Technical Bulletin No. 11

MANUAL FOR ACCLIMATIZATION OF MICROPROPAGATED CYMBIDIUMS

National Research Centre for Orchids
(Indian Council of Agricultural Research)
Pakyong – 737 106, Sikkim, India

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Manual for
Acclimatization of Micropropagated Cymbidiums

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Cover Photographs

Front : Cymbidium culture bottles

Back : Cymbidium plantlets under secondary hardening at Darjeeling Campus of NRCO

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Foreword

Cymbidium is one of the most promising floriculture crops for hilly regions of India because of suitability of agroclimatic conditions, average product selling prices, capacity to create employment opportunities among small and marginal farmers. Further, no competition for the product from the plains which would have rendered the cost of the product higher by adding transportation cost. Despite of, many advantages, the full potential of this crop must not yet been utilized for the benefits of people living in hilly regions of the country. However, states like Sikkim, Arunachal Pradesh, Nagaland and West Bengal have made significant progress in cultivation of cymbidiums. The results of introductions in Uttrakhand, Himachal Pradesh and Hilly regions of Tamil Nadu have also shown positive results. This crop has tremendous potential for cultivation in areas lying from 1500-2000 MSL in Himalayan region. In my view, area under cultivation can be increased if the quality of planting materials is available to the cultivators at reasonably low cost. In the production of micropropagated plantlets, acclimatization of tissue cultured plantlets costs approximately 60 percent of the total cost of production. This bulletin is an outcome of practical experience of authors who were part of the programme on production of 'quality planting material and utilization in northeast'. Under this programme, over 60,000 plantlets were propagated and acclimatized for setting up demonstration in northeastern states of India. We experienced if the acclimatization is carried out at cultivators' level, the cost of

planting materials brought down at sufficiently lower levels. Moreover, the cultivators would be able to take advantage of liberalization policies of the government with respect to importation of good quality planting materials in culture vessels, which are cheaper and easier to import that fully grown plants. I am sure the current publication, "A Manual for Acclimation of Micropropagated Cymbidiums" will be useful to the scientists and entrepreneurs interested in micropropagation of cymbidiums as well as to the farmers interested in growing cymbidiums as a commercial crop for higher productivity and profitability.

R. P. Medhi
Director

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1. INTRODUCTION

Micropropagation is a wonderful tool in the hands of plant propagators that enable's them to clone large number of plants in a very short period of time, eliminate inherent diseases, and overcome on limitations of seasonal dependency. The plantlets in a bottle are easy to handle, transport and are available at much lower unit cost than acclimatized or hardened off plantlets. However, widespread use of this technology has been restricted as a substantial number of micropropagated plants fail to survive when they are transferred to glasshouse conditions. The plantlets produced in tissue culture laboratory show abnormal morphology, anatomy and physiology. Primarily these plantlets have less developed cuticle, low deposition of epicuticular wax, incomplete vascular connection between the root and shoot, poor stomatal regulation mechanism, reduced development of photosynthetic tissues etc. These abnormalities are induced due to culture environment within culture vessels and culture room. Transferring these plantlets without substantial care to greenhouse environment would cause desiccation or wilting resulting death of the plantlets. Hence, these are gradually exposed *ex vitro*. The new conditions have lower humidity, higher light intensity, and more extreme temperatures. This is one of the most difficult tasks in plant propagation as the propagator has to keep his plants alive until they become fully independently functional. During this period, plantlets undergo many morphological, physiological and anatomical changes leading to control over the loss of water, self-sufficiency in manufacture of food, growth and development of plantlets etc. The success in acclimatization of micropropagation plantlets

determines commercial production and economic viability of the enterprise.

Cymbidium is considered as the most promising floriculture crop for hilly regions of India. It is a high value and low volume crop ideally suits to hilly region of the country because of a large distance between production and consumption centers. At present, cymbidiums are commercially cultivated in Sikkim, Arunachal Pradesh, Nagaland and hilly tract of West Bengal. The results for introduction of these orchids in Uttrakhand, Himachal Pradesh and Hilly regions of Tamil Nadu have shown encouraging results. It has been observed that areas lying from 1500-2000 MSL in Himalayan region offers congenial climate for growing of these orchids. To meet the requirement of planting materials, several tissue culture laboratories have been setup under public and private sector initiatives to meet the demand of planting materials. However, the cost of planting material and its quality are still causing concern in expanding cymbidium cultivation in the country. A substantial number of micropropagated plantlets do not survive on transfer from *in vitro* to greenhouse or field environment thereby rendering planting material cost higher. The cost of planting materials can be reduced by reducing mortality during transfer from *in vitro* to *ex vitro* environment. The successful acclimatization would require proper understanding morphological, anatomical and physiological characteristics of micropropagated plants induced by culture environment and *ex vitro* environmental conditions where the plantlets are to be transferred. The present bulletin is an outcome of Department of Biotechnology funded programme on 'production of quality planting material and their

utilization in north east'. Under this programme, 60,000 plantlets of cymbidium orchids were produced and successfully acclimatized for setting up demonstration units in Sikkim.

2. UNDERSTANDING PROCESS OF MICROPROPAGATION

Micropropagation is a technique of producing a large number of plants rapidly by aseptic culture of meristematic regions under controlled nutritional and environmental conditions. For the production of disease free planting materials, the meristem are either derived from a disease free plant or pathogens are eliminated by following standard plant virus elimination techniques. The process of micropropagation begins with the selection of the mother plant or clone to be propagated. The explants, usually shoots or auxiliary shoots are collected from the mother plants. These are washed with mild detergent in running tap water and then surface sterilized with 0.01 % of mercuric chloride or commercial bleach and finally 3-4 rinsing with distilled water. The shoot tips are isolated and cultured on defined medium aseptically. The culture medium consists of amino acids, vitamins, plant hormones, minerals and carbohydrates gelled with agar. Murashige & Skoog (MS) medium is commonly used for establishing aseptic cultures of cymbidium orchids. Under favorable conditions, responsive explants undergo organogenesis or somatic embryogenesis. In organogenesis, microshoots and roots are differentiated over a period of growth and development. Usually microshoots are produced when the explants are cultured on cytokinin rich medium. Subsequently, rooted plantlets are obtained by culturing microshoots on auxin rich medium to give rise to

plantlets. The direct organogenesis dependent on various factors like genotype, age and maturity of donor plant, *in vitro* conditions, quality and quantity of plant hormone present in the culture medium etc. The embryogenically-competent somatic cells produce somatic-embryos. The somatic embryos are similar to zygotic embryos having both shoot and root pole which develop into the shoot and root of the plantlets. For commercial propagation of cymbidium orchids, shoot tip culture is employed.

3. ADVANTAGES OF MICROPROPAGATION

Micropropagation has several advantages over conventional methods of propagation. The most important ones are listed as below:

- « It is the fastest method for producing genetically identical and physiologically uniform plants.
- « The technique helps in production of disease free plants and can be used to rejuvenate the farms having old and disease planting stocks.
- « Plants can be produced throughout the year without seasonal constraints in a small space.
- « Since the plants are grown in culture bottles/packs are easy to transport at distant places.
- « Micropropagation can be employed for producing homozygous plants, reducing gestation period requiring for exploitation of a new variety and conserving germplasm *in vitro*.
- « Micropropagated plants are vigorous and come quicker

in flower than seeded plantlets.

« Genetically modified cells or cells after protoplast fusion can be regenerated using micropropagation technique.

4. UNDERSTANDING *IN VITRO* ENVIRONMENT

The environment inside the culture room is different from the outside environment. Generally, *in vitro* environment is characterized by constant temperature, low light level and fixed light and dark cycle. Understanding *in vitro* environment and its effects on growth and development are necessary for successful acclimatization of plantlets *ex vitro*.

4.1 Environment culture conditions of culture vessels

The environments under which the plantlets grow within the culture vessels can be classified in aerial and root zone environment. The culture vessels have low head space and very limited air exchange because the culture vessels are capped to maintain aseptic conditions. The aerial environment within the culture vessels is characterized by high relative humidity (90-100%), fluctuating CO_2, and low light intensity. The gaseous atmosphere in culture vessels is consisting of mainly nitrogen, oxygen, carbon dioxide and ethylene. In addition to, root zone environment determined by nutrient concentration, pH, sugars, plant hormones, compactness, osmotic potential etc of the culture media. The plants developed in such environmental conditions are different in morphology, physiology and anatomy from the plants raised in *ex vitro* conditions. These anomalies are more often referred as culture induced

phenotypes [For details see characteristics of micropropagated plants]. This is the reason why tissue cultured plants require special attention during acclimatization.

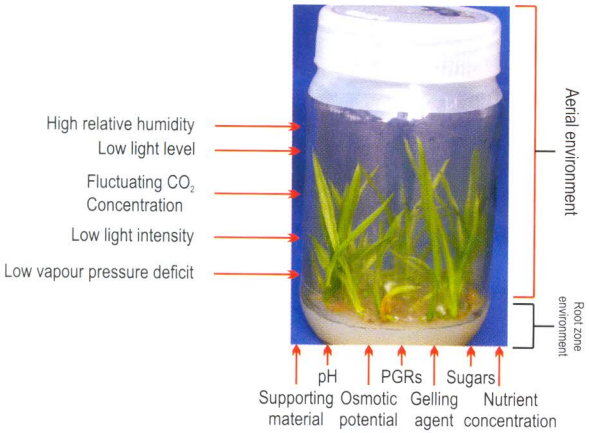


Plate.1 Showing *in vitro* environment of the culture vessel

4.2 Environment conditions of culture room

Environmental conditions of culture room like the light (source, duration and intensity), temperature affects growth and development of plants growing in culture vessels. Primarily, fluorescent lamps are used in tissue culture laboratories as light source. The spectrum of florescent lamps matches with the requirement of plants and provide uniform horizontal distribution of light. In some tissue culture laboratories, different types of light emitting diodes are used to emit red, blue and far-red light to control morphogenesis. Nowadays, LED lamps are also used as light source in plant tissue culture laboratories. Usually, plants are grown under 16: 8 hour light and dark cycle with 1000-2000 lux intensity. The temperature of culture room is adjusted as per crop requirement and stage of development. Generally it ranges between 22-25^0 C. The

temperature inside the culture bottles is slightly higher than ambient temperature of culture room.

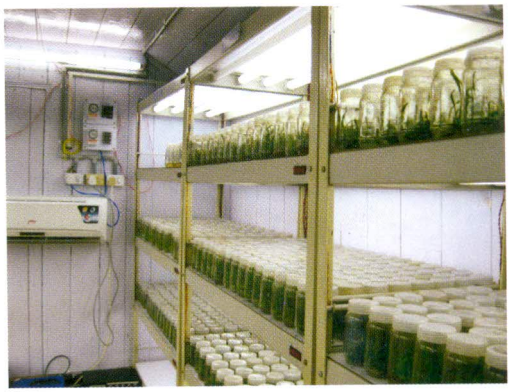


Plate.2 Showing environmental condition of culture room

5. CHARACTERSTICS OF MICROPROPAGATED PLANTS

It has been demonstrated that environmental conditions of culture room and culture vessels are responsible for development of plants with abnormal morphology, anatomy and physiology. These anomalies are corrected during acclimatization of plants. The principal anomalies in tissue culture plants are given as below:

5.1 Morphological characteristics

The morphology of plantlets is related to growth and survival of micropropagated plants. The morphological features like plant height, leaf number, leaf width, leaf area, roots number and root surface area affect the survival and further growth of *in vitro* grown plantlets in *ex vitro* environment. The plants with well developed roots are helpful in maintaining internal water whereas plants with poorly developed roots directly affect the

absorption of nutrients from the potting mix. Tall shoots with many active roots are beneficial for survival and plant growth during hardening. In general, the roots of *in vitro* raised plantlets are vulnerable and do not function properly when the plants are established *ex vitro*. However, in cymbidiums *in vitro* developed roots are functional and also new roots are formed readily. They also produced new roots from microcuttings if proper care has been exercised during acclimatization.

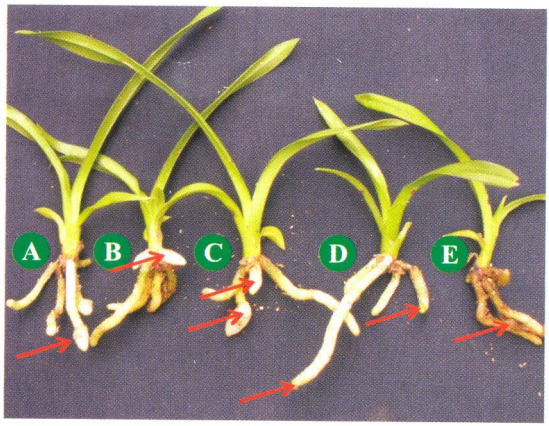


Plate.3 Showing root development in cymbidium plantlets A. revival of *in vitro* roots, B. initiation of new roots, C. both, initiation of new roots and revival of *in vitro* roots, D. revival of *in vitro* root tips and E. no root activity, 30 DAP during acclimatization.

5.2 Physiological characteristics

One of the major hindrances to the growth of micropropagation industry is very high mortality of *in vitro* plantlets either during acclimatization phase or during transfer to the field conditions. The plantlets shifted to *ex vitro* environment very often desiccate and wilt leading to low rate of survival. It has been estimated that roughly only 25 percent of the *in vitro*

regenerated plantlets are successfully transplanted *ex vitro*. This is due to some intrinsic features of *in vitro* propagated plants. Some of such features contributing to low survival of micropropagated plantlets *ex vitro* are described below:

5.2.1 Poor photosynthetic activity

The photosynthetic activity of tissue cultured plants is chiefly affected by sugars in culture media, culture vessel conditions leading to anatomical and biochemical changes in the plantlets cultured *in vitro*. The added sugar and culture vessel conditions like low light level, low concentration of CO_2, inadequate gas exchange, compel the plants to grow hetero or mixotrophically. In result, high sugars are accumulated in the leaves which inhibit the activity of photosynthetic enzymes. It has been observed that *in vitro* produced plants have poorly organized grana in the chloroplast; smaller and fewer palisade cells to use light effectively. It is necessary plantlets become autotrophic upon transferring them into *ex vitro* environment. The photosynthesis of the plants growing in culture vessels can be increased by using CO_2 permeable films, lowering humidity, reducing sucrose concentration and increasing gas exchange.

5.2.2 Impaired stomatal mechanism

In vitro propagated plants have poorly malfunction stomata that are unable to close under adverse conditions. The stomatal density is also higher in tissue cultured plants as compared to plants grown under field conditions. The impaired mechanism of the stomata has been due to abnormal orientation of microfibrils and high deposition of Ca+ and Na+ in guard cells which may interfere with the movement of K^+. The stomata of

plants cultured *in vitro* are raised, with rounded guard cells whereas these are sunken with elliptical guard cells in normal plants. When the plantlets are taken out from the culture vessels and planted in *ex vitro* environment they may desiccate or wilt due to excess water loss through stomata. A sufficient humidity around the plants the plantlets protect them from dehydration.

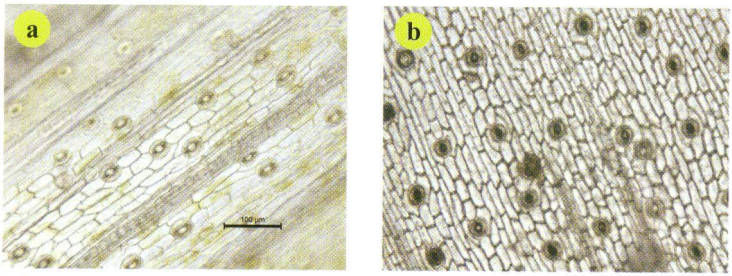


Plate. 4 Showing stomata on lower surface of leaves of a. *in vitro* raised plantlets and b. hardened plantlets of Cymbidium hybrid

5.2.3 Water stress

The retarded development of cuticle, epicuticular waxes and poorly functional stomatal apparatus in *in vitro* raised plantlets cause high stomatal and cuticular transpiration when they are taken out of the culture vessels. The excessive water loss is linked with failure of stomata to regulate transpiration from aerial parts of the plantlets or insufficient or no absorption of water from the roots leading to desiccation or wilting of plants. The permeability of water through the cuticle is determined by the structure and amount of cuticular and epicuticular waxes present on the leaves. The high humidity in culture vessels prevents synthesis of cuticular and epicuticular wax. The reduced amount of epicuticular wax is directly correlated with increased water loss from the leaves. The tissue cultured plants

also have poor vascular connection between shoot and root may reduce translocation of water.

5.2.4 Pathogenic infections

Plants in culture vessels grow under controlled and aseptic conditions without any exposure to fungal or bacterial contaminants. The plants grown under such conditions are vulnerable to pathogenic infection as their defense mechanism has not yet developed well. Harsh climatic condition under *ex vitro*, untimely acclimatization, injuries during removal of plantlets washing and cleaning may cause bacterial or fungal infections during acclimatization and thereby dying of plantlets. The plantlets should be removed from the culture vessels during the season of active growth and all possible care should be taken to avoid physical injuries to the plantlets during removal, washing and cleaning of plantlets.

5.3 Anatomical characteristics

Micropropagated plantlets show different anatomical characteristics than *ex vitro* propagated plants. These include poorly developed cuticle and cuticular wax and poor vascular bundles.

5.3.1 Poor development of cuticle and cuticular wax

The high humidity in the tissue culture bottles obstructs the development of cuticle and cuticular wax on the emerging leaves. When the plantlets with poorly developed cuticle are transferred to *ex vitro* environment they undergo desiccation due to loss of water because of relatively high temperature fluctuation, low relative humidity, and higher light intensities in

ex vitro environment.

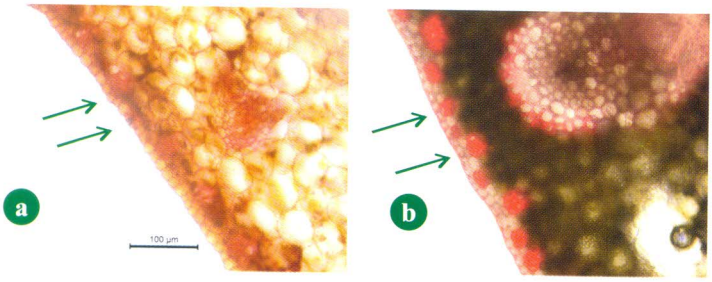
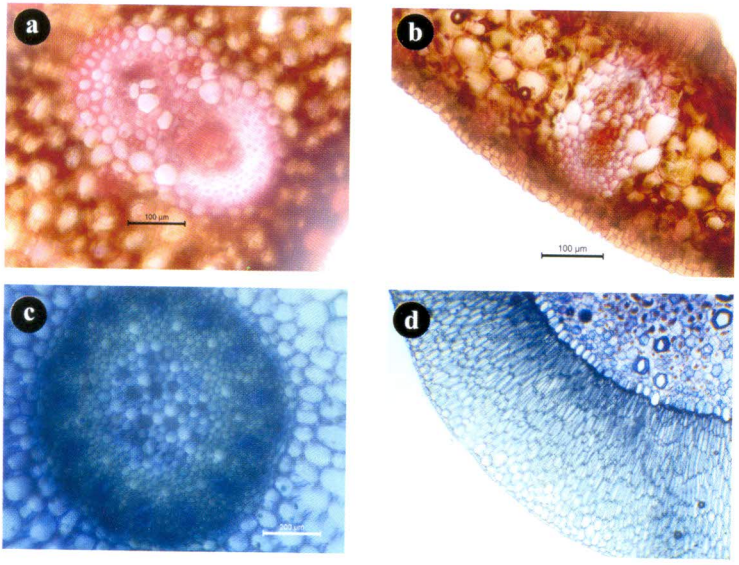


Plate.5 Showing difference in cuticle development on leaves of a. tissue culture raised plantlets b. acclimatized plantlets of cymbidium hybrid

5.3.3 Poor vascular connection

In vitro raised plantlets have poorly developed vascular bundles. Micropropated plantlets have poor vascular connection between the root and shoot thereby hindering the absorption of water through the roots and its proper transportation to the shoot.



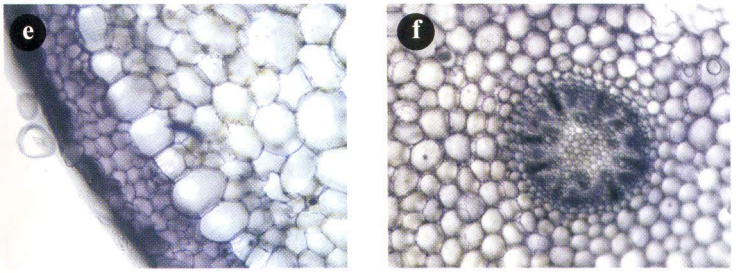


Plate. 6 Showing vascular bundles in leaves and roots of acclimatized and *in vitro* raised plantlet a. acclimatized leaf b. *in vitro* leaf c & d. acclimatized roots and e & f. *in vitro* roots

6. STRATEGIES FOR ACCLIMATIZATION

The good techniques of acclimatization of tissue cultured plants lead to regulate the mechanism of stomatal closure, autotrophic growth, low water loss, and establishment of plants in septic environment. There are several strategies adopted to increase the rate of survival of micropropagated plantlets during acclimatization. The strategies adopted to increase the rate of survival may be broadly classified in to two, *in vitro* and *ex vitro* strategies.

6.1 *In vitro* strategies

The *in vitro* strategies are adopted when the plantlets are still growing in culture vessels. These include reduction in levels of energy source, carbohydrate, reduction in levels of humidity, increasing gas exchange, increasing CO_2 concentration and light intensity etc.

« The reduction in levels of sucrose in culture medium increases the photosynthetic ability of plantlets thereby enhances the rate of survival tissue cultured plantlets during *ex vitro* acclimatization.

- The researches have shown that chlorophyllous plantlets have a better ability to photosynthesis but due to the presence of higher amount of sucrose and low CO_2 they prefer to grow hetero or mixotrophically. The reduction or removal of sugars from the culture medium and providing higher concentration of CO_2 changes their mode of nutrition hetero to autotrophic. Such plants have greater survival and eliminate the cumbersome procedure of *ex vitro* acclimatization.
- The closures of culture vessels affect the gaseous as well as light environment within the culture vessels. The use of gas permeable microporous polyethylene films for vessels closure system increases exchange of gases thereby reducing the harmful effect of culture induced phenotypes.

6.2 *Ex vitro* strategies

The *ex vitro* strategies are adopted when the plantlets are taken out of the culture vessels. These are adopted to reduce water loss from the aerial part of the plantlets, preplant treatments, medium or substrate in which plantlets are transplanted, and fertilizer solutions applied to feed the plantlets.

Reducing water loss from the *in vitro* raised plantlets for first few weeks is necessary to maintain the integrety of plantlets and to circumvent desiccation or wilting. It is accomplished by planting tissue cultured plantlets in high humidity chambers.

7. PROCEDURE FOR ACCLIMATIZATION OF MICROPROPAGATED CYMBIDIUMS

The acclimatization of plantlet in *ex vitro* is the last stage in the process of micropropagation. It involves nearly 60 percent of total production cost. The viability and profitability of a micropropagation laboratory depends upon success in *ex vitro* acclimatization plantlets. Acclimatization of micropropagated plantlets requires a preplanned strategy and installations. For convenience, the acclimatization process can be divided into four steps namely; preoperational activities, operational activities, transplanting of micropropagated plantlets and monitoring and caring transplants. The detailed activities under each step are summarized as below:

7.1 Step 1: Preoperational activities

Before the beginning of the actual process of acclimatization, manpower should be trained and installations required for the acclimatization should be constructed. The size of installations depends on the quantity of material is to be handled. A detailed time table of all the activities is as follows:

7.1.1 Selecting and training workers

The persons engaged for acclimatization of micropropagated should be qualified and have a proper understanding of the physiology of micropropagated plants. If not, workers should be trained about the basics of acclimatization. The numbers of workers are needed depend on their experience and the quantity of plantlets entering into acclimatization unit. A qualified and experienced worker can roughly plant two to three times more plantlets than a novice worker.

7.1.2 Planning and construction of installations

Generally three installations, transfer area, glasshouse and polyhouse are required for acclimatization unit. The size or space of these installations is determined by the quantity of plantlets entering for the acclimatization.

7.1.2.1 Transfer area

The transfer area in an acclimatization unit consists of a store for storing potting substrate and other inputs, place autoclaving/sterilizing the potting substrate, a room for keeping culture vessels received from the micropropagation laboratory, surface for mixing pot mix, washing area and working tables for transplanting of plantlets. This space should be cool, protected from direct sunlight and strong winds.

7.1.2.2 Greenhouse/ Glasshouse

Glasshouse or greenhouse is required for primary hardening of micropropagated plants. It should have provisions for controlling temperature, light and relative humidity. The microsprays is suspended either over the tables or from the roof to control the temperature and relative humidity. The natural light is useful in acclimatizing the plantlets. The plantlets may be exposed to the morning sunlight. However, these should be saved from midday or afternoon direct sunlight for first few days of acclimatization. Installing protective screen on the top or below the roof curtails unwanted sunlight. The humidity chambers

Plate.7 A green house with climate control

can also be constructed under these greenhouses where the plantlets are kept for 3-4 weeks.

7.1.2.3 Polyhouse

A naturally ventilated polyhouse is required for secondary hardening of micropropagated cymbidiums. Microsprays can be installed for maintaining relative humidity around the plantlets. These can be installed over the benches or suspended from the roof. The plantlets are planted in 5 cm diameter plastic pots before they are taken to polyhouse for secondary hardening. The pots are transferred to secondary hardening room on benches. The plantlets remain in secondary hardening house for about 2-3 months. An insect net may be provided at the sides to prevent the entry of insects.

Plate.8 Secondary hardening of micropropagated cymbidiums under naturally ventilated polyhouse at Darjeeling Campus of NRCO

7.1.3 Procuring equipment, materials and inputs

To acclimatize the micropropagated plantlets, the following elements are required:

« Pot mix, sphagnum moss and equipment for sterilizing

or fumigating the pot mix.

- « Fertilizers, plant protection chemicals, balance, EC meter, pH meter, flask washer, scissors, plastic or bamboo trays.
- « Container for receiving plantlets with which agar have been removed.
- « Buckets, spade, wheelbarrow and garden spade hose and irrigation.
- « Plastic trays, pots (2") with perforation for planting of tissue cultured plantlets.
- « Field book, permanent marker, pencils, plastic tags for identification.

7.2 Step 2: Operational activities

The operational activities include obtaining micropropagated plantlets from the tissue culture laboratories, acclimatizing them in culture bottles, preparing potting substrate for planting and other preparation for transplanting of micropropagated plants like preparing humidity chambers, disinfecting benches transfer area etc.

7.2.1 Obtaining *in vitro* plantlets

The *in vitro* propagated plants coming from the tissue culture laboratories are usually supplied in boxes containing bottles or flasks. After reaching the bottles/flasks to acclimatization units, these should be unpacked immediately. The bottles should be placed at intervals under cool fluorescent or indirect sunlight. The flasks should never be placed in direct sunlight or in the glasshouse. The plantlets from infected bottles should be

removed and potted immediately. The flasks may be counted and their numbers may be recorded according to the variety.

7.2.2 Acclimatizing plantlets in culture vessels

The process for acclimation of micropropagated plants starts when they are still in the laboratory. This achieved by lowering or eliminating sucrose from culture media, increasing CO_2 concentration in combination with high light intensity, using antitranspirants, controlling gas exchange etc. Upon receiving, the culture bottles in acclimatization unit, the humidity in the headspace of culture vessels is lowered by adding of desiccants in culture vessels or by bottom cooling. This can also be achieved by loosening of closure for few days to adjust in *in vivo* conditions and then removing the closures from the culture vessels and keeping them in sterile conditions for 3-4 days. During *in vitro* acclimatization of plantlets, the roots of micropropagated plants can be infected with symbiotic fungi for better survival and growth upon transferring them in the field.

7.2.3 Preparation of soil substrate

A potting medium for proper growth and development of micropropagated cymbidiums should have a good balance between its moisture-holding and drainage characteristics. Such substrate allows the roots to obtain sufficient water without draining out. White moss is common substrate used for acclimatization of micropropagated cymbidiums. However, its collection from natural habitats has been banned by Ministry of Environment and Forestry, Govt. of India. The experiments conducted at NRCO have shown that white moss can be

replaced with common sphagnum moss for acclimatization of cymbidiums. The potting substrate should be uniformly moistened before placing it to nursery containers. Moistening of substrate helps in positioning the roots in the container while planting. The plants should be water immediately after planting and substrate should not be allowed to dry or too wet. Too wet environment in the root zone may cause root rot or bacterial rot leading to death of plantlets.

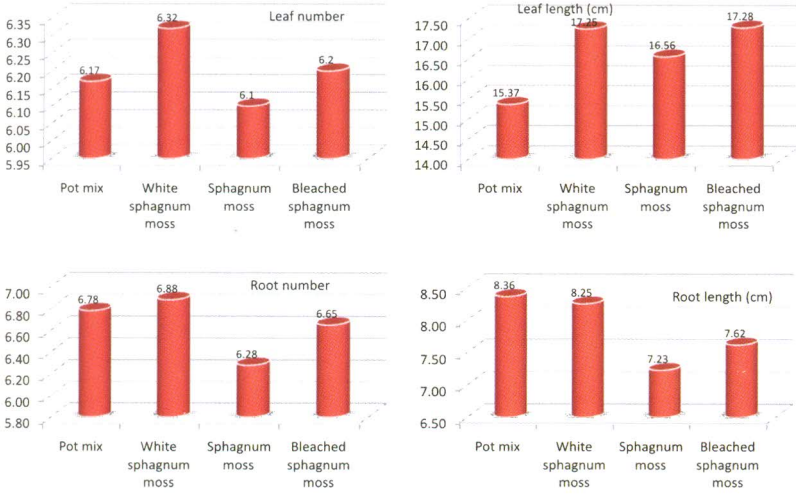


Plate 9. Effect of various potting substrates on growth of micropropagated cymbidium plantlets during acclimatization

7.2.4 Preparing for transplanting

Before the beginning of actual transplanting all the installations should be disinfected, the planting trays are filled with moist potting substrate, a mixture of fungicide is prepared for disinfecting the plantlets and fertilizer mixture is prepared to irrigate the plantlets. If necessary, the humidity chambers may be prepared. The personnel may be re-trained in transplanting

of the plantlets.

7.2.4.1 Disinfecting and cleaning of the site

The installations and working benches should be disinfected with sodium hypochlorite and the equipments and implements should be organized in their places. The benches, planting trays and instruments likely to be used during transplanting should be disinfected. The humidity chambers where the plantlets are to acclimatized should also be disinfected.

7.2.4.2 Preparing the trays/compots

Plantlets of cymbidium can be transplanted in plastic trays, compots or pots (10 cm dia.). A plastic tray measuring 29x23x9cm accommodates about 16-25 plants. The trays or pots can nearly half filled with moist sphagnum moss and then sphagnum moss is wrapped around the roots and placed in the tray or pots. In trays, the plants are tightly secured with the help of sphagnum moss.

7.2.4.3 Preparing humidity chambers

Humidity chambers are enclosed area/ chamber e.g. polythene tents under the greenhouse. These are constructed to maintain sufficiently high humidity around the plants. These can be like a low height tunnels or can be made by clear transparent bag. The plants require high humidity for the first few weeks. The extended periods under high humidity encourages fungal growth and hence, humidity should be reduced to a normal level earliest as possible.

7.3 Step 3: Transplanting of micropropagated plantlets

Transplanting of micropropagated plants is a very stressful stage for the plantlets, especially when it is carried out by unqualified or inexperienced workers. When the plantlets are taken out from the flasks and transferred to the humidity chambers they undergo microclimatic stress, water and nutrient stress due to change in environmental conditions. The inescapable damage caused during removal of plantlets from culture bottles and preparing them for transplantation further cause stress to the plantlets. The plantlets removed from the culture bottle should be transplanted immediately. Carefully transplanted plants have better survival rate than carelessly planted ones.

7.3.1 Taking out plantlets from culture bottles

For removing plantlets from the culture bottles, plastic tape and the cover is removed and warm water is added to the flask and swirled. Further, by holding the flask in one hand while gently thumping it with the other to loosen the agar from the flask's walls. If the separation is difficult spatula may be used for taking out the plants without causing any damage to the roots. The plantlets can be taken by holding the bunch of shoots together as shown in fig 10. The artificial, gel-like agar medium from the roots should be washed softly in running water. It is necessary because agar containing sucrose and other nutrients would serve as a medium for growth of pathogens. The plantlets should be washed in soapy water of mild detergent and rinsed 3-4 times with plain water to ensure that no traces of agar-medium are present on the plantlets. The plantlets are wetted off by paper towel or air dried on newspapers under shade.

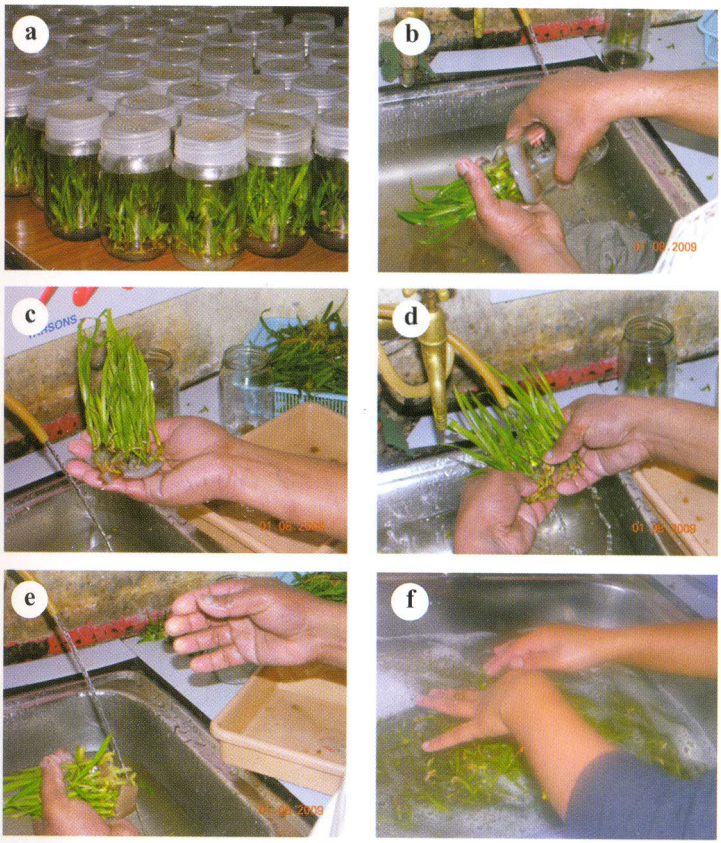


Plate10. Showing the method of taking out plantlets from culture vessel a. bottles ready for deflasking b. taking out plantlets c. plantlets out of culture bottle d & e. removing the agar f. washing of plantlets.

7.3.2 Grading of plantlets

After washing and wetting off by paper towels, the plantlets are graded on the basis of plant height, leaf number and roots present on them. Plants bearing healthy shoots but no roots should be taken out and their basal portion should be dipped in NAA @ 10 mg/lt for 30 min and planted. This will encourage

rooting. However, the concentration may vary variety to variety which can be worked out for a particular variety. Large plantlets with well developed shoot and root (3-4 leaves and 2-3 roots) grow rapidly upon transplanting them into potting substrate. Small poorly formed or weak plantlets should be discarded.

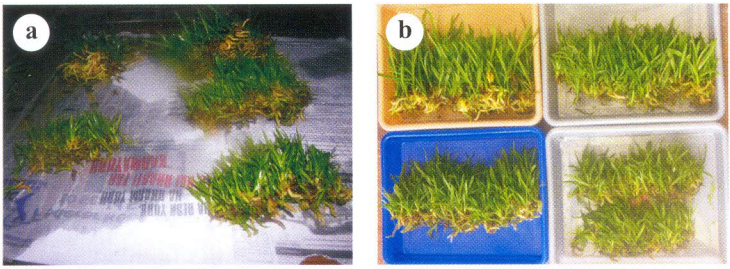


Plate11. Grading of cymbidium plantlets after washing

7.3.3 Fungicidal treatment

After grading, plantlets are soaked in a solution containing bavistin (2.0 g/lt) for 5 min. Thereafter, plantlets are taken out and spread on paper towel or newspaper for wetting off excess moisture. The plants are now ready for planting. These can be transplanted in pots or trays. The photograph below showing the plantlets soaked in a solution of bavistin.

Plate12. Plantlets soaked in solution containing Bavistin

7.3.4 Transplanting in to plastic trays/community pots

The trays are filled nearly half with moist sphagnum moss and then the roots of individual plantlets are wrapped with moist sphagnum moss and placed in trays or in pots. The plantlets are tightly secured with sphagnum moss after placing requisite

number plantlets in trays or pots. A plastic tray measuring 29x23x9 cm accommodates about 16-25 plants. The trays are watered with nutrient solution and placed in humidity chambers for 2-3 weeks. The trays are marked with a label on which should appear the variety's name, number of humidity chamber, date and hour of transplanting and the transplanter's name.

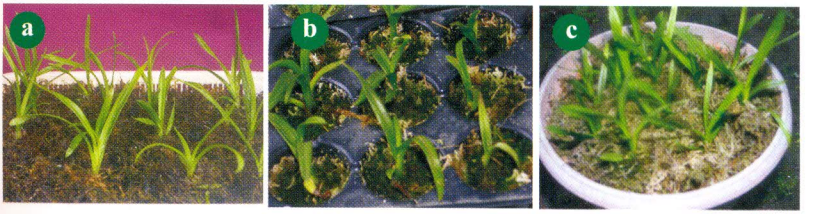


Plate13. Cymbidium plantlets planted in a. perforated plastic tray b. plug tray and c. earthen pot

7.3.5 Transferring to humidity chambers

Transferring plantlets to humidity chambers start real process of hardening. The objective of placing the plantlets in a humidity chamber is to protect plantlets from excessive water loss arising out of physiological and anatomical abnormalities in micropropagated plantlets. The preparation of humidity chambers is described under 7.2.4.3. The humidity chambers are prepared/placed in hardening house where temperature (25 - 27 ^{0}C) and relative humidity (70-80 %) are maintained around the humidity chambers by intermittent misting.

7.4 Step 4: Monitoring and caring transplants

After transplanting the plantlets need to be monitored regularly for microclimatic conditions, moisture availability, wilting or drying symptoms, nutrient deficiency, occurrence of diseases and pests. First 2-3 weeks are very crucial and a great care needs

to be excised with respect to management of relative humidity and light conditions. The plantlets are gradually exposed to lower humidity and higher light levels. The light is curtailed by using shade cloth or shade nets.

7.4.1 Microclimate in humidity chamber

The plantlets placed in humidity chambers are very prone to water loss and pathogenic infections. Light, temperature and relative humidity of the humidity chambers is the most important factors during acclimatization. If relative humidity is too low and light level is high plantlets will desiccate. Very high humidity around within humidity chamber would cause bacterial soft rot. The plantlets are kept in humidity chambers for 2-3 weeks. In the following weeks, these should be exposed to 50 % shade and the plastics of humidity chambers are removed when the plantlets have adapted to the micro environment of the greenhouse.

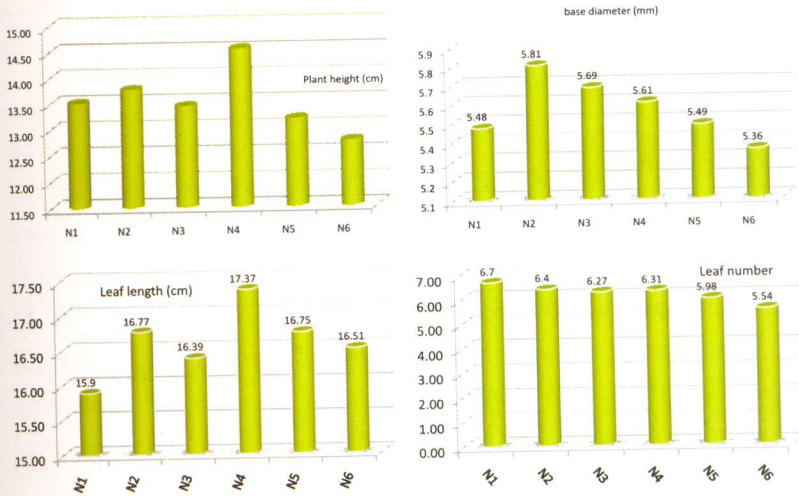
7.4.2 Irrigation

The plantlets irrigated with the correct quantity of nutrient solution once before placing them in a humidity chambers would not require further irrigation till they are in humidity chamber. However, if the symptoms of physiological wilting or drying of substrate are noticed, the plantlets may be irrigated. The plants are irrigated after removing from the humidity chambers at a regular interval considering the microclimatic conditions of the polyhouse and growth stage of plantlets. Irrigating plantlets with micrsprays is useful. When the plantlets are to be irrigated with microsprays, these should be rigorously monitored for phytopathlogical problems in the

plantlets. The excess moisture in the substrate would cause root rot or bacterial soft rot and algal growth on the substrate and pots. The potting substrate should not be soggy because the plants have thin and tender cell walls in their collar region and can quickly become infected and die.

7.4.3 Fertilizer application

Fertilizing the plantlets with weak nutrient solutions is beneficial. The fertilizers may be applied through microsprays or using hose but it should be ensured that water on the plantlets dries before the sun is set. We tested six nutrient solutions and found that salts (macro and micro) of Nitsch medium increase the plant height and leaf length (Fig-----). The plantlets should be watered twice a week with week fertiliser solution depending on the moistness of the substrate and prevailing weather conditions.



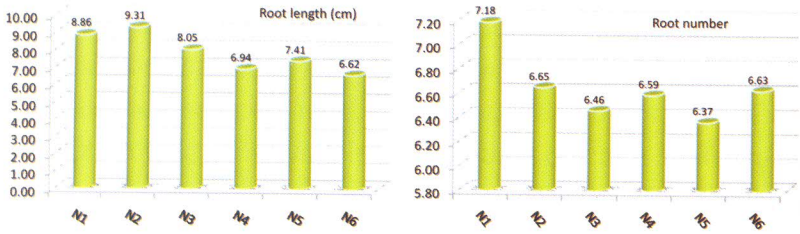


Plate 14. Effect of various nutrient solutions on growth of micropropagated cymbidium plantlets during acclimatization

7.4.4 Pest management

Microclimatic conditions of the glasshouse are favourable for building up of harmful insect and pest population. If they are not managed properly, may cause severe losses to plantlets growing in the greenhouse. Greenhouse floors, walls, benches or any other hiding places should be kept clean. The clean cultivation discourages building up of insect and pest population. The major insects causing damage to plantlets during hardening are given as below:

7.4.4.1 Red spider mites

Mites are a serious problem in cymbidium orchids. They suck the cell sap from the underside of the newly emerged leaves giving rise silvery appearance. Hot and dry weather favours build up red spider mite population. The incidence of insects can be discouraged by providing humidity around the plants. At the initial stage these can be controlled by spraying neem oil (Azadirachitin) @ 5 ml/lt of water. In severe cases spraying with Imidacloprid (Confidor) @ 0.05 % at 10-15 days interval is useful in controlling mites.

7.4.4.2 Scales

The scales stick on the under surface of leaves and suck the sap from the cells causing loss of vigour and deformation in infested plantlets. The plants infested with scale insects should not be introduced in hardening house. Scales can be removed by rubbing the scurf encrustation with a soft tooth brush dipped in 70 % methylated spirit or by spraying with Malathion 0.05 percent.

7.4.4.3 Snails

These nocturnal creatures remain hiding in debris, under the benches or even in potting media during the day and cause damage to plants by feeding on tender parts of the plants like buds, flowers and tender leaves during nights. They can be hand picked or trapped. The application of baits containing metaldehyde (1%) on the greenhouse floor, benches effectively controls this pest. Clean cultivation discourages build up snail population.

7.4.5 Disease management

High humidity and optimum temperature favours development of many fungal and bacterial diseases in greenhouse grown plants. The major diseases encountered during acclimatization of cymbidiums are described as below:

7.4.5.1. Tip burn

The leaves of cymbidium first turn to light brown in colour and later dark brown. The exact reason of tip burn is not known but it is believed that accumulation of salts causes tip burn. Flush out the pots to remove the accumulated salts. The cultivars having

Cymbidium devonianum in their back ground are susceptible to this disorder.

7.4.5.2 Anthracnose

It is a fungal disease caused by *Collectotrichum gloeosporioides*. Oblong to oval, sunken, reddish brown to grey coloured spots appear on the leaf tips of infected plants. The affected part of the leaves should be removed with sterilised cutting tools. The pots and benches should be sprayed with 2 percent Formalin. Clean cultivation prevents spread of this disease. Spraying with Mancozeb and Carbendazim @ 1 g/l at weekly interval controls this disease.

7.4.5.3 Black rot

A devastating disease of cymbidiums caused by a fungus which could get introduced through unsterilised potting ingredients especially leaf mould. The water soaked symptoms develop on the aerial parts of the plants that turn brown. The affected shoots can be pulled out with slight pressure and rotting portion gives out a foul smell. The drenching of potting mixes with Metalyxyl @ 2.0 g /litre is useful.

7.4.5.4 Bacterial soft rot

This disease causes severe losses between transplantation to initiation of roots. High humidity coupled with high temperature favours development of this disease. The disease is caused by *Erwinia* sp. The affected plantlets show water soaked greyish-green lesions. The tissues of the infected area disintegrate and produce foul smell. The disease can be controlled by spraying tetracycline hydrochloride @ 1000 ppm.

7.4.5.5 Viral diseases

Cymbidiums in India are infected by three viruses namely Cymbidium mosaic virus (Cym MV), Odontoglossum ringspot virus (ORSV)and Orchid Fleck Rhabdovirus. Cym MV and ORSV are most common found in India. The affected show irregular or concentric yellow patches on the leaves. The viruses spread through cell sap during cutting of leaves, cleaning and handling of plantlets. Always use disease free planting material for commercial cultivation and prevent the spread of disease (read pamphlet 'prevention of virus transmission in cymbidium' published by NRC for Orchids).

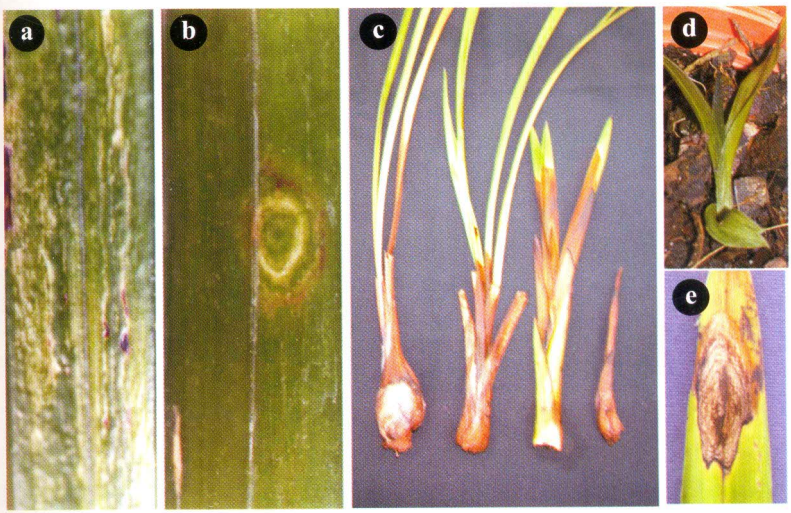


Plate15. *Cymbidiums* plantlets affected with a. Cym MV b. ORSV c. black rot d. bacterial rot e. anthracnose

8. VARIATION IN MICROPROPAGATED PLANTS

Micropropagation is usually referred as art and science of cloning selected genotypes in a short period of time. The plantlets regenerated through this technique may show phenotypic variations called as somaclonal variations. These variations are either genetic or epigenetic in origin. The genetic variations are inheritable and arise due to pre-existing variations in somatic cells of explant or caused by mutations and changes at DNA level. However, the epigenetic changes are non-heritable and induced mainly due to exposure to plant growth regulators and culture environment. Somaclonal variations are undesirable where the objective is to produce genetically identical (true-to-type) plantlets for commercial cultivation. Nevertheless, somaclonal variations have been useful in the creation of genetic variability, producing disease resistance lines, increasing yield and quality, etc. Somaclonal variants of cymbidium show variegation in leaf, flower color and shape. In addition to variegation flower size and inflorescence has also been reported in *Phalaenopsis*.

9. SUMMARY

Cymbidiums propagate very slowly through conventional propagation methods like division and propagation through backbulbs etc. However, in recent years micropropagation has become a standard tool for producing a large number of plantlets in a short span of time. At commercial scale, the success or failure of micropropagation technique is often

depending upon acclimation and hardening of micropropagated plants rather than laboratory. In their early stage, micropropagated plants are very sensitive to changes in environmental conditions, but they perform just any other propagule after the roots become active. It is advisable that farmers should purchase tissue cultured plants at pre hardened stage, unless they have mastered techniques for acclimatization. After acquiring sufficient knowledge and practice, they may procure culture bottles and harden at their own farm. This would reduce the cost on planting materials as the tissue culture laboratories need not incur the cost on hardening of plantlets which roughly 60 percent of the total cost of production,

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