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An Efficient *In vitro* Hardening Technique of Tissue Culture Raised Plants

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Abstract: Micropropagation has been extensively used for the rapid multiplication of many plants species. However, its wider use is restricted often by the high percentage of plant loss or damaged when transferred to *ex vitro* condition. To acclimatize the micropropagated plants, different workers have employed different approach toward successful establishment of *in vitro* raised plants under *ex vitro* condition. In the present study, a successful attempt has been made to acclimatize the tissue culture raised plants which is cost effective compare to other existing hardening technique. Tissue culture raised orchid seedlings were acclimatized and hardened *in vitro* by using 1/10th liquid MS basal medium subsequently replaced by tap water with chips of charcoal, bricks and decayed wood as an alternate substratum. The newly formed roots under this condition firmly attached to the charcoal chips with the passage of time, similar to velamenous root attach to the trunk of a tree. Charcoal and moss substratum was more suitable than bricks for epiphytic species and decayed woods for terrestrial. The researchers report the novel and efficient one step hardening technique for tissue culture raised orchid seedlings which will reduce the production cost.

Key words: Acclimatization, alternative substratum, field establishment, orchid micropropagation, plant tissue culture, survival frequency

INTRODUCTION

Micropropagation has been extensively used for the rapid multiplication of many plants species. However, its wider use is restricted often by the high percentage of plant loss or damaged when transferred to *ex vitro* condition. This is due to regenerates has 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-stem connections (Fila *et al*., 1998). Acclimatization of regenerates will overcome this threat with gradual lowering in air humidity (Bolar *et al*., 1998; Lavanya *et al*., 2009). These days many acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow controlled by computer (Hayashi *et al*., 1988; Pospíšilová *et al*., 1999), but this comes at a price.

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in the soil (Saxena and Dhawan, 1999). To acclimatize the micropropagated plants, different worker have employed different approach toward successful establishment of *in vitro* raised plants under *ex vitro* condition. The concentration of sucrose and agar in the medium is also said to have an effect on subsequent acclimatization to *ex vitro* conditions (Genoud-Gourichon and Sallanon, 1996; Hazarika 2003; Synkova, 1997; Lavanya *et al*., 2009). A major part of the production cost; labour cost, rooting and acclimatization of plantlets accounts approximately 60% (Hazarika 2003), therefore an efficient acclimation technique is necessary for *in vitro* raised plants. So, in quest of a new efficient and cost effective technique of *in vitro* hardening (one step process), the authors demonstrate the effectiveness and viability of the new technique on three different orchid species (*Arachnis labrosa* (Lindl. ex Paxt.) Reichb.f., *Cleisostoma racemiferum* Lindl. and *Malaxis khasiana* Soland ex. Swartz) and its advantages over the conventional technique. This novel technique of one step hardening of orchid was successfully employed and established with high degree of efficiency.

MATERIALS AND METHODS

The present study was carried out in the Department of Botany, Nagaland University, Nagaland, India during April 2002 to December 2008. The present investigation was carried out with three different orchids (two epiphytic monopodial orchids viz. *Arachnis labrosa* (Lindl. ex Paxt.) and *Cleisostoma racemiferum* (Lindl.) Garay and one terrestrial orchid viz., *Malaxis khasiana* Soland ex. Swartz.

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The plantlets (size: ~3-4 cm long with 3-4 roots) were raised from different explants sources of all the three species following the protocols given by Deb and Temjensangba (2005, 2006a, b) and Temjensangba and Deb (2005a-c, 2006). The fully differentiated plantlets with 3-4 roots from the axenic culture condition were selected for the present investigation. The plantlets were maintained in a highly reduced level of MS (Murashige and Skoog, 1962) salt solution (1/10th strength) devoid of any plant growth regulators and sucrose or any other organic carbon sources. In the culture vials different types of matrix/substratum were used as an alternative substratum. Alternatively plantlets were also maintained without MS salt solution i.e., with only water. The different matrix/substrate like charcoal pieces (~5-7 mm size), small brick chips (~5-7 mm size) and mosses were used for the epiphytic orchids whereas, decayed wood/chopped forest litter was used along with charcoal pieces, brick chips and moss for terrestrial orchid. The matrix was either used singly or in combination (in 1:1 ratio). The substratums were chopped and air dried before use. In the culture vials different substratum were added and mixed with MS salt solution/water [~8-10 mL in each test tube (25×150 mm size) and ~20-25 mL in each conical flask (250 mL size)] and cotton-plugged before autoclaving at 121°C of 1.05 kg cm^{-2} for 30 min. In another set of the experiment the substratum were incorporated without autoclaving. The rooted plantlets from the axenic sources were taken out washed off the traces of agar using thin brush followed by the rinsing in luke-warm water. The plantlets were then inoculated in the culture vials and in each culture vials two plantlets were cultured. The cultures were maintained at 24±1°C and under normal laboratory light condition (40 μmol/m^2/sec) provided with fluorescent tube and 12/12 h (light/dark) photoperiod. Initially the cultures are maintained for about one month and culture vials are plugged and thereafter the cultures vials were watered wherever culture vials were dried up and culture vials caps were kept open. The cultures were maintained for about three months to acclimatize and hardened the plants. A part from the above some cultures were also maintained on reduced MS medium containing sucrose (2%) (w/v) accompanying no plant growth regulators and maintained in the normal laboratory condition as practiced in conventional method.

The hardened plants from all the three species were transplanted to Community Potting Mix (CMP) (charcoal pieces: brick chips: moss at 1:1:1 ratio for epiphytes and charcoal pieces: brick chips: moss: forest litter at 1:1:1:1 ratio for terrestrial orchids) along with the content of the culture vials. The potted plants were maintained in the poly house as well as directly in the natural condition. Initially the transplanted plants were watered at regular interval. The plantlets were transplanted at different seasons of the year both in the natural condition as well as in the poly house. A part of the hardened plants were maintained in the culture vials for last three year and are watered at regular interval. The experimental design was completely randomized all the experiments were repeated at least thrice.

RESULTS AND DISCUSSION

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Direct transfer of tissue culture raised plants to field/wild is not possible due to high rate of mortality, as the regenerates in the culture condition has been cosseted environment with a very high humidity, varied light and temperature condition and being protected from the attack of microbial and other agents. Direct transfer to sunlight also causes charring of leaves and wilting of the plants (Hiren *et al*., 2004; Lavanya *et al*., 2009). In other words the survival percentage is determined by the hardening of the plantlets. It is therefore, necessary to accustom the plants to a drier or natural atmosphere by a process called acclimatization or hardening.

In the present investigation, the plantlets were raised from different explants like immature embryos, foliar explants and aerial roots following the protocol given by Deb and Temjensangba (2005, 2006a, b), Temjensangba and Deb (2005a-c, 2006). The fully differentiated well rooted plantlets from the regeneration media were used for the present study (Fig. 1a-c). Among the different types and combination of substratum used for hardening, a combination of charcoal pieces and mosses (at 1:1 ratio) was found superior for epiphytic orchids while for terrestrial a mixture of moss and decayed wood/forest litter (1:1 ratio) was preferred (Fig. 1d-f). Similarly, different matrix or substrates with manipulation in salt solution were employed for hardening of *in vitro* raised plants by various workers viz.: soilrite for *Catica papaya* (Agnihotri *et al*., 2004), soaked cotton for *Saccharum offinarum* (Gill *et al*., 2004), etc. However, no report on the use of other substrate was recorded as studied in present investigation.

In present study, initially MS salt solution was used as nutrient source for one month thereafter MS salt solution was replaced by normal tap water.

Two significant observations were made during the hardening processes which are as follows:

- Initially the culture vials were caped/cotton plugged but at later stages the culture vials were left unplugged. During this period the cultures were very

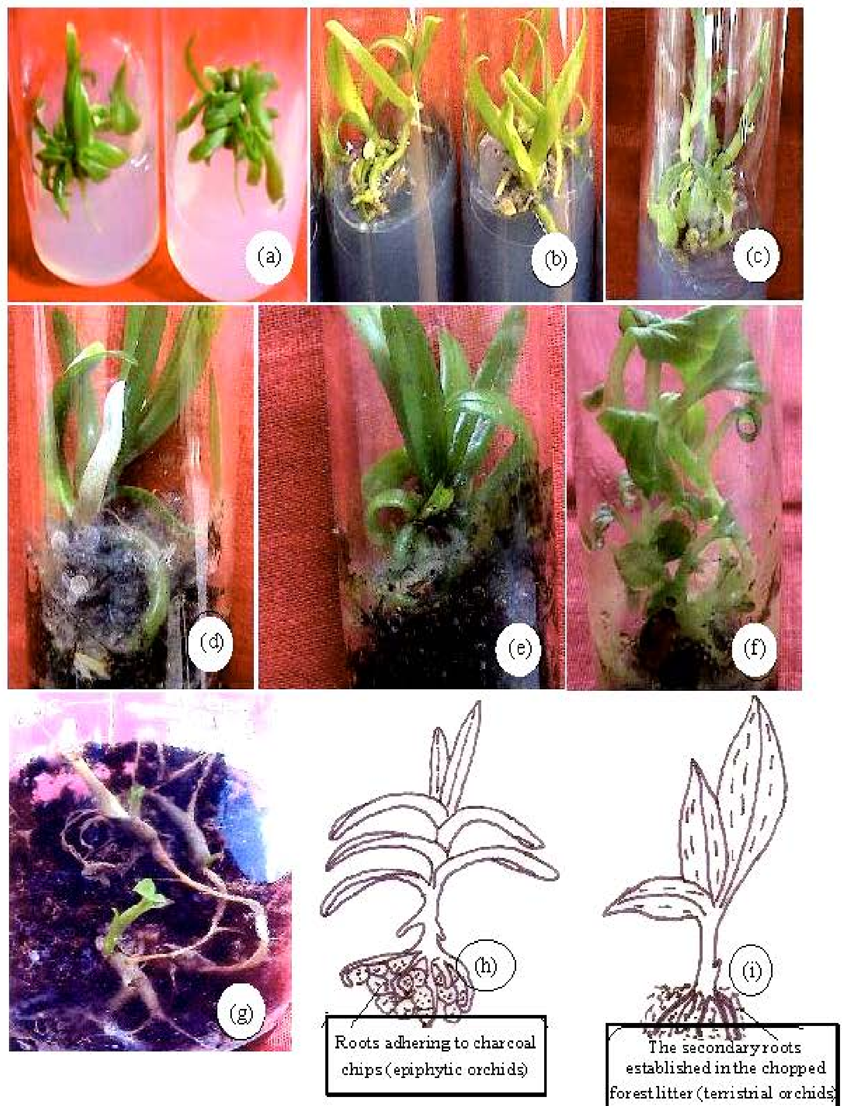

Fig. 1: (a-c) Regenerated plantlets from the regeneration medium used for *in vitro* hardening. a: *Arachnis labrosa*, b: *Cleisostoma racemiferum* and cL *Malaxis khasiana*, (d-f) The plantlets established on *in vitro* hardening medium. d: *A. labrosa*, e: *C. racemiferum* and f: *M. khasiana*, g: *M. khasiana* plantlets under *in vitro* hardening showing formation of secondary shoot buds and roots (Cultures do undergo dehiscence under *in vitro* condition and resume normal growth after the dehiscence period), (h- i) The roots of the fully established plantlets in the *in vitro* hardening condition are adhering to the substratum in the culture vessels (Line drawings), h: The roots of epiphytic orchids are adhering to the charcoal chips and i: The secondary roots of the terrestrial orchid established in the chopped forest litters

healthy and in some cases multiple shoot buds and roots developed (Fig. 1g). The roots were adhered to the substratum. It was observed that roots gets adhered to the substratum (Fig. 1h, i)

- The well dried substratum could also be used with equal efficacy compare to autoclaved substratum

During the process of hardening the regenerates gets acclimatized for its subsequent transfer to soil or CPM. The cultures were maintained under normal laboratory temperature and photoperiod for about three months. The hardened plants were transferred to the potting mix and maintained in the poly house as well as directly in the wild

Table 1: Comparative survival frequency of transplanted regenerates of *A. labrosa*, *C. racemiferum* and *M. khasiana* following two different hardening techniques with control

Species name	Survival frequency of transplants (%) (±SE)*		
	Control	Conventional technique	New technique
A. labrosa	13 (±1.50)	40 (±1.75)	80 (±2.25)
C. racemiferum	15 (±1.75)	45 (±2.50)	80 (±2.50)
M. khasiana	16 (±1.50)	40 (±2.25)	85 (±1.50)

*±SE: Standard error

and watered regularly at initial phase. The hardened plantlets of both epiphytic and terrestrial orchids from new technique showed about 95% survival under poly house condition. However, the plantlets hardened through conventional technique exhibited high rate of mortality on their transfer to CPM. The survival frequency was as low as 40, 45 and 40% under conventional technique while, in control it was 13, 15 and 16%, respectively. And transplants from new technique registered about 80 and 85% (epiphytic and terrestrial, respectively) survival in natural condition after three months of transfer (Table 1). A similar response was recorded in *Phoenix dactylifera*, where transplants exhibited more than 50% mortality rate after the 1st week transfer but survival rate increased considerably up to 92%, when plantlets were passed through pre-acclimation phase (Bhargava *et al.*, 2003). On the other hand, the survival frequency recorded a remarkable increase from the plantlets hardened through the new protocol developed in our study. Agnihotri *et al.*, (2004) also reported 80% transplant success of plants hardened in soilrite.

The newly potted plants were watered near the root systems i.e., avoiding the leaf with 1/10th MS salt solution at one wk interval, which proves to be beneficial for better growth. In *Rubia cordifolia*, the potted plants were reported to have irrigated with 1/2 strength MS medium without vitamins and sucrose thrice a week for 15 days for 30 days (Shrotri and Mukundan, 2004). Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth (Mukherjee, 1983; Kumaria and Tandon, 1994).

The new one step hardening technique proves to be highly cost effective and efficient alternative to conventional hardening technique for acclimatization of *in vitro* raised plants. Significantly, the survival frequency of the new technique is considerably higher after transplantation in comparison to the conventional technique. This technique of *in vitro* hardening will reduce the production cost of tissue culture technique. The technique may also be used for other non-orchid groups with partial modification and works are in progress in our laboratory.

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