MASSIVE *IN VITRO* CLONING OF SANDALWOOD (*SANTALUM ALBUM* LINN.) USING CULURED NODAL SEGMENTS

Short Title: Regeneration from nodal segments in Sandalwood

ABSTRACT

Nodal segments of sandalwood were cultured on MS medium with diverse fortifications to search out higher *in vitro* response leading to plantlet regeneration *via* (somatic embryogenesis and/or organogenesis) was persuaded significantly. Medium combination MSTd.5N (MS + 1.0 mgl^{-1}TDZ+ 0.5mgl^{-1}NAA) induced direct somatic embryogenesis. Higher frequency of somatic embryo per explants was observed with media combination, MSB.5N/MS2B.5N (MS + 1.0 mgl^{-1}BA+ 0.5mgl^{-1}NAA/MS+2.0 mgl^{-1}BA+ 0.5mgl^{-1}NAA). Nutrient media combination MS4Kn.5N (MS+ 4.0 mgl^{-1}Kn+ 0.5mgl^{-1}NAA) resulted higher plantlet regeneration *via* direct organogenesis was recovered on regeneration medium MSTd.5N (MS + 1.0 mgl^{-1}TDZ+ 0.5mgl^{-1}NAA) while plantlet regeneration *via* indirect organogenesis was attained in higher ratio on regeneration medium MSTd.5GA.5N (MS +1.0 mgl^{-1} TDZ+1.0 $mgl^{-1}GA_3$+0.5 NAA). The plantlets were transferred to pots and hardened in Environmental Growth Cabinet and Net House during preliminary weaning period and transferred to field successfully. Morphologically normal plants were recovered.

Keywords: *Santalum album*, nodal segment culture, organogenesis, somatic embryogenesis, plantlet regeneration.

Abbreviations: MS-Murashige and Skoog medium; BA-6-benzylaminopurine; TDZ-Thidizuron; Kn-Kinetin; NAA-α-Naphthalene acetic acid; 2, 4-D- 2, 4-dichlorophenoxyacetic acid; 2, 4, 5-T - 2, 4, 5- trichlorophenoxyacetic acid; IBA-Indole-3-butyric acid; GA_3: Gibberelic acid.

INTRODUCTION

Sandalwoods are medium-sized hemiparasitic trees and notable members are Indian sandalwood (*Santalum album* L.) and Australion sandalwood (*Santalums picatum*). Indian sandalwood belongs to the family santalaceae which is one of the important tree species of tropical forests because it produces essential oil in the heartwood which is used extensively in the incense and perfumery industry (Loneragan, 1990; Rao and Bapat, 1995). Sandalwood oil is one of the oldest known perfume materials with 4000 year of uninterrupted use. Major constituents of sandalwood oil are α- santalol (60 %) ß- santalol (30%), α- and ß - santalene. The world famous East Indian Sandalwood oil is extracted from the strongly scented heartwood of this tree. Sandalwood oil is used primarily in perfumery because of its outstanding fixative properties. It is used in preparing all types of perfume compositions especially Indian attars like Hina, Gulab, Kewda and Jasmine in which the natural essential oils from distillate of floral distillation is absorbed in sandalwood oil. With neem oil, it is used as contraceptive. It is used for healing wounds and blisters caused by the smallpox vaccination. Besides, it is used as cardiotonic, diuretic, moisturizer, astringent, antifugal, antimicrobial and coolent. Sandalwood is also one of the finest woods for carving. Wood is smooth with uniform fibers. Saw dust from heartwood is mostly used in incense for scenting cloths and cupboards.

Production of sandalwood has fallen sharply over the past decades due to the escalating of illegitimate sandalwood processing units. Conventional breeding of sandalwood for introgression of new genetic information can be an expensive and difficult task because of its long generation time, sexual incompatibility and heterozygous nature (Rugkhla, 1997). Moreover a major threat to sandalwood trees is the spike disease which has a devastating effect and very often completely eliminates the plantation. Efforts to control and eliminate the disease have been unsuccessful. As Such, it is, imperative to develop alternative techniques for rapid and large-scale multiplication of the species. Investigations were undertaken on sandalwood with the main objective of developing techniques for clonal multiplication of the elite species which are also disease free as such trees are known to exist in sandalwood plantations. *In vitro* and particularly somatic embryogenesis, technology has been used for quite some time in sandal wood for the regeneration of plants. Tissue culture techniques can be used to encounter difficulties of conventional propagation methods by microcloning of elite lines.

Considerable work have been carried out in sandalwood by many researchers throughout the world using diverse explants such as embryo (Rangaswamy and Rao, 1963; Tripathi *et al*.,

2017), hypocotyls (Bapat and Rao, 1979; Lakshmi Sita *et al.,* 1979; Rao and Bapat, 1992; Tripathi *et al*., 2017), shoot tip (Lakshmi Sita, 1980; Lakshmi Sita and Raghava Ram, 1995), nodal segment (Bapat and Rao, 1979; Lakshmi Sita *et al.,* 1979; Lakshmi Sita,1980; Rao and Bapat, 1992; Rao and Bapat,1995; Rugkhla and Jones, 1998;Sarangi *et al.,* 2000; Sanghamitra and Chandni, 2010), leaf disc (Mujib,2005; Bele *et al*., 2012), seedling (Chaturani *et al.,* 2006), endosperm (Bapat and Rao, 1979; Lakshmi Sita *et al.,* 1979; Rao and Bapat, 1992),cotyledons (Tripathi *et al*., 2017), protoplast (Smith and McCown, 1983; Bapat *et al*., 1985; Russell and McCown, 1986; Rughkla, 1997) and cell suspension cultures (Bapat *et al*., 1990; Dey, 2001) with varying degree of success. Keeping in view the current status present attempt was made to compute the optimum quantity of plant growth regulators to be added in culture medium and other physical factors exhibiting higher *in vitro* morphogenesis with 'elite' lines of M.P. and adjoining areas by means of nodal segment culture because it is available throughout the yearand could be used as explant source.

MATERIALS AND METHODS

'Elite' cultivar of sandalwood was selected for the present study using nodal segment as an "explants" Experimental materials were collected from 5-10 years old plants, planted at Bahadri Farm, KNK, College of Horticulture, Mandsaur M. P. India. To begin with a preliminary experiment, two different fortifications of basal media *viz*: MS (Murashige and Skoog, 1962) and WP (Llyod and McCown, 1980) were experimented to perceive better *in vitro* response. During the beginning period MS basal medium was found more amenable than WP medium (data not presented),therefore, for subsequently experimentations basal MS medium was employed. To begin with a preliminary experiment in respect to plant growth regulators, nodal segments were inoculated on MS media fortified with two different auxins, *namely*: 2,4-D and NAA alone as well as three diverse cytokinins *viz*: BAP, kinetin and TDZ as sole in varying concentrations to find out better *in vitro* response. During preliminary experiments, it was scrutinized that an auxin or a cytokinin alone is not adequate for inducing morphogenesis in higher frequencies (data not presented). Accordingly, for concluding experiment basal MS medium was amended with different concentrations of BAP, TDZ and Kn in combination with NAA and 2,4-D in varying concentrations. Apart from MS basal macro and micro salts, vitamins, all initial culture media were supplemented with 30.0 gl^{-1} sucrose and the final volume was made to 1000 ml and pH was

adjusted to 5.8 ± 0.1 with 1N KOH solution. After adjusting the pH, agar powder @ 7.5 g l^{-1} was added to the media as a semi-solidifying agent. Warm culture media, still in liquid state were poured into baby food bottles (50-60 ml / bottle) followed by autoclaving at 121^{o}C under 15 psi pressure for 20-25 min. Readymade basal media, plant growth regulators and other ingredients were procured from Hi-media Laboratories, Mumbai, India.

The indeterminate shoots were excised and collected in distilled water from orchard planted plants. Top 10 to 20 nodal segments were trimmed to 0.5-1.0 centimeter length and washed under running tap water for 30 min to remove adhering dirt particles. Then nodal segments were placed into double distilled water containing 2% Tween 20 (v/v) for 30 min to remove the adhering fine particles. The cleaned segments were then treated with 70% (v/v) ethanol for 2 min followed by treatment with different concentrations of Bavistin® (BASF, Germany) and aqueous solution of two different surface sterilizing agents *i.e.* $HgCl_2$ and Ca $(OCl)_2$ in different concentrations and combinations for diverse durations with initial vacuum of 100 psi (Table 1). Finally, the segments were subjected to treatment with 4-5 rinsing with sterile double distilled water. In cultured baby food bottles, one piece of nodal segments were plated and sealed with Parafilm® and incubated under complete darkness at 25±2°C for one week. Later *in vitro* cultured explants were subjected to photoperiod regime of 16 h light and 8 h dark at an intensity of 2000-lux luminance provided by PAR lamps. For histological studies, somatic embryos of different stages and periods of culture showing morphogenesis were fixed in FAA and processed in alcohol-xylol series. Sections were cut at 10 μm and stained with safaranin-fast green.

After 4-5 weeks of initial culturing, somatic embryoids and calli were transferred to MS regeneration medium fortified with different concentrations and combinations of BAP, TDZ, Kn, NAA and GA_3, 20.0gl^{-1} sucrose and 7.5gl^{-1} agar. However, in cases where organ formed directly on explants surface were sub-cultured on initial medium for regeneration. Cultures were kept at 25±2^{o}C for 12 hr photoperiod. When root formation was not attained on regeneration medium, plantlets were subsequently transferred to MS rooting medium supplemented with different concentrations of IBA, NAA, BA, Kn and GA_3 alone as well as in combinations, 15.0 gl^{-1} sucrose and 7.5 gl^{-1} agar. For regeneration and rooting, reduced level of sucrose was used on the basis of work conducted by various scientists as well as preliminary experiments of this laboratory.

Plants de-flasked from cultures and thoroughly washed with running tap water to remove the adhering agar and planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed in Environmental Growth Cabinet under 30±2°C temperature and 65±5% relative humidity regimes for 15-20 d for acclimatization. Acclimatized plants were then transferred to Net House for 30 d for hardening before transplanting to the field. Observations were recorded for number (s) of direct and indirect somatic embryo inducing explants, average number of somatic embryos, number of shoots/ embryoid, number of direct and indirect organogenic calli forming explants and number (s) of shoots/ direct organogenic calli and indirect organogenic calli. The experiment was laid out in completely randomized design to find out the significance of different culture medium with two parallel experiments (replications). Approximately 100-120 explants were cultured on each media. Arc-sine transformation (where values lies below than 100%) and log transformation (where values exceeded more than 100%) was made as per requirement before analysis of data. The data were analyzed as per method suggested by Snedecor and Cochran (1967).

RESULTS AND DISCUSSIONS

In nodal segment cultures, the nodal segment excised from top 1-10 nodes could not induce higher bud break, which may be due to tender nature of buds. The exposure of explants with sterilants may kill these soft buds. Maximum bud break was observed from 10-15 nodes, possibly due to juvenile state and capacity to withstand the toxic effect of sterilants. These results are in accordance to the findings of Tran Than Van (1975) and Mishra *et al*. (1999) who found higher morphogenic response followed by plantlet regeneration that appears to be highly dependent on position of explant. Studies have revealed that lower or mid portion of the branch are easier to establish *in vitro* than upper part of the branch (Bonga, 1987). Explants from young shoot tips were used as inoculums to regenerate shoots from the meristemetic tissue through direct organogenesis. Internodal stem segment measuring 1 cm and retaining one node without leaves was found to be the most potential explant for shoot organogenesis, in comparison to the apical meristem (2-3 mm from the shoot tip) and shoot tip of 1-2 cm length along with shoot apex. This finding is accordance with finding of Sarangi *et al*. (2000).

Nodal segment explants of sandalwood were cultured on MS medium with various PGRS treatments plants regeneration *via* two pathways: direct (somatic embryogenesis/organogenesis)

and indirect (somatic embryogenesis/organogenesis).In direct mode, somatic embryoids shoots developed from explants surface without intervening callus phase, shoot bud meristemoids appeared on surface of on callus mass in indirect mode The results reported here showed that most cells in sandalwood, which were still actively dividing, were embryogenically competent which followed two general patterns of embryogenesis. It has been well reported that direct embryogenesis occurs from pre-embryogenic determined cells, while indirect somatic embryogenesis requires the induction of embryogenically determined cells (Sharp *et al.,* 1980; Williams and Maheswaran, 1986).

Initial response of cultured nodal segments was similar after 4-7 d on most of the media combinations tried. All explants became swollen and no callus appeared as swollen explants. Callus proliferation usually started from the portion in contact with the medium after 10-14 d of culture (Fig.1F; Fig.2 D-E). In direct embryogenesis, embryo like structures initiated directly on the entire surface of explants. Embryoid formation started appearing after 7 d from initial culturing (Fig.1A-F). Whereas, in indirect embryogenesis embryo start appear it started after 14 d (Fig. 1G) on the callus mass. Embryoid like structures were round with irregular out lines frequently appeared as shining structures in clusters (Fig.1A-B). the combination of various media treatments greatly affected embryoid differentiation and in few cases embryoids developed after 35d of first sign of callus formation, During present investigations, somatic embryogenesis followed four developmental stages *i.e*. globular (Fig.1A-B), heart (Fig.1C), torpedo (Fig.1D) and cotyledonary (Fig.1 E) and germination (Fig.1F). Secondary embryogenesis could be observed on the entire surface of primary embryos (Fig.1B) and produced plantlets in enormous numbers (Fig.1F). Histological analysis revealed that the initial green translucent globular structure originated from epidermal and sub-epidermal layers of the explant. Clear bipolar, it's with shoot and root poles as well as continuous procambial strands and distinct epidermis was observed without vascular connections with the explant (Fig.1I). A pre-embryo was found which appeared to develop from a single cell of sub epidermal cells. The section also showed a secondary globular structures which grew on the surface layer of primary somatic embryos (Fig.1J-L). As division in superficial cells progressed, the cortical parenchyma cells actively divided in periclinal and anticlinal directions, which partly led to the nodular appearance; they later became the multicellular suspensor of the somatic embryos (Fig.1J). It is likely that a single epidermal cell had undergone anticlinal division before the uppermost cell

differentiated into the embryogenic mass. It was not clear if secondary somatic embryos originated from single cells. As superficial cells formed somatic embryos, new pre-embryo developed at adjacent deeper zones of the explant and subsequently, parenchymatous cells of cortex also formed pre-embryo (Fig.1K-L).

During direct organogenesis, adventitious shoot primordia appeared after 7-10 d on explant surface. Green adventitious primordial formation started appearing in some cases. However, the appearance of adventitious structure could be observed after 28 d (Fig.2 A-C). In indirect organogenic mode, shootlets developed from the on the surface of the callus (Fig.2 F-H). Shoot differentiation usually started meristemoids 10 d from initial culturing (Fig.1E). However, the duration varied from culture to culture and in a few cases shoots started appear after 45 d. Most of the calli, after prolonged culturing on the induction media gave rise to plants. However, transfer into regeneration medium allowed higher plant formation and growth rate (Fig.2 H). In cases, where there was no root formation was attained, shootlets were subsequently transferred into the rooting medium (Fig.2 I). Rooted plantlets were elongated after transferring into elongation medium. Well-developed plantlets were kept under 28±2^{o}C and 60±5% relative humidity for 20-25 d in a Environmental Growth Cabinet and subsequently (Fig.2 J). to the Net House/ Poly House (Fig.2 K) for 25-30 d for hardening before actual transfer in the field (Fig.2 L).

A wide range of basal medium have been employed such as MS medium (Laxmi Sita *et al*., 1979; Bapat and Rao, 1984; Rugkhla and Jones, 1998; Sarangi, 2000; Mujib, 2005; Sangmitra and Chadni, 2010; Chandni *et al*., 2010; Bele *et al*., 2012; Tripathi *et al*., 2017), WP medium (Mujib, 2005; Chandni *et al*., 2010) and White medium (Sarangi *et al*., 2000). The present results showed that, basal MS medium used throughout the experiment was more responsive as compared to WP medium in course of preliminary experiments. Furthermore, as observed during present studies it is clear from recorded data composition of culture media does not seem to play major role in *in vitro* response as much as the type and concentration of plant growth regulators, hence for nodal segment culture, three different sets of culture media were formulated by supplementing different auxins as alone, diverse cytokinins as sole as well as auxins 2,4-D and NAA in combinations with cytokinins: BA, TDZ and Kn to basal MS media to achieve the best *in vitro* response.

Use of, auxin 2, 4-D favoured callus formation in higher frequencies at a concentration ranging from 1.0-7.0 mg l^{-1}. The callus enlarged with an increase level of 2, 4-D up to 7.0 mg l^{-1}. Beyond this concentration the calli proliferation turned into dark black colour necrosed subseal and the best embryogenic response in terms of percentage of cultures forming somatic embryos and average number of somatic embryos per responding explant, was acquired with addition of 0.5-5.0 mgl^{-1} 2, 4-D. Increasing the 2,4-D concentration up to 5.0 mg l^{-1} 2,4-D did not improved the embryogenic response (data not presented).Auxin 2,4-D initiated direct somatic embryogenesis in higher frequencies at concentration ranging from 0.5-3.0 mgl^{-1}. Indirect somatic embryogenesis and organogenesis were higher with culture media supplemented with 2, 4-D in the range of 1.0- 4.0 mgl^{-1}.However, direct organogenesis and plantlet regeneration efficiency was found to be quite low with application of 2, 4-D alone irrespective of all tested concentrations. Auxin NAA was able to induce somatic embryo formation at moderate frequency. However, organogenic response was found to be higher with application of NAA in range of 0.5-5.0 mgl^{-1}. Culture medium supplemented with NAA alone, produced calli in lower to higher frequencies depending upon the concentrations (1.0-5.0 mgl^{-1}). Maximum calli initiated on culture medium supplemented with NAA at the concentration of 4.0 mgl^{-1}. Beyond this concentration ratio of non-morphogenic calli increased subsequently. NAA promoted direct organogenesis at concentration ranging from 1.0-4.0 mgl^{-1}. Plantlet regeneration efficiency was also found to be higher with application of NAA as compared to other auxins tested.

Among tested cytokinins, a higher proportion of explants responded well on media containing TDZ than with BA or kinetin. Supplementation of different concentrations of TDZ as alone in the medium exhibited poor callus induction and organogenic calli formation. However, culture medium supplemented with TDZ enhanced direct somatic embryo induction and plantlet regeneration efficiency as compared to media supplemented with NAA and/or 2, 4-D. BAP and kinetin performed poorly. Cultures produced in media supplemented with kinetin resulted in the formation of shoots of higher length with lesser numbers and low morphogenic frequency in advance phase of cultures.

Initial studies to launch plant growth regulator type, combinations and concentrations revealed that auxins as well as cytokinins alone were not effective for achieving higher *in vitro* response for nodal explant culture. Either formation of morphogenic calli or plantlet regeneration efficiency was investigated low to moderate. Therefore, while deciding final experimentation

auxins NAA and 2,4-D in combinations with cytokinins: BA, Kn and TDZ in varying concentrations was applied for achieving the best *in vitro* response. The analysis of variance presented in Table 2-3 revealed highly significant ($p<0.05$) differences among the response of different culture media combinations in terms of number of direct and indirect somatic embryo inducing explants, average number of somatic embryos, number of shoots/ embryoid, number of direct and indirect organogenic calli forming explants and number of shoots/ direct organogenic calli and indirect organogenic calli. It indicates the presence of considerable amount of variability amongst different culture media combinations.

The effect of plant growth regulators its combination is presented in the Table2 and 3. Higher proportion of direct somatic embryogenesis, average number of somatic embryo per explant and plantlet regeneration *via* direct organogenesis were recorded on nutrient medium containing a moderate concentration of TDZ (1.0 mgl^{-1}) in combination with a lower concentration of NAA (0.5 mgl^{-1}). Earlier studies on somatic embryogenesis of white ash (Bates *et al.,* 1992) walnut, watermelon, muskmelon, geranium, grape (Lu, 1993; Huetteman and Preece, 1993), peanut (McKently, 1995) and sandalwood (Rugkhla and Jones, 1998; Bele *et al*., 2012; Tripathi *et al*., 2017) have also been investigated significant role of TDZ. In, the optimum concentration of TDZ for somatic embryo induction was quite low in sandalwood as compared to other species. TDZ at low concentrations (0.5-1.0 mgl^{-1}) has been reported to be more efficient in inducing organogenesis or somatic embryogenesis than other cytokinins, particularly in recalcitrant woody species (Huetteman and Preece, 1993). The reasons for the high activity of TDZ in woody species have not been investigated at the physiological or molecular level. A carbon isotope study showed that TDZ was very stable in the culture media and persistent in plant tissue (Mok and Mok, 1985). It has been suggested that TDZ helps to establish the internal optimum balance of cytokinin and auxin required for induction and expression of somatic embryogenesis (Saxena *et al.,* 1992; Lu, 1993).

A relative higher concentration of BAP (1.0-2.0 mgl^{-1}) combined with a lower concentration of NAA (0.5 mgl^{-1}) promoted indirect somatic embryogenesis and organogenesis. Ratio of organ formation directly from surface of cultured explants was recovered from culture medium fortified with a higher concentration of kinetin at the concentration of 4.0 mgl^{-1} in combination with a lower concentration of NAA (0.5 mgl^{-1}). Maximum plantlets regenerated *via* somatic embryogenesis (direct and/or indirect) on regeneration medium supplemented with 2.0

mgl^{-1}TDZ in combination with 1.0 mg $l^{-1}GA_3$, while plantlets in higher frequencies *via* indirect organogenesis was attained with regeneration medium amended with comparative lower concentration of TDZ (1.0 mg l^{-1}) in combination with 0.5 mgl^{-1} GA_3 and 0.5 mg^{l-1} NAA. The system described here required different hormonal combinations and concentrations from those previously reported by Rao and Bapat (1995) who used media containing IAA and BAP to obtain the most normal somatic embryos, multiplication, germination and conversion into plantlets and noted the problem of low conversion frequency. In the present study, TDZ alone or with NAA can spontaneously induce embryogenesis at a higher frequency and with greater reproducibility. This finding is an accordance to the finding of Rugkhla and Jones (1998) who also reported 100% somatic embryo induction with application of TDZ alone as well in combination with 2, 4-D. However in this study, NAA performed better than 2,4-D. Mature somatic embryos needed supplementation of GA_3 for germination, conversion and elongation of plantlets, otherwise many abnormal somatic embryos were obtained and this resulted in a low conversion into plantlets.

Confirmation of somatic embryogenesis is based on histological evidence that the structure is bipolar and has no vascular connection to the explant (Haccius, 1978). The most suitable regeneration systems for transformation are direct or repetitive production of somatic embryos or *de novo* shoot organogenesis, which originate from single cells of the epidermal layer (Marcotrigiano, 1986; Raemakers *et al.,* 1995).Histological observations indicated that differentiation of maternal tissue led to a suspensor-like structure of primary somatic embryos. The presence of a narrow suspensor indicated a single cell origin and a broad suspensor area indicated a multicellular origin of somatic embryos (Williams and Maheswaran, 1986). Both narrow and broad suspensors were found in the present study. Similar reports have also been documented by Rugkhla and Jones (1998) in sandalwood. Somatic embryos of single or multiple cell origin derived from the epidermal layer were found in *Medicago sativa* (Dos Santos *et al.,* 1983) and from epidermal and cortical cells in *Trifoliumrepen* (Maheswaran and Williams, 1985). The single cell origin of secondary somatic embryos was noted in walnut (Polito *et al.,* 1989) and used successfully in developing solid transgenic plants (McGranahan *et al.,* 1988).

Regenerated shoots, 3-4 months old and 1-2 inch height were isolated from the mother tissue and transferred on various media for rooting (Table 4). Different hormonal treatments using to MS basal agar gelled and liquid media. Falled to develop root even affects prolonged

/incubation under different culture conditions. It is interesting to note all media treatments shootlets were implanted on MS /agar gelled medium tried to induce roots, favored healthy growth of shootlets with low frequency (1-3%), of rooting. Combination of IBA with kinetin induced formation of white friable calli under at the cut end; without root. Different combinations of IBA with BA rather induced adventitious shoot formation instead of rooting. Agar gelled medium with different concentrations of IBA in combination of BA/ kn and GA_3 also did not induce root formation in the excised shoots. These findings are in close agreement with earlier report of Sarangi *et al*. (2000) who also failed inducing *in vitro* rooting in sandalwood despite of a long and repetitive effort.

Plantlets regenerated *via* direct somatic embryogenesis have more possibility to be true to the type. Whereas with, Plants regenerated *via* callus cultures possibility to gel variation may not be ruled out. Plantlets also originated through direct organogenesis (*via* auxiliary bud proliferation). More possibility exists for the formation of the identical clones of the donor plants of such plantlets, since they are not exposed to the factors imposing somaclonal variations due to bypassing long callus phase.Sarangi *et al.* (2000), Bele *et al*.(2012) and Tripathi *et al*. (2017) obtained similar results for different explants cultures in sandalwood. During present study, organogenesis in form of root never lead to viable plants, whereas shoot forming cultures were able to produce complete plants by subsequent transferring into rooting media. Regeneration of plants *via* somatic embryogenesis is preferred to organogenesis as embryoids usually arise from single cell, genetic manipulations could be carried through subsequent generations.

The present investigation points that under appropriate conditions nodal segment culture of sandalwood were more competent in contrast to other woody species. Higher number of shoots, embryogenic frequency and the conversion rate of embryoids into plantlets obtained during present experimentation were higher as compared to earlier findings. The present described procedures provide effective strategy for genetic transformation (direct and/ or *via* vector). As such present plant regeneration procedure in sandalwood is available, which has immediate potential for breeding and biotechnological studies. Rooting is intermittent, and further experimentations are in progress to get sustainable *in vitro* rooting system.

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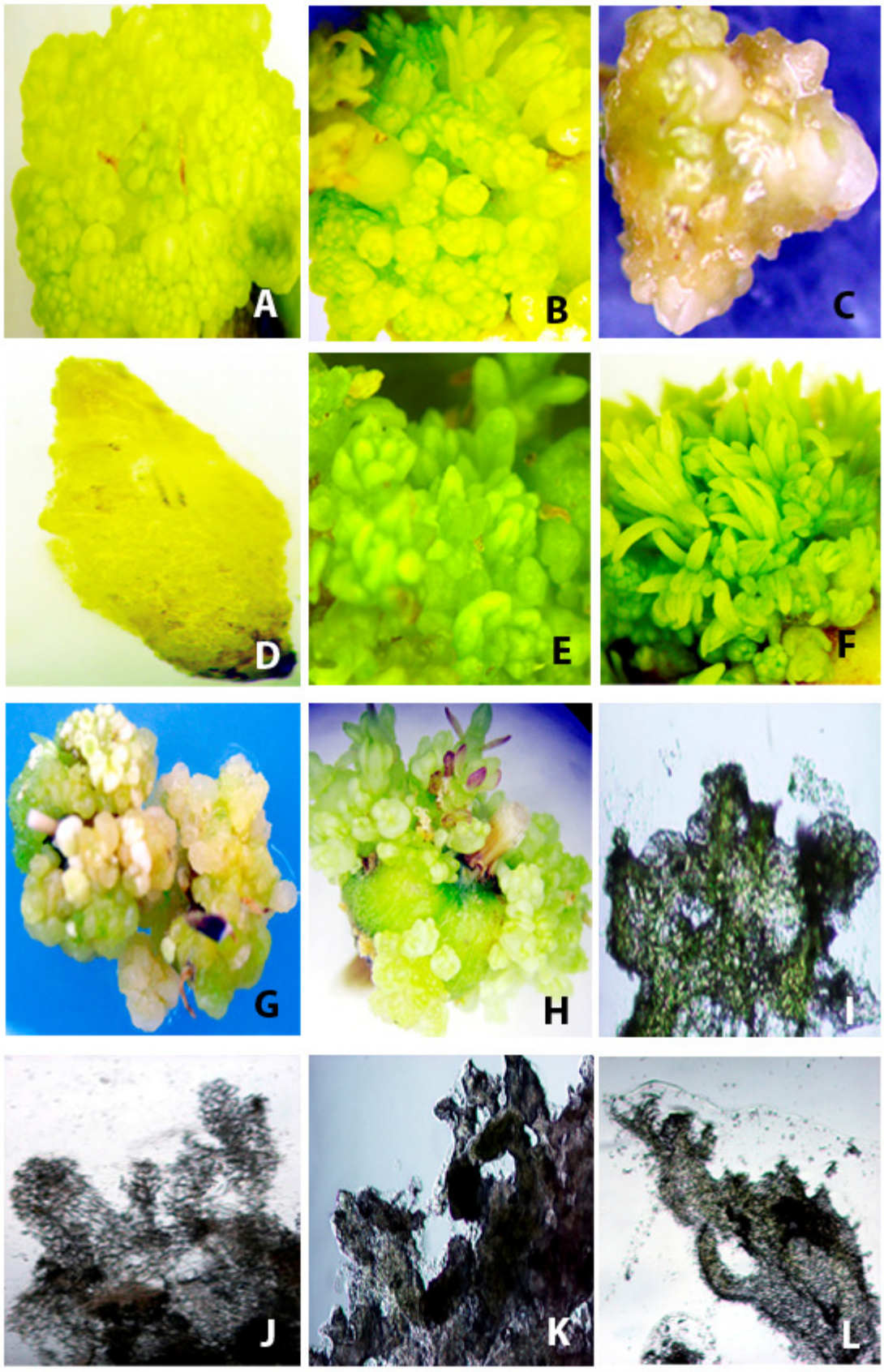
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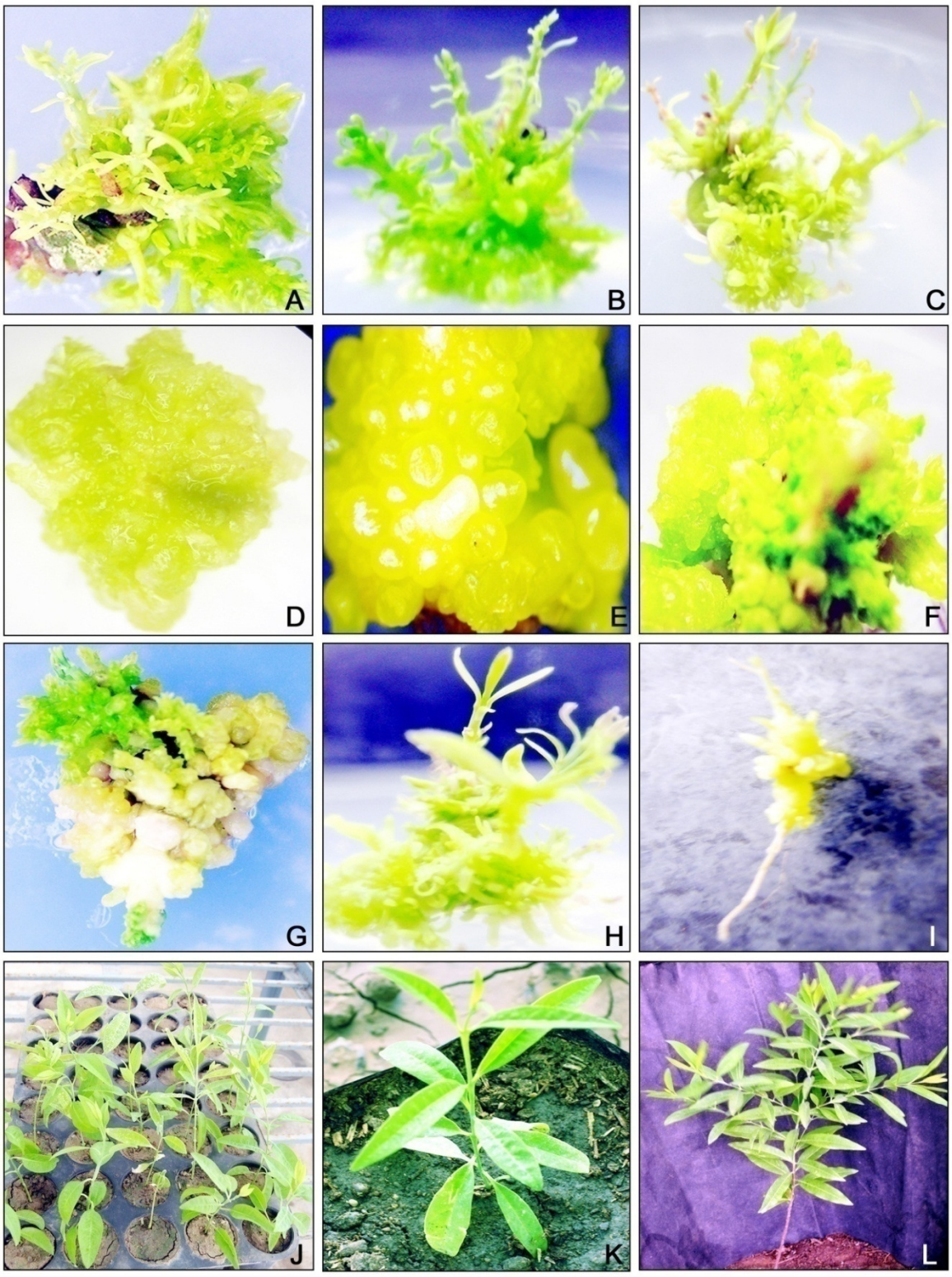
LEGENDS FOR FIGURE 1

Fig. 1 Plant regeneration in *Santalum albumvia* somatic embryogenesis: **A.**Somatic embryo of globular stage; **B.** Formation of secondary embryos; **C.** Somatic embryo of heart stage;**D.** Somatic embryo of torpedo stage; **E**. Somatic embryo of cotyledonary stage; **F**. Germination of somatic embryo; **G.** Formation of globular stage somatic embryo *via* indirect mode; **H.** Plant regeneration from indirect somatic embryogenesis; **I.** Longitudinal section through green globular somatic embryo showing bipolarity by developing procombial strand with a close radicular and without vascular connections with the mother tissue; **J.** Somatic embryos differentiated from cortical parenchyma; and **K-L**. Development of leaf primordia with initiation of secondary somatic embryos.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LEGENDS FOR FIGURE 2

Fig. 2 Plant regeneration in *Santalum albumvia* organogenesis: **A-C.** Formation of multiple shoots *via* direct organogenesis; **D-E.** Callus formation; **F-G**. Initiation of multiple shoots; **H**. Formation of multiple shoots; **I.** *In vitro* rooting; **J.** Regnerants transferred in Environmental Growth Cabinet; **K.** Regenerants transferred in Net House for hardening; and **L** Plantlets transferred in field.

Table1Effects of different surface sterilizing and antifungal agents on recovery of aseptic culture in sandalwood.

Treatments	Concentration (%)	Exposure Time (in min)	Aseptic culture (%)	Survival of explants (%)
Ca $(OCl)_2$	5	10	12.96^{p} (21.08)	26.54^{m} (30.99)
Ca $(OCl)_2$	5	15	15.07^{o} (22.82)	26.62^{m} (31.04)
Ca $(OCl)_2$	5	20	26.34^{n} (30.86)	26.67^{m} (31.08)
Ca $(OCl)_2$	10	10	36.14^{m} (36.94)	33.72^{l} (35.48)
Ca $(OCl)_2$	10	15	37.96^{lm} (38.02)	46.41^{j} (42.92)
Ca $(OCl)_2$	10	20	44.23^{i} (41.67)	58.10^{g} (49.64)
Ca $(OCl)_2$	15	10	$40.07j^{k}$ (39.26)	34.88^{l} (36.18)
Ca $(OCl)_2$	15	15	43.17^{i} (41.06)	46.59^{j} (43.03)
Ca $(OCl)_2$	15	20	46.49^{h} (42.97)	49.14^{i} (44.49)
Ca $(OCl)_2$	20	10	48.36^{g} (44.04)	52.11^{h} (46.19)
Ca $(OCl)_2$	20	15	49.12^{g} (44.48)	57.34^{g} (49.20)
Ca $(OCl)_2$	20	20	52.36^{f} (46.33)	61.35^{f} (51.54)
$HgCl_2$	0.1	2	38.30^{kl} (38.22)	68.50^{b} (55.84)
$HgCl_2$	0.1	5	44.23^{i} (41.67)	68.43^{b} (55.79)
$HgCl_2$	0.2	2	48.06^{gh} (43.87)	67.39^{b} (55.16)
$HgCl_2$	0.2	5	49.64^{g} (44.78)	62.22^{ef} (52.05)
$HgCl_2$	0.2	10	49.64^{g} (44.78)	27.55m (31.64)
Bavistin+Ca $(OCl)_2$	0.5+10	30	40.25^{j} (39.36)	38.16^{k} (38.13)
Bavistin + Ca $(OCl)_2$	0.5+15	30	57.01^{e} (49.01)	49.45^{i} (44.67)
Bavistin + Ca $(OCl)_2$	0.5+20	30	75.95^{c} (60.61)	68.43^{b} (55.79)
Bavistin+ $HgCl_2$	0.5+0.1	20	77.95^{b} (61.97)	75.93^{a} (60.60)
Bavistin+ $HgCl_2$	0.5+0.2	20	82.23^{a} (65.05)	63.13^{de} (52.59)
Bavistin + $HgCl_2$	0.5+0.1	30	68.46^{d} (55.81)	64.15^{c} (53.20)
Bavistin+ $HgCl_2$	0.5+ 0.2	30	69.80^{d} (56.64)	61.04^{f} (51.36)
Mean			**]48.07(43.80)**	**51.41 (45.78)**
CD (0.05%)			**1.830**	**1.791**

- Ca $(OCl)_2$: Calcium hypochlorite, $HgCl_2$: Mercuric chloride
- Figures in parenthesis are transformed values (Arc-sine transformation).
- Values within column followed by different letters are significantly differed at 5% probability level.

Table 2. ***In vitro*** **morphogenesis in cultured nodal segments on different fortification of MS media**

Culture Media ▼	Plant growth regulators mg.l^{-1}					Direct somatic embryogenesis	Indirect somatic embryogenesis	*Average no. of somatic embryos	Direct organogenesis	Indirect organogenesis	*Plantlet regeneration *via* direct organogenesis
	2,4-D	NAA	BA	TDZ	Kn						
MS.5D.5Td	0.5	-	-	0.5	-	22.43^{ij} (28.25)	7.50^{e} (15.89)	251.14^{l}(2.40)	20.35^{j} (26.80)	6.56^{f} (14.83)	80.96^{u} (1.91)
MSD5Td	1.0	-	-	0.5	-	25.08^{gh} (30.04)	8.34^{e} (16.78)	266.38^{k}(2.43)	28.12^{e} (32.01)	9.78^{d} (18.22)	112.13^{h} (2.05)
MS2D.5Td	2.0	-	-	0.5	-	20.97^{jk} (27.24)	10.16^{d} (18.56)	295.94^{i}(2.47)	26.15^{f} (30.740	9.65^{d} (18.09)	104.22^{k} (2.02)
MS3D.5Td	3.0	-	-	0.5	-	17.53^{lm} (24.73)	11.17^{c} (19.50)	326.12^{f}(2.52)	28.24^{e} (32.08)	9.63^{d} (18.07)	112.92^{g} (2.05)
MS4D.5Td	4.0	-	-	0.5	-	15.96^{m} (23.53)	8.94^{e} (17.39)	335.06^{e}(2.53)	21.32^{ij} (27.480	9.19^{e} (17.64)	84.35^{t} (1.93)
MS5D.5Td	5.0	-	-	0.5	-	6.74^{n} (15.04)	5.51^{f} (13.57)	270.65^{j}(2.44)	17.55^{kl} (24.75)	7.17^{f} (15.53)	68.41^{w} (1.84)
MS.5B.5N	-	0.5	0.5	-	-	26.44^{g} (30.93)	18.25^{a} (25.27)	116.58^{u}(2.07)	24.48^{g} (29.64)	13.04^{b} (21.15)	96.18^{o} (1.98)
MSB.5N	-	0.5	1.0	-	-	31.55^{de} (34.16)	19.18^{a} (25.56)	135.80^{o}(2.13)	27.35^{e} (31.52)	17.56^{a} (24.76)	108.04^{j} (2.04)
MS2B.5N	-	0.5	2.0	-	-	33.08^{d} (35.09)	17.56^{a} (24.76)	345.83^{d}(2.54)	19.44^{jk} (26.14)	18.56^{a} (25.50)	76.23^{v} (1.88)
MS3B.5N	-	0.5	3.0	-	-	35.55^{c} (36.58)	14.09^{b} (22.03)	317.29^{g}(2.51)	22.35^{hi} (28.20)	12.14^{bc}(20.37)	88.94^{q} (1.95)
MS4B.5N	-	0.5	4.0	-	-	32.62^{d} (34.81)	10.23^{d} (18.62)	78.24^{w} (1.90)	36.49^{c}(37.150)	11.79^{c} (20.06)	144.16^{d} (2.16)
MS5B.5N	-	0.5	5.0	-	-	29.23^{f} (32.71)	14.54^{b} (22.41)	28.38^{x} (1.45)	16.07^{l} (23.62)	9.63^{d} (18.05)	64.50^{x} (1.81)
MS.5Td.5N	-	0.5	-	0.5	-	33.55^{cd} (35.38)	7.43e^{f} (15.81)	315.74^{h}(2.50)	26.37^{ef} (30.88)	10.12^{d} (18.52)	104.15^{l} (2.02)
MSTd.5N	-	0.5	-	1.0	-	44.12^{a} (41.61)	9.11^{e} (17.53)	368.68^{a}(2.57)	27.11^{e} (31.36)	13.54^{b} (21.57)	212.10^{a} (2.33)
MS2Td.5N	-	0.5	-	2.0	-	39.63^{b} (39.00)	10.15^{d} (18.55)	354.35^{b}(2.55)	21.63^{i} (27.700	12.55^{b} (20.73)	84.56^{s} (1.93)
MS3Td.5N	-	0.5	-	3.0	-	30.18^{ef} (33.31)	11.18^{c} (19.51)	348.56^{c}(2.54)	22.08^{i} (28.01)	11.78^{c} (20.05)	88.28^{r} (1.95)
MS4Td.5N	-	0.5	-	4.0	-	28.59^{f} (32.31)	8.25^{e} (16.660	128.32^{q}(2.11)	26.59^{e} (31.02)	9.18^{e} (17.63)	104.02^{m} (2.02)
MS5Td.5N	-	0.5	-	5.0	-	25.14^{g} (30.07)	7.08^{f} (15.43)	118.38^{t}(2.08)	30.33^{d} (33.40)	7.72^{f} (16.10)	120.26^{f} (2.08)
MS.5Kn.5N	-	0.5	-	-	0.5	18.65^{l} (25.55)	6.33^{f} (14.57)	116.38^{v}(2.07)	48.53^{b} (44.14)	6.56^{f} (14.83)	192.40^{c} (2.29)
MSKn.5N	-	0.5	-	-	1.0	21.41^{j} (27.54)	8.29^{e} (16.73)	125.76^{r}(2.10)	23.72^{gh}(29.13)	9.12^{e} (17.57)	92.93^{p} (1.97)
MS2Kn.5N	-	0.5	-	-	2.0	23.06^{hi} (28.68)	9.15^{de} (17.58)	131.06^{p}(2.12)	24.56^{fg} (29.69)	10.04^{d} (18.45)	108.94^{i} (2.04)
MS3Kn.5N	-	0.5	-	-	3.0	20.23^{k} (26.71)	10.48^{cd} (18.86)	150.83^{m}(2.18)	35.54^{c} (36.58)	10.52^{cd} (18.90)	140.22^{c} (2.15)
MS4Kn.5N	-	0.5	-	-	4.0	19.16^{kl} (25.94)	7.53^{e} (15.920	142.52^{n}(2.15)	53.63^{a} (47.06)	9.27^{de} (17.72)	96.28^{n} (1.98)
MS5Kn.5N	-	0.5	-	-	5.0	15.69^{m} (23.31)	5.54^{f} (13.61)	118.68^{s} (2.08)	49.44^{b} (44.66)	7.93^{ef} (16.35)	196.23^{b} (2.30)
Mean						**25.69 (30.11)**	**10.25 (18.39)**	**216.11 (2.27)**	**28.23 (31.82)**	**10.54 (18.78)**	**111.73 (2.03)**
CD (0.5%)						**2.0591**	**1.960**	**0.027**	**1.899**	**1.920**	**0.0271**

- Figures in parenthesis are transformed values (Arc-sine transformation).
- *Figures in parenthesis are transformed values (Log transformation).
- Values within column followed by different letters are significantly differed at 5% probability level.

Table 3. Effect of different plant growth regulators on plantlet regeneration from cultured nodal segments *via* indirect somatic embryogenesis and organogenesis

Culture media ▼	Plant growth regulator mg.l^{-1}					Plant regeneration *via* somatic embryogenesis	Plant regeneration *via* indirect organogenesis
	BA	TDZ	Kn	GA_3	NAA		
MS.5B.5GA	0.5	-	-	0.5	-	241.58^{r} (2.39)	146.50^{o} (2.17)
MSB.5GA	1.0	-	-	0.5	-	246.30^{q} (2.40)	153.64^{m} (2.19)
MS2B.5GA	2.0	-	-	0.5	-	289.38^{j} (2.47)	167.04^{k} (2.23)
MS.5BGA	0.5	-	-	1.0	-	250.86^{p} (2.40)	82.68^{v} (1.92)
MSBGA	1.0	-	-	1.0	-	314.74^{g} (2.50)	120.68^{t} (2.08)
MS2BGA	2.0		-	1.0	-	327.54^{d} (2.52)	179.76^{i} (2.26)
MS.5Td.5GA	-	0.5	-	0.5	-	267.50^{n} (2.43)	133.53^{p} (2.13)
MSTd.5GA	-	1.0	-	0.5	-	281.58^{l} (2.45)	189.58^{g} (2.28)
MS2Td.5GA	-	2.0	-	0.5	-	331.10^{c} (2.52)	198.92^{d} (2.30)
MS.5TdGA	-	0.5	-	1.0	-	313.56^{h} (2.50)	127.50^{q} (2.11)
MSTdGA	-	1.0	-	1.0	-	324.24^{f} (2.52)	157.58^{l} (2.20)
MS2TdGA	-	2.0	-	1.0	-	348.24^{a} (2.54)	198.82^{e} (2.30)
MS.5Kn.5GA	-	-	0.5	0.5	-	102.88^{x} (2.01)	125.63^{r} (2.10)
MSKn.5GA	-	-	1.0	0.5	-	129.54^{v} (2.12)	148.56^{n} (2.18)
MS2Kn.5GA	-	-	2.0	0.5	-	170.54^{t} (2.23)	120.88^{s} (2.09)
MS.5KnGA	-	-	0.5	1.0	-	122.52^{w} (2.09)	90.78^{u} (1.96)
MSKnGA	-	-	1.0	1.0	-	167.30^{u} (2.23)	35.84^{x} (1.56)
MS2KnGA	-	-	2.0	1.0	-	192.58^{s} (2.29)	58.66^{w} (1.77)
MS.5B.5GA.5N	0.5	-	-	0.5	0.5	262.08^{o} (2.42)	183.12^{h} (2.27)
MS.5Td.5GA.5N	-	0.5	-	0.5	0.5	301.14^{i} (2.48)	284.07^{b} (2.46)
MS.5Kn.5GA.5N	-	-	0.5	0.5	0.5	278.58^{m} (2.45)	174.30^{j} (2.24)
MSB.5GA.5N	1.0	-	-	0.5	0.5	283.52^{k} (2.46)	203.26^{c} (2.31)
MSTd.5GA.5N	-	1.0	-	0.5	0.5	325.04^{e} (2.52)	289.44^{a} (2.47)
MSKn.5GA.5N	-	-	1.0	0.5	0.5	340.64^{b} (2.54)	198.16^{f} (2.30)
Mean						**258.87 (2.39)**	**157.04 (2.16)**
CD (0.5%)						**0.019**	**0.026**

- Figures in parenthesis are transformed values (Log transformation).
- Values within column followed by different letters are significantly differed at 5% probability level.

Table 4 Response of regenerated shootlets on MS basal medium with different concentrations of plant growth regulators tried for induction of *in vitro* rooting

Culture media combinations ▼	Plant growth regulators mgl^{-1}					*In vitro* rooting response
	NAA	IBA	Kn	BA	GA_3	
MS.1N	0.1	-	-	-	-	Healthy for 1 month, no rooting, dried.
MS.5N	0.5	-	-	-	-	As above
MSN	1.0	-	-	-	-	Growth + healthy for 2 months, no rooting
MS5N	5.0	-	-	-	-	As above
MS10N	10.0	-	-	-	-	Dried within one month, no rooting
MS.1IB	-	0.1	-	-	-	Healthy for 1 month, no rooting, dried
MS.5IB	-	0.5	-	-	-	As above
MSIB	-	1.0	-	-	-	Healthy for 3 month, no rooting
MS5IB	-	5.0	-	-	-	Healthy for 4 months, no rooting
MS10IB	-	10.0	-	-	-	Growth + healthy for 6 months, no rooting
MS.1Kn	-	-	0.1	-	-	Growth + callusing at the cut end, no root formation
MS.5Kn	-	-	0.5		-	Adventitious shoots formed, no rooting
MSKn	-	-	1.0		-	Hairy roots
MS5Kn	-	-	5.0		-	Hairy roots
MS10Kn	-	-	10.0		-	Hairy roots
MS.1IB.5B	-	0.1	-	0.5	-	Growth + healthy for 4 months, adventitious shoots formed, no rooting
MS.5IB.5B	-	0.5	-	0.5	-	As above
MSIB.5B	-	1.0	-	0.5	-	As above
MS5IB.5B	-	5.0	-	0.5	-	Healthy for 4 months, no rooting
MS10IB.5B	-	10.0	-	0.5	-	Growth + healthy for 6 months, no rooting
MS.1IB.5Kn	-	0.1	0.5	-	-	No growth, healthy for 1 month, no rooting
MS.5IB.5Kn	-	0.5	0.5	-	-	As above
MSIB.5Kn	-	1.0	0.5	-	-	As above
MS5IB.5Kn	-	5.0	0.5	-	-	Healthy for 4 months, no rooting
MS10IB.5Kn	-	10.0	0.5	-	-	Growth + healthy for 6 months, no rooting
MS.1IB.5Kn.5G	-	0.1	0.5	-	0.5	Healthy for 1 month, no rooting
MS.5IB.5Kn.5G	-	0.5	0.5	-	0.5	As above
MSIB.5Kn.5G	-	1.0	0.5	-	0.5	Healthy for 3 months, no rooting
MS5IB.5Kn.5G	-	5.0	0.5	-	0.5	Healthy for 4 months, no rooting
MS10IB.5Kn.5G	-	10.0	0.5	-	0.5	Growth + healthy for 6 months, no rooting