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SHORT COMMUNICATION

Photoautotrophic System: A Review and Potential Applications in Plant Micro Propagation

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Abstract

Standard methods for *in vitro* plant propagation often involve the use of closed tissue culture vessels containing agar media with macronutrients, micronutrients, and sucrose. The closed bottle culture system is usually kept in controlled temperature and light conditions, differing from the natural sunlight. It often results in high relative humidity and limited or no gas exchange within the culture vessels. Plantlets produced from *in vitro* cultures show malfunctioned stomata, undeveloped cuticles and lower leaf chlorophyll levels, and hyper-hydration of the plantlets. Photoautotrophic tissue culture is *in vitro* propagation without or with a reduced sugar level in the culture media; therefore, the growth or accumulation of carbohydrates of the explants is dependent upon photosynthesis and inorganic nutrient uptake. This approach is usually combined with ventilation or CO_2 enrichment. Recently, incorporating porous materials such as vermiculite, gum and paper pulp to the agar media has been shown to promote rooting of the plantlets. This article discusses the advantages and disadvantages of the photoautotrophic micropropagation in comparison with the standard micropropagation methods. The paper also provided the results of the photoautotrophic micropropagation studies conducted at Laboratory of Tissue Culture II at the Department of Agronomy and Horticulture, Bogor Agricultural University, Indonesia.

Micropropagation System

Micropropagation is a method used to grow and propagate plant cell, tissues or organs under aseptic condition. Micropropagation is more advantageous to traditional propagation methods, including mass production of true-to-type clones, further multiplication of disease-free materials, and rescue of unseeded plants.

The standard method for *in vitro* plant micropropagation is often to deal with tightly closed culture vessels or containers which help avoid undesired contamination, in the presence of agar or other gelling media containing macronutrients and micronutrients and sucrose as a source of carbon for the explants. The closed-vessel culture is usually kept in a controlled environment with set temperature and light conditions. These conditions are usually insufficient for plant growth and are different from the natural environment.The standard micro propagation method, however, has been successfully used to micropropagate many crops.

In vitro environment is greatly different from *ex vitro* one as it lacks of air circulation and present high relative humidity within the culture vessels. Also, the closed system provides a very low CO_2 concentration. Therefore, *in vitro* cultures result in malfunctioned stomata, undeveloped cuticles and lower leaf chlorophyll levels, and hyper-hydricity of the plantlets (Pospisilova et al., 1999). The roots of the plantlets arisen from *in vitro* cultures cannot grow to their best (Majada et al. 2002); as water supply to the plantlets is limited due to a low hydraulic conductivity of the root to stem connection (Fila et al., 1998), resulting in low survival rate after transfer to the *ex vitro* condition. *In vitro* plantlets need a period of acclimatization to adjust their anatomical abnormalities and to enhance their physiological performance to ensure survival under *ex vitro* conditions. Often, the acclimatization can take several weeks, depending on the plant species. Plantlets have to adapt rapidly from a heterotrophic or photo-mixotrophic into an autotrophic growth, and to develop a better control of transpiration through their stomata and cuticle (Da Silva et al., 2017). Increasing light intensity and decreasing relative humidity during acclimatization can facilitate this transition (Da Silva et al., 2017). The percentage of survival during acclimatization stage differs between

different crop species and it is highly dependent on acclimatization environment. However, survival rate can be lower than 50% in some cases. The anatomical, morphological and physiological factors that accounts for the fragility of cultured plants have been described by Hazarika et al. (2006), including the techniques that are most isfactory for acclimatization of *in vitro* cultured plants.

A temperature-controlled acclimatization method was reported was Hayashi et al. (1988) where temperature, humidity, irradiance, CO_2 concentration and air flow rate were optimized to allow *ex vitro* growth of plantlet. Under optimal acclimatization environment, leaf thickness increases, leaf mesophyll progresses into palisade and spongy parenchyma through differentiation, while stomatal density decreases and stomatal form changes from circular to elliptical (Pospisolova et al., 1999). Development of leaf cuticle, epicuticular wax, and stomata to regulate transpiration are very important for plantlet survival after transferring to *ex vitro* environment (Pospisilova et al., 1999).

Carbohydrate Reduction in Photoautotrophic Micropropagation Systems

A micropropagation without or with a reduced sugar level in the culture media is called photo autotrophic. With this system the growth or accumulation of carbohydrates of the explants is dependent upon photosynthesis and inorganic nutrient uptake. Therefore, in the photoautotrophic system, plantlets are promoted to photosynthesize during the *in vitro* stage. The ability to photosynthesize at the early stage allows the plantlets to produce and accumulate carbohydrate independently; therefore these explants can develop morphologies which are similar to those grown *ex vitro*. Explants with fully-developed and functional stomata and leaf cuticle are likely to have a higher survival after transplanting to the *ex vitro* environment. In addition to providing sugar-independent media, photoautotrophic micropropagation is set to have a better gas exchange by providing sufficient ventilation, while keeping the cultures free from contaminants through the ventilation holes.

The photoautotrophic system has many advantages over the traditional *in vitro* culture; the absence of sugar results in the media reduces microbial contamination. In our past studies, providing ventilation to teak culture promoted faster growth (Figure 1) and promoted better root development (Krisantini, 2009, unpublished report). Similar results were reported in chrysanthemum (Hardiani, 2015) and potato (Rai, 2015). The plantlets that have the ability to photosynthesize during the *in vitro* stage potentially have higher adaptability and survival during the transition to the *ex vitro* environment.

Despite the advantages mentioned above, photoautotrophic micropropagation system has its limitations. Understanding *in vitro* environment and environmental control that promotes photosynthesis, transpiration and nutrient uptake are very important for a successful photoautotrophic micropropagation (Kozai et al., 2005). Techniques to maintain CO_2 concentration and air current speed inside the culture vessels can be quite complicated. In addition, the photoautotrophic micropropagation will need better regulation of temperature and light to maximize photosynthesis of the plantlets.

The first report on sugarless *in vitro* culture was reported in potato (Kozai et al.,1988) and later on strawberry (Kozai and Sekimoto, 1988). Exclusion of sugar will initially cause slower growth, but the explants will grow normally. Slow culture growth could also be useful for *in vitro* conservation. According to Martin and Pradeep (2003), sugarless media facilitated the storage of *Ipsea malabarica,* an endemic and endangered orchid from for 27 months with zero contamination. Slower growth of explants in sugar-free medium minimizes sub-culture and reduced the risk of contamination. Rai (2015) studied the effects of different levels of sugar in the media on potato culture and reported that in reduced sugar (5 to 10 $g.L^{-1}$), roots were formed later and the explants had fewer roots compared to those grown in 20 to 25 $g.L^{-1}$ sugar in the media. Combining sugarless or reduced sugar level in the media with increased CO_2 concentration inside the flasks, and providing quality light can further promote growth of culture or plantlets.

CO_2 Enrichment in Photoautotrophic Systems

The standard micropropagation technique uses air-tight vessels or containers to avoid contamination. The closed system, however, results in a limited or no air flow and very low CO_2 level in the bottle. CO_2 enrichment in a photoautotrophic culture can be achieved either by using a gas permeable cover and increasing CO_2 concentration around the culture vessels (Tichá, 1996), or by forced ventilation to supply CO_2 into the culture vessels (Yue et al., 1993).

Fujiwara et al. (1987) described ventilation rate as the number of air exchanges per hour, which depends on the pressure and temperature gradients between the internal and external environment, concentration gradients of CO_2, H_2O, and the air movement outside the bottle culture. CO_2 concentration inside a culture vessel depends on the volume of the vessels,

biomass of the explants and their photosynthesis characteristics, and the culture room environment (Kozai et al., 2005). Therefore, knowledge on these characters is important to develop a better photoautotrophic micropropagation system.

A simple photoautotrophic system has been developed and tested at Tissue Culture Laboratory II of Bogor Agricultural University. Chrysanthemum (*Dendranthema grandiflora* Tzevelev; Figure 2) and potato (*Solanum tuberosum*) were grown from one-node segments in flasks with ventilation holes covered with micropore tapes (3M) and explant growth of both species was significantly promoted with ventilation. However, a certain level of sugar (10 or 15 $g.L^{-1}$) was required to optimize the growth of the explants in both studies (Hardiani, 2015; Rai, 2015). Research by Mitra et al. (1998) demonstrated that CO_2 enrichment of 2% (v/v) promoted chrysanthemum growth in a photo autotrophic culture similar to those grown with sugar. In addition, the number of branches and internode length of the explants grown in photoautotrophic were also comparable to the photo mixotrophic culture (Mitra et al., 1998). Promoted growth of branches in explants is a highly desired characteristic for mass propagation.

CO_2 concentration, air diffusion or ventilation of the culture vessel can be elevated using gas permeable membrane disks. Providing ventilation to the culture flasks reduces the relative humidity, hence promoted transpiration, and a better nutrient and water uptake (Cui et al., 2000). The benefitsof using gas permeable membrane has been demonstrated in potato (Kozai et al., 1988; Rai, 2015), coconut (Samosir and Adkins, 2014), and in high economical value cut flower species carnation (Kozai and Iwanami, 1988), gerbera (Liao et al., 2007), statice (Ziao and Kozai, 2006). Samosir and Adkins (2014) applied the conventional micro propagation system for coconut micro propagation, but added an extra step where coconut was cultured in a photoautotrophic system with CO_2 enriched air at 1600 $\mu mol.mol^{-1}$ during the light phase, and ambient air of 350 $\mu mol.mol^{-1}$ during the dark phase of propagation. The coconut grown with the additional photoautotrophic system had a greater fresh weight and leaf area, thicker, a higher stomatal density and chlorophyll *a* content, and a better rooting system compared to those from the conventional culture (Samosir and Adkins, 2014). In addition, the percentage of coconut survival after transferred to the *ex vitro* environment increased from 40 to 100% with photoautotrophic system.

A photo-mixotrophic system produced plantlets with widely opened and circular shaped stomata, with negative daily CO_2 exchange rate (Kozai et al., 2015). In addition, CO_2 exchange rate increased from 16% in a photoautotrophic environment without CO_2 enrichment to 27% with CO_2 enrichment (Kozai et al., 2005). Hardiani (2015) reported that the guard cells surrounding the stomatal pore in the chrysanthemum culture from photo autotrophic culture can open and close in response to the environmental changes; this character was not noted from the explants from conventional *in vitro* system. The two highly specialized guard cells from explants grown in the photoautotrophic system demonstrated the ability to integrate environmental to control the stomatal aperture and thereby promoted a better gas exchange. In line with these findings, Rai (2015) reported that reducing the sugar in the media combined with ventilation will produce explants with greater number of stomata in potato leaves.

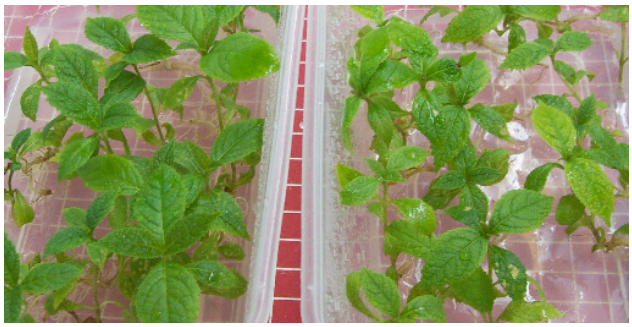

Figure 1. Teak (*Tectona grandis*) propagation on a modified MS media at four week after culture with ventilation (left) grow better and faster compared to without ventilation (right). Ventilation was provided by making holes along the container walls covered with micropore tapes.

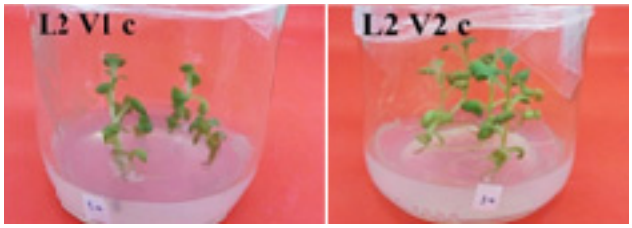

Figure 2. Chrysanthemum (*Dendranthema grandiflora*) at eight weeks after culture on a modified MS media with two ventilation holes (L2V2 c, right) grew better compared to one ventilation holes (L2V1 c, left). Ventilation was prepared by making holes on the plastic cover of the flask and sealed with micropore tapes. Photo by Hardiani (2015).

Addition of Porous Materials to the Agar Media in Photoautotrophic Systems

Addition of porous materials to the agar media was

beneficial to the root development of the plantlets through providing a better aeration to the root zone (Kirdmanee et al., 1995). Plantlets grown on agar usually have thin and fragile roots which are easily damaged during transplanting, resulting in growth inhibition or death of the plantlets (Roberts and Smith, 1990). Root growth needs sufficient oxygen in the rooting zone; therefore, combining porous supporting materials to the agar media will promote root development. High porosity of the culture medium improves water and nutrient absorption (Fujiwara and Kozai, 1995) and anatomical characteristics of the roots (Afreen-Zobayed et al., 1999) thus allowing a survival rate following transfers to greenhouse or field conditions.

Afreen-Zobayed et al. (1999) examined five types of supporting materials to grow sweet potato *in vitro*, i.e. Florialite, a mixture of vermiculite and cellulose fibers; Sorbarod, vermiculite, gellan gum, and agar. Addition of Florialite to a liquid medium significantly improved root development in sweet potato (Afreen-Zobayed et al., 1999) and sugarcane culture (Xiao et al., 2003). Furthermore, Afreen-Zobayed et al. (2000) tested vermiculite mixed with 30% (w/w) of paper pulp in sweet potato culture, and resulted in higher shoot and root mass than control (agar only). The net photosynthetic rate per plantlet was also higher with the addition of vermiculite and paper pulp (Afreen-Zobayed et al., 2000). Woody species which are generally difficult to root are likely to benefit from addition of supporting materials into agar media as reported in *Eucalyptus* (Kirdmanee et al., 1995) and coffee (Nguyen et al., 1999). Nguyen et al. (1999) also reported an increase in photosynthetic ability of the coffee plantlets grown on agar and supporting material.

The protocols for the *in vitro* growth of plantlets under photoautotrophic conditions are still to improve, particularly in the developing countries where latest technology is not always available. Nearly 50 plant species, including some woody plants, had successful propagation rate under photoautotrophic conditions (Kozai et al., 2005, Kozai 2010).

Conclusion

This study indicates tremendous potentials to develop the photoautotrophic system, including modification of the system to make it more cost-effective and widely applicable. Further studies on addition of porous materials to the conventional agar media should be undertaken. Simple method for CO_2 enrichment should be explored for possible application in the area where advanced technology is not available at affordable costs.

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