PART - III

Hardening and Artificial Seed Technology

CHAPTER - 4

Hardening of *in vitro* regenerated plantlets in *Arundina* and *Geodorum*

4.1 INTRODUCTION

Orchids are healthy competitors in the international cut flower market (Hew and Clifford 1993, Hew 1994). The small proportion (<5%) of the total international cut flower trade currently occupied by orchid highlights their potential to take over even greater share of the market (Hew 1994). The development of Knudson's asymbiotic method has vastly improved the germination of orchid seeds. Following Knudson's discovery to date improved tissue cultural techniques using different plant parts like root, shoot, leaves, inflorescence have been adopted making orchid cultivation faster and easier (Arditti and Ernst 1993). There is an active market for micropropagated orchid plantlets which are produced at a rate of 11 million units per year (Jones and Sluis 1991). But due to some factors like slow growth of orchid plantlets coupled with lower multiplication rate, vitrification, poor rooting, high mortality of plantlets during acclimatization and high cost of explant establishment pose substantial limitation for successful micropropagation of orchids (Kozai 1991). However, acclimatization and field transfer of *in vitro* grown plantlets is perhaps the most difficult step in plant tissue culture. Transferring of plantlets from culture vessel to potting mix requires very careful and step wise procedure. Moreover, it is difficult because the *in vitro* grown plantlets are deficient in photosynthetic efficiency and mechanism to control water loss (Dhawan 1993). As the culture media is enriched in sucrose and other essential nutrients, the plantlets though appear green do not have photosynthesizing efficiency and due to high humidity inside the culture vessel (close to 95%), the plantlets lack the protective wax layer. The stomatal opening and closing mechanism is not proper in the *in vitro* raised plants (Dhawan 1993). The roots are mostly non-functional as the whole body surface can absorb nutrients easily from the medium (Pierik 1993). Therefore, if the plantlets are directly transferred to potting mix, they will immediately show wilting symptom and ultimately die. A careful acclimatization is thus very important for proper hardening and field transfer of *in vitro* grown plantlets.

Recent advances in plant tissue culture techniques have revolutionised the tissue culture industry in general and orchid industry in particular. But constraints of high labour and other expenses like nutrient medium, hardening and delivery of in vitro raised plants are yet to overcome (Rao *et al* 1996).

In this context artificial seed technology assumes greater importance because of its low cost and high volume propagation system. The discovery of artificial seed technology has opened new vistas in plant biotechnology. It has been demonstrated that the artificial seeds, prepared by encapsulating the somatic embryos or vegetative propagules or PLBs (in orchids) can withstand external strains and stresses and thus can be used as an universal delivery system (Datta, Kanjilal and De Sarker 1999, Redenbough *et al* 1988) . Researches in various laboratories have revealed that if the plant propagules or the PLBs or somatic embryos are encapsulated by incorporating fungicide, bactericide and food preservative, they could be a useful delivery system for tissue cultured plants by eliminating the tissue hardening steps by directly sowing them in soil (Bapat and Rao 1988, Bapat *et al* 1987, Redenbough 1988, Fizi *et al* 1994). Althogh some successful attempts have been made in this direction by using the artificial seed technology but it is still at its developmental phases. Hence, the present study was undertaken to find out a suitable process for hardening the *in vitro* raised plantlets of *Arundina graminifolia,* and *Geodorum densiflorum* by using the conventional method as well as by artificial seed technology.

4.2 MATERIALS AND METHODS

Selection and preparation of plantlets prior to their transfer to pots

15-16 week old plantlets of *A. graminifolia* and *G. densiflorum* obtained through *in vitro* seed culture were used as ready source of material for the present study.

Healthy plantlets having a height of 1-8 cm with 5-6 well developed leaves and 3-4 roots were deflasked and washed very carefully with double distilled water. Upmost care was taken during washing so that no agar or media should remain attached with the plantlets. After that they were treated with fungicide captan (4 mgl^{-1}) and kept on a sterile petridish.

Preparation of community pots

Earthen pots measuring 2" diameter X 1.5" height were brought from local market. The pots were filled with a mixture of charcoal, small brick peices, garden soil and sand at a ratio of 1:1:1:1. The potting media used in this experiment consisted of locally available materials.

Transfer of plantlets to community pots

After filling up the pots with potting medium the plantlets were very carefully transfered and planted in the carthen pots in groups. Half strength basal VW and KnC media were used in *A. graminifolia* and *G. densiflorum* respectively. The pots were then covered with inverted beakers. Distilled water was regularly sprayed in the beakers to keep high humidity around the plantlets. The pots were kept at culture room condition i.e at 25 ± 2°C under 16 hr photoperiod for at least 3 weeks. After that the pots were transferred to green house and the beakers were removed. In the green house the pots with plants were kept for another 2 weeks under natural photoperiod and humidity, Finally after 2 weeks the plantlets were transferred to large earthen pots filled with garden soil: sand (1:1). Watering was done at regular intervals.

4.3 OBSERVATIONS

Plantlets of various sizes on transfer to community pot did not show induction of growth upto the end of second week. During this period high humidity was maintained to prevent the plantlets from dessication. Survival percentage of the plantlets greatly depends on the size of the plantlets selected for acclimatization. Larger the size of the plantlets, greater the survival percentage. The effect of the size of plantlets on survival percentage during acclimatization and hardening was recorded, summarised and shown in Tables 4.3.1 and 4.3.2 . Most of the plantlets died when 1-3 cm long plantlets were transferred to pots from culture vessels resulting in a very low survival percentage. Gradual increase in size resulted in increase in survival percentage and it was significantly higher when 5-8 cm long plantlets were transferred.

Only 8% and 12% plantlets survived in *A. graminifolia* and *G. densiflorum* respectively when 1-3 cm long plantlets were transferred to the potting mix. The survival percentge gradually increases with the increase in plantlet size (Table 4.3.1 and 4.3.2). 54% and 62% plantlets were successfully hardened in *A. graminifolia* and *G. densiflorum* respectively when 5-8 cm long plantlets with 6-8 leaves and 3-4 roots were transferred to the community pots (Plate 14).

Plantlets on transfer to soil did not show any growth upto the end of second week. Most of the *in vitro* grown roots were found damaged. New roots appeared at the end of third week or at the middle of fourth week which is followed by the development young leaves in both the investigated genera. It has been observed that the growth of the plantlets were better if they were supplied with half strength of their respective liquid basal media initially after transfer . After the appearence of new leaves the pots containing plantlets were transferred to the green house for further growth. When the plantlets were large enough, they were transferred to the garden.It was noted that if the plantlets were transferred in group rather than singly, the survival percentage and the growth of the plantlets were better in both the studied genera.

Plate 14

It has been noted that if the plantlets, immediately after transfer to community pots, are subjected to a sharp and sudden decrease in humidy, they died immidiately due to dessication. However, gradual decrease in humidity resulted in a better survial percentage of the plantlets. Only 4-6 % plantlets survived if the plantlets are subjected to a sudden and sharp decrease in humidity percentage. It has been noted that plantlets, transferred in groups always have a better chance of survival than those transferred singly.

Table 4.3.1 Effect of the size of plantlets on survival percentage of seedlings in *A. graminifolia* during hardening. Each treatment had 5 replicates and repeated thrice.

Size of plants (cm)	No. of plants given	No. of plants survived*	Percentage of plants transferred to field*
1-3	50	4.00 ± .30	8.00 ± 1.09
3-5	50	19 .00 ± .94	38.00 ± 2.00
5-8	50	27.00 ± 1.33	54.00 ± 1.78

* Values are mean of 5 samples

Table 4.3.2 Effect of the size of plantlets on survival percentage of seedlings in *G. densiflorum* during hardening.

Size of plants (cm)	No. of plants given	No. of plants survived*	Percentage of plants transferred to field*
1-3	50	6.00 ± .46	12.00 ± 2.00
3-5	50	22.00 ± 1.26	44.00 ± .70
5-8	50	31.00 ± 2.89	62.00 ± 1.58

* Values are mean of 5 samples

Table 4.3.3 Effect of humidity on acclimatization and hardening in *A. graminifolia* and *G. densiflorum*. Each treatment had 5 replicates and repeated thrice.

Initial humidity % (at the time of transfer)	Humidity % after 1 week	% of plant survived *A. graminifolia*	*G.densiflorum*
95	95	54 ± 2.91	60 ± 1.64
95	90	52 ± 1.70	54**± 2.00
95	85	46***±3.90	50***±1.00
95	80	42 ***±3.00	36***±1.00
95	70	26***±0.90	22***±2.79
95	60	08***±0.30	11***±2.36
95	50	02***±1.54	03***±0.80

Values followed by asterisks in each treatment within the same column are significantly different from control (95% humidity after one week), using Student's t- Test at *5% level, **1% level and *** 0.1% level.

4.4 DISCUSSION

This part of the investigation deals with the development of a suitable method for acclimatization and field transfer of the *in vitro* raised plantlets in two critically endangered orchids of North eastern Himalayas, namely *Arundina graminifolia* and *Geodorum densiflorum*. Although various methods have been developed for micropropagation of orchids since past few years, the problems of high labour and hardening are yet to overcome (Rao *et al* 1996). Due to the lack of information in this regard, the present study was directed to find out a suitable method for hardening. It has been reported earlier that the *in vitro* raised plants are difficult to acclimatize because these are deficient in photosynthetic efficiency, proper mechanism to control water loss (Dhawan 1993). Moreover, the root regeneration *in vitro* appeared to be vulnerable as they are weak and often die in soil. (Pierik 1993)

The results obtained in the present study clearly showed that the survial percentage of the plantlets greatly depends on the size of the plantlets selected. Moreover, it has been noted that plantlets transferred in group survived better, a point finds support in earlier report (Singh 1993). However, high mortality rate may be due to the inefficiency of the leaves to start photosynthesis. Moreover, the *in vitro* grown roots were dried when they were transferred to soil and growth of the plantlets only started with the development of new roots. This inactivity of roots induce high rate of transpiration and ultimately results in the loss of many plantlets (Pierik 1993).

The effect of humidity on acclimatization has been investigated in both the taxa. It has been noted that if the transferred plantlets are subjected to sudden change in humidity, the plantlets died immediately resulting in a sharp decrease in survival percentage. But gradual decrease in the same yielded better result. If after transfer the humidity is decreased gradually, a better result was obtained. This may be due to the fact that the gradual decrease in humidity may result in a better cuticular wax formation and hence less cuticular transpiration (Pierik 1987)

Due to the very special environment *in vitro* it is very difficult to produce plants which are well adapted to living outside the test tube (Pierik 1993). The method, described in this chapter will help to harden the *in vitro* raised plantlets better and with increased viability rate.

4.5 SUMMARY

This part of the present investigation deals with the acclimatization and field transfer of the *in vitro* raised plantlets of *Arundina graminifolia* and *Geodorum densiflorum*, the two critically endangered orchid taxa of North Eastern Himalayan region.

In vitro grown plantlets of various sizes were selected as a ready source of material. They were very carefully washed with sterilized double distilled water and treated with 4% Captan solution. Finally the plantlets were transferred to community pots having a size of 2" in diametre and 1.5" in height. The community pots were filled with a mixture of charcoal, small brick pieces, garden soil and sand at a ratio of 1:1:1:1. After transferring the plantlets, the community pots were kept at culture room condition for at least two weeks. High humidity (around 95%) was maintained around the plants by covering them with inverted beakers. Distilled water was sprayed at regular intervals to maintain the humidity.

Plantlets on transfer to community pots did not show any growth upto second week following transfer. Most of the *in vitro* grown roots were died during this period. New roots appeared at the begining of the third week which is followed by the development of new leaves. The survival percentage of the plantlets greatly depends on the size of the plantlets selected for acclimatization. Larger the size of the plantlets, higher the survival percentage. Very low survival percentage was recorded when 1-3 cm long plantlets were transferred. Gradual increase in survial percentage was oberved with increase in plantlet size. After the emergence of new roots and leaves the plantlets were transferred to green house and kept there for further two to three weeks after which they were finally transferred to the field.

Effect of humidity on survival percentage of the plantlets was also recorded. It has been observed that if the plantlets were subjected to sudden change in humidity, they died immediately resulting in a sharp decrease in the survival percentage of the plantlets. only 4-6% survival percentage was recorded when plantlets were subjected to sharp decrease in humidity. However, in the contrary, if there is a gradual decrease in humidity, they showed optimum survival percentage.