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A Review on Efficient Micropropagation by Ex vitro Rooting

G Indravathi*
*Dept. of Biotechnology, Govt. College for Men, Cluster University, Kurnool, Andhra Pradesh, India
*Corresponding author: gindravathi@gmail.com

Abstract*—The morphological and anatomical anomalies of in vitro rooted plants during micropropagation, like the absence of functional stomata, poorly developed cuticle and weak root system subject the plantlets to desiccation. Efforts made to improve these characteristics by controlling the stressful culture conditions greatly contributes to better rooting and acclimatization of the tissue culture grown plantlets. Introduction of ex vitro rooting during micropropagation overcomes these limitations. Since ex vitro rooting and acclimatization were done at the same time, gradual adaptation to the external environment, transition of the root system from a non-functional structure to a functional one, and finally habituation of plantlets to ambient relative humidity and light irradiance takes place simultaneously. Ex vitro rooting simplifies micropropagation protocol, reduce labour, production cost and helps in the easy adaption of scientific technology from lab to land.*

***Keywords*—** *Acclimatization, ex vitro rooting, in vitro rooting, micropropagation.*

I. INTRODUCTION

In the early days of micropropagation, *in vitro* rooting was the general method used for obtaining plantlets, but in the present scenario *ex vitro* rooting is used in commercial laboratories as it eliminates one culture stage and reduces the overall cost of micropropagation [1,2,3,4,5]. Roots of plantlets produced *in vitro* are usually very weak and without root hairs [6]. The anatomical, morphological and physiological characteristics of the *in vitro* developed micro shoots, for the majority of the woody species, have a great impact on subsequent rooting and survival after transfer to greenhouse conditions. During the early acclimatization period, the roots do not function normally to support the plants in uptake of water and nutrients. In the rehabilitation phase, it is necessary to improve the root system, not only for strong growth due to the strong absorption of water and nutrients, but to replace the water loss of the shoots. Any effort to improve these characteristics by controlling the stressful culture conditions undoubtedly contributes to better rooting and acclimatization of the plantlets. The acclimatization process will begin while the microplants are still under *in vitro* conditions. Since *ex vitro* rooting and acclimatization were done at the same time, gradual adaptation to the external environment, a transition of the root system from a non-functional structure to a functional one, and finally habituation of microplants to ambient relative humidity and light irradiance takes place simultaneously [7,8].

Microshoots can be rooted in two ways [9]. In complete *in vitro* rooting auxins were mixed into the medium for the whole culture period, while in the *ex vitro* experiment the micro shoots were dipped exclusively in auxin solution for a short period and directly planted in potting mix. Roots produced by *ex vivo* rooting were more branched than roots produced by auxin supplementation in the culture medium. Furthermore, during *in vitro* rooting, auxins can be photo oxidized by exposure to light (Fig.1). When auxins are applied continuously, that is even after root induction it promotes callus formation and inhibits the outgrowth of the root primordia [10].

II. *IN VITRO* ROOTING - LIMITATIONS

Rohr *et al*. (2003) discussed the morphological and anatomical anomalies of *in vitro* rooted plants, like the absence of functional stomata, poorly developed cuticle and weak root system subject the plantlets to desiccation [11]. The direct transfer of *in vitro* developed plantlets from *in vitro* to glasshouse conditions results in 100% mortality in cork oak [12] and 50% in *Albizia amara* [13]. Weitz et al (1989b) performed physiological studies on Camellia japonica roots grown on agar and soil and found that soil-grown roots had higher trace elements than agar-grown roots, probably due to the thickening of the cell walls and the accumulation of phenolic compounds [14]. *In vitro* produced roots lack good vascular connection, develop weak casparian strips, and the absence of phi-thickenings, which promotes a limited water transport to aerial parts of the plantlets [15,16,17,18]. *In vitro* formed roots struggle hard to adapt to the new environment and undertake diverse changes during the acclimatization of micropropagated plants. Under *in vitro* conditions, the basal part of the stems, where the roots are being formed, is stuck into the medium which prevents easy exchange of gases leading to accumulation of gases (ethylene and CO_2) and partial anerobiosis. Ethylene is also inhibitory during root induction phase, possibly because it interferes with the establishment of polarity in the meristem [19].

III. EX VITRO ROOTING - ADVANTAGES

Roots grown under *ex vitro* conditions, exhibit well-defined phloem and xylem vessels in the vascular cylinder at the maturation zone, casparian strips in both endodermis and exodermis, and phi-thickenings in the cell walls of the cortex close to the endodermis. These characters match with a functional structure able to supply water and selective nutrients to the aerial part of the plant by blocking the apoplastic transport [20]. On the other hand, *ex vitro* roots are formed in an opaque and well-aerated substrate without any external source of sugar and nutrients, therefore, roots are

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dependent on the shoot to obtain the necessary nutrients and energy to grow. In return, water uptake from the roots accelerates and water flows to the branch. The *ex vivo* results are good for the yield and adaptation of Rhododendron species [21,22]. Scarpa *et al.* (2000) mentioned that direct rooting of Myrtle plantlets in soil has a high survival rate compared to *in vitro* rooting [23]. *Ex vitro* rooting of micropropagated shoots of *Rhododendron ponticum* resulted in a higher survival rate of the plantlets during the acclimatization period than that of *in vitro* rooting [24].

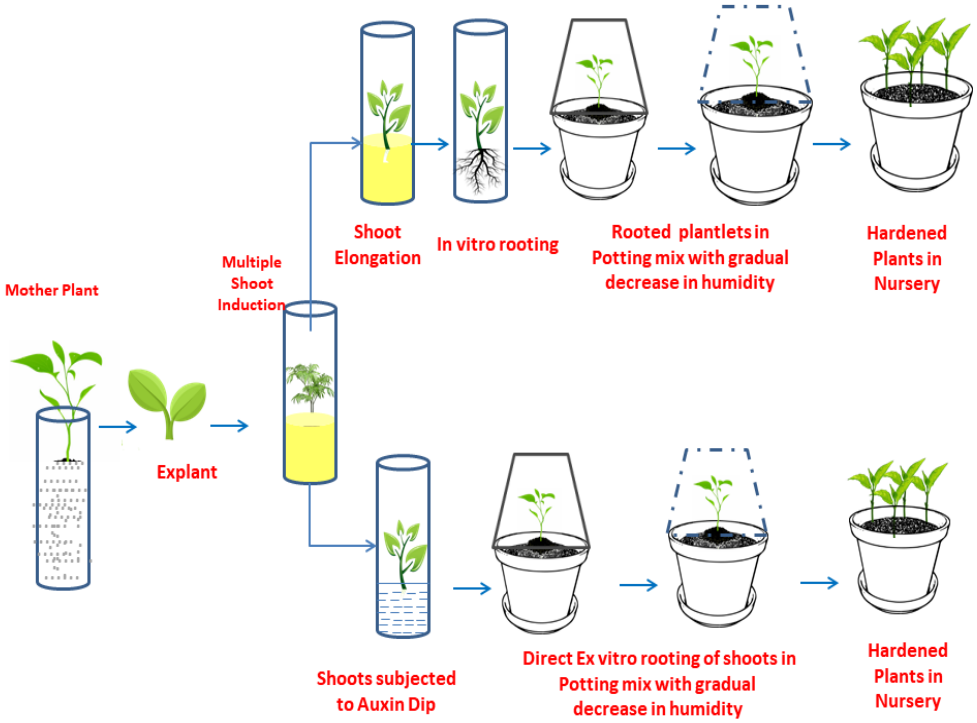

Figure 1: Schematic representation of *in vitro* and *ex vitro* rooting during micropropagation

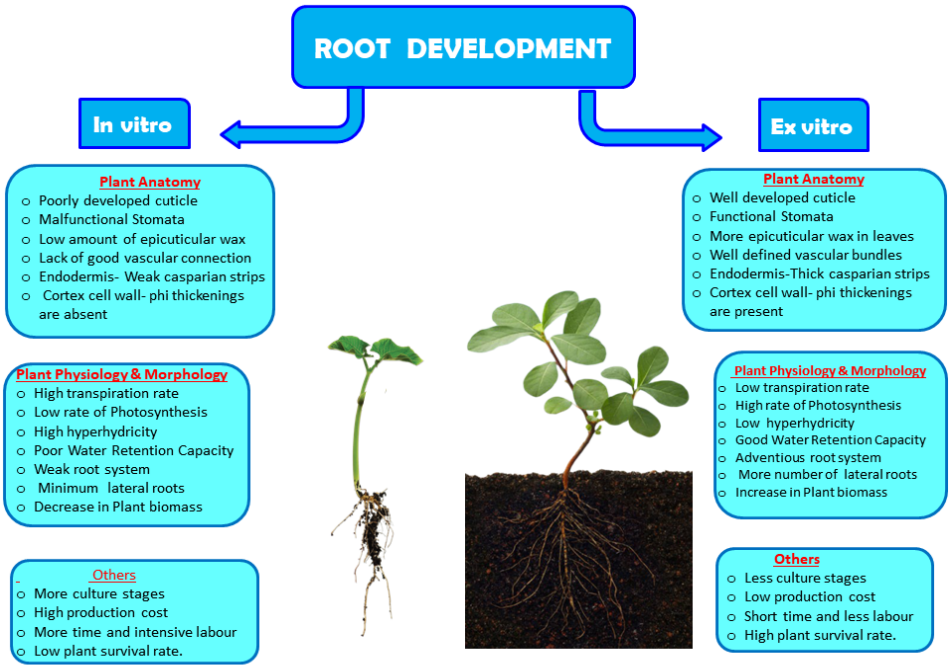

Figure 2: Comparitive studies between *In vitro* and *Ex vitro* Rooting during micropropagation

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Ex vitro rooting was applied to simplify the micropropagation protocol and to reduce production costs. It is a promising method as there is a reduction in cost by avoiding the *in vitro* rooting culture stage, reduction in labour, and the time of establishment from laboratory to soil (Fig.2). Thus, acclimatization and hardening could be accomplished as a one-step procedure within a short period before transplantation. Many commercial laboratories avoid complete rooting *in vitro*, because of intensive labour and high cost, and prefer rooting *ex vitro* in inert substrates or rooting plugs [25,26,27]. Thus, attempts made to economize and simplify the micropropagation technique could help in the easy adoption of scientific technology from the lab to land.

A. Combination of Both In vitro and Ex vitro Rooting:

Contrary to complete *in vitro* and *ex vitro* rooting, a combination of both is used which is root induction *in vitro* followed by root development *ex vitro*. Today, the trend is to avoid the whole rooting process *in vitro*; instead of this, the practice is at a first step to induce root primordia *in vitro* and at a second step to promote extensive rooting *ex vitro* [28,29]. Debergh *et al*. (2000) reported that micro-cutting of many woody plants can be transferred to greenhouse conditions with only root primordia as planting becomes more convenient, and can even be easily automated [30]. Fabbri and Bartolini (1985) established that it is important to transfer shoots to the acclimatization substrate before roots emerge because roots developed *in vitro* are structurally different from those developed in soil [31].

IV. FACTORS INFLUENCING EX VITRO ROOTING

The response of stem cuttings for rhizogenesis is dependent upon the plant material ontogenetic age, the size of microshoots, the auxin nature, the contact duration, and its application time [32]. Other factors that influence the planting of mechanical cuttings include relative humidity, light conditions and the type of treatment equipment.

4.1. Auxins:

4.1.1. Auxin Treatment:

The success of the transplant and the survival of the plants depend greatly on the quality of the roots. Auxins can be applied to the shoot explants in different ways which include, dipping the basal ends of shoots in high concentrated auxin solution (or) auxin powder for a short period. Auxin may be applied for several days or weeks at a low concentration (micromolar range), or for several seconds or minutes at a high concentration (millimolar range) [33]. It can also be done by saturating the potting mix/ inert substrate with auxin solution. Most of the *ex vitro* rooting techniques are based on rooting the shoots by dipping them in a concentrated solution of auxin and subsequently planting them directly in the potting mix. The roots formed by dipping were more branched than the ones resulting by auxin addition to the culture medium.

The process of adventitious root formation can be divided into at least two developmental stages: the initiation of primordia following cutting or wounding, and the stage of root emergence and growth. It is considered that in the first stage auxin acts as gene activator, i.e., triggers the early formation of root primordia. Auxin enters cuttings predominantly via the cut surface, and the addition of auxin immediately after cutting initiates the first cellular divisions and formation of root primordia [10]. For root elongation, exogenous auxin is usually not required or is even inhibitory [34]. High auxin concentrations are necessary only for the first stages of histogenesis as they can inhibit the outgrowth of root primordia, growth of roots, and growth of cuttings [35,36]. When the auxins are added at too lower doses rhizogenesis is not stimulated and some times even retarded [29]. Further in the rooting region excessive induction and proliferation of new roots is blocked by inhibitors formed in the tips of growing roots themselves [37] and it was also shown that not all primordia initiated after auxin treatment develops into emergent roots [38].

4.1.2. Choice of Auxin:

Most reports of adventitious root induction of woody species have involved treatment with exogenous auxin such as IBA, NAA, IAA [39,40]. Heloir *et al*.(1996) reported successful germination of walnut shoots with exogenous auxin [41]. The three auxins IBA, NAA, IAA exhibit differential stability. Nissen and Sutter (1990) have shown that in tissue culture media IAA is photo-oxidized rapidly (50% in 24h) and IBA slowly (10%) [42]. NAA is very stable [43]. The three auxins work differently as they have different affinities for auxin receptors, differences in uptake, transport, and metabolism [44]. Selection of the auxin type and optimization of its concentration results in a differential response in root induction [45]. However, the actual concentration of free auxin in the cells from which the roots develop does not reflect concentrations of the three auxins in the medium [46]. Since NAA is not destroyed by auxin-oxidase, it might be the preferable auxin in crops with a high activity of auxin-oxidase, in particular when the auxin is only applied as a short initial treatment [10]. De-Klerk *et al*. (1997) and Peeters *et al*. (1991) found that the rate of auxin uptake varied [44,47]. They observed that NAA uptake was six times faster than IAA, and Vander Krieken *et al*. (1993) found that IBA uptake was four times faster than IAA [48].

Martin (2003) reported the survival of 75% of *Rotula Aquatica* micro shoots [4] in the soil after auxin dip using 0.5 mg/1 NAA. In *Albizia amara* of the different auxins tested maximum root induction (92%) was achieved when shoots were dipped in 60mg/l NAA for one hour [29]. Similar observations of high auxin dip using NAA was found beneficial by a few workers in woody species such as *Rotula aquatica* [4], *R. ponticum* [24] and oil palm [49]. The in vitro yield of tea microshoots treated with IBA was successful at the split ends [50, 51]. IBA (500 mg/l for 30 min) was used for treating the cut ends of tea shoots before transfer to soil mix in Hikko trays [52, 53]. Enhanced rooting was observed in Green Ash shoot-tip explants treated with a 15-seconds dip in 1 mM IBA [54]. Bates *et al*. (1992) observed 80% *ex vitro* rooting of micropropagated White Ash shoots after a quick dip in 1 mM IBA [55]. The rooting rate was increased to 91.7 % when

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Iranian Myrtle micro shoots were subjected to auxin dip using a solution of l.5 mg/1 IAA + 0.31 mg/1 IBA [5]. Dipping in a solution of 1 g l-1 IBA increased 94 % rooting of *R. ponticum* [24].

4.2. Size of Microshoots:

The genetic background and the physical form of the mother plant and the size of the plant taken for rooting are important in the interaction with hormones and environmental conditions. *Ex vitro* rooting requires shoots of high quality. According to De Klerk (2000), the rooting process was divided into phases for improving the rooting treatment of micro-cutting [56]. During micropropagation, two opposite processes occur rejuvenation and maturation where plants not only rejuvenate but also make the opposite transition from juvenile to adult. When maturation prevails, the micro-cutting will have a low ability to root, so young shoots are preferred for rooting. The hormonal regulation and the factors controlling the phase change at the molecular level need to be studied deeply [57]. Microshoots of short length (2-3 cm height) used in most of the *ex vitro* rooting experiments, as they result in lower production costs (shoot elongation step avoided), higher rooting ability, and shorter exposure time to pathogenic fungi [58]. Further, mini-cuttings produce roots of better quality and morphology (taproot-like) than those produced from macro-cuttings using auxin dip [59,60].

4.3. Darkness:

Light is often considered an inhibiting factor in root formation of many species [61] especially at the induction phase of root primordia, whereas, darkness during rooting has a rather stimulating effect on root formation [62]. Root induction in continuous light was less as compared to dark in many reports. Darkness applied during the first week of rooting enhanced rooting percentage, several roots developed per micro-cutting and length of roots increased in several woody species such as apple, wild cherry, chestnut, rhododendron, cork oak, etc. [12,62,63]. The positive effect of darkness is sometimes emphasized by raising the temperature [29,65]. The optimal length of the dark period varies between 3 and 10 days depending on the species and cultivars [7,8, 66].

Shoots kept in dark were slightly pale, thin, and lengthy with narrow leaves. But, they attained good growth when transferred to light. It has further been observed that a high auxin dip of micro shoots followed by few days dark incubation in the auxin-free medium was better in root induction [67]. Druart (1997) observed that continuous darkness during the root induction phase, increases peroxidase activity resulting in high rooting rate [68]. During the first week of rooting, darkness combined with high temperatures (26-28^{0}C) followed by maximal lightening, low temperature and high wetness under *ex vitro* conditions favour rhizogenesis.

4.4. Relative Humidity:

Survival of micro shoots *ex vitro* depends on its ability to withstand water loss and carry out photosynthesis, which is enhanced by gradual acclimatization and hardening. Under *in vitro* conditions plantlets are grown in a small vessel in a closed environment and the air exchange rate between outside and inside is very low and the relative humidity is very high [69]. High relative humidity was the dominant factor for the condition of hyperhydricity in micropropagated plantlets [30]. Thus, microenvironment of the culture vessel and accumulation of gases such as ethylene and carbon dioxide was found to be responsible for hyperhydricity [70]. High relative humidity, makes the plantlets unable to resist water stress after transplanting from *in vitro* to *ex vitro* conditions. These hyperhydric tissues further induce hypoxia stress which drastically affects the plant metabolism [71]. But under *ex vitro* conditions humidity is gradually reduced by using large culture vessels and special closures that facilitate water loss there by improving the internal structure of plantlets.

4.5. Hardening Substrates:

The type of potting mixture used during acclimatization is one of the important factors determining the survival percentage of the plants under *ex vitro* conditions. During this stage, the plantlets change its metabolism from heterotrophic to the autotrophic mode and are very sensitive to external climatic conditions and pathogens. At the same time, photosynthesis is still limited and carbon balance may become a limiting factor because it is supported mainly by the reserves accumulated from the *in vitro* substrate. Hardening the *in vitro* raised shoots to make them adapted to the natural environment is a critical process due to their anatomical and physiological peculiarities. Physical, chemical, and biological properties of a potting mixture are important for the establishment of plantlets.

The general methods used for *ex vitro* acclimatization use various types of containers filled with different types of potting mix (peat, perlite, soil, vermiculite individually or in optimized combination) as transplanting substrates as well as artificial culture areas (greenhouses, tunnels, growth rooms) where humidity is maintained at high levels so that plants grown under laboratory conditions do not dry out.

4.5.1. Coco Peat:

Cocopeat has been considered as a substitute for natural peat in potting media. The particular structure of coconut fibers, their physical and chemical properties, make them suitable for hardening. Cocopeat contains equal portions of lignin and cellulose and is rich in potassium and the micronutrients Fe, Mn, Zn and Cu [72]. Manjusha and Sathyanarayana (2010) recorded a survival rate of 75% for the stevia micro cuttings at the hardening phase on cocopeat [73]. *Garcinia indica* showed 76% survival rate when hardened on cocopeat as a potting mixture [74]. Sivaram and Mukundan (2003) recorded a survival rate of 70% for the stevia microcuttings during hardening phase using cocopeat [75].

4.5.2. Vermicompost:

Vermicompost was shown to be the most suitable planting substrate for hardening which ensured 96% survival in *Tylophora indica* [76]. Kansara *et al*. 2013 reported successful hardening in Castor using vermicompost due to the presence of rich organic matter providing strength and essential nutrients for plant survival [77].

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4.5.3. Vermiculite:

Vermiculite has been used as a substrate to acclimatize papaya plantlets *ex vitro* [78,79]. Papaya shoots hardened on vermiculite were of better quality and more conducive to acclimatization [80]. Vermiculite treatments produced finer root systems with more lateral branches and root hairs. Yu *et al.* (2000) reported 90% rooting of Papaya on vermiculite [79]. The use of vermiculite containing medium improved the rooting of *J. nigra* x *J. Regia* hybrids and *J. Regia* clones [50].

4.5.4. Rooting Plugs:

The highest rooting and survival rate *ex vitro* was observed when Hazlenut micro shoots were cultured directly in jiffy peat plugs after auxin dip [81]. Improved rooting in Raspberry with the use of rooting plugs is perhaps attributable to improved aeration. Aeration was previously shown to play an important role in raspberry root development using foam substrates [82]. Rooting plugs with a liquid medium may provide a suitable balance between aeration and moisture availability (humidity) at the base of the shoot-tip explant, and thereby promote root initiation and development.

4.5.5. Potting Mix Combination:

An efficient one-step hardening technique for tissue culture raised orchid seedlings was reported on chips of charcoal, bricks, and decayed wood as an alternate substratum [83]. Successful acclimatization of papaya plants was reported using perlite, peat, polystyrene beads in a 1:1:1 ratio [84]. High percent plant survival rate was obtained in Banana when potting mixture containing soil, sand, and Farm Yard Manure was used in 2: 1: 1 ratio improved biological properties of the soil and aeration [85,86].

4.5.6. Float Hydroculture Using Perlite:

Successful *ex vitro* rooting and acclimatization was achieved in *Rubus fruticosus* and Rosa hybrid cultivars using floating cell trays [87]. This method is derived from the technique of acclimatization in flooded perlite taken in plastic trays. On the surface of the nutritive solution, there are floats made of polystyrene or other materials that sustain the plants [88,89]. The technique of rooting *ex vitro* in floating cell trays was inspired by a method used for lettuce, tomato, and tobacco seedlings [90,91,92]. This method eliminates the necessity of using special, sophisticated installations for air humidification. Also this method is easy, cost effective and it can be done by using locally available materials without fertilizers and plant growth regulators. Application of perlite provided porosity and drainage, in the potting mix which stimulated the root dry mass accumulation in kiwi plants [93].

Thus by the use of the well-aerated potting mix, the formation of callus in the shoot base will be reduced and a good vascular connection with the root system was achieved [41] .The roots formed *ex vitro* in an opaque and well-aerated substrate without any external source of sugar are dependent on the shoot to obtain the necessary nutrients and energy to grow, so that they easily adjust to greenhouse conditions and show higher percent survival than *in vitro* rooted plants [94, 95, 96].

V. CONCLUSION

The technique of *ex vitro* rooting was good in terms of rooting and acclimatization as it simplifies the micropropagation protocol and reduces production costs. Successful *ex vitro* rooting was promoted by dipping basal ends of the shoots in a concentrated solution of auxin and subsequently planting them directly in the potting mix. The roots formed by dipping were more branched than the ones resulting by auxin addition to the culture medium. Further, auxins should be distinctively applied during different stages of root formation (induction, initiation, root emergence, and elongation). The effect of light and darkness has considerable importance during the stages of root formation, including the sequence of these regimes, intensity, and quality of light. Genetic background and physiological state of the mother plant and the size of the explant taken for rooting are important factors in the interaction with hormones. Thus all the environmental factors such as light quality, photoperiod, relative humidity, temperature, plant growth regulators, and substrate nutrients can have dramatic effects on rooting processes.

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