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# Regeneration of Complete Plantlets from Callus Culture of *Azadirachta indica* A. Juss using Immature Flower Buds

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**Abstract**
Plant regeneration via callus culture was achieved using immature flower buds of mature tree of *Azadirachta indica* A. Juss on semi-solid MS medium enriched with 9.05µM 2,4-D + additives. The regenerated callus were amplified on lower concentration of 4.52 µM 2,4-D + additives for micro-shoot regeneration study, the best micro-shoot regenerated from callus was achieved on MS medium supplemented with 8.88 µM BAP with 3.78±0.50 numbers of shoot and 3.66±0.57 shoot length. The micro-shoots were amplified on 8.88 µM BAP+ 0.27 µM NAA with 5.22±0.53 numbers of shoot and 4.62±0.59 shoot length respectively. Micro shoot were successfully rooted on MS medium supplemented with IBA 4.92 µM with rooting percent (100%), 4.35±0.22 root number and 3.95±0.23 root length. The callus derived plantlets were hardened *in vitro* and *ex vitro* and acclimatized in poly house followed by agro-shade house. The efficiency of the protocol will be helpful for propagation of *Azadirachta indica* plants on a mass scale.
Abbreviations: MS: Murashige & Skoog, 2, 4-D: 2, 4-dichlorophenoxy acetic acid, BAP: 6-Benzyl aminopurine, NAA: α-napthalene acetic acid and IBA: indole-3-actic acid.
**Keywords**:*Azadirachta indica*, flower buds, callus culture.

## Introduction

Neem (*Azadirachta indica*) is a versatile Indian tree of great importance. Neem is an untapped natural resource as far as its genetic potential is concerned. *Azadirachta indica* is one of the most valuable arid zone trees belonging to the family Meliaceae. A native of dry forest areas of India and the subcontinent, it is widely cultivated in the arid, nutrient-deficient regions of India and Africa. Besides being a popular avenue tree with a large crown, the wood of neem has been used as timber for house building, furniture and other domestic and agricultural tools. The timber is reported to work well with hand and machine tools (Tewari 1992).

Its wood resembles teak wood in strength and is more resistant to shock, fungi and insect attack; it is immune to termites and durable even outdoors (Thengane, 1995). Neem tree is known to increase the soil fertility and its water holding capacity as the tree has a unique property of calcium mining which changes the acidic soil into neutral. The tree is resistant to high temperatures and drought and has been employed for afforestation of dry localities, reforesting bare ravines and checking soil erosion (Gill et al., 1996). Besides, various parts of the neem tree, particularly leaves, bark and seeds have been traditionally used in India in ayurvedic medicines. The seed oil has been used as antimalarial, anthelminthic, vermifuge, antiseptic, antimicrobial and is also known to cure various skin disorders.

*Azadirachta indica* A. Juss is renowned for its insecticidal properties and neem seed extracts show great potential as environmentally acceptable bioinsecticides for crop protection (Jacobson 1988; Mordue (Luntz) and Blackwell 1993; Schmutterer 1990). The first commercial neem insecticide, Margosan-O, was registered by the Environmental Protection Agency (EPA) in 1985 for use on non-food crops (Jacobson 1988; Larson 1993) and since then the number of commercial and experimental neem insecticides has increased markedly with Azatin (Agridyne Technologies, USA) recently receiving EPA approval for use on food crops (Johnson et al. 1994). Neem owes these properties due to the presence of several bioactive compounds; the most prominent one being Azadirachtin.

In order to meet the economic demand of the neem tree, an efficient propagation technique is required which could result in large quantities and good quality of planting materials. Vegetative propagation of an adult neem tree by conventional methods is difficult (Kaushik 2002). Therefore, it is normally grown from seeds but the seeds are of recalcitrant type; they loose its viability within 2-3 weeks (Mohan et al. 1996). Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant owing to cross-pollination and enormous heterozygosity. Moreover, the reproductive phase in neem normally begins after 5 years of seed propagation (Koul et al. 1990 and Schmutterer 1995). So, one has to wait for a long time to obtain seeds and fruits.

Micropropagation method would provide the best means for mass clonal propagation of selected elite trees, where as tissue culture is the only methodology that can produce a large quantity of clonal plants in short time with high phytosanitary quality (Silva et al. 2014). Azadirachtin, which shows antifeedant and growth-regulatory activities against a wide range of insects, is the major bioactive chemical present in neem kernels (Ley et al. 1993) with the average yield from seeds being around 2–4 mg $g^{-1}$ dry weight seed kernels (National Research Council 1992). Azadirachtin yields are, however, variable, being dependent on plant ecotype and environment (Ermel et al. 1986; Singh 1986). The development of simple and rapid methods for the production of uniform neem populations with high azadirachtin yields is desirable.

To meet the economic demand of the neem tree, an efficient propagation technique is required which could result in large quantities and good quality of planting materials. Vegetative propagation of an adult neem tree by conventional methods is difficult (Kaushik 2002). Therefore, it is normally grown from seeds but the seeds are of recalcitrant type; they loose viability within 2-3 weeks (Mohan et al. 1996). Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant owing to cross-pollination and enormous heterozygosity. Moreover, the reproductive phase in neem normally begins after 5 years of seed propagation (Koul et al. 1990 and Schmutterer 1995).

So, one has to wait for a long time to obtain seeds and fruits. Micropropagation method would provide the best means for mass clonal propagation of selected elite trees in a short period of time. Due to the difficulty in germinating

neem seeds, *in vitro* propagation techniques are important for this plant, as they allow for selection and rapid multiplication of high-value phenotypes (Quraishi et al. 2004). *In vitro* propagation of neem has been reported via both direct (Ramesh and Padhya 1990; Joshi and Thengane 1996; Zypman et al. 1997; Eeswara et al. 1998) and indirect (Zypman et al. 1997; Salvi et al. 2001) morphogenetic pathways.

In present investigation, the main objective of this study was to establish a procedure which can be used routinely to produce complete micropropagated plantlets from neem immature flower buds explants of plants from mature trees.

## Materials and methods

### Plant Materials

*Azadirachta indica* (Neem) plants selected on the basis of phenotypically and morphologically characters. In present investigation, immature flower buds used as explants for culture initiation and regeneration of plantlets. Immature Flower buds collected from mature trees located at Forest Genetics and Tree Breeding Division, Arid Forest Research Institute, Jodhpur during flowering season in April to May 2012.

### Explants Preparation and Surface Sterilization

Immature flower buds collected from mature trees were washed under running tap water to remove dirt and superficies impurities. Explants washed in RO water (Milipore RiOSS) having 2-3 drops of Tween 80 for gentile shaking for 5-10 mint, then rinsed with autoclaved RO water for 3-4 times. In aseptic condition, explants treat with 0.1% (w/v) Bavestin (BASF India limited, Mumbai, India) and 0.1% (w/v) streptomycin for 8-10 min to reduce the chance of fungal contamination. After treatment rinsed with autoclaved RO water for 3-4 times. Finally treat with 0.1% (w/v) $HgCl_2$ for 6 min for surface sterilization of explants. After treatment rinsed with autoclaved RO water for 3-4 times.

### Nutrient Media and Culture Conditions

Murashige and Skoog's (MS: Murashige and Skoog 1962) medium supplemented different concentration 0.45, 2.26, 4.52, 9.05 and 18.1 µM) of 2, 4-D (2,4-Dichlorophenoxy acetic acid; Duchefa biochemie, Postbus, Netherlands) with sucrose (3%) and additives (567.76 µM ascorbic acid, 260.24 µM citric acid and 484.38 µM adenine sulphate) was used for culture initiation (Figure 1A). Agar-agar (Hi-media, India) was added to media as gelling agent at the concentration of 0.6% (w/v). The pH of the medium adjusted to 5.8±0.2 with 1N NaOH and medium was autoclaved at 15 psi and 121°C for 15 min. the culture initiation and amplification were done aseptically in laminar air flow hood. Culture were incubated in tissue culture racks in aseptic growth room having a temperature of 26±2°C in 16 H light and 8H dark photoperiod and 1600 lux intensity light via white cool florescent tube (Philips, India).

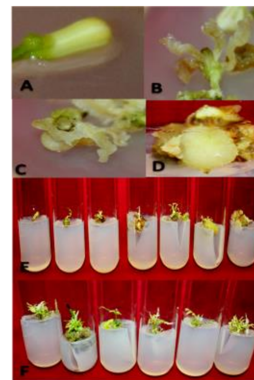


Figure 1. Callus induction and regeneration of micro-shoots of Azadirachta indica. A Immature flower bud inoculated on MS medium supplemented with 2, 4-D. B & C Callus induction on MS medium supplemented with 9.05 µM 2, 4-D+additives from sepals 0f flower buds, D Callus amplification on MS medium supplemented with 4.52 µM 2,4-D+additives, E&F Regeneration of micro-shoot from callus on MS medium 8.88 µM BAP +additives.

### Callus Induction

Immature closed flower buds were used as explants material (Figure 1A). During the process, development of callus from the flower buds. The swelling of flower buds starts with the development of callus all along the sepals. During the formation of callus the swelling of callus was observed resulting into the opening of flower buds. However the callus development occurred only through the development of sepals (Figure 1B&C).

Once the callus development process initiated the remaining parts of the immature flower buds starts drying living behind brown colored that tissue. The callus initiated from sepals grows rapidly within two weeks. Into two weeks sufficient callus which was latter subculture on MS medium supplemented with lower dose of 2, 4-D (4.52µM). On this medium optimal callus growth was obtained and callus was maintained subsequently on this callus multiplication medium (MS+2,4-D 4.52µM). (Figure 1B, C, D). Cultures were incubated in dark.

### Regeneration of Micro-shoots and their multiplication

For shoot regeneration studies, small piece of regenerated calli were subculture on MS medium supplemented with different concentration (0.44, 2.22, 4.44, 8.88 and17.75 µM) of BAP (6 – Benzylamino purine, Duchefa biochemie, Postbus, Netherlands) + additives. After 2 -3 weeks of culture small green spots (shoot primordia) appears on the calli. The callus piece with clearly differentiated shoots and leaves of approximately 1.5 – 2.5 cm in length and 3-4 leaves was scored as regenerating callus (Table 2 and3). Micro Shoot was amplified on MS medium supplemented with BAP (0.44, 2.22, 4.44, 8.88 & 17.75µM) in combination with NAA (0.27 µM) (Figure 1E,F & 2 A,B).

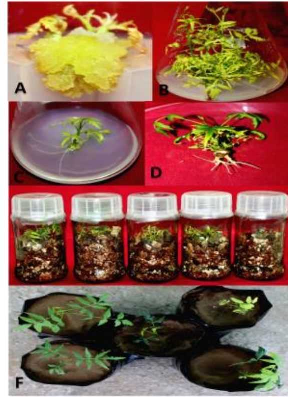


Figure 2. A Regeneration of micro-shoots on MS medium 8.88 µM BAP +additives. B Multiplication of micro-shoots on MS medium 8.88 µM BAP +0.27µM NAA+additives, C &D in vitro rooting on 4.92 µM IBA +additives, E Hardening and acclimatization of in vitro raised plantlets in vermiculite, F Regenerated plantlets in poly beg.

***In vitro rooting of micro-shoots***

*In vitro* regenerated micro-shoots from callus culture (longer then 2-4 cm) were harvested from clumps and transfer to medium for rooting. Individual micro-shoots transfer to MS medium enriched with IBA (0.49, 2.46, 3.94, 4.92 & 9.84 µM) + additive (86.85µM Proline & 48.96µM Tryptophan) with sucrose (3%). *In vitro* rooting was best achieved in IBA 4.92 µM after 6 – 10 days on culture.

The *in vitro* rooted micro-shoots were removed from culture vessels and washed with autoclave RO water to remove adhered nutrient agar. These were carefully transferred to jam bottles containing autoclaved vermiculite moistened with ½ MS salts. The bottles were capped and kept for 2 weeks for *in vitro* hardening under growth room conditions. After 2 weeks, jam bottles containing regenerated plants kept in poly house initially near the pad section (RH 80 – 90 % and temperature 28±2°C) in order to harden the plantlets (Figure 2E).

***Hardening and Acclimatization of in vitro regenerated plantlets***

After one week of transfer of *in vitro* raised plantlets to poly house, the caps of jam bottles were gradually opened over a period of 2 weeks and finally removes. Bottles containing plantlets were the shifted from the pad section towards the fan section to provide growing conditions of low humidity (50 – 60%) and high temperature (30±2°C). after 4 weeks, acclimatized plantlets were transferred to poly begs containing a mixture of soil+sand+FYM (Farm yard manure) (2:1:0.5). such plantlets were kept in the poly house for 3 – 4 weeks and then shifted to agro shade house then open environment (Figure2F).

***Experimental design and statistical analyses***

Data were analyzed through one way analysis of variance using statistical package for social sciences (SPSS 8.0). Auxin and cytokinin their concentrations taken as independent (fixed) factor with five levels. Whereas Shoot number, shoot length and rooting percentage (%) were taken as dependent variables. For the analysis, under the null hypothesis, that the dependent variables were not affected significantly by the fixed factors and their interactions. All the significant main effects and their interaction were studies.

Duncan Multiple Range Test (DMRT) $P$ = 0.05 was used to compare the means from main effects. Minimum of 4 replicates with 5 samples (one explant) were taken per treatment and with repetition of experiments thrice. Degree of variations was shown by Mean and standard error. Data given in percentages were subjected to arcsine √X) transformation (Snedecor and Cochran 1967) before statistical analysis.

**Results and Discussion**

The immature flower buds were used as explants for callus induction and regeneration of complete plantlets of *Azadirachta indica.* The experiment was carried out to study the effects of 2, 4-D concentration of callus induction. The maximum callus intensity was observed in MS medium enriched with 9.05µM 2, 4-D + additives (567.76 µM ascorbic acid, 260.24 µM citric acid and 484.38 µM adenine sulphate) after 10-12 days of experiment. The initiated callus were amplified on lower concentration of 4.52 µM 2, 4-D + additives (567.76 µM ascorbic acid, 260.24 µM citric acid and 484.38 µM adenine sulphate) (Table1).

Table 1. Effect of 2, 4-D concentration on *in vitro* callus induction from flower buds of *Azadirachta indica* on MS medium + additives.

| 2,4 – D (µM/l) | Callus Intensity | Remarks |
|---|---|---|
| **Control** | + | Poor Growth, white callus |
| **0.45** | + | White fragile callus |
| **2.26** | ++ | White fragile callus |
| **4.52** | +++ | White fragile callus |
| **9.05** | ++++ | White, Pale, Yellow and responsive callus |
| **18.1** | + | Brown slow growing |

The well-formed micro-shoot were with leaves were regenerated on different concentration of BAP. One way analysis of variance (ANOVA) showed that the BAP highly significant affect the mean shoot number ($F_5$ =5.35, $P<0.05$) and mean shoot length ($F_5$ =7.26, $P<0.05$) (Figure 1) (Table 2).

Table 2. Effect of BAP concentration on *in vitro* micro-shoot regeneration from callus of *Azadirachta indica* on MS medium + additives.

| BAP (µM/l) | Shoot Number Mean±SE | Shoot Length (cm) Mean±SE |
|---|---|---|
| **Control** | $1.14 \pm 0.14^{c}$ | $0.21 \pm 0.01^{c}$ |
| **0.44** | $1.90 \pm 0.18^{bc}$ | $1.27 \pm 0.14^{bc}$ |
| **2.22** | $2.20 \pm 0.13^{bc}$ | $1.61 \pm 0.15^{b}$ |
| **4.44** | $2.38 \pm 0.26^{b}$ | $3.13 \pm 0.35^{a}$ |
| **8.88** | $3.78 \pm 0.50^{a}$ | $3.66 \pm 0.57^{a}$ |
| **17.75** | $2.64 \pm 0.36^{b}$ | $2.31 \pm 0.45^{ab}$ |
| **Mean** | 2.56±0.17 | 2.38±0.22 |
| | F=5.35** | F=7.26** |

Values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test (p ≤ 0.05) Each value represents mean (± standard error).

The results showed that different concentration of BAP significantly affect the plant regeneration parameters i.e. shoot number and shoot length. The highest micro-shoot regeneration was showed on 8.88 µM BAP +additives in terms of 3.78±0.50 shoot number and 3.66±0.57 shoot length. The table 3showed that the different concentration of BAP in combination with NAA showed significant effects on the micro-shoot amplification parameters i.e. shoot number and shoot length. The best shoot amplification was observed on 8.88 µM BAP+ 0.27 µM NAA+ additives with 5.22±0.53 shoot number and 4.62±0.59 shoot length (Figure2).

Table 3. Effect of BAP+NAA concentration on *in vitro* shoot multiplication of *Azadirachta indica* on MS medium + additives.

| BAP + NAA (μM/l) | Shoot Number Mean±SE | Shoot Length (cm) Mean±SE |
|---|---|---|
| **Control** | 1.29±0.18[c] | 0.26±0.03[a] |
| **0.44+0.27** | 2.90±0.28[b] | 2.09±0.19[b] |
| **2.22+0.27** | 3.00±0.37[b] | 2.22±0.49[b] |
| **4.44+0.27** | 3.94±0.55[ab] | 4.13±0.29[a] |
| **8.88+0.27** | 5.22±0.53[a] | 4.62±0.59[a] |
| **17.75+0.27** | 3.93±0.57[ab] | 3.32±0.63[ab] |
| **Mean** | 3.73±0.25 | 3.21±0.25 |
| | F=5.49** | F=8.21** |

Values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test (p ≤ 0.05) Each value represents mean (± standard error).

The individual micro-shoots, regenerated from callus, were tested for their ability to form roots. The rooting treatment consisted of MS medium supplemented with IBA. One way analysis of variance (ANOVA) showed that the IBA highly significant affect the mean root number ($F_5$ =21.22, $P<0.05$) and mean root length ($F_5$ =30.02, $P<0.05$) (Figure 3).

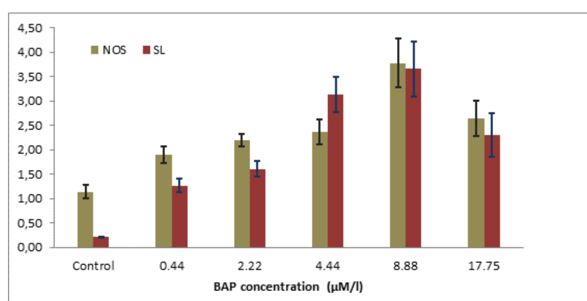


Figure 3. Regeneration of micro-shoot on MS medium supplemented with different concentration of BAP+Additives.

The results showed that different concentration of IBA significantly affect the plant regeneration parameters i.e. root number and root length. The highest rooting in micro-shoot was showed on 4.92 μM IBA +additives (86.85μM Proline & 48.96μM Tryptophan) with 100% rooting percent, 4.35±0.22 root number and 3.95±0.23 root length (Table 4).

Table 4. Effect of IBA concentration on *in vitro* rooting of micro-shoot of *Azadirachta indica* on MS medium + additives.

| IBA (μM/l) | Roting% | Root Number | Root Length (cm) |
|---|---|---|---|
| **Control** | 10.00±5.77 (13.92)*[d] | 1.50±0.50[b] | 0.30±0.00[c] |
| **0.49** | 20.00±0.00 (26.56)[c] | 2.00±0.00[b] | 0.48±0.11[c] |
| **2.46** | 55.00±5.00 (47.89)[b] | 2.18±0.18[b] | 1.01±0.06[c] |
| **3.94** | 70.00±5.77 (57.11)[b] | 2.36±0.17[b] | 2.20±0.25[b] |
| **4.92** | 100.00±0.00 (88.72)[a] | 4.35±0.22[a] | 3.95±0.23[a] |
| **9.84** | 80.00±0.00 (50.77)[b] | 3.75±0.19[a] | 3.81±0.25[a] |
| **Mean** | 55.83 (47.49) | 3.21±0.15 | 2.75±0.19 |
| | F=80.13** | F=21.22** | F=30.02** |

*Arc Sine values in parentheses. Values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test (p ≤ 0.05) Each value represents mean (± standard error).

The neem callus growth was remarkably affected by growth regulator type and concentration. While there was a little extension growth of the explants, no callus growth was observed during the culture period in the basal medium without growth regulator (Control). However, callus developed on the surface of the immature flower bud cultured on MS basal medium supplemented with varying concentrations of 2, 4-D.

Unlike previous reports on tissue culture of neem (Shrikande et al. 1993; Su et al. 1997; Murthy and Saxena 1998), the present report demonstrates for the first time a highly reproducible callus culture production system from immature flower bud tissues collected from mature tree. Exogenous supply of PGR inhibited the callus induction process in immature flower buds, whereas inclusion of auxin (2,4-D) in the medium was necessary for inducing callus in neem. At present there is no report on callus culture from immature flower buds. Callus induction was found to be affected by the source of the original explants. The immature cotyledon explants (i.e. those from the small, pale green fruits) turned black and dried out after two weeks with essentially no callus formed on all the callus induction media tested. When moderately mature cotyledons (yellowish-green, 7 to 12 mm in length) or hypocotyls segments were used, callus formation was achieved in all hormone combinations. The amount of callus formed and their morphological characteristics, however, vary substantially with the auxin used. IAA induced compact nodular calluses, which was also noted in the study of Shrikhande et al. (1993). In comparison with the IAA calluses, 2,4-D callus were very watery, unorganized and proliferated more rapidly (1 week vs. 3 week incubation to obtain ca. 2–3 mm thick callus on the hypocotyl explants). Calluses formed with NAA were more compact than those with 2, 4-D and growth was not as fast.

Several studies have been undertaken to develop tissue culture protocols for neem. *In vitro* multiplication of neem was achieved by using nodal segments (Drew 1993; Yasseen 1994) and leaf explants (Eeswara et al. 1998). Plant regeneration in neem was reported from callus derived from leaves (Narayan and Jaiswal 1985), anthers (Goutam et al. 1993) and cotyledons (Muralidharan and Mascarenhas 1989). Roots could be an excellent source of explants for rapid multiplication of neem through cyclic and continuous production of seedlings. However, use of roots as explants has rarely reported. In this paper, regeneration efficiency of shoot tip and root tip explants has been discussed for rapid multiplication of neem. In an early study on production of nimbin in *A. indica* callus cultures, Sanyal et al. (1981) demonstrated that IAA stimulated formation of roots in the neem calluses. Schulz (1984) described callus initiation from young neem leaves and petioles and subsequent shoot formation. Ramesh and Padhya (1990) reported *in vitro* propagation of *A. indica* via production of adventitious shoot buds from leaf discs. Development of shoots and roots in anther-derived callus of *A. indica* was demonstrated by Gautam et al. (1993). In a recent study, Joshi and Thengane (1996) reported *in vitro* propagation of neem by shoot proliferation.

Application of neem callus cultures for the production of useful secondary metabolites such as azadirachtin has been studied by Kearney et al. (1994) and Allan et al. (1994). The secondary metabolites from *A. indica* possess no known acute mammalian toxicity, and they are biodegradable (Mordue (Luntz) and Blackwell 1993). To the authors' best knowledge, the only report on successful neem regeneration via somatic embryogenesis was by Shrikhande et al. (1993). In that study, somatic embryos were induced from immature cotyledonary tissues via indirect somatic embryogenesis, and the experiments were conducted using semisolid media. No information was given on suspension culture of neem somatic embryos. In this paper, a new culture protocol modified from that of Shrikhande et al. (1993) is discussed for the induction of somatic embryogenesis in *A. indica* which involves the use of both agar and liquid media.

Moreover, callus culture is an excellent tool to induce somaclonal variation can be useful source of new variation for genetic breeding (Carvalho et al. 2013), and mainly to obtain strains more efficient to production of secondary metabolites.

**Conclusion**

The present experiments have shown that it is possible to induce shoot differentiation and complete plantlet development from immature flower bud explants of neem. The neem callus growth was remarkably affected by growth regulator type and concentration. While there was a little extension growth of the explants, no callus growth was observed during the culture period in the basal medium without growth regulator (Control). However, callus developed on the surface of the flower bud cultured on MS basal medium supplemented with varying concentrations of 2, 4-D.

The system developed may be used for rapid mass multiplication of elite Neem trees may be for high producing neem oil or for high azadirachtin contents. Since the production of neem trees are through sepals and flower pedicle the superior characters may be inherited into the progenies. However clonal variation may be occur due to the intermediate callus phase, which may not be ruled out. Rapid replication or propagation of mature trees (25-30 year old) is very difficult and present protocol describes a means for propagation of mature neem. Tree of any age which produce flower may be propagated through this protocol.

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