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# Photoautotrophic micropropagation an emerging new vista in micropropagation-A review

**R.P. Kaur***

Central Potato Research Station,
Post Bag No. 1, Jalandhar-144 003, India.

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**ABSTRACT**

Photoautotropism is a natural mode of nutrition in plants. However, this mode of nutrition is supressed in plants where photomixotrophy is induced under tissue culture laboratory conditions and provision of readymade carbon in the form of sugar in nutrition medium. The micropropagation procedure is carried out in aseptic conditions and the micropropagated plantlet needs to be acclimatized before its transfer to the external environment, as most of the necessary plant structures for the survival of the plant are lacking. However with the advent of the photoautotrophic micropropagation there is much scope for development of less labour intensive, automated tissue culture procedure leading to development of hardened plants which will not require acclimatization. Photoautotrophic micropropagation is carried out using the natural ability of plant to prepare its own food in controlled environment. Various factors that affect photoautotrophic micropropagation like carbon source, light intensity, humidity, support material and $CO_2$ concentration have been elaborated.

**Key words:** Acclimatization, Hardening, Photoautotrophic micropropagation, Photomixotrophy.

Plants are photo autotrophic organisms which have a natural capacity to produce their own food (carbohydrates) by utilizing freely available inorganic substances by the process of photosynthesis. *In vitro* grown plantlets are continuously exposed to a unique microenvironment which provides minimal stress and optimum conditions for plant multiplication. The plantlets are developed within the culture vessels under low level of light, aseptic conditions, a medium containing ample sugar and nutrients allowing heterotrophic growth and in an atmosphere with high level of humidity. These contribute a culture-induced phenotype which supresses the natural ability of the plants to carry out photosynthesis. The regenerated plants cannot survive the external environmental conditions when directly placed in a greenhouse or field (Hazarika *et al*., 2000, Kozai and Kubota, 2005, Park *et al.*, 2011). Thereby resulting in weak plants with underdeveloped stomata and lack of cuticle on leaves. The plants once transferred to net house come under water stress attributable to poor leaf structure which is unable to restrict water loss and malformed roots which are unable to effectively conduct water from the soil to the plant parts (Fila *et al*., 1998, Santamaria and Kerstiens, 1994). *In vitro* leaves are often characterized with abnormal morphology, anatomy and physiology, such as reduced number of palisade cell layers, irregular stomata, chloroplast degeneration, absence of or thick cuticle (Chakrabarty *et al.*, 2006, Dewir *et al.*, 2006, Ivanova and Van Staden, 2010) which is an important cause for low establishment rates. Hyper hydracity is another common culture induced problem afflicting tissue culture plantlets where leaves show altered leaf anatomy and aeration stress in cell. In most cases, after transplantation the *in vitro* developed leaves are shed off and replaced by well-developed newly formed leaves (Preece and Sutter, 1991, Diettrich *et al*., 1992). Alternatively, the retained leaves may get modified to adapt to the *ex vitro* environmental conditions with well-developed leaf structure and stomata, thicker cuticle, epidermal hairs and enhanced sensitivity of the stomatal guard cells regulating transpiration (Sallanon *et al.*, 1991, Diaz-Perez *et al.*, 1995, Noe and Bonini, 1996). Another important factor affecting acclimatization is photosynthesis in the plantlets, differences have been recorded with regard to the chlorophyll contents (Kadlecek *et al.*, 1998) and net photosynthetic rates (Baroja *et al*., 1995, Van Huylenbroeck, 1995). Subjecting the tissue culture plantlets to high irradiance has been observed to cause chlorophyll photobleaching which results in lower photosynthesis rates (Van Huylenbroeck, 1994, Baroja *et al.*, 1995, Van Huylenbroeck, 1995). In the tissue culture process maximum losses are attributable either due to contamination or to the transplantation stage (stress induced) of the microplants from culture room to field conditions. The survived plants are able to develop necessary structures to carry out all the physiological activities normally thereby overcoming stress.

*Corresponding author's e-mail: kaur.rp@gmail.com.

An intermediate hardening stage or acclimatization is therefore necessitated to improve survival rates of such *in vitro* developed plants. During this acclimatization all the factors including air humidity, temperature, irradiance, $CO_2$ concentration and air flow rates need to be controlled so as to prepare the plant for the *ex vitro* conditions (Deng and Donnelly, 1993, Bolar *et al.*, 1998, Kanechi *et al.*, 1998).

Sugar/carbon is a major component of the culture medium. The elimination of the same provides a major factor for transition from heterotrophic to a photoautotrophic mode of nutrition complemented with drastic reduction in culture contamination and plantlet losses. This kind of micropropagation procedure carried out in the absence of sugar has been suggested as an effective means to overcome the acclimatization stress. Therefore micropropagation conditions wherein the plantlets grow on medium without saccharides, with elevated $CO_2$ concentration and higher irradiance enabling development of fully functional photosynthetic apparatus are the main characteristics of photoautotrophic micropropagation (PAM) ( Ticha, 1996, Kubota *et al.*, 1997, Pospisilova *et al.*, 1997).In this kind of micropropagation, the provisions for reduction in oxygen levels (reduces photorespiration), elimination of carbon source from medium, provision of increased optimum $CO_2$ levels, lower humidity and higher photon influx of illumination in the culture room are provided. The process is cost effective and less labour intensive than the routine tissue culture process and can be carried out effectively even in a non-sterile environment without involving any specialized technician. The major controlling factor for carrying out PAM is environmental control involving humidity, $CO_2$ concentration, oxygen concentration, medium composition, photosynthetic irradiance (Desjardins *et al.*, 1995, Kozai and Smith, 1995, Pospisilova *et al.*, 1997).

The concept of PAM was developed two decades ago with the observation that fluctuation in $CO_2$ levels occurred in culture microclimate, which indicated low levels of photosynthetic activity inside the culture vessels (Fujiwara *et al.*, 1987). The tissue culture which was designated then as the heterotrophic system changed its perception to a photomixotrophic system where the tissue culture plants were capable of utilizing not only the sugar provided in the medium for metabolism but also synthesize their own from inorganic material in medium in presence of light. This shifted the research to development of completely photoautotrophic systems. With pioneer work done by Fujiwara *et al.* (1988) and Kozai and Iwanami(1988) in tissue culture plants grown photoautotrophically. Initially, it was developed as a means of reducing production cost and for automation/robotization of the micropropagation process. It has been estimated that the total labour costs involved in tissue culture amount to approximately 60% of the total production costs involved in conventional micropropagation systems, thereby requiring automation and robotization for reduction in overall costs (Kozai and Iwanami, 1988). Further, the plants regenerated by photoautotrophic micropropagation are more vigorous than the tissue culture developed plants in well regulated photoautotrophic systems and the plant is able to endogenously develop the required optimum amounts of growth regulators, vitamins and other organic substances. The application of these procedures have the ability to facilitate micropropagation *in vitro* and also transfer of plants from lab to field thereby enhancing their survival rates, obviating the hardening regime. The cultural requirement of such PAM allows the scope for utilization of larger vessels where automization/robotization may be applicable. Table 1 lists the number of species where PAM has been successfully used and the parameters considered in the study.

However, the work on photoautotrophic nutrition is limited and its practical application at a larger scale is lacking. The article focuses on the innovative technique of PAM and the major development in the area and explores the reasons of its limited adaptation.

The microclimate of PAM can be controlled to enhance and harness higher micropropagation rates. Several studies have been conducted on changes in culture microclimate over the two decades of PAM (Zobayed *et al.*, 2004, Yoon *et al.*, 2009, Kozai, 2010). The review has been divided to cover some of the important regulators of a good photoautotrophic systems and the influence each has on the growth of cultures.

**Support material:** The supporting material has a crucial role to play in PAM. The survival of plants in external environment depends on the quality of the plants being transplanted, which in turn depends on the availability of nutrients, water uptake and good roots development. All these are dependent on the supporting material of the plant in micropropagation. It can be compared to the vital role of the soil structure on plant growth in external environment. Different indicators and parameters such as SWC (soil water content), SOM (soil organic matter), bulk density and porosity (Bouma and Droogers, 1998, Grant *et al.*, 2001, Lampurlanes and Cantero-Martinez, 2003) and simulation model prediction have been found to be appropriate for describing soil quality and agricultural productivity (Pulleman *et al.*, 2000). Percent porosity has been used as a parameter for describing good supporting material (Afreen-Zobayed *et al.*, 1999). Physical

**TABLE1:** Studies carried out in Photoautotrophic micropropagation

| Plant | Researcher | Factors studied |
|---|---|---|
| *Coffea arabusta* | (Afreen *et al.*, 2002, 2002) | Somatic embryos photoautotrophic micropropagation |
| Melon | (Adelberg *et al.*, 1992) | |
| Sweet potato | (Afreen-Zobayed *et al.*, 1999, Afreen-Zobayed *et al.*, 2000) | Supporting material |
| | (Heo and Kozai, 1999) | Forced ventilation |
| *Pinusradiata* | (Aitken-Christie *et al.*, 1992) | Nutrient media composition |
| *Solanumtuberosum* | (Cournac *et al.*, 1991) | Aeration, sucrose supply and $CO_2$ concentration |
| | (Fujiwara *et al.*, 1995) | $CO_2$ concentration |
| | (Hayashi *et al.*, 1995) | lightining cycle |
| | (Kubota and Kozai, 1992) | forced ventilation |
| | (Niu and Kozai, 1997) | Simulation of the growth |
| Chrysanthemum | (Cristea *et al.*, 1999) | Chlorophyll (Chl) and carotenoids, Chl a/b ratio, net photosyntheticrate and ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase activities |
| *Rehmanniagluticosa* | (Cui *et al.*, 2000) | air exchanges, sucrose concentration, photosynthetic photon flux and differences in photoperiod and dark period temperatures |
| Red Rasberry | (Donnelly and Vidaver, 1984a, 1984b, Deng and Donnelly, 1993, Deng and Donnely, 1993) | $CO_2$ enrichment, reduction in humidity, reduced medium sucrose concentration, pigment content, gas exchange and leaf anatomy |
| | (Doi *et al.*, 1992) | |
| | (Laforge *et al.*, 1991) | |
| Grapevine | (Fournioux and Bessis, 1993) | $CO_2$ enrichment |
| Rose | (Genoud-Gourichon *et al.*, 1996) | sucrose, agar, irradiance and $CO_2$ concentration |
| | (Hayashi *et al.*, 1993) | CO2 enriched conditions |
| | (Langford and Wainwright, 1987) | sucrose concentration |
| Orchid (Cybidium) | (Heo *et al.*, 1996) | CO2 concentration, PPF and sucrose concentration |
| *Dianthus caryophyllus* | (Jeong *et al.*, 1996) | photosynthetic photon flux and $CO_2$ concentration |
| | (Kozai and Iwanami, 1988) | $CO_2$ enrichment and sucrose concentration, high photon fluxes |
| Cauliflower | (Kanechi *et al.*, 1998) | carbon dioxide enrichment, natural ventilation and light intensity |
| Eucalyptusplantlets | *(*Kirdmanee *et al.*, 1995) | $CO_2$ concentration and supporting material |
| Carnation | *(*Kozai *et al.*, 1988) | basal medium composition |
| Tomato | *(*Kubota *et al.*, 2001) | Comparison of Photoautotrophic and Photomixotrophic Micropropagation |
| | *(*Le *et al.*, 2001) | exogenous sucrose |
| Strawberry | *(Laforge et al.*, 1991) | Effect of light intensity and $CO_2$ enrichment |
| Asparagus | (Laforge *et al.*, 1991) | Effect of light intensity and $CO_2$ enrichment |
| Turf grass | (Seko and Kozai, 1996) | $CO_2$ enrichment and sugar-free medium |
| *Gardenia jasminoides* | (Serret *et al.*, 1996) | photoautotrophy and photoinhibition |
| *Doritaenopsis* | (Shin *et al.*, 2013) | sugar metabolism, photosynthesis, and growth |
| Tobacco | (Ticha *et al.*, 1998, Ticha *et al.*, 1999) | sugar medium, stomatal morphology |
| *Phalaenopsis* | (Yoon *et al.*, 2009) | CO2 enrichment and sugar deprivation |

and chemical nature of rooting substrate has been observed to appreciably affect rooting in Papaya (Kataoka, 1994).

Kirdmanee *et al.*(1995) reported enhanced growth and rooting, higher net photosynthetic rate of plantlets of eucalyptus grown on vermiculite as compared to other supporting materials used *viz*. agar matrix, gellan gum, and plastic net.

Structural abnormalities of roots grown on agar (Kataoka, 1994) lack of root hairs and their death on transplantation (Debergh and Maene, 1981). The shoots

rooted in non-agar supporting material produced roots *in vitro* that are phenotypically more similar to roots grown in soil. Afreen-Zobayed *et al.* (1999)reported that sweet potato plantlets when cultured photoautotrophically gave best response Florialite was used as the supporting material as compared to others including Sorabrod, mix of vermiculite and cellulose fibre, agar matrix and gellan gum. From the studies it was inferred that a suitable non-agar medium can be used to carry out PAM.

**Use of large culture vessels:** One of the major advantages of PAM which may be harnessed in mechanisation/ robotization of the labour intensive tissue culture work is use of larger vessel size, due to reduced risk of contamination. Larger vessels of various sizes have been successfully used by several workers 2.6 L vessel by (Kubota and Kozai, 1992), 2.8L vessel (Heo and Kozai, 1999), 100 L vessel by (Kirdmanee *et al.*, 1995).

**Photosynthetic photon flux:** The *in vitro* grown plants are generally characterized by low photosynthetic ability which is the main cause of their low survival and growth rates in acclimatization stage (Grout, 1978, Grout and Aston, 1978, Preece and Sutter, 1991). Low photosynthetic rates were observed for Rasberry plants transplanted to *ex vitro* (Donnelly and Vidaver, 1984a and 1984b) whereas enhanced net photosynthetic rates of photoautotrophic Eucalyptus plantlets were observed to increase its survival percentage to *ex vitro* conditions (Kirdmanee *et al.*, 1995) PAM is now being widely used in commercial transplant production and entwined for a wide range of plant species (Roche *et al.*, 1996).

Light quality and intensity have been characterized as important factor for plant growth and development *in vitro*(Macedo *et al.*, 2011).High photosynthetic flux of photoautotrophicallymicopropagated plant, cultured under high PPF and high $CO_2$ concentration has been observed in several species. Kubota *et al.* (2001) observed that PAM using explants with leaves under high PPF, high $CO_2$ concentration and high ventilation rate promoted both growth and photosynthesis without reducing multiplication rates whereas photomixotrophic micropropagation under similar conditions induced greater growth by reduced photosynthetic ability. Mills (2009)reported that in micropropagation of *Cistusincanus* enhanced root and shoot biomass could be achieved even in the absence of sugar with increase in irradiance indicating a vital role of photosynthesis in the same. The low intensity light in culture rooms is comparable to shady conditions where leaves undergo adaptive physiological changes to enable efficient use of limited light resource and therefore predisposed to photo damage by sudden exposure to highlight conditions in the external environment. This generally occurs when *in vitro* plants are transferred to external greenhouse conditions. This may lead to reduced low net $CO_2$ assimilation after transfer to high light conditions, possibly for the rest of plants life yielding low photosynthetic output and weaker plants(Van Huylenbroeck, 1994, Van Huylenbroeck *et al.*, 2000, Mills, 2009). Specifically when carrying out PAM, high light intensities are beneficial to plants by making them more photosynthetically active and adaptive to the external environmental conditions.

**$CO_2$ concentration:** While carrying out photosynthesis plants fix external $CO_2$ in the presence of chlophyll and light to form carbohydrates. Therefore $CO_2$ concentrations are the most vital component in a PAM system (Buddendorf and Woltering, 1994). Shin *et al.*, (2013) reported from their study on comparison between photoautotrophic, heterotrophic and photomixotrophicmicropropagation of *Doritaenopsis* that photoautotrophic growth *in vitro* could be significantly promoted by increasing $CO_2$ concentration and light intensity in culture vessels. High $CO_2$ absorption and its fixation in the form of carbohydrates/ cell mass was observed in photoautotrophic cultures placed in high light. In conventional *in vitro* grown heterotrophic micropropagation low photosynthetic rate of plants *in vitro* have been attributed to low $CO_2$ concentration in culture vessels (Park *et al.*, 2011, Xiao *et al.*, 2011). Increased $CO_2$ concentration can be provided to the cultures using gas permeable closures to culture vessels, increased $CO_2$ concentration around the culture vessels and direct supply of $CO_2$ into the vessels called as forced ventilation (Ticha, 1996, Solarova and Pospisilova, 1997). Solarova and Pospisilova (1997) cultured carnation plants photoautotrophically in absence of any sugar and increased $CO_2$ concentration from 0.6 to 10 or 40 gm/$m^3$ during last three weeks of *in vitro* culture or during first three weeks of acclimatization or both. The application of this increased $CO_2$ concentration promoted the growth of carnation plants.

Effects of increased $CO_2$ concentrations was individually studied by (Figueira *et al.*, 1991, Fournioux and Bessis, 1993) and in combination with other factors like irradiance and media composition (Kozai and Iwanami, 1988, Kozai *et al.*, 1988). It was observed that the use of increased $CO_2$ concentrations during *in vitro* growth of plantlets, improved the establishment rates and vigour of plantlets on transfer to field (Laforge *et al.*, 1991, Deng and Donnelly, 1993, Kirdmanee *et al.*, 1995)

Seko and Kozai, (1996), Niu and Kozai, (1997), Nguyen *et al.* (1999) also concluded that high Photosynthetic photon flux and $CO_2$ enrichment drastically enhanced the

growth of coffee, carnation, cymbidium, turfgrass and potato cultured *in vitro* under photoautotrophic conditions.

**Sucrose concentration:** Sugars have essential functions in plant metabolism serving as carbon skeletons, energy sources, osmotic agents and signal molecules. The main role of sugar in *in vitro* conditions is to ensure regular supply of sugar to plants due to low photosynthetic activity in presence of low irradiance and low $CO_2$ concentration which determine the plant sink source equilibrium (Mills, 2009). Different studies have shown opposite effects of endogenously applied sucrose to both plantlets and seedlings grown *in vitro*. Whereas some studies have shown its promontory effects, others its inhibitory. These contradictory results may be attributed to the differences in the irradiance and $CO_2$ concentrations determining plant sink source equilibrium. The endogenously applied sucrose under source stimulation contributes to biomass production and development of photosynthetic apparatus. But however in case of sink limitation, sugar increases plant susceptibility to feedback inhibition (Le *et al.*, 2001). Deng and Donnelly, (1993) reported that sucrose in the medium promoted plantlet growth but depressed photosynthesis and reduced *in vitro* hardening. Root hairs were more abundant and longer on rooting of photoautotropic plantlets than mixotropic plantlets.Preconditioning by addition of high concentration of sucrose was reported to influence the *in vivo* rooting and establishment of cuttings (Wainwright and Scrace, 1989) but lowering the sucrose concentration in the medium increases the photosynthetic ability, thereby improving plantlet survival in rose plant (Langford and Wainwright, 1987).

Koroch *et al.* (1997) reported that preconditioning at different sucrose concentration prior to acclimatization had no effect on plant establishment, but influenced plant quality. Sucrose is essential in the medium for many species. In some cases, independent growth could not be achieved on a medium without sucrose during rooting (Grout and Price, 1987). Singh and Shymal, (2001) evaluated different requirements for *in vitro* rooting of hybrid tea rose cv. Sonia and Super-star and found that both the cultivars differed in their demand for sucrose during rhizogenesis. Alleviated sucrose levels favoured rooting and root quality, but a slight declining trend was noted with the highest level (40 g $l^{-1}$) studied. (Hazarika *et al.*, 2000) reported that *in vitro* preconditioning of citrus microshoots with sucrose concentration of 3% was found optimum for subsequent *ex vitro* survival and growth. There was a linear increase of biochemical constituents, *viz.* reducing sugar, starch, total chlorophyll on addition of sucrose to the medium.

Induction of multiple shoots using shoot tips of gerbera was accomplished on MS medium supplemented with 3% sucrose and other phytohormones and almost 100% survival rate was obtained after transfer (Aswath and Choudhury, 2002).

Ticha *et al.* (1998) reported that plant growth, dry matter accumulation and total leaf area were higher under photomixotrophic than photoautotrophic conditions. Not only biomass formation, but photosynthesis was also positively affected by exogenous sucrose. Photomixotropic growth of plantlets prevented the occurrence of photoinhibitory symptoms and the plant response is species-dependent.Studies on gardenia (Serret *et al.*, 1996) showed that sucrose-feeding increases the susceptibility to photoinhibition.

**Reduction in relative humidity:** The plants that develop under lower relative humidity have fewer transpiration and translocation problems *ex vitro*, and persistent leaves that look like normal ones. The low deposition of surface wax, stomatal abnormalities and a non-continuous cuticle are typical anatomical features of herbaceous plants growing under conditions of abundant moisture. This typical *in vitro* anatomy can be prevented by increasing the vapour-pressure gradient between the leaf and the atmosphere (Hazarika, 2003). Lowering the relative humidity *in vitro* has been done experimentally with varying results. A range of methods have been used including the use of desiccants, by coating the medium with oily materials or both (Sutter and Langhans, 1982), (Ziv *et al.*, 1983) by opening culture containers (Brainerd and Fuchigami, 1981), adjusting culture closures or using special closures that facilitate water loss (Fari *et al.*, 1987) or by cooling container bottoms (Vanderschaeghe and Debergh, 1988). A relative humidity of 85% decreased the multiplication rate of carnation but increased the number of glaucus levels. Increasing the sugar or agar concentration or adding osmotic agents such as polyetheleneglycol to the medium will also lower the relative humidity and in some cases, serve the same purpose as desiccants. (Wardle *et al.*, 1983) in their studies using silica gel and lanolin oil to reduce humidity in chrysanthemum, recorded high mortality and less roots. Improved plant survival rates after transplantation have been promoted by the reduction of relative humidity using growth retardants like Paclobutrazol, Flurprimidol, triapenthenol, chlorphonium chloride, uniconazol, ancymidol (Smith *et al.*, 1992). Humidity inside the culture vessel has been reduced to improve the internal structure of plantlets and give a more successful establishment in the glasshouse61. (Short *et al.*, 1987) reported that optimum growth and *in vitro* hardening of cultured cauliflower and

chrysanthemum occurred when plantlets were cultured at 80% relative humidity. Leaves of chrysanthemum and sugar beet, which were initiated and developed at relative humidity below 100%, displayed increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration (Ritchie *et al.*, 1991).

## CONCLUSION

PAM has been identified as micropropagation without sugar in culture medium, where carbohydrate accumulation of tissue cultures and subsequent growth is dependent fully on photosynthesis and inorganic nutrient uptake (Zobayed*et al*., 2004, Arigita*et al.*, 2010, Kozai, 2010). According to Xiao *et al.* (2011) it is a photosynthetic micropropagation in sugar free medium. PAM can be used not only *in vitro* using higher CO2 concentration and light intensities but also used to aid acclimatization of microplants before their transfer to external environmental conditions. A major advantage of the technique is drastic reduction in contamination due to non-inclusion of sugar in the medium and use of non-agar supporting matrix. This further indicates reduction in stringency of culture process and relaxed asceptic environment. The PAM allows for use of larger culture vessels which indirectly imply robotisation in the overall process and scope of higher throughput. Although several reports on the process are available, there is less acceptance and adaptation at commercial level. One of the major causes of the same might be the requirement of higher light intensities, $CO_2$ concentrations and temperature control required in PAM. However with the advanced technologies now available with regard to climate control and robotization in the process, this emerging technology will find a stronger hold and commercial adaptation.

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