INTEGRATION OF ARBUSCULAR MYCORRHIZAL TECHNOLOGY WITH MICRO-PROPAGATION

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One of the important applications of modern biotechnology in agriculture is its use in tissue culture. *In vitro* micropropagation techniques are increasingly being applied to large scale production of quality planting materials especially in fruit crops and woody timber trees. There are four important stages in the micropropagation of plants irrespective of the various techniques employed for it. They are the regeneration in *in vitro*, proliferation of the regenerates, rooting in *in vitro* and for transplantation of the plantlets in the soil or fields. Of these, the last stage is very crucial and important with respect to the establishment of the *in vitro* derived plantlets. It has been established that tissue culture plantlets have very divergent leaf anatomy and physiology and hence require an acclimatization period during the transition from culture to green house, from a total unnatural system to the very natural environment. In the *in vitro* condition, plantlets are heterotrophic and get very high favourable conditions for their growth. But in the *ex vitro* situation, the plantlets have to switch over to autotrophic nutrition, involving normal photosynthetic activity and water relations. The plantlets may not be able to withstand such sudden shocks of the environmental changes, mostly due to some aberrant characteristic features of *in vitro* derived plantlets.

Physiological features of tissue culture plantlets

One of the major impediments to the success of micropropagation is the very high mortality rate of *in vitro* plantlets either during acclimatization phase or during transfer to the field conditions. Generally, most of the *in vitro* derived plantlets fail to survive. Very often desiccation and wilting are the main causes of low survival. It has been estimated that roughly only 25 percent of the *in vitro* regenerated plantlets has been successfully transplanted

ex vitro and still fewer transferred to the field. Such a disappointing state of affairs has been attributed to certain underlying causes, of which the aberrant features characteristic of *in vitro* derived plantlets are significant. Some of such features are as follows.

1. Leaves with poor or no development of cuticular wax:

The high humidity in the culture vessels hinders the development of cuticle and epicuticular wax on the newly emerging leaves. When such plantlets are planted out they undergo desiccation and drying. It has been noticed that the palisade cells of leaf surface are poorly developed and pronounced mesophyll airspaces.

2. Impaired stomatal mechanism:

The impaired stomatal mechanism has been attributed to the abnormal orientation of microfibrils and high deposition of calcium in guard cells as well as high levels of Na^+ in guard cells which may interfere with the movement of K^+

3. Poor photosynthetic activity:

Because of the availability of enough sugar in the medium, the *in vitro* growing plantlets are not truly photoautotrophic but mixo or heterotrophic. Poor organization of grana in the chloroplasts of the *in vitro* growing plantlets along with etiolated effect produced by ethylene in the glass vessels greatly contribute to their decreased photosynthetic ability.

4. Vitrification of shoots:

Vitrification of shoots to different extent due to non development of cuticular wax and associated poor vascular tissue differentiation.

5. Poor vascular connection:

Rooting of shoots preceded by intervening callusing and poor association of conducting tissues of root and shoot (poor vascular connection between root and shoot) hinder the absorption of water through the roots and its proper transportation to the shoot.

6. Root hair development

Lack of proper root hair development in general directly affects the absorption of nutrients from the soil.

7. Dehydration and pathogenic infection:

Poor development of cuticular wax together with the defunct stomatal system leads to excessive dehydration of tissue culture plantlets. The micropropagated plantlets often fail to compensate this water loss through effective water absorption and transportation. The absorption organ, viz., the root hair is not properly developed and vascular connection between root and shoot is defective. This ultimately ends up with heavy mortality due to dehydration and wilting. Further, plantlets developed under controlled aseptic condition without any exposure to microflora are highly vulnerable to microbial infection as its defence mechanism has not been triggered earlier. All these characteristics together with other factors

such as untimely processing of plantlets, exposure to unfavourable seasonal conditions etc. are contributing in increasing the mortality during transplantation. Efforts to overcome these problems by adopting new technologies met with varying degrees of success. Successful hardening and *ex vitro* establishment of plantlets are very often achieved by inoculation with Arbuscular Mycorrhizal Fungi (AMF) at the time of planting out. AM fungi have been shown to play a very crucial role in supporting the survival, growth and establishment of micropropagated plants..

Mycorrhizal Association

The term mycorrhizae denotes "fungus roots". It is a symbiotic association between host plants and certain group of fungi at the root system, in which the fungal partner is benefited by obtaining its carbon requirements from the photosynthates of the host and the host in turn is benefited by obtaining the much needed nutrients especially phosphorus, calcium, copper etc., which are otherwise inaccessible to it, with the help of the fine absorbing hyphae of the fungus. Based on the type of association formed by the ubiquitous fungi two broad groups, viz., Ectomycorrhizae and Endomycorrhizae have been recognized.

Endomycorrhizal fungi grow intra and intercellularly forming specific fungal structures in the cortical region of the host. These fungi are further divided into three subgroups. They are Ericoid, orchid and the ubiquitous and large group of arbuscular mycorrhizal (AM) fungi. Ericoid mycorrhizal fungi have septate hyphae and colonize plants belonging to Ericaceae, Whereas orchid mycorrhizal fungi are aseptate with clamp connections, and colonize orchidaceous plants. The mycorrhizal fungi having aseptate hyphae are grouped under vesicular arbuscular mycorrhizal fungi (AMF)

Characteristics of arbuscular mycorrhizae

AM fungi are ubiquitous in nature and colonize most of the cultivated crops except members of Chenopodiaceae, Cruciferaceae

and Caryophyllaceae. They are obligately symbiotic and colonize the cortical region of the very fine absorbing roots of host plants. All soil fungi that form arbuscule are placed under order Glomales. The order Glomales belongs to class Zygomycetes of the subdivision Zygomycotina. Of the six recognized genera, Glomus, Sclerocystis, Enterophosphora and Acaulospora form both vesicles and arbuscules. Gigaspora and Scutellospora do not have the intraradical vesicles. They posses the following characteristic features in general;

Vesicles and arbuscules:

AM fungi have aseptate mycelium and upon colonization do not change the morphology of plant roots. On root colonization the AM fungi produce two specialized structures known as vesicles and arbuscules in the cortex region of root. Arbuscules are complex structures similar to haustoria produced within the host cells. They serve as sites of nutrient exchange between host and the fungus. The vesicles are terminal, ovate to globose structures that contain drops of yellow oil. It is reported that vesicles are thin walled, act as temporary food storage organs, but when vesicles remain thickwalled they might function as resting spores.

Obligate symbionts:

Arbuscular mycorrhizal fungi are always found associated with a host plant. All efforts to culture the fungi on laboratory media have been found to be futile.

Form morphologically distinct resting spores:

The fungus produces resting spores (chlamydospores) in soil which vary in size ranging from 100-600 µm and are recovered from soil by wet sieving. They produce typical AM infection in axenic cultures.

Colonize the cortical region of feeder roots:

AM fungi are only found associated with cortical tissues of

very fine feeder roots. No visible tissue damage could be produced. Growth of mycelium is inter and intracellular.

External mycelium :

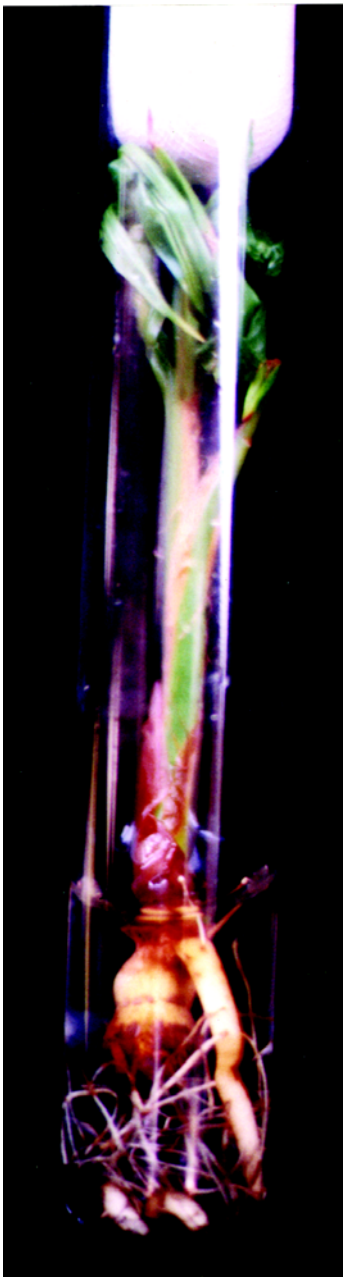
The external mycelium of the fungus is comprised of coarse hyphae and a tuft of much finer thin walled absorption hyphae. The absorption hyphae are important structures with regard to plant nutrition. It is comprised of strategically placed network of additional absorbing surface which enable the plant to tap soil nutrients and water beyond the depletion zone, which are otherwise inaccessible to plant roots. Nutrient ions taken up by the hyphae are transported through the hyphae and released in the root cells by means of arbuscules.

Beneficial attributes of AMF to plants

It is a well documented fact that the host plant is enormously benefited due to AM association. This includes the enhanced uptake of phosphorus and other nutrients, imparting resistance to stress conditions such as drought, high salt concentration and heavy metal toxicity, assisting in the enhanced nitrogen fixation by leguminous plants, resistance to soil borne diseases, improved water relations, early establishment and growth of nursery seedlings, preventing soil erosion etc.

Improved nutrient uptake

Irrespective of their type, the most important role of AM fungi is to absorb nutrients from the soil in tropics are not deficient and transfer them to their hosts. Although all the soils in tropics are not deficit in phosphorus, its unavailability due to phosphorus fixation is found to be a major constraint. This is true in the case of many agricultural lands in India, particularly the acidic soils of Kerala. The presence of AM fungi in roots of crops grown in such soils is found useful with reference to their phosphorous uptake. Mycorrhizal structures effectively take up phosphorus from lower concentration



at which normal plant roots fail. Further, the AM fungi increase the surface area of absorptive system of plant and explore the soil by the external hyphae beyond the root hairs and phosphorus depletion zone. Absorbed phosphorus is converted to polyphosphate granules in the external hyphae and passed to the arbuscules for transfer to the host. The same mechanism also helps the uptake of potassium, zinc, iron, copper, magnesium and calcium. Effect of AM in aiding the uptake of nutrients, particularly phosphorus, has been well documented in studies conducted in cassava, black pepper, cashew and legumes grown in phosphorus fixing soils of Kerala.

Better water relations

AM fungi play an important role in the water economy of plants. Their association improves the hydraulic conductivity of the root at lower soil water potentials and this improvement is one of the factors contributing towards better uptake of water by plants. Also, leaf wilting after soil drying, did not occur in mycorrhizal plants until soil water potential was considerably lowered (approx. 1.0 M. Pa) Leaflets of Leucaena plants inoculated with VA mycorrhizae did not wilt at a xylem pressure potential as low

as −2.0 MPa.

Drought tolerance

Colonization by AM fungi can improve drought resistance of plants. This has been demonstrated in green house studies in cultivated crops such as wheat, onion Capsicum and red clover as well as several other plant species. Field grown corn plants showed a remarkable resistance to water stress when inoculated with AM. Mycorrhiza induced drought tolerance can be related to factors associated with AM colonization such as improved leaf water and turgor potentials and maintenance of stomatal functioning and transpiration, greater hydraulic conductivities and increased root length and development.

Well developed root system

Due to endomycorrhizal inoculation, the micropropagated plants have changed root morphology. It caused increase in lateral root number and root length. The AM plants develop a more economical root system which is more efficient in absorption of nutrients and water. Mycorrhizal inoculation stimulates rooting and growth and thereby transplant survival of cuttings and seedlings raised in the nursery media. They also help in establishment of pant cover on mine soils, eroded soils, industrial waste. Where the plant cover is diffcult to establish.

Enhanced phytohormone activity

The activity of phytohormones like cytokinin and indole acetic acid is significantly higher in plants inoculated with AM. Higher hormone production results in better growth and development of the plant.

Increased photosynthesis

Increased efficiency in photosynthesis due to AM fungi has been observed. The reduction in stomatal and mesophyll resistance to carbon dioxide uptake, increased chlorophyll content and better

hydration of plants brought about by AMF colonization favours the carbon dioxide fixation.

Salt and heavy metal tolerance

Certain plants that are known to be halophilic are colonized by AM fungi. Some of these plants show high tolerance to osmotic stress. Onion and bell pepper plants showed improved growth in saline soils when inoculated with AM fungi. However, the salinity of soil was marked by change in the species distribution among AM fungi. Several studies indicate that colonization of plants by AM fungi confers protections against toxic metals. AM grasses grown in Zn polluted soil showed reduced Zn toxicity. Apparently, mycorrhizae can enhance plant uptake of toxic metals, but they may also afford protection from these metals.

Soil structure

The mycorrhizae are important contributors to soil stability. Obviously, by increasing nutrient uptake, they improve plant cover and root proliferation. The large amount of AM fungal hyphae in soil serves to bind soil particles together and maintain the stability. Thus AM fungi play a role in erosion control. In low fertility eroded soil the utilization of selected AM fungi along with starter fertilizer can increase the plant establishment and found useful in afforestation programme. Soil compaction results in reduced plant growth an root development, water content and soil aeration. In such soils mycorrhizal inoculation is found to alleviate some of the ill effects of compaction.

Interaction with soil pathogens

The soil borne pathogens such as certain fungi and nematodes are known to be inhibited by the AM fungi in plant root system. In many crops an inverse relation between AMF association and the incidence of fungal and nematode pathogens are observed. Artificial inoculation of seedlings and cuttings with an appropriate

AM fungus in nursery is found to reduce the attack of fungal and nematode pathogens. This has been observed in many crops such as black pepper, cardamom, ginger turmeric, solanaceous vegetables, amaranthus and legumes in the studies conducted in Kerala Agricultural University. Species of Pythium, Phytophthora and Rhizoctonia are responsible for important diseases in the nursery and field of major crops of Kerala. Ability of AMF to induce tolerance to these pathogens is very well established.

Enhanced phenol activity

The mechanism involved in the mycorrhiza induced disease tolerance may be the changed physiological and biochemical nature of the host plant. Increased production and activity of phenolic compounds due to AMF colonization has been observed. Tissssue culture plantlets inoculated with AM fungi had a higher ortho dihydric phenol in the root tissue. Higher phenolic content increases the defence mechanism of the host plant and thereby imparts resistance to various diseases.

Integration with other soil micro-organisms

There is a positive interaction between AM fungi and nitrogen fixing bacteria such as Rhizobium in legumes, Asospirillum and Azotobacter in non legumes and frankia in actinorrhizal plants. AMF association remarkably increases the multiplication, persistence and nitrogen fixation rate of these bacteria. In legumes, particularly the AM dependent crops like subabul and red gram two to three fold increase in nitrogen fixation due to co-inoculation of Rhizobium and AM fungi has been noticed. A similar positive interaction exists between AMF and phosphorus solubilizing micro-organisms.

Crop response to AMF inoculation

Effect of mycorrhizal fungi on growth of their host has been studied with a spectrum of crops, including micropropagated plants and soil conditions. The most remarkable consequence of AM

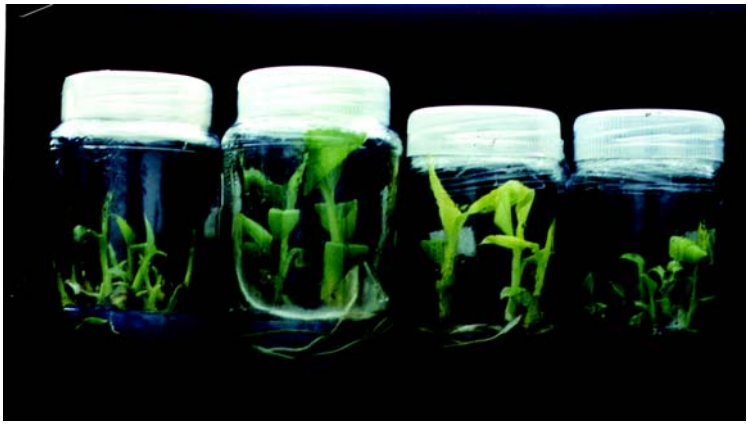
colonization is the enhanced host growth and yield. This effect is most marked when crops are grown in phosphorus deficient soils. Increased growth or yield due to artificial inoculation with AM fungi has been well documented through green house and field studies in vegetables, particularly brinjal, tomato and chillies, legumes such as cowpea, pigeon pea and soybean, important cereals like seasamus and groundnut etc. Studies conducted at the Kerala Agricultural University emphasize the importance of AMF technology in the regions. Beneficial effects of AMF have been established in black pepper, cardamom, cashew, cocoa, ginger, turmeric, cassava, sweet potato, mulberry, transplanted vegetables, legumes and low land rice, flower plants like Zinnia and marigolds. The response to inoculation varies with crop, soil and AM fungi. Hence, it is necessary to identify and appropriate combination to achieve maximum benefit of AMF inoculation.

Association of AMF on tissue culture plantlets

Inoculation with AMF on micropropagate plantlets improves its establishment and growth in the field. Unfortunately not much work has been conducted in this line. But the few convincing reports decisively prove that successful hardening and *ex vitro* establishments of plantlets could be achieved by inoculation with AM fungi at the time of planting out.

One of the earliest reports one the effect of AM fungus on apple clones suggested that the growth and leaf mineral content of two apple clones propagated *in vitro* were increased substantially. Similar enhanced effect on the growth of *in vitro* cultured strawberry platlests could be achieved due to the association of AMF. The rooting of plantlets of garlic regenerated from callus was significantly enhanced due to inoculation with Glomous mosseae. The transplant success and growth of Robus idaeus and Pistacia integerrima were achieved with mycorrhiza inoculation..

The effect of arbuscular mycorrhizal inoculation was evaluated



in micropropagated *Populus deltoids* (Poplars) to determine whether AM inoculation helps in the hardening, survival, establishment and growth of micropropagated plants and the correct stage at which micropropagated plants are to be inoculated. A mixed inoculum comprising of *Glomus* spp. and *Acaulospora* spp.isolated from natural soils was used for the study. The isolate mixture was maintained on *Zea mays*. The spores were isolated from these soils, surface sterilized using 0.4 percent streptomycin sulphate and 200 mg per litre chloramin T solution and washed repeatedly with sterile water. A spore suspension was made in sterile water with a spore density of 200 spores per ml. Two ml suspension was used for inoculation. The controls were inoculated with 2 ml sterile water. The effect of AMF at three different stages of growth of micropropagated plants viz., a) S_1-at the stage of the *in vitro* plants given rooting stimulus, b) S_2- rooted but non hardened plants and c) S_3-rooted and hardened plants placed in sterile sand with AMF spores were evaluated. The arbuscular mycorrhizal colonization after two weeks and prior to transplanting were estimated.

A positive response of micropropagated plants due to AMF inoculation was noticed in all the three stages. Though the response was marked for the S_1 ande S_2 plants, they did not survive during the extended period of the trial. The results show the stage three

(S_3) plants are best suited for AMF inoculation. The colonization and positive response of AM association were manifested in terms of active root growth. Comparison over the extended period of time showed better survival of AM inoculated plants. Control plants could not withstand the outdoor ambient temperature and none of them survived.

Effect of AMF on tissue culture flower plants is also encouraging. In a different study at Vellayani, evaluated the effect of AMF in enhancing of survival, growth characteristics and uptake of nutrients by micropropagated anthurium. AMF was incorporated into the rooting medium at the time of planting out through infected root bits. The study was conducted with Glomus constrictum and G. etunicatum. Observations on AMF colonization, growth parameters and nutrient uptake were recorded upto 45 days after treatment. The results showed positive response due to AMF association like increased survival percentage, enhanced growth and nutrient uptake and early flowering.

Micropropagated avocado plants generally exhibited low survival and very slow rate of growth during acclimatization. Inoculation of G. *fasciculatum* on micropropagated avocado plantlets improved the formation of well developed root system, shoot growth, shoot-root ratio and NPK content in plant tissues which helped plants to tolerate environmental stress transplanting. It was concluded that inococulation of AMF seems to be the key factor for subsequent growth and development of micropropagated plants of avocado

Microporopagated *Annona cherimola* are more dependent on mycorrhizal formation for optimum growth than plants derived from seeds. The greatest effect of AMF on growth was observed when they were introduced after acclimatization period. Apart from improving the survivability and quality of tissue culture plantlets, micropropagation provides stress tolerance to plantlets. Enzymes alleviating stress are thought to be the important factor involved. The response of fruit and ornamental plants to inoculation with G.

intraradices varied with varieties. This is attributed to difference in root colonization. When transplanted to field, the mycorrhizal plants

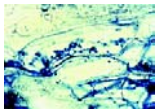
were consistently healthier than non-mycorrhizal plants.

In the case of rose, the plantlets regenerated by *in vitro* technique could not survive the outdoor conditions. In the hardening experiment the plantlets showed desiccation and wilting, and after fourth week 100 percent mortality was observed. Attempt made to establish the rose plantlets with AMF inoculation at the time of planting out was met with remarkable success. All the six species of AM fungi tested in the study significantly increased the survival, growth and establishment of rose plantlets. Non- mycorrhizal control plants could not withstand the outdoor condition for a long period and showed heavy mortality from third week onwards and surprisingly, a 100 percent mortality was noticed by fourth week. Survival rate, growth characteristics and time required for flowering varied with AMF species. G. etunicatum was found to be most effective and recorded 65 percent establishment along with better plant growth and early flowering.

The effect of AMF on the survival of *in vitro* generated jack plants and the resultant change in phenol content under *ex vitro*

Spores of
AMF fungi

Vesicles of AMF in
the root cortex



Arbuscules of AMF
in the root cells

Growth of banana TC
plantlets (c-control, M-AMF
inoculated at the time of
planting out)

Root growth in TC
plantlets of banana
inoculated with AMF

Growth of Alocasia TC
plantlets (c-control,
M-AMF inoculated

Rose plants, control
plants in the process
of wilting

plantlets established
with AMF

Flowering in
established plants

Anthurium TC
plantlets established
with AMF inoculation

Growth of banana
plantlets inoculated with
AMF and Azospirillum (MA)
and control

Growth of Alocasia
plantlets inoculated with
AMF and Azospirillum

conditions were evaluated using five arbuscular mycorrhizal fungal species and found that in addition to the beneficial effects, the total and orthodihydric phenol content were also significantly higher in AMF inoculated plantlets. This higher phenol content, especially the orthodihydric type, accounts for the higher tolerance to soil borne diseases.

Althougth the *ex vitro* establishment is not a major problem in banana and Alocasia plantlets, inoculation with AMF at the time of planting out, in addition to the geneal increase in survival rate, remarkably improved the growth and vigour of plantlets. Effect of AMF was more evident on banana. Shoot and root weight were significantly higher in treated plants. The dual inoculation with Azospirillum augmented the biomass of banana and Alocasia by 39 and 17 percent over inoculation with AMF alone. The synergistic interaction of AMF and Azospirillum helps to reduce hardening period and improve *ex vitro* growth and development of plantlets.

Inoculation of AM fungi significantly improves the survival percentage, growth characteristics and uptake of nutrients of *in vitro* derived plantlets in the exvitro condition. The technology is viable and useful to tackle the survival and establishment problem associated with tissue culture plantlets and also to produce energized tissue culture plantlets.

The probable mechanism of protection:

Every positive aspect of AMF is useful to correct or compensate the physiological defect of micropropagated plantlets which can be summarized as follows:

Consequent to the poor development of cuticular wax and the defunct state of stomata, there is an excessive dehydration from plantlets. Lack of normal development of root hair and vascular connection between shoot and root further aggravate the situation as these factors directly affect the absorption and transport of water and other nutrients. Axenic conditions followed for developing

plantlets make it highly sensitive to microbial infection. Such plantlets, when planted out to out door conditions, very often meet with high mortality due to desiccation and microbial infection.

Mycorrhizal hyphae functioning synonymous to root hair not only compensate the deficiency in root hair development but also remarkably increase the overall uptake of water and nutrient ions. Hyphae distributed in the root cortical region though not proved may bridge the defective vascular connection and aid in the transport of water and mineral nutrients from root to shoot. Physiological

and biochemical changes brought about by AMF association render the plantlets more resistant to microbial infection and stress conditions. Thus, the major defects of tissue culture plantlets, viz., poor absorption and transportation and sensitivity to microbial infection could be corrected with desirable traits of AMF association. Further, the vigorous growth and development of plant due to AMF colonization will also contribute to the higher survival and establishment of plantlets.

Mycorrhizal inoculum and inoculation of plantlets

Choice of AMF fungus :

Appropriate AM fungus may be chosen from among various native AMF isolates of the desired plant species, after a previous selection for functional compatibility. If such efficient native isolates are not available, make use of any AM fungus which is proved effective on plant species under study.

Multiplication of AMF cultures :

Mass multiply the AM fungus in sterile sand : soil (1:1) mixture or any other suitable growth medium using Rhodes grass or Guinea grass as host plant. Ruakura or Hoagland plant nutrient solution containing half of the recommended phosphorus is given once in a week. After 90 days of growth, extract sufficient number of viable spores from the growing medium following the wet sieving and decanting procedure.

Preparation of inoculum :

Surface sterilize the spores with streptomycin sulphate (0.04%) and chloramine T (200 mg l^{-1}). Wash thoroughly with sterile water to remove all the disinfectant. Suspend the spores in sterile water with an approximate density of 50 spores ml^{-1} to use as spore inoculum. Well colonized mycorrhizal roots free from soil particles can also be used as inoculum, after proper surface sterilization.

Inoculation time :

Inoculation can be given at the *in vitro* stage of rooting, planting out for acclimatization and transplanting to the field after acclimatization. If the plant species is having survival and establishment problems, then the VAM inoculation has to be given either during rooting stage or at the time of planting out. If inoculation is at the later stage ie., transplanting to the field, it will help only in the field establishment and growth.

Inoculation :

Fill three-fourth of the small plastic pots usually used for planting out the micropropagated plants for acclimatization with sterile planting medium. Apply 4ml of spore suspension or one gram of mycorrhizal root bits of 3-5 mm size as a layer over the medium. Place the plantlet above the mycorrhizal layer and cover with planting medium. This facilitates a close contact of inoculum with the root and early colonization.

Growing condition :

The inoculated plants are grown in green house or mist chamber as it is being followed for acclimatization with required humidity and light. However, an extended day length of 16 hours with cool white fluorescent lamp favours the mycorrhizal development.

Conclusion

The convincing reports of the effect of arbuscular mycorrhizae in enhancing the survival and establishment of the micropropagated plantlets emphasize the importance of AMF in plant tissue culture technology.

Micropropagation has been commercially exploited in woody perennial tree crops. Under such situation AMF inoculation at the hardening stage itself has more relevance because an AMF colonized plant at the early stage itself has got a better chance of growth and

survival under stress conditions such as infection by soil borne pathogens, drought etc. when compared to plants colonized by native AMF at a later stage of its growth or to a non-mycorrhizal plant. However, it is still doubtful how far biotechnologists and microbiologists have recognized this fact. Plant pathologists and microbiologists engaged in mycorrhizal research may be unaware of the problems of micropropagation. Similarly, biotechnologists may not be well informed of the potentialities of AMF in micropropagation. Sooner or later this powerful tool will be recognized and the technology will be integrated with micropropagation.