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HARDENING AND ACCLIMATIZATION OF AN ENDANDGERED FOREST TREE *GIVOTIA ROTTLERIFORMIS* GRIF.

M. RAMBABU[1], D. UJJWALA[2] AND N. RAMASWAMY[3]

[1].*Department of Botany, Kakatiya Govt. College,Hanamkonda,Warangal, (T.S.)*
[2] *Department of Botany, TSWRDCW, Kothagudem, Bhadradri Kothgudem, (T.S.)*
[3] *Department of Biotechnology, Kakatiya University, Warangal, (T.S.)*
Email: rambabudrbot@gmail.com
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Givotia rottleriformis is an economically important forest tree and especially used in toys making industry. The acclimatization of the *in vitro* developed plants is a crucial stage to make them survive under the ex-vitro conditions Hardening is a method in which the tissue culture plants developed in artificial media are habituated to grow in natural environment. Afterwards the plants are transferred to polybags filled with potting mixture and grown under shaded house for 6 – 8 weeks. In the present investigation, hardening and acclimatization experiments were carried out both on *in vitro* and *ex vitro* rooted plantlets. The potted mixtures containing red soil + sieved sand + vermicompost (1:1:1) showed good survival for *in vitro* and *ex vitro* rooted plants. Maximum percentage of survival rate was observed in the plantlets developed from *ex vitro* rooting.

Key Words: *Givotia rottleriformis*, *In vitro, Ex vitro,* Hardening, Acclimatization.

Micropropagation has been extensively used for the rapid multiplication of many plants' species. However, its wider use is restricted offers by the high percentage of plants lose or damage when transferred to soil. This is due to regenerants have to adjust to many abnormalities in *ex- vitro* environment like high level of irradiance low humidity and water is limiting due to low hydraulic conductivity of roots and root system connections (Fila *et al.* 1998). Acclimatization of regenerates will over come this threat with gradual lowering in air humidity (Bolar *et al.* 1998, Lavanya *et al.* 2009).

Hardening / acclimatization plays an important role in tissue culture technology for establishment of plantlets developed *in vitro*, for Lab-to-Land Program. In conservation and multiplication of especially endangered, medicinal and economically important forest tree species, this technique plays a vital role for an establishment of plantlets.

The *in vitro* developed plants are to be transferred to field. Prior to the transfer into the field, the *in vitro* regenerated plantlets are to be acclimatized in order to withstand the external environmental conditions. Hardening process is very useful in increasing the survival rate of *in vitro* regenerated plantlets on their transfer to field (Saxena and Bhojwani 1993, Ahuja 1993a, b). Arya and Shekhawat (1987) have also reported the hardening in woody plants. Micropropagation is involved many critical stages for its success, in woody plants especially at hardening level (Purohit *et al.* 1998).

During this process plants have to be adapted to new environmental conditions such as lower relative humidity, higher light intensity, fluctuating temperatures and constant disease-stress (Preece and Sutter 1991). The intrinsic plant quality, produced *in vitro* is one of the most important factors, governing the success rate during the transition to *ex vitro* conditions. In this regard, excessive water loss by transpiration and an impaired photosynthetic apparatus are the two major problems (Van Huylen-Broeck and Debergh 1996).

Givotia rottleriformis, Grif. (Vern. Tel. Tella Poniki) is an economically important forest tree. The wood of this species is very light, soft and white used for toys, carving images, light packages. Seeds yield oil for lubricating fine machinery (Gamble 1967), Nayer

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Ramamurthy and, Agarval (1998), Somasundaram (1980) and Sharfudhin Khan (1953). The species is almost on the verge of extinction for the over exploited for its timber in toys making industry. (Murthy JSN 1992, Babitha Rao Meru and Sreenivasulu 2002)

MATERIALS AND METHODS

Hardening/acclimatization experiments were carried out both on *in vitro* and *ex vitro* rooted plantlets.

Hardening of *in vitro* rooted plantlets: After induction of roots *in vitro*, the plantlets with 3-5 nodes and having atleast 5-6 roots of 5-6 cm length were washed carefully with sterile distilled water to remove traces of agar. Later these plantlets were transferred to plastic pots containing different sterilized substrates for hardening (Fig 1a-c). The plastic pots were covered with plastic bags to prevent desiccation and to avoid rapid changes in the environment. The plantlets were acclimatized in the walk-in-chamber at 25^0-27^0C temperature. During the hardening procedure, plastic bags were gradually perforated after 15 days and after one month they were removed. After removing the plastic bags the plantlets were transferred to earthenware pots containing garden soil and maintained under shady conditions in the research field.

Hardening of *ex vitro* rooted plantlets: After formation of roots in *ex vitro*, the plantlets of *G.rottleriformis* were transferred to different substrates for hardening. Hardening was done in two stages:

(I) The *ex-vitro* rooted plantlets after roots were visible, the plantlets were shifted from low temperature (29^0C) / high relative humidity (80% RH) regime of green house to the region which experienced relatively high temperature (35-38^0C) and low RH (50-60%).

The *ex-vitro* rooted plantlets were shifted to pots containing different substrates viz., red soil only, red soil + sieved sand (1:1), red soil + sieved sand + farmyard manure (1:1:1) and red. soil + sieved sand + vermicompost (1:1:1) These plantlets were kept in green house for hardening. Low RH (50-60%) and high temperature (29-35^0C) was maintained in green house.

(ii) After 45 days these plantlets were shifted to fresh pots containing red soil + compost (1:1). These plantlets were maintained under shady conditions in the research field.

RESULTS

The *in vitro* rooted plantlets from shoot tip and nodal cultures were hardened on four different types of soils mixtures viz., red soil, red soil + sieved sand (1:1), red soil + sieved sand + farmyard manure (1:1:1) and red soil + sieved sand + vermicompost (1:1:1) (Table-1).Out of four potting mixtures used for hardening procedure, the survival percentage was maximum (70%) in red soil + sieved sand + vermicompost compared to all other substrates used. New leaves were formed after 12 days after transplantation in the same soil mix. Low percentage of survival (16%) was recorded in only red soil and the new leaf appeared after 21 days. Remaining substrates showed moderate percentage of plantlet survival (40 & 66%) and new leaves appeared after 19 & 16 days respectively. After one month, polythene bags were removed and the plantlets were shifted from walk-in-chamber to earthenware pots containing garden soil. These plantlets were maintained under shady conditions in the research field. The acclimatized plants were normal, healthy and showing similar morphological features as that of donor plants (Fig 1a-c).

The *ex-vitro* rooted plantlets were hardened on four different types of potting mixtures viz., red soil, red soil + sieved sand (1:1), red soil + sieved sand + farmyard manure (1:1:1) and red soil + sieved sand + vermicompost (1:1:1). The survival percentage was found to be maximum (90%) in red soil + sieved sand + vermicompost (1:1:1) in comparison to all other substrates used. New leaves formation was observed after 10 days of

Table1: Effect of different substrates on hardening of *in vitro* rooted plantlets in *G. rottleriformis*

Substrates	No. of *in vitro* plants transferred for hardening	No. of plants survived	Survival percentage	Days required for new leaf formation
Red soil	30	05	16	21
Red soil + sieved sand (1:1)	30	12	40	19
Red soil + sieved sand + farmyard manure (1:1:1)	30	20	66	16
Red soil + sieved sand + vermicompost (1:1:1)	30	21	70	12

transplantation. The survival percentage was found less (40%) in red soil and duration for new leaves formation was 15 days. These acclimatized plants are normal, healthy and morphological characters are also similar to donor plants.

DISCUSSION

Acclimatization of *in vitro* and *ex vitro* rooted plantlets has been successfully established in *G. rottleriformis*. The potted mixtures containing red soil + sieved sand + vermicompost (1:1:1) showed 70% survival for *in vitro* rooted plants and 90% survival for *ex vitro* rooted plants. Maximum percentage of survival rate was observed in the plantlets developed from *ex vitro* rooting. Similarly, Saxena and Bhojwani (1993) found a simple hardening process in bamboo which is very useful in increasing the survival rate of tissue culturally raised plantlets on their transfer to field. Arya and Shekhawat (1987) have reported on the hardening phenomenon in which they observed the successful acclimatization of the plantlets after gradual reduction of humidity. Canstantine (1978) found the importance of light during the hardening period for growth of the plant.

Hardening is a critical step prior to transplantation of plants to the soil. The *in vitro* plantlets survive in 100% relative humidity and they also dependent on the medium for supply of sugar and other nutrients. Plants are, therefore, allowed to grow on rooting media for about one month after root initiation. During this phase the nutrients in the culture go on gradually depleting and plants become sturdy and easy to acclimatize in walk-in-chamber (Ahuja 1993a, b).

Little is known about the physiological changes which occur during the acclimatization/hardening process. Studies related to acclimatization were most often limited to observation on visual plant quality, rooting and histochemical changes and plant survival (Van Huylenbroeck and Debergh, 1996). Three important factors are involved in physiological changes during acclimatization. They are: Photosynthesis, carbon metabolism and the activity of enzymes related to oxidative stress. Light intensity too plays an important role in acclimatization. *In vitro* plantlets are grown under low photosynthetic photon flux density (PPFD). A sudden increase in light intensity during acclimatization leads to photoinhibition and severe stress (Van Huylenbroeck, 1994; Van Huylenbroeck and De Riek, 1995). Higher CO_2 levels can have a positive effect during acclimatization of micropropag ated plantlets (De Riek and Van Huylenbroeck 1994).

Fig 1(a-c): Effect of different substrates on hardening of *in vitro* rooted plantlets in *G.rottleriformis*.

The benefits of higher sucrose concentrations *in vitro* on the acclimatization process are attributed to the accumulation of starch as a nutrient and energy source reserve (Capelladse *et al.,* 1991). Sucrose metabolism differed depending on the degree of carbon assimilation at transplanting. In photoautotrophic *Spathiophyllum* plants, sucrose, fructose and glucose levels remain constant or even increased before root emergence, indicating the plants were able to assimilate enough carbon to sustain energy demand by growing or developing tissue (Van Huylenbroeck and De Riek 1995). Mixtrophic shoots had higher initial sucrose concentrations, but showed higher sucrose synthase activity directly after transplanting corresponding with an observed decrease in sucrose levels. Once a functional photosynthetic apparatus was developed no difference in sugar and starch metabolism could be observed between mixtrophic or autotrophic starting material (Van Huylenbroeck and Debergh 1996).

Anti-oxidant enzymes can also have the influence at the time of acclimatization. During acclimatization an increase in enzymatic activity of both catalase and superoxide dismutase was observed in *Spathiophyllum* and *Calathea* (Van Huylenbroeck and Debergh 1996). They suggest that during acclimatization the changes in humidity combined with light intensity can generate activated oxygen species (AOS). The physiological function of the observed changes can be considered as a protective mechanism against AOS.

In the present investigations the hardening of *in vitro* rooted plantlets of *G.rottleriformis* has been developed with 70% survival and hardening of *ex vitro* rooted plantlets has been developed with 90% survival. Thus, *ex vitro* rooted plants. According to our observations, *ex vitro* rooting is preferable in tree species. These protocols developed for acclimatization can be used for rapid *in vitro* multiplication and conservation of *G.rottleriformis.*

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