Acclimatization of neem microshoots adaptable to semi-sterile conditions

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The hardening of in vitro propagated microshoots of neem (Azadirachta indica A. Juss.) was carried out using 3 methods under semi-sterile conditions in low cost mini-polytunnels and a shade house. The percentage survival and rooting response was 16.25% in the first (2:1, v/v, sand and soil with 1” × 1” central cylindrical cocopeat plugs) and second method (1:1, v/v, cocopeat : bio-fertilizer), but was 100% in the third method (2:1, v/v, sand and soil with 1:1, v/v, cocopeat:biofertilizer and addition of Trichoderma viride). During the acclimatization process, the chlorophyll content in leaves gradually increased from 0.97 (stage I) to 1.35 (stage II), 1.56 (stage III) and 2.14 mg/g (stage IV), indicating a shift in the mode of nutrition from heterotrophic through myxotrophic to autotrophic. Similarly, the percentage water loss from the leaves of plantlets decreased from 90.38 (stage I) to 46.83% (stage IV), indicating stomatal development and progressive hardening.

Ex vitro rooting and use of the bio-control agent could bring down the cost of production and make micropropagation of neem feasible and to be adopted as a rural enterprise.

Keywords: Bio-control agent, hardening, micropropagation, neem, Trichoderma

Introduction

Neem is a multipurpose tree with agricultural, medicinal and social uses. The recent spurt of interest in this tree is due to its spectacular biological activity, which has resulted in growing demand for its seed. There have been many reports on significant variability for the azadirachtin content and other desirable traits in neem trees¹. In view of the problems associated with poor seed viability and likely variability with sexually propagated progeny, micropropagation has been suggested for producing true to type planting material². Tissue culture has been investigated for rapid propagation of superior lines as well as uniform planting material. Although tissue culture techniques are widely adopted for the propagation of tropical multipurpose trees, there are certain limiting factors, such as, low survival rate of in vitro regenerants in the field and high cost of propagules raised via in vitro technology. Therefore, ex vitro rooting and low cost hardening methods can help in overcoming these limitations.

Tissue cultured plants possess certain characteristic features, i.e., culture induced phenotype (CIP) due to their acclimatization to special environment in vitro³.

Transplants must undergo a period of acclimatization, more specifically, a period of transitional development in which both anatomical characters and physiological performance overcome the influence of in vitro culture conditions⁴. Much research has been conducted to solve the problems related to acclimatization. Brainerd and Fuchigami⁵ examined response of micropropagated plants to low relative humidity. Sutter and Hutzell⁶ used humidity tents and antitranspirants to increase the survival percentage of micropropagated plants under low relative humidity. Hardening is a time-consuming and labour-intensive process contributing to major portion of the production cost. Although, in the early days of micropropagation, in vitro rooting was the general method used for obtaining plantlets, ex vitro rooting is now used in commercial laboratories as it eliminates one culture stage and reduces the overall cost of micropropagation⁷.

The successful ex vitro acclimatization of micropropagated plants determine the quality of the end-product and, in commercial production, economic viability of the enterprise⁸. Successful result can be achieved by careful environmental control during acclimatization. Considering physiological changes in the micropropagated plants, Kozai⁹ stated that many
problems of acclimatization remain unsolved, needing further study. The present study is carried out to establish *ex vitro* hardening of neem in low-cost mini polytunnels under shade-house with semi-sterile conditions, which make micropropagation of neem more feasible and to be adopted as a viable technology at village level bio-centers. An attempt is made to analyze chlorophyll content, stomatal conductance and analysis of stomatal size during various stages of hardening from *in vitro* microshoots to plants ready for field transfer.

Materials and Methods

Plant Material

Two elite neem varieties of *in vitro* raised microshoots were used in the experiment, viz., PUT-1 with high azadirachtin content (0.97% in seed kernel) from Plant Biotechnology Laboratory, Department of Biosciences, Sri Sathya Sai University, Anantapur Campus, Andhra Pradesh, and CRIDA-8 neem with azadirachtin content of >0.75% obtained from Central Research Institute for Dryland Agriculture, Hyderabad (Courtesy: Dr B Venkateswarlu).

Hardening Technique

Three different methods were employed with slight variations. In all the methods, *in vitro* regenerated micro shoots were treated for *ex vitro* rooting with 1000 ppm IBA for 10 min and planted in polybags containing sandy soil (sand and soil in 2:1 ratio) with a central hole of 1”×1” diameter and depth. In the first method, microshoots were planted in a cylindrical plug of 1”×1” containing cocopeat, whereas in the second method the planting was done in the cylindrical plug consisting 1:1 ratio of cocopeat and biofertilizer Annapoorna [Glomus mosae 2.0 CFU/g, Trichoderma viride 2.1×10^4 CFU/g, Azospirillum 3.5×10^3 CFU/g, Pseudomonas 1.2×10^6 CFU/g, P-Solubilizer of phosphate solubilizing bacteria (Bacillus sp. 2×10^6/g), neem cake 10%, castor cake 10%, and Pongamia cake 10%). In the third method, microcuttings were planted in the plugs after a dip in 0.1% *T. viride* for 1 min as in the second method, but with addition of 2 mL of 0.1% *T. viride* in the mix, followed by weekly foliar sprays. In all the three methods, the polybags were placed in low cost mini polytunnels under shade net (75% RH), maintaining required humidity levels with occasional moistening and semi-sterile conditions were maintained in the shade house.

Stages in Hardening

Primary Hardening

The micro cuttings were subjected to primary hardening in closed mini polytunnels with 95-100% RH for 4-5 wk (stage II) and partially opened mini polytunnels with 65% RH for 4-5 wk (stage III).

Secondary Hardening

It was done under shade net with 45% RH for 1 month (stage IV).

The response of micro cuttings in various rooting media was recorded as budding, rooting and percentage survival.

Assessment of Hardening

The physiological status of plants during hardening was assessed at the following stages:

- **Stage I**—Microshoots in the culture vessels (100% RH)
- **Stage II** (Fig. 1a)—Microshoots in closed mini polytunnel (95-100% RH)
- **Stage III**—Plantlets in partially opened polytunnels (65% RH)
- **Stage IV** (Fig. 1b)—Plants under shade net (45% RH)

Analytical Methods

Chlorophyll estimation was done in 80% acetone extract by calculating absorption coefficient. Rate of water loss from the leaves during different stages of hardening was determined by method of Brainerd *et al.* Stematal analysis was carried out by measuring length, width and vestibule width using stage and occular micrometer. The data obtained were subjected to statistical analysis by Duncan’s Multiple Range Test.

Results and Discussion

The response of microshoots of neem to three different rooting methods is presented in Fig. 1. In the first method, using a rooting mix of sand:soil in 2:1 ratio with central cocopeat plugs, the survival rate was 16.5% with 20% budding and 16.25% rooting. The low rate of survival was presumably due to fungal infection. In the second method, there was no significant improvement in rooting and survival rate, but budding increased to 53.3% when biofertilizer was mixed along with cocopeat in the cylindrical plug in equal proportions. The third method involving the use of biocontrol agent in cocopeat plugs gave 100%
survival with 100% budding and rooting. Biofertilizer Annapoorna with 20% *T. viride* used in the second method appears to be below the effective minimum level for preventing fungal infection as well as initiation of rooting. Dip in *T. viride* followed by additional amount in rooting mix and weekly sprays could be effective not only to control fungal infection, but also to enhance root initiation and root growth, and the rooting response was advanced by 15 d. The dip in *T. viridae* seems to have stimulated root proliferation by its ability to colonize at the base of microshoots. This technique proved to be 100% successful in hardening the *in vitro* microshoots under semi-sterile conditions Fig. 2.

Harman reported that bio-control agent *T. viride* is a good additive to greenhouse potting mix\(^1\). It can reduce fungicide use in greenhouses by limiting root-borne diseases and protect transplants in the field by virtue of its ability to stimulate root proliferation and root colonization. The present investigation confirms the above report. The periodical foliar spray addition of *T. viride* for the control of disease proved to be effective not only in controlling the disease, but also inducing root formation even from the base of leaf (Fig. 1c), which could later be established for further growth. University of Connecticut reports that *T. viride* grows on the surface of roots where it helps in disease control and enhances root growth\(^12\). Once *Trichoderma* colonises the root, it improves growth in two ways, first it kills pathogenic fungi causing root rot and, secondly, it protects the roots from physical stresses enabling them grow faster. Unlike the existing reports which emphasize on increasing the biomass of root system, the present investigation shows the profound effect *T. viride* on root initiation from *in vitro* micropropagated shoots.

**Assessment of Hardening**

Survival of microshoots *ex vitro* depends on its ability to withstand water loss and carry out photosynthesis, which is enhanced by gradual acclimatization and hardening. This fact can be supported by assessing the anatomical and physiological changes during various stages of hardening (Table 1).

**Chlorophyll Content**

The chlorophyll content increased from *in vitro* to plantlets and to fully hardened plants of both PUT-1 and CRIDA-8 varieties. The enhancement in the level of chlorophyll a and total chlorophyll were parallel to each other, though the amount of chlorophyll b remained less consistent. The lower level of chlorophyll in stage I indicated poor photosynthetic ability due to heterotrophic mode of nutrition. Earlier reports by Grout *et al*\(^13\) showed that photosynthetic apparatus of newly transferred cauliflower plantlets were insufficient for achieving a net positive carbon balance, mainly because of lower levels of chlorophyll. The average values of chlorophyll a and total chlorophyll are significantly different from stage I to stage II indicating a rise in chlorophyll pigment and a shift to mixotrophic nutrition. Stage IV plantlets scored highest chlorophyll content indicating improved photosynthetic efficiency as hardening progresses.

**Stomatal Analysis**

It is clear from data that stomatal length and width increased from stage I to stage IV, whereas vestibule width decreased (Table 1). The length and width of stomata in stage I and II were not significantly different, but varied in stage III and IV. Vestibule width was larger in stage I in both PUT-1 (2.89 µ) and CRIDA-8 (2.89 µ) varieties, whereas it was least in
stage IV, i.e. 1.99 and 2.08 µ in PUT-1 and CRIDA-8, respectively; a clear indication of improvement in stomatal anatomy. Blanke et al. report that stomata in in vitro raised plants are abnormally shaped and raised to the surface of the leaf instead of being sunken and have wide vestibule.

**Rate of Water Loss**

The data on rate of water loss from leaves of plantlets hardening at various stages in PUT-1 and CRIDA-8 are presented in Table 1. The mean decrease in percentage of water loss ranged from 90.38 to 46.83% in PUT-1 and 89.86 to 47.51% in CRIDA-8 from stage I to IV. In vitro plant leaves are more susceptible to water loss than ex vitro plants. One reason for plants to lose water rapidly in the initial stages of hardening could be due to less wax on leaves. The failure of leaves to develop surface wax seems to be mainly due to in vitro environment and is not directly related to the ingredients of the culture medium. Leaves formed in culture may produce some additional wax once they progressively began to be hardened, which may be the reason for reduction in water loss in hardened plant of stage IV. Poor stomatal anatomy and its function in in vitro shoots may also contribute to high water loss during initial stages of hardening.

Neem being a hardy tree of tropics thrives well in drought prone area. In order to adapt to such climate, it should withstand water loss. Tissue cultured neem plants to perform well in field must regulate water loss. Acclimatization of the in vitro neem plants to field conditions involves gradual change in anatomy and physiological function as analysed in the present study.

**Cost Reduction in Hardening Technology**

In the hardening technique, attempts were made to economize the production process and simplify the technique with less sophistication, which could be adopted at village biocenters in order to extend the scientific technology from lab to land. Ex vitro rooting eliminated the cost of additional culture stage in rooting media. Single step rooting where shoots are directly transferred into polybags filled with rooting media instead of two step process where rooting in plastic trays with soilrite followed by transferring to ploy bags for hardening had made rooting could be avoided. The other infrastructure required for hardening also cost relatively less with a mini polytunnel (around Rs. 200/-) and shade net (75% RH) and irrigation facilities, which made hardening of micro-propagated neem more feasible to be adopted at village level.

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