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Research Article

An Improved *in Vitro* Micropropagation Technique for Teak (*Tectona grandis* L.)

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ABSTRACT

An *in vitro* clonal propagation protocol has been developed for teak (*Tectona grandis* L.) plants with two kinds of media such as Murashige and Skoog's (MS) and B_5 (Gamborg's) along with the supplementation of various plant growth hormones and Activated Charcoal (AC). Node and shoot tip explants were used for callus induction and plantlet regeneration studies. Maximum percentage of callus formation was observed in B_5 with 2,4-D 2.0 mg l^{-1} (88.6%) than MS medium (85.5%) with activated charcoal. Minimum callus formation percentage was observed in MS medium with 2,4-D 2.0 mg l^{-1} (23.8%) than B_5 medium (41.8%) in without activated charcoal. Organogenic callus was observed in only medium supplemented with BAP 2.0 mg l^{-1} (53.5%). Multiple shoots were induced from nodal segments through axillary bud proliferation. Maximum shoot multiplication percentage (83.4%) was achieved on B_5 medium than MS medium (79.8%) supplemented with 6-benzyladenine (BAP) (2.0 mg l^{-1}) and α-naphthalene acetic acid (NAA) (1.0 mg l^{-1}) and activated charcoal. Cultures have been continuously transferred to new medium with same hormone treatment in every 3 weeks. In general B_5 medium is more efficient than MS medium for woody tree species. Multiple shoots of high quality were produced *in vitro* from node explants. An average of about 13 shoots/uninodal explants was obtained in 3 weeks of culture on B_5 medium. When shoots were rooted by supplemented with indole-3-butyric acid (IBA) 1.0 mg l^{-1}, the frequency of rooting of shoots previously grown on either hormone-free medium or 0.5 mg l^{-1} of BAP. More than 95% of shoots were developed strong and highly ramified roots on MS medium containing IBA (1.0 mg l^{-1}). *In vitro* raised plantlets were regenerated on liquid MS medium supplemented with 0.5 mg l^{-1} of NAA and acclimatized on vermiculate before transferring them to field. Shoots were successfully rooted and acclimatized to greenhouse conditions. The plantlets were acclimatized first in a mist chamber and then in poly-bags in a mixture of soil, sand, and farmyard manure (1:1:1 v/v). After 3 months, plants were transferred to field conditions. This method has been resulted in 97.9% survival of the plantlets.

Keywords: Tectona grandis L.f., Organogenic callus, Multiple shoots, Activated charcoal, Plantlet regeneration efficiency

[I] INTRODUCTION

Tectona grandis L.f. (Verbinaceae) is one of the most valuable structural timber species of the world. It is indigenous to Burma, Thailand and Central and South India. Teak has been introduced into all agro-climatic regions of Central and South India has naturalized in almost all of these regions [1]. The tree is characterized by a tall clear bole with a rounded crown. Its

leaves are broadly elliptical and physical quality of its timber which is also resistant to termite attack due to the presence of sesquiterpenes. Teak wood is used for making fine furniture, shipbuilding, and decorative objects [2]. Teak is conventionally reproduced through seeds, but germination is often difficult because the hard seed coat limits the production of a large number of seedlings in a defined time. The propagation of teak via cuttings and other techniques have also been reported [3, 4], but this method has several limitations and only provides a few propagules from selected individuals.

In vitro propagation technique has become an efficient way for producing plants as uniform as possible on a large scale and in a short time for the plantation industry. However, although numerous authors have been experimenting to establish an efficient, reproduciable and simple system for micropropagation of teak [5], it still remains problematic due to the poor capacity of shoot proliferation, high susceptibility of shoots to vitrification and browning and the low frequency of *in vitro* rooting. None of the published protocols fully satisfies the requirements for commercial application in spite of the progress made on tissue culture of teak since the 1970s [6 - 8] Akram and Aftab 2008). However, micropropagation of teak from mature trees has remained problematic. Poor explants response and rapid explants browning are major hurdles. Tissue browning *in vitro* can be a serious problem when mature shoots of trees are used as a source material for micropropagation studies [9].

Teak plantlets have been were achieved through multiple shoot formation from nodal segments and shoot tip explants [10]. Because teak exhibits phenolic exudation, removal of these compounds by treating explants with certain antioxidants may be a practical approach to enhance the rate of growth of teak *in vitro*. *In vitro* propagation technique has become an efficient way for producing plants as uniform as possible on a large scale and in a short time for the plantation industry [11]. Teak is traditionally reproduced through seeds, but in most cases, germination is difficult due to the hard seed coat, low seed quality and late seed production. Poor germination rate leading to a low production of seedlings further contributes to the paucity of planting material [12, 13].

In general, use of activated charcoal in tissue culture could be dealt with providing a dark environment [14], adsorption of undesirable or inhibitory substance, adsorption of plant growth regulator [15], releasing macro and micro elements and inhibition of sucrose hydrolysis [16]. Additionally, it has been suggested that AC absorbs inhibitory metabolic by-products of growth, such as ethylene and phenolic compounds. The beneficial effects of AC may be attributed to its irreversible adsorption of inhibitory compounds in the culture medium and substantially reduce the toxic metabolites, phenolic exudation and exudate accumulation [17]. Activated carbon (AC) was initially added to tissue culture media in an attempt to simulate soil conditions: today it is routinely included in many tissue culture media formulations. The benefits of using activated carbon often include greater plant survival, greater plant growth, and improved plant quality and vigor [13].

Moreover, adsorption of undesirable substance such as ethylene released from explants or culture media by AC can enhance microtuber growth [14]. Activated charcoal (AC) was found to significantly reduce the total phenol content of media by 53%, compared with ascorbic acid (AA) in tissue culture of *Strelitzia reginae* [18].

The browning and subsequent death of cultured explants is a major problem that is usually dependent on the phenolic compounds and the quantity of total phenols. Phenolic compounds occur as secondary metabolites in all plant species [19, 20]. The phenols are synthesized by the plants and in many cases excreted and then oxidized. In tissue culture studies, phenolic

substances, especially oxidized phenols generally affect *in vitro* development negatively. Activated charcoal at (0.5 and 1 %), induced *in vitro* microtuber formation and significantly increased size at higher levels under light condition in potato (*Solanum tuberasum* cv. Agria) [21]. Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and subsequent lethal browning of explants [22]. Tissue culture attempts of this plant have had limited success due to the oxidative browning of explants [23]. This crucial problem was also frequently encountered in genera related to *Strelitzia*, namely *Musa* and *Ensete* spp. [24 - 26]. This paper describes a method for the micropropagation of teak using node and shoot tips explants in different media such as MS medium and B_5 medium along with the supplementation of plant growth hormones and activated charcoal.

[II[MATERIALS AND METHODS

2.1. Explants source and sterilization

Fresh and young plant seedlings were maintained in the departmental nursery, Government Arts College, Dharmapuri-636705, Tamil Nadu, India. From the seedlings as well as healthy mature trees about 25 years old plants were used as source of explants. Shoot tips and single nodes of young twigs were excised and they were initially surface sterilized with ethanol (70% v/v) for 30 s and then dipped in 5-10% commercial bleaching solution containing 5% sodium hypochloride (NaOCl) and a few drops of Tween-80 for 5-10 min. After rinsing three times with sterile distilled water, the explants were dipped in a 0.20% mercuric chloride solution for 5-7 min, followed by three rinses in sterile distilled water.

2.2. Culture establishment

The node and shoot tip explants were cultured in test tubes (25 mm × 150 mm) with MS medium [27] and B_5 medium [28] containing 3% sucrose and solidified with 0.8% agar, supplemented with 6-benzylamino purine (BAP) and α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA), and indole-3-butyric acid (IBA) in different concentrations. Because of *in vitro* phenolic exudation, pretreatment of shoots was carried out before the explants preparation. For this purpose, softwood shoots (4-6 cm) were further cut to 2 to 4 cm long segments and pretreated in 0.3% of activated charcoal (AC). Activated charcoal (Sigma, USA), 3 g l^{-1}, was added to the medium to reduce phenolic browning. The pH of the medium was adjusted 5.8 before solidifying with 0.8% agar. The cultures were maintained at 27±2°C temperature and a photoperiod of 16/8 light/dark under fluorescent light.

2.3. Shoot multiplication

The callus and multiple shoots initiated were subcultured on fresh media, for further growth once every 25 days, maintaining the same concentrations of hormones used in initial cultures for 3 subcultures to produce a large number of shoots. The percentage of explants initiating shoots, the number of shoots per explants, number of nodes per shoot, shoot length and basal callus fresh weight were recorded after 4 weeks of culture. Callus was removed from the shoots before weighing. The micropropagation cycle consisted of a monthly subculture of nodal segments after removal of the new shoots onto fresh medium. Data were collected three times at a two month interval and subjected to Mean ± Standard Deviation.

2.4. Rooting

Elongated shoots derived from nodal explants were transferred to MS and B_5 medium supplemented with different concentrations of IAA, IBA and NAA for their rooting. The rooting medium consisted of MS and B_5 with 3% sucrose and solidified with 0.8% of agar. Indole-3-butyric acid (IBA) was tested either alone (at 0.5, 1.0, 1.5, 2.0 mg l^{-1}) or in combination with BAP (0.5 mg l^{-1}).

The aseptic shoots were cut into single nodes with their respective 2 leaves and placed, randomly distributed, into the different proliferation media. In general, from each shoot 4-5 nodes were obtained. The cultures were initially maintained for 6 days under dark and then exposed to light and temperature as mentioned above. Eight shoots were inserted with their basal endes into the medium. Multiple shoots were rooted by following the procedure of Akram and Aftab [29]. All experiments were repeated two times. The percentage of rooted shoots, the total number of roots and root length for each rooted microcutting were evaluated after 4 weeks of culture on the rooting medium.

2.5. Micropropagation and acclimatization

The regenerated young plants were removed from the culture vials and washed thoroughly with tap water. They were acclimatized in a culture room a mixture of soil, sand, and farmyard manure (1:1:1 v/v) for a period of two weeks. Initially plantlets were covered with a polyethylene film, which was gradually eliminated in two weeks time. Plants were transferred to pots and placed in a greenhouse (28±2ºC, RH 90%) for completing their acclimatization. The hardened plants were transferred to a greenhouse before transferring them in to the field. Photographs were taken using a Nikon F3 camera with a macro lens with or without a bellows attachment.

2.6. Data analysis

A completely randomized design was used for all experiments. Callus formation, multiple shoot initiation, root induction and plantlet regeneration were carefully calculated based on the number of explants used. Data of all experiments were statistically analyzed and expressed as Mean ± Standard Deviation.

[III] RESULTS

3.1. Callus formation

In vitro response of teak explants such as node and shoot tips were cultured on MS and B_5 medium with 3% sucrose, 0.8% agar and 0.3% activated charcoal and without charcoal. The results of two explants cultured on two different culture media to identify the better media for mass propagation of teak. The explants were inoculated in the medium supplemented with auxin (2,4-D, IAA, NAA) alone at the concentration of 0.5-2.0 mg l^{-1}. Shoot tip and node explants were respond the same frequencies in all different hormones treatment. Callus induction, multiple shoots initiation and plantlet regeneration were presented in **[Table-1]**.

About 90% of the node and shoot tip explants remained aseptic without any contamination and remaining of the 10% of the explants were contaminated by either bacteria or by over secretion of phenolic compounds. Three to five days after incubation on establishment medium, the node and shoot tips were started to respond first to callus formation then shoot induction. Percentage of callus initiation was calculated based on number of explants inoculated. Callus initiation and proliferation was noticed in all explants on both medium with and without activated charcoal. Maximum percentage of callus formation was observed in B_5 medium with activated charcoal supplemented with 2,4-D 2.0 mg l^{-1} (88.6%), followed by 70.5% in 1.5 mg l^{-1}, 42.8% in 1.0 mg l^{-1} and 31.5% in 0.5 mg l^{-1} than MS medium (85.5%), followed by 66.7%, 39.2% and 27.6%, respectively **[Fig. 1a-b]**.

When compare to other hormones treatments in both media BAP and NAA (0.5 to 2.0 mg l^{-1}) showed less percentage of callus formation. Increasing the concentration of hormones, the response of cultures also increases upto 2.0 mg l^{-1} above the concentration explants and cultures responds abnormally.

Very limited amount of callus formation (23.8%) was observed in IAA 2.0 mg l^{-1} without activated charcoal amended. Well-developed callus from both explants were sub-cultured on medium supplemented with same concentration of hormone supplemented as well as in combination

of BAP (0.5-2.0 mg l^{-1}). After three to five sub-culture, the nodular calli were turned to as organogenic calli **[Fig. 1c]**. From these organogenic calli multiple shoots were developed by subsequent subcultures.

3.2. Multiple shoots formation

Initially multiple shoots were developed from the organogenic calli as well as shoot tip cultures after second passage. Two shoots per node, four to five shoots shoots were produced in shoot tip explants and all the shoots were able to elongate in subsequent cultures. The multiple shoots initiation and multiplication was observed in medium supplemented with BAP (1.0-2.0 mg l^{-1}) in combination of NAA and IAA (0.5-1.0 mg l^{-1}) with and without activate charcoal.

After inoculation of nodal as well as shoot tip explants were showed the high intensity for phenolic compounds in all cultures were observed. In most cultures, exudation of phenolic compounds from the nodal explants turned the medium completely brown and nodal explants thus could not survive and turned necrotic under the experimental conditions. Maximum number of shoot (83.4%) were induced on B_5 medium supplemented with BAP 2.0 mg l^{-1} and NAA 1.0 mg l^{-1} and activated charcoal, and (80.6%) in without charcoal.

This result was compared to MS medium cultures on same hormone concentration treatments showed 79.8% and 71.5%, respectively. Similarly the initiation of multiple shoots was also achieved in other combination of BAP (1.0-2.0 mg l^{-1}) in combination of IAA (0.5-1.0 mg l^{-1}) with and without activated charcoal **[Fig. 1d-f]**. The frequency of shoot formation was less (63.2% and 58.4%) in B_5 medium with and without charcoal and in MS medium with same treatment showed in 62.4% and 53.5%, respectively **[Table-1]**.

However the latter appeared more suitable for proliferation since it showed the highest number of shoots per nodal segment and relatively high number of nodes per shoot, giving the highest multiplication factor reported for teak in the literature. The shoots derived from this medium could be rooted or cut into single node segments to continue the shoot multiplication cycle. In this paper provided a protocol for improve an average of shoots per explant in 8 weeks, but the shoots required further subculture onto elongation medium. In an attempt to save time by avoiding an additional subculture step, we used GA_3 in the proliferation medium to produce shoots with longer internodes therefore, preparing the new shoots to be used for rooting, and at the same time, the basal callus size was reduced.

3.3. Rooting

After four to five weeks of shoot cultures were transferred on the rooting medium, rooting took place only in the presence of auxin. For rooting of shoots two different medium (MS and B_5 medium) supplemented with two axins IBA and NAA (0.5-1.0 mg l^{-1}) alone in different concentrations. When compared to medium as well as auxin treatments the best rooting was observed in IBA 1.0 mg l^{-1} in B_5 medium (65.4%) than NAA 1.0 mg l^{-1} in MS medium (41.2%). Rooting was significantly affected with the increasing concentration of either IBA or NAA. IBA alone induced 60% rooting depending on its concentration, producing swollen and brittle roots.

Rooting reached 100% when medium alone without activated charcoal **[Fig. 1g]**. Callus formation at the base of shoots increased with increasing concentrations of IBA (1.5-2.0 mg l^{-1}). The number of roots per explant was low or absent with the lowest IBA (0.5 mg l^{-1}) concentration. The use of a low concentration of IBA is advisable since it does not interfere with shoot growth, root elongation and keeps the basal callus **[Fig. 1h]**.

However, it induces only a few thin roots without lateral ramifications. Roots emerged at the base of shoots after 2 weeks. An additional 2 weeks on this medium increased the number of roots and promoted shoot elongation.

3.4. Micropropagation and acclimatization

After root initiation, the regenerated plantlets **[Fig. 1i]** were removed from the culture vials and washed with sterile distilled water to remove agar sticking in the roots. Plantlets with fully expanded leaves and well-grown roots (21 d) were transferred to polythene bags containing sterile soil, sand, and farmyard manure (1:1:1 v/v). *In vitro* raised plantlets were regenerated on liquid MS medium supplemented with 0.5 mg l^{-1} of NAA and acclimatized on vermiculate before transferring them to field. Shoots were successfully rooted and acclimatized to greenhouse conditions.

The plantlets were acclimatized first in a mist chamber and then in polythene bags in a After 3 months, plants were transferred to field conditions. This method has been resulted in 97.9% survival of the plantlets. For acclimatization of micropropagated plants were kept under 16/8 photoperiod at 26±2°C and regularly poured half strength MS solution for one month. Relative humidity was maintained at 75-80% by covering them with polythene bags. All the transferred plant were then hardened and later established in the field successfully.

[IV] DISCUSSION

Teak explants of nodes and shoot tips were cultured on MS and B_5 medium with 3% sucrose, 0.8% agar and 0.3% activated charcoal and without charcoal for callus initiation, multiple shoot formation and plantlet regeneration with suitable hormones. Some of the explants were contaminated by either bacteria or by over secretion of phenolic compounds. Callus initiation and proliferation was achieved in activated charcoal supplemented with 2,4-D. similarly stem callus proliferation was obtained on MS medium supplemented with 2.0 mg l^{-1} of 2,4-D in *Withania somnifera* [30].

Maximum callus growth was noticed within 6 weeks of culture in MS medium supplemented with BAP 2.0 mg l^{-1} and KIN 1.0 mg l^{-1} in *Acacia catechu* [31]. Plant hormone 2,4-D has been found very effective callus formation and organogenesis in *Saccharum officinarum* variety Co 740 [32]. However, culture browning and therefore necrosis remains a serious threat during micropropagation of teak using tissues derived from mature trees. Phenolic compound exudates cause necrosis and death in some explants in *in vitro* condition. It is, hence, a prerequisite to remove these compounds from explants before culturing to avoid medium darkening [33]. The process of teak culture establishment in this experiment correlated with Devi et al. [10]; Gyves et al. [34] and Gupta et al. [35], who reported that axillary shoots developed after 7-10 days and with Tiwari et al. [12] Thomas [17] who observed axillary shoots after 6 weeks on several nodes.

The multiple shoots initiation and multiplication was observed in medium supplemented with BAP (1.0-2.0 mg l^{-1}) in combination of NAA and IAA (0.5-1.0 mg l^{-1}) with and without activate charcoal. From the results of the present investigation, it is evident that shoots from large stem segments is a strong possibility for recalcitrant woody plant species of tropical origin. It is well known that the woody tree species of temperate or tropical origin respond differently at different temperatures and the optimum conditions including temperature may vary from species to species. Usually, the optimum temperature is lower for temperate or higher for tropical tree species [36].

In the present study, the greenhouse and its warmth thus remained one of the key factors in order to promote more number of shoots that may simply be due to the fact that “teak grows best in warm, moist tropical climates” seems to have been triggered by the high relative humidity [37]. The process of teak culture establishment in this experiment coincided with Devi et al. [10], who reported that axillary shoots developed after 7-10 days and with Tiwari et al. [12] who observed axillary shoots after 6 weeks on several

nodes. According to the literature, BAP has been the most commonly used in micropropagation of teak alone or with kinetin [38, 39]. However, high concentrations of BAP can be inhibitory to the growth of axillary bud sprouts and can present some risks of unexpected abnormalities of new shoots after this treatment such as vitrification. The synergistic effect of cytokinin and auxin has been re-ported in teak [7]. Activated charcoal provides darkened environment, adsorption of inhibitory substances and release of growth promoting substances in the medium [15]. Shirin et al. [6] also achieved highest number of multiple shoots on BAP and NAA using nodal explants from mature teak shoots. Gangopadhyay et al. [40]; Nissen and Sutter [14] carried out rooting in a liquid medium supplemented with IBA and IAA, but the percentage of the survival of plants after transplantation was relatively low. The best rooting was observed in IBA in B_5 medium than NAA in MS medium. Rooting was significantly affected with the increasing concentration of either IBA or NAA. Our results offer better performance in terms of rooting percentage and plant survival and rapid growth. Singh et al. [41] (2006) used coppice shoots that were rooted with IBA and thiamine with 38.3% rooting response. In all the above reports, only IBA was used for rooting of coppice as well as mature shoot cuttings of teak. Activated charcoal under long photoperiod condition induced microtuberization of potato plantlets. The positive effect of AC on microtuberization might be due to adsorption of ethylene (a strong inhibitor for tuberization) and reduction of light in root medium. Reduction of light at the base of a shoot can provide a conductive environment to the accumulation of photosensitive auxin or cofactors [42, 15]. Similarly, activated charcoal enhanced shoot growth in both light and dark conditions. Some properties of this compound includes inhibition of sucrose hydrolysis during medium autoclaving and its ability to adsorption of phenolic compounds released from explants [15], which maybe contribute the promotion of shoot growth. Our results showed that more than 800 shoots can be obtained in a similar period of time, through the dissection of the shoots into nodal explants avoiding a further subculture onto shoot elongation medium to prepare shoots for rooting phase. A protocol for micropropagation of teak using MS basal medium supplemented with BAP and kinetin was suggested by Goswami et al. [39].

[V] CONCLUSION

This study provides an efficient *in vitro* propagation method which could be commercially feasible for teak, by providing a protocol for producing genetically uniform plants from selected genotypes. Investigations were showed the highest number of micropropagated shoots reported for teak, up to now, in the literature and in a relative short period of time, producing about 4 shoots with 4 nodes within 4 weeks. Cytokinin in combination with low concentration of auxin, in rooting medium, was essential in stimulating high rooting percentage with high quality of roots, resulting in fast growing plantlets during acclimatization phase, reaching 100% of plant survival. The hormones treatment method of their administration, resulting in a reduction of the process time from the usual 4-5 months reported in the literature to less than 3 months including the greenhouse acclimatization period.

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Table-1. Effect of plant growth regulators in MS and B_5 medium for callus induction and plantlet regeneration from node and shoot tip explants.

Hormones (mg/l)	MS medium				B_5 medium			
	Without AC		With AC		Without AC		With AC	
	% callus	Ave. callus	% callus	Ave. callus	% callus	Ave. callus	% callus	Ave. callus
2,4-D 0.5	23.8	44.5±5.2	27.6	57.8±4.2	28.3	48.5±2.1	31.5	61.4±5.2
1.0	32.5	60.6±4.1	39.2	62.3±5.1	36.2	64.3±3.5	42.8	66.3±2.3
1.5	54.1	72.4±6.3	66.7	74.5±6.3	60.4	75.9±5.5	70.5	79.2±4.6
2.0	72.6	86.8±5.7	85.5	89.9±6.7	80.2	90.1±6.8	88.6	95.5±6.8
BAP 0.5	18.7	8.1±0.4	20.2	10.2±1.0	23.1	12.5±1.2	24.6	14.6±1.2
1.0	26.5	9.5±0.7	30.1	13.4±1.1	31.3	14.8±1.3	34.5	14.2±1.0
1.5	45.2	11.4±1.0	49.2	14.5±1.3	50.2	18.9±1.5	53.7	22.1±1.4
2.0	48.4	13.5±1.2	53.5	19.9±2.7	58.6	22.4±2.5	60.4	26.3±1.6
NAA 0.5	4.7	18.5±1.2	7.7	21.2±2.2	11.5	23.1±2.1	12.1	27.1±2.3
1.0	9.5	21.6±2.1	24.3	24.3±2.1	25.3	25.1±2.3	28.5	29.5±2.1
1.5	15.4	24.4±2.3	36.6	27.5±2.3	32.4	28.6±2.4	40.1	33.4±3.0
2.0	31.2	26.8±3.2	49.8	30.9±2.7	37.9	30.2±2.2	54.5	35.0±3.2
IAA 0.5	7.6	15.3±1.4	11.8	21.2±2.2	10.2	19.4±1.6	15.8	25.4±2.3
1.0	18.3	16.4±1.7	25.4	24.3±2.1	25.4	20.7±1.8	29.5	26.4±2.1
1.5	22.2	19.4±2.0	32.7	27.5±2.3	33.7	23.6±2.2	36.4	27.6±2.3
2.0	23.8	21.5±2.2	39.3	30.9±2.7	41.8	25.6±2.4	44.9	30.1±2.5
BA 1.0 + NAA 0.5	22.3	32.5±3.2	28.2	47.8±4.1	31.8	35.6±3.4	33.6	40.6±3.8
BA 1.0 + NAA 1.0	36.4	40.6±4.1	42.4	52.3±5.8	44.6	45.0±4.6	46.1	50.4±4.6
BA 2.0 + NAA 0.5	45.8	52.4±5.3	52.5	64.5±6.9	51.5	56.2±3.6	58.6	61.5±5.8
BA 2.0 + NAA 1.0	71.5	66.8±5.7	79.8	79.9±7.7	80.6	70.5±6.5	83.4	74.9±6.8
BA 1.0 + IAA 0.5	17.6	27.2±2.2	23.7	30.7±3.5	23.4	31.2±3.0	27.3	35.6±3.3
BA 1.0 + IAA 1.0	32.9	29.3±3.1	38.9	32.3±3.8	38.2	33.5±3.1	42.5	39.4±3.5
BA 2.0 + IAA 0.5	40.4	32.4±3.3	46.6	34.5±3.9	49.5	36.9±3.3	51.8	50.8±3.6
BA 2.0 + IAA 1.0	53.5	46.8±3.4	62.4	59.9±3.7	58.4	40.5±3.9	42.1	63.2±4.2

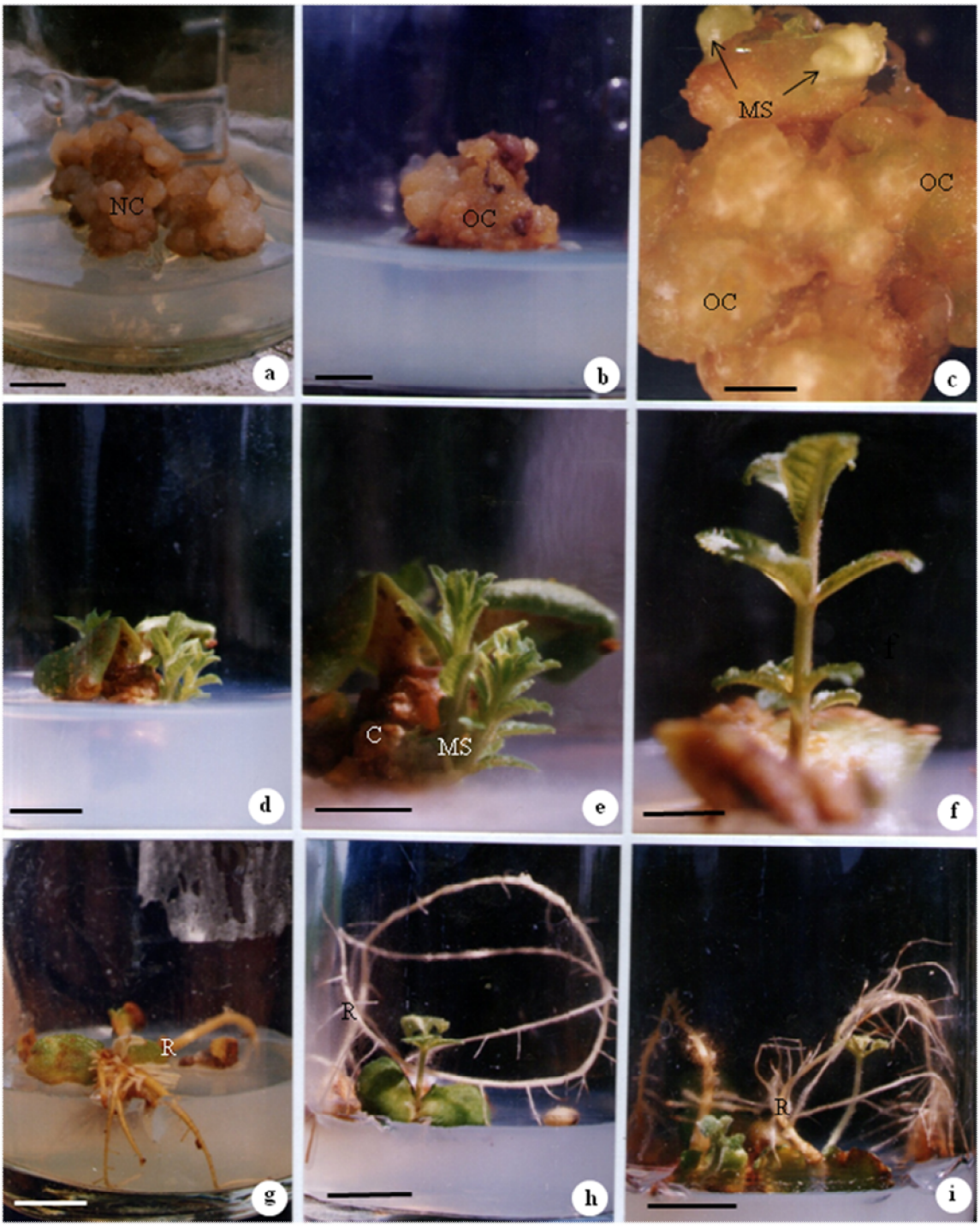

Figure-1. Callus induction, multiple shoot and plantlet regeneration from node and shoot tip explants. a. Nodular callus developed from node. b. Callus proliferation from shoot tip. c. Multiple shoots initiated from shoot tip derived organogenic callus. d. Multiple shoots developed along with callus from *in vitro* derived multiple shoots. e. Well-developed multiple shoots after 3 subcultures. f. Isolated individual shoot on rooting medium. g-h. Initiation and branched root development from single shoot. i. *In vitro* regenerated plant on regeneration medium. (bars in a-d: 1 cm) (C-callus, OC-organogenic callus, NC-nodular callus, MS-multiple shoots, R-root).