DE NOVO SHOOT AND ROOT ORGANOGENESIS IN LEAF DISC CULTURES OF A*ZADIRACHTA INDICA* A. JUSS.

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ABSTRACT

Leaf explants of *Azadirachta indica* A. Juss. were obtained from in vitro maintained plantlets established from nodal segment cultures of a 35-year-old tree. Leaf discs of uniform size were made through 5mm size cork borer and planted on Murashige and Skoog (MS; 1962) medium supplemented with either cytokinin (BAP/Zeatin/TDZ Kinetin) or auxin (NAA/IAA/IBA) at a fixed concentration of 5 µM. MS basal medium alone did not show any response; presence of at least one auxin or one cytokinin is mandatory for caulogenic induction. Whereas 6-benzylaminopurine (BAP) was found to be most beneficial for shoot organogenesis in these calli, root organogenesis could be achieved on -naphthaleneacetic acid (NAA) supplemented medium. Thus, the kind of growth regulator has a significant effect on shoot/root organogenesis in these differentiating calli. On MS + 5µM BAP, compact, brown and dark-green callus was developed at the cut end of the explants, after 5 weeks, in more than 76% cultures, which subsequently redifferentiated into an average of 7 green shoots per explant after 10 weeks. Shoots were elongated well at a lower concentration of BAP (0.5µM) and rooted on ¼ MS (major inorganic salts reduced to quarter strength) medium supplemented with 0.5µM IBA. Conclusively, a right presence of auxin or cytokinin has been found obligatory for induction of cells and organogenesis from them.

Keywords: Adventitious shoot proliferation, Leaf-disc culture, Neem, Organogenesis, Regeneration, Totipotency.

1. INTRODUCTION

Plant tissue culture provides an ideal condition to elicit cellular totipotency of plant cells. This unique capacity of plant cells has found many applications in plant improvement, propagation and conservation other than basic and applied plant sciences. It also favors large-scale propagation of plants, irrespective of seasonal and geographical constraints. The present investigation deals with the standardization of procedures to raise uniform plantations of *Azadirachta indica* A. Juss., commonly known as neem, a member of the

family Meliaceae. It is believed to be a native to the Indian subcontinent and Myanmar. Various parts of the neem tree, particularly leaves, bark and fruits have been traditionally used in India in Ayurvedic medicines, and the seed-oil has been used as an antimalarial, antihelminthic, vermifuge, antiseptic, antimicrobial, wound healing agent, and also to cure various skin disorders. The plant possesses insecticidal, antifeedant, growth inhibiting and antifertility activities against a broad spectrum of insects. The tree is well known for its drought tolerance and prevention of soil erosion, thus, is valued for reforestation of degraded land. Natural propagation of neem occurs by seeds which are usually available once or rarely twice in a year. Seeds are recalcitrant and lose their viability after 2-3 weeks, which can be extended to 6-8 weeks by cold storage (Dogra and Thapliyal 1996; Koul et al. 1990; Schmutterer 1995). To overcome such limitations, and to meet the market demand owing to its immense medicinal properties, in vitro micropropagation system is desirable to produce adequate planting material of plus trees. Accordingly, the present work is undertaken to establish an efficient and reproducible protocol for high frequency shoot regeneration via adventitious shoot proliferation from leaf explants of an adult 35-year-old neem tree. A number of factors are involved to oversee this process under *in vitro* conditions; presence of the kind of growth regulators in the medium is one of the critical factors.

2. MATERIALS AND METHODS

2.1. Plant Material

Leaf explants were obtained from in vitro shoots established from nodal segments of a 35-year-old Neem tree. Leaf discs of uniform size were made through 5mm size cork borer and implanted in the culture tube (25mm x 150mm; Borosil, India) with their abaxial side in contact with the medium. Each culture tube consisted of one explant per 20ml of medium.

2.2. Organogenesis and Culture Conditions

Murashige and Skoog (1962; MS) medium containing 30gl^{-1} sucrose and solidified with 8 gl^{-1} agar was used throughout the experiments. The medium was supplemented with either cytokinin, 6-benzylamino-purine (BAP), Zeatin, Thidiazuron (TDZ), Kinetin (Kn), or auxin, α-naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), individually, at a fixed concentration of 5µm for the induction of organogenic callus. The pH of the medium was adjusted to 5.8 before autoclaving at 121^{0}C and 1.06 Kgcm^{-2} for 20 minutes. All the cultures were maintained at 25±2°C temperature and 50-60% relative humidity under a 16/8 hour (light/dark) photoperiod provided by diffuse light (1000-2000 lux). Twenty-four cultures were raised for each treatment and each experiment was repeated at least three times. Observations were recorded at weekly intervals.

2.3. Shoot Elongation, Rooting and Hardening

Regenerated shoots were excised from calli when they reached a size of 1-2 cm and transferred onto MS medium containing 0.5µM BAP for elongation. For rooting, elongated shoots (about 3cm long) were excised, and placed vertically into quarter-

strength MS basal medium (1/4 MS; major salts reduced to $1/4^{th}$ strength) containing 0.5μM IBA.

For elongation and rooting, all the cultures were maintained at 25±2°C temperature and 50-60% relative humidity under a 16/8 hour (light/dark) photoperiod provided by diffuse light (1000-2000 lux). Twenty-four cultures were raised for each treatment and each experiment was repeated at least twice. Observations were recorded at weekly intervals.

After 4 weeks, rooted shoots were washed with water to remove the agar and were transferred to the plastic pots (5cm size) containing a mixture of autoclaved vermiculite, perlite and garden soil (1:1:1) and maintained at standard culture room conditions. The plants were acclimatized by covering the pots with polythene bag to maintain high humidity for 6-7 days. After 7 days, 3-4 small holes were made in the bag and the potting mixture was drenched with half strength MS major inorganic salts through these holes, at frequent intervals. After 25 days, polythene bags were removed and the acclimatized plants were transferred to a shaded area under natural conditions.

3. RESULT AND DISCUSSION

3.1. Callus induction and organogenesis

In the present work, a beneficial effect of auxin and cytokinin had been studied on in vitro organogenesis in neem. Leaf-disc explants were cultured on MS medium supplemented with individual treatment of different cytokinins (BAP/Zeatin/TDZ/ Kn) or auxins (NAA/IAA/IBA) at equimolar concentration of 5μM. In individual cytokinin treatment, Zeatin evoked maximum callus induction (100%) and produced profuse brownish-green, compact callus from all over the surface of the explant. TDZ also responded to callus induction and produced yellowish-brown and compact callus in only 40% of the cultures. On both the media, calli remained unorganized even after 3 subcultures, each of 5 weeks duration. Explants did not respond to kinetin containing medium. Organogenic, brownish-green and compact callus was developed in 76% cultures from leaf disc explants when the medium was supplemented with the cytokinin BAP. On MS + 5μM BAP, the compact calli developed at the cut end of the explants turned into brown and dark-green, nodulated callus after 5 weeks of culture initiation, which subsequently organized into well developed shoots after 10 weeks (**Fig.1A**). On this medium, an average of 7 green shoots were developed per explant.

Although various auxin treatments responded to massive callus proliferation, shoots did not develop from these calli. However, rhizogenesis from calli was observed in medium fortified with 5μM NAA. On MS + 5μM NAA, brown and green, nodulated callus was developed within 3 weeks in 85% of the cultures which later organized into distinct roots (**Fig. 1B**).

Regeneration from leaf explants, derived from adult trees was first reported by Narayan and Jaiswal (1985). Leaf-disc formed unorganized callus on a medium containing 2,4-D + BAP. Calli when transferred to a medium containing BAP produced shoot-buds (5-8 shoots/explant) while those on NAA supplemented medium developed into roots always. This in agreement with the present findings where NAA amended medium supported root organogenesis whereas shoot organogenesis could be achieved on BAP supplemented medium. Further, Narayan and Jaiswal (1985) noticed that addition of NAA ($0.05 mgl^{-1}$) in conjunction with BAP enhanced the frequency of shoot differentiation from 54% to

62.5%. In the present study, we have achieved a significantly better regeneration frequency where 85% of the cultures responded for shoot regeneration from calli in a single induction medium, from leaf discs of a mature 35-year-old tree.

3.2. Micropropagation

The shoots**,** regenerated from leaf callus, did not grow much while attached to the explant. Therefore, 1-2cm long individual shoots were detached from the callus and transferred MS medium supplemented with 0.5µM BAP, for elongation **(Fig. 1C)**. On this medium, shoots attained a height of 5cm with each shoot having an average of 4-5 nodes after 3 weeks.

For rooting, ¼ -strength MS medium supplemented with 0.5µM IBA was tested. On this medium, 79% of the shoots were rooted with 4-5 roots per shoot within 4 weeks **(Fig. 1D)**. By following the protocol as described in material & methods, the plantlets were acclimatized and successfully established in soil **(Fig. 1E)**. They were indistinguishable morphologically from seedlings obtained via sowing.

CONCLUSION

The findings provide a practical approach to satisfy the expected increased demand for large scale planting material of neem. The *de novo* differentiation of adventitious shoots could be achieved from leaf disc explants of a mature 35-year-old tree with a frequency as high as 7 shoots per explant in every 10 weeks. The frequency of micropropagation can be enhanced further by cutting each solitary shoot into 4-5 nodes at the end of multiplication cycle and culturing individual node onto MS + BAP (1µM) + CH (250 mgl^{-1}) medium, a described by Chaturvedi et al (2004), where pre-existing axillary bud may again give rise to a long multinodal shoot. Following this protocol, >68 lakhs plants per annum can be made available starting from a single explant.

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FIGURE

LEAF CULTURE

Shoot regeneration, elongation and rooting

A. 8-week-old culture of leaf disc on MS + BAP (5.0µM) showing shoot differentiation from brown and dark-green, nodulated, compact callus developed at the cut end of the explant (x 3).

B. 5-week-old culture of leaf disc on MS + NAA (5.0µM) showing rhizogenesis from nodulated callus (x3).

C. An individual shoot from **A**, 4 weeks after transfer to MS + 0.5µM BAP. The shoot has elongated well and developed into a multinodal structure (× 1.0).

D. An elongated shoot from **C**, rooted on ¼ MS + 0.5µM IBA. Healthy and branched roots have developed directly from the basal cut end (× 1.1).

E. A shoot from **D**, two months after transfer to soil (× 1.1).

Figure 1