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# SOMATIC EMBRYOGENESIS IN *NARDOSTACHYS JATAMANSI* DC—AN ENDANGERED MEDICINAL HERB OF HIMALAYAS*

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*Callus cultures of* Nardostachys jatamansi *DC (Family Valerianaceae) were established from petiole explants on a MS basal medium, supplemented with 3.0 mg/l α-naphathaleneacetic acid (NAA) and 0.25 mg/s kinetin (kn). Somatic embryogenesis was evident when the callus was sequentially subcultured through a series of media with gradually decreasing levels of auxin (from 3.0 mg/l down to 0.25 mg/l NAA) and a concomittant but gradual increase in the levels of cytokinin (from 0.25 mg/l to 2.0 mg/l in combination with 2.0 mg/l level of kn over a period of seven months. Fully developed somatic embryos were obtained when the concentration of NAA was reduced upto 0.25 mg/l in combination with 2.0 mg/l level of kn. The formation of plantlets* via *somatic embryogenesis in* Nardostachys jatamansi *is important in context with the conservation of this endangered medicinal and aromatic herb of the wet and high Himalayan ranges.*

*Nardostachys jatamansi* DC., commonly known as *Jatamansi* or the Indian spikenard, is a primitive member of the family Valerianceae. The plant has a restricted distribution from Garwhal Himalayas extending to Bhutan and Sikkim in the eastern Himalayas. It occurs on rocky edges and crevices at high altitudes ranges from 3,300m to 5,700m (Weberling, 1975). *Jatamansi* is a perennial rhizomatous herb with simple radical leaves and a compound dichasial inflorescene. The pentamerous sympetalous flowers give rise to indehiscent fruits that contain non-endospermous seeds. *Nardostachys* finds its use in medicine and perfumery, and place in Greek literature and holy Bible (Shah, 1982). The Indian

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Ayurvedic system of medicine recommends the use of *jatamansi* roots in various psychiatric disorders such as epilepsy and hysteria (Arora and Arora, 1963). The tranquilizing and anti-arrhythmic properties of the plant extract are attributed to the presence of a saturated bicyclic sesquiterpene ketone - jatamansone (Houghton, 1988) while the perfumery value of the oil is due to the presence of volatile iso-valeric esters and jatamanshic acid.

The widespread use of *jatamansi* in traditional medicines, perfumes, and incenses (Shah, 1982) has resulted in the indiscriminate exploitation of its wild populations. Very little efforts were made for its systematic cultivation. The plant is enlisted as an endangered species in Indian Plant Red Data Book (Jain and Sastry, 1984). Need for systematic approach towards conserving the natural populations of *jatamansi* and devising rapid propagation procedures for replenishment is, therefore, evident. Under the aegies of an All India co-ordinated project on the consevation of some endangered plant species through tissue culture (sponsored by the Department of Environment, New Delhi), the present work was undertaken to develop rapid *in vitro* propagation procedure in *jatamansi*. This communication enumerates the experimental protocol for the high frequency induction of somatic embryogenesis in petiole-derived calli of *N. jatamansi*. This is the first report on somatic embryogeny in a genus of the family Valerianceae and this stresses upon the efficacy of sequential and gradual alterations in the levels of exogenously applied growth regulators for achieving morphogenesis in otherwise recalcitrant systems.

## MATERIALS AND METHODS

Plants of *N. jatamansi* DC. (2n=26) were collected from Milam locality of Pithoragarh, Uttar Pradesh, and were maintained in a growth chamber (Percival Inc. USA, at 22°C, 70 per cent relative humidity-RH and 14 hr photoperiod 4000 lux). Petiole explants (2-3mm long) were excised and surface sterilized for 10 min. in a 2 per cent (v/v) solution of sodium hypochlorite. The basal medium (BM) consisted of Murahsige and Skoog's (1962) medium salts, 3 per cent sucrose, 100 mg/l myo-inositol and 0.7 per cent agar. For callus initialiation and subsequent morphogenetic studies, various auxins namely IAA (Indole-3-acetic acid), IBA (Indole-3-butryic acid), NAA (α-napthaleneacetic acid), or 2, 4-D (2,4-dichlorophenoxyacetic acid) and cytokinins such as kn (kinetin), BAP (6-benzyladenine), 2-iP (2-isopentyladenine) and Z (zeatin) were incorporated into the basal medium in concentration, combination and sequence as detailed in the results. The pH medium was adjusted to 5.8±0.02 before autoclaving at 15 psi and 121°C for 15-20 minutes. Cultures were

incubated at 25 ± 2°C and 14/10 hr light/dark period (2000 lux) using cool, white, fluorescent lights.

**Sequential subculturing**

Experiments on sequential subculturing of callus on gradually decreasing auxin (NAA) and concomittantly increasing cytokinin levels were done using 18 hormonal combinations. These include 3.0, 2.0, 1.0, 0.5, 0.25 or 0.00 mg/l NAA with 0.25, 0.50. 1.0 or 2.0 mg/l of either kn (Medium No. 1-6) or BAP (Medium No.7-12) or 2-iP (Medium No. 13-18), respectively. At the end of each culture with the passage of 4-6 weeks duration on a particular medium, the callus was divided into three equal parts. One part was recultured on to the fresh medium of similar composition, second was placed on a hormone free BM and the third part was carried forward to the next medium in the sequence, i.e., from medium No. 1 (MS+ 3.0 mg/l NAA+ 0.25 mg/l kn) to medium No. 2 (MS+ 2.0 mg/l NAA+ 0.25 mg/l kn) and so on. In order to assess the effect on callus age on morphogenesis, the callus from the stock cultures was also directly transferred to the above mentioned 18 media at the time of each sequential subculture.

**Histological studies**

For histological observations, tissues were fixed in FAA (formalene: acetic acid : ethyl alcohol : : 95:5:5) and dehydrated through a graded tertiary butyl alcohol/xylene series. Observations were made on 10 µm thick serial sections which were double stained with saffranin and aniline blue. For Scanning Electron Microscopy (SEM), the embryogenic callus tissue was fixed in 4 per cent (v/v) glutaraldehyde, followed by 1 hr treatment with 1 per cent (w/v) osmimum tetraoxide in 0.1M phosphate buffer. Samples were then dehydrated in a graded ethanol series, critical point dried and gold - coated before viewing under a scanning electron microscope (JEOL 36, Japan)

## RESULTS AND DISCUSSION

For callus initiation, the petiole explants were cultured on BM supplemented with 1.0 - 4.0 mg/l NAA, IAA, IBA or 2,4-D with or without 0.25 - 1.0 mg/l kn (Table 1). From amongst the four auxins, NAA was most effective in initiating callusing in *N. jatamansi*. While IAA and IBA could induce only a limited callus response at 4.0 mg/l level, supplementation of 2, 4-D in BM resulted in early browning and death of the explants. The optimum callusing occurred in medium having 3.0 mg/l of NAA in combination with 0.25 mg/l of kn. Calli obtained on medium having less than 3.0 mg/l NAA showed a tendency to develop small roots within 2 weeks of its initiation, while levels higher than 3.0 mg/l resulted in browning and senescense of the freshly induced callus. Presence of kn in the auxin-containing media, though not

**Table 1. Efficacy of various auxins with or without Kn for inducing callus response from petiole explants in *N. jatamansi***

| Kn (mg/1) | Auxin (mg/1) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IAA | | | | | IBA | | | | NAA | | | | 2, 4-D | | | | |
| | 0 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | |
| 0.0 | — | — | — | — | $C^{+}$ | — | — | — | $C^{+}$ | $C^{+}$ | $C^{++}$ $R^{+}$ | $C^{+++}$ | $C^{+++}$ B | B | B | B | B | |
| 0.25 | — | — | — | — | $C^{+}$ | — | — | — | C | $C^{++}$ | $C^{++}$ $R^{+}$ | $C^{++++}$ | $C^{+++}$ B | B | B | B | B | |
| 0.50 | — | — | — | — | — | — | — | — | — | $C^{+}$ $R^{+}$ | $C^{++}$ $R^{+}$ | $C^{+++}$ | $C^{+++}$ B | $C^{+}$ | B | B | B | |
| 1.00 | — | — | — | — | — | — | — | — | — | $C^{+}$ | $C^{+}$ | $C^{++}$ | $C^{++}$ B | $C^{++}$ | $C^{+}$ | B | B | |

(—) No visible response; (C) Callusing; (R) Rooting from callus; (B) Browning and death; (+) Increasing number of '+' sign denotes the intensity of response and rate of subsequent growth

essential for initiating a callus response, improved upon the proliferation of the induced callus. Hence, a callus stock was developed and maintained through repeated subcultures on BM + 3.0 mg/l NAA + 0.25 mg/l kn. From third subculture onwards, callus tissue from stock cultures was used for organogenetic studies. For this, various single and double combinations of IAA, IBA or NAA (0.20-1.0 mg/l) with kn, BAP, Z or 2-iP (0.5-2.0 mg/l) were tested and the results are summarised in Fig. 1. Three distinct types of response were visible on these 140 medium combinations, i.e., continued callus proliferation only, callus growth was accompanied with differentiation of numerous small roots or the development of dark green nodular zones on the callus surface. The first two types of responses were prevalent in media having IAA, ABA or NAA alone or high levels of these auxin (>0.5 mg/l) with low level of kn, BAP, Z or 2-iP (< 1.0 mg/l). The appearance of green nodules was envinced by the presence of higher levels of these cytokinins (>1.0 mg/l) in all the auxin-containing medium. Histological examination of these green nodules revealed the presence of meristematic zones consisting of densely cytoplasmic cells with prominent nucleus and nucleolus. These zones were interspred with well-organised tracheids. The meristematic zones, however, remained quiscent and did not differentiate further into

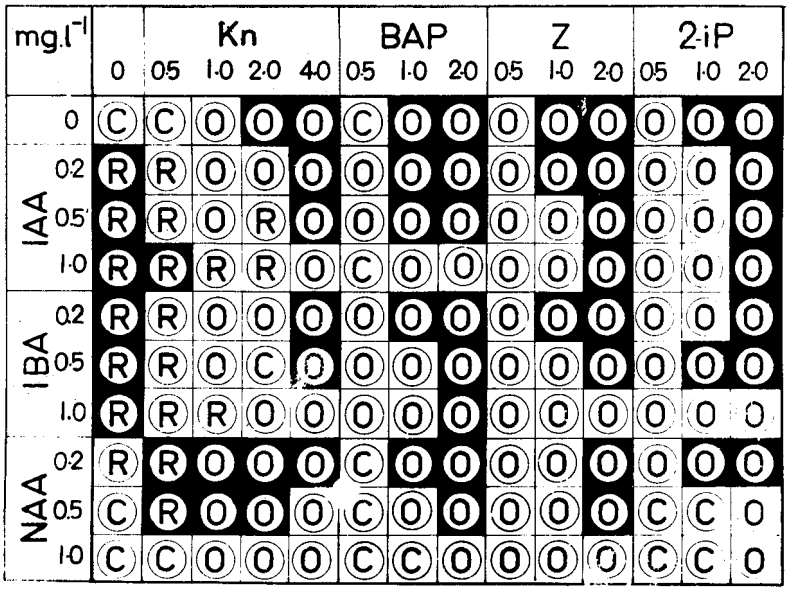


Fig. 1. Morphogenetic responses of callus culture of *N. jatamansi* after 6 wks of growth on BM supplemented with double combinations of various auxins and cytokinins (C-callus proliferation; R-root formation; O-production of dark green nodules; -moderate response; - extensive response).

organised structures upon repeated subculturing onto the fresh medium of similar composition as the one on which these nodules were induced. Certain other manipulations in the medium like removal of growth regulators, lowering of nutrient strength of the BM to half and one fourth, and addition of 10-20 per cent (v/v) cococut milk, proved ineffective in inducing any organised development from these nodules.

Experiments were then initiated to assess the applicability of sequential culturing of calli through a graded series of auxin/cytokinin ratio for inducing organogenesis in *N. jatamansi*. Such an approach was based upon for the inferences of the past studies (Steward *et al.*, 1967; Steward and Mapes, 1971; Krikorian and Kann, 1981; Srinivasan and Mullins, 1980) in *Asparagus, Hemerocallis* and *Vitis* spp., respectively. These workers emphasised the significance of balanced synergistic use of planned and sequential combination of auxin and cytokinin to foster organogenesis in otherwise recalcitrant cell cultures that exhibit arrest of growth at the early stages of differentiation, a situation very similar to that encountered in *N. jatamansi*. Hence, a graded series of decreasing NAA (3.0, 2.0, 1.0, 0.5, 0.25 and 0 mg/l) in combination with increasing cytokinin levels (0.25, 1.0, and 2.0 mg/l kn, BAP or 2-iP) were tried for sequential transfer of the callus cultures. Amongst these, only NAA/kn series proved effective in bringing about the somatic embryogenesis. The stock callus continued to proliferate on media 1 and 2, i.e., BM + 3.0 mg/l NAA = 0.25 mg/l kn and BM + 2.0 mg/l NAA + 0/25 mg/l kn (Fig. 2a, B). When calli were transferred from medium 2 to medium 3 i.e., BM + 1.0 mg/l NAA + 0.5 mg/l kn, dark green sectors became evident within 2 weeks of transfer (Fig. 1C). These green sectors organized into compact nodules on medium 4, i.e., BM + 0.5 mg/l NAA + 1.0 mg/l kn (Fig. 2D,d). On medium 5, i.e.,BM + 0.25 mg/l NAA + 1.5 mg/l kn, numerous somatic embryos with distant radicular and plumular axes were produced (Fig. 2E,e). Further lowering of auxin/cytokinin ratio in the medium 6, i.e., BM + 0.25 mg/l NAA + 2.0 mg/l kn, allowed the embryo to plantlet conversion to take place with rapid development of cotyledonary leaves and new shoots (Fig. 1F). The plantlet conversion frequency ranged from 10-15 per cent on this medium. The root growth on this medium was slow. The radicular axis became flattened and recallused. Numerous secondary embryoids also originated from this freshly induced callus. The frequency of *in vivo* establishment of somatic embryo-derived plantlets under glass house conditions was very low (3-5%). This was possibly due to abnormal root development as mentioned above.

The calli that were recultured or maintained throughout the entire duration of the experiment on a particular medium in the sequence

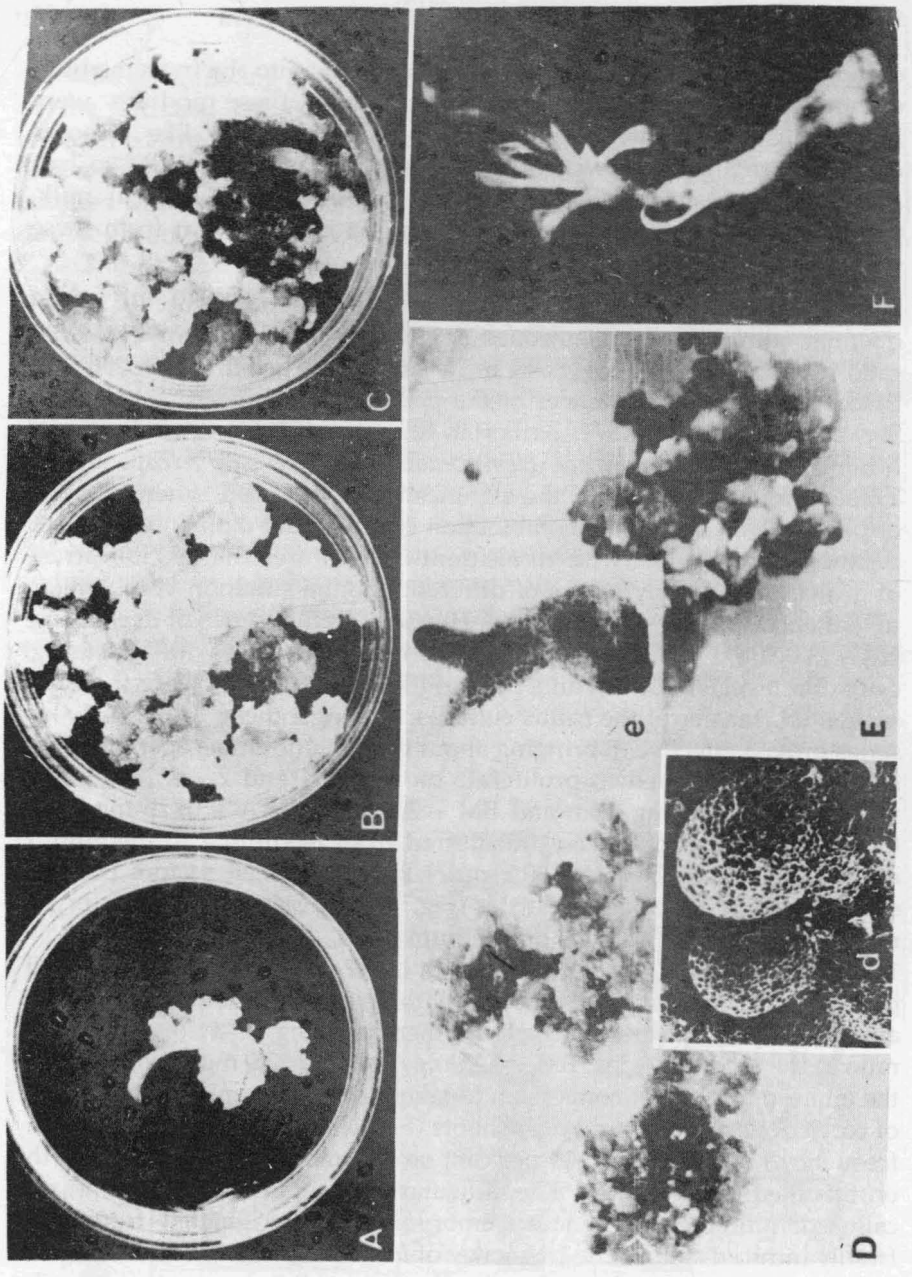


Fig. 2. Induction of somatic embryogenesis and plantlet development upon sequential subculturing of callus of N. jatamansi through a graded series of increasing NAA and concommitantly increasing levels of Kn in BM

A, Callus initiation on BM+3.0 mg/l NAA+0.25 mg/l Kn; B, Rapid callus proliferation on BM+2.0 mg/l NAA+0.25 mg/l Kn; C, Appearance of green compact regions on the callus on BM+1.0 mg/l NAA+0.5 mg/l Kn; D-d, Differentiation of dark green nodules on BM+0.5 mg/l NAA+1.0 mg/l Kn; E-e, Formation of complete embryoids on BM+0.25 mg/l NAA+1.5 mg/l Kn; F, Plantlet development on BM+0.25 mg/l NAA+2.0 mg/l Kn.

retained their respective stage of differentiation and did not grow into the next stage. Further, direct transfer of stock calllus to Medium 4, 5, or 6 at the time of each sequential subculture also failed to evoke an embryogenic response, indicating thereby the embryogenesis obtained *via* sequential culture was not a function of callus age. Substitution of kn by BAP or 2-iP as a cytokinin source in the medium (Medium Nos. 7-18), at concentrations similar to that employed in NAA/kn series, did not allow differentiation to proceed beyond the green nodule stage.

Somatic embryogenesis is a plastic response of cultured cells, controlled by numerous cultural and environmental variables (Ammirato, 1983, 1987; Ammirato and Steward, 1971; Tran Thann Van, 1981). Among others, the role of a proper balance between nature and concentration of auxin and cytokinin at different stages of somatic embryo development, i.e., initiation, maturation and hardening, has been frequently documented in a large number of plant species (Evans *et al.*, 1981; Rangaswamy, 1986). While auxins like 2,4-D, IAA and NAA have been shown to influence more directly the initial proliferation of embryogenically competent and committed tissue (Halperin, 1970; Fujimura and Komamine, 1980) and establishment of bipolar organisation (Ammirato and Steward, 1971; Christianson, 1987), cytokinins such as kn, BAP, Z and 2-iP are more critical requirements of embryo maturation and plantlet conversion phases (Kavathekar ***et al.***, 1978; Krikorian and Kann, 1981). The present study substantiates the above inferences and also emphasises upon the importance of sequential change of hormonal regimes in the medium for the complete development of somatic embryos in *N. jatamansi*. It has been earlier enunciated that as in the case of sexual embryogenesis *in vivo* gradual shifts in nutritional/ hormonal requirements also characterize the various developmental stages of somatic embryogenesis *in vitro*, (Steward *et al.*, 1967). Such gradual shifts in exogenously harmonal concentrations have proved successful in the complete differentiation of somatic embryos in many recalcitrant systems like *Panax ginseng* (Chang and Hsing, 1980) and *Hemerocallis* (Krikorian and Kann, 1981). The present study is of consequence for the rapid propagation of *N. jatamansi*. Efforts are now underway to enhance the embryo to plantlet conversion frequency with concomittant suppression of recallusing of the regenerated roots for better establishment of the plants in the soil.

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