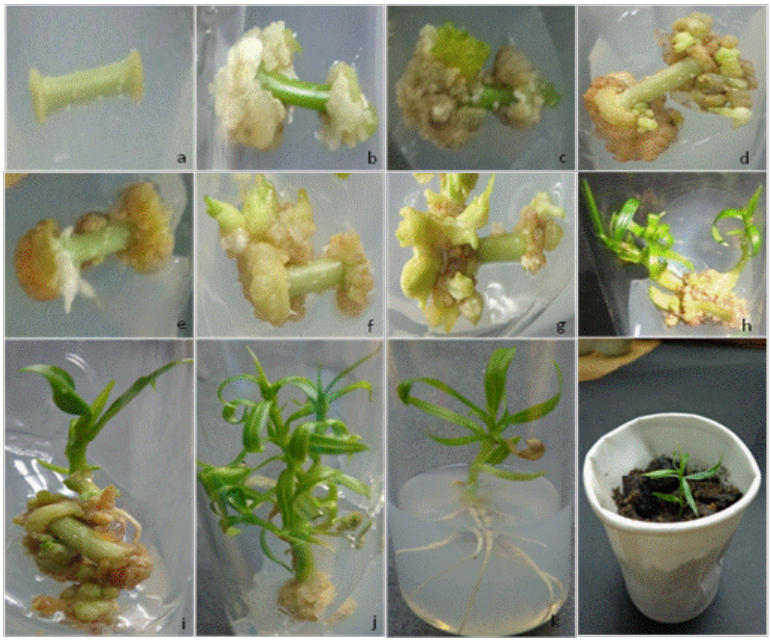

Fig. 1: Micropropagation of plantlets from internode explants of *Thevetia neriifolia* on MS medium fortified with various plant growth regulators.

Internode explant showing callus initiation and proliferation in 2,4-D+KIN (0.5+1.0mg/L^{-1}), c) Embryogenic clumps in same medium, d) Shoot meristemoids in IBA+KIN (0.5+1.0mg/L^{-1}), e) Root initiation in IBA+KIN (2.0+1.0mg/L^{-1}), f) Shoot bud initiation in IBA+BA

(0.5+1.0mg/L^{-1}), g-h) Shoot bud proliferation and regeneration in BA (1.0mg/L^{-1}), i) Organogenesis in IBA+BA (0.5+1.0mg/L^{-1}), j) Direct shootlet regeneration from shoot apex-node cluster with axillary and terminal buds in BA (1.0mg/L^{-1}), k) *In vitro* rooted plant in IBA (1.0mg/L^{-1}) ready for hardening, k) plant let in paper cup during acclimatization.

Slightly elevated level of BA (1.0-2.0mg/ L^{-1}) favored shoot formation (fig. 1. f). However, an optimum combination for organogenesis was observed as IBA+BA (0.5+1.0mg/L^{-1}) for internodes with an average of 2.722±0.752 shoots/explant, in addition to root initials from the same callus (fig. 1. I). Similar combinations were used to induce shoot bud multiplication from *Jatropha curcas* stem and leaf explants [11]. Although an increase in BA concentration initiated the formation of shoot buds, maximum number of shoot bud initiation was recorded when BA (1.0mg/L^{-1}) was used alone in the absence of any auxins (fig. 1. g-h). Here, callus proliferated rapidly and vigorously and an average of 6.091±1.231 shoots per explant were developed. The present results support the findings of Haq *et al*, [12] that BA (1.0 mg/ L^{-1}) alone was proved to be very effective with an average number of shoots (7.8±1.09) from nodal explants of *Vinca rosea*, a close relative of *Thevetia.* However, the addition of auxin at low concentration significantly enhanced shoot proliferation in *Rauwolfia serpentine* and was reported to be very important for the mass scale propagation [13]. Among the node, internode and shoot tip explants, the best response for shootlet regeneration was achieved from internode segments in MS medium supplemented with BA (1.0mg/L^{-1}) independently.

Table 1: Effect of various plant growth regulators on the internode segments of *Thevetia neriifolia*

Plant growth regulators		Callus morphology after 8 weeks of culture in same medium	no. of shoots *
2,4-D + KIN	0.0+1.0	Creamy white, granular	0.00±0.00
	0.5+1.0	Creamy white, friable	0.00±0.00
	1.0+0.5	Creamy white, friable	0.00±0.00
	1.0+1.0	Creamy white, friable	0.00±0.00
	1.0+2.0	Creamy white, friable	0.00±0.00
	2.0+1.0	Creamy white, friable	0.00±0.00
IBA + KIN	0.5+1.0	Creamy light green, granular	1.333±0.500
	1.0+0.5	Creamy light green, granular	0.400±0.548
	1.0+1.0	Creamy light brown, compact	0.667±0.516
	1.0+2.0	Creamy light green, granular	0.857±0.378
	2.0+1.0	Creamy light brown, compact	0.00±0.00
IBA + BA	0.0+1.0	Light greenish creamy, granular	6.091±1.231
	0.5+1.0	Light greenish creamy, granular	2.722±0.752
	1.0+0.5	Light greenish creamy, granular	1.111±0.333
	1.0+1.0	Pale green, compact	1.286±0.756
	1.0+2.0	Light green, less compact	1.400±0.516
	2.0+1.0	Light brown, compact	0.00±0.00

*no. of shoot initials or buds/explant after II subculture in IBA+BA (0.5+1.0mg/L^{-1}) (12weeks)

Subculturing: The organogenic capabilities were assessed by subcultuing the callus in the same medium followed by a combination of IBA+BA and BA alone. Since a good response for shoot differentiation was observed at lower concentrations of IBA+BA (0.5+1.0mg/L^{-1}), further subculturing from IBA+KIN and IBA+BA combinations for shoot initiation were done in this medium. For further maturation and regeneration, they were given a final transfer to BA (1.0mg/L^{-1}) medium. The initiated shoot buds in IBA+KIN combinations can be successfully regenerated using the above combination. When the explants were removed from IBA+KIN medium to BA rich medium, an increase in shoot bud formation and regeneration was observed, reveals the superiority of BA over KIN in caulogenesis. In general, the frequency of shoot regeneration was lower in IBA+KIN callus than IBA+BA callus (fig. 2).

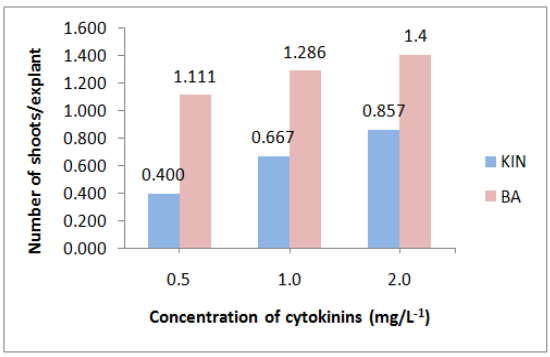

Fig. 2: Effect of different concentrations (0.5-2.0mg/L^{-1}) of cytokinins (KIN and BA) on shootlet initiation of *T. neriifolia* internode explants when IBA remain constant (1.0mg/L^{-1}).

Indirect organogenesis refers to the formation of shoots indirectly from an intermediary callus that first develops on explants. Plant growth regulators are the most important inducing signal for organogenesis. Type of plant growth regulators and their interaction play an important role in dedifferentiation, induction and development of shoots or roots [14].

An indirect shoot regeneration system helps to identify or induce somaclonal variability and to develop transgenic plants following genetic transformation of plant cell [15]. It is generally believed that cytokinins are beneficial for shoot formation, while auxins stimulate callus formation, root induction and somatic embryogenesis.

The ratio of cytokinins to auxins is also critical in determining shoot versus root formation as revealed from the experiment. In conclusion, shoots were successively regenerated after 2-3 subcultures in the presence of BA. Among the two cytokinins studied. BA was more efficient than KIN in promoting shoot formation.

Rooting and acclimatization

Maximum number of roots were produced in normal strength MS medium fortified with 1.0mg/L^{-1} IBA than lower concentrations (0.1-0.5mg/L^{-1}) and half strength MS medium. Auxin NAA (1.0mg/L^{-1}) also gave favorable results in rootlets formation in number and length of roots in the same plant [16].

Healthy matured rooted plantlets (fig. 1. k) were transferred into paper cups containing autoclaved sand soil mixture. After two weeks, acclimatized plantlets were transferred to glass house conditions for further development (fig. 1. l). Around 72% of the rooted plantlets were acclimatized in the above conditions and they did not exhibit any morphological variations.

CONCLUSION

To minimize the impact of over exploitation by therapeutic industries, an efficient protocol has been developed for the vast multiplication of yellow oleander plants *via* indirect organogenesis. An average number of 2-3 plantlets can be created at a lower concentration of IBA+BA (0.5+1.0mg/L^{-1}) and shootlet multiplication can be further enhanced up to 6-7 per internode explant using cytokinin BA alone (1.0mg/L^{-1}). This rapid and effective method of micropropagation definitely helps to promote the mass cultivation of this valuable medicinal plant.

CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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