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REVIEW

Development and application of photoautotrophic micropropagation plant system

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Abstract Research has revealed that most chlorophyllous explants/plants in vitro have the ability to grow photoautotrophically (without sugar in the culture medium), and that the low or negative net photosynthetic rate of plants in vitro is not due to poor photosynthetic ability, but to the low CO_2 concentration in the air-tight culture vessel during the photoperiod. Moreover, numerous studies have been conducted on improving the in vitro environment and investigating its effects on growth and development of cultures/plantlets on nearly 50 species since the concept of photoautotrophic micropropagation was developed more than two decades ago. These studies indicate that the photoautotrophic growth in vitro of many plant species can be significantly promoted by increasing the CO_2 concentration and light intensity in the vessel, by decreasing the relative humidity in the vessel, and by using a fibrous or porous supporting material with high air porosity instead of gelling agents such as agar. This paper reviews the development and characteristics of photoautotrophic micropropagation systems and the effects of environmental conditions on the growth and development of the plantlets. The commercial applications and the perspective of photoautotrophic micropropagation systems are discussed.

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Introduction

Micropropagation is one of the tissue culture technologies for rapidly multiplying genetically superior, physiologically uniform, and developmentally normal plantlets that cannot be propagated by seeds or whose vegetative propagation efficiency is low in a limited time (Jeong et al. 1995). However, the widespread use of micropropagation is still limited due to high production costs (Aitken-Christie et al. 1995). These costs are mostly attributed to a low growth rate, a significant loss of plantlets in vitro due to microbial contamination and physiological and morphological disorders, poor rooting, low percent survival at the *ex vitro* acclimatization stage, and high labor costs (Kozai 1991; Kurata and Kozai 1992; Majada et al. 2002).

Most of the above factors are directly or indirectly related to the heterotrophic or photomixotrophic characteristics of plant growth in vitro in a conventional micropropagation system, in which sugar in the culture medium is the main or sole source of carbon and energy for plant growth. The conventional micropropagation technique is mostly carried out using small culture vessels with agar or other gelling agents containing nutrients and sucrose as a carbon source for the plantlets at a low photosynthetic photon flux (PPF). The in vitro environment in a typical conventional micropropagation system is characterized by high relative humidity, high ethylene concentration, stagnant air, and a low CO_2 concentration in the vessel during the photoperiod (Fujiwara and Kozai 1995). This in vitro

environment, which is entirely different from the ex vitro environment such as the greenhouse environment, often causes malfunction of stomata, poor epicuticular wax development, elongated shoots, low chlorophyll concentration, hyperhydration of plantlets, low growth rate, little rooting, callus formation at the base of explants and low percent survival ex vitro (Kozai 1991; Majada et al. 2002; Serret et al. 1996). In order to overcome these problems, photoautotrophic micropropagation was developed (Kozai 1991).

Photoautotrophic micropropagation is narrowly defined as the micropropagation without sugar in the culture medium, in which the growth or accumulation of carbohydrates of cultures is dependent fully upon photosynthesis and inorganic nutrient uptake (Kozai 1991; Zobayed et al. 2004). Thus, it can also be called photosynthetic micropropagation, inorganic micropropagation, or micropropagation in sugar-free medium (Kozai et al. 2005). Maintaining the in vitro environmental conditions (e.g., levels of PPF, CO_2 concentrations, etc.) at optimal ranges is critical. This paper aims to review the characteristics of photoautotrophic micropropagation system and its latest development and to discuss its commercial application and the perspective of scaling up to closed plant production system, a new concept of producing high quality transplants under controlled environment.

Development of photoautotrophic micropropagation

In late 1980s while studying the environmental conditions of tissue culture vessels containing leafy green plantlets, a sharp decrease in CO_2 concentration inside the culture vessel shortly after switching from dark period to photoperiod was observed (Fujiwara et al. 1987). The decreases in CO_2 concentration during the photoperiod indicated that the plantlets are capable of photosynthesis. This finding led to the development of the concept of photoautotrophic micropropagation. In the following year (1988) several studies were reported in which photoautotrophic micropropagation, that is, growing plantlets in vitro in sugar-free medium was achieved. First of all, Kozai et al. (1988) successfully cultured potato (*Solanum tuberosum* L.) plantlets in sugar-free medium with a goal to develop an automated mass propagation system for producing disease-free seed-potato tubers and disease-free potato plantlets. In another study, strawberry (*Fragaria* × *ananassa*) plantlets were cultured successfully under photoautotrophic conditions by Kozai and Sekimoto (1988). Carnation (*Dianthus caryophyllus* L.) (Kozai and Iwanami 1988) and tobacco (*Nicotiana tabacum* L.) plantlets (Pospisilova et al. 1988) were also grown photoautotrophically in the same year (1988). Since then the trend of developing protocols for the in vitro growth of plantlets under photoautotrophic conditions started and is still continuing. So far, there are nearly 50 plant species being reported successful under photoautotrophic conditions (Kozai et al. 2005). In fact, not only plantlets, chlorophyllous culture such as leafy explants (Kozai 1991) and somatic embryos of cotyledonary stage (Afreen et al. 2001) can be cultured photoautotrophically, provided that CO_2 concentration and other environmental conditions are not at limiting levels. Kubota et al. (2005) summarized the plant species that have been successfully micropropagated photoautotrophically. Table 1 is a supplemental list for plant species cultured photoautotrophically in the past 5 years. Figure 1 demonstrates some representative photoautotrophic micropropagation plant system.

As described by Kozai and Kubota (2005), photoautotrophic micropropagation has many advantages over conventional micropropagation with respect to improvement of plantlet physiology (biological aspect) and operation/management in the production process (engineering aspect). Advantages of biological aspects include (1)

Table 1 List of plant species successfully micropropagated photoautotrophically since 2006 in supplementing to the table listed by Kubota et al. (2005)

Scientific name	Common name	Main controlled factors	Main measured factors	Authors
Gerbera jamesonii	Gerbera	Sucrose concentration	Growth, quantum yield	Liao et al. (2007)
Dendrobium candidum	–	Vessel ventilation, light intensity, CO_2 conc.	Growth, photosynthesis, chlorophyll content, stomata density	Xiao et al. (2007)
Hypericum perforatum	St. John's wort	Vessel ventilation, light intensity, CO_2 conc.	Growth, stomata density	Couceiro et al. (2006)
Momordica grosvenori	–	Vessel ventilation, light intensity, CO_2 conc.	Growth, photosynthesis, chlorophyll content	Zhang et al. (2009)
Myrtus communis	Myrtle	Gas permeable lid, Chlorophyll, photosynthesis	Leaf ultrastructure	Lucchesini et al. (2006)

Fig. 1 The plantlets grown in photoautotrophic micropropagation systems. **a** Effect of four levels of light intensity: 25, 50, 100, 200 µmol m^{-2} s^{-1} on the growth of *Momordica grosvenori* plantlets cultured on sugar and NAA-free medium on day 26. The control is a photomixotrophic culture using sugar- and NAA-containing medium (Zhang et al. 2009). **b**, **c** *Coffea arabusta* somatic embryos at the late cotyledonary stage when cultured photomixotrophically under conventional culture conditions (**b**) and photoautotrophically under high CO_2 and PPF conditions (**c**). Hyperhydricity was noticed in leaves of photomixotrophic embryos (Afreen et al. 2001). **d** Roots of *Eucalyptus camaldulensis* plants cultured for 6 weeks on sugar-free MS medium under CO_2 enriched conditions using 4 different supporting materials (Kirdmanee et al. 1995a). *Upper left* Agar, *Upper right* Gelrite, *Lower left* Plastic net, *Lower right* Vermiculite. **e** Photoautotrophic growth of in vitro *Paulownia fortunei* plants as affected by a high/low CO_2 concentration and PPF on day 28 (Nguyen et al. 2001); **f** A-20 l larger vessel with *Eucalyptus camaldulensis* plantlets grown photoautotrophically (Zobayed et al. 2000a); **g** Photoautotrophic growth of in vitro neem (*Azadirachta indica*) plants as affected by PPF on day 40 (Nguyen and Kozai 2001); **h**, **i** Growth promotion of gerbera (*Gerbera jamesonii*) plantlets in large vessels by using photoautotrophic micropropagation system with forced ventilation (Xiao et al. 2005)

promotion of growth and photosynthesis; (2) high survival percentage/smooth transition to ex vitro environment; (3) elimination of morphological and physiological disorders; (4) no callus formation at the base of explant; and (5) little loss of plantlets due to microbial contamination. Advantages of engineering aspects include (1) flexibility in the design of the vessel (larger vessels); (2) increased annual productivity per floor area; (3) reduction in labor cost; and (4) simplification of the micropropagation system.

The following items are often considered as disadvantages of photoautotrophic micropropagation: (1) relative complexity of techniques and knowledge required for controlling in vitro environment; (2) expense for lighting, CO_2 enrichment, and cooling; and (3) limitation of application to multiplication systems using multiple buds/shoots.

For successful photoautotrophic micropropagation, understanding the in vitro environment and basics of environmental control for promoting photosynthesis, transpiration and nutrient uptake of plantlets are critically important. The carbohydrate for growth has to be produced by the culture itself in a photoautotrophic micropropagation system. Therefore, the primary goal of environmental control in photoautotrophic micropropagation is to promote photosynthesis of plantlets. Maintaining the in vitro environment such as CO_2 concentration and air current speed inside the vessel, temperature, and PPF at optimal ranges for maximum photosynthesis of the plantlets is critical. The headspace of the "closed" culture vessel is the site in which temperature, light, and gases interact to determine the in vitro environment. Temperature and light control in a culture room is relatively easy. Therefore, the most challenging task is to increase the ventilation rate of the culture vessel while keeping pathogen-free conditions to improve the in vitro environment and to minimize the differences in aerial environment between the in vitro and ex vitro.

Natural ventilation of small culture vessels

Regardless of size, almost all culture vessels are not completely air tight. In other words, gas exchange occurs by natural ventilation through the gaps of the contact surfaces of the vessel. Natural ventilation is based on diffusion through the air gap between inside and outside air of the vessel or through a gas permeable membrane filter attached on the lip or on the wall of the vessel. The driving forces of the gas exchange in a culture vessel under natural ventilation are: (1) pressure and temperature gradients between the inside and the outside environment, (2) concentration gradient of gases (CO_2, H_2O, etc.) between the inside and the outside environment, and (3) the current velocity and pattern of the air movement surrounding the vessels. To improve the ventilation of small culture vessels, gas permeable membrane filters are used on the lip or wall of the vessel. Apparently, the CO_2 concentration inside a culture vessel is dependent on a number of factors: the vessel itself (volume, air tightness, number of gas permeable membrane filters, etc.), the plantlets (biomass or number of plantlets and the photosynthetic characteristics of the plantlet), and culture room environment (CO_2 concentration in the culture room, PPF, air current speed, etc.). The ventilation rate of a culture vessel under natural ventilation is expressed by a parameter called number of air exchanges per hour (Fujiwara et al. 1987).

When the photosynthetic characteristics of plantlets are known, the time course of diurnal changes of CO_2 concentration inside the vessel can be predicted or simulated under various conditions. Niu et al. (1996) simulated the effects of ventilation rate of the vessel, volume of vessel, lighting cycle, and the CO_2 concentration of the culture room on the diurnal changes of CO_2 concentration inside the vessel. Some of the simulated results were verified experimentally by culturing potato plantlets photoautotrophically (Niu et al. 1997; Niu and Kozai 1997).

By using the gas permeable membrane disks, air diffusion or natural ventilation of the culture vessel can be improved and the CO_2 concentration inside the vessel is elevated during photoperiod, resulting in enhanced photosynthesis, growth rate and hence a shorter production period (Cui et al. 2000; Kitaya et al. 2005). Meanwhile, relative humidity inside the vessel is reduced, which leads to increased transpiration and nutrient and water uptake of the plantlets. Numerous studies have showed the benefits of using gas permeable membrane disks in enhanced plantlet growth and quality from increased vessel ventilation rates such as the early studies by Kozai et al. (1988) in potatoes, Kozai and Iwanami (1988) in carnation, and more recently by Nguyen and Kozai (2001) in banana (*Musa* spp.), Xiao et al. (2003) in sugarcane (*Saccharum* spp.), Lucchesini et al. (2006) in myrtle (*Myrtus communis* L.), Liao et al. (2007) in gerbera (*Gerbera jamesonii* L.), Xiao and Kozai (2006) in statice (*Limonium latifolium*), and Zhang et al. (2009) in *Momordica grosvenori*.

Simply removing sugar from the culture medium without increasing PPF and CO_2 concentration inside the vessel would not promote growth of culture or plantlets. For example, shoots of *Nopalea cochenillifera* were cultured on MS medium in a $2 \times 2 \times 2$ factorial experiment (with or without sugar, flasks with aluminum-paper covers or with cotton covers, and cultivation in a growth room or greenhouse) (Houllou-Kido et al. 2009). In this case, growth and multiplication rate were higher in sugar containing medium under both culture room and greenhouse conditions and sugar was essential for explant development. Insufficient supply of CO_2 during photoperiod

inhibits plantlet photosynthesis as supported by recent studies on myrtle plantlets (Lucchesini et al. 2001; Lucchesini et al. 2006).

Instead of using plastic and glass culture vessels, Tanaka et al. (1998a, b) developed a disposable film culture vessel called "Culture Pack" (CP) to overcome the disadvantages in conventional micropropagation and to facilitate the application of photoautotrophic micropropagation. Due to the high gas permeability of the film, the aerial environment inside this culture vessel is improved through natural ventilation between the inside and outside air. The CP system was shown to be suitable for growth under CO_2 enriched conditions with a low PPF at 45 μmol m^{-2} s^{-1} on sugar-free medium and rock-wool multi-block or agar substrates for *Spathiphyllum* (Tanaka et al. 1992), *Eucalyptus* (Nagae et al. 1996), *Azadirachta excelsa* (Kool et al. 1999), banana (*Musa* spp.) (Nhut et al. 2002), and strawberry (Nhut et al. 2003). Another important feature of this film culture vessel is the high light transmittance. At PPF of as low as 45 μmol m^{-2} s^{-1}, high net photosynthetic rate was achieved in the above mentioned plant species. High PPF results in high electricity costs for lighting as well as additional heat in the culture room, which in turn increases the additional cost of cooling. Tanaka and his colleagues continued developing and improving the culture vessels by trying different films and frame materials in order to lower the costs. Recently, a vessel called "Vitron" made of TPX (4-methyl-1-pentane polymer) and CPP (polypropylene), a relatively low cost film vessel, was developed. Using this Vitron vessel, the growth and quality of *Eucalyptus* plantlets cultured in sugar-free liquid medium and oasis substrate were greatly enhanced (Tanaka et al. 2005). DaSilva et al. (2006) also reported that best growth of *Spathiphyllum* cv. Merry was obtained by culturing the plantlets in sugar-free liquid medium using Vitron vessel under a CO_2 concentration of 3,000 μmol mol^{-1} and a PPF of 45 μmol m^{-2} s^{-1}.

Large culture vessels with forced ventilation

Forced ventilation is more effective in gas exchanges and can be achieved by pumping a particular gas mixture with an air pump into the culture vessel through gas permeable filter disk (Kozai et al. 2000; Zobayed et al. 1999). Forced ventilation rate can be easily controlled during the production period by using an airflow controller, while natural ventilation rate is difficult or not possible to change without changing the number of gas permeable filter disks (Aitken-Christie et al. 1995). The control of the ventilation rate is an important part of achieving optimum conditions for growth of plantlets in vitro. The ventilation rate of a culture vessel should be adjusted according to the magnitude of net photosynthetic rates of the cultured plantlets inside the vessel for the optimization of the aerial environment and thus maximization of growth (Kozai and Kubota 2001).

Fujiwara et al. (1988) developed a large culture vessel (0.6 m long, 0.3 m wide and 0.1 m high) with a forced ventilation system for enhancing the photoautotrophic growth of strawberry explants and/or plants during the rooting and acclimatization stages, where CO_2 gas was mixed with air and pumped into the vessel. This was a kind of aseptic micro-hydroponic system with a nutrient solution control system.

Kubota and Kozai (1992) showed that the net photosynthetic rate and photoautotrophic growth of potato plants cultured in a 2.6-l polycarbonate vessel with forced ventilation, containing a multi-cell tray with rock-wool cubes, were greater than those cultured using a conventional (small) culture vessel with natural ventilation.

Heo and Kozai (1999) developed a forced ventilation micropropagation system with a vessel containing a multi-cell tray widely used for plug seedling production. The cells were filled with sterilized vermiculite or cellulose plugs. The photoautotrophic growth of sweetpotato (*Ipomoea batatas*) plants cultured with this system was several times greater than the photomixotrophic growth of plants cultured with conventional or small culture vessels containing sugar and with natural ventilation. However, in either of the forced ventilation system mentioned above, the growth in the vessel was not uniform, with larger plants near the air inlet and comparatively smaller plants near the air outlet.

Zobayed et al. (2000a) and Heo et al. (2001) developed large culture vessels with air distribution pipes for forced ventilation. The major aim of the system was to provide an air current pattern which enables uniform distributions of CO_2 concentration and relative humidity as well as those of air current speeds, and thus the uniform plantlet growth. Their aims were achieved successfully.

The feasibility of forced ventilation systems has been tested for both *Eucalyptus* (Zobayed et al. 2000a, 2001) and *Coffea* (Nguyen et al. 2001). The net photosynthetic rate was enhanced, normal stomatal closing and opening were observed, and the epicuticular leaf-wax content was significantly higher than in conventionally cultured photomixotrophic plants (Zobayed et al. 2001). Carbohydrate status in the leaves was investigated by Wilson et al. (2001) and the plants cultured in the large vessels with forced ventilation were considered acclimatized in vitro and their transpiration rates and percent water loss remained lower than those of the conventional plants when transplanted to ex vitro conditions.

Xiao and Kozai (2004) developed a photoautotrophic micropropagation system (PA system) using five large culture vessels (volume: 120 l each) with a forced

ventilation unit for supplying CO_2-enriched air for commercial production of calla lily (*Zantedeschia elliottiana*) plantlets. The culture period of plantlets in the PA system was reduced by 50%, compared with that in a conventional, photomixotrophic micropropagation system (PM system) using sugar-containing medium. Percent survival ex vitro of plantlets from the PA system was 95%, while that from the PM system was 60%. The production cost of calla lily in the PA system was reduced by 40%, compared with that in the PM system. The sales price of ex vitro acclimatized calla lily plantlets was increased by 25% due to higher quality, compared with plantlets produced in the PM system.

This PA system has also been improved and applied for commercial production of gerbera plantlets (Xiao et al. 2005). Growth of the plantlets in the PA system was compared with that in the PM system. The number of leaves, the leaf area, and the shoot and root dry weight were, respectively, 1.7, 5.2, 4.6, and 3.8 times greater in plantlets grown in the PA than those in the PM system. The net photosynthetic rate and chlorophyll concentration of the PA-grown plantlets were, respectively, 9.2 and 2.2 times greater than those of the PM-grown plantlets. Rooting percentage in vitro and survival percentage ex vitro were, respectively, 98 and 95% for the PA plantlets, and 62 and 57% for the PM plantlets. The total productivity of the PA system was 6.9 times higher than that of the PM system. Therefore, the PA system can be used to produce a large number of high-quality plantlets with less space, simplified operation, and high productivity.

Porous supporting materials

The rooting and photosynthetic ability of plantlets is usually affected by the physical and chemical nature of supporting material (Zobayed et al. 2000b). When agar was used as supporting material, root growth of many plant species was found to be poor and the roots were usually thin and fragile (Roberts and Smith 1990). These roots were often damaged during transplanting, resulting in low growth or death of the plantlets (Debergh and Maene 1981). Use of porous supporting materials improves the root zone environment and thus enhances rooting. High porosity of culture medium increases the oxygen concentration around the root system, which improved development of the root system, and enhanced water and nutrient absorption of the plantlets (Fujiwara and Kozai 1995). Moreover, the extensive root system produced in vitro appeared to contribute to the higher percent survival of plants during acclimatization to greenhouse or field conditions.

Replacing conventional agar gel with porous materials significantly affects the root zone environment and therefore the anatomical characteristics of roots. Afreen-Zobayed et al. (1999) compared the root growth of sweetpotato plantlets cultured photoautotrophically in five supporting materials: Florialite (a mixture of vermiculite and cellulose fibers), Sorbarod, vermiculite, gellan gum, and agar. They found that root system developed in Florialite with liquid medium had numerous lateral roots, compared with the other supporting materials. The well-developed root system helps nutrient and water uptake and promotes overall growth of the plantlets (Afreen-Zobayed et al. 1999). Plantlets grown in such porous supporting materials survive well in ex vitro environmental conditions. High survival percentage of *Eucalyptus* plantlets ex vitro was highly correlated with the enhanced root development due to improved root zone environment (Kirdmanee et al. 1995a, b, c). Similar results, in terms of correlation of improved root system, enhanced growth and high survival percentage were observed with other crops including acacia (*Acacia mangium*; Ermayanti et al. 1999), coffee (*Coffea arabusta*; Nguyen et al. 1999a and b), *Eucalyptus* (Zobayed et al. 2000a, 2001), mangosteen (*Garcinia mangostana*; Ermayanti et al. 1999), and sweetpotato (Afreen-Zobayed et al. 1999). Woody plants are generally difficult to root, and therefore, a supporting material such as vermiculite or Florialite would be beneficial to those crops.

Recent advancement in photoautotrophic micropropagation

A number of commercially important medicinal plants have been successfully cultured photoautotrophically in recent years. Leafy nodal cuttings of St. John's wort (*Hypericum perforatum* L.), a medicinal plant native to Europe and Asia with a long history of being used for treatments of neurological disorders and depression, were cultured on a sugar-free MS medium under CO_2 concentration of 1,000 μmol mol^{-1} in the culture room, PPF of 150 μmol m^{-2} s^{-1} and number of air exchanges of the vessel at 3.9 h^{-1} (Couceiro et al. 2006). The growth and quality of the St. John's wort plantlets were enhanced compared to those cultured photomixotrophically (with sugar in the medium, under ambient CO_2 concentration and no gas permeable membrane disc on the lip of the culture vessel) (Couceiro et al. 2006; Mosaleeyanon et al. 2005).

Dendrobium candidum, a peculiar and valuable Chinese herb and crassulacean acid metabolism (CAM) plant, was cultured for 45 days on a sugar-free modified MS medium in Magenta-type vessels with the number of air exchanges of the vessel of 3.2 h^{-1}, at a PPF of 80 μmol m^{-2} s^{-1}, and CO_2 concentration of either 400 μmol mol^{-1} (PA treatment) or 1,000 μmol mol^{-1} (PAC treatment) in the culture

room during the photoperiod (Xiao et al. 2007). Leaf area, fresh and dry weight, and stem diameter were significantly greater in both photoautotrophic treatments (PA and PAC treatments) than those in the conventional or photomixotrophic treatment on a sugar-containing MS medium with the number of air exchanges of the vessel of 0.2 h^{-1} and CO_2 concentration of 400 μmol mol^{-1} in the culture room. Moreover, the photoautotrophic plantlets had higher net photosynthetic rate, greater chlorophyll content, normal stomata and less microbial contamination, compared to those of the photomixotrophic plantlets. The photomixotrophic plantlets had widely opened stomata with circular shape in the photo- and dark periods. The daily CO_2 exchange rate (CER) of the plantlets was negative in the photomixotrophic treatment. The plantlets in the two photoautotrophic treatments (PA and PAC) had positive CER but no significant differences were observed in the growth values and CER between PA and PAC. The ratio of daily CER in the photoperiod was 16% in the photoautotrophic treatment without CO_2 enrichment, and 27% in the photoautotrophic treatment with CO_2 enrichment.

Momordica grosvenori Swingle, a traditional Chinese medicinal plant, has been used to treat colds, coughs, sore throats, and gastrointestinal disorders for many years. The fruit contains a natural sweetener called Mogroside V, which is 300 times sweeter than cane sugar and extremely low in calories. The plantlets were cultured in vitro for 26 days on sucrose- and hormone-free MS medium with enhanced ventilation (3.6 h^{-1}), and four levels of PPF, namely 25, 50, 100 or 200 μmol m^{-2} s^{-1}, and a CO_2 concentration of 1000 μmol mol^{-1} in the culture room (Zhang et al. 2009). The control treatment was a photomixotrophic culture on a MS medium containing sucrose and NAA with a CO_2 concentration of 400 μmol mol^{-1} in the culture room, and a PPF of 25 μmol m^{-2} s^{-1}. Based on the results, a second experiment was conducted to investigate the effects of NAA and sucrose on callus formation. In this second experiment, plantlets were grown in the absence and presence of either NAA or sucrose. Compared to the control, the photoautotrophic plantlet had a well-developed rooting system, better shoot, greater chlorophyll content and higher electron transport rate, and the *ex vitro* survival percentage was increased by 31%. Both sucrose and NAA stimulated callus formation on the shoot bases of control plantlets, whereas calluses did not form on the plantlets grown in sucrose- and hormone-free medium. The higher light intensities increased the fresh and dry weight of the photoautotrophic plantlets. A PPF of 100 μmol m^{-2} s^{-1} was more suitable for the growth of *M. grosvenori* plantlets. Therefore, photoautotrophic plantlets grown at high light intensities would be better suited to the intense irradiance found in sunlight.

Two genotypes of C_4 grass sea oat (*Uniola paniculata* L.) responded differently to photoautotrophic micropropagation (Valero-Aracama et al. 2007). Sea oats required an initial source of carbon from the medium until they are capable of using CO_2 from the vessel headspace as their main carbon source, which is consistent with the findings of Arigita et al. (2002) with kiwi explants (*Actinidia deliciosa* Chev. Liang and Ferguson 'Hayward'). Similar to many C_3 species, sea oats benefited from photoautotrophic micropropagation in that plantlet growth and development of photoautotrophy were enhanced during in vitro culture (Valero-Aracama et al. 2007).

Arbuscular mycorrhizal fungi (AMF) are multifunctional microorganisms and play important roles in ecosystems as biofertilizer, bioprotectors, and bioregulators (Lovato et al. 1996). Liu and Yang (2008) reviewed the studies on photoautotrophic micropropagation and inoculant cultivation of AMF. They suggested the integration of AMF and photoautotrophic micropropagation to alleviate the injuries of plantlets in vitro and *ex vitro* caused by environmental stresses are feasible. This integration may provide an opportunity to improve the in vitro root zone environment of photoautotrophic micropropagation system. However, proper selection of plant species and AMF species is essential to achieve the integration and produce high quality plantlets and transplants.

Scaled-up micropropagation system to aseptic culture room

The idea of the forced ventilation micropropagation system can be further extended to the use of an aseptic culture room, considering the room itself as a large culture vessel containing many small sterile trays with plants on culture shelves. This kind of micropropagation system can be also considered as a transplant production system utilizing small cuttings under disease-free conditions or a closed vegetative propagation and transplant production system with artificial light (Kozai 1998; Kozai et al. 1998; Kubota and Chun 2000). In this system, no one is permitted to enter the culture room for handling the trays with plants and for environment control in normal production modes. Thus, both tray transportation and environmental control in the culture room must be automated. To supply the large number of transplants (20–40 billion plants per year) needed for re-afforestation, large scale photoautotrophic micropropagation systems with fully automated environmental control and transportation systems may be feasible and would contribute to compensating the loss of forests and natural resources worldwide. With this system, energy and mass exchanges between the inside and outside of the system can be minimized for maximizing water, CO_2, light and other resource utilization efficiencies and reducing electric energy consumption. Ohyama and Kozai (1998) showed that electric energy

consumption per transplant was estimated to be 0.1 MJ when a plug tray (30 wide × 60 cm long) with 72 cells is used and the cost in Japan is 2–3 Yen (1.5–2 US cents) per transplant, which was acceptable considering the market prices of micropropagated plants (about 100 yen per plant) at that time. The cost could be reduced by 50 or 70%, respectively, when a plug tray with 150 or 300 cells was used (Nishimura et al. 2001). Research and development of such a scaled-up photoautotrophic micropropagation system are still underway.

Conclusion

Numerous studies have been conducted on improving the in vitro environment and enhancing the growth and development of cultures/plantlets on nearly 50 species since the concept of photoautotrophic micropropagation was developed more than two decades ago. Although large scale commercialization has not been achieved worldwide, the research efforts from small culture vessels with gas permeable membrane discs to large culture vessels with forced ventilation have proved the feasibility of scaling up the photoautotrophic micropropagation system. The step by step progresses in photoautotrophic micropropagation have led to the development of another new idea. That is, the closed systems with artificial lights for high quality transplant production (Kozai et al. 2005). By combining the concepts of closed production system and photoautotrophic micropropagation, a large number of high quality transplants can be produced in a limited time with high energy and water efficiency.

The closed transplant production system is not aseptic but fairly clean and insect-free. The closed system was commercialized in 2004. In 2010, this system has been used at about 100 sites in Japan for production of seedlings, cuttings and scions/stocks for grafting. For the commercialization of such a transplant production system with artificial light, a considerable reduction in electricity consumption for lighting and air conditioning has been achieved. Currently, remarkable reductions in electricity consumption (0.4 MJ per transplant) and its cost (less than 1 US cent per transplant) have been achieved by improving the lighting and air-conditioning systems as shown by Kozai and Xiao (2005) and Kozai (2007).

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