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Module : 06 Micropropagation-I

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Module: Micropropagation I

Learning objectives

1.1 Introduction

Cloning in plants, usually refers to asexual multiplication of plants from the somatic tissues. It is often a common practice followed by horticulturists for preserving the unique required characters of particular genotypes. Conventionally, this can be attained by cuttings, air layering, splitting, grafting, etc. Though, for most of the plant species, specifically various species of tress and the orchids, these approaches are either not available or are extremely slow. Further, most tree species lose ability to root as they age and thus by the time they can be evaluated for the elite nature, they have crossed the age when they can be vegetatively propagated. In the early 1960's, the process of totipotency in plant cells was recognized and thus the re-generation of a huge plant number beginning from very small tissue sections was achieved, tissue culture was assessed as an alternative method for fast clonal propagation of plants (Murashige, 1974). As time passes, scientists were able to develop various formulations of media with different and varying concentrations of inorganic salts, vitamins and growth regulators thus making possible regeneration of plants from a large number of species. *In vitro*, clonal propagation of plants is commonly known as micropropagation ever since the the process is miniaturization, it has now become an industrialized technology and is utilized worldwide for multiplication of ornamentals, orchids, fruits and forest species.

Micropropagation has few advantages above the conventional methods for clonal propagation as: (a) By using a small explant a huge plant can be produced with relatively short space and time.

(b) Small plant tissue pieces are required to induce aseptic cultures so that selected mother plant is not damaged, (c) As compared to the conventional methods of vegetative propagation, the rate at which multiplication takes place is faster in cultures, as growth regulation and nutrient levels as well as temperature and light can be easily manipulated, (d) It has applications to those species wherein the vegetative propagation methods are not

available, (e) Since it is all done under artificial conditions, it canbe carried out around the entire year,

(f) Since stock plants that are free of viruses are protected from re-infection, and thus these micropropagated plants are easily exported with no quarantine problems, (g) These plants are devoid of any microbial infestation, (h) If the selected mother plant is infected with virus, it can be freed of the same by applying various techniques such as heat therapy and shoot-tip grafting. Since a relatively larger number of plants could be multiplied thereafter, the procedure proves to be economically viable, (i) *In vitro*, the production can be intended to ensure availability of plants when required, and (j) The newer micropropagated plants tend to acquire a few desirable traits, for eg: ornamental plants with bushy habit; strawberry with large number of runners; more tillers in sugarcane, early fruiting in fruit crops etc.,thus making them more desirable.

1.2 General Micropropagation Technique

Micropropagation is a very well defined five step process, each step having its specific requirements: *Stage 0*: The preparatory stage deals to provide good quality of explants; *Stage 1*: Establishment of aseptic culture conditions; *Stage 2*: Multiplication; *Stage 3*: *In vitro*, rooting of shoots; and *Stage 4*: Transfering plants to the field/greenhouse conditions (transplantation).

1.2.1. Stage 0: Preparatory Stage

Contamination is one of the most serious problem for establishment of aseptic cultures. For successful establishment of aseptic cultures, the mother plant should be in good hygienic conditions. Most often, the explants that are obtained from the plants are preserved in greenhouse providing higher percentage of aseptic culture. Mother plants are given basal irrigation to reduce contamination problems. Some times, growing shoots are loosely covered with a polythene so as to avoid direct contact with the pathogens

Stage 0 involves the manipulation of temperature and light regimes according to which the mother plants are preserved. Also, application of growth regulators, especially cytokinins favours better bud break in cultures.

1.2.2. Stage 1: Initiation/Establishment of Cultures

The aim of this stage is to allow the aseptic growth of plant cultures that are be micropropagated by the use of suitable explants. The success of this stage depends on the

choosing the appropriate explants, the proper sterilizing procedures as well as appropriate media that favours further growth.

(i) *Explant.* The criteria for choosing an explant depends on the mode of regeneration and the amount of desired multiplication. The most frequently used explant for micropropagation is the apical bud or the nodal segment with an axillary bud. As the focus is on virus elimination the explant is sub millimetre shoot /meristem tip. The propagation from axillary/apical buds has the benefit of true-to-type progeny as it is pre-formed meristem which is activated for further growth. The relative explant's choice enlarges once the objective is *de novo* re-generation of shoots or else that of SE (somatic embryogenesis). On the basis of the principle of totipotency, indirect or direct adventitious bud formation can be obtained either from nucellus explants, stem, leaf and root. Somatic embryogenesis has been obtained in species of tress, legumes and cereals, using young and zygotic embryos that are immature. Nucellus explant is used for obtaining somatic embryos in mango, citrus & cashew.

In monocots, the regeneration of huge number of adventitious buds without callus formation has been done by using intercalary meristems at the base of bulb scales or young leaves.

(ii) *Sterilization.*

The explants are prepared by cutting stems explants into 2 – 3 cm long pieces, at least with one axillary bud with a small piece of petiole attached. They give a quick rinse with 70% ethyl alcohol so as to remove waxes present on the surface. For field grown hardy material, this is followed by washing with mild soap solution (3 - 5 min.) with constant stirring and then washing under running tap water for varying duration (1 – 4 hrs.). The above listed treatments prepare the explant for responding to further surface sterilization treatment. Usually explants one sterilized with 0.1% Sodium hypochlorite solution for 15 minutes. Mercuric chloride solution is also found to be effective, but most laboratories avoid its usage because of the problems associated with disposal of mercuric chloride. The concentration and duration of the treatment are dictated by the nature of the explant (hard explants such as seeds can be tolerated much rigor us treatment compares to the soft nodal explants from the fresh growths). The objective is to kill the microbes present on the surface without harming the explant tissue. After sterilization, explants are therefore rinsed thrice with sterilised distilled water in laminar airflow cabinet.

1.3.3. Stage 2: Multiplication

Accomplishment of the micropropagational protocols mainly depends on the efficacy of this stage as it has been observed that multiplication folds are achieved at the end of each cyle. The multiplication of shoot is attained by: (i) callus regeneration, (ii) the explant producing direct adventitious buds, and (iii) forced axillary branching. Every method has benefits and drawbacks.

(i) *Regeneration from Callus:* Vaious plant cells are accomplished to develop callus under most appropriate culture conditions. On suitable media, this callus can be made to regenerate shoots through organogenesis or to form somatic embryos. These methods have the potential for the production of a huge number of plant species, the second being more effective, both as a process and genetic uniformity of plants.

Somatic embryogenesis is the most promising approach that has the potential of producing a huge number of embryos germinating similar to embryos coming out of the seed (zygotic embryos) and thus producing plants possessing a primary root system. This is unalike as compared to a separate rooting step which is mandatory for the shoots produced *in vitro*. Furthermore, low cost production of huge number of plants can be achieved if the standardization of protocol for somatic embryogenesis is done and scaling up for bulk production in the bioreactors is done.

While there are many benefits offered by somatic embryogenesis, yet an effective & productive protocol for SE (somatic embryogenesis) is not available for the majority of species. Furthermore, it is not possible to synchronize embryogenic cultures; and the poor development of embryo into plants is due to physiological and morphological defects. The major demerit of somatic embryogenesis is an intermediate callus phase, that induces variability. It is worth mentioning that many commercial laboratories in India and overeas, are producing banana in bioreactors through somatic embryogenesis.

(i) *Adventitious bud formation*: The buds of shoot develop from a different plant parts, apart from the axillary or apical bud are known as adventitious buds. Moreover, adventitious nature is found in the regenerated shoot buds developing from callus. Most of the plants of horticultural origin have the potential to form adventitious shoots from leaf pieces (*Saintpaulia, Begonia*) and root cuttings (raspberry, blackberry), that can be explored for further clonal propagation. The remarkable advantages of *in vitro* propagation by adventitious shoot formation

are: (i) enhancement in the number of shoots developing per propagule, (ii)shoot buds are produced in cultures by very smaller pieces of tissues that are unable to survive *in vivo*, and (iii) majority of plants perform very well *in vitro* that are unable to allow adventitious shoot formation in nature. Liliaceae and Iridaceae species have been very well explored for Adventitious bud formation. The use of outer bulb scales with small segments, as many as 100 bulblets can be obtained from a single scale of lily.

Better genetic fidelity is observed in plants that have been obtained from direct adventitious shoot bud formation as compared to those plants that are regenerated from callus.However, there are many known examples where plants derived from adventitious buds showed off types (potato; *Brassica*). The two major risks :splitting of chimeras and regeneration of pure types are associated with the multiplication of genetic chimeras. Depending on the nature of cell of the chimeric tissue, solid forms of plants are obtained.

(ii) *Forced axillary branching:* Usually buds that exsist in axil of the leaves are suppressed because of apical dominance. The growth of the axillary bud into shoot by the removal of the apical meristem removal is well exploited for the developing hedge of plants. In horticulture, for vegetative propagation by cutting the stems, the shoots develop from the axillary bud present at the node and the lower end of the cutting formed the shoot. Though, this method of clonal multiplication is very slow and has the limitation of the number of cuttings (10"-12" long with 2-3 nodes) that are obtained from the mother plant. In cultures, 2 – 3 cm long cuttings with a single node, in the pressure of growth regulators, specifically a cytokinin, that forces the growth of axillary bud even if the terminal bud is present, which may result in cluster of shoots. Exploitation of forced axillary branching is done for carrying out *in vitro* clonal propagation of plants. Particularly, this is the most renowned method for commercial micropropagation for obtaining the desired plants. In vitro, uninterrupted multiplication by using axillary branching can be attained by the culturing of nodal explants in the medium containing an appropriate concentration of cytokinin with/ without an auxin. Since cytokinins are consistently present inside the medium, During culturing, shoots develop from the axillary buds present on the nodal cuttings.

The entire procedure becomes repetitive untill the initial explant gets transformed into a clump of branches (Fig. 17. 5A). On the other hand, the limiting factors such gradual depletion of the growth regulator/s and nutrients in the culture medium, influence the proliferation of shoot proliferation in a single multiplication cycle.

Therefore, further shoot multiplication stops after 3-4 weeks.

To carry out another cycle of shoot multiplication, the clump of shoot is cut into pieces, each with at least one well formed shoot/an explant (with a minimum single node) is transferred to fresh medium with similar composition.

The repetition of the procedure is done numerously, untill considerable shoot number is attained. It is suggested that there must be a limitation to the number of multiplication cycles so as to avoid risks of introducing abnormalities because of the culture environment. For banana, seven multiplication cycles are recommended. In most commercial laboratories there is practise of initiating fresh cultures each year thus restricting the multiplication cycles to approximately 18 each of 3 week duration. In certain plants, like Neem, *Feijoa*, potato, *Leucaena*, the phenomenon of apical dominance is so strong that even in the constant presence of cytokinins the axillary buds grow into a single long unbranched shoot. In these cases at the passage end the solitary shoot is cut into single nodal segments followed by transferring them to fresh medium. The rate of multiplication would depend on the number of nodal cuttings that can be prepared from the solitary shoot at the passage end.

In most plant species, 3-4 fold multiplication is achieved every three weeks. Initially the forced axilliary branching method may be slow, but with each passage there is a logarithmic increase in the number of shoots, and within a single year a large number of shoots are produced beginning from a single nodal cutting. This shoot multiplication method is the most desired method for *in vitro* clonal propagation in plants and is considered to be most trustworthy as far as the genetic uniformity in the micropropagated plants. Furthermore, forced axillary branching allows successful clonal multiplication of chimerical plants .

1.3.4. Stage 3: Shoot Elongation and Rooting

Somatic embryos are bipolar structures with shoot and root primordia and germinate to develop into complete plants. However, it has been observed that for the development of a complete plant; the shoots that are formed are regeneration from callus, directly by formation of adventitious buds or by forced axillary branching further need an supplementary step of rooting.

Sometimes, the shoots that are developed *in vitro* by any of the three methods discussed above, are repeatedly exposed to cytokinin, may be short and further require an intermediate elongation step before it is transfered to rooting medium. The medium for elongation may be

either liquid medium of similar composition as used for shoot multiplication or may be with reduced zero level of cytokinin. For uniform elongation of the shoots and furthermore to reduce the cost of handling it is quite logical to allow cluster transfer of shoots to the selected medium for elongation. The presence of cytokinins in the medium prevents the formation of roots, thus, the transferring of shoots to an appropriate rooting medium is very essential. Rooting is commonly accomplished by the transfer of specific shoots (about 2 cm long) into a medium supplemented with an suitable auxin. A more economical aspect is observed in onion and garlic, if the shoot clusters are utilized as a only unit for this step. This stage of rooting is labour intensive, which accounts for around 70% of total price of the plants that are micropropagated. Thus, the rooting percentage should be higher (>95%). In order decrease the rooting cost of micropropagated shoots, most of the commercial companies resort towards *in vivo* rooting. Thus, the micropropagated shoots are often preserved as micro-cuttings, after the treatment of the basal end cut with a commercial rooting mix or an appropriate solution of auxin, they are planted directly in potting mix.

In vivo rooting offers several benefits: (i) *In vitro* generated roots die after transplantation then the newly formed roots sustain the plant, (ii) the vascular association between the *in vitro* formed shoot and the roots is not very well established, (iii) *in vitro* developed roots lack root hair making them less effective when transplanted, (iv) Unskilled labourers commonly perform transplantation and the roots produced *in vitro* get damaged as the plants to be transferred are quite large. (v) During *in vitro* rooting, the callus development at the junction of shoot and roots is often a problem. *In vivo* rooting decreases the cost, as well as avoids the above discussed problems related with them. However, *in vivo* rooting is not achieved in many species. Further, sophisticated greenhouses required for hunidit control.

1.3.5. Stage 4: Transplantation and Acclimatization

Final successful micropropagation usually depends on development of plants in the potting mix as well as in the soil. The growth of *in vitro* grown plants that are exposed to the artificial environment of the culture vial are characterized by the culture medium rich in organic and in-organic nutrients, growth regulators and sucrose, higher humidity levels, lower light conditions and poor gaseous exchange.

Usually stable plant growth is visualized under unnatural conditions but these plants suffer from many morphological, cytological, anatomical and physiological abnormalities, which

calls for their cautious acclimatization to *in vivo* conditions. The two main abnormalities observed in these plants are poor control of water loss and heterotrophic mode of nutrition because the culture medium is rich in organic nutrients. The leaf culture vials exposed to high humidly show poorly developed cuticle, deposition of wax is scanty, stomata is large and abnormal, which remains closed even when exposed to ABA, higher CO_2 levels/ dark treatment, the mesophyll tissue differentiation is poor, which predominantly comprises of spongy parenchyma, and chloroplasts that are developed poorly with lower content of chlorophyll and grana is disorganized (Bhojwani and Razdan, 1996). The chances of survival are reduced because of excessive water loss on transplantation due to absence of cuticle and impaired stomatal movements. Hence, the *in vitro* plants must be hardened carefully (acclimatization) prior to transferring them to field conditions.

1.3.5.1. Acclimatization

Fact underlying the acclimatization of the *in vitro* plants, which are in the habit to grow under low light and high humidity heterotrophic conditions is to make them adaptable to grow under high light and low humidity autotrophic conditions. Usually the *in vitro* plants take 4-6 weeks for hardening so that they are able to show normal conditions survival. A single micropropagated plant is initially obtained from the agar medium, then washing of roots is done thoroughly for removal of agar/medium followed by individually planting them in pots containing appropriate potting mix (peat, vermiculite, perlite, coco-compost or polystyrene beads and soil). Irrigation of plants is done with a mild nutritive solution such as MS salt solution or Knop's solution with ¼ strength. Initially fertigation dose is kept low. Transplanted plants are covered with with bags made of plastic that are perforated with minute holes for air circulation. This is easiest method for maintaining higher range of humidity all over the transplanted plants. For 15-20 days the plants are preserved in shade or low light intensity then they are slowly acclimatized to low humidity conditions. The polybags are gently removed everyday for fewer hours initially and then gradually increasing exposure time till the plants are capable to tolerate the entire cover removal. This is the most crucial stage when the plants are tough enough to survive on inorganic nutrients and the reactivation of their photosynthetic machinery so that they may become autotrophic. Only after the formation of the roots and leaves that the plants are capable to withstand field conditions. In a commercial laboratories, a entirely different approach is applicable where a larger number of plants are involved. Transplantion of plants in pro-trays / germination trays with 96 holes is done. It becomes easy for handling for irrigating/ transfering purposes and

considerably decreases on labour cost for handling the plants. Fogging or Misting helps to maintain high humidity. Fogging, with very minute droplets (ca 20 µm), is desirable. In tropical countries, conditions of higher humidity, cold temperatures and lower light conditions are kept in large polyhouses wherein the fitting is done with heavy duty exhaust fans at one end and pads, that are continuously drenched with water, are kept at the other end (fan and pad system). Within the polyhouse in a period of 4 weeks, plants (in portrays) are shifted from higher humidity region near the pad end to lower humidity region near the fan end. At this time the plants are irrigated with nutritive solution having high phosphorous content to promote rooting and when pro-trays are allowed to shift towards the fan, fertilization is altered with a solution containing equal amount of NPK. Repeated spray of insecticides & fungicides is essential as high humidity situation is conducive for the growing pests and pathogens. Any dead plant / leaf must be immediately removed as any dead tissue becomes source of attracting fungi.

Consecutively, the rooted plants can be partially hardened *in vitro* by reducing the humidity in the culture vessel by slowly unscrewing the lid of the culture jar over a period of 3-4 weeks. However, this should be done under strict monitoring as culture media, being a rich source of nutrients may also attract microbes (bacteria / fungi) which due to their fast growth can even kill the plantlet. Culture vials are preffered having microporous closure allowing exchange of gases and the lowermost part of the culture vesselsis cooled. Furthermore, humidity can be reduced by the use of desiccants. The ability of the plants to photosynthesize in cultures can be improved by improving the irradiance to about 80 µmol m^{-2} s^{-1}. Adding growth retardants like ancymidol or paclobutrazole, in the rooting or the shoot proliferation medium is known to increase desiccation tolerance and consequently the abilty of palnts to survive on transplantation. Reducing sugar level also helps in the shift from heterotrophic to autotrophic mode, but is not always feasible as rooting is high energy requiring stage.

1.3.5.2. In Vitro Formation of Storage Organs

Species of plants possessing the ability to produce storage organs such as corm, tuber, bulb, rhizome in nature are explored to do the same in cultures in order to promote transplantation with high rates of survival. The process eliminates the the overall rooting step. It is easy to store or ship storage organs and can be planted by using machines or manually. The formation of storage organs i*n vitro* has been accomplished in *Gladiolus*, *Crocus*, *Dioscorea* spp., *Freesia*, potato, *Dierama luteoalbidum* by increasing the concentration of sucrose, and modifying the growth regulators and temperature and light treatments. The most classical

example extensively studied for *in vitro* formation of tubers is Potato. The specific conditions that help in the promotion of tuberization of various cultivars of potato *in vitro* are recognized and the entire processes have been developed to the commercial production level are also a subjected to automation.

In general, shoot multiplication takes place during long day conditions in a medium with 2-3% sucrose and microtuberization *in vitro* is stimulated by short day conditions/ conditions of complete darkness with elevated sucrose levels (8-9 %) in the cultural medium. Microtubers that are harvested are either sowed in trays (plastic) or followed by aeroponic technology to produce minitubers, these minitubers are used as seed tubers for crop production. This technology has replaced true potato seed (TPS) technology and most of the seed production of potato is done through TC/ aeroponic technology in India.

1.4. FACTORS AFFECTING MICROPROPAGATION

1.4.1. Initiation of Cultures and Shoot Multiplication

The best extensively used basal media for, cultural initiation and shoot multiplication is MS (Murashige and Skoog, 1962) medium. It has been variously improved for diverse systems.
Full strength MS medium is quite toxic for a number of plants and needs to be decreased to half strength or even lesser. Bamboos, like *Dendrocalamus,* show improved shoot proliferation with ½ strength than with full strength MS basal medium. To Carry out multiplication in *Pingicularia* even ½ strength MS medium was toxic, and the required concentration of basal medium was reduced to $1/5^{th}$.
The most prominent result of decreased concentration of salt of MS can be as a result of decreased nitrogen strength, especially the ammonium salt. You have already learnt about standardizing tissue culture media.

For initiation and later shoot multiplication, the medium has to be supplemented with appropriate growth regulator/s. After setting up suitable experiments it is releaved that whether a cytokinin alone is sufficient or an auxin is also required. It has been observed that a cytokinin alone is not adequate enough to stimulate responses at both initiation level and shoot multiplication stage. Out of the numerous commercially available cytokinins (kinetin, BAP, 2iP, zeatin, TDZ), BAP is the one that is used widely. On the other hand, for few plant species additional cytokinins are proved to be more beneficial. For example, as per *Rhododendron*, *Ficus benjamina*, garlic and blueberry are considered, 2iP has proved to be

more superior to BAP. Zeatin isn't recommended for commercial micropropagation since it is expensive. Thidiazuron (TDZ), is a urea-derived cytokinin, has been demonstrated to be useful for hard to regenerate species of trees.

Topolins are most recent known aromatic group of cytokinins. They have been introduced in the tissue culture studies and have given quite encouraging results with many systems. *m*T (*meta*-Topolin) has improved acclimatization as well as *ex vitro* survival rate in *Spathiphylum* spp, multiplication and control of hyperhydricity in *Aloe polyphylla* and the multiplication of plantains. *m*TR (*meta*-Topoline riboside) improved the survival rate of potato cultures, and *m*T derivatives improved histogenic stability and anti-senescence effects in *Petunia* and rose cultivars, respectively. It has been observed that *m*TR has known to be superior to BAP as far as rate of multiplication and the quality of shoot is considered in *Baleria greenii.*

The concentration in between the range of 1-2 mg L^{-1} of cytokinins is considered to be most effective though higher concentrations are also used. At lower concentrations in the range of 0.002-0.10 mg L^{-1}, TDZ is found to be effective. Though, much high concentrations of cytokinin influence callusing and which may bring morphological abnormalities, like hyperhydration. It is recommended to choose lower concentrations as chances of getting abnormal shoots increases with increased cytokinin concentrations. To increase regeneration in few plant species, cytokinins has to be complemented with an appropriate auxin. The most ordinarily used auxins are IBA and NAA that are in the range of 0.1 to 1 mg L^{-1}.

2, 4-D is most desirable choice for induction of somatic embryogenesis but is avoided where shoot multiplication is avoided is desired. In some species as *Gardenia*, GA_3 improve the rate of multiplication and the formed shoot quality. For initiation of cultures and shoot multiplication, the media that is gelled with agar or gelrite is used. However, multiplication and even survival of a large number of plant systems is better in liquid cultures. In certain orchids, like *Cattleya,* cultures are developed only in liquid cultures. The most suitable pH of culture medium is between 5.7 to 5.8.

However, few plants, such as *Dianthus* and *Magnolia,* highly acidic pH of 3.5 and 4.5, respectively, is preferred for improved shoot multiplication rates. Since at low pH, gelling cannot be achieved & thus liquid media is used. The cultures are maintained in light in order to promote shoot multiplication. With varying duration of 8 – 16 hr. the light intensity of 3000 lux is maintained. Yellow light is favoured over white light.

1.4.2. Rooting

In vitro, the majority of the micropropogated plants have the ability to root on full strength MS medium that has been improved with suitable auxin concentration. However, certain plant species were unable to root in full strength MS medium. For increasing the *in vitro* percentage of rooting *Rhododendron* and *Narcissus* usually prefer half strength MS medium. Furthermore, in case of shoot multiplication, ammonium ions were necessary for *in vitro* rooting. It was observed in apple that the Shoots of few cultivars rooted only when the concentration of ammonium nitrate decreased to half of its strength or was entirely removed from the medium. Auxins are the most essential component for herbaceous monocots, dicots as well as woody trees. IBA is mostly preferred as compared to NAA or IAA. In the culture medium IAA has a short life as a result of photooxidation. However, NAA induces rooting but usually promotes callusing at the base. Nevertheless, auxins are preferred for rooting in woody plants. IAA-induced rooting in the presence of bi- or polyphenolic substances, such as phloroglucinol and ferulic acid is promoted in few species of trees, specifically in rosaceous fruit trees,.

IAA is protected by phenolic compounds from damages caused by oxidative decarboxylation. The period of exposure of plants to auxins varies from species to species. It is observed that woody species require longer or constant exposure to the auxin while herbaceous plants and soft woody species require exposure of shorter duration. Auxin exposure for longer duration induces callusing in the base or leading to leave's chlorosis thus making it difficult for acclimatization. The usual concentration for rooting by auxin is 0.1-1.0 mg L^{-1} but woody plants need high concentrations. Longer length of root usually gets damaged during the transplantation time thus root length of 5-10 mm is considered satisfactory which is commonly achieved within 10 to 15 days. It must be kept in mind that auxins are required for root induction and are not required for further growth. Also, most frequently, *in vitro* formed roots die later and are required to provide initial support to the plant, till the time new leaves & root appear.

1.5. SUGGESTED FURTHER READING

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