Int.J.Curr.Microbiol.App.Sci (2020) *9*(11): 3321-3329

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 11 (2020)**
Journal homepage: http://www.ijcmas.com

Review Article https://doi.org/10.20546/ijcmas.2020.911.397

Ex-vitro Establishment of Tissue Cultured plants in Fruit Crops-A Review

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Keywords

Heterotrophic, Acclimatization, Growth, *Ex-vitro*, Greenhouse

Article Info

Accepted:
22 October 2020
Available Online:
10 November 2020

A B S T R A C T

In vitro multiplied plants possess the poor physiology due to heterotrophic nature and cannot survive in the harsh environmental conditions such as high irradiance, low relative humidity, high & low temperatures and the septic conditions when transferred from lab to land. Therefore, most of the *in vitro* grown species requires the acclimatization process under *ex-vitro* conditions to ensure the maximum survival and vigorous growth. The acclimatization is a gradual process and often carried out inside the greenhouse. This review emphasizes on the physiological, anatomical and morphological factors that are responsible for delicacy of *in vitro* cultured fruit crops and the ways to overcome these problems.

Introduction

Micropropagation is a very popular technique of producing a large no. of plants under aseptic conditions and has been applied for commercial plant production over 30 years. Initially it was limited to certain ornamental crops but nowadays it encompasses fruit crops as well as other horticultural crops. The problems lie with tissue cultured plants are fragile nature, abnormal morphology, physiology and anatomy and inability to withstand the unfavourable environmental conditions due to the heterotrophic mode of nutrition (Hazarika, 2006). Micro-propagated plants lack the cuticle layer, stomata closure, chlorophyll content and have poorly developed roots which are then accompanied by the transpirational losses, low humidity and pest & diseases when transferred to *ex vitro* conditions (Bhatia, 2015; Chandra *et al.*, 2010). Hence, hardening of the cultured plants under ex vitro conditions must be carried out to get the desired outcome. Acclimatization of regenerated plantlets is accomplished primarily in mist chamber or greenhouse and then in secondary hardening chambers like shade net houses under partial shade. Generally, the regenerated plantlets are separated completely from the existing MS medium and thorough washing of roots is done in order to remove the medium. Plantlets

are then transferred to the pots carrying the hardening substrate cocopeat, sand, soil and FYM in combination or alone. The relative humidity is maintained near about 95% and plants are kept covered with plastic sheets or inverted glass jars to avoid transpirational water losses. In order to make the plants acclimatized, moisture content and the amount of nutrients is reduced gradually and the plants are transited to shade net house for further hardening (Bhalsing *et al.,* 2001).

By this process, wax layer is formed, and leaf thickness increases and the density of stomata decreases resulting in stabilization of water status (Pospíšilová *et al.,* 1999). Tissue cultured plants generally undergo the transplantation shock, as a result, high mortality is experienced (Dhawan and Bhojwani, 1986). Therefore, the plantlets can be partially hardened off *in vitro* by decreasing the RH and increasing the light intensity before removing them from the culture medium (Maene, 1985). The transplantation shock can also be alleviated by the addition of ABA immediately to the substrate (Pospíšilová *et al.,* 1998). It is evident from the studies that, mycorrhizal fungi have great role in terms of enhancement in gas exchange and nutrient uptake during acclimatization of fruit crops (Luna *et al.,* 2000; Schubert and Lubraco, 2000; Quatrini *et al.,* 2003). Increased concentration of sucrose supplemented in culture medium increases the carbohydrate content and decreases size of stomata during *ex vitro* establishment (Jo *et al.,* 2009).

Physiological, morphological and anatomical factors affecting the *ex-vitro* survival of micro-propagated plants

Cuticle

Plant cuticle is a protective covering of the leaf epidermis, young shoots and other aerial parts of plant. This layer is made up of insoluble cuticular membrane and filmed with soluble epicuticle waxes. Cuticle serves as the barrier for the water permeability and prevents the water loss from epidermal surface by the evaporation. Studies indicate that the crystalline structure of epicuticle waxes on surface of *in vitro* raised plants varies significantly from the *ex vitro* grown plants (Preece and Sutter, 1991). However, *in vitro* grown plantlets can resemble greenhouse raised plants in terms of anatomy of leaves under the modified environmental conditions of lower RH (75%) and higher light levels *in vitro* (Capellades *et al.,* 1990).

Chlorophyll content

Chlorophyll is a green pigment of plant leaves which is very essential for the photosynthesis. The heterotrophic conditions and prolonged dark period alter the biosynthesis of chlorophyll in the plants resulting in poor photosynthetic capacity (Donnelly and Vidaver, 1984). Similarly, due to the lack of light quality, mineral nutrition (especially N, Mg & Fe) and chemical metabolites produced in plants, chlorophyll activity and formation is also influenced (Ahmad *et al.,* 2018). As a result, the plantlets appear pale yellow and do not participate actively in photosynthesis when transplanted to *ex vitro* conditions. Dobranszki and Drienyovszki, (2014), reported the increased chlorophyll content during *in vitro* culture of apple with BA. However, the BAP induced the long term defects in the *Aechmea blanchetiana* during *in vitro* propagation (Martins *et al.,* 2018).

Stomata

Stomata of *in vitro* grown plants are circular in shape and are invariably opened which may be caused by higher relative humidity inside the incubation room (Wetzstein and Sommer, 1983; Wardle and Short, 1983). Due to the

presence of these deformed stomata, excessive loss of water from the plants occurs under the field conditions (Ziv *et al.,* 1987). Some growth regulators such as Abscisic acid act as strong inihibitor of this stomatal collapse but this event can be reversed with the exogenous application of IAA and cytokinins (Wright and Hiron, 1969; Pemadasa, 1982; Blackman and Davies, 1983).

Root growth

Micro-propagated plants have relatively poor root growth than those plants cultivated in vivo. The reason may be the presence of shallow culture medium which inhibits the root elongation. The roots of *in vitro* cultured plants generally have the underdeveloped vascular system than the *ex vitro* raised plants (McClelland *et al.,* 1990). However, maximum root initiation (90-100%) can be obtained in apple with the medium containing coarse sand, perlite, rotated liquid medium and agar but root growth is reduced in agar (Hutchinson, 1984). IBA is a promising growth regulator in terms of root induction and is used at large scale. Application of IBA @ 10^{-6} M significantly increased the number and length of roots in jackfruit under 12h light period (Rahman and Blake, 1988). Likewise, Dinesh *et al.,* (2019), obtained the higher *ex vitro* rooting of pomegranate than *in vitro* conditions with 1476 µM IBA treatment and maximum survival during acclimatization.

Ex vitro establishment of micro-propagated fruit crops

Banana

In-vitro cultivated plants are raised in a protected environment and shifting these cultured plantlets in to the main field in open conditions requires some preconditioning of the plants-generally referred to as hardening. *In-vitro* culture of banana plants is in vogue as banana is marred with a host of viral diseases. So orchardists now-a-days are relying more on micro-propagated banana plantlets which are virus free. To cater to the increasing demand for tissue cultured banana plants, a lot of bio-tech labs are now producing tissue cultured plants following standard procedures so as to reduce the mortality rate of the saplings when these are shifted to the open field conditions. Bari banana 1 cultivar showed maximum survival up to 95-100% after 15-20 days of *in vitro* culture on IBA (0.4-0.6 mg/L) with 7 days hardening at room temperature (Molla *et al.,* 2004). Similarly, it was observed that micro-propagated saplings of *in vitro* propagated Red banana may be hardened by using red soil + sand + coco peat (1:1:1 v/v) combination (Uzaribara *et al.,* 2015) which resulted in maximum survival up to 95%. Grand Naine, a prominent cultivar of banana showed highest survival rate (100%) of micro-propagated plants during acclimatization with the potting mixture soil: sand: FYM (2:1:1) and covering the plants with glass beakers (Ahmed *et al.,* 2014). Similarly, other potting mixtures were tried for hardening of the banana plants and it was found that the best combination of potting medium for hardening of *in vitro* cultured banana cv. Amritpani was soil: FYM: sand in the proportions of 1:1:1. This combination also increased plant height and root growth (Maharana *et al.,* 2017).

Culture medium augmented with antibiotic rifampicin for difficult to-establish banana cultivar Elakki Bale during *in vitro* cultivation helps in maintaining the aseptic culture and resulted in higher survival (96.3%) of plantlets during *ex vitro* establishment (Bohra *et al.,* 2014). 'Williams' banana treated with topolines synthesized the higher amount of phenolics in greenhouse-acclimatized plants when compared with cytokinins, which led to

increased survival rates in the banana plants (Aremu *et al.,* 2012).

Apple

Desiccation of the plantlets due to the lack of proper closure of stomata is commonly found when the plants are separated from the hetrotrophic conditions (Brainerd and Fuchigami, 1982). The stomata of *in vitro* raised apple plantlets lose their regulatory ability which is the possible reason of excessive water loss from plant through transpiration (Blanke and Belcher, 1989). Therefore, the exposure of *in vitro* grown apple shoots to high relative humidity (90%) and high boundary layer conductance may result into the development of functional stomata (Shackel *et al.,* 1990). In micropropagated apple, rooting of plantlets is a difficult task and leads to the death of plant when not subjected to acclimatization. Acclimatization of *in vitro* derived apple micro-cuttings resulted in more number and length of the roots in the phototron units than in the greenhouse under fog system (Skirvin and Sriskandarajah, 1993). *In vitro* propagated apple rootstock M9 grown in coco peat exhibited the maximum survival than in the soil and achieved 95% hardening during October-March (Modgil *et al.,* 2009).

IBA treated apple shoots followed by acclimatization in plastic covered pots gave 70% to 100% vigorously growing shoots in the greenhouse (Bolar *et al.,* 1998). Similarly, emergence of new leaves and shoots in the apple plants was observed on the fourth day of acclimatization at 95% relative humidity inside a transparent plastic box (Juan *et. al.,* 1995). Pre-acclimation of cultured plants at 90% RH one week before the actual hardening results in less transpirational losses and higher survival rate of the plants (95%) after transplanting (Ko *et al.,* 2017).

Inoculation of the micropropagated apple plants with mycorrhiza during the *ex vitro* hardening also enhances the growth and nutrient uptake. Tissue cultured plants of the apple rootstock MM 106 inoculated with *Glomus mosseae* (*Arbusicular mycorrhiza*) resulted in heavy colonization of fungi with the roots of plants and increased P uptake under different substrates (Schubert and Lubraco, 2000).

Grapevine

In grapevine, hardening of plants using potting mixtures has been demonstrated for survival (Dev *et al.,* 2019). Most suitable potting mixture for the hardening of micropropagated grape cultivars constituting coco peat + vermiculite + perlite (2:1:1) resulted in highest survival (85.97%) of the plants within shorter period of 24 days. Similar conclusion was made by Jamwal *et al.,* (2013), with substrates sand: soil: FYM: vermiculite (1:1:1:1), resulted in 73.33% plants survival. Higher rooting of non-rooted *in vitro* raised grape cultivar 'Norton' with 1000 ppm IBA under *ex vitro* acclimatization has been demonstrated by Bigger, (2010). Application of bio-agents in the acclimatization process of young *in-vitro* grown plantlets has been demonstrated (Krishna *et al.,* 2005). Inoculation of *in vitro* cultured grapevines with arbuscular mycorrhizal fungi (AMF) during hardening showed improved physiological conditions and nutrient status as well as higher photosynthetic rate. The best substrate for hardening of grape rootstock Paulsen 1103 and for growth of mycorrhiza was soil, compost and sand (Zemke *et al.,* 2003). Whereas, Barreto and Nookaraju, (2007) reported the best rooting medium for two grape cultivars 2A-Clone and Red Globe, composed of Coco-peat in combination with sand and soil for quick establishment and hardening of plantlets.

Higher *ex vitro* survival rate (>90%), number of shoots per plant, vigorous growth and viability of table grapevine cv. Napoleon is obtained with *in vitro* application of BA (2 mg/L) (Ibañez *et al.,* 2005). In grapes, new leaves which are formed during *ex vitro* acclimatization generally have higher photosynthetic rate than those formed *in vitro* (Carvalho *et al.,* 2001).

Strawberry

Root formation of three strawberry cultivars namely Senga Sengana, Kent and Kama between *in vitro* and *ex vitro* conditions was compared and reported that the largest root system is found under *ex vitro* conditions (Borkowska, 2001). However, *in vitro* grown strawberry plants on sucrose medium may have better photosynthetic rate under both *in vitro* as well as *ex vitro* conditions (Yue *et al.,* 1993). Garfias *et al.,* (2006), obtained 90% survival of *in vitro* grown strawberry in the greenhouse with intermittent misting (80% RH).

Citrus

Application of anti-transpirants can propel the hardening process and open field establishment of the *in vitro* raised plants in citrus (Hazarika and Parthasarathy, 2002). The reduced water loss and maximum *ex vitro* survival of citrus with *in vitro* application of antitranspirant 8-hydroxyquinoline (2 mg/L). Hazarika *et al.,* (2001) recorded increased plant height, reduced size of stomata and maximum survival rate (94.6% to 97.2%) of four citrus species with the *in vitro* application of paclobutrazol (1 mg/L). 100% survival of *in vitro* raised *citrus limon* plantlets during *ex vitro* weaning process is reported with mycorrhizal species *Glomus mosseae* along with increased plant height, root & shoot weight, phosphorous content, ratio of shoot/root and leaf area (Quatrini *et al.,* 2015). Similar results were obtained by the Wu *et al.,* (2011) in micropropagated citrus with *Glomus mosseae,* which led to the better *ex vitro* adaptation of plantlets, enhanced nutrient uptake and maximum photosynthetic rate.

Application of two derivatives of auxin (1.0 mg/L NAA + 1.5 mg/L IBA) together in the culture enhanced *in vitro* rooting, survival rate and shoot growth during acclimatization stage in citrus cultivars Orange Pear and Rangpur Lime (Soares and Miranda, 2016).

In conclusion the *ex-vitro* establishment of tissue cultured plants is a mandatory process without which field survival of the plants is almost impossible. Acclimatization modifies several physio-morphological parameters in plants such as reduction in the stomatal numbers, increased leaf thickness, increased chloroplast numbers and vigorous root growth. It is, however, labour and time intensive process but ensures the maximum growth and endurance of *in vitro* raised fruit crops.

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How to cite this article:

Rohit Mahendra, Nidhi Chauhan, Jyoti Bharti Sharma, Kanchan Rana and Manish Bakshi. 2020. *Ex-vitro* Establishment of Tissue Cultured plants in Fruit Crops-A Review. *Int.J.Curr.Microbiol.App.Sci*. 9(11): 3321-3329. doi: https://doi.org/10.20546/ijcmas.2020.911.397