PHOTOAUTOTROPHIC (SUGAR-FREE MEDIUM) MICROPROPAGATION AS A NEW MICROPROPAGATION AND TRANSPLANT PRODUCTION SYSTEM

Edited by
T. Kozai, F. Afreen and S.M.A. Zobayed

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Preface

This book provides two basic concepts on plant propagation and value-added transplant production in a closed structure with artificial lighting: 1) photoautotrophic (sugar-free medium, photosynthetic or inorganic nutrition) micropropagation systems, and 2) closed transplant production systems with minimum resource consumption and environmental pollution. This book also describes the methodology, technology and practical techniques employed in both systems, which have been commercialized recently in some Asian countries such as China and Japan.

We often use a closed structure such as a tissue culture vessel, a culture room, a growth chamber, a plant factory with lamps, and a greenhouse to propagate plants and produce transplants. Main reasons why we use such a closed structure is: 1) higher controllability of the environment for desired plant growth, 2) easier protection of plants from damage by harsh physical environment, pathogens, insects, animals, etc, 3) easier reduction in resource consumption for environmental control and protection, and 4) higher quality and productivity of plants at a lower cost, compared with the plant propagation and transplant production under rain, wind and sunlight shelters and in the open fields.

Thus, there should be some knowledge, discipline, methodology, technology and problems to be solved on plant propagation and transplant production common to those closed structures, regardless of the types and sizes of the closed structure. Currently, however, there are not much discussion and consideration common to those closed structures, and there are few academic information and personnel exchanges among researchers in the fields of plant tissue culture, micropropagation, plant factory, greenhouse, etc. This is an ideal book that spans topics from physical state of culture environment to commercial application of photoautotrophic micropropagation. The book aims at providing the concepts, methodology, technology and practical techniques common to various kinds of plant propagation and transplant production.

Generally, in plant tissue culture and micropropagation, sugar, vitamins and amino acids are added to the culture medium, and explants and plants are grown *in vitro* heterotrophically or photomixotrophically. Fluorescent lamps are mostly used as the light source and maximum light intensity is around 100 μmol m^{-2} s^{-1}. Leafy part of an explant is often removed before transplanting on the culture medium, because it does not play an important role for its further growth and development. Most research papers on plant tissue culture deal with effects of different combinations of plant growth regulators and organic nutrients in the culture medium on plant growth and development *in vitro*.

On the other hand, in plant production using a greenhouse, a nursery and a plant factory, inorganic nutrient components only are supplied to the soil, substrate or water, and plants are grown photoautotrophically (or photosynthetically). Sunlight is mostly used as the sole light source and maximum light intensity under sunlight is about 1,000 μmol m^{-2} s^{-1}. A leaf is an essential part of seedlings and leafy cuttings for their photosynthetic growth. There is not much research on the effects of plant growth regulators supplied in the soil or substrate on the plant growth and development. Why

materials and methods in greenhouse crop research are so different from those in plant tissue culture and micropropagation research?

This is the first book which resolves these questions and also presents the unique combination of biological and engineering perspectives on photoautotrophic or sugar-free medium micropropagation system. The chapters involve the current innovative researches on control and optimization of the *in vitro* microenvironment, plant production in closed structure, and advances evidence on how some underrated aspects of the process actually determine the status of the final product. Chapter 2 provides information about the appropriate use of SI units, symbols and terminology for the environmental studies of plant tissue culture. Chapter 6 illustrates the physiological and anatomical features, which have a correlation with the physical factors of microenvironment and determines the quality of transplants. In chapter 14 a handful of plant propagation and cost efficiency related questions and answers are included to encourage readers to "have a go".

I have been a greenhouse researcher for the past 35 years, specializing in environmental control. Besides being so, I started a research project on measurement, analysis and control of physical environment in plant tissue culture for promoting plant growth *in vitro* 20 years ago. Recently, I have been working also on environmental control of plant factories using lamps as the sole light source.

When I started the research project on micropropagation, I wondered why all tissue culturists were using the culture medium containing sugar and other organic nutrients, even when they grew green-colored, leafy plants. Because, the plants were similar in size to the seedlings or transplants with unfolded cotyledonary leaves and leafy cuttings grown in a greenhouse and in the open fields. I was surprised that most plant tissue culturists were not interested in controlling the light intensity, relative humidity, CO_2 concentration and air movement in the culture vessel, which were the most important environmental factors in greenhouse environment control.

These ideas prompted us to find out the environmental factors restricting *in vitro* plants to grow on the sugar-free medium under pathogen free-condition. Then, we discovered that low CO_2 concentration in the vessel during the photoperiod was the main factor restricting the photosynthesis of plants *in vitro*. Step by step, we improved the relative humidity, light intensity, air movement, etc. in the vessel to promote the plant growth and enhance the plant vigor. Thus, I became confident that we could grow the green-colored, leafy plants *in vitro* on the sugar-free medium by properly controlling the *in vitro* environment for promoting photosynthesis. Finally, we developed a photoautotrophic micropropagation system using a large, forcedly ventilated and/or CO_2 enriched culture vessel containing sugar-free and inorganic medium, which looks like a miniature greenhouse, but under disease-free and artificial light conditions. These aspects are illustrated in Chapter 4 and 9.

During the research, we got an idea of a closed transplant production system. This system is a warehouse-like structure covered with opaque thermal insulators, in which ventilation is kept at a minimum, and artificial light is used as the sole light source for plant growth. The closed system is ideal for growing disease-free and vigorous transplants or small plants under pathogen-free and optimized environmental conditions, using minimized resource including fossil fuels, water, labor, time, and space. A detailed appraisal of which is given in Chapter 17.

I hope that this book will prove to be a useful one to researchers, graduate students and industry people in the fields of tissue culture, micropropagation, greenhouse horticulture, plant factory, controlled environment agriculture and forestry, readers who are interested in plant propagation and value-added transplant production for solving global and local problems on environmental conservation, food, feed and biomass production, and fossil-fuel resource saving by use of bio-resources.

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Toyoki Kozai
July, 2004

Chapter 1

T. KOZAI

INTRODUCTION

Key words: Chlorophyllous cultures, closed production systems, CO_2 concentration, environmental pollution, photoautotrophic micropropagation, phytomass, quality transplant.

The world population in the year 2004 is about 6.4 billions and has been predicted to reach 9 billions by the middle of the 21st Century. Recent annual rate of population increase is nearly 3% in Asian, African and South American countries. In those countries, the environmental pollution and the shortages of food, feed, phytomass (plant biomass) and natural resources including fossil fuels and usable fresh water will become more and more serious on a larger scale in the forthcoming decades.

The difficulty with solving these global issues on food, energy, phytomass and environmental pollution is that we need to solve these issues concurrently based upon one common and innovative concept and methodology created from broad and long-term viewpoints, and to develop a new industry, which is strongly related to agriculture, horticulture, forestry and aquaculture and also to other manufacturing industries, but is not the same as any of those industries (Kozai et al., 2000 a,b).

The reason why we need to solve those issues concurrently is that solving only one issue separately from the other two often makes the situations of the other two even worse. For example, the worldwide spread of 'advanced' agricultural technology for increasing 'crop yield' may often make the environmental pollution worse, increase the atmospheric CO_2 concentration, and cause shortages of fossil fuels and other natural resources. This is because the modern agricultural technology is heavily dependent upon the oil-derived products such as chemical fertilizers, chemical pesticides, plastics, and fuels for machines.

Increase in phytomass in Asian, African and South American countries is also essential to stabilize their climates and to conserve their ecosystems or environments, while the forest area in tropical countries has recently been decreasing at an annual rate of 0.7% with a yearly net decrease in area of about 13 million hectares for years 1990 to 1995. For re-afforestation of this area, 25-40 billions of transplants (2,000 - 3,000 transplants per hectare) are required annually. In addition, the decrease in phytomass due to desertification in arid regions is considerable (World desertification area is 5-8 million hectares every year). Such local and global decreases of the vegetation areas, and consequently the decrease in phytomass, are

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possible factors causing recent meteorological changes on different geographical scales.

In order to solve these global issues in the 21st Century, we are requested to develop a concept, a methodology and an industry to produce billions of plants every year not only for food, feed and environmental conservation, but also for alternative raw materials to produce bio-energy, bio-degradable plastics and many other industrial products. By using plant-derived products, we can minimize the environmental pollution and the use of fossil fuels and atomic power. It has been predicted that, in the forthcoming decades, demands for transplants for use in afforestation and re-afforestation will rise sharply in the pulp, paper, timber, energy, plantation, horticulture and furniture industries, and in the desert rehabilitation for environment conservation (Kurata and Kozai, 1992; Aitken-Christie et al., 1995). Use of phytomass in those industries is essential to reduce the consumption of fossil fuels and to lower the atmospheric CO_2 concentration, and to stabilize local and global climates. A large number of high quality transplants, woody and herbaceous horticultural plants, are also needed every year for people living in cities to improve their quality of life or green amenities. The same is true for medicinal plants.

Quality of transplants is determined by their genetic, physiological and morphological characteristics. High quality transplants with superior physiological and morphological characteristics can be produced only under carefully controlled environments. “Photoautotrophic micropropagation systems” and “closed transplant production systems”, discussed in this book, are expected to be useful concepts and methods to produce a large number of high quality transplants at a low cost with use of minimum resources. These ideas were originated from our research backgrounds as environmental control engineers and environmental physiologists specializing in greenhouse, plant growth chamber and plant factory.

Micropropagation is one of the plant tissue culture technologies for producing a large number of genetically superior and pathogen-free transplants in a limited time and space. However, the widespread use of micropropagated transplants is still restricted because of its high production costs, mostly attributed to its low growth rate and a significant loss of plants *in vitro* by microbial contamination, poor rooting, low percent survival at the *ex vitro* acclimatization stage and high labor costs (Kozai, 1991).

Most of the factors bringing about the high production costs are directly or indirectly related to the *in vitro* environment, which is characterized by high relative humidity, low light intensity, low CO_2 concentration during the photoperiod, high ethylene concentration, restricted air movement, sugar-containing culture medium with low air porosity, etc. 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 plants *in vitro*, resulting in low growth rate and low percent survival *ex vitro*. In order to overcome these problems, we developed a photoautotrophic micropropagation system.

1. INTRODUCTION

Photoautotrophic micropropagation is a pathogen-free micropropagation method using sugar-free medium, in which the growth of cultures or accumulation of carbohydrates in cultures is dependent upon the photosynthesis and inorganic nutrient uptake of cultures. Thus, it can also be called photosynthetic, inorganic or sugar-free medium micropropagation (Kozai, 1991; Aitken-Christie et al., 1995). With this method, we could shorten the culture period by about 30%, solve the physiological disorders such as stomatal malfunction and hyperhydration, improve percent survival up to nearly 100%, save the labor cost by over 30% by using large culture vessels.

The concept of photoautotrophic micropropagation is derived from recent research that revealed relatively high photosynthetic ability of chlorophyllous cultures such as leafy explants, somatic embryos of cotyledonary stage and plantlets *in vitro*. Recent research has also revealed that the growth of such cultures and percent survival of plantlets *ex vitro* are considerably improved by increasing ventilation rate of the culture vessel, exhibiting the importance of gaseous (CO_2, H_2O, C_2H_4, etc.) composition of air in the culture vessel (Aitken-Christie et al., 1995). The chlorophyllous cultures can grow vigorously without sugar in the culture medium by improving the *in vitro* environment to promote photosynthesis, transpiration and inorganic nutrient uptake of the cultures. Commercialization of photoautotrophic micropropagation has recently been expanding especially in China since 1998 and partly in Vietnam and Thailand, but there is little interest in photoautotrophic micropropagation in European and American countries so far.

In this book, aspects of physical environments *in vitro* and their effects on plant growth and development are discussed with respect to the general characteristics of the *in vitro* environment, responses of plants to the *in vitro* environment, general responses of plants to the *ex vitro* environment, reason for the difficulty of the *ex vitro* acclimatization, environment control for the *ex vitro* acclimatization, *in vitro* acclimatization, and scaling-up of the photoautotrophic micropropagation system using a large culture vessel with forced ventilation.

Now, in general, the concept of 'closed production systems' is to develop a production system of any product with minimum use of resources and with minimum environmental pollution. By applying this general concept of closed production systems and by combining it with the concept of photoautotrophic micropropagation system into a multi-purpose transplant production system, we developed a 'closed transplant production system' with artificial lighting for seedlings and cutting propagation for producing billions of high quality transplants with minimum use of resources and with minimum environmental pollution. Research and development of the closed transplant production systems has been creating a new field of bioengineering and bioindustry.

A 'closed plant production system' or simply a 'closed system' has been commercialized for production of transplants in Japan since 2002, mainly based upon our research. The closed system is defined as a warehouse-like structure covered with opaque thermal insulators, in which ventilation is kept at a minimum, and artificial light is used as the sole light source for plant growth (Kozai, 1998; Kozai et al., 1998, 2004).

Advantages of the closed system over a greenhouse for producing high quality plants include: 1) rapid and efficient growth of plants mainly resulting from a considerably higher light utilization efficiency (2-3 times) of plants due to optimized growth conditions, 2) the significantly higher quality plants produced under uniformly controlled environments in the protected area, free from pest insects/pathogens and the disturbance of outside weather, 3) the higher (about 10 times) productivity per floor area per year, mainly due to the use of multi-layered shelves (e.g., 4-5 shelves) with the ratio of planting area to floor area of 1.2-1.5, a high planting density per tray area (1,500 plants/m^2), a high percentage of salable plants (>95%), 10-20% higher sales price due to their higher quality and uniformity of plants, and 30-50% shorter production period, 4) the drastically higher utilization efficiencies of water, CO_2, (about 15 times for water and 10 times for CO_2) and fertilizers mainly due to the minimized ventilation and recycling use of dehumidified water by air conditioners, resulting in little waste water to the outside, 5) virtually no requirement of heating cost even in the winter because of its thermally insulated structure 6) the lower labor cost (50% or less) due to the smaller floor area, the worker-friendly shelves, comfortable working environments, and 7) the easier control of plant developments such as stem elongation, flower bud initiation, bolting and root formation.

High electricity cost and initial investment are often mentioned as disadvantages of the closed system. However, the electricity cost for lighting and cooling per plant was found to be lower than 5% of total production cost in case of transplant production of leafy and fruit vegetables. And, since only about 10% of greenhouse floor area is required to produce the same amount of transplants, initial cost per annual transplant production in closed systems is lower than that in greenhouses. By using a closed system with a floor area of 150 m^2 with 60 shelves having 960 plug trays in total, about 10 million transplants can be produced annually. The closed system can also be applied for production of medicinal and ornamental plants and leafy vegetables, if their height is lower than about 30 cm.

This book describes rationales of photoautotrophic micropropagation and closed plant production systems and their commercial applications with related basic scientific explanations from aspects of environmental physics, physiology and engineering.

1. INTRODUCTION

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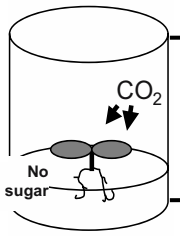
Chapter 2

T. KOZAI & C. KUBOTA



UNITS AND TERMINOLOGY USE FOR THE STUDIES OF PHOTOAUTOTROPHIC MICROPROPAGATION

Contents

Key words: *Cymbidium, in situ*, modelling, osmotic potential, photoautotrophic micropropagation, photosynthetic photon flux, simulation, water potential.

1. INTERNATIONAL SYSTEM OF UNITS (SI UNITS)

The International System of Units, abbreviated as SI units, has recently been widely adopted in plant science and related fields to express a physical quantity with a numerical value and a unit. On the other hand, in plant tissue culture, some non-SI units are still being used, which makes it difficult to compare numerical values of physical quantities with each other. In this chapter, recommended SI units and other acceptable units to be used for environmental studies of plant tissue culture especially photoautotrophic micropropagation are briefly given, along with comparable non-SI units. Subsequently, technical terms with respect to light and humidity, which are often used incorrectly, are defined briefly. More information on SI units and terminology used in plant physiology are given in Salisbury (1996) and Appendix I.

Table 1 gives recommended SI units for environmental factors in contrast with non SI units, which can be still found in the literature and should be discontinued. In Table 1, L and l, used for liter and C for degrees Celsius are not SI units. However, they are acceptable to use with SI units. 'PPM', which is often used in older literatures, is a misleading term. It defines a concentration by mass/volume as mg l^{-1}

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or mg L^{-1} in the case of plant growth regulators in culture medium, while it defines a concentration by volume/volume (volumetric parts per million) in the case of CO_2 and C_2H_4 gases.

In expressing concentrations of sugars, minerals and gelling agents such as agar, the unit of g l^{-1} (or g L^{-1}) and mol l^{-1} (or mol L^{-1}) are preferable. Percent (%) cannot be substituted for g l^{-1} or mol 1^{-1}, because the numerical value of percent can be calculated only when units of numerators and denominators are the same to each other. However, sugar and agar concentrations are often mistakenly expressed as 2% instead of 20 g 1^{-1}. When concentrations of sucrose and glucose are, respectively, 34.2 g 1^{-1} and 18.0 g l^{-1}, their molar concentrations are the same (0.1 mol 1^{-1}), because sucrose (molecular weight: 342) is a disaccharide and glucose (molecular weight: 180) is a monosaccharide. Preferred unit (g l^{-1} or mol l^{-1}) depends upon the purpose of work.

Table 1. Recommended SI units for environmental factors and non SI units, which are still used in the literature.

Environmental factor	*SI unit*	*Non SI unit*
PPF (photosynthetic photon flux)	mol m^{-2} s^{-1}	E m^{-2} s^{-1}
PAR (photosynthetically active radiation)	W m^{-2}	cal $cm^{-2}min^{-1}$, kcal m^{-2} h^{-1}
Illuminance	lux or lx	ft-c (foot candle)
Concentrations		
CO_2, O_2 and C_2H_4	mol mol ^{1}	% or PPM
Sugar and gelling agent	g l^{-1}, g L^{-1} mol L^{-1} or mol l^{-1}	%
Growth regulators	mg l^{-1} or mg L^{-1}	PPM
Ion, etc.	mol L^{-1}, mol kg^{-1}	M
Water vapor concentration	kg kg^{-1} (DA) or mol m^{-3}	
Water vapor partial pressure	Pa	bar, mm Hg, mm H_2O
Water and osmotic pressure	Pa	bar, mmHg, mm H_2O
Temperature	K	C

Note: L and l indicate liter and C indicates degrees Celsius. They are not official SI units but allowed to be used with the SI units.

Some relationships between SI units and non SI units, which may be useful for plant tissue culture studies are as follows (see also Table 1): 1 W m^{-2} (= 1 J m^{-2} s^{-1}) = 14.33 (= 0.2389 x 60) cal m^{-2} min^{-1} = 860 (= 14.3 x 60) cal m^{-2} h^{-1}, 1 lux = 0.093 ft-c, 1 μmol mol^{-1} = 1 PPM (volume/volume, common for gases), 1 g 1^{-1} = 0.1% (mass/volume, common for solutes in solvents), 1 mg l^{-1} = 1 PPM (mass/volume, common for liquids), 1 mol 1^{-1} = 1 M and 1 Pa = 1x10^{-5} bar (1 MPa = 10 bar). In older literatures where non SI units are used, the unit E m^{-2} s^{-1} is used where E stands

for Einstein, to express mol of photons, i.e., 1 mol m^{-2} s^{-1} (photon) = 1 E m^{-2} s^{-1} (photon).

In SI units, kg is a unit of mass, not a unit of weight. Weight is a measure of force produced by gravity. Thus, mass of an object does not change with latitude and altitude of location, but its weight does. However, weight is still widely used instead of mass (e.g., fresh weight in place of fresh mass).

Numerical superscript can be used only with a SI unit. However, g $plant^{-1}$ and g $vessel^{-1}$ are incorrect. These should be g/plant (or g per plant) and g/vessel (or g per vessel), respectively, because plant and vessel are not SI units.

In scientific expression, 'content' means an amount (often per plant, per leaf, or per vessel, etc.) which is expressed as the unit, g, L, or mol, etc. 'Concentration' means an amount (content) per volume, mass, etc. Thus, for example, the meaning of chlorophyll content is completely different from that of chlorophyll concentration. However, the former is often used to express the latter. Correct usage of content and concentration are recommended in this chapter.

The units expressing hour and day are 'h' and 'd', respectively. 'hr' and 'day' should not be used as units. Units related to light and humidity are perhaps the most confusing and require more extensive explanation in the following sections.

2. LIGHT ENVIRONMENT

The term 'light' is often defined as the electromagnetic radiation perceivable by human eyes (wavelength: 380-780 nm). On the other hand, in the field of physics, it refers to a wider range of electromagnetic radiation ranging from ultraviolet (100-380 nm) to infrared (1500 nm). In this chapter, the term 'light' is defined as the electromagnetic radiation (simply called 'radiation' hereafter) which causes photochemical reactions of plants: photosynthesis and photomorphogenesis (germination, root and bud initiation, stem elongation, etc.).

Photosynthetic reaction of plants occurs in the wavelength band ranging from about 400 to 700 nm with a peak wavelength for action spectrum of 660-680 nm. Photomorphogenesis occurs in the wavelength bands of 400-500 nm (blue) with a peak of 450-470 nm, 600-700 nm (red) with a peak of 660-670 nm and 700-800 nm (far-red) with a peak of 730-740 nm. The blue region is absorbed by photoreceptors of high energy reaction systems in leaves. The red and far-red regions are absorbed by phytochrome systems in leaves.

Radiation with wavelengths between 400 and 700 nm is called 'photosynthetically active radiation' and is often abbreviated as PAR. The unit of radiant flux for instance, PAR flux expressed as W m^{-2} or J $m^{-2}s^{-1}$ (W = J s^{-1}). Thus, radiant flux or irradiance by the unit W m^{-2} means a flow of radiant energy per square meter per second. In the above discussion, radiation is considered as a 'wave' characterized by its wavelength.

Light is also treated as small particles, called photons or quanta, each containing a discrete amount of energy. A photon is characterized by its wave number which is the number of wavelengths per centimeter (cm^{-1}), or by its frequency which is the

number of wavelengths that passes by a given point per second (s^{-1}). Thus, wave number (cm^{-1}) = 1 x 10^7/wavelength (nm). Energy in a photon with wave number of 2.50 x 10^4 cm^{-1} (blue: 400 nm) is 1.75 (= 700/400 = 2.50/1.43) times that in a photon with wave number of 1.43 x 10^4 cm^{-1} (red: 700 nm). Figure 1 gives an amount of energy contained in each photon as a function of wave number. It is often written in articles as PPF (wavelength: 400-700 nm). However, it should be written, strictly speaking, as PPF (wave number: 1.43 x 10^4 - 2.5 x 10^4 cm^{-1}). The term PAR should not be used with the unit of mol m^{-2} s^{-1}.

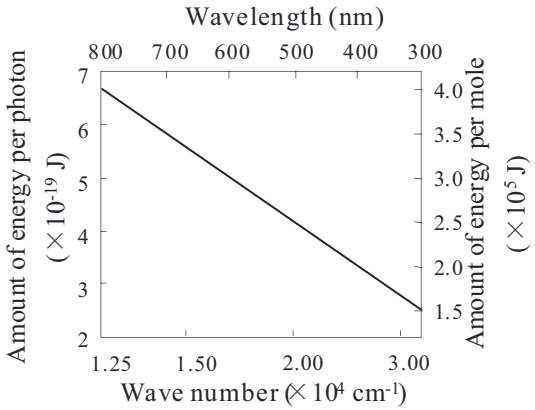

Figure 1. Amount of energy per photon and per mole as a function of wave number.

The unit of photon flux is μmol m^{-2} s^{-1} (one μmol is equal to 1x10^{-6} mol and one mol equals to 6.022 x 10^{23}, photons). Photosynthetic photon flux, often abbreviated as PPF, refers to a flow rate (per square meter per second) of photosynthetic photons with wave numbers ranging from 1.43 x 10^4 to 2.5 x 10^4 cm^{-1}. PPFD (photosynthetic photon flux density) is also frequently used to express the same in literature, but PPF is more preferable than PPFD. In plant tissue culture, maximum PPF would be 1,000 μmol m^{-2} s^{-1} or 1 mmol m^{-2} s^{-1} in most cases.

Photosynthesis and photomorphogenesis are photochemical or photon-based reactions, not thermal- or energy-based reactions. In other words, photosynthetic photons containing different amounts of energy per photon play the same role in photosynthetic systems. A photon with wave number of 1.43 x 10^4 cm^{-1} (red: 700 nm) is 1.74 (= 700/400 = 2.5/1.43) times more energy-efficient than a photon with wave number of 2.5 x 10^4 cm^{-1} (blue: 400 nm), because a red photon with less energy than a blue photon has the same function as a blue photon, in case of photosynthesis. The same applies for the photons affecting photomorphogenesis. This is why photon flux is preferred to radiant flux in the fields of plant physiology. On the contrary, radiant flux is preferred to photon flux when energy balance or energy budget of plants or a plant canopy is studied.

Illuminance with the unit of lux (= lumen m^{-2}) means a luminous flux on a

surface as evaluated by human vision. Figure 2 shows the standard spectral luminous (or photopic) efficiency curve showing the relative capacity of radiant energy for various wavelengths to produce visual sensation in human eyes. Human eyes are more sensitive to yellow-green region than to blue and red regions. This action spectrum for human vision is different from the action spectra for photosynthesis and photomorphogenesis.

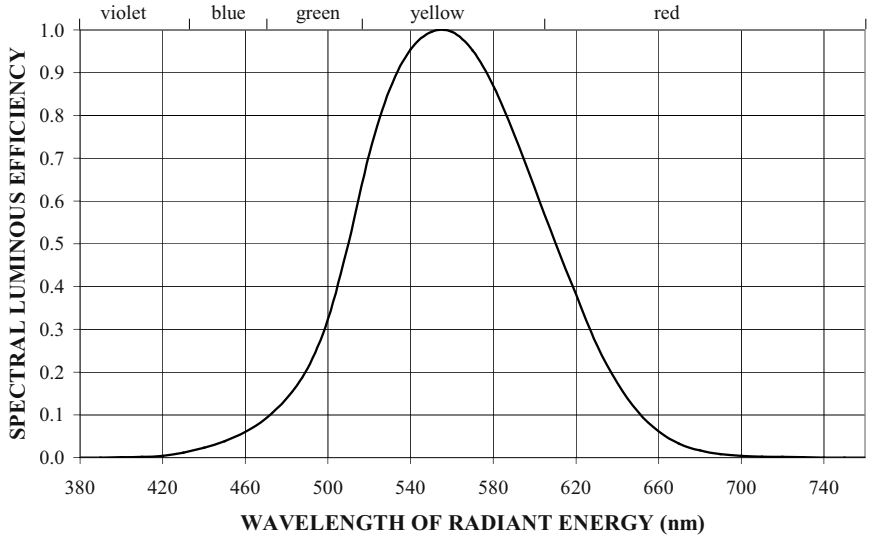

Figure 2. Spectral luminous efficiency of humans. Human eyes are the most sensitive to the wavelength of 555 nm (yellow). Spectral sensitivity of a lux meter for measuring the illuminance is just the same as shown here. Thus, a lux meter is not suitable for measuring PPF and PAR.

Conversion factors from radiant flux to photon and luminous fluxes depend upon the wavelength (or wave number), and *vice versa*. Thus, the value of a conversion factor can be determined only when the spectral distribution of a specific light source is given. Table 2 is a conversion table for some typical light sources.

3. GAS ENVIRONMENT

3.1. Water vapor

Relative humidity is a measure of water vapor concentration in air, expressed as a fraction of actual water vapor concentration in air to saturation water vapor

concentration at the same air temperature. Relative humidity multiplied by 100 is the percent relative humidity, but this, too, is often called relative humidity. In this chapter, relative humidity means percent relative humidity.

Table 2. Conversion factors for some typical light sources (Thimijan and Heins, 1983)

	μmol m^{-2} s^{-1} (W m^{-2})$^{-1}$	lx (μmol m^{-2} s^{-1})$^{-1}$	
Light source	400 – 700 nm	400 – 700 nm	400-850 nm
Sun and sky, daylight	4.57	54	36
Blue sky only	4.24	52	41
High pressure sodium	4.98	82	54
Metal halide	4.59	71	61
Warm-white fluorescent	4.67	76	74
Cool-white fluorescent	4.59	74	72
Incandescent	5.00	50	20

For more details, see also Thimijan and Heins (1983)

Saturated water vapor concentration increases exponentially with increasing air temperature (Figure 3). Actual water vapor concentration in air is called absolute humidity expressed by the unit of mol m^{-3} or kg kg^{-1} (DA), where DA stands for Dry Air. Thus, when the absolute humidity is x kg kg^{-1} (DA), the total air mass is (1+x) kg. Water vapor concentration is also expressed by its partial pressure using the unit of Pa.

Water vapor saturation deficit of air, or simply saturation deficit, is defined as the difference between saturated and actual water vapor concentrations in air at a given air temperature. This saturation deficit is a driving force of water vapor movement from liquid water surface as evaporation and transpiration. The potential evaporation and transpiration rates are proportional to the saturation deficit. At a given relative humidity, the saturation deficit and thus potential evaporation and transpiration rates are greater at higher air temperatures. Air humidity is an incorrect term. The term humidity is used only for air and therefore air humidity is a redundant term. More importantly, it is not clear if air humidity means relative humidity or absolute humidity.

3.2. Number of air exchanges of the vessel

The culture vessel is often fairly airtight to maintain aseptic conditions. When considering the changes in concentration of a gaseous component in the vessel, the magnitude of air infiltration or ventilation is conveniently expressed by the number of air exchanges of the vessel per hour. This number is defined as hourly ventilation rate divided by the air volume of vessel (volume of vessel minus volume of culture medium). In general, the difference in concentration of each gaseous component between inside and outside the vessel decreases with increasing the number of air exchanges of the vessel. The number of air exchanges is typically around 0.1 h^{-1} for test tubes covered with aluminum foil, 0.5 h^{-1} for normal Magenta vessels, and 2-5 h^{-1} for Magenta vessels with gas permeable film.

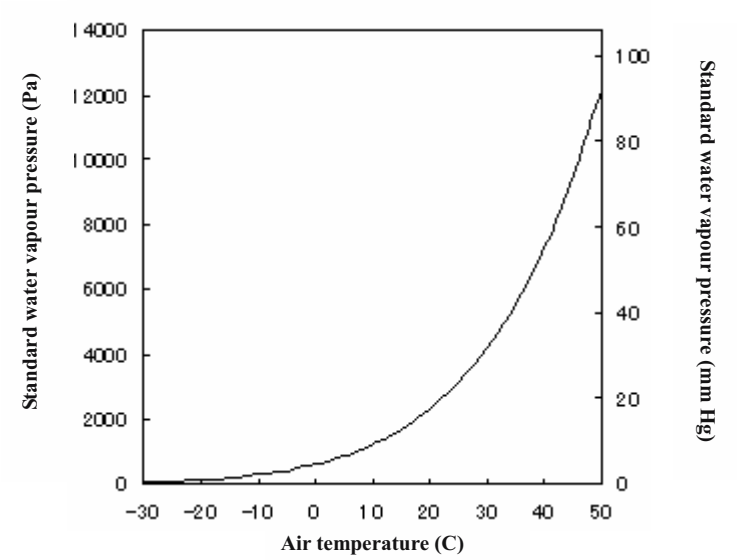

Figure 3. Saturation water vapor concentration or partial pressure as a function of air temperature.

4. WATER AND OSMOTIC POTENTIALS

Water flows from a point with higher water potential to a point with lower water potential regardless of its phase (liquid, gas or solid). Water potential difference, with a unit of pressure (Pa), is a driving force for water movement from culture medium to the air through a plant in the culture vessel. The same applies for the water in solution.

Water potentials of pure water and of the air saturated with water vapor (100% relative humidity) at 25 C are 0 Pa. Figure 4 shows the water potential of air at

T=273, 300 and 323 K as a function of relative humidity. The equation for calculation of water potential of air is given in Appendix I.

Water potentials of a solution and of the unsaturated air have a negative value. The water potential of a solution is also called osmotic potential. Water potential caused by the matrices with capillary suction such as gelling agent and porous supporting material is also called matric potential. The water potential of a medium can be expressed as the sum of the osmotic and the matric potentials.

Osmotic potential of a solution with different solutes is approximately proportional to the sum of molar concentrations of the solution. An equation to calculate the osmotic potential of an ideal solution is given in Appendix I. Table 3 gives osmotic potentials of some well-known culture medium formulations.

Matric potential is zero for liquid medium and has negative values for gelling agents and porous solid materials such as a porous soil block, although the water potential of gelling agents are nearly zero, compared with that of porous solid materials. Osmotic pressure of a solution has a positive value with an absolute value equal to that of osmotic potential. The same applies for the relationship between matric pressure and matric potential.

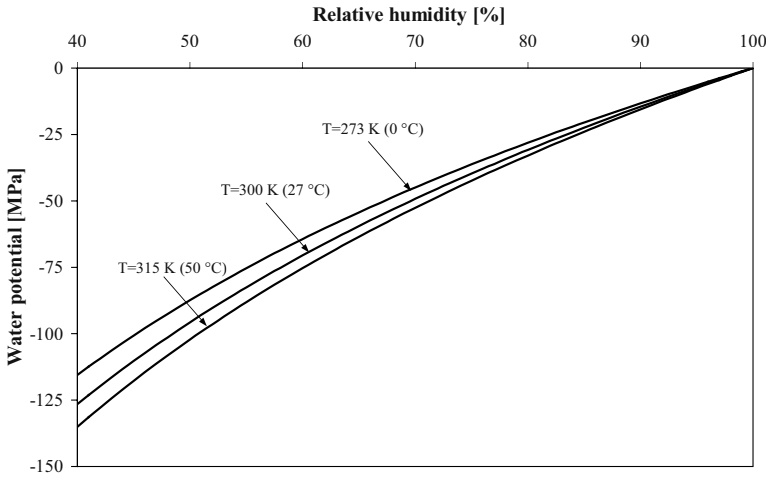

Figure 4. Water potential of air as a function of relative humidity at 273, 300 and 323 K. Water potential of air with 100% relative humidity is 0 Pa. Water potential decreases exponentially with decrease in relative humidity at a given air temperature.

5. STATE AND RATE VARIABLES

State variables characterize and quantify all conserved properties of a system such as amounts of sugar, minerals, water, etc. in the culture medium. Rate variables

characterize and quantify the rate of change over time of the state variables. It should be noted that the unit of a rate variable is amount (volume, mass, area, etc.) per unit time or per unit area/mass/volume per unit time. In this chapter, 'rate' is used only for rate variables such as respiration rate, net photosynthetic rate, growth rate, transpiration rate, multiplication rate and germination rate. Respiration rate of a plant is defined as the amount of CO_2 gas in mol or kg produced by the plant as a result of respiratory activity per unit time (or per unit time and per unit mass or leaf area). Similarly, germination rate is defined as the number of germinated seeds or somatic embryos per unit time.

Table 3. Water (or osmotic) potential values caused by the basic components of some widely used culture media in liquid form and nutrient solution for hydroponics. The values are converted for use at 25 C. The basic components consist of inorganic salts, vitamins and the other organic substances excluding sugar (Fujiwara and Kozai, 1995).

Basic component	Water potential (kPA)
B5 (1968)	-143
Heller (1953)	-89
Modified LP (1984)	-171
MS (1962)	-212
Nitsch and Nitsch's (1969)	-106
N6 (1975)	-177
R2 (1973)	-219
SH (1972)	-153
White (1943)	-37
Enshi-Shoho (1966)[1]	-76
Knop (1965)[1]	-49
Knudson (1946)[1]	-69

[1] Nutrient solution for hydroponic culture

The term germination rate is sometime used in the literature to express the percent of germinated seeds. In this case, the term percent germination is recommended instead of germination rate to distinguish its meanings from the germination rate with regard to unit of time. The same applies for survival rate, etc. Germination frequency is also used to mean percent germination in some cases.

However, in the actual sense, the term frequency means the number of occurrences or the ratio of the number of occurrences to the number of possible occurrences, and therefore percent germination is recommended if it is used with regard to the unit of percent. The same applies for conversion rate and conversion frequency of somatic embryos into regenerated plants.

The term multiplication rate means the number of shoots, stem cuttings, germinated somatic embryos, bulblets, etc., which are excised from a plant as usable explants, propagules or transplants obtained in a unit time (per week, month or day). The term multiplication ratio should be used if it means the ratio of the number of usable explants excised from a plant, without considering the multiplication period. Numerical values of multiplication ratios can be compared directly only when their multiplication periods are the same to each other.

6. MISCELLANEOUS

The term 'solid' or 'semi-solid' should not be used to explain a gelled medium using agar or other gelling agents. The term 'solid medium' should be used to specify supporting materials such as vermiculite, cellulose fibers and other porous supports, which are really 'solid'.

The term vitrification is sometime still used to describe organs and tissues having an abnormal morphological appearance and physiological function. However, more correct scientific definition of vitrification in plant tissue culture is the transition from liquid to solid, i.e., the formation of unorganized (non-crystallized) ice during low temperature storage of *in vitro* cultured cells, tissues and organs. It is therefore recommended that the term vitrification should no longer be used to define cultures having an abnormal morphological appearance and physiological function and should be substituted by the term 'hyperhydricity' (Degergh et al., 1992).

The term 'parameter' is often used to simply denote 'variable'. Fresh and dry mass, leaf area, etc. are often better called growth variables rather than growth parameters. Relative growth rate, leaf area index, etc. can be termed parameters when used as factors characterized by each of its value as of curves, surfaces or functions in plant growth equations.

Surplus numbers of significant digits are sometimes provided to present the data. For example, a value of 345.7 mg means a value greater than or equal to 345.65 and less than 345.75 with a precision of 0.05 mg (50 μg). Thus, a value with four significant digits should be given only when the least significant digit is correct and meaningful. For example, percent survival is 18%, not 18.5% when 12 plants out of 65 plants survived. A large number and a small number with a few significant digits are expressed as, for examples, '1.2×10^4' instead of 12,000 and '1.2×10^{-4}' instead of 0.00012.

In this chapter, SI units and technical terms frequently used in plant science and related fields are introduced.

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APPENDIX I: Calculation of osmotic potential and water potential of air

Osmotic potential of an ideal solution, W_s, is approximated by:

$$W_s = (RT/V_w)\ ln\ (x) \qquad (A.1)$$

where R is the Universal gas constant (8.314 J mol^{-1} K^{-1}), T is temperature (K), V_w is the partial molar volume of water (18.07 x 10^{-6} m^3 mol^{-1} at 298 K), and x is the molar fraction of the components. '*ln*' denotes natural logarithm.

Water potential of air, W_a, is approximated by the following equation:

$$W_a = (RT/V_w)\ ln(e/e_o) \qquad (A.2)$$

where e and e_o are actual and saturated water vapor partial pressure at air temperature of T. For symbols R, T, V_w and *ln*, refer to Equation (A.1). (Note a similarity in the equation form between (A.1) and (A.2)).

Water potential of sugar (sucrose, glucose, fructose, sorbitol and mannitol) in the culture medium, P_s (kPa), can be approximated by using the following practical equation (Fujiwara and Kozai, 1995).

$$P_s = -7.8\ C_d - 14.7\ C_m \qquad (A.3)$$

where C_d is the concentration of sucrose (g l^{-1}), C_m is the total concentration of monosacarides (g l^{-1}). Sucrose in the medium is hydrolyzed into glucose and fructose in the presence of invertase (an enzyme) produced by in-vitro plantlets. Mannitol and sorbitol are sometimes added to the culture medium simply to lower the water potential of the culture medium. P_s is – 234 kPa at the sucrose concentration of 30 g l^{-1}, and is – 441 kPa at the same concentration (30 g l^{-1}) of a monosaccaride. Water potential of MS solution containing 30 g l^{-1} of sucrose is – 444 kPa but the water potential of MS solution containing 30 g l^{-1} of glucose is – 662 kPa. It should be noted that the water potential of medium changes over time as the medium components are absorbed by plantlets and sucrose is hydrolyzed into glucose and fructose in the medium.

Chapter 3

T. KOZAI & C. KUBOTA

CONCEPTS, DEFINITIONS, VENTILATION METHODS, ADVANTAGES AND DISADVANTAGES

Contents

Key words: Aerial environment, air movement, CO_2 concentration, ethylene, forced ventilation, natural ventilation, relative humidity.

1. CONCEPTS AND DEFINITIONS

A tissue culture vessel is a system for growing small plants under aseptic conditions. Generally, the culture vessels are made of glass or clear plastics suitable for transmission of light into the vessel. In this sense, tissue culture vessels can be considered as miniature greenhouses. The difference between a tissue culture vessel and a greenhouse as a system for plant production is that greenhouses are equipped with systems for controlling the environment (temperature, radiation, photoperiod, etc.), while, conventionally, tissue culture vessels are not. Consequently, tissue culture vessels must be placed in a growth room with environmental control systems. Nevertheless, environmental conditions surrounding plantlets are not directly controlled in tissue culture. There has been much research done on environmental control in greenhouse crop production, while very few for *in vitro* environmental control. Greenhouse environmental control contributes to improved growth and quality of plants. Can *in vitro* environmental control be similarly beneficial for the plantlet growth and improve the quality of plantlets? In nature, plants fix atmospheric carbon in the presence of light. Can *in vitro* plantlets grow without sugar in the medium as a carbon source? Concepts of photoautotrophic micropropagation and methods of *in vitro* environmental control have been developed through researches initiated from such simple questions universally asked by anyone having worked in micropropagation.

Photoautotrophic micropropagation often refers to micropropagation with no

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 19-30.

sugar added to the medium. Narrowly defined, photoautotrophy is a nutritional type where living organisms grow without any additional exogenous organic components as nutrients. When defined in this narrow sense, media in photoautotrophic micropropagation should exclude all organic components. As in hydroponics, media for photoautotrophic culture consist exclusively of inorganic components. Vitamins, growth regulators, and gelling agents should not be added to the medium in photoautotrophic micropropagation. Instead of gelling agents, porous substances like vermiculite should be employed as supporting materials in photoautotrophic micropropagation. As will be discussed in later chapters, use of such supporting materials along with liquid nutrient solution under controlled environment has beneficial effects on the growth and development of *in vitro* plantlets.

Ideally, photoautotrophic micropropagation should be segregated from sugar-free micropropagation. However, in this chapter, while we will define photoautotrophy as the plant nutritional type where only endogenous carbohydrate is used as the energy source, for all practical purposes photoautotrophic micropropagation refers to micropropagation with no sugar added to the medium. Sugars and other carbohydrates may be significant components of agar and other gelling agents, but perhaps it is reasonable not to consider it as an exogenous carbohydrate source in the practical definition of photoautotrophic micropropagation.

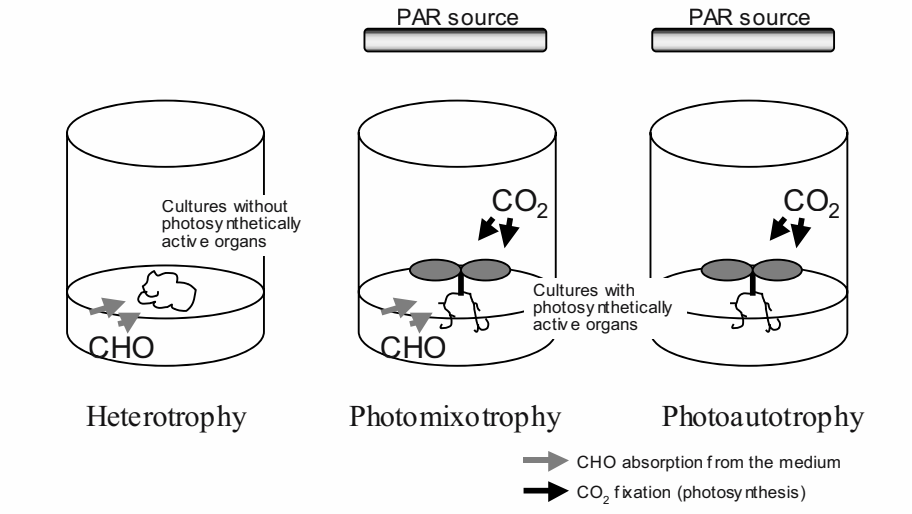

Figure 1. Classified nutritional types of in vitro cultures.

There are often misconceptions or misusages of the terminology for the nutritional types of *in vitro* cultures. Classified nutritional types of *in vitro* cultures are shown in Figure 1. Any chlorophyllous cells, tissues, organs, or plantlets

(chlorophyllous cultures) growing under conventional conditions using sugar-containing medium grow photomixotrophically. Photomixotrophy is the nutritional type where living organisms use not only endogenous but exogenous carbohydrates as an energy source. Thus regardless of the degree of dependence on sugar in the medium, chlorophyllous cultures growing on media containing sugar should be considered as photomixotrophic. Heterotrophy is the nutritional type where exogenous (medium) carbohydrate is the sole source of energy. Any cultures without chlorophyll development are heterotrophic and grow heterotrophically.

For successful photoautotrophic micropropagation, understanding *in vitro* environment and basics of environmental control is critical. For plants growing photoautotrophically, promotion of photosynthesis is the primary way to enhance the growth rate of the plantlets. To promote *in vitro* photosynthesis, it is necessary to know the status of environmental conditions (for example, levels of PPF and CO_2 concentration) in the vessels and how to maintain them in optimum ranges for maximizing net photosynthetic rates of the plantlets. Lack of understanding of the *in vitro* environment or the interaction between plantlets and the *in vitro* and *ex vitro* environments, makes it more difficult to improve the micropropagation system by applying the photoautotrophic micropropagation method. Basics of *in vitro* environmental control and characteristics of *in vitro* environment are summarized in the later sections.

Successful photoautotrophic micropropagation also requires knowledge of when (or at which stage) cultures should transit from photomixotrophic into photoautotrophic status. Developmental and operational stages for conventional micropropagation are generally classified into four or five stages. For photoautotrophic micropropagation, the number of classified stages may be less than in conventional photomixotrophic micropropagation, since multiplication and rooting stages are often combined as one stage in photoautotrophic micropropagation by reproducing photosynthetically active, leafy nodal cuttings to be used as explants. Therefore, theoretically, only the introduction/initiation stage of the culture (Stage I) must be under heterotrophic/photomixotrophic conditions where virus (or pathogen) free cultures are established by culturing meristematic tissues. Once the chlorophyllous organs, able to conduct photosynthesis, are developed, the cultures are ready to move on to photoautotrophic micropropagation conditions. The acclimatization stage is often eliminated when plantlets are grown under optimal photoautotrophic conditions. Thus, a photoautotrophic micropropagation system could exclusively consists of two stages, initiation (Stage I) and multiplication/rooting (Stage II), while the conventional, photomixotrophic micropropagation requires four stages, initiation (Stage I) , multiplication (Stage II), rooting/preparation (Stage III), and acclimatization (Stage IV) (Figure 2).

2. TYPES OF NATURAL AND FORCED VENTILATION METHODS

Natural ventilation is a type of gas exchange of conventionally used vessels. Unless the vessel is specially designed to achieve a complete seal against air exchange, almost in all of the vessels used in conventional micropropagation some air

exchange occur by natural ventilation through the gaps of the contact surfaces of vessels, lids, and sealing tapes. The driving force of the natural ventilation is the difference in air pressure between inside and outside of the vessel, caused by the difference in air temperature (density) inside and outside of the vessel and/or the velocity and current pattern of the air surrounding the vessel. Therefore, the shape of the vessel, orientation of the lids and vents, and air current environment around the vessels will affect the number of air exchanges of naturally ventilated vessels. Air current speed around a vessel was experimentally confirmed to enhance air exchange rate of the vessel (Ibaraki et al., 1992). When the air, inside and outside of a vessel is stagnant, the difference in air pressure between inside and outside of the vessel is zero. Under such a condition, driving force of gas diffusion is a partial gas pressure difference.

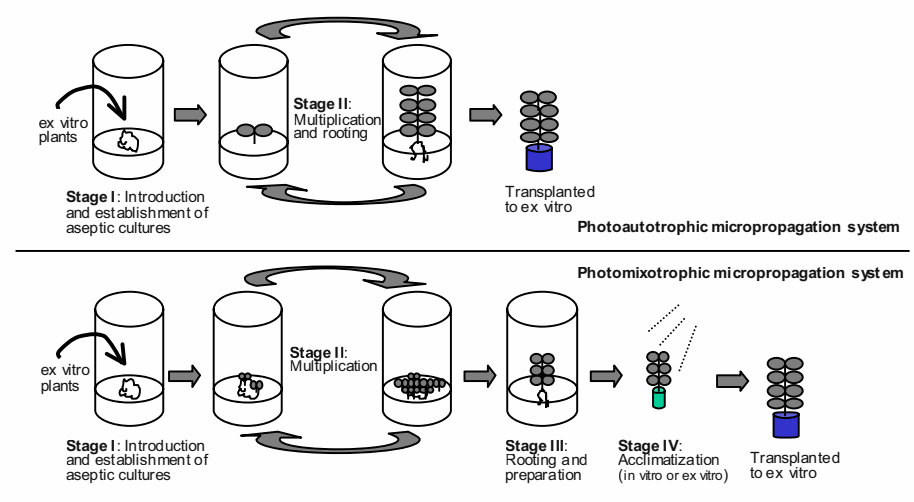

Figure 2. Photoautotrophic and photomixotrophic (conventional) micropropagation systems.

Enhanced ventilation of a vessel is desired, when attempting to improve the aerial microenvironment inside the vessel. One simple way to enhance the natural ventilation of a vessel is to use lids having gas permeable filters, or to use vessels having improved ventilation properties. There are numerous commercially available filters and vessel systems with high ventilation characteristics. A summary of the vessels with relatively high natural ventilation is given in Table 1 and Figure 3.

Optimization of the vessel aerial environment for maximizing photoautotrophic growth of plantlets requires that the ventilation rate should be adjustable according to the magnitude of net photosynthetic rates of the plantlets inside the vessel. A forced ventilation system is therefore more suitable than a natural ventilation system for adjusting ventilation rates by manipulating air flow rates through the vessel, and

therefore has been used as an optimized controlling system for *in vitro* plantlets (Fujiwara et al., 1988; Walker et al., 1989; Kubota and Kozai, 1992; Nakayama et al., 1991; Zobayed et al., 1999b; Erturk and Walker, 2000a; Heo et al., 2001).

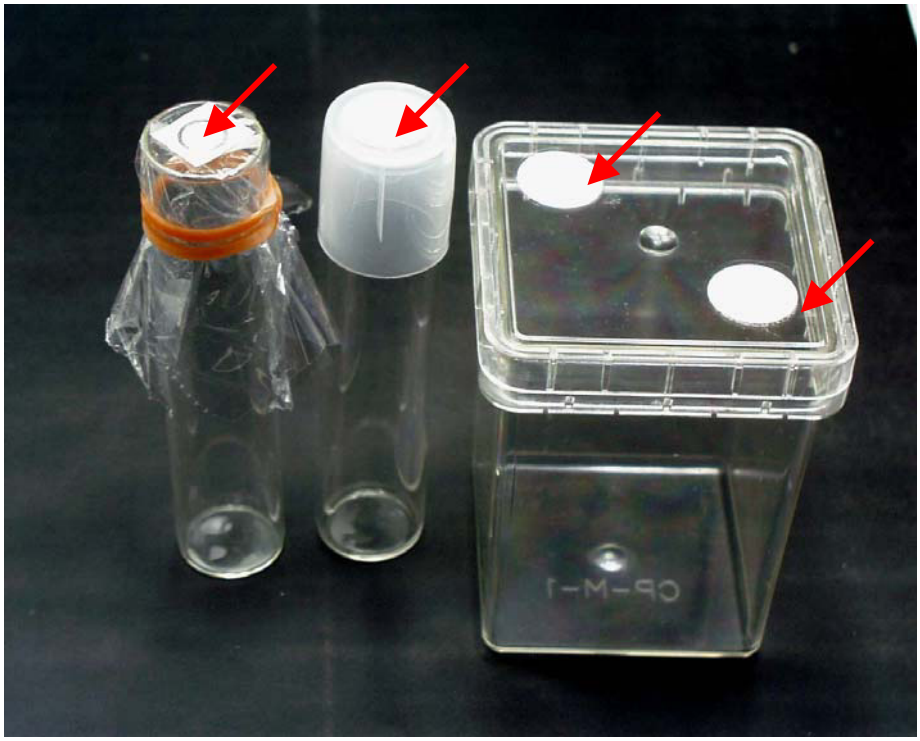

Figure 3. Varieties of vessel lids with enhanced ventilation; arrows indicate the filters for ventilation.

A typical ventilation system is a combination of tubings, filters, and an air pump. Figure 4 shows the system developed by Erturk and Walker (2000a) and highlights that sugarcane shoots were grown successfully using this system. As photoautotrophic micropropagation systems become more advanced, larger vessels using a forced ventilation apparatus have been developed. One of the advantages of forced ventilation is better control of the aerial environment inside the large vessels. However, in a large vessel with forced ventilation system, CO_2 concentration in the vessel is highest at the air inlet and is lowest at the air outlet if there is only one inlet and one outlet. Consequently, large horizontal gradients in CO_2 concentration between the inlet and outlet is created. Results obtained in early experiments (Kubota and Kozai, 1992; Heo and Kozai, 1999) made obvious the wide spatial variation and lack of culture uniformity due to the failure to create uniform environmental conditions inside large vessels using forced ventilation.

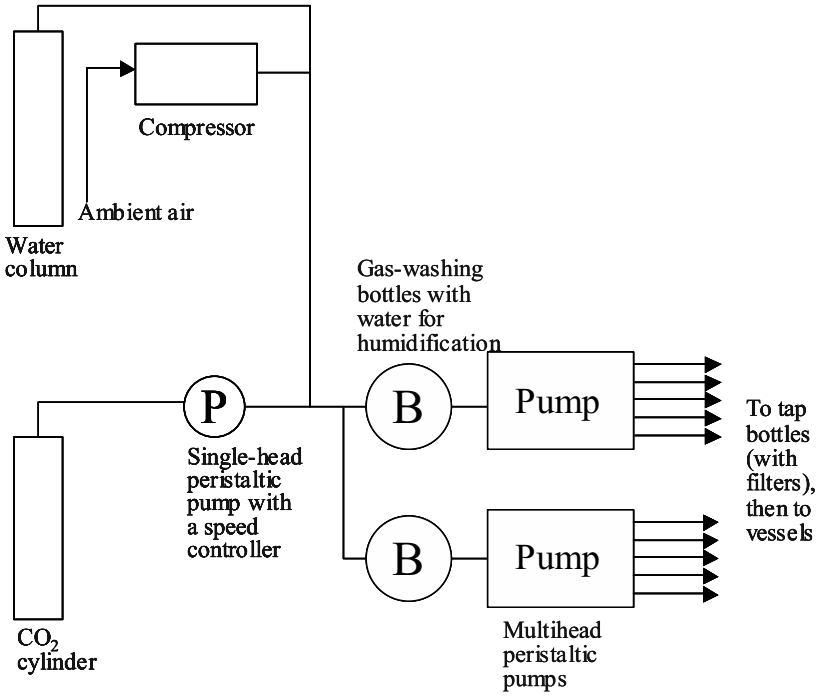

Figure 4. Basic components of the forced ventilation system developed by Erturk and Walker (2000a). CO_2 enriched air is pumped into each vessel (400 mL) through an air inlet and exited around the vessel lid.

Recent advances in the improvement of air distribution inside the large vessel have resulted in more uniform growth and quality of plantlets (Zobayed et al., 2000). In the bottom part of this large vessel is an air distribution chamber and the CO_2 enriched or non-enriched air is distributed through vertical pipes across the plug tray to obtain a uniform CO_2 distribution over plantlets in the vessel (Figure 5 and 6). Without such an air distribution system, the spatial distribution of CO_2 concentration tends to be uneven over the plantlets during the later growth stage of plantlets when the net photosynthetic rate per plantlet is high (Heo and Kozai, 1999).

3. ADVANTAGES AND DISADVANTAGES

Photoautotrophic micropropagation has many advantages with respect to improvement of plantlet physiology (biological aspect) and operation/management in the production process (engineering aspect).

Figure 5. A large culture vessel (volume: 20 L) with a forced ventilation system developed by Zobayed et al. (2000). The vessel was 610 mm long, 310 mm wide and 105 mm high.

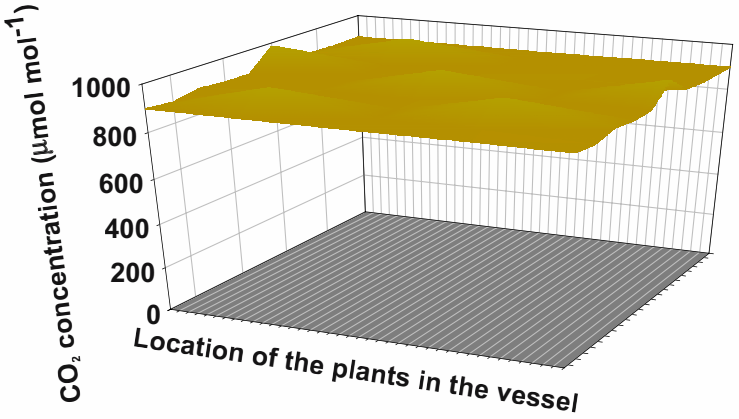

Figure 6. Horizontal distribution in the culture headspace of CO_2 concentration in the large culture vessel (Figure 5) containing 28-d old Eucalyptus plantlets (Zobayed et al., 2000). Note the uniform distribution of CO_2 concentration in the culture headspace.

Advantages of biological aspects include:

(1) Promotion of growth and photosynthesis
Net photosynthetic rate, and thus the growth rate of *in vitro* plantlets, is often enhanced when the plantlets are cultured photoautotrophically under a properly controlled environment, compared with those cultured conventionally. The positive effects of controlling environment are observed on growth of both photoautotrophic and photomixotrophic plantlets. The growth promotion is caused by the environmental conditions controlled to favour photosynthesis. Presence or absence of sugar in the medium affects the net photosynthetic rate of the plantlets. It has been observed that plantlets had higher net photosynthetic rates under photoautotrophic rather than under photomixotrophic conditions, perhaps due to enhanced RuBisCO activities as explained by Desjardins et al. (1995).

(2) High survival percentage / smooth transition to ex vitro environment
Photoautotrophically cultured plantlets are grown under such environmental conditions that enhance net photosynthetic and transpiration rates. High photosynthetic rate, normal anatomical structure and functional stomata (Zobayed et al., 1999a) contribute to enhanced survival percentages upon transfer to the *ex vitro* environment.

(3) Elimination of morphological and physiological disorders
A good example of the morphological and physiological disorders that we often observe in conventional micropropagation but not in photoautotrophic micropropagation is hyperhydricity (vitrification). Hyperhydricity is reportedly caused by physical and chemical factors including high relative humidity and ethylene concentration inside the vessel.

(4) Little loss of plantlets due to contamination
An advantage of the absence of sugar in the medium is the reduced chance of microbial contamination. Because of lower plantlet loss, production costs will be reduced by the reduction of cultures that the production manager may need to schedule as a compensation for contamination loss. The conceptual separation of microbial contamination and pathogen infection is possible in photoautotrophic micropropagation since, to some extent, microbes can be accepted as long as they do not overgrow the cultures or are not pathogen. The advantage of lower plantlet loss due to contamination emphasizes the advantages in engineering (operational and management related) aspects described below.

Advantages of engineering aspects include:

(1) Flexibility in the design of the vessel (larger vessels)
In photoautotrophic micropropagation, selection of vessel size and material is flexible, whereas it is limited due to risk of contamination in conventional micropropagation. Based on the concept of pathogen-free plant propagation, it is not necessary to grow plantlets under aseptic conditions as long as pathogens are

excluded. Thus, the surface sterilization method for vessels might be simpler (or less expensive) than that for the conventional micropropagation. Figure 7 shows a surface sterilization system using hot water at 60 C, designed to sterilize the polystyrene-foam (Styrofoam) panels or trays used in hydroponics. Such a system could be introduced for sterilizing culture vessels used for photoautotrophic micropropagation.

Figure 7. Surface sterilization system using hot water.

(2) Automation

Since vessel size is not a limitation in the photoautotrophic micropropagation system, automation for handling plantlets (cutting and transferring into new vessels) can be introduced. Furthermore, automatic plug transplanters are now commercially available, and as such, technologies developed for plug seedling production can be introduced with very simple modifications to fit the micropropagation-based plug transplant production system. However, successful use of automation in the *in vitro* and *ex vitro* stages of micropropagation demands uniformity of cultures and plantlets. Photoautotrophic micropropagation with environmental control will contribute to the production of uniform plantlets that can be handled by robots.

(3) Simplification of the micropropagation system

Due to the reduced probability of contamination in photoautotrophic systems, micropropagation systems can be simplified, pecially those employing larger

vessels. Additionally, the multiplication stage can be combined with the rooting stage by using leafy nodal cuttings as explants (Figure 2). Furthermore, the acclimatization stage, necessary in conventional micropropagation can be often eliminated or shortened.

The following items are often considered as disadvantages of photoautotrophic micropropagation:

(1) Relative complexity of techniques and knowledge required for controlling in vitro environment

Several industries have successfully introduced photoautotrophic micropropagation (i.e., Long, 1997) but the application is still limited. One of the reasons for this is that photoautotrophic micropropagation requires the use of technology and expertise to control the *in vitro* environment. Without fully understanding the relationship between the physiology of the *in vitro* plantlets, *in vitro* environment, *ex vitro* environment, and the physical or structural characteristics of the culture vessels, it will be almost impossible to optimize the photoautotrophic micropropagation system, maximizing the growth of the plantlets while maintaining high quality, yet minimizing inputs of energy and resources. The complexity of controlling the photoautotrophic micropropagation system may contribute to the failure of the establishment of successful photoautotrophic micropropagation. More discussion will be made on this in the later chapters.

(2) Expense for lighting, CO_2 enrichment, and cooling

Photoautotrophic micropropagation often requires increased levels of photosynthetic photon flux (PPF) and concentration of CO_2 available to *in vitro* plantlets. The former can be achieved by increasing the number of lamps installed per unit shelf area, although improvement of reflection to obtain greater efficiency and other alterations of lighting methods can significantly increase the PPF received by plantlets inside the vessel. By improving the efficiency of providing CO_2 enrichment, lighting and cooling, increases in costs for photoautotrophic micropropagation can be minimized. More will be discussed in a later chapter on how to reduce the electricity costs and resource input in photoautotrophic micropropagation.

(3) Limitation of application to multiplication systems using multiple buds/shoots

Multiple bud/shoot formation is sometimes expected in conventional Stage II (multiplication stage) micropropagation (such as herbaceous perennials) and it is induced primarily by adding growth regulators. High multiple bud/shoot induction has not been achieved yet in photoautotrophic micropropagation (without sugar but with growth regulators as in Erturk and Walker (2000b) as compared to that in photomixotrophic micropropagation and it is often pointed out as a limitation when applying photoautotrophic micropropagation to the multiplication stage using conventional multiple shoots as explants (propagules). Comparison of explants' quality when obtained by photoautotrophic micropropagation and conventional photomixotrophic micropropagation is necessary, since the conventional photomixotrophic micropropagation often produces many buds/shoots with low

quality (too small in size, hyperhydrated, etc.), while photoautotrophic micropropagation produces fewer but better quality buds/shoots. Further understanding of physiological mechanisms and environmental limitations of enhancing bud development is necessary for wider application of photoautotrophic micropropagation.

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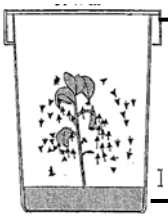
Chapter 4

T. KOZAI & C. KUBOTA



IN VITRO AERIAL ENVIRONMENTS AND THEIR EFFECTS ON GROWTH AND DEVELOPMENT OF PLANTS

Contents

Key words: Aerial environment, air movement, CO_2 concentration, ethylene, forced ventilation, natural ventilation, relative humidity.

1. INTRODUCTION

In this chapter, general characteristics of *in vitro* aerial environment and fundamental features of major environmental factors in photoautotrophic and conventional (photomixotrophic or heterotrophic) micropropagation are summarized and compared with the general characteristics of the greenhouse environment for plant propagation and transplant production. Effects of the number of air exchanges of the vessel on CO_2 concentration, C_2H_4 concentration and relative humidity in the

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vessel are also discussed. It is pointed out that the *in vitro* environment is considerably different from the greenhouse environment. Basic physical relationships governing the *in vitro* environment are introduced to facilitate understanding of the mechanism of *in vitro* environment and to indicate opportunities to improve the *in vitro* environment for photoautotrophic micropropagation. Basic ideas came from our observation that, in many cases, germinated seeds with cotyledons and stem cuttings with a leaf grow and develop faster in the controlled environment greenhouse than in the culture vessel in conventional micropropagation. Effects of environmental factors on the growth and development of plantlets *in vitro* are also discussed.

2. GENERAL CHARACTERISTICS

The environmental factors affecting the growth and development of cultures or plantlets *in vitro* are divided into aerial and root zone environmental factors (Figure 1). Most of the *in vitro* environmental factors are mutually influenced by, and also interact with, the plantlets *in vitro*. Moreover, the culture room environment and the physical characteristics of culture vessels affect the *in vitro* environment considerably (Figure 2, Kozai et al., 1995a).

Unique features of the *in vitro* environment, in contrast to those of the greenhouse environment, are the presence of sugar in the medium in conventional micropropagation and the absence or low density of microorganisms in the culture vessel. Asepsis of culture vessels containing cultures and medium is required in conventional micropropagation for two reasons. One is to obtain pathogen free plantlets, and the other is to prevent the rapid growth of microorganisms, including non-pathogenic ones, in sugar-containing medium, which can damage or kill the cultures. Thus, culture vessels are kept airtight to prevent microorganisms from entering. This use of airtight vessels with small air volume typically characterizes the *in vitro* environment in conventional micropropagation.

General features of the *in vitro* environment in conventional micropropagation are shown in Table 1, compared with those of the greenhouse environment. Notable characteristics of the *in vitro* environment are low flow rates of mass or materials (sugar, minerals, CO_2, etc.) and energy (heat and radiation including light) mainly due to the airtight culture vessels and a relatively stable culture room environment. Table 2 shows the typical values of major environmental factors in the vessel for conventional micropropagation in comparison with those in a controlled environment greenhouse for plant propagation and transplant production.

Due to the small volumes of air and culture medium per plantlet *in vitro*, low exchange rates of gases and medium components in the airtight culture vessel result in considerable changes in concentrations of the gases and medium components. This relationship is generally expressed as follows:

$$\Delta C = \Delta A/V \quad (1)$$

$$P=\Delta C /C \quad (2)$$

where ΔC is the change in concentration per unit time of a component in the air or medium in a vessel, ΔA is the change in amount of the component per unit time, and V is the volume of air or medium in the vessel. Thus, when ΔA is large relative to V, ΔC is considerable. C is the concentration of the component before it changes. P is the change in concentration of the component, ΔC, relative to C. V is generally small (50-500 ml) in conventional micropropagation compared with that in the greenhouse (600-6000 m^3 or 10 x 20 x 3 m^3 - 40 x 50 x 3 m^3), so that P has a high value when C is relatively low compared to ΔC. Actual change in ΔC of the gaseous component is affected also by the exchange rate of the component across the vessel, as described below.

2.1. CO_2, C_2H_4 and O_2 concentration

2.1.1. CO_2 concentration in the culture vessel with natural ventilation

It has been found that the CO_2 concentration in a relatively airtight vessel containing chlorophyllous cultures (green-colored or chlorophyllous somatic embryos of cotyledonary stage, shoots, leafy stem cuttings, plantlets, etc.) often decreases sharply to lower than 100 μmol mol^{-1} within a few hours after the onset of the photoperiod (Figure 3). This CO_2 concentration during the photoperiod is 270-330 μmol mol^{-1} lower than atmospheric CO_2 concentration of 370-380 μmol mol^{-1}, and is as low as just above the CO_2 compensation point of plantlets *in vitro* (50-100 μmol mol^{-1}). It remains the same until the dark period begins, and increases over time during the dark period up to 5 to 10 mmol mol^{-1}. It was also found that CO_2 concentration in a petri dish type vessel containing coffee (*Coffea arabusta*) somatic embryos of cotyledonary stage significantly decreased with time after the start of photoperiod, and a rate of change of CO_2 concentration is affected by PPF (Afreen et al., 2002).

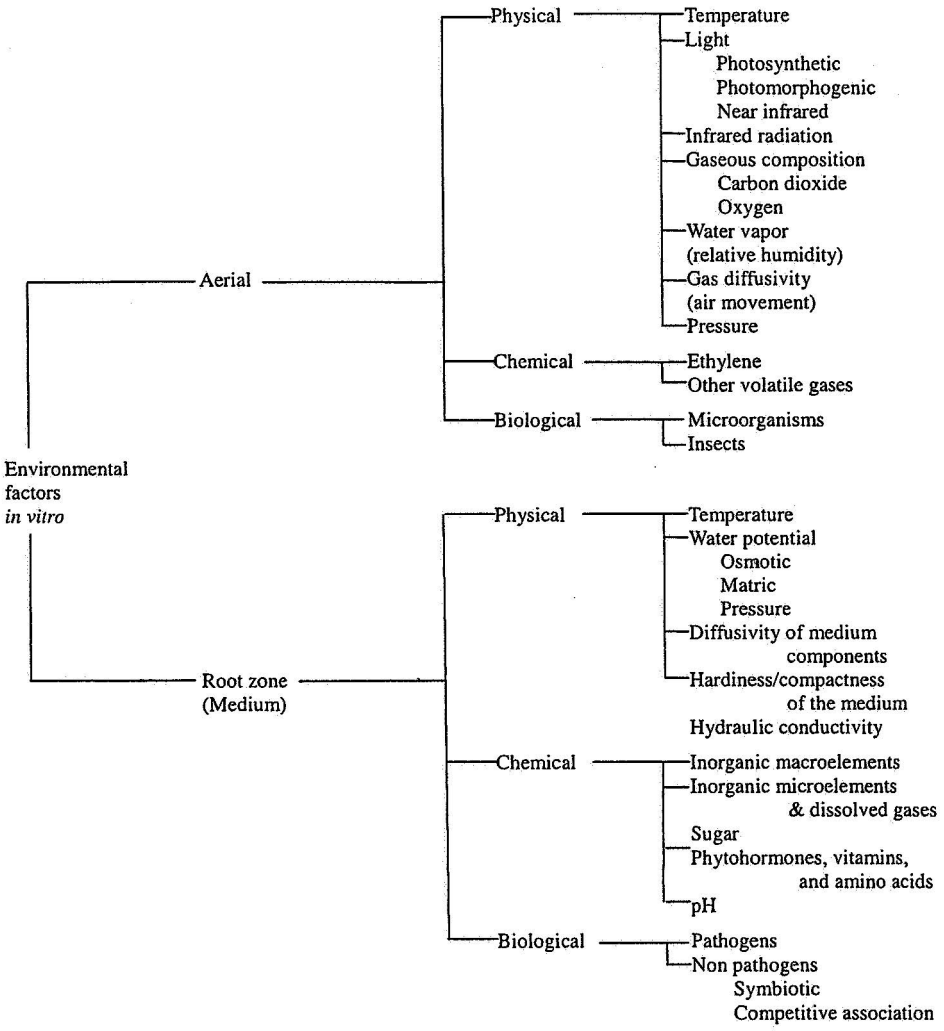

Figure 1. Classification of the in vitro environmental factors affecting growth and development of the cultures.

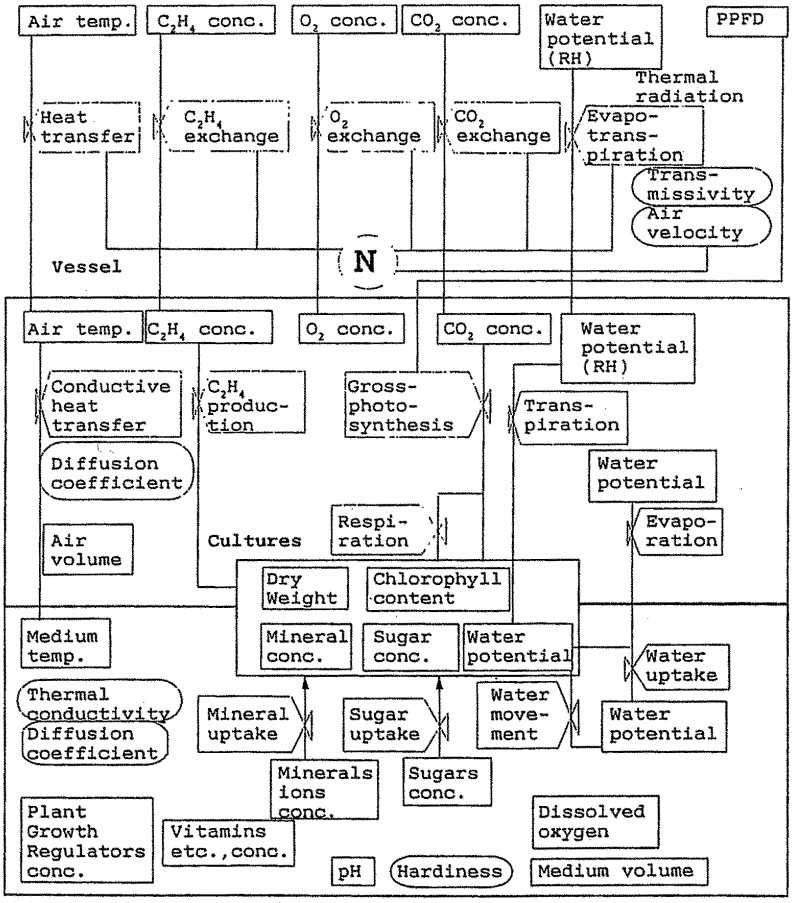

Figure 2. Schematic diagram showing the environmental factors inside and outside the culture vessel and their relationships. Lines indicate flows of energy and materials. Rectangle symbols denote state variables and valve symbols denote rate variables. Ellipse symbols denote coefficients and parameters. The letter N denotes the number of air exchanges of the vessel (Kozai et al., 1995b).

Table 1. General features of the in vitro environment in conventional micropropagation with respect to state and rate variables, compared with those of the ex vitro or greenhouse environment.

State variables	Rate variables
Aerial Environment	Flow of Material and Energy
1) High relative humidity (high water potential)	1) Low transpiration rate
2) Constant air temperature	2) Low air flow rate
3) Low CO_2 concentration in light	3) Low net thermal radiation flux
4) High CO_2 concentration in dark	4) Low net photosynthetic rate
5) High C_2H_4 concentration	5) Low photosynthetic photon flux
6) Small air volume per plant	6) High dark respiration rate
	7) Low exchange rate of mass between inside and outside the vessel
Root zone (Medium) Environment	
1) High sugar concentration	1) Low sugar uptake rate
2) High mineral ion concentration	2) Low mineral ion uptake rate
2) Low osmotic potential	3) Low water uptake rate
4) Low dissolved oxygen concentration	4) Low transport rates of medium components
5) High concentration of phenolic or other toxic substances	
6) Low density of microorganisms	
7) High concentrations of plant growth regulators	
8) Small medium volume per plant	

Definitions of state and rate variables are given in the text.

Table 2. Typical values of major environmental factors in the vessel for conventional micropropagation in comparison with those in controlled environment greenhouse for plant propagation and transplant production.

Environmental factors	In the vessel	In the greenhouse
PPF during photoperiod (μmol m^{-2} s^{-1})	10-100	100-1000
Air current speed during photoperiod (mm s^{-1})	1-20	10-1000
Temperature (C)	20-30	10-35
Relative humidity (%)	80-100	30-100
CO_2 concentration (μmol mol^{-1})	100-10000	250-400
C_2H_4 concentration (μmol mol^{-1})	0.0-1.0	<0.01

The decrease in CO_2 concentration in the vessel after the onset of the photoperiod shows that chlorophyllous cultures have photosynthetic ability. The resulting low CO_2 concentration during the photoperiod shows that photosynthetic activity of chlorophyllous cultures is restricted mainly by low CO_2 concentration. By definition, net photosynthetic rate of plantlets *in vitro* is zero at a CO_2 compensation point even when other environmental factors are favourable for photosynthesis of *in vitro* plantlets.

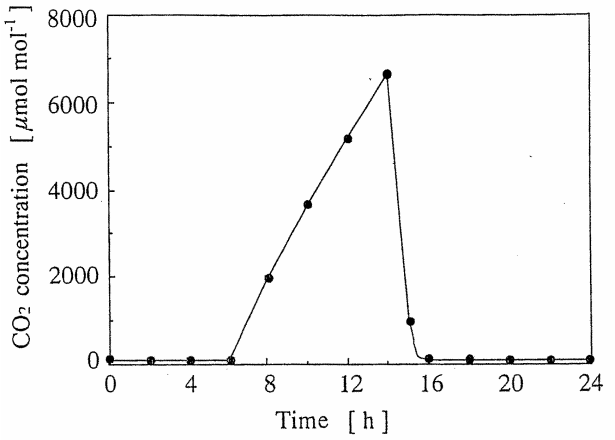

Figure 3. A diurnal change in CO_2 concentration in a culture vessel containing Ficus lyrata plantlets (Fujiwara et al., 1987). Dark period was from 6 to 14 h, and photoperiod was from 0 to 6 h and from 14 to 24 h. PPF at the culture level and air temperature in the culture room were 65 μmol m^{-2} s^{-1} and 25 C, respectively.

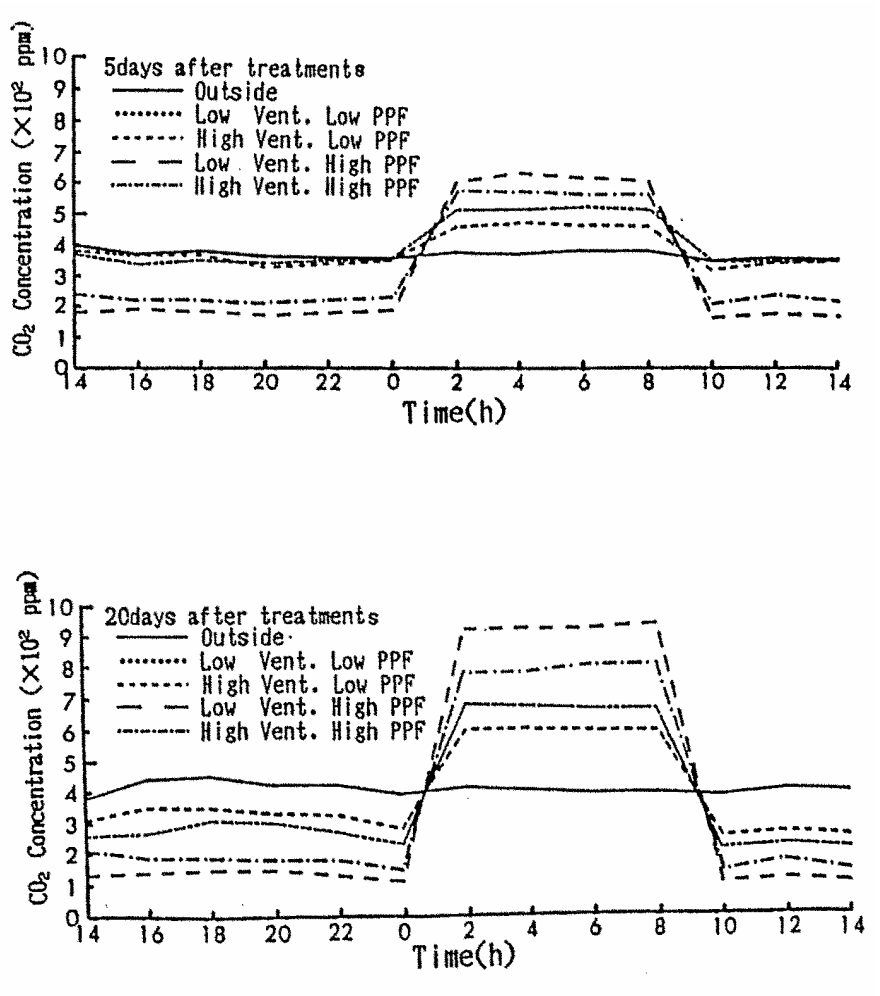

Figure 4. Diurnal changes in CO_2 concentration in the vessels containing strawberry plantlets cultured for 20 days as affected by the number of air exchanges of the vessel, N, and PPF on the empty culture shelf (Kozai and Sekimoto, 1988). Low N: N=1.5 h^{-1}, High N: N=2.7 h^{-1}, Low PPF: 34 μmol m^{-2} s^{-1}, High PPF: 133 μmol m^{-2} s^{-1}. Photoperiod: 8-24 h, Dark period: 0-8 h. Vessel air volume: 46 ml (test tube type vessel). Gas permeable film was attached on the lid for the high N treatment.

Decrease in CO_2 concentration in the vessel after the onset of photoperiod is more remarkable at lower number of air exchanges of the vessel and at higher net photosynthetic rates of cultures per vessel. Figure 4 shows the daily changes in CO_2 concentration in the vessel as affected by the number of air exchanges of the vessel and PPF. Steady-state CO_2 concentration in the vessel during the photoperiod is

lower at a lower number of air exchanges of the vessel, higher PPF and on day 20 than on day 5. This is because the net photosynthetic rate per vessel is higher at higher PPF and as a result of the greater leaf area of *in vitro* plantlets at later stages. High net photosynthetic rate of plantlets *in vitro* lowers the steady-state CO_2 concentration in the vessel and the low CO_2 concentration restricts the photosynthesis and growth of plantlets *in vitro*.

Figure 5 shows time courses of steady state CO_2 concentration during the photoperiod in vessels containing potato (*Solanum tuberosum* L.) plantlets cultured on sugar-free medium during a 15 d photoautotrophic culture period, as affected by the number of air exchanges of the vessels and the number of plantlets per vessel. In all the treatments, the steady state CO_2 concentration during the photoperiod decreased with the passage of days. It was lowest in LS treatment throughout the culture period because the number of air exchanges of the vessel was 0.75 h^{-1} in LS treatment, compared with 5.0 h^{-1} in LM and LL treatments. Steady state CO_2 concentration is lower in LM treatment than in LL treatment (although the numbers of air exchanges of the vessel are the same, 5.0 h^{-1}), because the number of plantlets per vessel is 2 in LL treatment and 4 in LM treatment. Net photosynthetic rate per vessel was greater in LM treatment than in LL treatment.

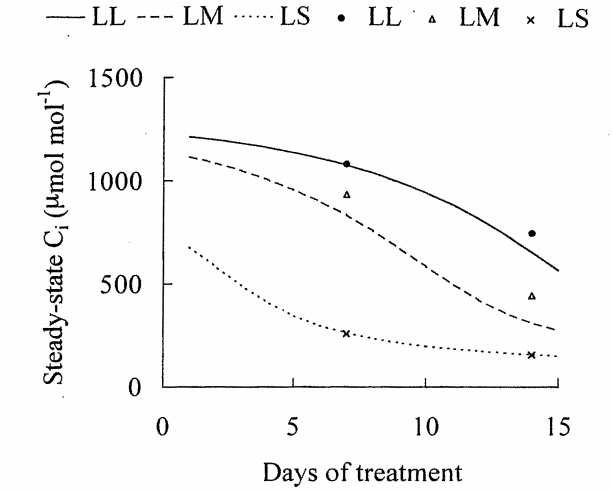

Figure 5. Time courses of steady state CO_2 concentration during the photoperiod in Magenta type vessels (air volume: 370 ml) culturing potato plantlets for the 15 d photoautotrophic culture period (Niu and Kozai, 1997). PPF: 105 μmol m^{-2} s^{-1} with a photoperiod of 16 h^{-1}. CO_2 concentration in the culture room: 1300 μmol mol^{-1}. Number of air exchanges: 0.75, 5.0 and 5.0 h^{-1} in LS, LM and LL treatments, respectively. Number of plantlets per vessel: 2, 4 and 2, respectively.

In order to keep the CO_2 concentration in the vessel during the photoperiod at a constant level over the culture period, either the CO_2 concentration in the culture

room or the number of air exchanges of the vessel or both must be increased with the passage of days. This is because, in general, net photosynthetic rate per vessel increases with increasing leaf area per vessel, especially at higher PPF. Mathematical relationships among the CO_2 concentrations inside and outside the vessel during the photoperiod, the number of air exchanges of the vessel, PPF and photosynthetic characteristics of plantlets *in vitro* are described in detail by Fujiwara and Kozai et al. (1995a) and Niu and Kozai (1997). Effects of CO_2 concentration on net photosynthetic rate and growth are described.

2.1.2. CO_2 concentration profile in a test tube type vessel

In a relatively small but long culture vessel like a test tube, the air movement due to free convection in the vessel is significantly restricted and thus a significant vertical variation of CO_2 concentration in the vessel is observed. Figure 6a shows the CO_2 concentration profiles during the photoperiod in a test tube with 25 mm in diameter and 120 mm in height, containing a sweetpotato (*Ipomoea batatas* (L.) Lam.) plantlet on sugar-containing MS agar medium at different PPF. The CO_2 concentration outside the test tube is 360 μmol mol^{-1}. At higher PPF, the vertical gradient of CO_2 concentration is steeper due to the higher net photosynthetic rate per vessel. CO_2 concentration just below the plantlet height was 180-190 μmol mol^{-1} lower than that just below the lid of test tube. Figure 6b shows the CO_2 concentration profiles during the photoperiod in the test tube at PPF of 100 μmol m^{-2} s^{-1} and different CO_2 concentrations outside the vessel. At higher CO_2 concentration outside the test tube, the vertical gradient of CO_2 concentration is steeper due to the higher net photosynthetic rate per vessel. These CO_2 concentration profiles show that restricted air movement resulting in limited diffusion of CO_2 gas in the vessel reduces the flow of CO_2 from the air into the plantlet, thereby reducing net photosynthetic rate. The vertical gradient of CO_2 concentration is not observed in a box type vessel like the Magenta vessel in which air movement due to free convection is more enhanced than in the test tube type vessel.

2.1.3. CO_2 concentration in the culture vessel with forced ventilation

Photoautotrophic micropropagation makes it possible to use a large vessel with an air volume of 10-100 L. On the other hand, it is difficult to achieve more than 3-5 h^{-1} air exchanges using gas permeable film for enhancing natural ventilation, because the number of air exchanges of the vessel per hour, N, is expressed by:

$$N=R/V \tag{3}$$

where R is hourly ventilation rate and V is the air volume of the vessel (note the similarity in form between Equations 1 and 3). In the case of a vessel with natural ventilation, R is almost proportional to the area of gas permeable film. As the V value increases, the area of gas permeable film must be increased at the same proportion to obtain the same value for N. This is difficult because V value increases

with 3rd power of the vessel size. Thus, we need to employ a forced ventilation system using an air pump to achieve a relatively high number of air exchanges of the vessel easily. Therefore, to practically achieve a relatively high number of air exchanges for the vessel, it is necessary to employ a forced ventilation system using an air pump.

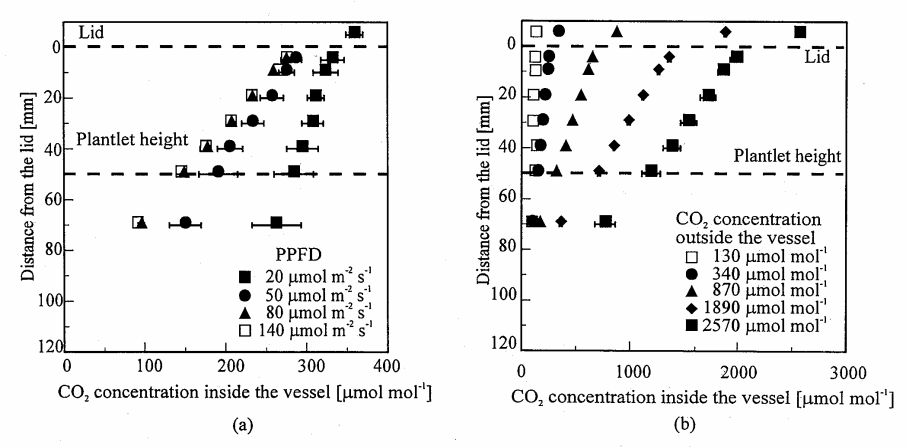

Figure 6. CO_2 concentration profiles in a test tube type vessel at (a) different PPFs and (b) different CO_2 concentrations outside the vessel (Ohyama and Kozai, 1997). The vessel contained a sweetpotato plantlet on sugar containing MS agar medium.

2.1.4. Ethylene (C_2H_4) concentration in the vessel with natural and forced ventilation

Ethylene (C_2H_4) gas, a phytohormone produced by plantlets, accumulates in an airtight vessel. In turn, C_2H_4 concentration affects the C_2H_4 production rate of the plantlet and the growth and development of plantlets *in vitro*. The number of air exchanges of the vessel also significantly affects C_2H_4 concentration in the vessel. Figure 7 shows the C_2H_4 concentration over time in the vessel with natural and forced ventilation, containing *Lagerstroemia thorellii* plantlets 30 days after transplanting. The vessels were uncapped and flushed with sterile air and then recapped at 0 h on day 30. C_2H_4 concentration in the vessel was then measured during the following 30 h. At the end of measurement (30 h), C_2H_4 concentrations in the vessels capped with a silicone rubber bung, cotton plug and polypropylene film were, respectively, about 1.20, 0.10, and 0.02 μmol mol^{-1}. On the other hand, it was undetectable (lower than 10 nmol mol^{-1}) for the vessel with forced ventilation. The numbers of air exchanges of the tested vessels were, respectively, 0.2, 1.1, 1.8 and 6.0 h^{-1}. C_2H_4 gas released from the vessels into the culture room does not raise the ambient C_2H_4 concentration because the air volume of culture room is more than

1000 times that of culture vessels. C_2H_4 gas is diluted with the culture room air resulting in a C_2H_4 concentration lower than one 0.01 μmol mol^{-1}.

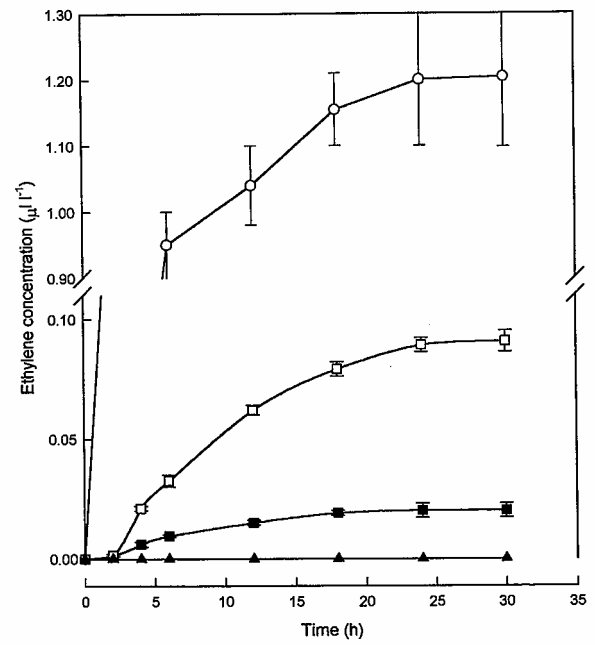

Figure 7. Time courses of C_2H_4 concentrations in the vessels containing Lagerstroemia speciosa plantlets during the photoperiod, as affected by the number of air exchanges of the vessel (Zobayed, 2000). Vessels were capped with silicone rubber bung (open circle), cotton bung (open square), polypropylene film (solid square) and forced ventilation (solid triangle). The numbers of air exchanges of those vessels were, respectively, 0.2, 1.1, 1.8 and 6.0 h^{-1}.

2.1.5. Oxygen concentration in the vessel

Oxygen concentration of atmospheric air is about 210,000 μmol mol^{-1} or 21% (volume/volume) which is approximately 60 times higher than the CO_2 concentration of atmospheric air. Decrease or increase in O_2 concentration in the vessel due to the respiratory and photosynthetic activities of plantlets is associated with the same degree of increase or decrease in CO_2 concentration in the vessel. A change in CO_2 concentration in the vessel from 100 μmol mol^{-1} (0.01% volume/volume) to 10 mmol mol^{-1} (1%) results in a change in O_2 concentration from 21 to 20%. This 1% decrease in O_2 concentration does not affect the respiratory and photosynthetic activities of plantlets. In an extreme case, CO_2 concentration in the vessel can reach 2-4% resulting in an O_2 concentration of 19-16%. Even so, this 2-

4% decrease in O_2 concentration does not affect the respiratory and photosynthetic activities of plantlets significantly.

Reducing O_2 concentration as low as 10% (volume/volume) reportedly increased the net photosynthetic rate and growth of photoautotrophically cultured *Chrysanthemum* plantlets (C_3 plants) (Tanaka et al., 1991). This is due to the reduced photorespiration rate by lowering O_2 concentration. *In vitro* O_2 concentration can be strategically controlled by forcedly ventilating the vessels with air of desired O_2 and CO_2 concentrations.

2.2. Relative humidity

Figure 8 shows the relative humidities and air temperatures in the vessels over time with and without gas permeable film, and with the absence or presence of potato plantlets *in vitro* 2 days and 24 days after transplanted on to the culture medium. Relative humidity of culture room air during the photoperiod is often relatively low (around 40%), because the room air is dehumidified (water vapor of the room air being condensed as liquid water) on a cooling panel of the air conditioner. It reaches 80-90% during the dark period, when the air conditioner is operated only intermittently.

On days 2 and 24, relative humidity in a vessel during the dark period is higher than 98% in all treatments. Inside a completely airtight vessel, the relative humidity during the dark period is, theoretically, a little lower than 100%. This is because the water potential of air should be the same as that of culture medium. The water potential of culture medium ranges between –300 kPa and –800 kPa, which is equivalent to relative humidity of 99.8-99.5%. Relative humidity in a vessel that is not completely airtight would be lower than 99.5% due to the exchange of air from inside the vessel with air outside the vessel where relative humidity is lower than inside. During the photoperiod, however, relative humidity in the vessel with gas permeable film and plantlets is around 80% on day 2 and 90% on day 24.

It is often believed that relative humidity is always nearly 100% in a conventional vessel. However, in Figure 8, the relative humidity during the photoperiod is around 90% even in a vessel without gas permeable film and with plantlets. As shown in Figure.8, air temperature is about 2 degrees Centigrade higher in the vessel than in the culture room because the light is absorbed by the vessel walls, lid, medium and plantlets, and thus heat is generated in the vessel. In this case, the vessel wall temperature is lower than the dew-point temperature of the inside air, and water is condensed on the inside surfaces of the vessel walls and lid. This condensation of water does not indicate that the relative humidity in the vessel is 100%. On the contrary, it indicates that water vapor in the air is dehumidified by the vessel walls and lid, and that the relative humidity is lower than 100%.

The transpiration rate of plantlets is proportional to the saturation deficit of water vapor and, at relative humidities higher than 80%, the saturation deficit is almost proportional to the difference between 100 and the actual relative humidity. Thus, the transpiration rate at relative humidity of 80% (100-80 = 20) is 10 times higher that those at relative humidity of 98% (100-98 = 2) at the same air temperature.

Effects of relative humidity on physiological and anatomical characteristics are described in a later chapter (Chapter 6).

Loss of water from the medium in the vessel with high number of air exchanges is generally significant and the medium tends to dry up when the culture period is longer than one month. This problem can be solved by two simple methods. One is to simply supply more volume of medium in the vessel. Another is to keep the relative humidity of culture room near 80% during the photoperiod. When the relative humidities of the room air is either 40% or 80%, and the relative humidity in the vessel is 90%, the difference in relative humidity between inside and outside the vessel are 50% and 10%, respectively. Consequently, the amount of water loss from the vessel in culture room air at 80% RH is approximately 20% of a vessel in culture room air at 40% RH. Relative humidity of 70-80% in the culture room can be achieved by using a humidifier and/or by raising the surface temperature of cooling panels of air conditioners to reduce condensation of water on it.

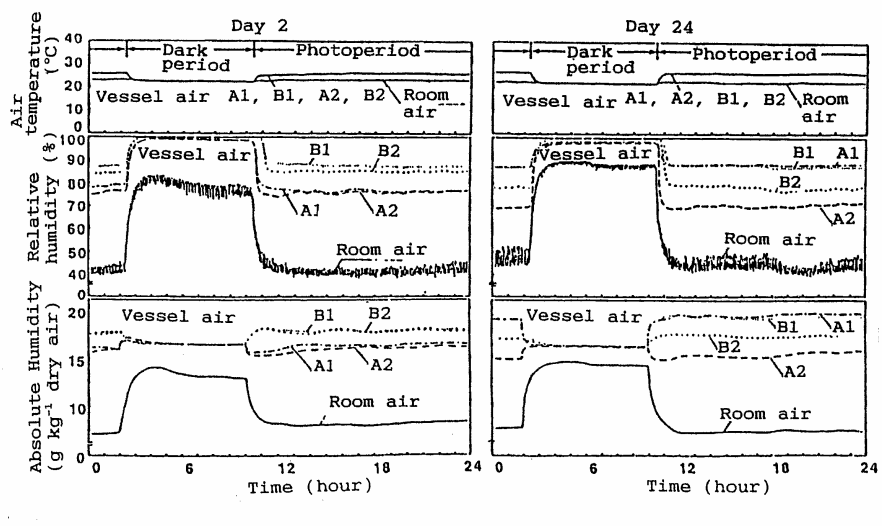

Figure 8. Daily changes of relative humidities, air temperatures and absolute humidities inside and outside the vessel with and without gas permeable film, and without and with in vitro potato plantlets 2 days and 24 days after transplanted in the vessel (Kozai et al., 1995b). A1: with filter and plantlets, A2: with filter and without plantlets, B1: without filter and with plantlets, B2: without filter and plantlets.

2.3. Light

Light environment needs to be considered with respect to photon flux, spectral distribution, lighting cycle (photoperiod/dark period), lighting direction (downward and sideward lighting), etc. Light environment for *in vitro* plantlets is affected by the optical characteristics of light source, vessels and their surroundings, and by the

geometrical relationships among light source, vessels and their surroundings. Effects of these light-related environmental factors on the growth and development of *in vitro* plantlets are discussed by Fujiwara and Kozai (1995b).

2.3.1. Increase in PPF by reflective sheet placed above the lamps

In micropropagation, straight-line fluorescent tubes, 120 cm in length and 25 or 32 mm in diameter (35-40 W), are used as the light source in most cases, which are the most cost-effective among many types of light sources. These straight-line fluorescent tubes emit light outward along the axis of each tube to all directions uniformly from each point of tube surface. Thus, 50% of light is emitted upward and the rest is emitted downward. Since the tubes are usually placed 30-40 cm above the culture shelf and the culture shelf is about 60 cm wide, about 50% of downward light or only about 25% of light emitted by the tube reaches the culture shelf directly (Figure 9). The rest of downward light goes to the outside of the culture shelf.

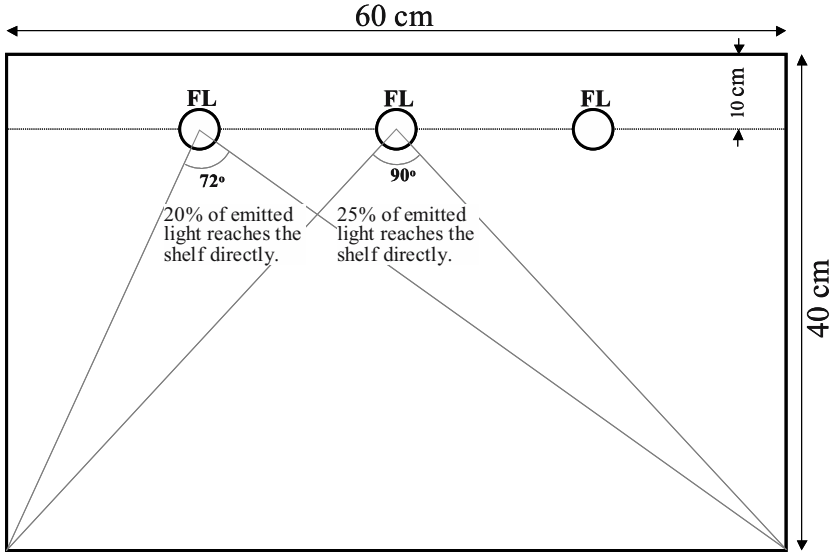

Figure 9. Schematic diagram of light emission from the lamp in a culture shelf. Less than 25% of light energy emitted from the fluorescent (FL) lamps reaches the culture shelf directly. Half of light energy emitted from the FL lamps goes upward. About 80% of upward light can be reflected downward at the inner surface above the lamps if the inner surface is white. About half of the reflected light energy reaches the culture shelf directly. About 25% of light energy that is emitted from the FL lamps and reaches neither the culture shelf nor the inner surface above the lamps directly can be partially reflected downward by the sides of the culture shelf and can reach the culture shelf if the sides of culture shelf are covered partially or totally with white material.

In order to increase the percentage of light received by the culture shelf, it is recommended to use a reflective sheet (white paper or aluminium foil sheet with reflectivity of 80-90%) above the lamps (below the upper culture shelf) to reflect the upward light downward. The PPF on the culture shelf is thereby increased by 40-50%. This is because 80% of the upward light (40% of emitted light = 50 x 80/100) is reflected back downward and 50% of the reflected light (20% of emitted light = 50 x 40/100) reaches the culture shelf. Thus, the total light received by the culture shelf is 45 (= 25 + 20) % of the emitted light. Overall, a 44% (= 100 x 20/45) increase in PPF on the culture shelf is achieved. The PPF on the culture shelf increases with decreasing distance between shelves and increasing reflectivity of reflective sheets. The use of a slightly inclined reflective sheet at the upper part of each shelf edge is also effective to reflect down the outgoing light to the culture shelf and, thus, to increase the PPF on the culture shelf (Figure 9).

Without the use of reflective sheets, the upward light is mostly absorbed by the bottom surface of opaque culture shelf above the fluorescent tubes. This light is converted into heat which raises the surface temperature of the culture shelf and thus the air temperature around the fluorescent tubes. In a case where a transparent glass sheet is used as shelf board, the upward light transmitted through the glass sheet above the lamps is absorbed by the culture medium on the upper shelf, resulting in a relatively high medium temperature. Therefore, the use of glass sheets should be avoided unless there is a definite reason for it.

2.3.2. Reduction in PPF due to vessels with closures

PPF values given in the literature are mostly measured on an empty shelf or at the top of a vessel placed on the shelf. Thus, the PPF at plantlet level in a culture vessel is significantly lower than the PPF at a top of vessel. Figure 10 shows the PPF measured in glass test tubes closed with different types of closures and placed upright in the stainless stand. Four hundred and fifty glass test tubes with and without closures were set on a shelf (90 cm by 120 cm) and PPF in each test tube was measured on its quarter section, using a small PPF sensor. The light source was one cool white fluorescent lamp (40 W) of 120 cm in length installed 33 cm above the shelf and above the line AD as drawn in Figure 10. The mean PPF in the test tubes without caps (38 μmol m^{-2} s^{-1}) is 64% of the PPF on the empty shelf (59 μmol m^{-2} s^{-1}). The PPF transmissivity of the polypropylene cap itself was 85%. The mean PPF in the test tubes with the polypropylene caps (33 μmol m^{-2} s^{-1}) is 2.4 times that of the test tubes with the aluminium foil caps (14 μmol m^{-2} s^{-1}), and is 3.8 times that of the test tubes with silicon foam rubber plug caps (9 μmol m^{-2} s^{-1}). The mean PPF in polycarbonate box type (Magenta type) vessels with polycarbonate formed lids was 38 μmol m^{-2} s^{-1} (64% of the PPF on the empty shelf: 59 μmol m^{-2} s^{-1}).

A large culture vessel with a lid having a high light transmissivity is essential to increase the PPF in the vessel with minimum number of fluorescent lamps and minimum electricity consumption.

As described above, PPF in the empty vessel with a transparent lid is about 65%. Consequently, 16% (= 25 x 65/100) of light emitted from the tube reaches the

culture medium surface of an empty vessel. Plantlets/explants *in vitro* absorb some percentage of the light transmitted into the vessel (probably less than 10% for explants, 40% and 80% for plantlets at the middle and end of multiplication stage, respectively). After all, only a small percentage of light energy emitted from the tubes is received by cultures in the vessels. The rest of the light energy is uselessly converted into heat energy and increases the cooling load of air conditioners and, thus, electricity consumption for lighting and cooling. Increasing the percentage of light energy received by the cultures is a key to reducing electricity consumption.

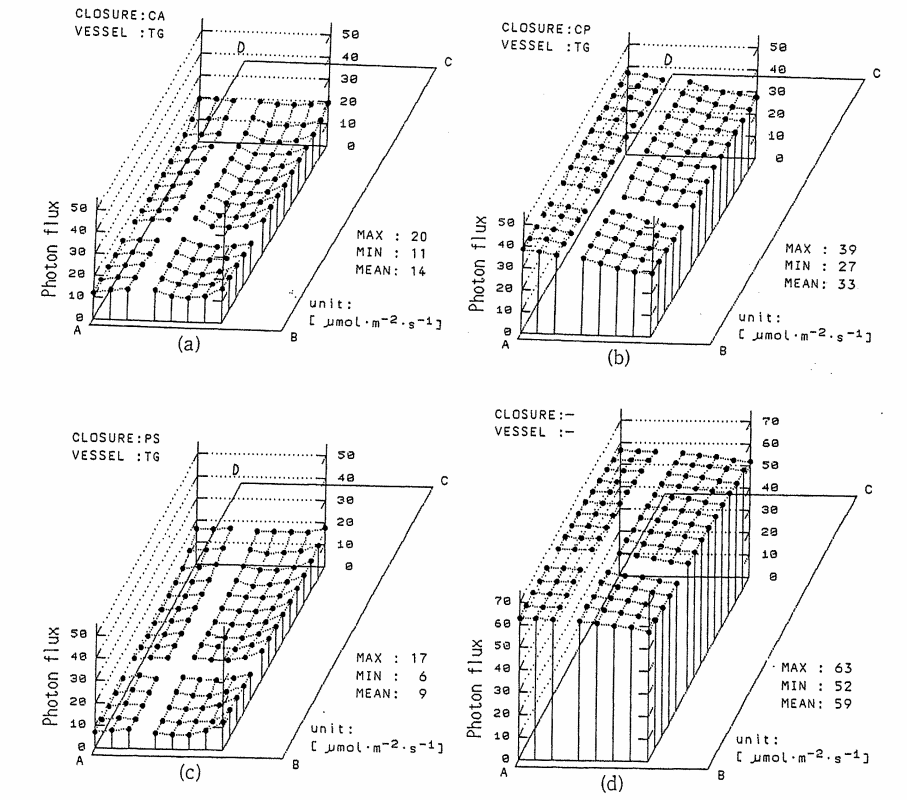

Figure 10. PPFs in the test tube type vessels as affected by types of vessel caps (Fujiwara et al., 1989). (a) aluminium foil caps, (b) polypropylene formed caps, (c) silicon rubber plugs, and (d) no caps. MAX, MIN and MEAN denote maximum, minimum and mean PPF, respectively.

2.4. Air movement

2.4.1. Air movement above the culture shelf

Warm air tends to move up due to its buoyancy. Thus, air temperature tends to be higher at the upper part of each culture shelf unit than at its lower part, and it tends

to be higher inside the shelf than outside. To avoid this uneven distribution of air temperature within the culture shelf and culture room, smooth air movement across the culture shelf needs to be enhanced. This enhancement can be realized by creating space gap at upper part of each shelf to let the warm air escape to the outside of the shelf or to the upper shelf. Fluorescent lamps are manufactured to give a maximum light output at a tube surface temperature of about 40 C. This tube surface temperature can be obtained when the air temperature is about 25 C and air current speed around the tube is around 5-10 cm s^{-1}.

2.4.2. Air movement in the vessel with natural ventilation

Air current speed in the culture vessel during the photoperiod is about 1-20 mm s^{-1} in most cases, which is low compared with that in the greenhouse (10-1000 mm s^{-1}) (Table 2). This low air current speed restricts the diffusion of CO_2, for photosynthesis, and of water vapor, for transpiration in the vessel. Air current speed in the vessel increases with the increase in PAR (or light intensity) at the top of the vessel (Figure 11) and/or with the increase in absorptance of transmitted radiation on the culture medium (Omura et al., 1995). This is because higher absorption of radiation at the medium surface raises the medium surface temperature and, thus, enhances free convection of air in the vessel. The addition of activated charcoal to the culture medium changes the medium color from white-translucent to black, increasing the air current speed in the vessel (Figure 11). Furthermore, air current speed increases when the plantlets are small, because smaller plantlets are less restrictive to air movement in the vessel. Of course, air movement is enhanced with an increased number of air exchanges of the vessel and the air current speed around the vessel. Shape of the vessel also affects the air current pattern in the vessel considerably. The smaller and thinner the vessel, the more restricted the air movement is in the vessel.

2.4.3. Air movement in large vessels with forced ventilation

Figure 12 shows the two dimensional patterns of air currents in a culture vessel with forced ventilation using air distribution pipes on the plug tray with sweetpotato plantlets 6 days after transplanting. Air moves up at the central part of the vessel and moves down along the sidewalls of the vessel. Air current over the plantlets ranged between 5 to 10 mm s^{-1}, which is approximately the same as the air current speed in the naturally ventilated vessel with plantlets. The forced ventilation rate was 13 ml s^{-1}, which corresponds to the number of forced air exchanges of the vessel being 3.9 h^{-1}. This number of air exchanges is difficult to obtain in the case of such a large culture vessel, if it is limited to natural ventilation using gas permeable films. Air current speed needs to be increased as the plantlets grow to reduce uneven spatial distribution of CO_2 concentration in the vessel.

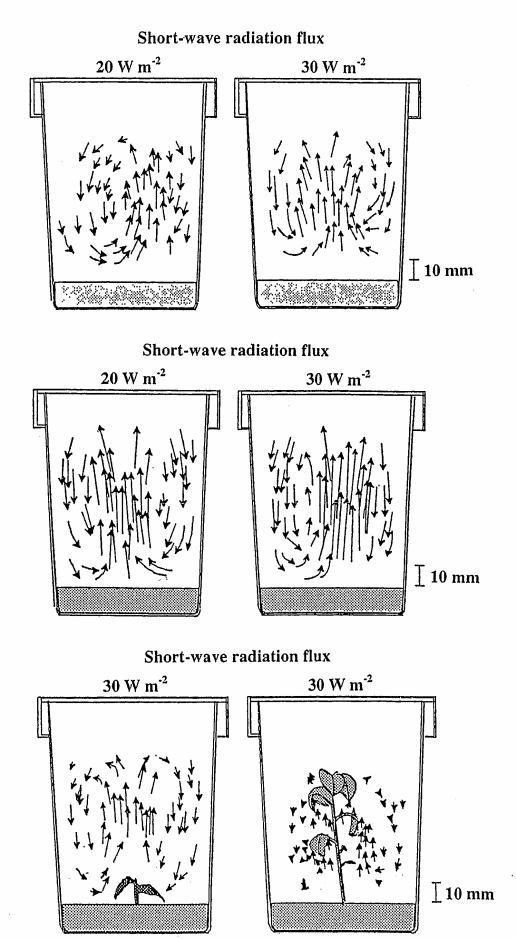

Figure 11. Air current pattern and speed per second in the Magenta type polycarbonate vessel (air volume: 370 ml) as affected by short-wave radiation flux, presence/absence of activated charcoal in the culture medium and the size of plantlets in the vessel (Kitaya et al., 1995). Upper vessel containing white-translucent agar medium with absence of activated charcoal and plantlet, middle vessel containing black-colored agar medium with presence of activated charcoal and without plantlets, and lower vessel containing black-colored agar medium with presence of activated charcoal and relatively small or large plantlet. The number of air exchanges of the vessel was 0.4 h^{-1} and the air current speed above the vessel was 1 m s^{-1}. Shortwave radiation fluxes of 20 W m^{-2} and 30 W m^{-2} correspond to PPF of 70 and 105 μmol m^{-2} s^{-1}, respectively. Air current pattern and air current speed were visualized by using a video camera for taking a moving picture of metaldehyde ($(C_2H_4O)_4$) particles used as tracers.

2.5. Temperature

Air temperature in the vessel during the dark period is almost the same as that in the culture room. However, air temperature during the photoperiod is 1-2 C higher inside the vessel than outside (Figure 13). The difference in air temperature between inside and outside the vessel increases with increasing shortwave or PAR radiation at the top of vessel. Practically, the number of air exchanges of the vessel does not influence the air temperature in the vessel. When activated charcoal is added to the culture medium, the medium surface temperature and, thus, air temperature in the vessel is raised due to its high absorptance of shortwave radiation.

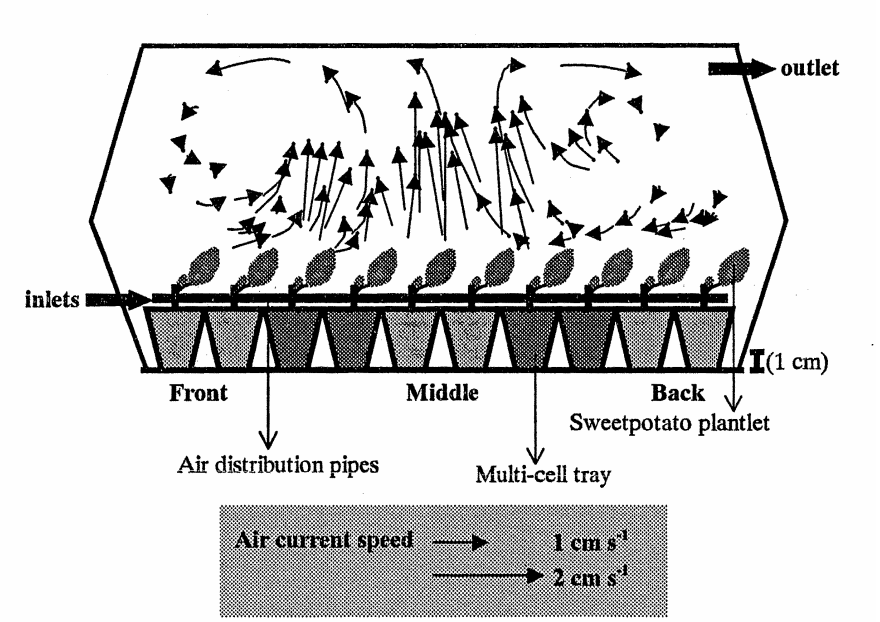

Figure 12. Two dimensional patterns of air currents in the culture vessel with forced ventilation using air distribution pipes on the plug tray with sweetpotato plantlets 6 days after transplanted (Heo, 1999). Vessel size: 368 mm long, 178 mm high and 170 mm wide, Air volume: 11.14 L. Forced ventilation rate: 13 ml s^{-1}. Number of air exchanges of the vessel: 3.9 h^{-1}. Air currents were observed as described in Figure 11.

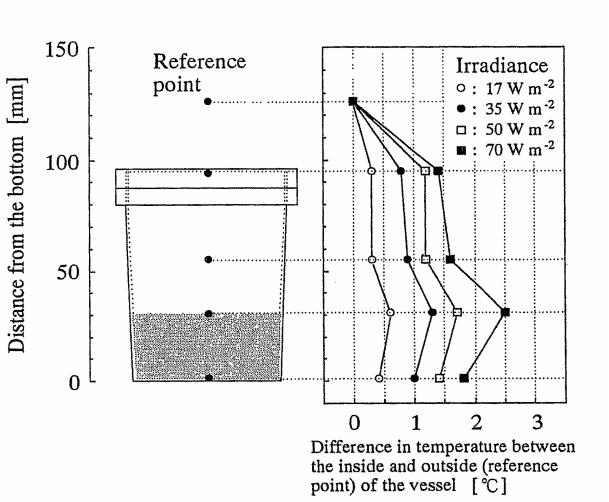

Figure 13. Temperature profiles above and inside a Magenta type vessel containing a gelled agar medium with 5 g l^{-1} activated charcoal at different irradiances (Fujiwara and Kozai, 1995b). The air temperature above the vessel was approximately 25 C. The irradiance was measured at the vessel lid level. Irradiances of 17, 35, 50 and 70 W m^{-2} correspond to, respectively, PPF of 60, 120, 175 and 260 μmol m^{-2} s^{-1} and illuminance of 5.5, 11, 16 and 24 klx. PPF of 260 μmol m^{-2} s^{-1} is considerably high even for photoautotrophic micropropagation. The temperature rise at the medium surface is lowered if activated charcoal is removed from the medium.

It has been suggested that the air temperature in the vessel rises extremely (3-5 C or higher) compared with the air temperature in the culture room when lamps are placed close to the vessels (e.g. 20 cm) even at a PPF of 150-200 μmol m^{-2} s^{-1}. This is due to neither the high PPF nor the short distance between the lamp and vessels, but rather to the high temperature of stagnant air around the vessels. Thus, enhancement of air movement above the vessels can lower the air temperature above the vessels and consequently in the vessels in most cases.

Air temperature in the vessel can be lowered 1-2 C by lowering the room air temperature by 1-2 C, which does not cause any significant increase in electricity consumption by the air conditioner.

The difference in air temperature between photoperiod and dark period (DIF) is known to affect stem elongation of many horticultural species (Heins et al., 1988). Similar effects of DIF on stem elongation of potato (*Solanum tuberosum*, Kozai et al., 1991) and mint (*Mentha rotundifolla*, Jeong et al., 1996) plantlets are observed under photoautotrophic culture conditions. In a later chapter, more will be discussed on the application of DIF as a tool for production of value added transplants.

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Chapter 5

T. KOZAI & C. KUBOTA

IN VITRO ROOT ZONE ENVIRONMENTS AND THEIR EFFECTS ON GROWTH AND DEVELOPMENT OF PLANTS

Contents

Key words: Hydroponic, nutrient medium, osmotic potential, oxygen concentration, photoautotrophic micropropagation, root zone, supporting material, water potential.

1. INTRODUCTION

In photoautotrophic micropropagation, plantlets must be grown under appropriate environmental conditions that promote photosynthesis. These conditions should include considerations of not only the aerial (headspace) environment, such as CO_2 concentration and photosynthetic photon flux, but also the root zone environment. While controlling the root zone environment is not easy, selection of supporting materials (i.e., replacing agar gel with porous supporting materials) that may create a better root zone environment (i.e., oxygen or nutrient availability) is relatively easy. Without sugar in the medium and with porous supporting materials and liquid medium, the system is more like a small hydroponic system, referred to as "microponics" (Hahn and Lee, 1996). Improvement of root zone environmental control in photoautotrophic micropropagation may well benefit from the expertise and techniques available from hydroponics.

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2. SUGAR CONCENTRATION

Effects of sugar in the medium have been well investigated and it is known that higher sugar concentrations in the medium suppress photosynthesis of *in vitro* plantlets. One exception is the enhanced net photosynthetic rate of cacti when cultured in sucrose-containing medium as compared with that in sugar-free medium (Malda et al., 1999). A good review of the effects of exogenous sugar on enzymatic activities, photosynthesis and growth of *in vitro* plantlets was written by Desjardins et al. (1995). Hdider and Desjardins (1995) showed that net photosynthetic rate of strawberry plantlets increased with time when they were transferred from sucrose-containing medium to sugar - free medium. Similarly, RuBisCO activity increased in those plantlets. Conversely, when the strawberry plantlets grown in the sugar-free medium were transferred to a sucrose-containing medium, the net photosynthetic rate and the carboxylase activity decreased with time. The rapid response of net photosynthetic rate within a period of several hours was also observed for potato explants transferred to media with or without sugar (Nakayama et al., 1991).

3. TYPES OF SUPPORTING MATERIALS

Replacing conventional agar gel with porous materials significantly affects the root zone environment and therefore the anatomical characteristics of roots. Figure 1 shows the structural comparisons of roots developed in conventional agar, gellan gum, vermiculite, Sorbarod® (a cellulose plug) and Florialite® (a mixture of vermiculite and cellulose fibers) (Afreen-Zobayed et al., 1999). The root system developed in Florialite® with liquid medium has 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., 1995). 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., 1999), *Eucalyptus* (Zobayed et al., 2000 and 2001), mangosteen (*Garcinia mangostana*; Ermayanti et al., 1999), and sweetpotato (*Ipomoea batatas*; 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.

4. PLANT GROWTH REGULATORS

In conventional micropropagation, different combinations and concentrations of growth regulators are added to the medium. The roles of growth regulators are basically the following three actions: (1) enhancing multiple bud or shoot development, (2) enhancing shoot elongation, and (3) enhancing rooting. Shoot and

root elongation can be achieved by controlling environment. In photoautotrophic micropropagation, use of multiple shoots is not necessary as long as a similar multiplication rate can be obtained by use of single node cuttings as explants (Aracama et al., unpublished). In conventional photomixotrophic micropropagation, information on medium composition and growth regulators is considered as the most critical to success. However, in commercial photoautotrophic micropropagation, systems and methods for environmental control and set point are considered as more critical information than the medium compositions.

5. NUTRIENT CONCENTRATION AND VOLUME OF THE MEDIUM

Most micropropagators and/or tissue culturists are concerned with the optimal strength of the medium and the optimal concentration of each medium component without regard for the medium volume per vessel and/or the number of explants per vessel and plantlet size. Figure 2 shows concentrations of some inorganic ions over time during the 24 d culture period, as affected by the culture volume per vessel and the strength of MS medium. As shown in Figure 2, change in concentration over time is significantly affected by the medium volume, even when the initial concentrations are the same. This means that the growth and development of cultures depend upon both the amount of ions per vessel or per plantlet and the concentration of ions. More attention should be paid to the total amount of ions rather than concentration when the medium volume is small. The same applies for sugar and other organic substances in the medium. When the medium volume is small, amount of a nutrient in the medium, not its concentration, determines the growth of plantlets *in vitro*.

6. WATER POTENTIAL

Water potential of a culture medium is practically expressed as the sum of water potential or osmotic potential of the nutrient liquid and water potential or matric potential of the supporting materials. Water potential of nutrient liquid is practically the sum of water potentials of inorganic salts, disaccarides (sucrose) and monosaccarides (glucose, fructose, mannitol and sorbitol) in the medium. Water potentials of organic substances other than sugar, such as vitamins, amino acids and plant growth regulators, are negligibly small because of their low molar concentrations. The water potential values of basic components in a solution of some widely used culture media and nutrient solutions for hydroponics are presented in the previous chapter (Chapter 2; Table 3). It is noted that water potential of MS (–212 kPa) is 3-6 times lower than those of White (–37 kPa) and Knudson C (-69 kPa). A practical equation for estimating water potential of sugar in the medium is shown in the previous chapter (Chapter 2; Appendix I).

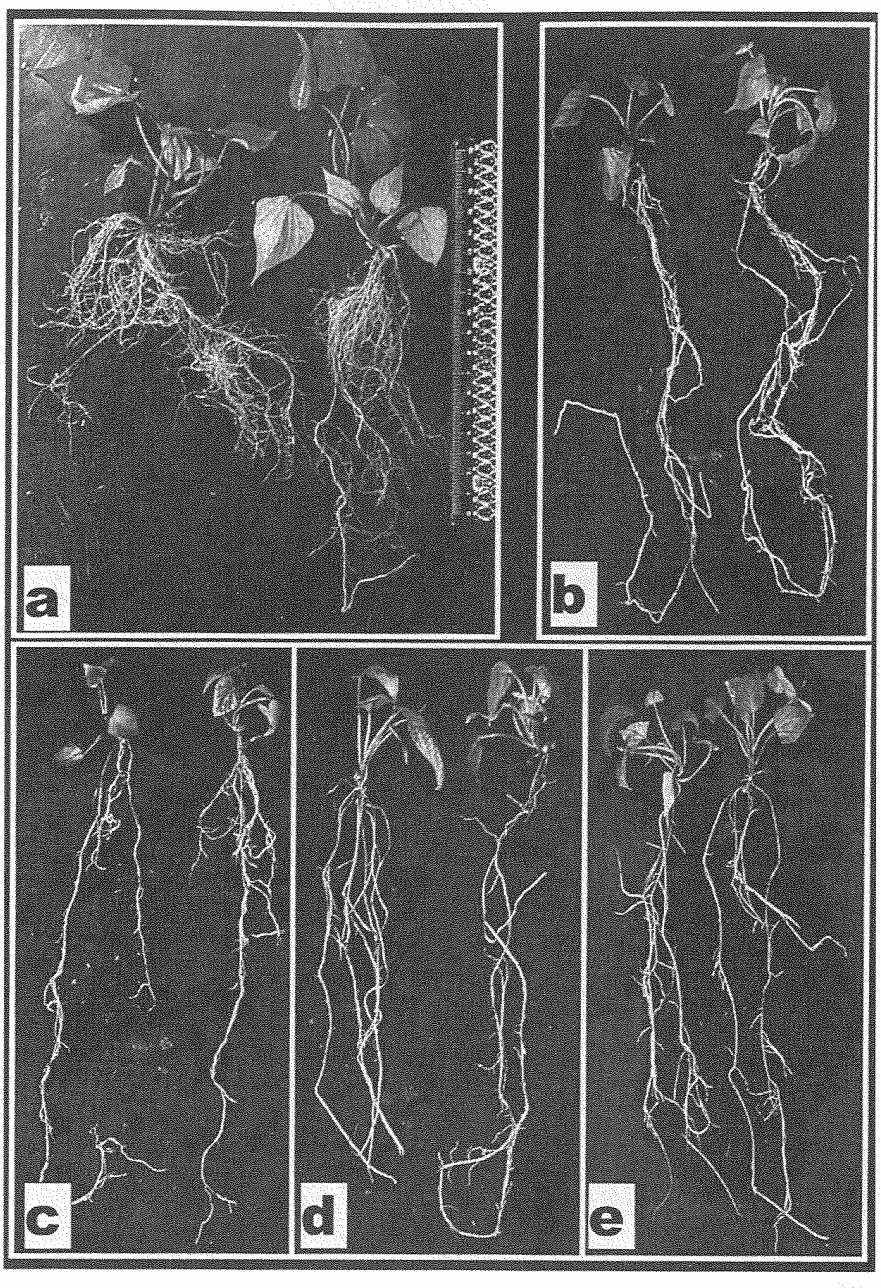

Figure 1. Root growth of sweetpotato plantlets cultured photoautotrophically for 21 days in different types of supporting materials: a) Florialite, b) Sorbarod, c) vermiculite, d) gellan gum, and e) agar (Afreen-Zobayed et al., 1999).

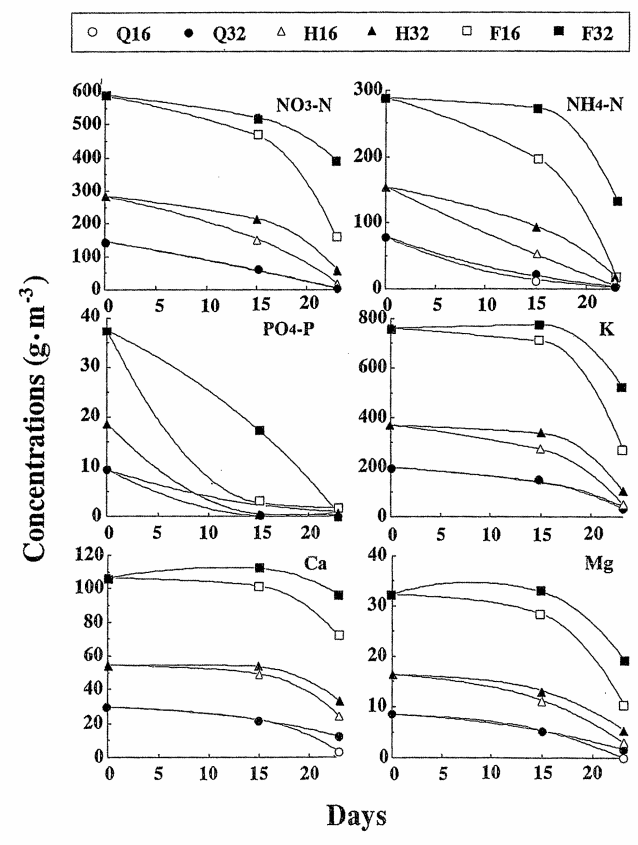

Figure 2. Changes in the concentration of NO_3, NH_4, P, K, Ca and Mg in the medium over 24 days of culture as affected by initial medium strength and volume (Kozai et al., 1995). The first letters of Q, H and F in the treatment codes denote quarter, half and full strength of MS medium. The numerical values of 16 and 23 following the Q, H or F denote the medium volume per vessel in vitro. Each vessel contained four leafy stem cuttings of potato (Solanum tuberosum L.) on sugar-free MS solution with fibrous supporting material.

Disagreements are numerous regarding the water (or matric) potential of gelling agents containing no sugar and no nutrients. Most researchers report that the water potential of a gelling agent is higher than –20 kPa, and that its absolute value is relatively small compared with that of the water potential of the liquid medium (- 200 to -700 kPa).

7. OTHER FACTORS

Symbiotic mycorrhization can generally confer several benefits to host plants, such as supplying nutrients or conferring resistance to pathogens. Attempts have been made toward establishment of *in vitro* mycorrhization that could benefit culture growth. Arbuscular mycorrhizal (AM) fungi have been established in photoautotrophic strawberry cultures (Cassells et al., 1996). The inoculation and establishment of colonization was achieved more successfully under photoautotrophic conditions than under conventional photomixotrophic conditions, especially when the non-sterile AM fungi were used. The system employed by Cassells et al. (1996) included the use of polyurethane foam as a medium supporting material replacing agar gel. Importance of controlling PPF and CO_2 concentration is recognized in the symbiotic effects of endomycorrhzation of plantlets (Louche-Tessandier et al., 1999). Further studies would be needed to identify ideal physical and environmental conditions that promote and maintain mycorrhization before the strategic use of symbiotic micorrhization in pathogen-free photoautotrophic micropropagation. Use of other beneficial microorganisms *in vitro* (Herman, 1996) also needs to be studied for use in photoautotrophic micropropagation.

Generally the tissue culture system is considered as a closed system and the concept of providing inputs to the system once it is closed is not widely regarded. In addition to medium ion availability, we should pay attention to the water availability or water usage in the closed system. The photoautotrophic micropropagation system developed by Zobayed et al. (1999) was designed to use 13.8 mL medium per plantlet during the 21 d culture of sweetpotato plantlets. The dry mass increase during the culture period was about 150 mg per plantlet. The volume of medium consumed per gram dry mass increase in the photoautotrophic system of Zobayed et al. (1999) is estimated roughly as one third of the volume of nutrient solution applied per gram dry mass increase of sweetpotato transplants grown *ex vitro* under artificial lighting conditions (estimated from values presented by Ohyama et al., 2000). The relatively low medium consumption per plantlet in the photoautotrophic micropropagation system suggests the possible accumulation and/or depletion of particular components in the system. A portion of water vapor in the headspace of the vessel leaves the vessel by ventilation and a significant portion of water vapor condenses on the sidewalls and is recycled (returned) to the medium. This may be one reason that such a small volume of medium used in photoautotrophic micropropagation as compared with an *ex vitro* transplant production system.

8. CALCULATION OF OSMOTIC POTENTIAL AND WATER POTENTIAL OF AIR

Osmotic potential of an ideal solution, W_s, is approximated by:

$$W_s = (RT/V_w)\ ln\ (x) \quad (1)$$

where R is the Universal gas constant (8.314 J mol^{-1} K^{-1}), T is temperature (K), V_w is the partial molar volume of water (18.07 x 10^{-6} m^3 mol^{-1} at 298 K), and x is the molar fraction of the components. '*ln*' denotes natural logarithm.

Water potential of air, W_a, is approximated by the following equation:

$$W_a = (RT/V_w)\ ln(e/e_0) \quad (2)$$

where e and e_o are actual and saturated water vapor partial pressure at air temperature of T. For symbols R, T, V_w and *ln*, refer to Equation (1). (Note a similarity in the equation form between (1) and (2)).

Water potential of sugar (sucrose, glucose, fructose, sorbitol and mannitol) in the culture medium, P_s (kPa), can be approximated by using the following practical equation (Fujiwara and Kozai, 1995).

$$P_s = -7.8\ C_d - 14.7\ C_m \quad (3)$$

where C_d is the concentration of sucrose (g l^{-1}), C_m is the total concentration of monosacarides (g l^{-1}).

Sucrose in the medium is hydrolyzed into glucose and fructose in the presence of invertase (an enzyme) produced by *in vitro* plantlets. Mannitol and sorbitol are sometimes added to the culture medium simply to lower the water potential of the culture medium. P_s is – 234 kPa at the sucrose concentration of 30 g l^{-1}, and is –441 kPa at the same concentration (30 g l^{-1}) of a monosaccaride. Water potential of MS solution containing 30 g l^{-1} of sucrose is – 444 kPa but the water potential of MS solution containing 30 g l^{-1} of glucose is –662 kPa. It should be noted that the water potential of medium changes over time as the medium components are absorbed by plantlets and sucrose is hydrolyzed into glucose and fructose in the medium.

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Chapter 6

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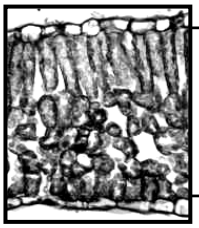

PHYSIOLOGICAL AND ANATOMICAL CHARACTERISTICS OF *IN VITRO* PHOTOAUTOTROPHIC PLANTS

Contents

Key words: Bioreactor, chlorophyll florescence, CO_2 concentration, forced ventilation, hyperhydricity, natural ventilation, net photosynthesis, photosynthetic pigments, Rubisco, somatic embryo, stomata, transpiration, wax deposition.

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1. INTRODUCTION

The physiological and anatomical characteristics of the *in vitro* plants are interrelated and chiefly governed by the microclimate of the culture vessel which includes mainly light, relative humidity, carbon dioxide, air movement inside the culture vessel and also the composition of the nutrient medium. All of these factors interact, influencing plantlet growth and production.

The conventional micropropagation technique is mostly carried out using small culture vessels with agar or other gelling agents containing nutrient medium and sucrose as a carbon source for the plantlets at a very low level of photosynthetic photon flux (PPF). The vessels are usually well sealed to ensure aseptic condition and also to prevent evaporative drying from plant and medium. The sealing of culture vessels inevitably interferes with the free exchange of gases between the culture microclimate and the culture room environment. Therefore this kind of microclimate exposes the culture to unnaturally high relative humidity, low exchange of gases between the culture vessel and the culture room which result in accumulation of gases like ethylene etc. Cultures enclosed in this microclimate will, inevitably, asphyxiate unless an alternative pathway of gas exchange between the culture vessel and the culture room environment is contrived.

In case of *in vitro* propagation when the growth of plantlet depends upon sugar in the culture medium (exogenous source) and CO_2 in the air of culture microclimate (endogenous source) then it is known as *photomixotrophic growth*. Growth which depends upon CO_2 in the air of culture microclimate (endogenous source) as the sole carbon source through photosynthesis is called *photoautotrophic growth* (Kozai and Zobayed, 2001). In conventional micropropagation, chlorophyllous plants are cultured on sugar-containing medium therefore they grow photomixotrophically and the method is called photomixotrophic micropropagation. The method of *in vitro* propagation of plantlets using relatively small chlorophyllous (leafy) explants in a sugar-free nutrient medium under pathogen-free condition where they can photosynthesize (autotrophic) and produce their own carbohydrates for their growth is known as photoautotrophic micropropagation.

Although the conventional method is commonly practiced mainly for the commercial micropropagation, recently there has been much debate on the question of whether the established culture method should continue to be used. As many reports pinpointed that the widespread use of conventionally micropropagated plantlets is often undermined by their poor physiological quality and growth; substantial percentage of plantlets cannot survive or cannot grow fast and vigorously after transplanting *ex vitro*, which limits the possible prospects of this unique propagation technique (Kozai, 1991; Zobayed et al., 2000).

This chapter reviews the causes of physiological and anatomical disorders of plantlets in the photomixotrophic system and discusses ways of overcoming the problems and improving the physiological and anatomical characteristics and quality of micropropagated plantlets by culturing the plantlets in photoautotrophic (sugar-free) medium and appropriately controlling the culture microclimate.

2. GENERAL CHARACTERISTICS OF *IN VITRO* MICROCLIMATE

Plant microclimate

Plant "microclimate" refers to the climate in the immediate vicinity of a plant or surrounding the plant inside the culture vessel. Plant microclimate is of primary importance to the plant and differs from the culture room environment. In photomixotrophic micropropagation, the *in vitro* plant microclimate has unique characteristics compared with the *ex vitro* or the greenhouse environment, whereas in photoautotrophic micropropagation the *in vitro* plant microclimate resembles that of the *ex vitro* or the greenhouse environment as will be discussed in the following sections.

2.1. Light

Photosynthesis literally means "synthesis using light". In nature, all chlorophyll-containing organisms use solar energy to synthesize organic compounds such as carbohydrate that cannot be formed without the input of energy. Energy stored in these molecules can be used later to power cellular processes in the plant and can serve as the energy source for all forms of life. In case of *in vitro* propagation, plantlets use artificial light to synthesize carbohydrate. Conventionally photomixotrophic micropropagation is done under a lower PPF of about 30–80 μmol m^{-2} s^{-1} compared to that of photoautotrophic, greenhouse or *ex vitro* environment. Thus, the low PPF coupled with the presence of sugar in the medium suppresses the need and opportunity for photosynthesis. Therefore photomixotrophically grown plants exhibit low carbon dioxide uptake rates and low net photosynthetic rates compared to those grown under high PPF and carbon dioxide enriched conditions (Kozai, 1991). However, *in vitro* grown plants have already been shown to have high photosynthetic abilities and thus may not need to grow in sugar-supplemented medium, alternatively they are grown in sugar-free medium and under high PPF and carbon dioxide enrichment (Kozai and Iwanami, 1988; Kozai et al., 1986; 1987; 1988 and 1990). Recent studies on the photosynthetic ability of coffee somatic embryos revealed that placing the embryos under a high PPF (100-150 μmol m^{-2} s^{-1}) for 14 days helps the production of chlorophylls, development of stomata and thus increases the photosynthetic ability (Afreen et al., 2001; 2002a and b).

2.2. Carbon dioxide concentration

The *in vitro* photomixotrophic plants start absorbing CO_2 at the onset of photoperiod, and thus the CO_2 concentration decreases steeply to a CO_2 compensation point (50-100 μmol mol^{-1}) and as a consequence the plants exhibit low net photosynthetic rates during rest of the photoperiod. This low net photosynthetic rate of photomixotrophic plants is mainly attributed to the low CO_2 concentration in the culture vessels during the photoperiod, and only partly to the low photosynthetic

ability of plants. As the plant continues to grow the need for CO_2 increases but in an airtight vessel CO_2 concentration cannot be raised according to the need of the plant which results in a restricted growth and development of plants. This problem is solved in photoautotrophic micropropagation system by firstly keeping the CO_2 concentration in the culture room significantly higher about 1000 μmol mol^{-1} or higher than the atmospheric concentration and secondly by increasing the number of air exchanges between the culture microclimate and the culture room environment either by using gas permeable film attached on the lid or side of the vessels or adopting loose-fitting lids which allow the diffusion of CO_2 inside the vessel, or by using forced ventilation which can mechanically move the CO_2 enriched air into the vessel. Thus, the CO_2 enrichment enhances net photosynthetic rate of plants and consequent growth, rooting and development.

2.3. Relative Humidity

The relative humidity inside the culture vessel is generally higher than 95% (Kozai et al., 1993) because the culture vessel, containing liquid nutrient, remained airtight and the temperature is approximately constant with time, although the reason for sealing the culture vessel is not to maintain a high relative humidity of the culture microclimate but to prevent microbes from entering the system. Evaporation from the culture nutrient medium and transpiration from leaf both increase the water vapor content in the culture microclimate of the airtight vessel. Thus high relative humidity is one of the negative side effects of sealing the culture vessel in the conventional micropropagation system. However, under photoautotrophic condition the relative humidity can be reduced by increasing the number of gas exchange either by enhancing the diffusive aeration of tissue (natural ventilation) or by contriving slow pressure-driven gas flow (forced ventilation) compared to the conventional system. By using forced ventilation the relative humidity in the vessel can be controlled precisely.

2.4. Air

All of the living tissues of cultured plants, not only the photosynthetic organs, require unimpeded air exchange to function normally. Severe impediment to air exchange more than a few hours is usually fatal to growing cells. The air volume of the culture vessel in conventional system is small and the number of air exchanges per hour of the culture vessel is low. Therefore the air temperature is relatively constant throughout the day, typically 25±3C and the ethylene concentration is high. Ethylene concentration only above 0.1 μmol mol^{-1} in the microclimate is known to inhibit plant growth and resulted in morphological disorders such as hyperhydricity in many plants. Under photoautotrophic conditions, due to the increased ventilation the number of air exchanges with the outer environment is high and so the air exchange between the tissue and the surrounding air enhanced significantly which overcomes some of the problems (listed above) of the conventional

photomixotrophic system.

2.5. Temperature

Generally, temperature affects photosynthesis of plantlets and oxygen sensitivity of photosynthesis increases with temperature. Leaf or shoot tip temperature has more direct effects than air temperature, which has only an indirect effect on the physiological response of the plantlets. In conventional photomixotrophic culture system, as the vessels are sealed therefore the temperature is relatively constant with time. However, in the photoautotrophic system, the vessel is aerated either naturally or forcedly and thus the temperature is not govern by the sealing system of the vessel rather is depended on the air temperature of the culture room. The effects of temperature on the physiological and morphological response of plants are discussed in the later section.

2.6. Substrate or supporting medium

The diffusion coefficient of the substrate or supporting medium surrounding the tissue quantifies the relative ease with which the gas diffuses through a given medium such as air, water and tissue to the cultured tissue or cell. For O_2, ethylene and CO_2 the diffusion coefficient in water is almost 10,000 times smaller than in air which makes water a principle barrier of tissue aeration, again the presence of agar or similar gelling agent increases the barrier to gas diffusion even more (Jackson, 2003). Low dissolved oxygen concentration, especially in gelled agents is a characteristic of photomixotrophic supporting medium. The use of fibrous supporting materials with high air porosity such as Florialite (Afreen et al., 2000), vermiculite (Kirdmanee et al., 1995), rockwool (Kozai et al., 1991) cellulose plugs (Kirdmanee et al., 1995) generally gives better root development and subsequent enhancement of plant growth under photoautotrophic conditions.

3. GENERAL PHYSIOLOGICAL CHARACTERISTICS OF *IN VITRO* PLANTS

3.1. Net Photosynthetic rate

Understanding photosynthetic characteristics is very important for successful environmental control in photoautotrophic micropropagation. Net photosynthetic rate of *in vitro* plantlets is described as gross photosynthetic rate minus respiration rate (photo and dark respirations). The magnitude of each term is different depending on whether the type of photosynthesis is C_3, C_4 or CAM (crassulacean acid metabolism). Theoretically, photoautotrophic micropropagation should apply to all plant species that grow photoautotrophically in nature. Failure, if any, should be attributed to inappropriate environmental conditions caused by the respective specific photosynthetic characteristics interacting with the general physical and chemical features of the *in vitro* environment.

In photomixotrophic micropropagation the *in vitro* plants show low gross photosynthetic rate due to the low CO_2 concentration and low ability of photosynthesis. The photomixotrophic plants exhibit low or negative daily net photosynthetic rate (negative daily CO_2 balance) due to low gross photosynthetic rate and high dark respiration rate.

Much research has been done to better understand the photosynthetic characteristics of *in vitro* plantlets. *In situ* measurement of gross or net photosynthetic rate is desired for such research objectives, but there are some difficulties and limitations in the measurement of gas exchange rates of plantlets without disturbing other environmental conditions. *In situ* measurement is especially important to understand the status of photosynthesis of the plantlets under the culture conditions since some of the unique *in vitro* environmental conditions such as high relative humidity, minimal air current, etc., are difficult to reproduce in the open-flow based assimilation chambers that are widely used for measurements on greenhouse grown plants. One possible method for *in situ* measurement of net photosynthetic rate is to measure $^{14}CO_2$ incorporation after a pulse of few seconds in a vessel as in De Riek et al. (1991).

The estimation of *in situ* net photosynthetic rate can be done by applying Fujiwara's method (Fujiwara and Kozai, 1995) as well. One of the advantages of Fujiwara's method is its simplicity, although many assumptions are made to make it as simple as it is. Based on Fujiwara's method, a photosynthetic response curve can be obtained by creating different equilibrium (or steady state) conditions by altering CO_2 concentration and PPF in the growth chamber. In such measurements, net photosynthetic rate curves can be easily obtained by using the CO_2 concentration inside the vessel as the independent variable. However it is difficult to obtain the curves using PPF as the independent variable because the CO_2 concentration inside the vessel can only be controlled indirectly in this method.

In conventional micropropagation systems, the CO_2 concentration inside the vessel containing plantlets during photoperiod approaches the CO_2 compensation point, where gross photosynthetic rate is balanced with respiration rate (dark respiration plus photorespiration rates). Under such conditions, light is no longer a limiting factor and increment in PPF would not be expected to increase net photosynthetic rates of the plantlets *in vitro*. Under such compensation conditions that give null net photosynthetic rates, photoautotrophic plantlets do not maintain positive carbon balance, while photomixotrophic plantlets do so by sugar uptake from the medium. Therefore, knowing the compensation conditions, i.e., the combination of environmental variables inducing null net photosynthetic rate, is critical for photoautotrophic micropropagation.

3.2. Estimation of in situ net photosynthetic rate

Fujiwara's equation (Fujiwara and Kozai, 1995) of steady state net photosynthetic rate is derived from the CO_2 balance inside the culture vessel. The equation is as follows:

$$V \cdot dC_{in} = -l \cdot P \cdot dt + N \cdot V \cdot (C_{out} - C_{in}) \cdot dt \qquad (1)$$

where V is the volume of air in the vessel (L), C_{in} is the CO_2 concentration in the vessel (mol mol^{-1}), l is the conversion coefficient from CO_2 moles to volume (L) at the air temperature inside the vessel, P is the net photosynthetic rate of the plantlets inside the vessel (mol CO_2 h^{-1}), N is the number of air exchanges of the vessel (h^{-1}), and C_{out} is the CO_2 concentration outside the vessel (mol mol^{-1}). The dC_{in} implies the difference in C_{in} for a short time interval dt. Assuming that the C_{out} and C_{in} are stable i.e. $dC_{in}/dt \approx 0$ (in steady state or equilibrium conditions) as is often observed in late photoperiod, the following equation 2 will be derived

$$P = k \cdot N \cdot V \cdot (C_{out} - C_{in}) \qquad (2)$$

where k ($k = 1/l$) is the conversion coefficient from CO_2 volume to moles at the air temperature inside the vessel (e.g., 4.09 x 10^{-2} mol L^{-1} at 25C). The equations are true only under the equilibrium conditions where both C_{in} and C_{out} do not change with time. Both equations 1 and 2 rely on an assumption of "perfect diffusion", that no gradient in CO_2 concentration exists inside the vessel, and therefore, any possible gradient generates some error in the estimation of net photosynthetic rate. To reach the status of equilibrium of C_{in} and C_{out}, the culture room should be free of such disturbances for instance, human access or temperature fluctuations for a few hours before measurement. During the sampling of the air inside and outside of the vessel, care should be taken not to disturb the equilibrium of C_{in} and C_{out}. Quick sampling of headspace air in the vessel through a silicon bung over a small hole using airtight syringes is recommended for the measurement of C_{in} in equation 2. Smaller volumes of air samples are also preferred, and the use of gas chromatography satisfies the accuracy required to measure at an order of 1 μmol mol^{-1} of CO_2 concentration using a small amount of sample (0.2 to 0.5 mL). The equation 2 can be used for estimation of dark respiration as long as C_{in} and C_{out} are stable. In the dark period, C_{out} is lower than C_{in}, and thereby, the equation 2 gives a negative P.

For the measurements of *in situ* net photosynthetic rates of *in vitro* plantlets another simple system was developed by Niu et al. (1998). Niu et al. combined Fujiwara's steady state method with modeling of net photosynthetic rate of the plantlets and generated net photosynthetic response curves against PPF as an independent variable by incorporating data of net photosynthetic rates, under different combinations of CO_2 and PPF conditions (Niu and Kozai, 1997). An open-flow based system has been used extensively for measurement of *in vitro* (not *in situ*) net photosynthetic rates with various PPF or CO_2 concentrations (Grout and Aston, 1978; Donnelly and Vidaver, 1984; De et al., 1992, 1993a and b; De la Vina et al., 1999; Van Huylenbroeck et al., 2000).

3.3. Photosynthetic characteristics

3.3.1. Temperature

The biochemical process of photosynthesis is governed by enzymes and thus is strictly temperature dependent. As shown in Figure 1, the general response of plant growth to temperature includes minimum temperature and optimum temperature. Low temperatures may inhibit photosynthesis to the zero point by affecting the enzymatic activity directly. As discussed in previous sections any decline in temperature can reduce the CO_2 diffusion rate and therefore decrease the rate of net photosynthetic indirectly.

Similar curves are obtained for number of leaves developed in the photoautotrophic micropropagation of sweetpotato as affected by air temperature (Kozai and Watanabe, unpublished), where there is a significant difference in number of leaves by 2-degree differences in temperature. When nodal cuttings are used as explants, number of leaves (nodes) represents the multiplication ratio, and therefore, the primary environmental factor for multiplication ratio is temperature. It should be noted that a small difference in number of leaves as affected by temperature would generate a large difference in number of plantlets produced after many cycles of multiplication.

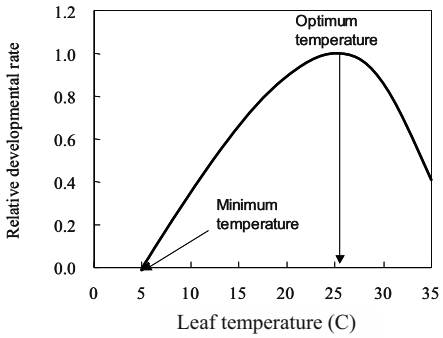

Figure 1. General response of plant developmental rate to temperature.

3.3.2. Leafy explants and photosynthesis

In photoautotrophic micropropagation, leafy explants are commonly used. Therefore, photosynthetic ability of explants is critical, especially regarding the initial growth of plantlets. As shown in Figure 2, sugar concentration of the medium affects the net photosynthetic rate of leafy single node cuttings of potato as soon as they are transferred to the medium (Nakayama et al., 1991). In forcedly ventilated vessels, net photosynthetic rates of the explants are promoted, compared with those in naturally ventilated vessels (Nakayama et al., 1991). The enhanced net photosynthetic rates of explants could stimulate new root and axillary shoot

development from the explants.

Leaf area is an important quality variable of explants. The photoautotrophic growth of potato plantlets is affected by the leaf area of the explants; explants with larger leaves give greater initial net photosynthetic rates per plantlet, and therefore provide greater growth rates (Miyashita et al., 1996). Explant leaf removal is a common practice in conventional micropropagation, but it is noted that tomato explants retaining leaves generated almost twice as much dry mass after 3 weeks as those with leaves removed under conventional, photomixotrophic culture conditions (Kubota et al., 2001). Criteria for selecting usable explants must be more strict for photoautotrophic than for photomixotrophic micropropagation. The quality of explants in conventional micropropagation will not necessarily meet the criteria for photoautotrophic micropropagation.

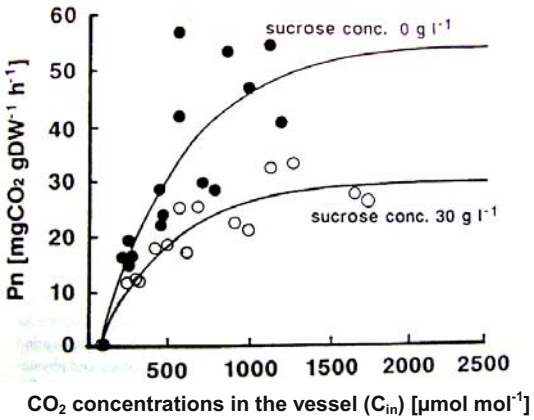

Figure 2. Net photosynthetic rates per leaf dry weight (Pn) of potato explants as affected by CO_2 concentration in the vessel and sugar concentration in the medium The Pn were measured as soon as explants were transferred to medium with or without sugar. (after Nakayama et al., 1991).

3.3.3. Somatic embryos and photosynthetic efficiency

Somatic embryogenesis is a key technology for mass production of elite clones and it has been introduced commercially, producing transplants for planting in clonal forestry. One of the challenges preventing the wider application of somatic embryos is low percent germination of somatic embryos and conversion to plantlets. Photoautotrophic micropropagation may contribute favorably in this area since somatic embryos have chlorophyll in their cotyledons and/or newly emerged true leaves, in which active photosynthesis can be expected. Long (1997) also suggested

the possibility of using photoautotrophic methods for improving germination of somatic embryos. The preferable photoautotrophic environmental conditions that enhance germination and conversion of somatic embryos may be different from those for explants or plantlets. In cacao somatic embryos, high percentages of conversion to plants were obtained under high CO_2 concentration of 2000 μmol mol^{-1} compared with that under ambient CO_2 concentration (Figueira and Janick, 1993). Based on the lower number of stomata and, perhaps, high resistance to CO_2 diffusion into somatic embryos, the high level of CO_2 concentration reported in Figueria and Janick (1993) may be necessary for enhancing the net photosynthetic rate of somatic embryos.

Development of photosynthetic ability of somatic embryos has been intensively studied in recent years. Rival et al. (1997) compared photosynthetic parameters (photochemical activities, CO_2 exchange and carboxylase enzymatic activities) among different developmental stages, from somatic embryos to plantlets. Afreen et al. (2002a and b) examined the photosynthetic ability of different developmental stages of coffee somatic embryos and found that cotyledonary and germinated embryos have photosynthetic ability. The uniqueness of their approach is the 14 days pretreatment of cotyledonary and germinated somatic embryos under increased PPF conditions which stimulated the development of photosynthetic abilities and increased the CO_2 uptake rate in those somatic embryos. Therefore, when grown under photoautotrophic conditions those embryos showed growth increments (compared with initial growth) and the dry mass was almost double the initial dry mass when grown under enriched CO_2 (approx. 1100 μmol mol^{-1}) and high PPF (100 μmol m^{-2} s^{-1}). Those findings also support the hypothesis that germination of somatic embryos will be improved significantly by controlling the environmental conditions that promote photosynthesis. Photoautotrophic growth of somatic embryos is discussed in much more detail in Chapter 7.

3.4. Chlorophyll fluorescence (photosynthetic ability)

Photosynthesis involves the conversion of light energy into chemical energy mediated by light sensitive chlorophyll molecules in the leaf. Over the last decade, the measurement of chlorophyll fluorescence kinetics has provided considerable information on the organization and function of the photosynthetic apparatus. During the process of photosynthesis, each quantum of light absorbed by a chlorophyll molecule raises an electron from the ground state to an excited state. Upon de-excitation from a chlorophyll *a* molecule from excited state 1 to ground state, a small proportion (3-5% *in vivo*) of the excitation energy is dissipated as red fluorescence. The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative pathways of de-excitation which are primarily photochemistry and heat dissipation. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation.

In the photoautotrophic growth of plants, biomass accumulation is totally relative to the contribution of photosynthesis. Chlorophyll fluorescence parameters such as F_v / F_m, F_v / F_o etc. are generally used to study the organization and function of photosynthetic apparatus (Gently et al., 1989). These parameters are the ratios of variable chlorophyll fluorescence ($F_v = F_m - F_o$) to either maximal (F_m) or ground (F_o) chlorophyll fluorescence. F_v / F_m ratio is used to evaluate the photosystem II (PS II) in the dark-adapted state with fully open PS II reaction centers (Serret et al., 2001). This value is highly correlated with the quantum yield of net photosynthesis in intact leaves (Bjorkman and Demmig, 1987). On the other hand, the ratio F_v / F_o is a reliable indicator of the potential photosynthetic capacity of leaves (Serret et al., 2001).

Serret et al. (2001) studied the association between the degree of photoautotrophy and the photosynthetic capacity of micropropagated gardenia plantlets by using chlorophyll fluorescence parameters. At each micropropagation stage, varying degrees of photoautotrophy were achieved by changing the types of closure, sucrose content in the growing medium and the PPF. Afreen et al. (2002a) assessed the photosynthetic ability of different stage coffee somatic embryos grown in the photomixotrophic and photoautotrophic conditions by using the chlorophyll fluorescence parameters. A fibre-optic based chlorophyll fluorimeter (Hansatech, King's Lynn, Norfolk, UK) was used to analyse the photochemical activity of the somatic embryos. In dark-adapted samples (2 h), the maximal quantum yield of photochemistry through PSII (ϕ_p^{max}) was calculated from the ratio $(F_m - F_o)/F_m$ (Kitajima and Butler, 1975). The actual quantum yield (ϕ_p) of PSII photochemistry in light-adapted leaves was calculated from the steady-state level of chlorophyll fluorescence (F_s) and maximal fluorescence level: $\phi_p = (F_m - F_s)/F_m$ (Havaux et al., 1991). Values of both ϕ_p^{max} and ϕ_p of different stage embryos measured after 60 days of culture were not significantly different between the photoautotrophic and photomixotrophic treatments. In general, among the different stages values were greater in cotyledonary and germinated embryos in both treatments.

3.5. Photosynthetic pigments

Pigments are chemical compounds which reflect only certain wavelengths of visible light. Chlorophylls, the most important pigment in nature, are greenish in color, and contain a stable porphyrin ring around which electrons are free to migrate. Because the electrons move freely, the ring has the potential to gain or lose electrons easily, and thus has the potential to provide energized electrons to other molecules. This is the fundamental process by which chlorophyll "captures" the energy of sunlight. It is capable of channeling the energy of sunlight into chemical energy, that is, during the process of photosynthesis, green plants use light energy to produce chemical energy and chlorophyll is essential for this process. There are several kinds of chlorophyll, the most important being chlorophyll *a*. This is the molecule which makes photosynthesis possible, by passing its energized electrons on to molecules which will manufacture sugars. All plants, algae, and cyanobacteria which photosynthesize

contain chlorophyll *a*. A second kind of chlorophyll is chlorophyll *b*, which occurs only in green algae and in the plants. Chlorophyll *a* is generally 3-times higher than chlorophyll *b* in normal *in vivo* plants.

In the photoautotrophic micropropagation, photosynthesis is the sole source for the carbohydrate accumulation and thus to grow under photoautotrophic conditions explants or plantlets must be chlorophyllous. Compare to photomixotrophic or sugar-containing culture medium, growing plantlets under photoautotrophic conditions do not alter the leaf chlorophyll content significantly, if the other environmental parameters especially PPF and the number of air exchanges of the vessel remain same. For example, while growing *Rehmannia glutinosa* plantlets in culture medium containing different concentrations of sugar (10, 15, and 30 gL^{-1}), Cui et al. (2000), found no significant difference in chlorophyll contents. In their study, the PPF (70 μmol m^{-2} s^{-1}), temperature (25C), ambient CO_2 concentration (1000 μmol mol^{-1}) and the number of air exchanges of the vessel (4.4 h^{-1}) remained same in all the treatments. However, in the same study, chlorophyll contents increased significantly when the number of air exchanges in the vessel was increased. Previously, forced ventilation has been found to increase the chlorophyll concentration compared to that of the natural ventilation or airtight system (Zobayed et al., 1999a). In airtight system or vessel having low number of air exchanges, generally ethylene accumulation take place which is known to decrease the leaf chlorophyll content. While culturing *Prunus avium* in closed vessels, Righetti (1996) found shoots were irregularly shaped, hyperhydrated with curled leaves, low chlorophyll content which decreased after 15-18 days of culture. Under these conditions the lowest dry-matter percent production and the highest ethylene synthesis were also observed. In a recent study, Park et al. (2004) observed that the chlorophyll content of the hyperhydrated shoots of potato plantlets was significantly lower in the completely sealed vessel than those of the normal shoots of gas permeable vessels. A fivefold increase in chlorophyll content was also observed in normal shoots of carnation plantlets than those of hyperhydrated one (Jo et al., 2002).

3.6. Photosynthetic enzymes: Rubisco

Photosynthetic enzymes such as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) is the primary carboxylating enzyme used by C_3 plants during photosynthesis to incorporate CO_2 into sugars needed for growth and development. Even C_4 and CAM plants, which use PEP-carboxylase as their primary carboxylating enzyme, utilize Rubisco during subsequent secondary CO_2 assimilation events. As a carboxylase, it is involved in CO_2 fixation to the five-carbon sugar ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate which proceed to sugar production. Conventional photomixotrophic micropropagation involves the production of plants associated with low activities of Rubisco and the high net photosynthetic rate generally observed in photoautotrophic grown plants are perhaps due to enhanced Rubisco activities as explained by Desjardins et al. (1995). As pointed out by Roberts et al.

(1994) that cauliflower plants grown photomixotrophically under low light irradiance exhibited low net photosynthetic rate which was attributed to low levels of chlorophyll and ribulose bisphosphate carboxylase activity.

3.7. Transpiration

Transpiration rate is generally low in conventional *in vitro* plantlets and thus the low uptake rate of water and minerals are common. The prolonged exposure of the plantlets to high relative humidity, low CO_2 concentration and accumulated ethylene in the headspace of the vessel often causes the lack of development of a cuticular layer, or functional stomata (Figure 3) to control the transpiration or water loss (Figure 4) of the plantlets under low relative humidity (or high vapor pressure deficit) conditions. As described earlier, the water environment inside the vessel is affected by that of the culture room, transpiration from the plantlets, evaporation from the medium or condensed water, and condensation on inner surfaces of the vessel or on the medium. The work of Sallanon and Maziere (1992) illustrates the vapor pressure deficit and water potential of the plantlets in the vessels under different relative humidity conditions. Slight changes in water vapor deficit *in vitro* resulted in significant differences in growth and morphology of the plantlets (Sallanon and Maziere, 1992).

Under lower vapor pressure deficit, plantlets had higher multiplication ratios, larger leaf area, and longer shoot length. More axillary buds developed from the explant base under low vapor pressure deficit conditions while development occurred on the upper part of the explant under high vapor pressure deficit. The culture conditions examined were based on a conventional system (containing sugar in the medium).

Under photoautotrophic conditions especially under forced ventilation, *in vitro* transpiration rate can be increased due to i) comparatively low relative humidity in the culture vessel, ii) higher air current speed around the leaf and iii) functional stomata. Increased transpiration can stimulate plant growth by enhancing acropetal transport of dissolved nutrients and evaporation from the cuticle draws wax precursors to the leaf surface (Roberts et al., 1994). Transpiration or control of water loss is also important during the early stage of *ex vitro* acclimatization. A photoautotrophic culture system with forced ventilation can significantly improve the development of normal functional stomata, and the formation of large amounts of epicuticular wax which contribute to better control of transpiration and result in less water loss after transplanting *ex vitro*, thereby conferring a high survival percentage compared to conventional *in vitro* (Zobayed et al*.,* 2000).

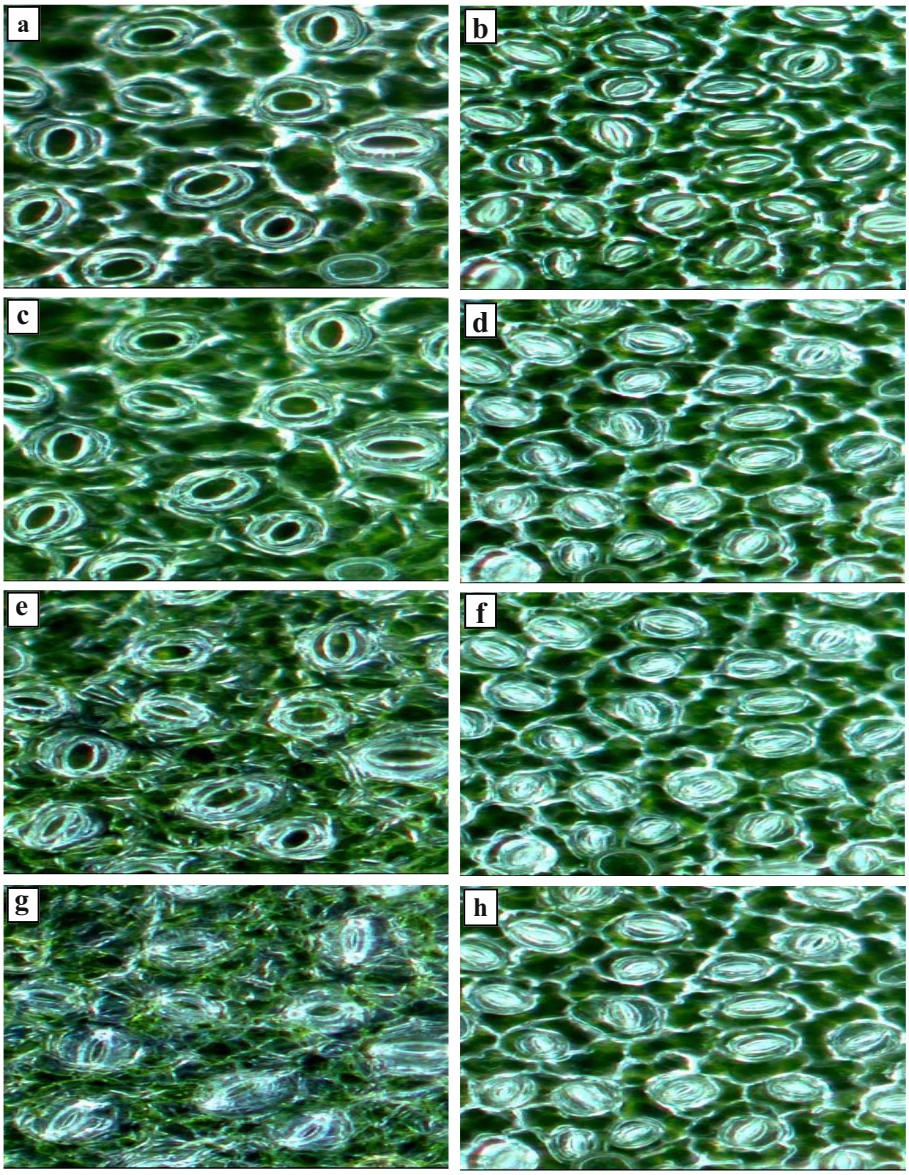

Figure 3. Stomata on the abaxial surface of fully expanded 3rd/4th leaves from the apex of 21 days old sweetpotato plantlets cultured photomixotrophically under natural ventilation (a, c, e, g) and photoautotrophically under forced ventilation (b, d, f, h). Photographs were taken 1 (a, b), 2 (c, d), 30 (e, f) and 60 min. (g, h) after transplanting ex vitro. (after Zobayed et al., 2000)

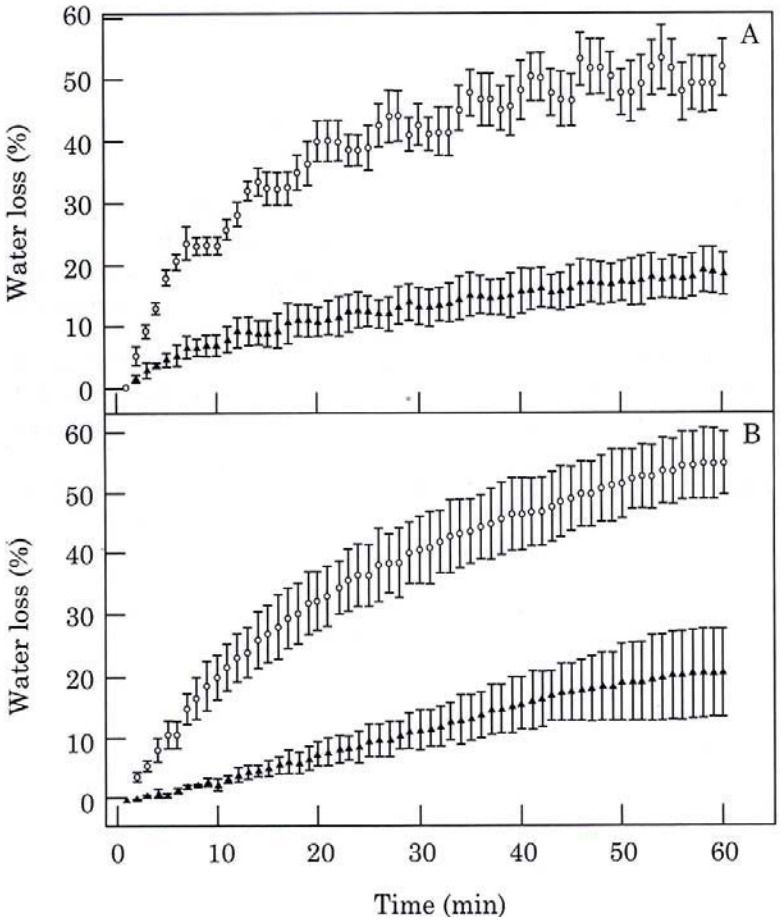

Figure 4. Percent water loss of excised (A) and attached (B) leaves of sweetpotato plantlets immediately after transplanting ex vitro. Plantlets were cultured photomixotrophically under natural ventilation (○) and photoautotrophically under forced ventilation (▲). Each point represents mean ± s.e. of five replicates. (after Zobayed et al., 2000).

4. GENERAL ANATOMICAL CHARACTERISTICS OF *IN VITRO* PLANTS

Generally, anatomical characteristics especially the leaf anatomy of *in vitro* plants grown in the conventional micropropagation system has been studied intensively in the last few years. Conventionally, micropropagation is carried away using small, relatively airtight culture vessels containing nutrient media with 20–30 g L^{-1} sucrose (as a carbon source for the plantlets) and under a low PPF of about 30–80 µmol

m^{-2} s^{-1}. Most of the researches pointed out that under these conditions the anatomical features of the plants are at least to some extend different (abnormal) than the normal *in vivo* plants, they have a significantly higher content of carbohydrates, mostly in the leaves (Kozai and Zobayed, 2001). Thus the leaves have a poorly developed internal structure and become physiologically abnormal and simply act as a storage organ. It has also been concluded that the poor ventilation or restricted air exchange in the conventional culture vessel can lead to the development of abnormal anatomical features which could prevent or reduce the plant's ability to acclimatize *ex vitro* (Zobayed et al*.,* 2001a).

4.1. Mesophyll and palisade layer

The mesophyll layer of the leaf consists of parenchymatous tissues situated between the two epidermal layers of the leaf. It usually undergoes differentiation in order to form photosynthetic tissues and thus contains chloroplasts. Another important factor of the mesophyll layer is the presence of well-developed intercellular spaces which facilitates the exchange of gases. Thus for efficient photosynthesis not only the number of chloroplasts but the dimensions of intercellular spaces also play an important role. Zobayed et al. (2001b) showed that leaves of cauliflower and tobacco plants grown photomixotrophically in well sealed vessels exhibited a lack of well defined palisade and spongy mesophyll layers and the cells were more closely packed with smaller intercellular spaces compared to those grown in well aerated vessels (Figure 5). In contrast when grown photomixotrophically in aerated (diffusive and forced) vessels and *in vivo* both the species showed more structural integrity in the leaves, and had definite palisade and spongy mesophyll layers, the latter with large intercellular spaces. They also suggested that the chloroplast contents of the mesophyll layers in these leaves were greater compared to those of the sealed vessels ones. Similarly when *Eucalyptus* plants were grown photoautotrophically under forced ventilation had leaves (Figure 6a) that were thicker (723 µm), with well organized palisade and spongy mesophyll layers and the epidermal cells were well developed with an average depth of 42 µm. In contrast when grown photomixotrophically the *Eucalyptus* leaf thickness and epidermal cell thickness were 421 and 28 µm, respectively compared to those of photoautotrophically grown leaves (Figure 6b). The epidermis had irregular cells (Figure 6b) and smaller, irregular shaped oil cavities compared to that of photoautotrophic ones.

4.2. Wax deposition

Wax is a lipid compound which mainly consists of esters of long-chain fatty acids and long-chain monohydric alcohols. Waxes usually form protective coatings on the epidermis of leaves. The structure and amount of epicuticular wax affects the cuticular permeability and the degree to which a leaf surface can be wetted. The development of epicuticular wax is known to be advantageous for the plantlets

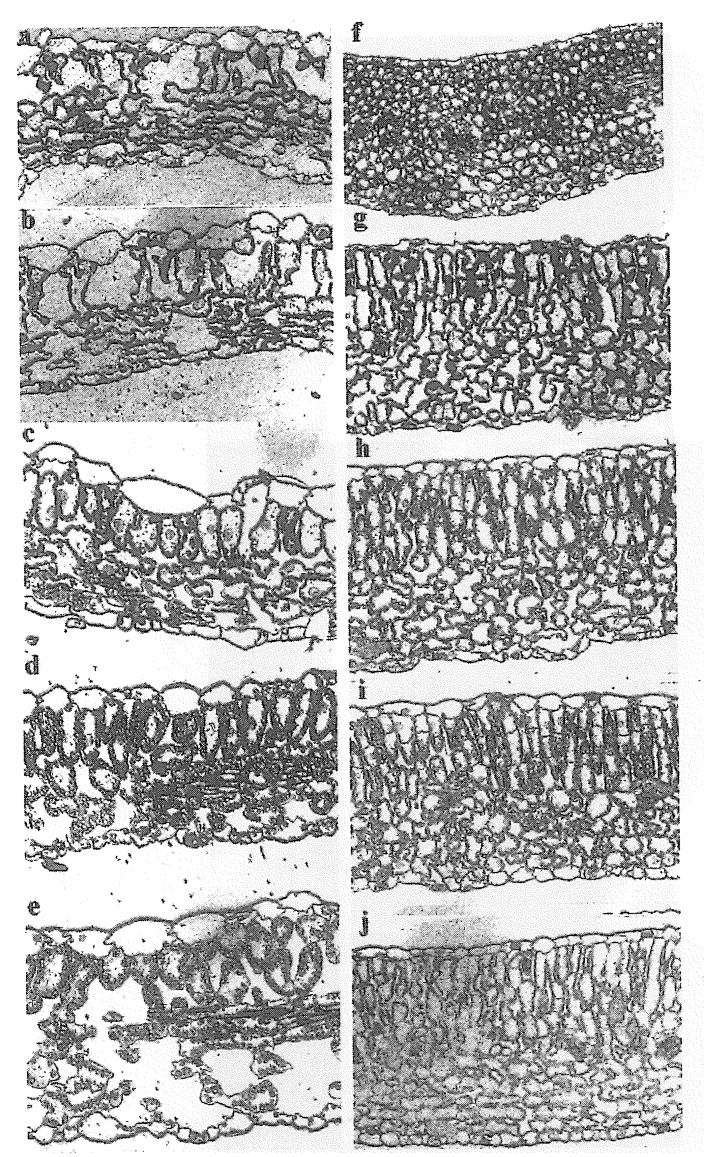

Figure 5. Transverse sections of 3rd and 4th leaves from apex of tobacco (a - e) and cauliflower (f - j) plantlets grown under different types of ventilation and also in vivo for 28 days (160 x). (a, f) Airtight vessel (sealed with silicone rubber bung); (b, g) diffusive ventilation (polypropylene disc); (c, h) slow forced ventilation (flow rate 5 cm^3 min^{-1}); (d, i) fast forced ventilation (flow rate 10 cm^3 min^{-1}); (e, j) in vivo (growth room conditions). Cultures were grown at ca. 25C with 16h photoperiods at PPF 150 µmol $m^{-2}s^{-1}$. (after Zobayed et al 2001b).

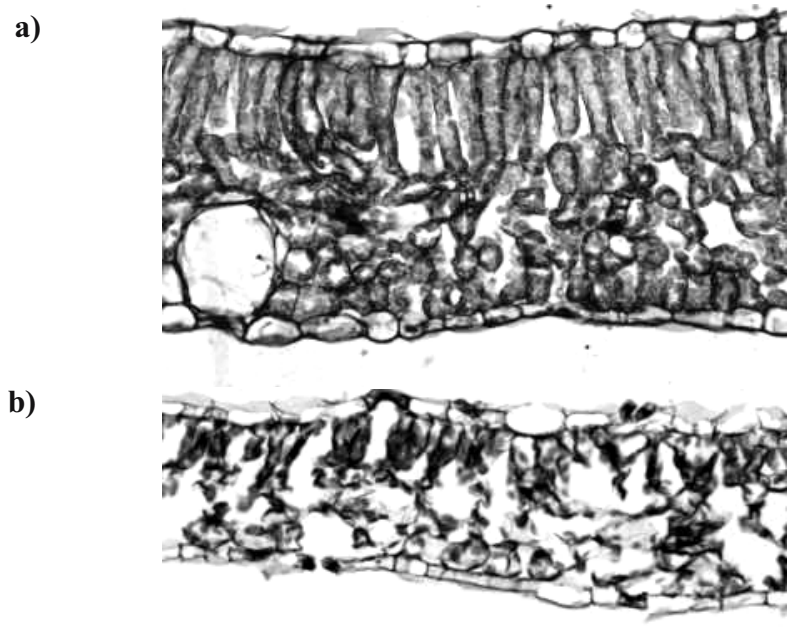

Figure 6. Transverse section of the 4th leaf from apex of 28 days old Eucalyptus plantlets grown (a) photoautotrophically in a scaled-up vessel under forced ventilation and (b) photomixotrophically in a Magenta vessel (control). oc, oil cavity; as, air space. (after Zobayed et al., 2001a).

especially during the acclimatization period (Grout, 1975; Sutter and Langhans, 1982) as it helps the plants from desiccation (Zobayed et al., 2001b), it also reduce the damage to photosynthesis and heat load of leaves by reflecting the light (McClendon, 1984). The degree of wax formation depends on the environmental conditions to which a plant is exposed. Lack of epicuticular wax formation was noticed in the leaves of cauliflower (Figure 7) and *Eucalyptus* (Figure 8) when grown in well sealed and poorly aerated vessels. On the contrary the plants from well aerated vessels and greenhouse showed intense epicuticular wax development which appeared as white powdery coating under the microscope (Zobayed et al., 2001b). They also noticed the formation of cuticular wax in well aerated and greenhouse grown plants.

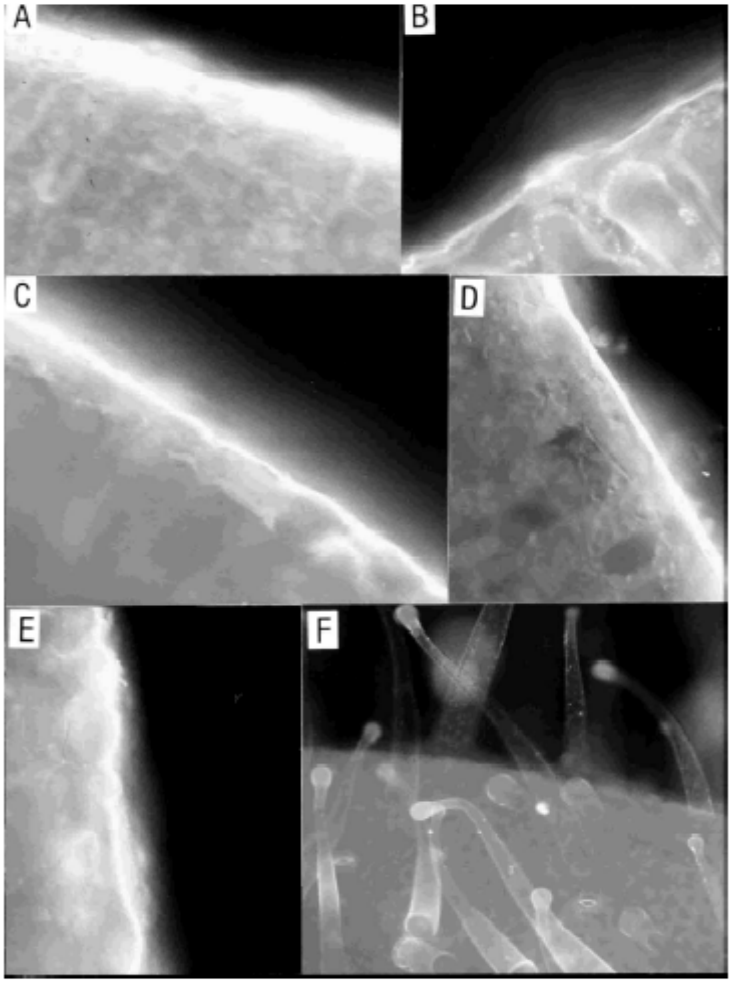

Figure 7. Transverse sections of upper epidermis of fresh 3rd or 4th leaf from apex of 28 days old cauliflower plantlets; sections were stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow (white in this photograph; 688 x). Cultures were grown at ca. 25C with 16 h photoperiods at PPF 150 µmol $m^{-2}s^{-1}$; RH: 26-32%. Plantlets were grown under: (A) airtight vessel (sealed with silicone rubber bung); (B) diffusive ventilation (polypropylene disc); (C) slow forced ventilation (flow rate 5 cm^3 min^{-1}); (D) fast forced ventilation (flow rate 10 cm^3 min^{-1}) and (E) in vivo (growth room conditions). Note in (A) and (B) the cuticles appeared thinner and fluoresced to a smaller degree than in (C, D, E). The lack of fluorescence was particularly obvious in (B). (F) Features of upper epidermis of fresh 3rd or 4th leaf from apex of 28 days old tobacco plantlets grown under forced ventilation (fast flow; rate - 10 cm^3 min^{-1}). Specimens were stained in 0.02% aqueous auramine and photographed under blue light to show waxes fluorescing yellow. Note the epidermal hairs have waxy walls and globular tips (150x). (after Zobayed et al., 2001b).

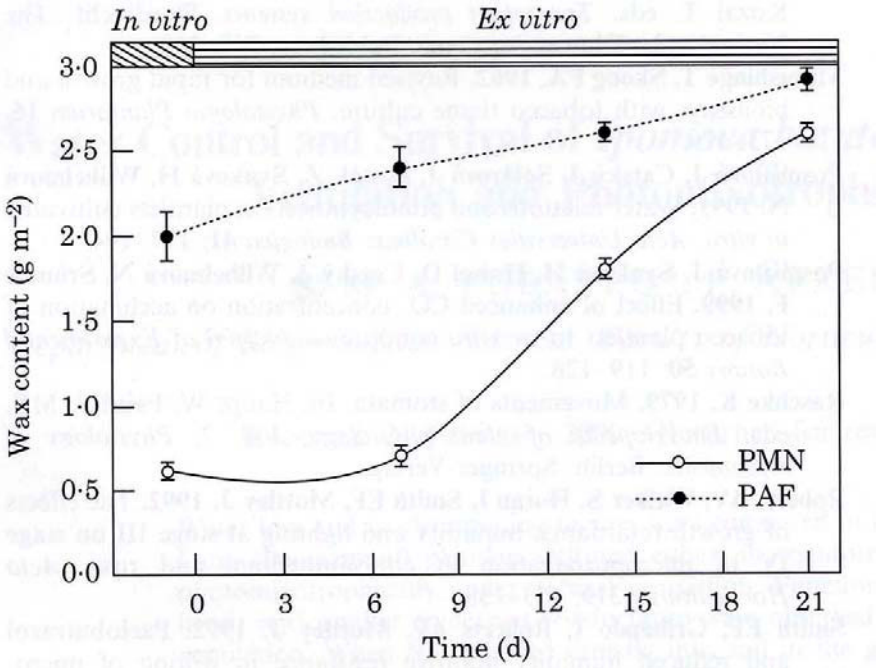

Figure 8. Epicuticular wax content of the leaves of sweetpotato plantlets cultured in vitro (day 21) and after transplanting ex vitro (day 7, 14 and 21). Plantlets were cultured photomixotrophically under natural ventilation (PMN) and photoautotrophically under forced ventilation (PAF). Bar represents mean ± s.e . of five replicates. (after Zobayed et al., 2000).

The direct correlation between the reduced amount of epicuticular wax and substantially increased water loss of plantlets after transplanting is discussed by Preece and Sutter (1991). In an earlier study Wardle et al. (1983) suggested the possibility of producing *in vitro* glaucous cauliflower plants with increased amounts of epicuticular wax by reducing the relative humidity using desiccants. The use of a culture vessel in which Tyvek inserts in the lid (Baumgartner Papiers) is shown to reduce the relative humidity from approximately 100% to 94% which resulted in an increased resistance to desiccation in chrysanthemum (Smith et al., 1990) and grapewine (Smith et al., 1992) plantlets. Later Zobayed et al. (2001a) noted that reducing the relative humidity to 84% by introducing forced ventilation in the culture vessel headspace the epicuticular wax content of *Eucalyptus* leaf was increased 3.4X in photoautotrophic conditions that of the control (photomixotrophic conditions under airtight capping system) (Figure 8).

4.3. Leaf hair

Leaf hair may play a role in insolation and light reflectance (Heide-Jorgensen, 1980). The formation of leaf hair could well be a reflection of the percentage of relative humidity of the plant microclimate, hairs being longest where relative humidity is lowest. Zobayed et al. (2001a) showed that the epidermal hairs were shortest on leaves in sealed vessels and increased in length with increasing efficiency of ventilation (Figure 7F). For instance, the length of hair from the mid-rib region of tobacco leaf was 2.6X as long in well ventilated vessel as those from the sealed vessel.

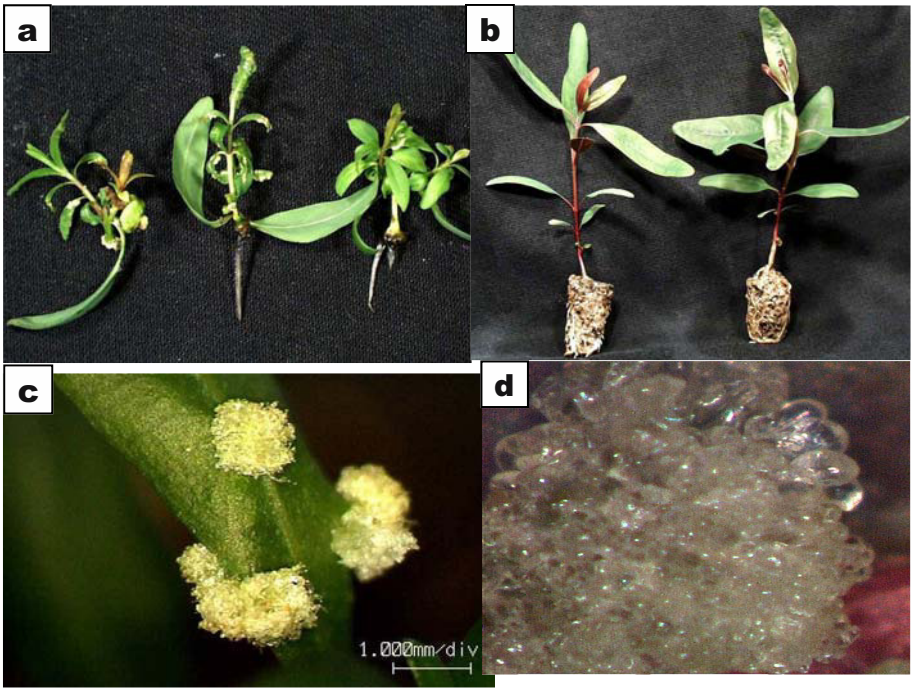

Figure 9 a) Eucalyptus plantlets grown photomixotrophically in a Magenta vessel with 20gL^{-1} sucrose for 28 days, b) Plantlets grown photoautotrophically in a large vessel with forced ventilation for 28 days; c) Callus (gall) formation on the leaf surface of plants grown photomixotrophically in a Magenta vessel.

4.4. Hyperhydricity

Hyperhydricity is a phenomenon of plant that occurs as a consequence of plant's response to non-wounding stresses when explants are placed in an unsuitable *in vitro* environment. The unsuitable environmental conditions include high relative humidity, constant air temperature, accumulation of ethylene, etc. gases, high osmoticity of the culture medium due to the presence of sugar and ammonium,

hormonal imbalance in the media, sealing of culture vessels etc. All of these factors are responsible for the morphological and physiological disorders of the *in vitro* plantlets.

Morphology: Hyperhydrated plantlets are so named because of their "glassy" appearance. They are chiefly characterized by their thick, translucent, and brittle leaves (Ziv, 1991) and thick and easily breakable stems (Park et al., 2004). Potato plantlets grown in conventional airtight vessel with sugar-containing medium were severely hyperhydrated exhibiting typical glassiness; shoot increment after 9 days of culture was only 33% and the shoots remained stunted, reduction in leaf number and significantly lower percent of shoot dry mass were observed (Park et al., 2004).

The low dry weight of the hyperhydrated shoots was attributed to the high water content of the shoots. High concentration of ethylene (0.18 ppm) was accumulated in the airtight vessel compared to 0.04 ppm in the gas permeable one only after 9 days of culture. They suggested that ethylene accumulation in the culture vessel was the major cause of hyperhydricity of the shoots. Other biochemical characteristics of hyperhydrated plants are less lignin deposition, less amount of cellulose which is associated to a low C/N ratio favouring the synthesis of amino acids rather than the sugar units for cellulose, less chlorophyll which causes translucency. *Eucalyptus* leaves grown photomixotrophically showed callus formation on the leaf surface (Figure 9a and c) which was hyperhydric and friable (Figure 9d). Plantlets grown under photoautotrophic conditions with forced ventilation did not produce any callus on the leaf surface (Figure 9b).

4.5. Stomata

Stomata are microscopic pores, each bounded by two crescent shaped cells known as guard cells. Stomata are usually found on leaf surface and occasionally on stems. They are able to open and close and act as portals for entry of carbon dioxide into the leaf for photosynthesis and an exit for water vapour from the transpiration stream. Their major function is to allow sufficient CO_2 to enter the leaf while conserving as much water as possible. The guard cells are usually connected to neighbouring cells via their dorsal walls and because of their relative isolation from the rest of the plant body, stomata are ideally suited for sensing and responding to environmental factors.

The most important anatomical abnormality reported by many researcher observed in a poorly ventilated sugar-containing (heterotrophic or photomixotrophic) medium is the non-functional stomata. As described earlier, the absence or reduction in leaf epicuticular and cuticular waxes, short leaf hair, all of these features combined with non-functional stomata would lead to abnormally and inherently high rates of transpiration, which could not be controlled during a period of acclimatization, an environment characterized by low relative humidity and high light, and which could jeopardize the plant's chances of survival.

Recent researches revealed that stomatal characteristics and leaf anatomy could be largely improved by using photoautotrophic micropropagation. For instance,

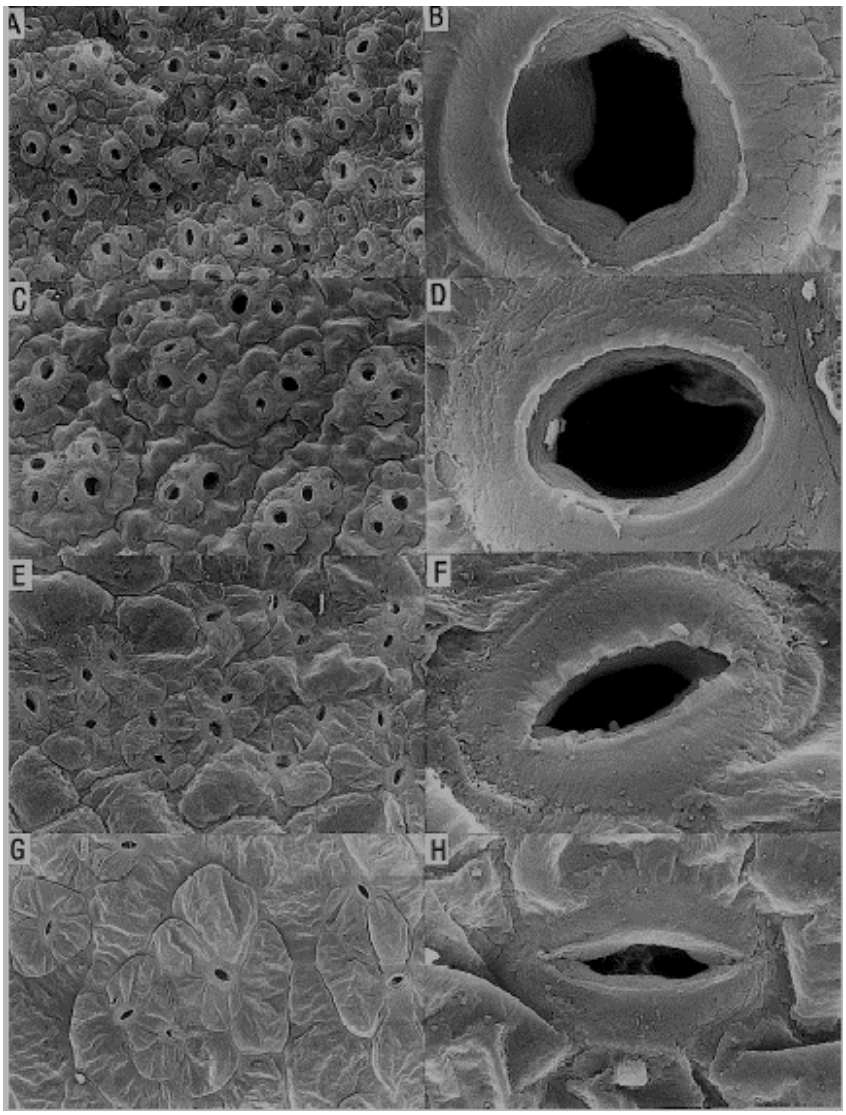

Figure 10. Stomata on lower epidermis of fresh 3rd or 4th leaf from apex collected during photoperiod from shoots of cauliflower plantlets after 28 days. Cultures were grown at ca. 25C with 16 h photoperiods at PPF 150 µmol $m^{-2}s^{-1}$; RH: 26-32%. Plantlets were grown under: (A, B) airtight vessel (sealed with silicone rubber bung); (C, D) diffusive ventilation (polypropylene disc); (E, F) slow forced ventilation (flow rate 5 cm^3 min^{-1}); (G, H) fast forced ventilation (flow rate 10 cm^3 min^{-1}). Note the degree of stomatal closing under different conditions. (after Zobayed et al., 2001b).

stomatal density of the photoautotrophic potato plantlets increased twofold compared to that of the photomixotrophic plantlets (Zobayed et al., 1999b).

Stomatal density is found to be increased significantly under photoautotrophic conditions with CO_2 enrichment (Kirdmanee et al., 1995). In *Eucalyptus* plantlets grown photoautotrophically, stomata opened during the light period and closed in the dark period, while in many of the photomixotrophic plants stomata remained widely open in both light and dark periods indicating abnormal functioning of stomata. The reduction of stomatal density has been reported in the hyperhydric leaves of regenerated carnarion (Olmos and Hellin, 1998). Lowering the relative humidity in the culture microclimate by using forced ventilation may greatly contribute towards the normal functioning of these stomata (Figure 10). Cauliflower plantlets grown in a well aerated vessel (forced ventilation) also showed functional stomata (Figure 10; Zobayed et al*.,* 2001b). The following table (Table 1 and Figure 11) summarizes the major characteristics of stomata from leaves of photoautotrophic and photomixotrophic plantlets.

Table 1. Major characteristics of stomata of leaves of plants grown under photoautotrophic and photomixotrophic conditions.

Stomata of the leaves of plants grown photoautotrophically	Stomata of the leaves of plants grown photomixotrophically
Functional stomata	Non functional stomata
High density	Low density
Smaller in size	Generally larger in size
Low stomatal conductance	High stomatal conductance
High ability of conserving water after transplanting ex vitro	Poor or low ability of conserving water after transplanting ex vitro

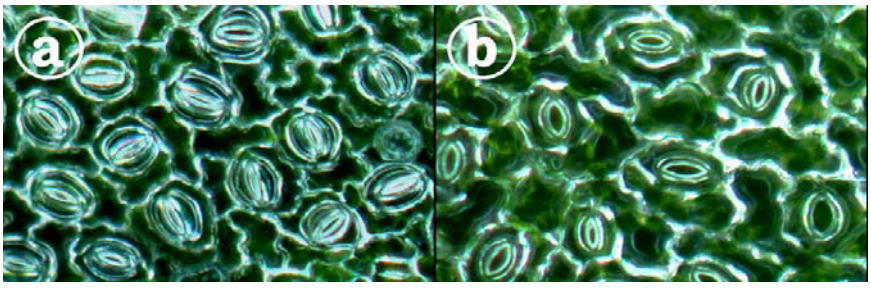

Figure 11. Stomata of the leaves of sweetpotato plantlets under dark condition a) Photoautotrophic. Note the high density and closed state of stomata and b) Photomixotrophic. Note the low density and opened state of stomata.

4.6. Root Formation

Improved root system is essential for plant growth *in vitro* not only because it plays a role in water and nutrient uptake but also replace water loss by the shoots especially during the acclimatization stage. An excellent study into the effects of different growth substrates (Florialite, vermiculite, sorbarods) and gelled media (gellangum and agar) on root formation and a mean of improving the root and /or shoot growth was that of Afreen et al. (2000). Among other things they concluded that selection of supporting material, in addition to controlling the culture microclimate, is very important for achieving better growth. In their case it was the use of Florialite (a mixture of vermiculite and cellulose fibre). In Florialite grown sweetpotato plantlets the main adventitious root gave rise to dense growth of fine lateral roots which could have resulted in a higher nutrient absorption capacity and can also be a major source of oxygen efflux. Thus the extensive root system was indirectly involved in the enhancement of growth, a higher net photosynthetic rate and higher dry mass accumulation and especially higher percent survival ex vitro. In contrast when grown in agar medium the main adventitious root produced sparse, short laterals, which explained the poor shoot growth of the plantlets *in vitro* followed by the poor survival percentage *ex vitro*. In both cases the plantlets were grown photoautotrophically. Chrysanthemum plantlets produced better root system when grown in cellulose plugs saturated with nutrient solution instead of gelling agents (Roberts et al., 1994).

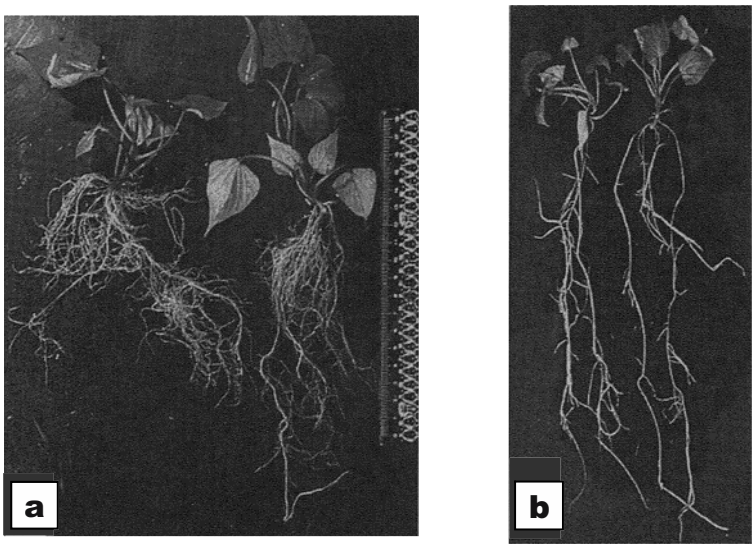

Figure 12. Root growth of sweetpotato plantlets cultured photoautotrophically for 21 days in a) Florialite and b) Agar matrix. (after Afreen et al., 2000).

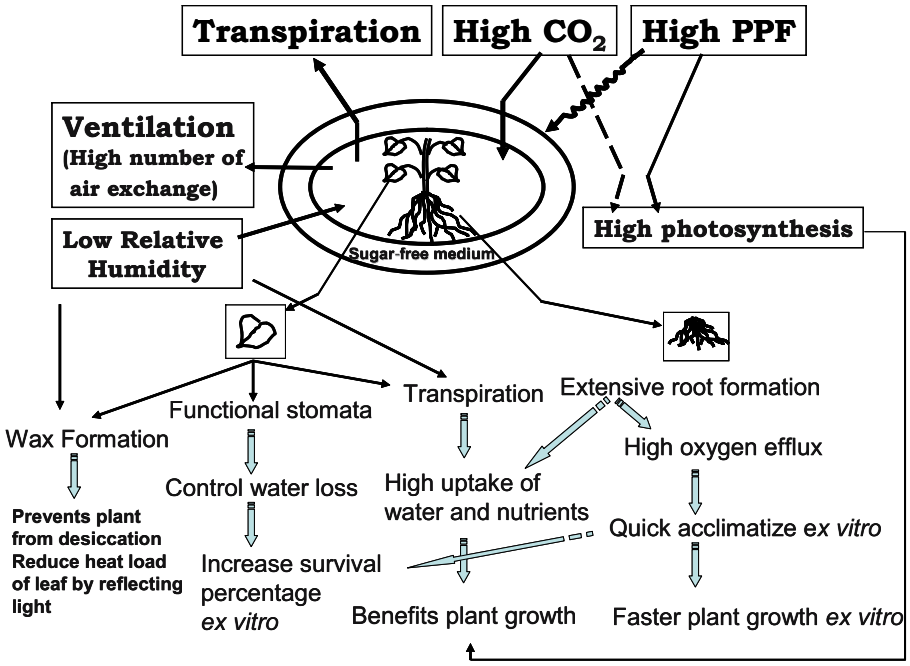

Figure 13. Schematic diagram of plant growth in photoautotrophic conditions.

5. CONCLUSION

The intrinsic quality of plants produced *in vitro* is one of the key factors governing the percentage of survival during the acclimatization to greenhouse or field conditions. The condition of a plant at anytime is the summation of the effects of all the environmental conditions it has experienced up to that time. It is therefore of the utmost importance that as many factors as possible should be under control at all stages of growth. Nevertheless, environmental conditions of the plant microclimate cannot be controlled directly in the conventional system of plant tissue culture as it can be in photoautotrophic system. In conventional photomixotrophic system the microclimate exposes the culture to unnaturally high relative humidity, low exchange of gases between the culture vessel and the culture room, especially the low PPF coupled with the presence of sugar in the medium suppresses the need and opportunity for photosynthesis. As a result morphological and physiological abnormalities such as malfunctioning of stomata, thin and unorganized palisade and mesophyll layer in the leaf, less or no wax deposition on the leaf surface etc. become obvious. The abnormalities and malfunctioning of plants in the conventional airtight system emphasize the need for the optimization of the *in vitro* culture condition. To overcome these biologically damaging side effects, the recommendation is

deduction of sugar from the growing medium, increment of light, CO_2 and ventilation of the culture vessel microclimate. The elimination of the *ex vitro* acclimatization stage, the enhancement of the latent photosynthetic ability of plantlets and simplification of the culture medium are the major advantages of photoautotrophic micropropagation (Figure 13). Photoautotrophic micropropagation of plantlets is rather a new technique and thus our knowledge of sugar-free medium micropropagation owes much to the researches done so far to unravel and better understand the underlying factors which regulate the growth and development of plantlets in the absence of exogenous source of carbohydrate.

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Chapter 7

F. AFREEN & S.M.A. ZOBAYED

PHOTOAUTOTROPHIC PLANT CONVERSION IN THE PROCESS OF SOMATIC EMBRYOGENESIS

Contents

Key words: Bioreactor, chlorophyll florescence, CO_2 concentration, forced ventilation, natural ventilation, net photosynthesis, somatic embryo, stomata.

1. INTRODUCTION

Somatic embryogenesis is a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (von Arnold et al., 2002). Somatic embryogenesis has emerged as an important tool for vegetative propagation, which offers the promise of a cost effective, large-scale propagation method and is considered as a unique alternative

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 91-122.

technique to overcome some of the limitations of conventional clonal propagation. Since the first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward et al. (1958) and Reinert (1958), the potential for somatic embryogenesis has been shown in a wide range of plant species. Starting with the first accounts of somatic embryogenesis in carrot, there have been a steadily increasing number of reports of embryogenic induction from somatic cells in a variety of plants, initially confined to members of the carrot family (Umbelliferae) and later extending to members of angiosperm and gymnosperm families. No consolidated listing of these plants is currently available, but separate listings of herbaceous dicots (Brown et al., 1995), herbaceous monocots (Krishnaraj and Vasil, 1995), woody angiosperms and gymnosperms (Dunstan et al., 1995), and angiosperms in general (Thorpe and Stasolla, 2001) have been published in recent years.

The most promising application of somatic embryo is in the field of genetic engineering where by means of somatic embryos specific and directed changes are introduced into elite individuals. As an embryo originates from a single cell or a group of cells, plants derived from somatic embryos tend to be genetically alike (Yasuda et al., 1985). In general, the production cost of plant propagation via somatic embryogenesis is potentially lower than that of microcuttings especially when bioreactors and automation procedures are introduced in the production process. The production advantages using somatic embryogenesis include: 1) a large number of uniform plantlets can be produced inexpensively; 2) production of both root and shoot meristems occur in the same propagation step; 3) easy and quick scale-up can be achieved via liquid culture; 4) long term storage via cryopreservation can be utilized; 5) there are opportunities for use of manufactured seeds (synthetic seeds) for easy handling and direct delivery to nursery. In forestry, the production of manufactured seeds throughout the year provides a complementary technology, which will reduce risks relative to seed orchards where seed production is limited and uncertain.

One of the main limitations for successful commercial application of this technology is transferring these embryos under greenhouse or field conditions. Somatic embryos do not survive if transplanted directly from *in vitro* to harsh *ex vitro*. Germinating the embryos *in vitro* is yet another costly step. The production of quality somatic embryos, which have the ability to survive *ex vitro*, is essential for the application of somatic embryogenesis technology. Efforts can be taken while embryos are still in culture to acclimatize them for transfer *ex vitro*. This chapter presents a review of somatic embryogenesis for the large-scale plant propagation with emphasis on the quality improvement of somatic embryos via photoautotrophic culture. Coffee (*Coffea arabusta*) will be the focus of this account, since much information on the mechanism of photoautotrophic transformation of somatic embryos is available in this species (Afreen et al., 2002a and b).

2. PROCESS OF DEVELOPMENT OF SOMATIC EMBRYOS

Somatic embryogenesis is a multi-step regeneration process starting with formation

of proembryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration. Production of somatic embryos from cell, tissue and organ cultures may occur directly which involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue with or without an intervening callus phase. The embryo usually germinates and grows as a normal plant, however, functionally is a clone of the parent (same genotype). There are two systems currently used for the production of somatic embryos, they are the use of gelled medium and the use of bioreactors. Both can be used in conjunction for the production of large numbers of propagules.

Somatic embryos originate from somatic cells that contain all the necessary genes to create a complete plant. Production of somatic embryos from a single cell or a group of cells is a result of down regulating somatic gene expression and turning on embryogenic genes. Such occurrences are notable in many species for instance in coffee (Figure 1) where globular, heart-shape, torpedo shape, precotyledonary, cotyledonary and germinated somatic embryos developed from leaf disc after 14 weeks of culture. Plant regeneration via somatic embryogenesis includes five steps:

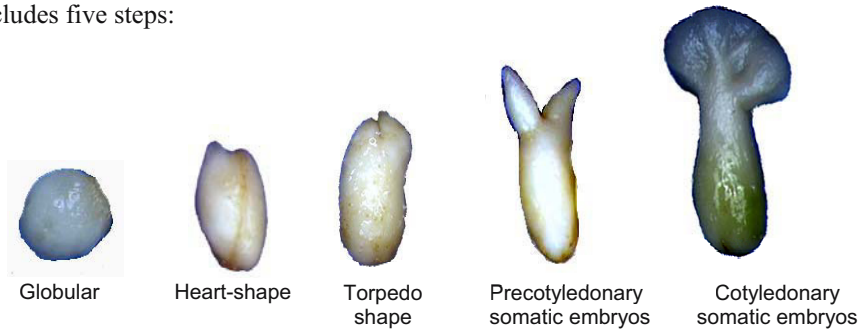

Figure 1. Different stages of coffee somatic embryos

2.1. Initiation of embryogenic cultures

Culturing the primary explant on medium supplemented with plant growth regulators, mainly auxin but often also cytokinin, is the first step where embryogenic cultures can be initiated. Somatic cells within the plant contain all the genetic information necessary to create a complete and functional plant. It has been proposed that plant growth regulators and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis.

2.2. Proliferation of embryogenic cultures

On solidified or liquid medium supplemented with plant growth regulators, proliferation of embryogenic cultures is the next step to develop somatic embryos. Once embryogenic cells are formed, they continue to proliferate, forming

pre-embryonic masses. Auxin is required for proliferation of pre-embryonic masses but is inhibitory for the conversion of pre-embryonic masses into somatic embryos (de Vries et al., 1988a and b; Nomura and Komamine, 1985). The degree of somatic embryo differentiation which takes place in the presence of auxin varies in different species. Globular, heart-shape, torpedo and cotyledonary are the different developmental stages of somatic embryogenesis (Figure 1).

2.3. Maturation of somatic embryos

During the maturation stage, somatic embryos undergo various morphological and biochemical changes. The storage organs, the cotyledons, expand concomitantly with the deposition of storage materials, the repression of germination and the acquisition of desiccation tolerance. Somatic embryos accumulate storage products that exhibit the same characteristics as those of the zygotic embryos.

2.4. Germination or embryo-to-plantlet conversion

Somatic embryogenesis is a complex process in which the quality of the final product, i.e. the survival and growth of regenerated plants, depend on the conditions provided at earlier stages, when mature somatic embryos are formed and germinate. Therefore, in order to develop mass propagation of somatic embryo plants, a better understanding of critical factors that might contribute to *ex vitro* performance of plants is required. Only mature embryos with a normal morphology and which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants. Somatic embryos usually develop into small plants, comparable to seedlings. When the plants have reached a suitable size they can be transferred *ex vitro*. In many reports, it has been shown that somatic embryo derived plants grow as seed plants do.

3. PROBLEMS RELATED TO EMBRYO-TO-PLANTLET CONVERSION AND GROWTH

The quality of a somatic embryo when used for commercial mass production of clonal transplants is determined by its maturation and germination ability (Afreen et al., 2001). One of the challenges preventing the wider commercial application of somatic embryogenesis to mass production of clonal transplants is the low percentages of embryo conversion to plantlets and problems related to *ex vitro* acclimatization. Although great progress has been made in protocol development in the field of somatic embryogenesis, it has been revealed that some treatments during embryo development and maturation, which increased yield of somatic embryo, can cause adverse effects on the embryo quality (Figure 2), thereby impairing germination and *ex vitro* growth of somatic embryo derived plants (Berthouly and Etienne, 1999). In the conventional system using sugar-containing medium in an airtight vessel, for embryo development and embryo-to-plantlet conversion and growth, a number of steps are involved (Gupta et al., 1993):

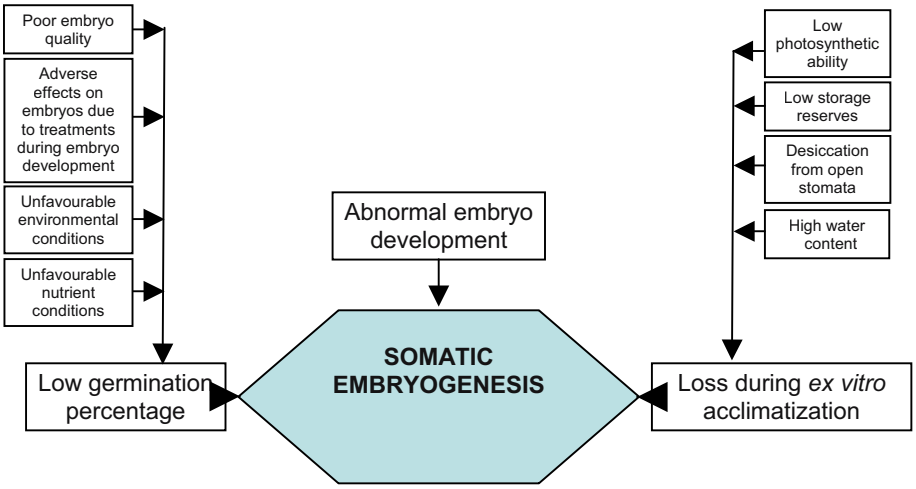

Figure 2. Schematic diagram showing the associated problems in the process of somatic embryogenesis.

i) Regeneration of somatic embryos
ii) Embryo maturation
iii) Embryo selection, occasionally desiccate and transfer on the germination medium; once embryos are formed each one must be excised to be germinated
iv) Germinated and rooted plantlet selection and transfer to soil and
v) Acclimatization *ex vitro*

However, these procedures are still time consuming and involve high labor cost. Figure 3 shows the predicted labor cost involved in different steps of somatic embryogenesis. Labor cost is generally low at the early stages i.e. during regeneration of somatic embryos and increased significantly during the later stages especially during the germination and *ex vitro* acclimatization. Once the embryos are developed, cotyledonary, late cotyledonary or germinated somatic embryos are usually selected individually by hand often under the stereomicroscope. The invention of machine vision and image analysis systems (Cazzulino et al., 1991) offer great potential for classifying and sorting embryos but the use is still limited. Selected embryos are transferred onto gelled medium for germination or seedling development. After 6-10 weeks of germination, plantlets with epicotyl are selected mostly by hand, transferred to soil in a greenhouse with frequent misting for acclimatization and growth. All of the procedures involve high labor cost although the germination percentage is often reported to be low in many species. One method to increase the percent germination and the vigour of plantlets from somatic embryos is to provide a synthetic endosperm as a coating to the somatic embryos

(Redenbaugh and Walker, 1990; Redenbaugh et al., 1988). This approach has achieved little success, perhaps because of the poor uptake of the added nutrients by the embryo axis, leaching of the nutrients during germination, or toxicity of the coating.

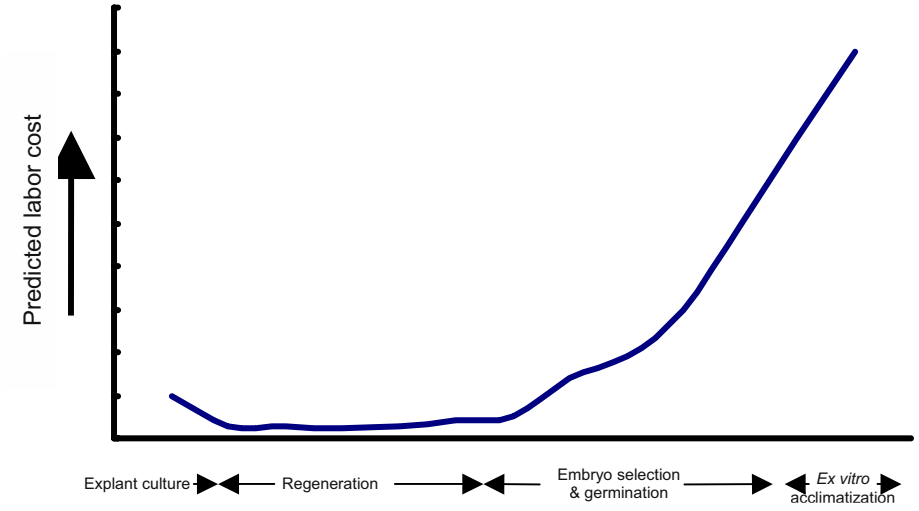

Figure 3. Predicted labour cost during the process of somatic embryogenesis.

A number of problems also remain in the *ex vitro* acclimatization of somatic embryo derived plants (Figure 2). Somatic embryos or the young plants derived from somatic embryos do not have sufficient storage reserves necessary for independent autotrophic growth; they usually have high water content and low photosynthetic ability, and rapid desiccation occurs through open stomata. In order to efficiently regulate the formation of plants via somatic embryogenesis it is important to understand how a somatic embryo develops and the influence of different physical and chemical environments on different developmental stages.

4. PHOTOAUTOTROPHIC CULTURE AND SOMATIC EMBRYOGENESIS

In the process of photosynthesis, plants use light to convert atmospheric carbon dioxide into carbohydrate i.e. sucrose. Sucrose is one of the major end products of photosynthesis. In the conventional plant micropropagation, sucrose is generally supplied to the growth medium and absorbed by plants through roots. The exogenous supply of sucrose makes the plant incompetent for the process of photosynthesis and thus the plant becomes completely or partly dependent on supplied sucrose for the source of carbohydrate. In photoautotrophic micropropagation the *in vitro* plantlets are grown in sugar-free medium, and the growth or accumulation of carbohydrates of plants is solely dependent upon photosynthesis (Kozai and Iwanami, 1988; Kozai et al., 1986, 1987, 1988, 1990,

1991 and 1999). The concept of photoautotrophic micropropagation is derived from the research that revealed high photosynthetic ability of chlorophyllous cultures such as leafy explants and plantlets *in vitro*. The beneficial impact of photoautotrophic micropropagation on plant growth, physiology, survival and commercial plant production are detailed in several chapters of this book.

The rapid progress of somatic embryogenesis research and its prospects for application to mass production of clonal transplants prompted us to investigate the possibility of growing *Coffea arabusta* somatic embryos under photoautotrophic conditions. Considerable effort has been devoted to full automation of somatic embryo development and micropropagation (Cervelli and Senaratna, 1995). The ability of somatic embryos to grow photoautotrophically will enable automation, and will lead to the reduction of production costs. Moreover, photoautotrophic growth can improve the quality of somatic embryos and possibly shorten and simplify the germination and plantlet development procedures. In the following few sections we will describe the photosynthetic ability of different stage coffee somatic embryos, and the protocol to culture cotyledonary or germinated somatic embryos under photoautotrophic conditions.

5. COFFEE SOMATIC EMBRYOS: A MODEL SYSTEM TO STUDY PHOTOAUTOTROPHY

Coffee, originally grew in African tropical forests, has been cultivating over a period of several hundred years. Records indicate that the actual roasting and grinding of coffee goes back to about 500 AD. Coffee beans were chewed raw for centuries in Ethiopia and Yemen. Currently, coffee plays a major role in the economy of many African, American and Asian countries. The coffee plant is an evergreen, woody perennial that belongs to the Rubiaceae family. Only two species of coffee are commercially important, *Coffea arabica* and *Coffea canephora*. The low caffeine content and fine aroma of *Coffea arabica* was combined with the pathogen resistance of *Coffea canephora* (Berthouly and Etienne, 1999) in a new species named *Coffea arabusta* (Capot, 1972). *C. arabusta* has been clonally propagated to obtain genetically uniform transplants using microcuttings. However, the growth of microcuttings *in vitro* is slow (Dublin, 1980) therefore somatic embryogenesis is considered to be an effective method for the mass clonal multiplication of *C. arabusta* (Dublin et al., 1991). Generally, somatic embryos developed in sucrose-containing medium are believed to be non-photosynthetic and heterotrophic. In one of our recent studies (Afreen et al., 2002a) we investigated the physiological status in relation to photosynthetic ability of coffee somatic embryos. The results are discussed below.

5.1. Physiological variables in relation to the photosynthetic ability of coffee somatic embryos

At different developmental stages of coffee somatic embryos, the physiological variables in relation to the photosynthetic ability investigated were: CO_2

concentration in the culture vessel headspace during the photoperiod, stomatal development, density and structure, chlorophyll a and b contents of the fresh tissues of somatic embryos, chlorophyll florescence including, potential activity of PSII (Φp^{MAX}) and the electron-transport activity (Φp).

5.1.1. Evaluation of CO_2 concentration in the culture vessel headspace

The CO_2 concentration in a culture headspace is the first indication of the photosynthetic ability of any chlorophyllous *in vitro* explant or plant. We measured the CO_2 concentrations in the culture headspace of different stages of coffee somatic embryos during the photoperiod (for methodology see Afreen et al., 2002a). The CO_2 concentration in the culture headspace during the photoperiod was normally lower than the ambient (370 µmol mol^{-1}) in germinated embryos and little above the ambient in cotyledonary stage embryos. This indicates that these embryos have photosynthetic ability and that CO_2 uptake rate or carbon assimilation rate of germinated embryo is positive (Table 1). In the case of torpedo and precotyledonary stage embryos, the occurrence of comparatively higher CO_2 concentrations than the ambient indicates that respiratory activity masked any photosynthetic assimilation probably because of there being few green chlorophyllous tissues and a significant amount of non-chlorophyllous tissues without any stomatal development.

Table 1. CO_2 concentrations and rates of CO_2 uptake/production of different stages of coffee somatic embryos.

	Headspace CO_2 concentrations (µmol mol^{-1})		Percentage of ambient CO_2 concentrations		CO_2 uptake/production rate cm^3 s^{-1} per embryo ($x10^{-6}$)	
Embryos	Light	Dark	Light	Dark	Light	Dark
Torpedo	510 ± 44	613 ± 45	130 ± 9	157 ± 12	–2.2 ± 0.3	–2.4 ± 0.4
Precotyledonary	425 ± 56	558 ± 65	109 ± 8	143 ± 9	–2.1 ± 0.2	–2.6 ± 0.5
Cotyledonary	434 ± 65	734 ± 76	111 ± 12	188 ± 12	–1.5 ± 0·1	–3.3 ± 0.5
Germinated	267 ± 43	876 ± 98	69 ± 8	225 ± 17	2.1 ± 0.2	–6.3 ± 0.8

Embryos were developed and grown under a PPF of 30 µmol m^{-2} s^{-1} for 16 weeks; each value represents a mean ± s.e. of ten replicates; negative values indicate CO_2 production (modified from Afreen et al., 2002a).

5.1.2. Stomatal study

Development of stomata is essential for the physiological processes of plants because stomata act as portals for entry of CO_2 into the leaf for photosynthesis (Willmer, 1983). Anatomical studies revealed that generally stomata did not form in torpedo (Figure 4a) and precotyledonary stage embryos (Figure 4b; Table 2) whereas well-developed stomata were noticed in the cotyledonary (Figures 4c, d and

Table 2) and germinated embryos (Figure 4e, f). In a very small number of cases, early stages of stomatal development were noted in the precotyledonary stage embryos. Stomatal density was highest in the germinated somatic embryos (Figures 4e, f and Table 2). The absence of stomata in torpedo and precotyledonary stages was reflected in the limitation of these somatic embryos to photosynthesize.

5.1.3. Chlorophyll contents

Chlorophyll is known to be crucial for photosynthesis of the green plants using light energy to produce chemical energy. In the current study, low chlorophyll concentrations were recorded in the torpedo and precotyledonary stage embryos. In the cotyledonary stage embryos, chlorophyll concentrations were higher compared with those in the torpedo or precotyledonary stage embryos; chlorophyll a and b were highest in the germinated embryos (Table 2).

5.1.4. Chlorophyll florescence

The potential activity of PSII (ϕ_p^{MAX}) estimated in the dark was low in the torpedo stage and increased in precotyledonary stage somatic embryos ($\phi_p^{MAX} = 0.69$) followed by cotyledonary stage somatic embryos ($\phi_p^{MAX} = 0.84$) and was highest in germinated somatic embryos ($\phi_p^{MAX} = 0.88$) (Table 2). The actual photochemical efficiency of PSII (ϕ_p) is considered to be a good estimate of the quantum yield of photosynthetic electron transport (Genty et al., 1992 and 1989). As expected from their low PSII activity, torpedo stage somatic embryos exhibited low electron-transport activity ($\phi_p = 0.1$). An increase in the quantum yield for electron transport was observed in the cotyledonary and germinated somatic embryos (Table 2). The results clearly indicated the occurrence of photochemical activity in the cotyledonary and germinated embryos.

The results therefore revealed that the cotyledonary and germinated somatic embryos showed photosynthetic ability, stomata did not fully develop in the precotyledonary stage embryos and were absent in the torpedo stage. Low chlorophyll concentrations were noted in the torpedo and precotyledonary stage somatic embryos, and increased in the cotyledonary and germinated somatic embryos. Therefore, we suggest that the cotyledonary stage is the earliest stage somatic embryo, which can be cultured photoautotrophically to develop plantlets.

5.2. Enhancement of photosynthetic ability of coffee somatic embryos

To develop an optimised protocol for the regeneration of somatic embryos from coffee leaf disc, cultures need to be incubated in dark or at a low light intensity (PPF: 20-30 μmol m^{-2} s^{-1}) (Afreen et al., 2002a; Nguyen, unpublished data). This is because endogenous plant growth regulator concentrations and/or the sensitivity of cells to growth regulator can alter in dark or light conditions and thus the embryogenesis (Zobayed and Saxena, 2003). Hutchinson et al. (2000) reported that continuous light treatment significantly reduced the amount of the endogenous plant

growth substances in geranium (*Pelargonium* x *hortorum*) tissues with concomitant inhibition of somatic embryogenesis. Croke and Cassells (1997) also reported promotive effect of dark incubation resulting in doubling of the embryo frequency in zonal geraniums. The positive influence of dark treatment to induce bud break and thus increased *in vitro* multiplication was reported in evergreen azalea (Hsia and Korban, 1998).

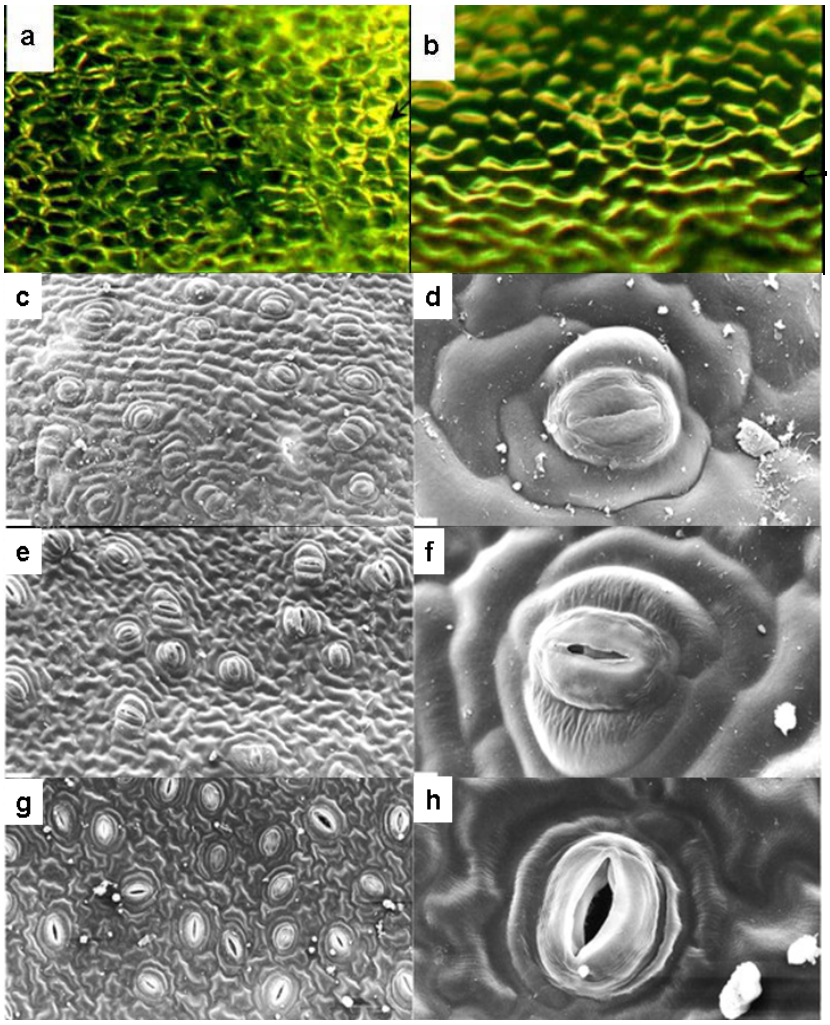

Figure 4. Images of the development of stomata in different stages of coffee somatic embryos: a) torpedo stage, b) precotyledonary stage, c and d) cotyledonary stage, e, f) germinated somatic embryos and, g, h) first true leaf of germinated somatic embryos. Note, no stomatal development in the torpedo (a) and precotyledonary stage (b) embryos.

Table 2. Maximal quantum yield (ϕ_P^{MAX}) of PSII photochemistry (in dark-adapted samples), actual quantum yield (ϕ_p) of PSII photochemistry (in light-adapted samples), stomatal characteristics and chlorophyll a and b concentrations of different stages of coffee somatic embryos.

Somatic embryos	ϕ_p^{MAX} $(F_m - F_o)/F_m$	ϕ_p $(F_m - F_s)/F_m$	Stomatal density (per mm^2)	Stomatal length (μm)	Stomatal width (μm)	Chlorophyll *a* (μg g^{-1} f. wt)	Chlorophyll *b* (μg g^{-1} f. wt)
Torpedo	0.50 ± 0.10	0.119 ± 0.006	–	–	–	39.2 ± 3.9	33.9 ± 4.9
Pre-cotyledonary	0.70 ± 0.07	0.127 ± 0.006	–	–	–	64.4 ± 4.5	60.9 ± 5.7
Cotyledonary	0.77 ± 0.07	0.178 ± 0.011	127 ± 19	24.5 ± 2	19.5 ± 2	203 ± 8.2	90.3 ± 5.6
Germinated embryos	0·86 ± 0·05	0.297 ± 0.008	170 ± 29	33.1 ± 5	29.5 ± 3	314 ± 57	118 ± 7.5

Average values of ϕ_p^{max} and ϕ_p of coffee leaves grown *in vitro* under 100 μmol m^{-2} s^{-1} PPF were 0·93 and 0.32, respectively; Each value represents a mean ± S.E. of ten (for chlorophyll florescence and chlorophyll concentrations) or 25 replicates (for stomatal measurements) (modified from Afreen et al., 2002a).

However, growing somatic embryos in dark or at low light intensity significantly inhibits or delays the development of photosynthetic pigments (Table 2). The development of photosynthetic pigments in the tissues of any chlorophyllous plants, organs, or explants requires light. For example, during the process of seed germination, early stage of seedlings usually does not have any chlorophyll and entirely depends on the reserve food materials stored in the endosperm (heterotrophic). As soon as the seedlings are exposed to the sunlight, the photosynthetic pigments starts to develop and the process of photosynthesis begins to produce carbohydrate and thus the plants became autotrophic.

In case of coffee somatic embryos we observed (Afreen et al., 2002a) that after the development of somatic embryos, they could be placed under a relatively high light intensity (PPF of 100 μmol m^{-2} s^{-1}) at least for 14 days for the development of photosynthetic pigments, functional stomata and consequent enhancement of photosynthetic ability (Figures 1, 5, 6 and 7). Placing the somatic embryos under high light intensity increased the chlorophyll concentrations (chlorophyll *a* and *b*) significantly in all stages (Figure 6). Light pre-treated embryos exhibited higher stomatal density (about 20% increases in cotyledonary somatic embryos) than those without light pre-treatment (Figure 5). Well-developed stomata were observed in the germinated embryos irrespective of light pre-treatment (Figure 4). Maximal quantum yield (ϕ^{MAX}) of PSII photochemistry (in dark-adapted samples) were also increased significantly especially in the cotyledonary stage embryos (about 10%). From these findings we concluded that pre-treatment of high PPF (100 μmol m^{-2} s^{-1}) increased the photosynthetic ability of the somatic embryos in almost all stages and the cotyledonary stage embryo is the earliest stage to grow photoautotrophically.

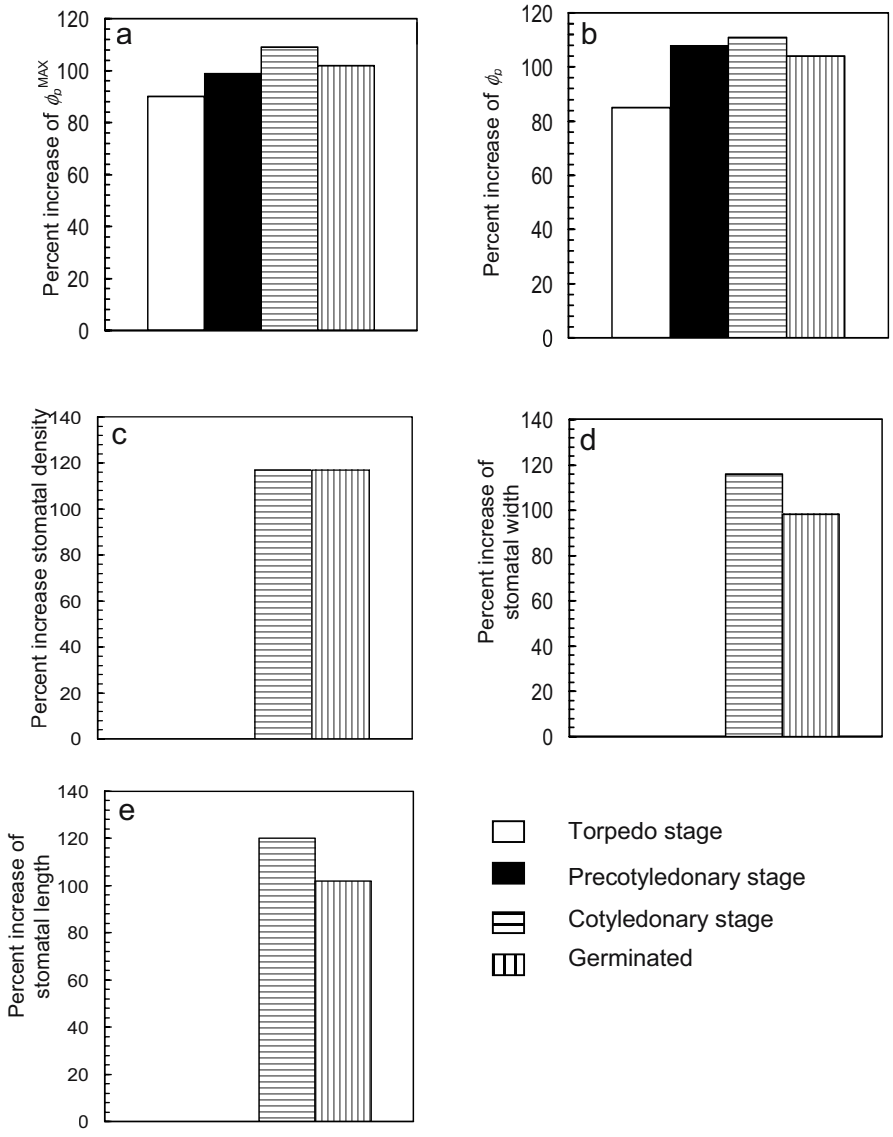

Figure 5. Percent increase of (a) ϕ_p^{MAX}, (b) ϕ_p, (c) stomatal density, (d) stomatal width and (e) stomatal length of different stages of coffee somatic embryos after 14 days high PPF (100 µmol m^{-2} s^{-1}) treatment.

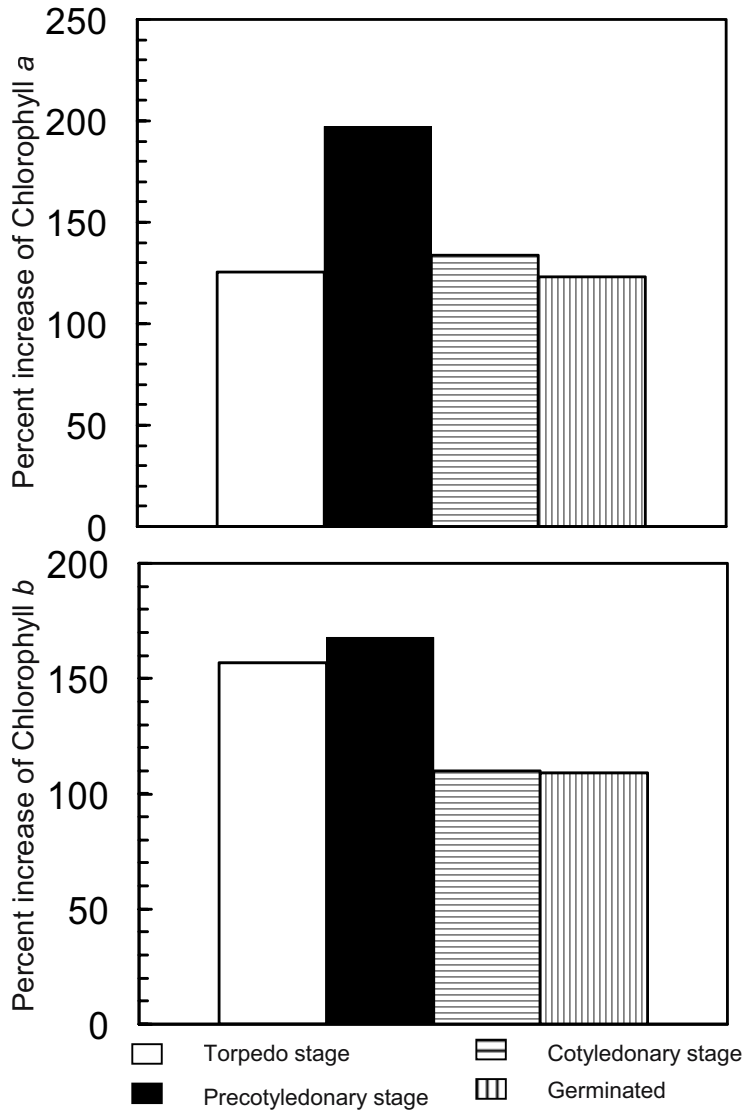

Figure 6. Percent increase of chlorophyll a and b of different stages of coffee somatic embryos after 14 days high PPF (100 µmol m^{-2} s^{-1}) treatment.

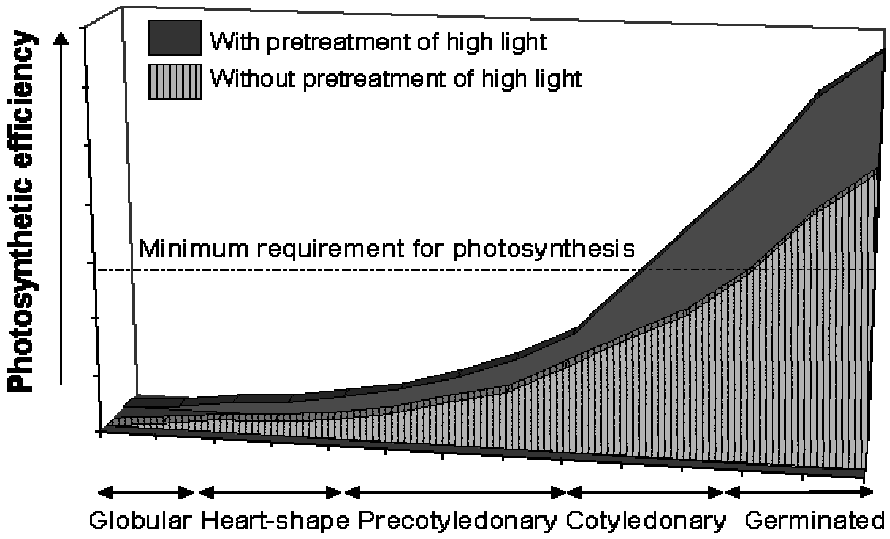

Figure 7. Enhancement of photosynthetic ability of different stages of coffee somatic embryos after 14 days high PPF (100 µmol m^{-2} s^{-1}) treatment (data source Figure 5).

5.3. Growth of different stages of somatic embryos under photoautotrophic conditions

Pre-treated (14 d with high PPF of 100 µmol m^{-2} s^{-1}) torpedo, precotyledonary, cotyledonary and germinated somatic embryos were transferred to plastic Petri dishes (volume 30 ml) containing sucrose-free MS medium gelled with agar and placed in a CO_2-enriched growth chamber (1000 µmol mol^{-1}) under 100 µmol m^{-2} s^{-1} PPF.

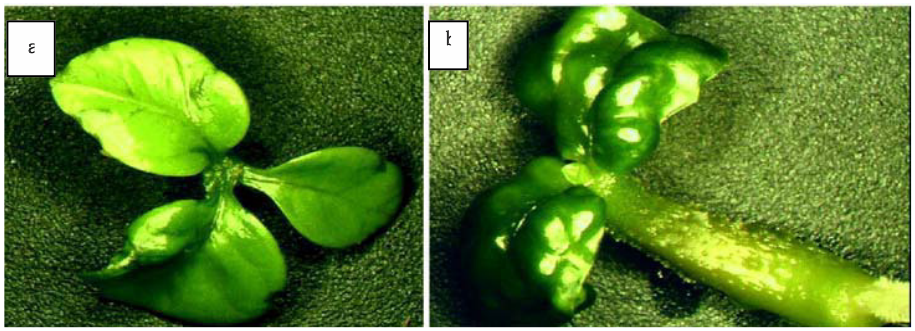

Figure 8. Coffee somatic embryos grown under (a) photoautotrophic and (b) photomixotrophic conditions (after Afreen et al., 2002a).

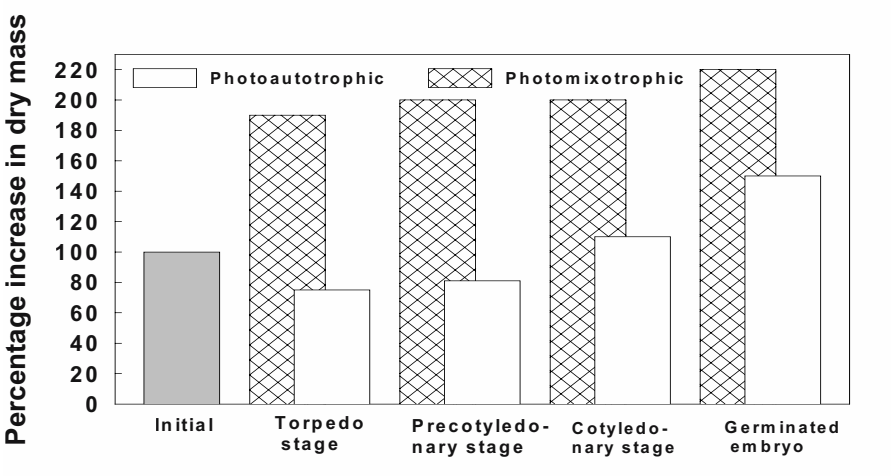

Figure 9. Percentage increase of dry mass (compared with that of the initial dry mass) of coffee plantlets developed photoautotrophically and photomixotrophically from different stages of somatic embryos after 60 d of culture. Embryos were developed and grown under a PPF of 30 µmol m^{-2} s^{-1} for 14 weeks followed by 2 weeks pre-treatment at high PPF (100 µmol m^{-2} s^{-1}) (after Afreen et al., 2002a).

The growth was compared with conventional photomixotrophic conditions (PM). In general, growth (fresh and dry mass) was greater in the PM than in the photoautotrophic (PA) conditions (Table 3; Figure 8). In case of torpedo and precotyledonary stages, fresh mass of somatic embryos were 1.9 and 1.7 times and dry mass were 2.3 and 1.8 times, respectively, under PM compared with those of PA. Similarly, the fresh mass of cotyledonary and germinated embryos were greater (40 and 42 mg per embryo, respectively) in PM than those of PA (25 and 32 mg per embryo, respectively). After 60 d of culture in the PA conditions, torpedo and precotyledonary stage embryos lost 20–25% of their initial dry mass (Figure 9). This loss could be due to continuous respiration and the low photosynthetic ability of the plant material (embryos were probably completely dependent on their own reserve food material). In contrast, in PM, the dry mass of each of the torpedo stage embryos increased by 200% of their initial dry mass (Figure 9). Under PA, the dry mass of cotyledonary and germinated embryos increased by 10 and 50%, respectively, of their initial dry mass (Figure 9).

Table 3 . Growth of different stages of somatic embryos under photoautotrophic and photomixotrophic conditions

	Total fresh mass (mg per embryo)	Total dry mass (mg per embryo)	ϕ_p^{MAX} $(F_m - F_o)/F_m$	ϕ_p $(F_m - F_s)$ $/F_m$	Chloro-phyll *a* (µg g^{-1} f. wt)	Chloro-phyll *b* (µg g^{-1} f. wt)
Photoautotrophic conditions						
Torpedo	5.1 ± 0.2	0.4 ± 0.1	0.71 ± 0.1	0.24 ± 0.05	274 ± 21	154 ± 11
Precotyledonary	6.3 ± 0.3	0.6 ± 0.1	0.76 ± 0.1	0.27 ± 0.03	343 ± 31	149 ± 09
Cotyledonary	24.9 ± 2.1	2.3 ± 0.2	0.93 ± 0.1	0.31 ± 0.06	417 ± 22	161 ± 12
Germinated embryos	32.7 ± 3.5	3.0 ± 0.4	0.92 ± 0.2	0.33 ± 0.04	405 ± 14	167 ± 19
Photomixotrophic conditions						
Torpedo	9.8 ± 1.3	0.95 ± 0.1	0.72 ± 0.1	0.25 ± 0.07	297 ± 27	145 ± 21
Precotyledonary	10.6 ± 1.0	1.1 ± 0.2	0.73 ± 0.1	0.26 ± 0.05	383 ± 23	153 ± 26
Cotyledonary	40.3 ± 2.8	3.6 ± 0.7	0.92 ± 0.7	0.33 ± 0.07	433 ± 33	165 ± 17
Germinated embryos	42.5 ± 1.4	3.2 ± 0.7	0.91 ± 0.6	0.32 ± 0.06	421 ± 26	172 ± 16
Factor A	***	***	N.S.	N.S.	N.S.	N.S.
Factor B	***	***	*	*	**	*
Factor A x B	***	*	N.S.	N.S.	*	*

Somatic embryos were developed and grown under a PPF of 30 µmol m^{-2} s^{-1} for 14 weeks followed by 2 weeks pre-treatment at high PPF (100 µmol m^{-2} s^{-1}). Factor A (different conditions, photoautotrophy or photomixotrophy) and factor B (different stages of somatic embryos); *P< 0.05; **P < 0.01; ***P < 0.001; NS, non-significant according to the Tukey test. Each value represents a mean ± s.e. of 10 replicates (after Afreen et al., 2002a).

These results are in agreement with previous findings that cotyledonary and germinated embryos show stomatal development, scavenge CO_2 and have high chlorophyll contents. In the case of cotyledonary and germinated embryos, the first true leaves to unfold were observed after 4–6 weeks of culture in both PA and PM. Precotyledonary stage embryos grown either under PM or PA conditions did not produce any true leaves; however, well-developed unfolded cotyledons were observed in somatic embryos after 6–7 weeks of culture in PM conditions. When grown photoautotrophically, about 54% somatic embryos also produced unfolded cotyledons, but these were smaller in size than those produced in the PM.

Table 4. Effects of different supporting media on the growth of cotyledonary stage coffee somatic embryos under photoautotrophic conditions.

Treatments	Number of true leaves	Total fresh mass (mg)	Total dry mass (mg)	Percentage rooting	Length of roots (mm)
Florialite	1.8 ± 0.4	61 ± 4[a]	5.3 ± 0.8[a]	69[a]	7.1 ± 2.1[a]
Vermiculite	1.8 ± 0.4	57 ± 5[a]	4.9 ± 0.7[a]	56[b]	8.2 ± 3.1[a]
Agar	1.7 ± 0.5	46 ± 4[b]	4.1 ± 1.0[b]	26[c]	1·7 ± 0.8[b]

Each value represents a mean ± s.e. of ten replicates. Means within a column followed by different superscripts are significantly different at $P < 0.05$ by the least significant difference test. All parameters except leaf number were significant at $P < 0.01$ (ANOVA) (after Afreen et al., 2002a).

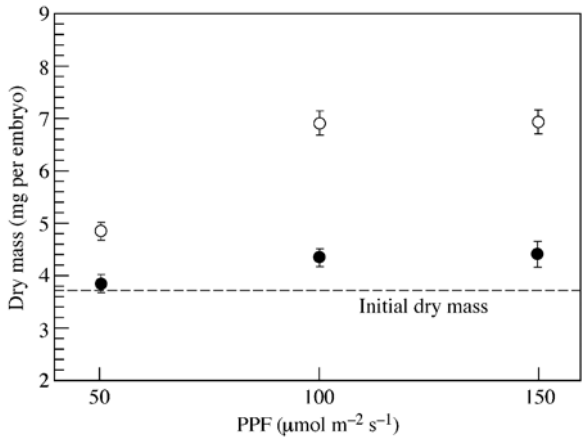

Figure 10. Dry mass of coffee plantlets developed photoautotrophically from cotyledonary stage somatic embryos grown under different PPF (50, 100 and 150 µmol m^{-2} s^{-1}) and CO_2 concentrations [400 µmol mol^{-1} (closed circles) and 1100 µmol mol^{-1} (open circles)]. Embryos were initially developed and grown under a PPF of 30 µmol m^{-2} s^{-1} for 14 weeks followed by 2 weeks pre-treatment at high PPF (100 µmol m^{-2} s^{-1}) (after Afreen et al., 2002a).

In general, normal formation of leaves was observed in embryos grown photoautotrophically; however, under PM, hyperhydricity of leaves was quite obvious (Figure 8). Interestingly, under PM, some torpedo and precotyledonary stage embryos produced new embryos from the base. Under PM, roots did not develop in torpedo or precotyledonary stage embryos, whereas 33 and 58% of cotyledonary and germinated embryos, respectively, produced roots. When embryos

were grown under PA, root formation was not observed in torpedo and precotyledonary stage somatic embryos. Some cotyledonary (15%) and germinated embryos (25%) produced roots. Taking into account the growth results, it becomes increasingly apparent that coffee somatic embryos can be grown under photoautotrophic conditions. Results also indicate that cotyledonary and germinated embryos show growth increments (compared with initial growth) when grown under photoautotrophic conditions. The values of both potential activity of PSII (Φp^{MAX}) and the actual photochemical efficiency of PSII (Φp) measured after 60 days of culture of different stage embryos were not significantly different between the photoautotrophic and photomixotrophic conditions (Table 3). In general among the different stages the values were greater in cotyledonary and germinated embryos in both the conditions. Again, the chlorophyll *a* and *b* contents of different stage embryos grown photoautotrophically were nearly the same with their photomixotrophic counterparts (Table 3).

5.4. Optimization of growth under photoautotrophic conditions

Attempts were made to culture somatic embryos under photoautotrophic conditions and to optimize different environmental conditions to maximize growth. Cotyledonary stage somatic embryos were grown photoautotrophically in three different types of supporting media: (1) agar (8 g l^{-1}; Kanto Chemical Co.); (2) vermiculite; and (3) Florialite [a mixture of vermiculite and cellulose fibre (described by Afreen et al., 2000; Nisshinbo Industries, Inc. Japan)]. The greatest total fresh and dry mass were recorded in the Florialite treatment, with values being 1.3 times those of the agar treatment. With the exception of rooting percentage, there were no significant differences among growth parameters between the Florialite and vermiculite treatments (Table 4). In agar-grown plantlets, percentage rooting was low; only 26% of embryos produced roots and those roots produced were very short. Conversely, in Florialite and vermiculite, 69 and 56% embryos, respectively, produced roots (Table 4).

To optimise the PPF and the CO_2 concentrations, cotyledonary stage somatic embryos were cultured under the following PPF and CO_2 concentrations:

(1) 50 µmol m^{-2} s^{-1} PPF and ambient CO_2 concentration of 400 µmol mol^{-1}
(2) 100 µmol m^{-2} s^{-1} PPF and 400 µmol mol^{-1} CO_2
(3) 150 µmol m^{-2} s^{-1} PPF and 400 µmol mol^{-1} CO_2
(4) 50 µmol m^{-2} s^{-1} PPF and 1100 µmol mol^{-1} CO_2
(5) 100 µmol m^{-2} s^{-1} PPF and 1100 µmol mol^{-1} CO_2 and
(6) 150 µmol m^{-2} s^{-1} PPF and 1100 µmol mol^{-1} CO_2

In general, the dry mass of cotyledonary stage embryos was enhanced when CO_2 was enriched (Figure 10). Increasing PPF further (150 µmol m^{-2} s^{-1}) did not lead to any change in the dry mass of embryos. In contrast, in low PPF (50 µmol m^{-2} s^{-1}) treatments there was hardly any increase in the dry mass (compared with initial dry mass). Therefore, results suggest that high PPF (100–150 µmol m^{-2} s^{-1}) and an

increased CO_2 concentration (1100 μmol mol^{-1}) are necessary for the development of plantlets from cotyledonary stage somatic embryos under photoautotrophic conditions.

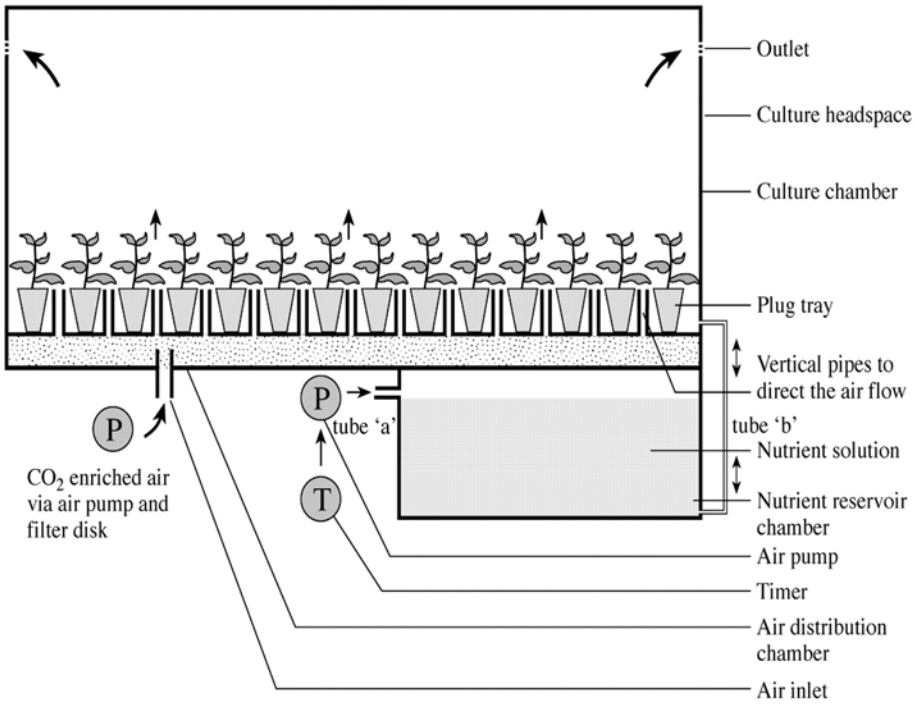

Figure 11. Schematic diagram of the Temporary root zone immersion bioreactor developed for growing cotyledonary stage somatic embryos under photoautotrophic conditions (after Afreen et al., 2002b).

5.5. Photoautotrophic culture of cotyledonary stage coffee somatic embryos: optimization of the production system

In the multi-stage somatic embryogenesis process of coffee (*Coffea arabusta*), cotyledonary stage is the earliest stage embryo, which is capable of photosynthesizing. However, the extent of plantlet heterotrophy, photomixotrophy or photoautotrophy is dependent not only on photosynthetic ability but also on medium composition, volume of culture vessels, their cross-sectional areas, and mode and amount of aeration of the vessel (Solarova et al., 1995). Therefore, we cultured cotyledonary stage coffee somatic embryos under photoautotrophic conditions in different culture systems with the aim of developing an optimized protocol for large-scale embryo-to-plantlet conversion and culture system. To achieve this objective, different *in vitro* culture systems (in solid and liquid culture

media) were compared for biomass production, photosynthetic efficiency, and percentage of germination. Pre-treated embryos were cultured under photoautotrophic conditions (in sugar-free medium with CO_2 enrichment in the culture headspace and high PPF) in three different types of culture systems:

i) Magenta vessel
ii) RITA-bioreactor with temporary immersion system (modified to improve air exchange), and
iii) Newly developed bioreactor with temporary root zone immersion system (TRI-bioreactor).

The design of the TRI-bioreactor has been described by Afreen et al. (2002b). In brief, the TRI-bioreactor consisted of two main chambers (Figure 11): the lower chamber was used as a reservoir for the nutrient solution, and the upper one for culturing embryos. A narrow air distribution chamber was located between these two chambers. Two air-inlet tubes (internal diameter 5 mm; length 10 mm) opened into the air distribution chamber and were connected directly to an air pump (Non noise S200; Artem Co. Ltd., Osaka, Japan) via a filter disc (pore diameter 0·45 μm, diameter 45 mm; Nippon Millipore Co. Ltd, Yonezawa, Japan) to prevent microbes entering the culture vessel. The top of the air distribution chamber had several narrow tubes that were fitted vertically in between the rows of the cell tray and opened in the culture chamber headspace. CO_2-enriched air entered the culture chamber from the air distribution chamber by means of these vertical tubes.

After 60 days of culture, results revealed that, in the TRI-bioreactor, almost 84% of the embryos produced plantlets, whereas in Magenta vessel and in modified RITA-bioreactor the conversion percentages were 53 and 20% respectively (Table 5). Embryos cultured in the TRI-bioreactor produced more vigorous shoot and normal roots than those grown in modified RITA-bioreactor and in Magenta vessel (Figure 12). The TRI-bioreactor grown plantlets exhibited a greater number of leaves and larger leaf area per plantlet than those noted in the modified RITA-bioreactor and Magenta vessel. Maximum leaf, stem and root dry mass were recorded in the plantlets grown in TRI-bioreactor (Table 5). In general, most of the growth variables of the plantlets grown in Magenta vessel were marginally different from those grown in the modified RITA-bioreactor. The most remarkable difference observed among the treatments was in the percentage of rooting. In the TRI-bioreactor, 90% of the plantlets developed roots; some roots produced lateral roots (Table 5). In the modified RITA-bioreactor, the roots, which developed in few plantlets, remained very small.

As mentioned above, in the TRI-bioreactor, forced ventilation, capable to control the flow rate of the inflow-air, was attached. As plantlets in the TRI-bioreactor grew, the CO_2 concentration in the culture headspace was controlled by increasing the air inflow rate and thus the number of air exchanges (Figure 13A). Thus, despite the increase in biomass, CO_2 concentrations were nearly the same throughout the experimental period (approx. 1280 μmol mol^{-1}); in contrast, in Magenta vessels and in the modified RITA-bioreactor, the number of air exchanges

could not be controlled, and were thus 3.3 h^{-1} throughout the experimental period (under natural ventilation). A reduction in CO_2 concentration was noted in the culture vessel headspace in the Magenta vessels during the photoperiod (Figure 13A). In the modified RITA-bioreactor, the CO_2 concentration in the headspace fell from 1278 μmol mol^{-1} on day 7 to 1266 μmol mol^{-1} on day 42 despite the low air exchange rate; possible reasons for this low consumption of CO_2 by plantlets include: (1) due to the small size of chlorophyllous plant materials, total CO_2 consumption is low; (2) total chlorophyll contents of the plantlets are lower than those of plantlets in other treatments; and most importantly (3), as the chlorophyllous plant material remained moist almost all the time due to immersion of whole plantlets every 6 h and the high humidity in the culture headspace, these plantlets were probably virtually unable to fix any CO_2 from the atmosphere for *in vitro* metabolism.

The highest net photosynthetic rate was observed in plantlets grown in the TRI-bioreactor (Figure 13B). In general, the highest chlorophyll content based on the fresh mass of leaves was observed in plantlets grown in the TRI-bioreactor (Figure 14A and B). Chlorophyll *a* and *b* contents were 606 and 241 μg g^{-1} fresh mass, respectively, in plantlets grown in the TRI-bioreactor, 2 and 1.6 times those of leaves of plantlets grown in the modified RITA-bioreactor. In the case of Magenta vessels, chlorophyll *a* and *b* contents of leaves were intermediate between those of plantlets grown in TRI- and modified RITA-bioreactors.

The potential activity of PSII (ϕ_p^{MAX}), as estimated in the dark, was nearly the same in leaves of plantlets grown in the TRI-bioreactor (ϕ_p^{MAX} = 0.89) and in Magenta vessels (ϕ_p^{MAX} = 0.83) (Figure 14C); in contrast, ϕ_p^{MAX} was low in leaves of plantlets grown in the modified RITA-bioreactor (0.76). A similar pattern was observed for actual photochemical efficiency of PSII (ϕ_p) (Figure 14D), which is known to be a good estimate of the quantum yield of photosynthetic electron transport (Genty et al., 1989 and 1992). An increase in the quantum yield for electron transport was noted in leaves of plantlets grown in both the TRI-bioreactor (ϕ_p reaching 0.35) and in Magenta vessel (ϕ_p = 0.32). As noted earlier, due to their low PSII activity, leaves of plantlets grown in the modified RITA-bioreactor exhibited comparatively lower electron transport activity (ϕ_p = 0.25) than those of plantlets in the other two treatments (Figure 14D).

Table 5. Plantlet conversion percentage and growth of plantlets from cotyledonary embryos of coffee grown photoautotrophically in a temporary root zone immersion bioreactor (TRI-bioreactor), a modified RITA-bioreactor and in Magenta vessels for 45 d (After Afreen et al., 2002b).

Treatment	Leaf number	Leaf area (cm^2)	Leaf f. wt (mg)	Leaf d. wt (mg)	Stem f. wt (mg)	Stem d. wt (mg)	Root f. wt (mg)	Root d. wt (mg)	Percentage rooting (%)	Plantlet conversion (%)
TRI-bioreactor	$6{\cdot}6\pm1{\cdot}4^{a}$	$2{\cdot}9\pm1{\cdot}2^{a}$	57 ± 19^{a}	$8{\cdot}2\pm2^{a}$	27 ± 7^{a}	$3{\cdot}7\pm1^{a}$	11 ± 7^{a}	$1{\cdot}2\pm0{\cdot}7^{a}$	90 ± 9^{a}	84 ± 1^{a}
RITA-bioreactor	$2{\cdot}6\pm1{\cdot}2^{b}$	$0{\cdot}8\pm0{\cdot}3^{b}$	20 ± 7^{b}	$2{\cdot}3\pm1^{b}$	18 ± 9^{b}	$2{\cdot}1\pm1^{a,b}$	$1{\cdot}7\pm0{\cdot}1^{c}$	$0{\cdot}1\pm0^{c}$	29 ± 2^{c}	20 ± 1^{c}
Magenta vessel	$3{\cdot}14\pm0{\cdot}9^{b}$	$0{\cdot}6\pm0{\cdot}4^{b}$	$12{\cdot}9\pm8^{c}$	$1{\cdot}3\pm0{\cdot}8^{b}$	$13{\cdot}6\pm8^{b}$	$1{\cdot}5\pm1{\cdot}0^{b}$	$4{\cdot}8\pm2{\cdot}1^{b}$	$0{\cdot}4\pm0{\cdot}1^{b}$	57 ± 5^{b}	53 ± 6^{b}

Each value represents a mean ± s.d. of 20 replicates. Means within a column followed by different superscripts are significantly different at $P < 0{\cdot}05$ by the least significant difference test. All parameters were significant at $P < 0{\cdot}01$ (ANOVA).

Figure 12. Coffee somatic embryos regenerated from leaf discs after 14 weeks of culture under low light (30 µmol m^{-2} s^{-1}) followed by 2 weeks under high light (100 µmol m^{-2} s^{-1}) (x0·5). B–D, 45-d-old plantlets developed from cotyledonary stage embryos under photoautotrophic conditions in a temporary root zone immersion (TRI) bioreactor (B, x0.2), a Magenta vessel (C, x0.7) and a modified RITA-bioreactor (D, x0.2). E–G, Stomata from the abaxial (lower) surface of the first true leaves of plantlets developed photoautotrophically in a TRI-bioreactor (E), a Magenta vessel (F) and a modified RITA-bioreactor (G). H and I, Individual plantlets immediately before transplanting ex vitro grown in a TRI-bioreactor (H) and a Magenta vessel (I). J–L, Root development of plantlets grown in a TRI-bioreactor (J), a Magenta vessel (K) and a modified RITA-bioreactor (L). M–O, On day 30 after transplanting plantlets previously grown in a TRI-bioreactor (M), a Magenta vessel (N) and a modified RITA-bioreactor (O) (after Afreen et al., 2002b).

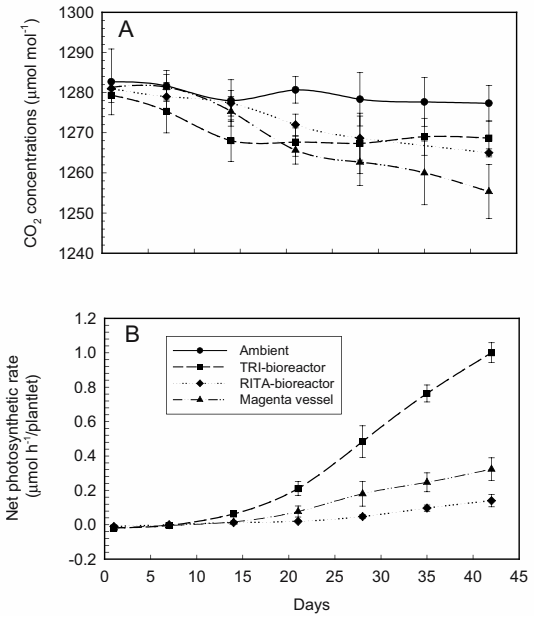

Figure 13. A, Carbon dioxide concentrations in the culture headspace of a TRI-bioreactor, Magenta vessel and modified RITA-bioreactor; B, net photosynthetic rates of coffee plantlets grown in a TRI-bioreactor, Magenta vessel and in a modified RITA-bioreactor (after Afreen et al., 2002b).

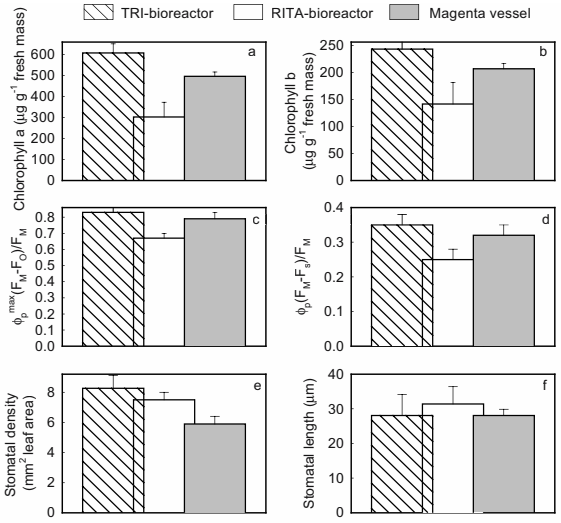

Figure 14. Chlorophyll a (A) and b (B) contents, chlorophyll fluorescence (C and D), stomatal density (E) and stomatal length (F) of leaves of 45-d-old coffee plantlets grown from cotyledonary stage embryos under photoautotrophic conditions (after Afreen et al., 2002b).

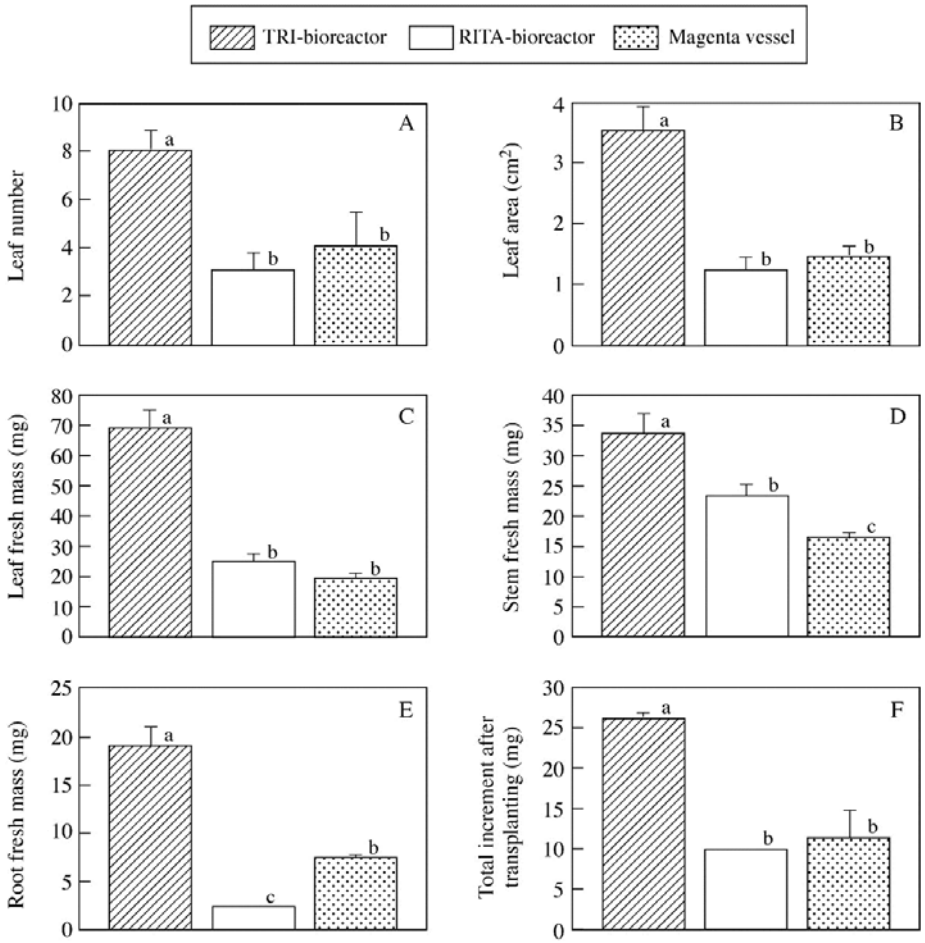

Figure 15. Growth parameters of 30-d-old coffee plantlets after transplanting ex vitro. A, Leaf number; B, leaf area; C, leaf fresh mass; D, stem fresh mass; E, root fresh mass; F, total increase of fresh mass after transplanting ex vitro. Significant difference between treatments at $P \leq 0.05$ indicated by a, b, c was determined by Student–Newman–Keuls test (after Afreen et al., 2002b).

The result clearly shows that in this treatment, the forced ventilation system provided the best conditions throughout the experiment for the assimilation of CO_2. As a result, the net photosynthetic rate, which is a closer reflection of normal *in vitro* metabolism, was greater than those of plantlets grown in the modified RITA-bioreactor and in Magenta vessels (Figure 13B). Moreover, growing plantlets under the optimum physical, chemical and most importantly the environmental conditions are also helpful to increase the chlorophyll concentration, which is essential to maximize the photosynthesis process.

Microscopy highlighted the differences among treatments with respect to stomatal density (Figure 12e–g), which was highest in leaves of plantlets grown in the TRI-bioreactor (8.3 mm^{-2} leaf area) followed by those of plantlets from the modified RITA-bioreactor (7.5 mm^{-2} leaf area) (Figure 14E). Compared with the other treatments, stomatal density was lowest in leaves of plantlets grown in Magenta vessels (5.9 mm^{-2} leaf area) (Figure 14E). Average stomatal length was nearly the same in leaves of all three treatments (Figure 14F). The most noticeable feature was that some stomata that developed in the leaves of plantlets grown in the modified RITA-bioreactor were open wide (Figure 12G), while others were distorted or still morphologically immature. It is possible that these stomata may not function properly, although no specific attempt was made to investigate this in the present study.

The results showed that for the plantlet conversion from cotyledonary stage embryos under photoautotrophic conditions, Magenta vessel and modified RITA-bioreactor resulted in the lowest growth regime. Our results also highlighted that for the embryo-to-plantlet conversion under photoautotrophic conditions the use of modified RITA-bioreactor was less effective at promoting shoot and root growth compared with the newly developed TRI-bioreactor system. This is most likely to be because in the modified RITA-bioreactor after every immersion of the plant material with nutrient solution, the entire plant became wet and, because the relative humidity inside the vessel is normally high (95-99%), the plant material either is never completely dried out or takes a long period to dry out. Thus, this thin layer of water surrounding the plant material acts as a liquid boundary layer, which impedes the exchange of gases between the plant and the surrounding environment and possibly prevents the CO_2 fixation in the chlorophyll-containing zones - clearly a key factor for the photoautotrophic growth of embryos. In case of conventional photomixotrophic systems, the media contain sugar and therefore the lack of air exchanges may not be as serious a consequence as it is for the plantlets which completely depend on CO_2 in the atmosphere for their photoautotrophic growth.

Again, it is emphasized that the RITA-bioreactor system has not been developed for culturing plantlets under photoautotrophic conditions. Also, in this study, the RITA-bioreactor was modified by attaching three gas permeable filter membranes on the lid, as was done for Magenta vessels. Thus, a completely different result can be expected if the original RITA-bioreactor with sugar-containing nutrient solution was to be used.

After transplanting under glasshouse conditions, a similar trend was noted in terms of survival percentage and growth (Figure 12M–O). *Ex vitro* survival, which was recorded on day 15 of transplanting, was highest (89%) in plantlets grown in the TRI-bioreactor. Plantlets grown in Magenta vessels had a survival percentage of 67%, although their growth was much slower than that of plants grown in the TRI-bioreactor. When plantlets from the modified RITA-bioreactor were transferred *ex vitro* only 33% survived. In terms of *ex vitro* growth, it was noticeable that plants from the TRI-bioreactor exhibited much faster growth (Figure 12M) and, as a consequence, after 30 d of transplanting almost all the growth parameters were significantly greater than those of plants grown in modified RITA-bioreactors and

Magenta vessels (Figure 15). The leaf number and leaf area of TRI- bioreactor grown plants were 2.7 and 2.8 times greater, respectively, than those of plants from the modified RITA-bioreactor, and 2.0 and 2.7 times greater, respectively, than those of plants from the Magenta vessel on day 30 after transplanting (Figure 15A and B). Similarly, leaf and root fresh mass were also enhanced and were 69 and 19 mg per plant, respectively, in the TRI-bioreactor grown plants, compared with 19 and 7.3 mg per plant, respectively, in the Magenta vessel grown plants and 25 and 2.4 mg per plant, respectively, in plants from the modified RITA-bioreactor (Figure 15C and E). During the present study, it became increasingly apparent that the vigorous growth (Figure 15F) and higher survival percentage observed in plants from the TRI-bioreactor could be the result of many environmental and physiological conditions during the *in vitro* culture period: for example, the relative humidity under forced ventilation was lower (85–90%) than that in the modified RITA-bioreactor (95–99%) or in Magenta vessels (95%). Smith et al. (1992) suggested that reducing the relative humidity in the culture headspace could improve resistance to wilting of micropropagated grapevine. In a previous experiment (Zobayed et al., 2000), we found that lowering the relative humidity in the culture headspace by introducing forced ventilation can increase the deposition of epicuticular wax on the leaf surface, which can, in turn, prevent water loss after transplanting and thus increase the chance of survival and subsequent growth.

6. ADVANTAGES OF PHOTOAUTOTROPHIC CULTURE OF SOMATIC EMBRYOS

Photoautotrophic micropropagation is the propagation of plantlets using relatively small chlorophyllous explants in a sugar-free nutrient medium under pathogen-free conditions where the plantlets can photosynthesize and produce their own carbohydrate for growth. Kozai et al. (1988) have successfully developed a system for photoautotrophic micropropagation by increasing CO_2 concentration and PPF (photosynthetic photon flux) in the culture vessel.

The major benefits of photoautotrophic micropropagation of somatic embryos are:

i) Photoautotrophic growth can improve the quality of somatic embryos. It can reduce the hyperhydricity and the development of abnormal embryos. It can possibly shorten and simplify the germination and plantlet development procedure. Photoautotrophic culture can improve the embryo-to-plantlet conversion percentage and acclimatization *ex vitro* (Afreen et al., 2002a and b; Figure 16).

ii) Relatively uniform growth in size and shape and uniform development are expected.

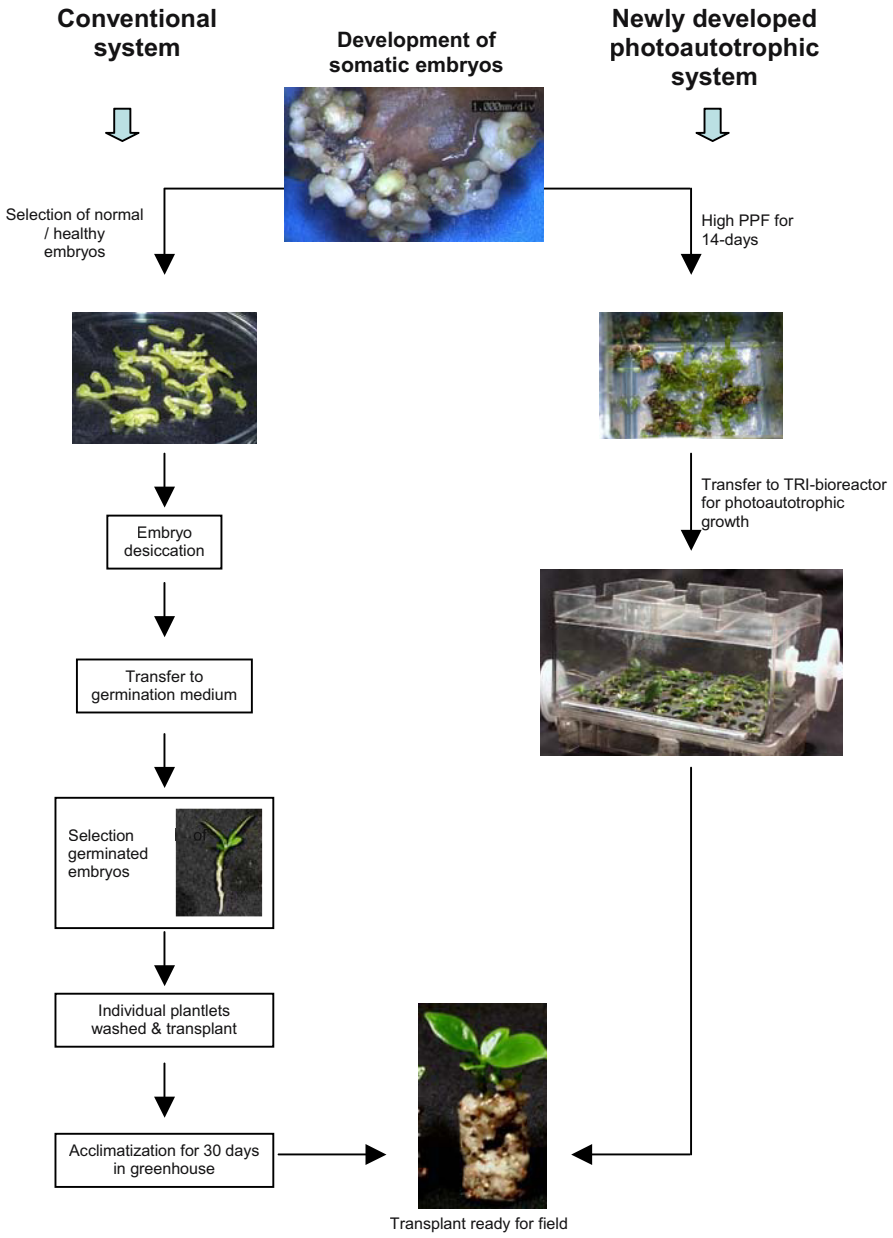

Figure 16. Schematic diagram showing the plant production protocol, both conventional (PM) and the photoautotrophic system (PA) of coffee through somatic embryogenesis.

iii) Application of growth regulators and other organic substances such as amino acids and vitamins to the culture medium can be eliminated or minimised. Use of some growth regulators is sometimes crucial in the conventional culture system to germinate somatic embryos.

iv) Problems related to synthetic seeds containing sugar and other organic nutrients can be overcome by using photosynthetically active somatic embryos.

v) Considerable effort has been devoted to full automation of somatic embryo development and micropropagation (Cervelli and Senaratna, 1995). The ability of somatic embryos to grow photoautotrophically will be beneficial for automation, robotization and computerization and contributes to reduction of production cost.

vi) Asepsis in the culture vessel for embryo-to-plantlet conversion may not be required if pathogen free status in the culture vessel is certified.

vii) Photoautotrophic culture of somatic embryos will contribute in reducing production costs specifically by reducing the labor input, and improving the plant quality. In the commercial micropropagation, labor usually accounts for about 70% of the total *in vitro* and *ex vitro* costs (Aitken-Christie et al., 1991).

7. CONCLUSION

The important consequences of the study are that the cotyledonary and germinated embryos have photosynthetic ability and that the light pre-treatment (PPF of 100 μmol m^{-2} s^{-1}) speeds up the process. Therefore, it is concluded that the cotyledonary stage is the earliest stage, which can be cultured photoautotrophically (with no supply of sugar to the culture medium) to develop plantlets. Afreen et al. (2002a) showed, for the first time, the photosynthetic ability of somatic embryos of Coffea arabusta and they successfully grew the somatic embryos photoautotrophically. We hope that the photoautotrophic culture system discussed here might also provide the basis of a useful model for the *in vitro* propagation by somatic embryogenesis of other important plant species.

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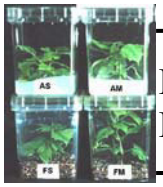
Chapter 8

Q. T. NGUYEN & T. KOZAI



PHOTOAUTOTROPHIC MICRO-PROPAGATION OF WOODY SPECIES

Contents

Key words: Air circulation, conventional vessel, forced ventilation, hyperhydricity, natural ventilation, root formation.

1. INTRODUCTION

The world population was predicted to be 8.3 billions by the year 2025 before hopefully keeping stable at about 11 billions toward the end of this century (Borlaug, 2002). The population growth would require at least that world food production increases by 2.6 billion gross tons (57%) between 1990 and 2025. It is obvious that an increase in human population is responsible for wilderness and forest destruction in the past decades. The total forest land in the tropics was decreasing at an annual rate of 0.7%, with a yearly net decrease of 12.6 million hectares, during the period 1990-1995 (FAO, 1999). The destruction in this area has

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 123-146.

become more serious recently when 17 million hectares of the forests have been deforested or destroyed per year (Morohoshi and Komamine, 2001). Moreover, desertification in arid regions has significantly decreased biomass of woody plants.

The destruction of forests and continuous use of coal and oil have led to a steady increase in CO_2 concentration in the atmospheric air, which is partly causing the recent climate changes at different geographical areas. Such serious damage of the world's environment together with shortages of food, feed, biomass and natural resources are threatening the safety of human life. It should be noticed that, in the 21st century, there will be a huge demand for harvested wood material used in pulp, paper, timber, and furniture industries as well as in houses and other constructions.

For solving the problems, the increase in plant biomass, with the aid of microorganisms and animals, is requested not only for environmental protection and conservation, or for lowering the atmospheric CO_2 concentration, but also for alternative raw materials to produce energy and many other industrial products. For reforestation of vast deforested areas in the tropical regions, there is an urgent need for 25-40 billions of quality transplants annually. However, the volume of transplants such as rooted cuttings supplied by traditional vegetative propagation methods cannot meet the demand of transplants in this century. The immediate need for mass production of quality transplants emphasizes the use of micropropagation methods.

Transplant production based on micropropagation methods has advantages over transplant production using seeds or cuttings with respect to genetic and phenotypic uniformity, enormous multiplication rate and scheduled-year-round production of disease-free or pathogen-free transplants (Aitken-Christie et al., 1995). However, the production costs for micropropagation of many plant species, especially woody transplants, were not as low as expected. The reasons for the high production cost of plants *in vitro* by conventional, heterotrophic or photomixotrophic micropropagation are: (1) a significant loss of plants *in vitro* by microbial contamination at the multiplication stage, (2) poor growth of plants *in vitro*, (3) morphological and physiological disorders which are partly due to the presence of plant growth regulators and/or unfavorable environmental conditions *in vitro,* (4) poor rooting partly due to callus formation at the plant base and thus, (5) excessive loss of these plants after transplanting in the greenhouse or field, (6) high labor cost, (7) high energy cost for lighting, cooling, sterilization, etc., (8) a significant cost for culture medium components and vessels, and (9) over- or underproduction of plants *in vitro*.

2. CONSIDERATIONS OF ENVIRONMENT *IN VITRO* FOR PLANT GROWTH UNDER HETEROTROPHIC OR PHOTOMIXOTROPHIC CONDITION

The environment *in vitro* in conventional micropropagation is generally characterized by high relative humidity (RH); low light intensity or photosynthetic photon flux (PPF); large variation in diurnal CO_2 concentration; constant air temperature; presence of sugar, vitamins, plant growth regulators and toxic

substances such as phenols in the medium; absence of microorganism; and accumulation of ethylene and other volatile gases in the vessel headspace.

In conventional micropropagation including heterotrophic and photomixotrophic culture methods, the amount of CO_2 uptake or net photosynthetic rate of plants *in vitro* is lower than plants in greenhouse or field controls (Donnelly and Vidaver, 1984). The net photosynthesis rate of birch plants regenerated *in vitro* was only one-third of those grown in the greenhouse, indicating the lack of full development of photosynthetic competency of plants *in vitro* (Smith et al., 1986). The low net photosynthetic rate of plants *in vitro* is attributed to the low RuBPcase activity (Grout, 1988), which was probably due to a high sucrose concentration in leaves of plants *in vitro* (Hdider and Desjardins, 1994).

On the other hand, CO_2 concentration in the culture vessels containing chlorophyllous or leafy plants *in vitro* decreases from a range of 3000 to 9000 μmol mol^{-1} in the dark period to lower than 100 μmol mol^{-1} in the photoperiod in conventional micropropagation (Kurata and Kozai, 1992). The low CO_2 concentration in the vessel during most of the photoperiod is partly due to a low ventilation rate of airtight vessels and a limited quantity of CO_2 in small culture vessels. The low CO_2 concentration inhibits the photosynthetic activity of plants *in vitro* and forces the plants to develop a heterotrophic or photomixotrophic growth by absorbing sugar from the culture medium as their main carbon source. Besides, at a CO_2 concentration around the CO_2 compensation point (50-70 μmol mol^{-1}), plants *in vitro* requires only a minimum PPF, because a higher PPF would not promote photosynthesis of plants *in vitro* (Kozai, 1988).

Using airtight vessels can prevent microbial contamination in *in vitro* cultures. However, the use of such culture vessels creates stagnant air, high RH and water condensation on the inner surface of the vessel. High RH in culture vessels is partly responsible for physiological and morphological disorders such as the lack of epicuticular wax formation (Brainerd et al., 1981) or hyperhydration of plants grown *in vitro* (Aitken-Christie et al., 1995). Hyperhydricity of plants *in vitro* is characterized by high water content of the leaf and stem tissues, thin palisade layers, and increase in the mesophyll air space of plants *in vitro* (Brainerd et al., 1981; Short et al., 1987).

Therefore, a reduction of RH in the culture vessel is known to reduce plant hyperhydricity. Under a low RH in the culture vessel, plants *in vitro* form normal wax, and promote the stomatal functioning. Thus, the acclimatization in the *ex vitro* stage may be shortened or eliminated.

In conventional micropropagation, ethylene accumulates at a high level up to 2 or 3 μmol mol^{-1} due to the airtight culture vessel (De Proft et al., 1985; Jackson et al., 1991). The accumulation of ethylene has an adverse effect on plant development, such as the decrease in leaf expansion and shoot length (Jackson et al., 1991). New shoot regeneration is also inhibited by a high ethylene concentration in the sealed vessel (Biddington, 1992).

The genetic fidelity is often problematic when a liquid medium containing plant growth regulators is used, and only a portion (often, 5-10%) of germinated somatic

embryos can actually be obtained as transplants. Also, the field performance of plants regenerated *in vitro* via calli and/or using bioreactors needs to be examined carefully. On the other hand, most (often, 90-95%) of plants regenerated *in vitro* from leafy single node cuttings under normal environmental conditions are considered to be genetically stable and can actually be used as transplants.

Development of new micropropagation systems for the control of *in vitro* environment is necessary to overcome the major shortcomings as described earlier for large-scale production of high quality plants *in vitro*. A great number of quality woody transplants can be produced at low cost with photoautotrophic (no sugar in the culture medium) micropropagation. Photoautotrophic or sugar-free micropropagation has become commonly used, especially in developing countries, for producing a large number of genetically superior and pathogen-free transplants at low production cost (Kozai and Nguyen, 2003).

3. NECESSITY OF PHOTOAUTOTROPHIC MICROPROPAGATION IN WOODY TRANSPLANT PRODUCTION

Photoautotrophic micropropagation requires the growth of chlorophyllous explants on a sugar-free medium under pathogen-free and favorable *in vitro* environmental conditions, which enable plants *in vitro* to photosynthesize and produce their own carbohydrates for growth. A photoautotrophic micropropagation system with the improvement of *in vitro* aerial and root zone environments has significantly enhanced the growth of plants *in vitro*, increased the multiplication rate and thus, shortened the multiplication period of plants *in vitro* (Kozai et al., 1988).

When plants *in vitro* are grown on sugar-free medium in a ventilated vessel under pathogen-free condition, loss of plants *in vitro* due to microbial contamination can be considerably reduced (microbes multiply rapidly only on the sugar-containing medium in most cases). On the other hand, by increasing light intensity and CO_2 concentration inside the culture vessel during the photoperiod, the net photosynthetic rate of plants *in vitro* on the sugar-free medium increases. Thus, the production costs of micropropagated plants can be reduced significantly if the increase in CO_2 concentration during the photoperiod and decrease in relative humidity in the vessel containing sugar-free medium can be achieved at a low cost.

Reduction of relative humidity and ethylene concentration in the vessel will promote the transpiration and the mineral uptake of plants *in vitro*, and improve physiological and morphological characteristics of plants *in vitro*, respectively (Kozai et al., 1995). In addition, plants *in vitro* are proved to grow better when gelling agent such as agar or Gelrite is replaced by air porous supporting material such as vermiculite or cellulose fibers, which improves rooting of plants *in vitro* by increasing oxygen or nutrient availability in the root zone environment.

The increase in CO_2 concentration and the decrease in relative humidity and ethylene concentration in the vessel can be achieved either by natural or forced ventilation. In the natural ventilation method, CO_2 in the vessel can be increased by enhancing the air diffusion (CO_2 and water vapor) through gas-permeable filters,

attached to the lid or sidewalls of the culture vessel. The CO_2 concentration in the vessel can also be increased by increasing the CO_2 concentration in the culture room (Kozai et al., 1987).

In the forced ventilation method, a particular gas mixture is flushed directly to the vessel with an air pump (Fujiwara et al., 1988). The advantage of this method is that a large culture vessel, which can accommodate thousands of plants *in vitro*, can be used for commercial mass propagation of plants *in vitro* (Xiao et al., 2000). The important factor in both methods is the rate of ventilation. The air circulation in the vessel increases with the increase in vessel ventilation rate and, thus, enhances the diffusion coefficient of the air in the vessel.

Photoautotrophic micropropagation offers many advantages over heterotrophic and photomixotrophic methods. The quality of plants *in vitro* produced and the lack of physiological problems with plants derived from photoautotrophic cultures are attributes with which anyone who has experience of this method can easily concur.

4. NATURAL VENTILATION SYSTEM USING SMALL CULTURE VESSELS UNDER DIFFERENT SUCROSE CONCENTRATION, CO_2 CONCENTRATION, PPF, PHOTOPERIOD AND SUPPORTING MATERIAL CONDITIONS

This section shows results on the photoautotrophic growth of woody plants using relatively small vessels like Magenta GA-7 with an air volume of 300-400 ml. For natural ventilation, microporous gas-permeable filters are attached on the hole of the lid or sidewalls of the culture vessel. The natural ventilation rate of the culture vessel is increased by the use of these filter discs. The number of air exchanges (defined as hourly ventilation rate divided by the vessel volume) of a Magenta-type vessel is about 0.15-0.2 h^{-1}. The number of air exchanges of Magenta-type vessels attached by one, two or three microporous gas filter discs (10 mm in diameter each) with a pore-diameter of 0.5 μm is about 2, 3, or 4 h^{-1}, respectively (Kozai et al., 1995). Thus, one filter disc attached on the lid of the vessel increases the vessel ventilation rate about 10 times compared to that of the conventional vessel without any filter disc. The method of measuring the number of air exchanges of the culture vessel is described in Aitken-Christie et al. (1995). By attaching one, two and three gas filter discs on the lid of the Magenta-type vessel, the CO_2 concentration during the photoperiod in the vessel containing plants increases to about 150, 200 and 250 μmol mol^{-1}, respectively, when the CO_2 concentration in the culture room is kept at 350-400 μmol mol^{-1}, as in the standard atmospheric air. Physical relationships among the ventilation rate, CO_2 concentrations in the vessel and the culture room, photosynthetic characteristics of plants *in vitro*, etc., are described using mathematical equations by Jeong et al. (1993), Aitken-Christie et al. (1995) and Kozai et al. (1995). If a CO_2 concentration in the vessel higher than 350 μmol mol^{-1} is needed during the photoperiod, CO_2 concentration in the culture room needs to be increased about 1,000 μmol mol^{-1} or higher, using a CO_2 controller, which is widely used for CO_2 enrichment with greenhouse crops. Gas permeable filter discs are currently rather expensive and its high price is restricting the commercialization of

photoautotrophic micropropagation. Reduction in price of the filter discs is expected for wider commercialization of photoautotrophic micropropagation.

4.1. Acacia (Acacia mangium)

Ermayanti et al. (1999) examined the growth *in vitro* and survival percentage *ex vitro* of acacia plants, culturing apical shoots either photomixotrophically (IBA added into a sugar-containing medium) or photoautotrophically (without IBA and sugar in the medium). A combination of high CO_2 concentration (1500 μmol mol^{-1}) in the culture room (CO_2 enrichment) and high natural ventilation rate (number of air exchanges: 6.7 h^{-1}) of the vessel increased the fresh and dry weights of plants *in vitro*, while the presence/absence of sugar and plant growth regulators did not have any significant effects (Table 1).

Table 1. Fresh (FW) and dry weights (DW), and percent of rooting of acacia (Acacia mangium) plants cultured in vitro for 28 days (Ermayanti et al., 1999). Means ± SD are shown.

Treatment					FW (mg/plant)	DW (mg/plant)	% rooting
Sucrose conc. (g l^{-1})	Growth regulator	CO_2 conc. (μmol mol^{-1})	No. of air exchanges (h^{-1})	Substrate			
30	Yes[z]	1500	6.7	Florialite	222 ± 89	40 ± 21	94
30	No	1500	6.7	Florialite	155 ± 21	32 ± 1	81
0	Yes	1500	6.7	Florialite	410 ± 145	62 ± 24	100
0	No	1500	6.7	Florialite	298 ± 4	41 ± 14	82
30	Yes	400	0.7	Florialite	152 ± 15	22 ± 2	38
30	No	400	0.7	Florialite	116[y]	17[y]	75
0	Yes	400	0.7	Florialite	110 ± 33	18 ± 4	82
0	No	400	0.7	Florialite	139 ± 38	20 ± 4	46
30 (control)	No	400	0.7	Agar	100[y]	14[y]	0
ANALYSIS OF VARIANCE[x]							
Sucrose concentration					NS	NS	NS
Growth regulator					NS	NS	NS
CO_2 enrichment and number of air exchanges (h^{-1})					**	**	NS

[z] Medium contained 1 mg l^{-1} IBA. [y] Only one replication for treatment with 30 g l^{-1} sucrose, without growth regulator and CO_2 of 400 μmol mol^{-1}.
[x] ANOVA (Analysis of variance) was applied for 9 treatments (except for the control treatment). NS, nonsignificant; **, significant at $p \leq 0.01$.

Moreover, acacia plants cultured by the conventional method (with sugar and IBA in the agar-containing medium and a low number of air exchanges of the culture vessel under CO_2 concentration of 400 μmol mol^{-1} in the culture room) showed the lowest fresh and dry weights, and no root formation (Table 1).

The rooting of plants *in vitro* was especially enhanced in Florialite treatments, a porous solid cube consisting of a mixture of vermiculite and cellulose fibers (Nisshinbo Ind. Inc., Japan), as a supporting material. Net photosynthetic rate was higher throughout the culture period in the treatment with sugar-free Florialite medium and a high number of air exchanges of the culture vessel under CO_2 concentration of about 1500 μmol mol^{-1} in the culture room.

4.2. Bamboo (Thyrsostachys siamensis Gamble*)*

Photoautotrophic growth of two-shoot clusters *in vitro* of bamboo (*Thyrsostachys siamensis* Gamble) cultured at different sugar concentrations (0 or 30 g l^{-1}), photoperiods (12 or 16 h d^{-1}) on the agar medium under ambient CO_2 concentration of 400 μmol mol^{-1} and PPF of 150 μmol m^{-2} s^{-1} was investigated by Nguyen et al. (unpublished). The number of air exchanges of culture vessels was 0.2 h^{-1} for bamboo shoots cultured on sugar-containing medium and 3.5 h^{-1} for those on sugar-free medium. On day 45, increased fresh weight and number of new, unfolded leaves of the treatment under photoautotrophic condition with larger photoperiod were the greatest among treatments.

A higher number of air exchanges combined with a larger photoperiod also increased the percent of root formation under photoautotrophic condition. After being transferred to the *ex vitro* stage for 21 days, bamboo shoots produced under photoautotrophic conditions had a survival rate of approximately 20 percent higher than those under photomixotrophic condition (data not shown).

4.3. Coffee (Coffea arabusta)

Nguyen et al. (1999a) examined the growth of single node cuttings of *Coffea arabusta* plants cultured *in vitro* at different sugar concentrations (0 or 20 g l^{-1}), types of supporting materials (agar or Florialite) and vessel ventilation rates or number of air exchanges (0.2 or 2.3 h^{-1}) at PPF of 200 μmol m^{-2} s^{-1}. Increased fresh weight, shoot length, root length and leaf area of plants cultured on Florialite with sugar-free nutrient solution under high number of air exchanges per hour were greater than those cultured on sugar-containing medium with agar or Florialite. At the end of a 40-day culture period, calli were observed at the shoot base of plants grown *in vitro* on sugar-containing medium with agar as a supporting material (Figure 1). The net photosynthetic rate of coffee plants *in vitro* was significantly increased when these plants were cultured on Florialite with sugar-free nutrient solution under high vessel ventilation rate per plant (Nguyen et al., 1999a). On the other hand, calli were not formed at the shoot base of plants grown *in vitro* on sugar-free Florialite medium (Kozai et al., 2000).

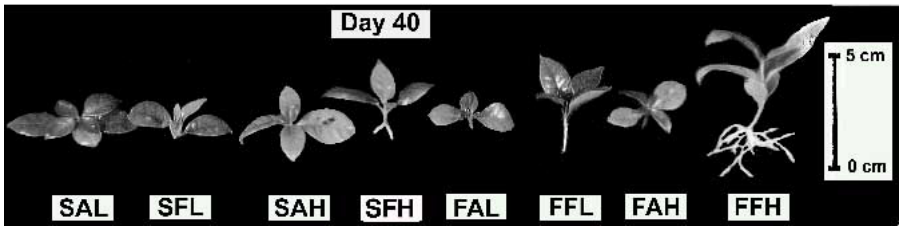


Figure 1. Coffea arabusta plants in vitro on day 40 as affected by presence/absence of sugar in the culture media, types (agar/Florialite) of supporting materials and high/low number of air exchanges of the vessel (Kozai et al., 2000).

Study on photosynthesis and related physiological parameters of coffee plants demonstrated that the CO_2 saturation point of *Coffea arabusta* plantlets was high (4500 to 5000 μmol mol^{-1}) but the light (PPF) saturation point was not as high as those of other plant species (Nguyen et al., 1999b). High CO_2 concentration significantly increased the fresh weight, shoot length, leaf area and net photosynthetic rate of coffee plants cultured *in vitro* photoautotrophically (Nguyen and Kozai, 2001).

Coffee (*C. arabusta*) somatic embryos at cotyledonary and germination stages could be cultured photoautotrophically and did not show any symptom of hyperhydricity in their leaves as compared with those cultured photomixotrophically (Afreen et al., 2002a). Pretreatment of somatic embryos at the cotyledonary stage under increased PPF conditions enhanced their photoautotrophic growth in the following culture period (Afreen et al., 2002b). These findings support the hypothesis that the growth of somatic embryos at cotyledonary stage can be enhanced significantly by controlling the environmental conditions for promoting photosynthesis (Afreen et al., 2001).

4.4. Eucalyptus (Eucalyptus spp.)

Eucalyptus (*Eucalyptus camaldulensis*) shoots with 2.2 cm long, in average, were cultured photoautotrophically *in vitro* for 6 weeks in Magenta-type vessels (air volume of 370 ml) under CO_2-nonenriched (400 μmol mol^{-1} in the culture room) or CO_2-enriched (1200 μmol mol^{-1} in the culture room) conditions (Kirdmanee et al., 1995). Each vessel contained one of four different types of supporting materials: agar, Gelrite, plastic net or vermiculite. Gas permeable filter discs (Milli-Seal, Millipore, Tokyo, Japan) were attached to the sidewalls of the vessel.

CO_2 enrichment significantly increased the growth (dry weight and number of primary roots) of plants on all types of supporting materials (Table 3, Figure 2A). The highest plant growth was obtained in the vermiculite, followed by the plastic net, Gelrite, and agar (in descending order) under either CO_2-nonenriched or CO_2-enriched conditions (Kirdmanee et al., 1995). The growth of plants *ex vitro* was the highest and the percent of damaged leaves/roots was the lowest in the vermiculite

under the CO_2-enriched condition. An extensive root system with many secondary roots was produced *in vitro* in the vermiculite (Figure 2B).

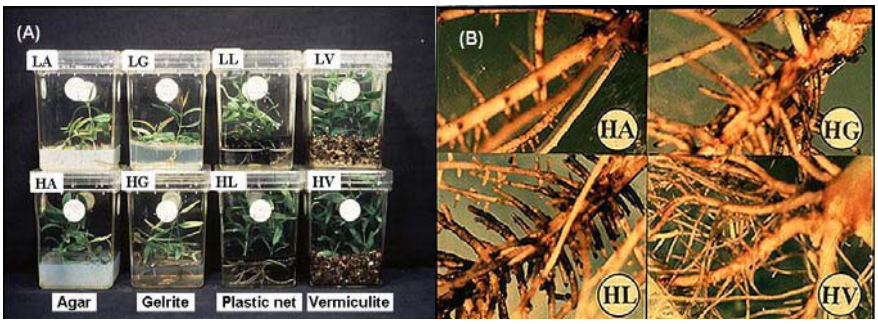


Figure 2. Photoautotrophic growth of eucalyptus (Eucalyptus camaldulensis) for 6 weeks (By courtesy of Kirdmanee C.). Eucalyptus plants in vitro under CO_2 non-enriched and enriched conditions, using 4 different supporting materials (A); and roots of eucalyptus plants in vitro under CO_2 enriched conditions (B). In the treatment legends, L and H at the left denote the CO_2 enriched and non-enriched conditions, respectively; A, G, L and V at the right denote agar, Gelrite, plastic net and vermiculite, respectively.

Table 2. Effects of CO_2 enrichment and supporting material in vitro on dry mass, leaf area, number of primary roots, and length of primary roots of eucalyptus (Eucalyptus camaldulensis) plantlets after 6 weeks in vitro (Kirdmanee et al., 1995).

CO_2 condition	Supporting material	Dry mass (mg)	Leaf area (cm^2)	No. of primary roots	Length of primary roots (mm)
Non-enriched	Agar	45	8	1	27
(400 µmol mol^{-1})	Gelrite	49	9	1	30
	Plastic net	64	11	4	39
	Vermiculite	82	12	5	42
Enriched	Agar	54	9	2	32
(1200 µmol mol^{-1})	Gelrite	62	10	2	37
	Plastic net	76	12	5	45
	Vermiculite	103	13	6	49
LSD $P \leq 0.05$		8	3	1	11
ANALYSIS OF VARIANCE					
CO_2 condition (C)		**	NS	*	*
Supporting material (S)		**	*	**	**
C x S		NS	NS	NS	NS

*, ** Significant at $p \leq 0.05$ and 0.01, respectively. NS Nonsignificant at $p \leq 0.05$

Sha Valli Khan et al. (2002) showed that after 28 days of culture there was no significant difference in growth of Eucalyptus tereticornis plants grown under conventional photomixotrophic (30 g l^{-1} sucrose, 0.3 mg l^{-1} BA) and

photoautotrophic conditions (no sucrose, high CO_2 concentration, high PPF, Florialite). Furthermore, hyperhydricity and basal callus formation were only observed with plants in the photomixotrophic condition. The net photosynthetic rate was negative at each measuring day (day 7, 14, 21 and 28).

4.5. Gmelina (Gmelina arborea Roxb.*)*

Single-node cuttings of *Gmelina arborea* Roxb. were cultured *in vitro* on the agar medium containing sugar (30 or 10 g l^{-1}) or without sugar in Magenta-type vessels with different ventilation rates or number of air exchanges (0.15, 1.5 or 3.5, respectively), under ambient CO_2 concentration of 400 µmol mol^{-1}, PPF of 180 µmol m^{-2} s^{-1} and photoperiod of 16 h d^{-1}. On day 35, plants grown on the sugar-free medium had significant greater fresh weight, shoot length and multiplication rate than those on sugar-containing medium (Table 3).

At the base of plants cultured under photomixotrophic (sugar-containing) conditions, callus was formed resulted in a retardant in root initiation and abnormal vascular systems compared with those under photoautotrophic (sugar-free) condition (Figure 3).

Table 3. Increased fresh weight, shoot length, and multiplication ratio of Gmelina arborea Roxb. plants cultured in vitro on day 35.

Treatment		Increased fresh weight (mg)	Shoot length (mm)	Multiplication ratio
Sucrose conc. (g l^{-1})	No. of air exchanges (h^{-1})			
30	0.15	194.4 [c y]	19.9 [b]	2.1 [b]
10	1.5	250.6 [b]	21.0 [b]	2.3 [b]
0	3.5	493.4 [a]	26.4 [a]	3.4 [a]
ANALYSIS OF VARIANCE [z]		**	**	**

[z] ANOVA was applied for 3 treatments. ** Significant at $p \leq 0.01$.
[y] Different letters on each column show a significant difference at the 1% level by LSD test.

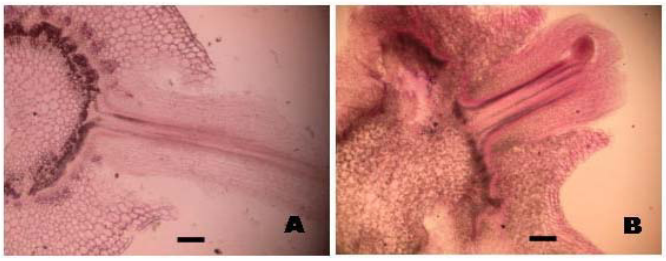


Figure 3. Root initiation (20x) of G. arborea plants cultured in vitro under photoautotrophic (A) and photomixotrophic (B) conditions observed on day 9 (Bar = 2.5 mm).

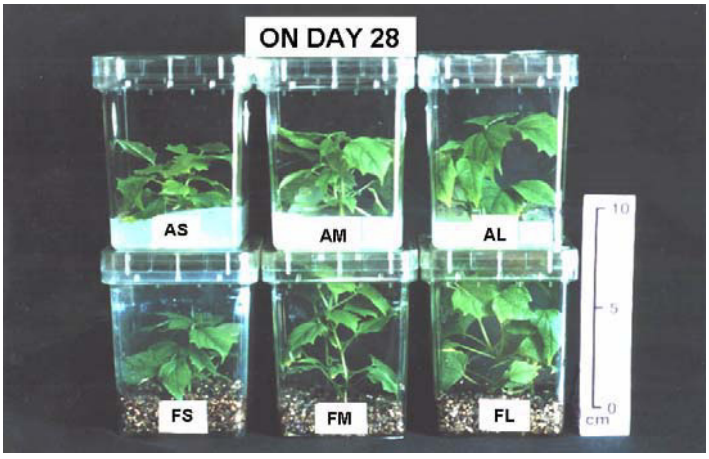


Figure 4. Photoautotrophic growth of Gmelina arborea Roxb. *Plants in vitro as affected by number of air exchanges of vessel and supporting materials on day 28. In the treatment legends, A and F at the left denote agar and Florialite, respectively. S, M and L at the right denote the number of air exchanges of 2, 3.5 and 4.2 h^{-1}, respectively (Nguyen and Kozai, 2001).*

Nguyen and Kozai (2001) demonstrated that the growth of single node *Gmelina arborea* Roxb. cuttings cultured photoautotrophically increased with the increase in number of air exchanges of the culture vessel and on the Florialite medium under ambient CO_2 concentration of 500 µmol mol^{-1} and PPF of 150 µmol m^{-2} s^{-1}. The increased dry weight, shoot length and leaf area of *Gmelina* plants *in vitro* were the greatest at the number of air exchanges of 4.2 h^{-1} compared with those of 2 or 3.5 h^{-1} on day 28 (Figure 4). The air porous supporting material such as Florialite improved root formation resulting in lower shoot/root dry weight ratios of all treatments using Florialite compared with those using agar. The development of lateral roots and

normal vascular systems was obviously enhanced by the use of high ventilation of the culture vessel and Florialite in the medium.

4.6. Mangosteen (Garcinia mangostana)

The photoautotrophic growth of mangosteen (*Garcinia mangostana*) was demonstrated by Ermayanti et al. (1999). Shoots excised from aseptically germinated seedlings were cultured on vermiculite or agar medium with the presence (30 g l^{-1}) or absence of sugar and plant growth regulators (10 mg l^{-1} of 2-ip and 1 mg l^{-1} of IBA) in the culture vessel with low (0.1 h^{-1}) or high (4.4 h^{-1}) number of air exchanges at a PPF of 110 µmol m^{-2} s^{-1} under high CO_2 concentration (1300 µmol mol^{-1} in the culture room) (Table 4).

Dry weight of mangosteen plants on day 30 was not significantly different among treatments. Addition of the growth regulator in the medium increased the number of leaves (Table 4). Twenty to forty percent of the shoots exhibited root induction in the treatments with a high number of air exchanges and vermiculite as supporting material, either with or without sugar/plant growth regulator. On the other hand, in the control treatment with a low number of air exchanges and sugar-containing agar medium, no rooting was observed. The CO_2 concentration was the lowest, and the net photosynthetic rate per leaf area in the control treatment was about ten percent of that in the treatments with a high number of air exchanges (Table 4).

Table 4. Dry weight (DW), percent (%) rooting, number of leaves (NoL) of mangosteen plants cultured for 30 days (Ermayanti et al., 1999). Means ± SD are shown.

Treatment					DW	%	NoL
Sucrose conc. (g l^{-1})	Growth regulators	CO_2 conc. (µmol mol^{-1})	No. of air exchanges (h^{-1})	Substrate	(g/plant)	rooting	
30	Yes[z]	1300	4.4	Vermiculite	0.30 ± 0.13	40	6 ± 1.0[NS y]
30	No	1300	4.4	Vermiculite	0.23 ± 0.18	20	4 ± 0.9[NS]
0	Yes	1300	4.4	Vermiculite	0.23 ± 0.10	20	6 ± 0.9[*]
0	No	1300	4.4	Vermiculite	0.20 ± 0.11	40	4 ± 0.9[NS]
30 (control)	Yes	400	0.1	Agar	0.21 ± 0.11	0	5 ± 0.9
ANALYSIS OF VARIANCE[x]							
Sucrose concentration					NS	NS	NS
Growth regulator					NS	NS	**

[z] Medium contained 10 mg l^{-1} 2-ip and 1 mg l^{-1} IBA.

[y] NS, *: Nonsignificantly or significantly different from the control treatment at $p \leq 0.05$ according to *t*-test, respectively.

[x] ANOVA was applied for 4 treatments (except for the control treatment). NS, nonsignificant; **, significant at $p \leq 0.01$.

Figure 5. Growth of neem plants in vitro as affected by sucrose concentration and supporting material on day 28.

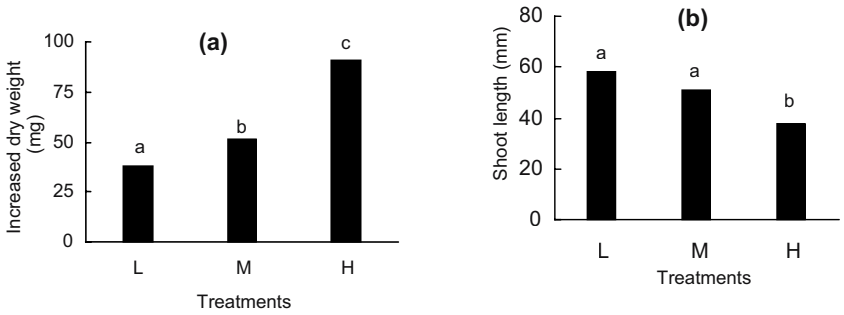


Figure 6. Increase in dry weight (a) and shoot length (b) of neem plants in vitro cultured photoautotrophically on day 40 (Nguyen and Kozai, 2001). In the treatment legends, L, M and H denote the light intensities of 70, 150 and 230 mol m^{-2} s^{-1}, respectively. Different letters on each column show a significant difference at the 1% level by LSD test.

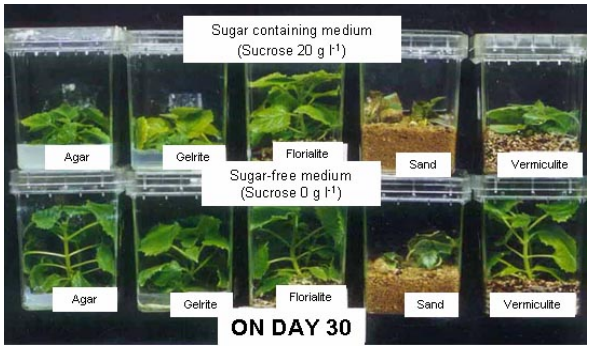


Figure 7. Growth of paulownia plants in vitro as affected by sucrose concentrations and supporting materials on day 30.

4.7. Neem (Azadirachta indica)

Single node cuttings of neem (*Azadirachta indica*) were cultured on agar or Florialite medium with presence (20 g l^{-1}) or absence of sucrose in Magenta-type vessels (air volume of 370 ml) having low (0.15 h^{-1}) or high (3.5 h^{-1}) number of air exchanges at CO_2 concentration of 450 μmol mol^{-1} and PPF of 100 μmol m^{-2} s^{-1} (Kozai and Nguyen, 2003). Leaf dry weight and total dry weight of neem plants *in vitro* were significantly greater when cultured on medium without sugar and with high number of air exchanges of the vessel than those with sugar and low number of air exchanges (Figure 5). Neem plants cultured on the Florialite medium developed significantly longer roots than those on the agar medium did.

Nguyen and Kozai (2001) demonstrated the effect of different light intensities (PPF of 70, 150, or 230 μmol m^{-2} s^{-1}) on the photoautotrophic growth of neem plants *in vitro* on medium with Florialite. Increased dry weight of neem plants *in vitro* on Florialite-based medium was significantly greater at PPF of 230 μmol m^{-2} s^{-1} than at PPF of 70 μmol m^{-2} s^{-1} (Figure 6). At high PPF (230 μmol m^{-2} s^{-1}) the shoot elongation of neem plants was suppressed, whereas the root elongation was promoted significantly on day 40 (Nguyen and Kozai, 2001).

4.8. Paulownia (Paulownia fortunei)

Nguyen and Kozai (2001) showed the *in vitro* growth of single nodal cuttings of *Paulownia fortunei* plants with two leaves for each cutting cultured without plant growth regulator in Magenta-type vessels under PPF of 120 μmol m^{-2} s^{-1} and CO_2 concentration of 450 μmol mol^{-1}. On day 30, *Paulownia* plants grew more vigorously under photoautotrophic (or sugar-free) condition than under photomixotrophic (20 g l^{-1} sucrose) condition, especially when vermiculite or Florialite was used as supporting materials in place of gelled medium such as agar or Gelrite (Figure 7).

The increased fresh weight of paulownia plants *in vitro* on the same type of supports was significantly greater under photoautotrophic condition compared with photomixotrophic condition (Figure 8). There was no significant difference in the multiplication ratio (number of cuttings for the next subculture per explant) when plants grew *in vitro* under the photoautotrophic or photomixotrophic condition on different kinds of supporting material (agar, Gelrite, Florialite, sand and vermiculite).

Kozai and Nguyen (2003) demonstrated that when paulownia plants *in vitro* were cultured photoautotrophically in a vermiculite-based medium for 28 days, high CO_2 concentration (1600 μmol mol^{-1}) and high PPF (250 μmol m^{-2} s^{-1}) significantly promoted their growth. High CO_2 concentration and high PPF enhanced the root initiation and normal root vascular development (Figure 9). Paulownia plants cultured under the CO_2-enriched and high PPF condition in the *in vitro* stage continued increasing their growth significantly during 15 days in the *ex vitro* stage.

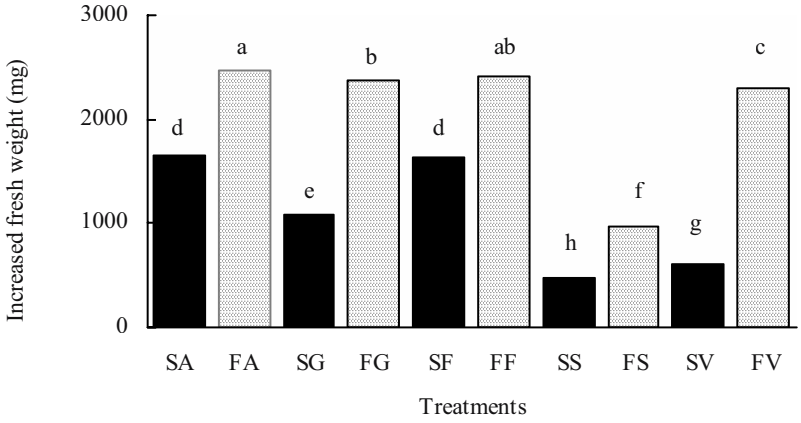


Figure 8. Increase in fresh weight of Paulownia plants in vitro as affected by sucrose concentrations and supporting materials on day 30. In the treatment legends, S and F at the left denote the present or absence of sucrose, respectively. A, G, F, S and V at the right denote agar, Gelrite, Florialite, sand and vermiculite medium, respectively. Different letters on each column show a significant difference at the 5% level by Duncan's multiple range test.

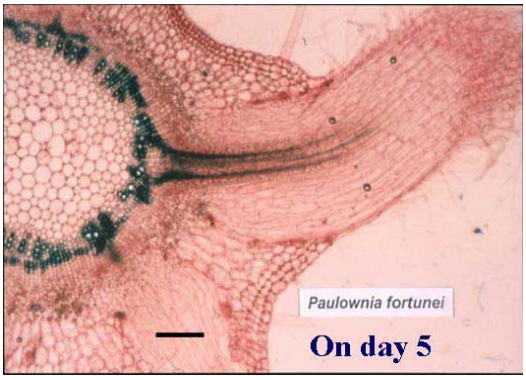


Figure 9. Transverse section at the root-shoot junction showing root initiation on day 5 of Paulownia plants grown in vitro under photoautotrophic condition with high CO2 concentration and high PPF (Bar= 5 mm).

Sha Valli Khan et al. (2003) showed that *P. fortunei* plants *in vitro* also developed a significantly large number of shoots under photoautotrophic CO_2-enriched condition. Stomata of leaf abaxial surface remained widely opened under the photomixotrophic condition, compared with those having narrow opening under the photoautotrophic condition.

4.9. Radiata pine (Pinus radiata)

The photoautotrophic growth of *Pinus radiata* shoots cultured *in vitro* for 49 days in a 500 ml vessel with 4.9 air exchanges per hour under 160 μmol m^{-2} s^{-1} PPF without CO_2 enrichment was demonstrated (Kubota, 1994). Rockwool multi-blocks were used as a supporting material with a liquid medium containing basal components of a modified LP (Aitken-Christie et al., 1988) medium. Dry weight of shoots cultured photoautotrophically was 24 percent greater than that of shoots cultured conventionally (0.1 h^{-1} air exchanges of the vessel, 60 μmol m^{-2} s^{-1} PPF, and gelled medium containing 30 g l^{-1} sucrose). Root formation did not occur in both treatments during the culture period. Under CO_2-enriched (850 μmol mol^{-1}) condition, radiata pine shoots were cultured photoautotrophically in a gelled medium with different basal compositions (Aitken-Christie et al., 1992). An addition of activated charcoal into the medium enhanced shoot elongation and fresh weight increment after 12 weeks of culture. However, no roots were formed during the culture period. As shown in other species, a combination of fibrous supporting materials and CO_2 enrichment may be beneficial for growth and rooting of radiata pine, but this hypothesis has not yet been examined.

5. AN INTERPRETATION OF PLANT GROWTH ENHANCEMENT IN PHOTOAUTOTROPHIC MICROPROPAGATION

The growth of chlorophyllous explants (shoots and leafy nodal cuttings) of the woody plant species examined above is greater under photoautotrophic conditions than under photomixotrophic conditions, in the presence of increased CO_2 concentration in the culture vessel during the photoperiod (which is achieved by the use of gas permeable filters in combination with CO_2 enrichment in the culture room). The increase in PPF is also required in most cases. Increases in CO_2 concentration and PPF promoted photosynthesis and thus carbohydrate accumulation of plants (Aitken-Christie et al., 1995; Jeong et al., 1995; Kozai et al., 1995). The use of fibrous or porous supporting materials in place of agar has been shown to be beneficial under high CO_2 concentration and PPF conditions for enhancing root initiation and function. These materials are especially effective in promoting the formation of lateral roots and normal vascular systems, which thereby benefits the overall growth and quality of the woody plants.

In addition, an increase in the number of air exchanges of the culture vessel enhances the air movement around the plants growing in the culture vessel. Then, it,

in turn, promotes the diffusion of CO_2 and water vapor around plants, resulting in the promotion of photosynthesis and transpiration of plants *in vitro* (Kitaya et al., 1996).

The increase in the number of air exchanges decreases the relative humidity in the culture vessel from nearly 100% to 85-90% (Aitken-Christie et al., 1995). The decrease in relative humidity in combination with the enhanced air movement in the culture vessel increases the transpiration rate of *in vitro* plants significantly, and thus increases water and nutrient uptake of plants *in vitro* (Aitken-Christie et al., 1995).

It should be also noted that calli are not formed at the base of the shoot when grown on sugar-free medium with porous supporting materials, which is an advantage of woody plant micropropagation. Physiological mechanism of this phenomenon needs to be studied in the future.

6. FORCED VENTILATION SYSTEMS WITH LARGE CULTURE VESSELS

As described above, the method of using gas permeable filter discs attached to the lid or the sidewalls of the culture vessel or increasing the gaseous concentrations in the culture room is a simple way to effectively increase the CO_2 concentration during the photoperiod and decrease the relative humidity and ethylene concentration inside a culture vessel. However, the CO_2 concentration and other gaseous concentrations in the culture vessel with natural ventilation are interrelated with a number of factors such as the metabolic activity of the plants *in vitro*, the plant size and leaf area, the number of air exchanges of the culture vessel and the culture room environment. Thus, the gaseous concentrations in the culture vessel with natural ventilation are often unpredictable and uncontrollable. Furthermore, it is difficult to provide a high number of air exchanges for a large culture vessel (Kozai and Nguyen, 2003).

Forced ventilation is a method involving the use of mechanical force generated by an air pump or an air compressor to flush a particular gas mixture directly into the culture vessel (often through microporous filters to prevent microorganisms from entering the vessel). In this system, the gaseous composition (CO_2, water vapor, etc.) of incoming air and ventilation rate and/or air current speed inside the culture vessel can be controlled relatively precisely by use of a needle valve, a mass flow controller or an air pump with an inverter (Aitken-Christie et al., 1995; Jeong et al., 1995). With a proper control of the gaseous composition in culture vessels, the growth of plants *in vitro* with forced ventilation can be enhanced significantly compared with natural ventilation.

Photoautotrophic micropropagation makes it possible to use large culture vessels with minimum risk of microbial contamination. In photoautotrophic micropropagation using a large culture vessel with forced ventilation, the labor cost could be reduced by nearly fifty percent as compared with that in conventional, photomixotrophic micropropagation (Xiao et al., 2000).

In 1988, Fujiwara et al. developed a large culture vessel with a forced ventilation system for enhancing the photoautotrophic growth of strawberry (*Fragaria* x *ananassa* Duch.) explants and/or plants *in vitro* 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) described a forced ventilation system using a polycarbonate vessel containing a multi-cell tray with rockwool cubes for photoautotrophic growth of potato (*Solanum tuberosum* L.) plants *in vitro*. The net photosynthetic rate of potato plants was significantly greater than plants grown in conventional (small) culture vessels with natural ventilation. Another forced ventilation micropropagation system was demonstrated by Heo and Kozai (1997). The photoautotrophic growth of sweetpotato plants cultured *in vitro* with this system was several times greater than the photomixotrophic growth of plants cultured in small culture vessels with natural ventilation.

However, in both of the forced ventilation systems mentioned above, the growth of plants *in vitro* in the culture vessel was not uniform. The plants were larger near the air inlet and comparatively smaller near the air outlet.

A new type of large culture vessel with air distribution pipes for forced ventilation was developed by Zobayed et al. (2000) with a major aim to provide an air current pattern that enables uniform distribution of CO_2 concentration and relative humidity as well as air current speed in the culture vessel, and consequently, the uniform growth of plants *in vitro*. The feasibility of this forced ventilation system has been tested for *Eucalyptus camaldulensis* (Zobayed et al., 2000) (Figure 10). A large culture vessel with a nutrient supply unit makes it possible to measure and control the pH, composition and volume of nutrient solution in the culture vessel. Moreover, the control of plant growth rate is relatively easy in such vessels with a nutrient control unit (Zobayed et al., 2000). Compared with eucalyptus plants *in vitro* cultured photomixotrophically in Magenta-type vessels, the plants cultured photoautotrophically in the large vessel with forced ventilation had a higher net photosynthetic rate, normal stomatal closing and opening, and significantly higher epicuticular leaf-wax content. Plants cultured in the large vessel with forced ventilation were, therefore, considered acclimatized *in vitro*, and their transpiration rates and percent water loss remained lower than those of conventional plants when transplanted to *ex vitro* conditions (Zobayed et al., 2001a, b).

Coffea arabusta plants *in vitro*, when cultured in a forced ventilation system, had significantly higher fresh and dry weights, shoot length and leaf area than those cultured with natural ventilation (Nguyen et al., 2001). The study included 40 days in the *in vitro* stage and 10 days in the *ex vitro* stage (Figure 11). Mean fresh and dry weights, leaf area, shoot and root lengths and net photosynthetic rate per plantlet were significantly greater in forced high rate treatments compared with those in natural and forced low rate treatments. PPF had a distinct effect on shoot length suppression and root elongation of coffee plantlets in forced high rate treatments. With the forced ventilation method in photoautotrophic micropropagation, the CO_2 concentration inside the culture box could be easily adjusted to promote the net photosynthetic rate of plants.

Figure 10. Scaled-up photoautotrophic culture vessel (picture taken after removal of the lid). The vessel was 610 mm long, 310 mm wide and 105 mm high (volume approx. 20 l) (Zobayed et al., 2000).

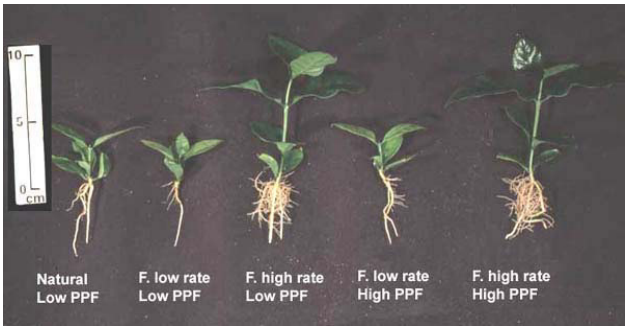


Figure 11. Coffee (C. arabusta) plants on day 10 of the ex vitro stage as affected by ventilation methods in the in vitro stage. In the treatment legends, Natural, Low rate and High rate denote natural and forced low and high rate ventilations (Nguyen et al., 2001).

The growth of shoots and roots of coffee (*C. arabusta*) somatic embryos cultured photoautotrophically in a large vessel (volume approx. 3 l) with nutrient supply system (TRI-bioreactor) were greater with forced ventilation than with natural ventilation (Afreen et al., 2002b). The percent survival was 98 percent for plants *ex vitro* derived from somatic embryos grown in the TRI-bioreactor with a forced ventilation system, while it was only 30 percent for those grown in the Magenta-type vessel system with natural ventilation. Furthermore, when cultured photoautotrophically, the cotyledonary embryos proved the highest embryo-to-plantlet conversion percentage in a TRI-bioreactor compared with those grown in Magemta vessel or modified RITA-bioreactor (Afreen et al., 2002b). The embryo-derived plants in the group grown in the TRI-bioreactor had the highest percent of

survival and a faster growth when transferred to the *ex vitro* condition, followed by those in modified RITA-bioreactor and Magenta vessel.

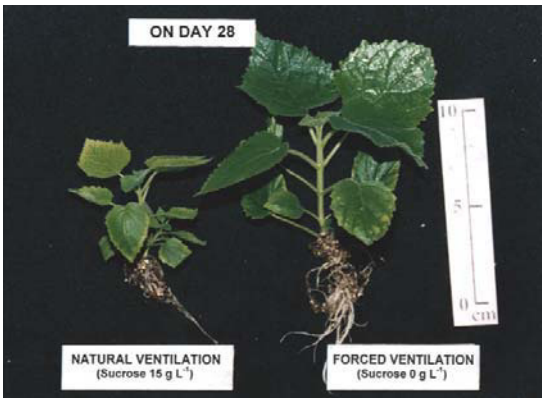


Figure 12. Effect of different types of ventilation on the growth of Paulownia (Paulownia fortunei) plants in vitro (Nguyen and Kozai., 2001).

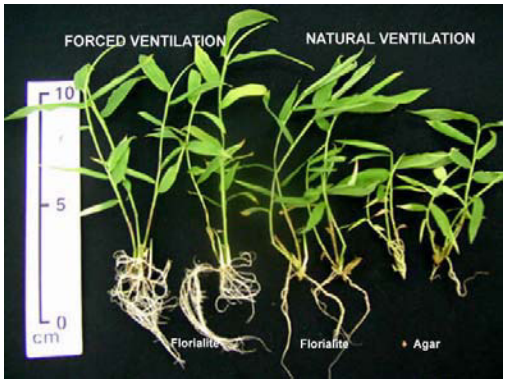


Figure 13. Growth enhancement of bamboo (Thyrsostachys siamensis Gamble*) plants cultured photoautotrophically in forced ventilation vs. natural ventilation systems on day 25.*

Another forced ventilation system for studying the photoautotrophic growth of *Paulownia fortunei* using a large vessel (volume approx. 17 l) was also developed (Nguyen and Kozai, 2001). The CO_2 uptake rate increased with the increase in the airflow rate over the culture period of 28 days, resulted in the increase in net photosynthetic rate. The growth of single-node leafy cuttings of *Paulownia* was

significantly greater under photoautotrophic condition with forced ventilation than under photomixotrophic condition with natural ventilation (Figure 12).

The forced ventilation system using a large vessel (volume approx. 13 l) has been recently proved to have a better effect on the photoautotrophic growth of bamboo (*Thyrsostachys siamensis* Gamble) plants *in vitro* for 25 days (data not shown). Increased dry weight, shoot and root lengths, percent of plants having roots and number of new shoots per explant were significantly greater in the forced ventilation treatment with Florialite-based medium compared with those in natural ventilation treatments with Florialite or agar-based medium in Magenta-type vessels. In the vessel with forced ventilation, bamboo plants developed a great number of lateral roots resulted in the growth enhancement of bamboo plants in the *in vitro* stage (Figure 13).

7. CONCLUDING REMARKS

In the near future, photoautotrophic or sugar-free micropropagation will be commonly used, especially in developing countries, for producing a large number of genetically superior and pathogen-free transplants at low production cost. In fact, the photoautotrophic micropropagation system has been commercialized widely in Kunming, China (Xiao et al., 2000) and partly in Vietnam. At the same time, the photoautotrophic micropropagation system will be adopted on a large scale as a useful means for solving the global problems of environmental conservation, food production and bio-resource production in the 21st century.

Photoautotrophic micropropagation can be done either by using small, conventional culture vessels with gas permeable filters for enhancing natural ventilation or large culture vessels with air pumps for forced ventilation. In a photoautotrophic micropropagation system using large vessels with forced ventilation, the production cost of plants *in vitro* was about 40 percent lower than that of plants in the photomixotrophic micropropagation system (Xiao and Kozai, 2004).

The concept of photoautotrophic micropropagation can be further expanded into the idea of ‘closed transplant production system’. With a closed transplant production system, the quality of transplants can be further improved; the consumption of artificial energy (electric energy and/or fossil fuels) and other resources (labor, facilities, supplies, etc.) can be decreased; and the release of environmental pollutants can be minimized (Chun and Kozai, 2000).

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Chapter 9

S. M. A. ZOBAYED

VENTILATION IN MICROPROPAGATION

Contents

Key words: Aeration, carbon dioxide, diffusion, ethylene, forced ventilation, large vessel, natural ventilation, photoautotrophic micropropagation, relative humidity.

1. INTRODUCTION

Micropropagation is a mass vegetative plant propagation system on an artificial nutrient medium under a control sterile environment to ensure pathogen-free, true-to-type and rapid production. The notion that the micropropagation technique can play a vital role in the production of pathogen free propagation was realized since the development of the 'totipotency' theory of the cell in the early twentieth century. Nearly three decades have elapsed since George Morel first proposed the application of plant tissue culture for the commercial clonal propagation of orchids. However, like many new technologies, progress in micropropagation is not as rapid as many expected, even now, relatively few crops are being produced commercially (Lumsden et al., 1994). The major reasons for the slow commercialization are i) loss of plants due to microbial contamination, ii) poor growth and development of *in vitro* plants, iii)

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 147-186.

morphological and physiological disorders of micropropagated plants, vi) low net photosynthesis due to the growth and development of plants on an exogenous carbohydrate-supplemented nutrient medium iv) slow *ex vitro* establishment and growth due to poor plant quality and finally, v) excessive loss of plants after transplanting to greenhouse or field.

It is long believed that the growth of *in vitro* plants depend largely on the composition of the nutrient medium and thus efforts are mainly made to improve the nutrient composition of the growing medium. However, recent researches revealed that the growth and development of plants or explants produced *in vitro* can be seriously affected by the composition of the gaseous atmosphere (Jackson et al*.,* 1987; Blazková et al*.,* 1989; Kozai et al*.,* 1986, 1989 and 1991). The conventional protective conditions, such as the use of screw caps, aluminium foils, transparent films, polypropylene disc etc. under which plant materials are grown to prevent microbial contamination and to retard desiccation of the tissues and the nutrient medium can cause unintentional restriction of the exchange of gases between the vessel atmosphere and the outside air (Buddendorf-Joosten and Woltering, 1994). Therefore, the gaseous environment *in vitro* is often abnormal when compared with the *ex vitro* environment. The major characteristics of the gaseous environment *in vitro* in conventional micropropagation system are high relative humidity, large diurnal fluctuations in CO_2 concentration and the accumulation of ethylene and other toxic substances (Kozai and Kubota, 1992). As a consequence the photosynthesis, transpiration, and uptake of water, nutrients and CO_2 can be suppressed and dark respiration enhanced, resulting in poor growth (Jeong et al., 1995) and physiological and morphological disorders of the *in vitro* plants (Debergh and Maene, 1984) including undesirable morphogenetic changes that are varied and species dependant (Jackson et al., 1987).

The method for supplementation of exogenous carbon, such as sucrose, in the culture medium is originally derived from the idea that the *in vitro* plants are heterotrophic and since they cannot synthesize their own carbon source therefore sucrose must be added in the medium. However, this erroneous idea has turned-out to be wrong after the development of photoautotrophic micropropagation system during the late-eighties (Kozai and Iwanami, 1988). Photoautotrophy is the process by which chlorophyllous organisms, such as plant, convert radiant energy into biologically useful energy and synthesize metabolic compounds using only carbon dioxide as a source of carbon. In the photoautotrophic micropropagation system, the culture medium is free of exogenous sugar and the vessel needs to be ventilated to enhance the CO_2 concentration at least upto the atmospheric concentration. After the development of photoautotrophic micropropagation system, there has been much interest on the ventilation of culture vessels (Table 1). Major aim to ventilate culture vessel is to improve the *in vitro* gaseous environment and to minimize the difference between the gaseous environment *ex vitro* and *in vitro*. The ventilation in the culture vessel has proved to have many advantages over the conventional airtight system (Table 1). In

this article we reviewed the natural and the forced ventilation system for the *in vitro* micropropagation. Development of forced ventilation system is comparatively a new technique; a historical overview of the chronological development of this system has also been reviewed along with its advantages for biomass production, environmental control and plant physiology.

2. NATURAL VENTILATION

To improve the air exchange and thus the growth, normality and quality of the plants, the vessel need to be ventilated i) naturally (natural ventilation) or ii) forcedly (forced ventilation). Natural ventilation is the energy-efficient process of bringing outer fresh air inside the culture vessel and extracting the same amount of air from the vessel. Natural ventilation generally takes place through the air gap between the vessel and the lid or through a gas permeable microporous film (pore diameter 0.2 – 0.5 μm; Figure 1a) attached on the lid (Figure 1b) or on the wall of the vessel. Driving force for gas exchange in a tissue culture vessel under natural ventilation are i) the pressure gradient between the inner and the outer environment ii) the temperature gradient between the inner and the outer environment and iii) the velocity and current pattern of the air surrounding the vessels. Therefore, the shape of the vessel, orientation of the lids and vents, air current and environment around the vessels will affect the number of air exchanges of naturally ventilated vessels (Kozai and Kubota, 2001). Air current speed around a vessel is experimentally confirmed to enhance air exchange of the vessel (Ibaraki et al., 1992).

Natural ventilation through the air gap between the vessel and the lid is probably the simplest way to improve the air exchange between the outer environment and the *in vitro* culture vessel environment. Loosely fitted lids have been found to improve the growth and quality of micropropagated plants (Jackson et al., 1991). However, this type of natural ventilation can increase the risk of microbial contamination especially when sucrose is used as a sole carbon source in the culture medium. Generally, the mass of medium, plant material and the air itself in the vessel is a little warmer than the surroundings, and thus, compared to the photoperiod, the temperature in the vessel is usually lower by 1-3 C during the dark period. This can create a partial vacuum, which pulls surrounding air into the vessel, and is one of the causes of exogenous contamination. Therefore, there is a need to vent the culture vessel with a reliable integral submicron membrane or the gas permeable film, which is part of a well-closed vessel.

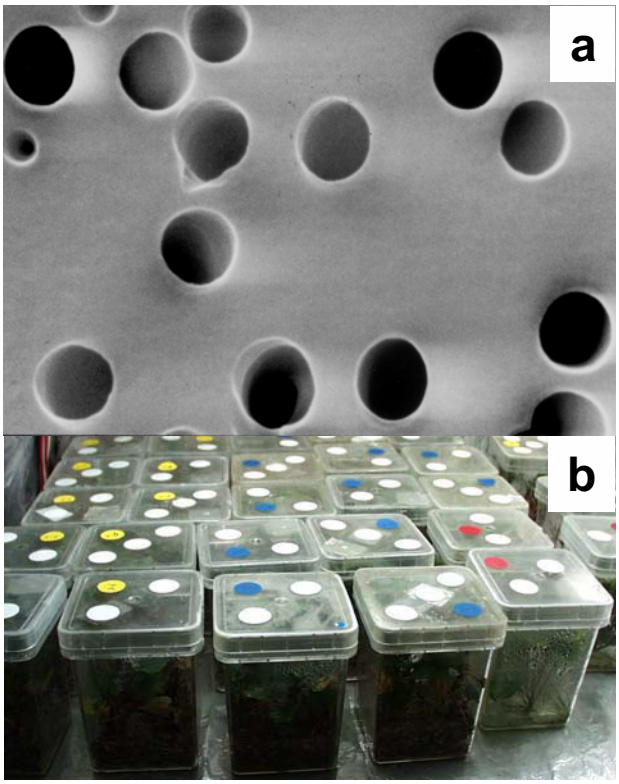

Figure 1. a) Scanning Electron micrograph of a Millipore filter disc; b) adhesive Millipore filter discs are attached on the lids of Magenta vessels to increase natural ventilation.

Currently, many types of gas permeable films are commercially available, for example, MilliSeal membrane (adhesive microporous filter disc, pore diameter 0.45 µm; Milli-Seal, Nihon Millipore Ltd.,Yonezawa, Japan; Figure 1a, b), MilliWrap membrane seal (Microporous sheet; pore diameter 0.45 µm; Millipore Corporation, USA), transparent polypropylene disc (thickness 25 µm; Courtaulds Films, Bridgewater, Somerset, UK), Teflon membranes (Vent Spots; pore diameter 0.5 µm; Flora Laboratories; Australia), Suncap closer (Sigma, USA), TQPL discs (adhesive microporous filter disc; TQPL supplies, UK). Ready-to-use vented vessels or lids attached with porous films/membranes are also currently available, such as, Culture Pack (culture box made of gas permeable transparent films; 25 µm in thickness; Daikin Industries, Japan), LifeGurad Sealed Vessel System (microporous filters attached to a transparent vessels; Osmotek Ltd., Israel), LifeLine Vented Lids (microporous filters attached to a semi

transparent lids; Osmotek Ltd., Israel) and Phytocap closure (capping system for test tubes with 20 mm or 25 mm diameter; Phytotechnology Laboratories, USA).

The diffusion rate of CO_2 through these gas permeable films is proportional to the difference in CO_2 and water vapour concentrations inside and outside the vessel and the gas conductance of the gas permeable film. The efficiency of these ventilation systems can be evaluated by measuring the time taken, (t_{50}), for half of an injected standard sample of a marker gas, e.g. ethylene, to escape from the vessel (Jackson et al., 1991; 1994). Figure 2 shows the results of t_{50} measured in the culture vessels capped with various capping system. The t_{50} for the removal of ethylene from a 120 ml glass vessel by attaching one adhesive microporous filter discs (filter pore diameter 0.45 μm; Millipore Corporation, USA) on the hole (8 mm) of the lid was only 10 min compared to 30 min for the Suncap closer (Sigma, USA), 147 min for the polypropylene disc, 195 min for the aluminium foil functioning diffusively and 285 min for the cotton bung. Microporous filter disc are available in different sizes and pore diameters and the ventilation rate can be varied with the size and shape of the culture vessels. Therefore, the key parameter to compare different types of vessel or the ventilation system is the number of air exchanges as proposed by Kozai et al. (1986). For example, a 25 mm test tube could have 8-10 numbers of exchanges from a 10 mm microporous filter, while the same filter on a Magenta vessel would be only 1-2 numbers of air exchanges. By increasing the number of filter disc attached on the vessel it is possible to increase the number of air exchange. Depending upon the plant species and number of plants, number of air exchange may be necessary to increase or decrease.

3. FORCED VENTILATION

The concepts of forced ventilation developed for plant micropropagation are only a decade old. Forced ventilation is the process of mechanically moving air from outside to inside a culture vessel and vice versa. In this process a particular gas mixture is flushed directly through the culture vessel by applying pressurized force. Forced ventilation is one of the most effective methods of ventilation and the basic principle behind this method of ventilation is to create a positive pressure inside the vessel. With this system, the gaseous composition (CO_2, water vapour or any other necessary gases) of the incoming air and forced ventilation rate and/or air current speed in the culture vessel can be controlled relatively precisely by using a needle valve, mass flow controller and an air pump with an inverter (Kozai et al., 1999).

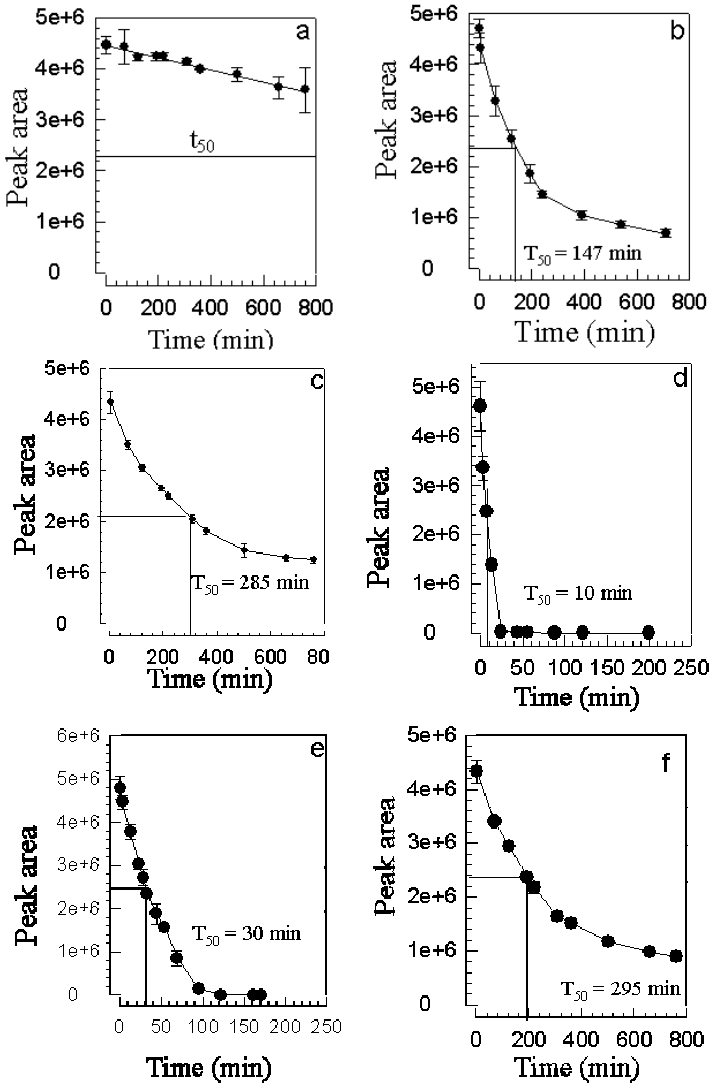

Figure 2. T_{50} measurements for the removal of injected ethylene from a 120 ml glass vessel capped in various ways. a) airtight system: sealed with silicone rubber bung; b) polypropylene disc; c) cotton bung; d) one adhesive microporous filter discs (filter pore diameter 0.45 µm; Millipore Corporation, USA) on the hole (8 mm) of the lid; e) Suncap closer (Sigma, USA) and f) aluminium foil functioning diffusively.

Table 1. Summarized results of the impact of ventilation on plant micropropagation.

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Ananas mangium	Natural ventilation; microporous filter membrane (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan)	NAE = 6.7 h^{-1} (photoautotrophy) and 0.6 (photomixotrophy)	Higher growth, root development, net photosynthesis in ventilated plants grown photoautotrophically	Ermayanti et al., 1999
Annona squamosa L.	Natural and forced ventilation; Suba-seal rubber puncture cap, Cling film (PVC), polypropylene discs, through-flow (forced) ventilation apparatus	t_{50} = 5587 min (suba-seal rubber), 1313 min (cling film), 94 min (ventilation apparatus)	Leaf production was greatly increased and ethylene-induced leaf fall considerably delayed under forced flow system	Armstrong et al., 1997
Annona squamosa L. *and* *Annona muricata* L.	Forced ventilation; non-mechanized humidity induced pressurized forced flow system compared with natural ventilation (polypropylene disc) and airtight vessel	FR = 5 ml min^{-1}; NAE = 0.1 (airtight), 1.5 h^{-1} polypropylene and 5 h^{-1} in forced ventilation system	Leaf and flower bud abscission was prevented and growth and multiplication enhanced under forced ventilation	Zobayed et al., 2002
Coconut	Natural and Forced ventilation; suba-seal rubber puncture cap, Cling film (PVC), Polypropylene discs, through-flow (forced) ventilation apparatus	t_{50} = 5587 min (suba-seal rubber), 1313 (cling film), 94 (ventilation apparatus)	In forced flow system calloid was more convoluted than under diffusive aeration and had a smooth distinct epidermal surface, clearly defined sub-epidermal meristematic nodules and resembled freshly initiated calloid from which regeneration of plantlets via somatic embryogenesis can be obtained	Armstrong et al., 1997

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Brassica oleracea L.	Forced ventilation; non-mechanized humidity induced pressurized forced flow system compared with natural ventilation (polypropylene disc) and airtight vessel	FR = 5 ml min^{-1} in a 60 ml vessel; NAE = 5 h^{-1}	Forced ventilation enhanced growth and chlorophyll content, flushed-out accumulated ethylene, enhanced CO_2 concentration, provide atmospheric O_2 in the headspace under forced ventilation	Zobayed et al., 1999a
Brassica oleracea L.	Forced ventilation; non-mechanized humidity induced pressurized forced flow system compared with natural ventilation (polypropylene disc) and airtight vessel	FR = 1-5 ml min^{-1} in a 60 ml vessel; NAE = 5 h^{-1}	Shoot culture (organogenesis): growth and chlorophyll content increased, flushed-out accumulated ethylene, enhanced CO_2 concentration, provide atmospheric O_2 in the headspace	Zobayed et al., 1999b
Chrysanthemum morifolium	Natural ventilation; Suncap closer (grown photoautotrophically) compared with polyethylene disc (sugar containing medium; control)	Not measured	Photoautotrophically grown plants with natural ventilation showed higher growth, multiplication, chlorophyll and carotenoids, Chl a/b ratio, net photosynthetic rate, ribulose - 1, 5-bisphophate, carboxylase/ oxygenase and phosphoenolpyruvate carboxylase activities than control	Cristea et al., 1998
Coffea arabusta	Forced ventilation in a temporary root zone immersion bioreactor compared to naturally ventilated bioreactors and Magenta vessel	FR = 50–200 ml min^{-1}; NAE = 1.6–5.8 h^{-1}	Enhanced growth, photosynthetic ability, percent germination of somatic embryos and survival *ex vitro* under forced ventilation	Afreen et al., 2002

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Coffea arabusta	Natural ventilation; 10 mm microporous filter membrane; 0.5 µm pore diameter (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan)	NAE = 0.2 - 2.3 h^{-1} (Kozai et al., 1986)	Growth and photosynthetic activity increased	Nguyen et al., 1999a
Coffea arabusta	Forced ventilation by using air-pump	FR = unknown; NAE = 1.1 - 5.9 h^{-1}	Growth increased	Nguyen et al., 1999b
Cymbidium PLB (cv. Lisa Rose No. 1)	Natural ventilation; vessel cap was punched (hole 0.8 cm^2) and attached a gas permeable membrane	NAE = 0.6 (control; photomixotrophy) and 3.5 h^{-1} (photoautotrophy)	Stomatal density, chlorophyll content and net photo-synthesis increased significantly compared to control; dry mass decreased; similar multiplication	Kirdmanee et al., 1992
Eucalyptus camaldulensis	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 20 L) headspace; plants grown photoautotrophically compared with photomixotrophic plants	FR = 12 – 210 L h^{-1}; NAE = 0.6 – 10.4 h^{-1}	Improved growth, uniformity and survival *ex vitro*	Zobayed et al., 2000a
Eucalyptus camaldulensis	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 3.4 L) headspace; plants grown photoautotrophically compared with photomixotrophic plants	FR = 30 – 400 ml min^{-1}; NAE = 0.5 – 6 h^{-1}	Improved growth photosynthesis and *ex vitro* survival, normal leaf anatomy and stomatal opening	Zobayed et al, 2001b
Fragaria X ananassa Duch. (cv. Ai-berry')	Forced ventilation by using air pump and CO_2 cylinder	FR = 12 x 10^4 ml h^{-1}	Enhanced growth and photosynthesis	Fujiwara et al., 1988

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Garcinia mangostana	Natural ventilation; attached two microporous filter membranes (Milli-Seal, Nihon Millipore Ltd.,Yonezawa, Japan); high and low ventilation (photoautotrophic) and low ventilation (control; photomixotrophic)	NAE = 6.7 h^{-1} (photoautotrophy) and 0.6 (photomixotrophy)	Greater rooting percentage and net photosynthetic rate under photoautotrophic conditions with high ventilation, dry mass not significantly different	Ermayanti et al., 1999
Gardenia jasminoides Ellis	Natural ventilation; tightly or loosely sealed caps; different capping system: cotton cap, aluminium cap, glass cap, parafilm	NAE unknown; t_{50} values varied from less then 1 h to 30 h in empty vessels (52 ml) and no significant difference in vessel containing medium	Development of autotrophy assessed by carbon isotope composition: higher autotrophy in root-induction stage than shoot-multiplication stage; loosely sealed tubes has stronger effect	Serret et al., 1997
Gynura pseudo-china	Natural ventilation; attached two microporous filter membranes (Milli-Seal, Nihon Millipore Ltd.,Yonezawa, Japan); high and low ventilation (photoautotrophy) and low (control; photomixotrophy)	NAE = 6.7 h^{-1} (photoautotrophy) and 0.6 (photomixotrophy)	Increased growth in high natural ventilation (photoautotrophic) than those of low (control)	Ermayanti et al., 1999
Ipomoea batatas L.	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 3.4 L) headspace; plants grown photoautotrophically compared with photomixotrophic plants	FR = 50-450 ml min^{-1}; NAE = 0.9-7.6 h^{-1})	Improved net photosynthetic rate, growth and uniformity of plants grown photoautotrophically under forced ventilation	Zobayed et al., 1999c

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Ipomoea batatas L.	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 4 L) headspace; plants grown photoautotrophically compared with photomixotrophic plants	FR = 0.8 – 7.5 ml s^{-1}; NAE = 0.9 – 7.6 h^{-1}	Increased stomatal density, leaf wax content, decreased stomatal size and area, reduced transpiration, epidermal conductance, and water loss during acclimatization and improved *ex vitro* growth and survival	Zobayed et al., 2000b
Ipomoea batatas L.	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 12.8 L) headspace	FR = 12, 24, 36 and 42 L h^{-1}; NAE = 0.9, 1.9, 2.8 and 3.3 h^{-1}	Growth and net photosynthesis increased under forced ventilation	Heo and Kozai, 1999
Ipomoea batatas L.	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 12.8 L) headspace	FR = 23, 17 and 10 ml s^{-1}; air speed 0.08, 0.06, 0.03 cm s^{-1}	Growth increased with uniform plant production under forced ventilation	Heo et al., 2001
Ipomoea batatas L.	Forced ventilation by using air-pump; uniform supply of CO_2 in the vessel (volume 12.8 L) headspace	FR = 23, 17 and 10 ml s^{-1}; NAE = 7.4, 5.5, 3.2 h^{-1}	Soluble sugar and starch content increased under forced ventilation compared to control	Wilson et al., 2001
Lagerstroemia thorellii Gagnep. and *Lagerstroemia speciosa* Pers.	Natural ventilation; polypropylene disc (thickness 25 µm; Courtaulds Films, Bridgewater, Somerset, UK) and cotton bung. Forced ventilation by using air-pump	NAE = 1.8 h^{-1} (polypropylene); 1.1 h^{-1} (cotton bung); 1.3 – 6 h^{-1} (forced ventilation)	Growth and *ex vitro* survival improved; flushed out accumulated ethylene	Zobayed, 2000

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Limonium latifolium	Forced ventilation using an air-pump attached with culture box (volume 125 L); small vessel with natural ventilation system (control) was compared	FR = 15 L min^{-1} after day 7; NAE = 7.2 h^{-1}	1500 plants cultured per box; growth and survival improved; cost was less than control	Xiao et al., 2000
Mentha piperita	Natural ventilation; attached two microporous filter membrane (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan); high and low ventilation (photoautotrophy) and low (control; photomixotrophy)	NAE = 6.7 h^{-1} (photoautotrophy) and 0.6 (photomixotrophy)	Increased growth in high natural ventilation (photoautotrophic) than that of low (control)	Ermayanti et al., 1999
Musa spp.	Natural ventilation; 10 mm microporous filter membrane; 0.5 µm pore diameter (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan)	NAE = 3.9 h^{-1} (Kozai et al., 1986)	Leaf area and leaf number increased, total dry mass decreased, multiplication ratio not significantly different	Nguyen and Kozai, 2001
Nicotiana tabacum L.	Natural ventilation using suncaps closures (Sigma, USA)	Not measured	Growth improved significantly	Ticha, 1996
Pogostemon cablin	Natural ventilation; attached two microporous filter membrane (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan); high and low ventilation (photoautotrophy) and low (control; photomixotrophy)	NAE = 6.7 h^{-1} (photoautotrophy) and 0.6 (photomixotrophy)	Increased growth in high natural ventilation (photoautotrophic) than that of low (control)	Ermayanti et al., 1999

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Rehmannia glutinosa	Natural ventilation by attaching membrane filters (10 mm diameter; Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan) and photoautotrophic culture; closed vessel with heterotrophic growth as control	NAE = 3.6 h^{-1} (Kozai et al., 1986)	Photosynthesis, stomatal conductance, controlled water loss during acclimatization, high chlorophyll fluorescence and chlorophyll content, rapid increase of carbohydrate content in *ex vitro* and high survival rate compared to control	Seon et al., 2000
Rehmannia glutinosa	Natural ventilation; microporous filter membrane 0.5 μm pore diameter; (0.8, 2.0. 3.8, 12.6 cm^2) (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan)	NAE = 0.8, 1.5, 1.9, 4.4 h^{-1} (Kozai et al., 1986)	Growth increased with the increase of number of air exchange under natural ventilation	Seon et al., 1999
Rosa hybrida Hort.	Natural ventilation; capped with aluminium foil, transparent polypropylene disc attached with microporous polypropylene disc	NAE = 0.3 and 3.8 h^{-1} (Kozai et al., 1986)	Growth improved significantly in the transparent polypropylene capping system both under photoautotrophic and photomixotrophic conditions	Hayashi et al., 1993.
Solanum tuberosum L.	Natural ventilation; capped with transparent polymethylpenten disc attached with microporous polypropylene disc	NAE = 5.6 h^{-1} (Kozai et al., 1986)	Natural ventilation with CO_2 enrichment and high PPF enhanced growth compared with non-ventilated vessel with CO_2 non-enrichment and low PPF treatment in both sugar free and sugar containing medium	Kozai et al., 1988

Species names	*Types of ventilation*	*Numbers of air exchange (NAE) or Flow rate (FR)*	*Major findings*	*References*
Solanum tuberosum L.	Natural ventilation, vessels attached with microporous filter membrane 0.5 µm pore diameter (Milli-Seal, Nihon Millipore Ltd., Yonezawa, Japan)	NAE = 0.75 and 5.0 h^{-1} (Kozai et al., 1986)	Both experimental data and computer simulation data showed increase of dry mass and net photosynthetic rate with the increase of ventilation	Niu and Kozai, 1997
Solanum tuberosum L.	Forced ventilation by using air-pump; plants grown photoautotrophically compared with photomixotrophic plants	FR = 10 – 30 ml min^{-1} in 385 ml culture vessel; NAE = 1- 4 h^{-1}	Improved leaf anatomy and ensure functional stomata, increased stomatal density and leaf thickness; reduced stomata pore size; enhance wax content; controlled water loss in *ex vitro*	Zobayed et al., 1999c
Solanum tuberosum L.	Forced ventilation using an air-pump attached with culture box (volume 125 L); small vessel with natural ventilation system was compared	FR = 15 L min^{-1} after day 7; NAE = 7.2 h^{-1}	2300 plants cultured per box; growth and survival improved; cost was less than control	Xiao et al., 2000
Zantedeschia elliottiana and Cunninghamia lanceolata	Forced ventilation using an air-pump attached with Plexiglas culture box (volume 125 L); small vessel with natural ventilation system was compared	-	1500 plants cultured per box Improved growth and survival, reduced production cost	Xiao and Kozai, 2004

In contrast, natural ventilation rate is difficult or not possible to change during the production period without dismantling the system. Moreover, it is difficult to obtain a high natural ventilation rate for a large vessel. The ventilation rate in a culture vessel should be adjusted according to the magnitude of net photosynthetic rate of the cultured plants inside the vessel for the optimization of the aerial environment and thus the growth (Kozai and Kubota, 2001). A forced ventilation system is therefore more suitable than a natural ventilation system for adjusting ventilation rates by manipulating airflow rates through the large vessel (Fujiwara et al., 1988; Walker et al., 1989; Kubota and Kozai, 1992; Kozai and Kubota, 2001; Zobayed et al., 1999a, b, c and 2001a; Erturk and Walker, 2000a; Heo et al., 2001).

3.1. Forced ventilation systems for relatively small vessels

Probably Horn et al. (1983) first proposed a forced ventilation system for the photoautotrophic culture of Soybean cell in suspension culture (250 ml flask). In 1988, Fujiwara et al. (1988) designed a forced CO_2 enrichment system using a specially equipped growth chamber. The chamber contains a CO_2 control unit consisting of a container with pure liquid CO_2, an electric solenoid valve with a relay for opening and closing the solenoid, and an infrared type CO_2 controller with an air pump for air sampling. Two identical transparent acrylic boxes containing culture vessels were placed in the chamber. In 1989 another apparatus was used by Walker et al. (1989) to determine the effect of ventilation on Stage II micropropagation of *Rhododendron* 'P.J.M.'. To provide 0, 300 and 1000 μmol mol^{-1} CO_2 gas treatments, gas mixtures were provided from different gas cylinders, and the atmospheric air was supplied by a diaphragm-type air pump. At the same time, Adkins et al. (1989) developed a continuous gas-flow system for the study of callus growth. The system allows for several gases to be mixed and passed through culture tubes containing callus on Miracloth boats placed on a filter paper bridge.

To generate low (30-65%), medium (70-95%) and high (97% and above) relative humidities in the culture vessels Kozai et al. (1990) developed a forced ventilation system. In this system a desiccant (silica gel) contained in a flask was used to dehumidify the air which was blown through it by an air pump. Fujiwara et al. developed another device in 1993 to study the physical environmental effects on growth and development of cultures. This device was 70 cm wide, 45 cm deep and 70 cm high. The upper part of this device consisted of a light source and a culture box containing culture vessels. The lower part was the control box with a control panel. The CO_2 was maintained at a certain level by adjusting the flow rates of pure CO_2 from the container (volume: 450 ml) and/or incoming air.

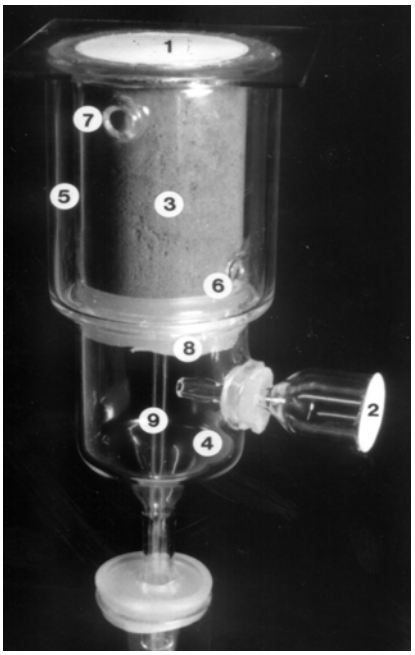

Figure 3. Humidity- induced forced ventilation apparatus (for details see Zobayed et al., 1999a). 1) inflow membrane; 2) outflow membrane; 3) inflow turret; 4) outflow turret; 5) water reservoir; 6 and 7) hole to pour water; 8) rubber bung to attach inflow and outflow turret; 9) glass tube to direct incoming gas.

Kitaya and Sakami (1993) engineered a system for CO_2 enrichment of chlorophyllous callus by utilizing the respiratory CO_2 produced by a crop of mushrooms. A micropropagation box was connected to a mushroom culture box using a semi-closed piping (silicone tube) system attached with ethylene absorbent, air pump, solenoid valve, etc. One important feature was that, unlike others, the source of CO_2 was free of cost and did not require any gas cylinder. To control relative humidities in the culture vessels Fujiwara et al. (1993) developed another system where the culture box connecting several vessels was attached through an inlet pipe to either distilled water or saturated salt solutions in large Erlenmeyer flasks. In the same year Yue et al. (1993) developed a forced ventilation system in which the relative humidity of the culture vessels could be controlled by adjusting the relative humidity of inlet outdoor air (385 - 420 ml l^{-1} CO_2) to each of four constant levels, i.e., 100, 91, 78 and 46%.

To ventilate culture vessels, a simple non-mechanised forced ventilation apparatus has recently been developed (for details see Armstrong et al., 1997; Zobayed et al., 1999a, b and Figure 3). The apparatus consisted of an inflow and an outflow turret (cylindrical glass chamber); the polycarbonate Nuclepore membrane, functioning as the inflow membrane (pore diameter = 0.05 μm), was attached to the edge of the inflow turret. At the centre of the turrets there was a thin glass tube which enters the culture vessel through a silicone rubber bung and directs the incoming gas flow to the culture vessel. The outflow membrane (pore diameter 0.2 μm) was attached to the hole (diameter 25 mm) of the outflow turret. Briefly, the operation of the apparatus depends on the humidity-induced diffusion of atmospheric oxygen and nitrogen through an inflow Nuclepore membrane. The diffusion occurs under the influence of a concentration gradient, maintained by the higher relative humidity of the inside of the apparatus relative to the outer atmosphere, comparatively free venting occurs through the outflow membrane. The apparatus is based upon humidity-induced diffusion and thus requires no complex and expensive mechanical parts such as pumps/cylinders, metering devices, etc. and it requires no external energy supply. The apparatus has already been shown to be beneficial for improving the growth and physiology of *in vitro* potato plants (Zobayed et al., 2001a), *Annona*, (Zobayed et al., 2002), cauliflower (Zobayed et al., 1999a, b) and culture of coconut callus (Armstrong et al., 1997) simply by increasing the gas exchange between the culture vessel and the external atmosphere. However, the major limitation of this system is that the flow rate is rather slow and difficult to control during the culture period.

3.2. Forced ventilation system for large culture vessels

3.2.1. Components of forced ventilation system with large culture vessels

The benefits of large culture vessel for micropropagation have recently been realized. One of the key advantages of photoautotrophic micropropagation (using sugar-free medium) is that it makes it possible to use large culture vessels with minimum risk of microbial contamination and thus almost all of the large vessels, so far designed, is based on the photoautotrophic micropropagation.

Figure 4 shows the major components of a large vessel attached with forced ventilation for photoautotrophic micropropagation (Zobayed et al., 2004). An ideal large vessel contained a) a culture box for propagating plants, b) an air pump located outside the vessel for supplying CO_2 enriched ambient (culture room) air via a flow meter in the culture headspace, c) especially designed air distribution pipes located inside the culture vessel for supplying uniform CO_2 in the culture headspace, d) air inlet and outlet located in the culture vessel lid or the side walls fitted with microporous filter disc, preferably with pore diameter of 0.2 to maximum 0.5 μm to protect the system free from microbial contamination

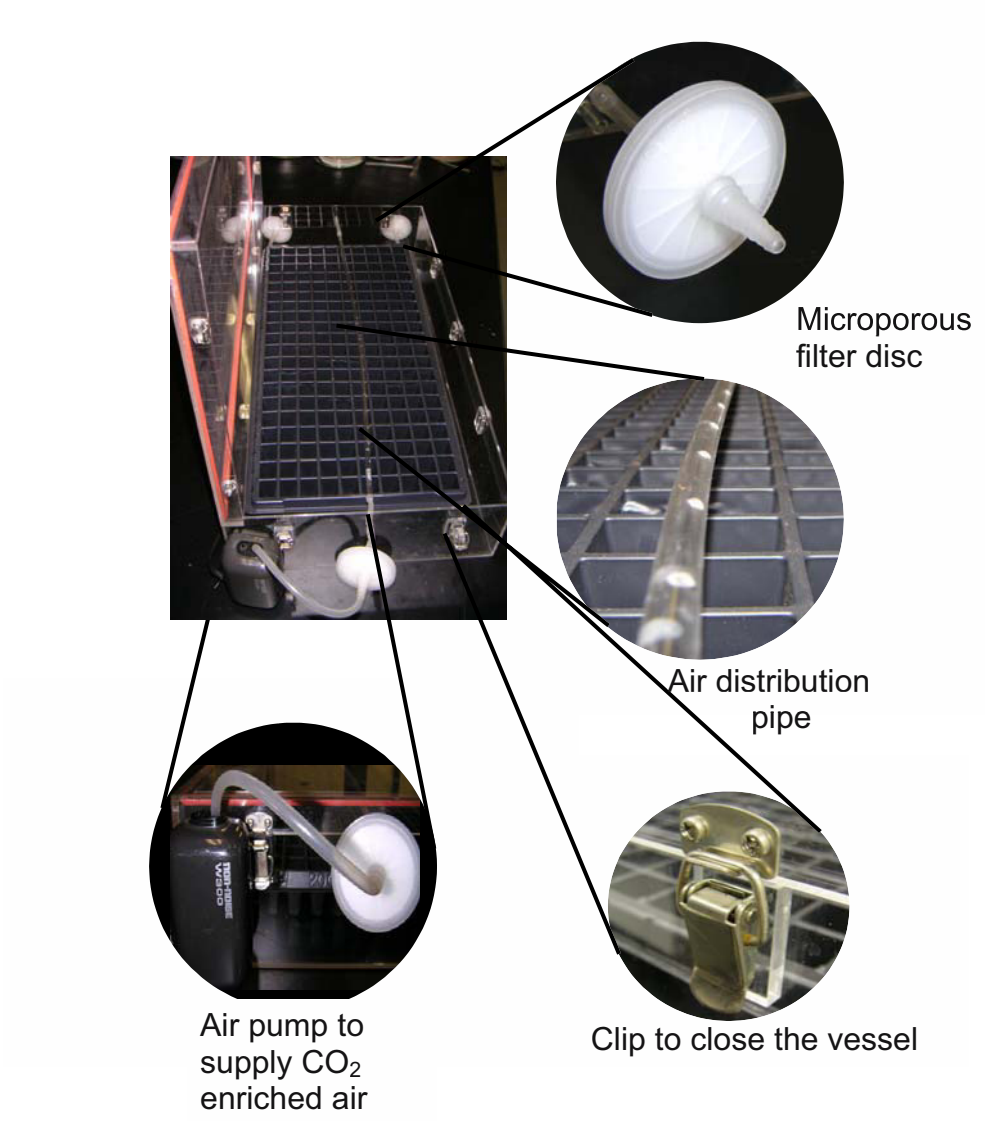

Figure 4. Different components of an ideal large vessel for photoautotrophic micropropagation (Zobayed et al., 2004).

e) an autoclaveable multicell tray for growing plants in the culture vessel. In case the culture room is not enriched with CO_2, each vessel should be connected directly to a CO_2 gas cylinder or a gas mixture chamber from where a desired concentration of CO_2 enriched air can be pumped into the culture vessel.

Sterilization of a large culture vessel is a major step for the successful establishment of aseptic mass propagation system in large vessels. For sterilization, autoclaving the vessel at 121C and 1.4 kg cm^{-1} for 20-40 min is a common procedure (Afreen et al., 2002, Nguyen et al., 1999a and b; Heo and Kozai, 1999; Heo et al., 2001; Wilson et al., 2001). When the vessel is too big to fit in the autoclave machine, for example, 125 L vessel used by Xiao et al. (2000) or when the vessel is made of non-autoclaveable plastics, such as acrylic sheet used by Zobayed et al. (1999c), the alternative procedure of sterilization is the surface sterilization by using disinfectants such as sodium hypochlorite solution, $KMnO_4$, formaldehyde etc. The step by step sterilization procedure for a 125 L vessel, described by Xiao et al. (2000) is as follows. (1) wash the culture vessel with clean water, (2) wipe the culture vessel with 0.2% sodium dichloroisocyanurate ($C_3O_3N_3C_{l2}Na$), a disinfectant, (3) stifle the culture vessel with $KMnO_4$ (5 g m^{-3}), formaldehyde (10 ml m^{-3}) for 10 hours, and (4) spray the culture vessel with 70% ethanol before transplanting. Trays were cleaned with water, and sterilized by dipping them into 0.2% sodium dichloroisocyanurate a disinfectant solution for twenty minutes.

Planting density is an important factor for the successful commercial scale micropropagation. In a large vessel planting density can be increased noticeably without significantly reducing the dry mass of plantlets. Moreover, designing of a large culture vessel should also consider to increase planting density by manipulating nutrient supply system, selecting suitable substrate, lighting system and uniform CO_2 supply. Recently, a number of reports showed that the planting density can be increased significantly in large vessels under photoautotrophic condition. For instance, 4600 potato (Xiao et al., 2000) 3000 *Limonium latifolium* (Xiao et al., 2000) and 2644 eucalyptus plantlets (Zobayed et al., 2000a) have been cultured per meter square culture area of large culture vessels. These planting densities are significantly higher compared to the conventional system of propagation in small vessels.

For photoautotrophic micropropagation in large vessels, the selection of a suitable supporting material is another important criterion not only for the optimum growth, easy to propagate and transplant *ex vitro* but also to make the system suitable for handling and sterilizing. There are evidences that roots growing in agar medium showed structural abnormalities of the tissues (Kataoka, 1994), often lacked root hairs and died shortly after transplanting, resulting in plantlets that ceased to grow (Afreen et al., 1999; Debergh and Maene, 1984). Perlite (Xiao et al., 2000) and vermiculite (Heo and Kozai, 1999) have been used for propagating plantlets in large culture vessels. However, these substrates are composed of fine granules, which are not easy to handle and thus may not be suitable as a

commercial rooting substrate for using in large vessel. Polyester fiber cube (Fujiwara et al., 1988), Cellulose plug (Heo and Kozai, 1999), Rock wool cube (Kubota and Kozai, 1992) and Florialite (Zobayed et al., 1999c, 2000a,b and 2001a; Wilson et al., 2001; Afreen et al., 2002) all are available in block form of different sizes and therefore are easy to handle in large vessel. However, in case of Cellulose plug, inoculation of explants is often difficult and sometime, because of its spongy texture, the inoculated explants are uprooted (Afreen et al., 1999). Moreover, root growth and morphology and *ex vitro* survival were found to be better in Florialite compared to Cellulose plugs or even vermiculite and agar medium (Afreen et al., 1999).

Figure 5. Micropropagation of Eucalyptus plantlets in a large vessel under photoautotrophic conditions (for details see Zobayed et al., 2000a)

3.2.2. Designing of a large vessel: a chronological development

Probably the first system of large culture vessel was developed by Fujiwara et al., (1988) where, a large culture vessel of approximate volume of 19 L (58 cm long, 28 cm wide and 12 cm high) with an attached air pump for the forced ventilation of the vessel was used to enhance the photoautotrophic growth of strawberry (*Fragaria* x *ananassa* Duch.) explants

and/or plants during the rooting and acclimatization stages. This was an aseptic micro-hydroponic system with a nutrient solution control system. Roche et al. (1996) developed a commercial-scale photoautotrophic micropropagation for potato micro-plants in which 100 nodal explants were cultured under natural ventilation in a stainless steel tray containing a block of polyurethane foam (85 x 300 x 25 mm) and enclosed with a polyethylene sleeve.

Figure 6. Coffee plantlets developed from somatic embryos are grown in a large vessel under photoautotrophic conditions (for details see Afreen et al., 2002).

Kubota and Kozai (1992) grew potato plantlets under forced ventilation in a large vessel (2.6 L) containing a multi-cell tray. Heo and Kozai (1999) developed a similar type of system using forced ventilation attached to a large culture vessel (volume 13 L), where 20 sweet potato plantlets were cultured photoautotrophically. However, the disadvantage of these large culture vessels commonly faced is the variation in growth of the cultured plantlets mainly due to the un-uniform distribution of CO_2 concentration and other environmental factors in the culture headspace. As CO_2 is the sole carbon source in a photoautotrophic micropropagation system, uniform distribution of CO_2 concentration in the culture headspace of a large vessel is an important factor to achieve uniform plant growth. In a large vessel system described above, CO_2 concentration in the vessel is highest at the air inlet and is lowest at the air outlet especially, if there is only one inlet and one outlet. Consequently, large horizontal gradients in CO_2 concentration between the inlet and outlet is created which resulted in a wide variation and lack of growth uniformity, with larger plants near the air inlet and comparatively smaller plants near the air outlet. This un-uniform growth is more obvious generally during the later growth stage of plantlets when the net photosynthetic rate per

plantlet is high (Kozai and Kubota, 2001). The reason for this un-uniform growth was due to the failure to create uniform environmental conditions inside the large vessels using forced ventilation. Generally, the larger the vessel volume, the more difficult it is to achieve uniform distribution of CO_2 in the vessel and thus the growth.

Zobayed et al. (1999c) engineered a forced ventilation system attached with a large vessel (volume 3.4 L; 40 plantlets per vessel) that supplies sterile nutrient solution throughout an extended culture period. The vessel was fitted with air distribution pipes (horizontally) inside the vessel for forced ventilation and 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. Heo et al. (2001) also developed a similar forced ventilation system with large vessel (13 L; 20 plantlets per box) horizontally fitted with air distribution pipes and successfully achieved uniform plantlet growth.

Figure 7. A photoautotrophic micropropagation system using large culture vessels attached with forced ventilation system applied for the commercial production of calla lily (Zantedeschia elliottiana) and China fir (Cunninghamia lanceolata) (Xiao and Kozai, 2004).

Zobayed et al. developed another large-scale micropropagation system by using a scaled-up vessel (volume 20 L; 500 plantlets per vessel; Figure 5a) with forced ventilation and air distribution chamber (Zobayed et al., 2000a). The bottom part of this large vessel has an air distribution chamber and the CO_2 enriched air was distributed through vertical pipes

across the plug tray to obtain a uniform CO_2 distribution over plantlets in the vessel and provide a uniform plantlet growth (Figure 5b). In a larger vessel (volume $\geq$20 L), without such an air distribution system, the distribution of CO_2 concentration tends to be uneven over the plantlets even when air distribution pipes were used (Zobayed et al., 2000a). This was probably due to the significant pressure drop (thus reduce flow rate) in the air distribution pipe as the distance from the source (air pump) increased.

Afreen et al. (2002) designed a large culture vessel with forced ventilation system (temporary root zone immersion bioreactor) for the photoautotrophic mass-propagation of cotyledonary stage coffee somatic embryos (volume: 9 L; Figure 6). In this system an automatic nutrient supply system was attached with the vessel for temporarily immersing the root zone of the plantlets with the nutrient solution. This temporary immersion system ensured the exposure of the roots to the air and improved the root quality.

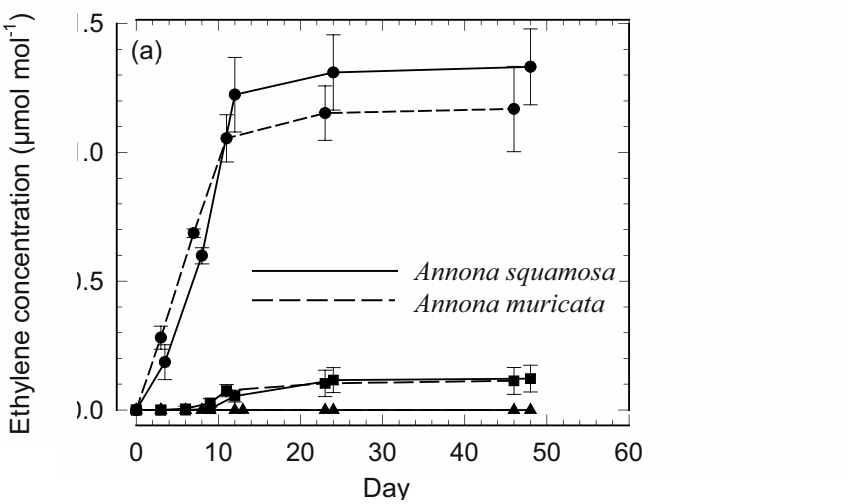

Figure 8. Ethylene concentrations over time in the vessel with airtight system, natural ventilation and forced ventilation containing Annona plantlets (Zobayed et al. 2002).

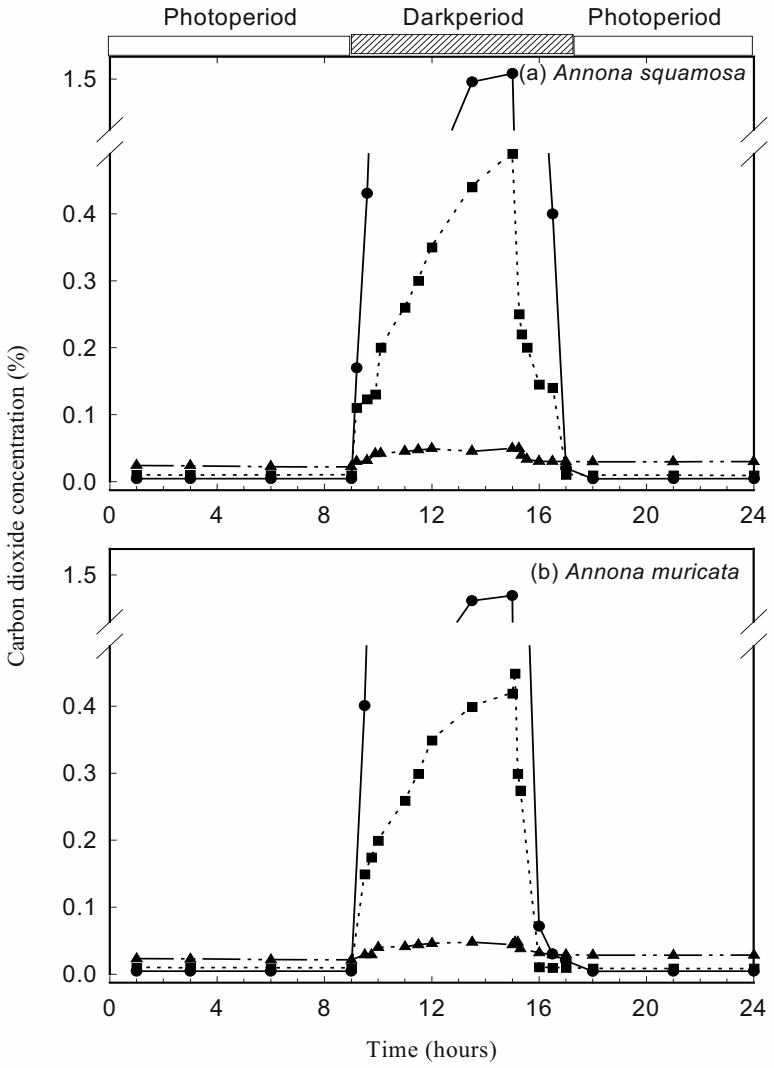

Figure 9. CO_2 concentrations in the culture vessel of 45 days old Annona squamosa L. (a) and Annona muricata (b) under forced ventilation (▲), natural ventilation (■) and in airtight vessel (●) (Zobayed et al., 2002).

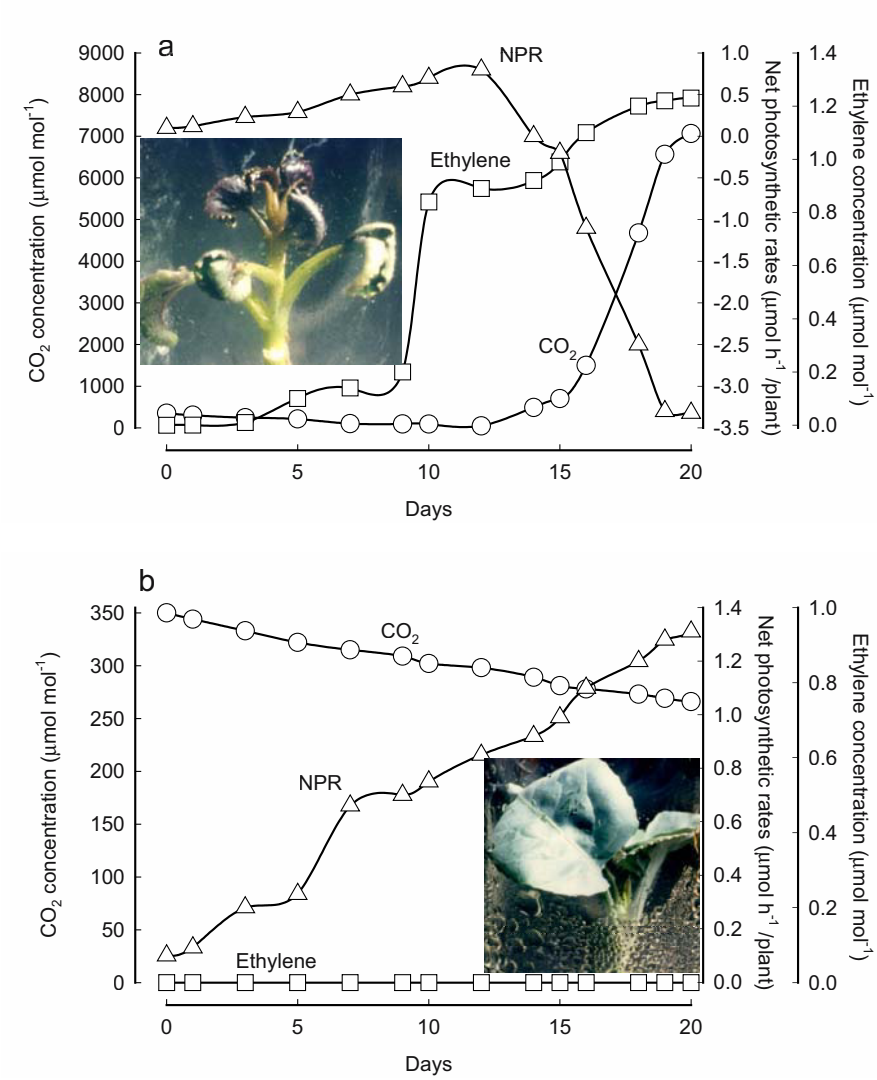

Figure 10. Net photosynthetic rates, CO_2 and ethylene concentrations in the culture headspace of cauliflower plantlets grown in a 60 ml glass vessel under natural ventilation (airtight) (a) and forced ventilation system (b).

Recently, a photoautotrophic micropropagation system using large culture vessels (volume: 120 L each; Figure 7) attached with forced ventilation system for supplying CO_2 enriched air was applied for the commercial production of calla lily (*Zantedeschia elliottiana*) as well as China fir (*Cunninghamia lanceolata*) (Xiao and Kozai, 2004). The system consisted of an airtight culture room (floor area: 20 m^2) equipped with nine culture modules (shelf) each contained five culture vessels. Each culture vessel consisted of a culture box (volume 120 L), made of plexiglass, with two air inlets (diameter: 5 mm) and six air outlets (diameter: 20 mm) for forced ventilation. Each air inlet was connected to an air-valve for controlling the number of air exchanges of the vessel. Gas-permeable microporous filters (diameter: 20 mm, pore diameter: 0.5 μm) were attached to each of the air outlets. Three multi-cell trays (48×36×7 cm) were placed in each culture box; vermiculite was used as supporting material. Modified MS (Murashige and Skoog, 1962) solution excluding sugar or vitamins and other organic elements was provided at the beginning of experiment. About 1,500 single leafy nodal cuttings were cultured for 15 days in each of five vessels. Growth and quality of plantlets produced in the large culture vessel were significantly improved compared with those of the plantlets in the conventional sugar containing photomixotrophic culture system in small vessels. The plantlets grown in photoautotrophic system had better growth, well developed root system and were survived well in *ex vitro* (Xiao and Kozai, 2004).

4. GAS CONCENTRATIONS IN CULTURE VESSELS IN RELATION TO VENTILATION

For healthy growth, all parts of a plant must exchange their internal gases readily with those in the surrounding air. Nevertheless, in conventional micropropagation systems the exchange of gases between tissues and the air is frequently restricted (Kozai et al., 1991; Jackson et al., 1987). Many plant species when grown *in vitro* release a variety of substances, which may accumulate and have significant effects on growth and development of plants *in vitro* (Heyser and Mott, 1980). The most widely studied gaseous product from cultures is ethylene.

4.1. Ethylene

Ethylene is a volatile that has received considerable study as a regulator of plant growth. Ethylene is known to accumulate in the culture vessel especially with a very low number of air exchange and to be associated with various physiological responses of *in vitro* grown plants during growth and development such as inducing epinasty (Zobayed et al., 2001a), causing leaf (Armstrong et al., 1997; Lemos and Blake, 1994) and flower bud abscission (Zobayed et al., 2002), reducing photosynthesis (Zobayed et al., 1999b) and thus plant growth (Jackson et al., 1991). Plants *in vitro* can be affected by ethylene and result in, for example, poor cell differentiation (Miller and Roberts, 1984), an absence of somatic

embryogenesis (Meijer and Brown, 1988; Purnhauser et al., 1987; Roustan et al., 1989), reduced shoot height and leaf area (Jackson et al., 1987 and 1991) and poor callus proliferation and growth (Adkins et al., 1990 and 1993).

The accumulation of ethylene in the culture vessels is also responsible for the hyperhydricity of a number of species (Jackson et al., 1991). The number of air exchanges of the vessel significantly affects ethylene concentration in the culture vessel and thus under forced ventilation, ethylene cannot accumulate in the culture vessel due to the continuous flushing out of air from the vessel.

Figure 8 shows ethylene concentrations over time in the vessel with natural and forced ventilation, containing *Annona squamosa* L. plantlets (Zobayed et al., 2002). The vessels were uncapped and flushed with sterile air and then recapped at 0 h on day 46. Ethylene concentration in the vessel was then measured during the following 48 h under airtight system, natural (polypropylene disc) and forced ventilation.

Ethylene concentrations increased rapidly in the airtight vessel and reached peak levels (1.3 μmol mol^{-1} in *A. squamosa* and 1.15–1.17 μmol mol^{-1} in *A. muricata*) within only 24 – 48 h of culture. For both the species, the pattern of the changes in ethylene concentration under natural ventilation was similar to that in the airtight vessels (Figure 8), and the peak concentration was only *ca*. 0.1 μmol mol^{-1} for both the species. Under forced ventilation, no ethylene accumulation was observed in the culture vessels, probably because any ethylene (or other toxic gases) produced by the plants was flushed out by the forced ventilation. It is interesting that even in the airtight vessels ethylene concentrations reached equilibrium: in this case net production also ceased rather abruptly and this strongly suggests the switching-in of a negative feedback mechanism.

4.2. Carbon dioxide

Where cultures are grown under a day: night regime, in a conventional micropropagation system with limited air exchange, CO_2 concentrations fluctuate due to the respiration and photosynthesis of the plants. During the dark period, when photosynthesis is not occurring, CO_2 concentrations increase through respiratory metabolism of glucose by glycolytic and tricarboxylic acid pathway (Lowe et al., 2003). During the photoperiod the photosynthetic activity of chlorophyllous plants in the culture vessel results in a decline in CO_2 concentration. Depletions of CO_2 concentration during the photoperiod is a common phenomenon as reported by many authors (Desjardins et al., 1988; Kozai et al., 1987; Kozai and Iwanami, 1988; Solárová et al., 1989; Zobayed et al., 1999a; Kozai et al., 1999). In airtight vessels the concentration may drop to levels that are generally considered to be limiting (Buddendorf-Joosten and Woltering, 1994 and 1996). During dark period CO_2 concentration in the culture vessel may remain much higher than the ambient in airtight vessel (De Proft et al, 1985; Fujiwara et al., 1988; Jackson et al., 1991). However the

concentrations of CO_2 strongly depend upon the ways in which the culture vessels are capped. Jackson et al. (1991) demonstrated that *Ficus lyrata* cultures with loose, intermediate and tightly sealed vessels, contained respectively 0.5. 3.4 and 8.5% CO_2 in the dark period. As shown in Figure 9, in *A. squamosa*, the CO_2 concentration in the headspaces of airtight vessels rose rapidly during any dark period and fell rapidly at the onset of any subsequent photoperiod (Zobayed et al., 2002). At the end of the dark period, the CO_2 was very high (approximately 1.5% or 15000 μmol mol^{-1}), while in the photoperiod it decreased approximately 40 μmol mol^{-1} and, because gas exchange with the outside atmosphere was restricted, this represents the CO_2 compensation point for these plants. In natural ventilation (capped with polypropylene disc) the CO_2 concentration decreased during the photoperiods to 88 μmol mol^{-1}. It should be noted that the rate of net photosynthesis at 88 μmol mol^{-1} is significantly less than at atmospheric CO_2 concentration. With forced ventilation, during the photoperiod, the CO_2 concentrations were sustained at even higher levels (200 μmol mol^{-1}) than in the natural ventilation system despite an appreciably higher rate of consumption (i.e. higher photosynthetic rates). The difference reflects the impedance to gas exchange of the polypropylene disc in the natural ventilation system. In the dark period, the CO_2 concentration in the natural ventilation system did not rise as steeply as in the airtight vessels but attained a concentration of 480 μmol mol^{-1}, and again reflecting the impedance to gas exchange of the polypropylene disc. However, the more effective gas-exchange induced by forced ventilation prevented the CO_2 concentrations from exceeding atmospheric levels. Thus, concentrations during the dark period with forced ventilation were 0.7× and 0.02× those in the natural ventilation and airtight systems, respectively. It has also been reported that CO_2 concentration in the culture vessel can be increased significantly during the photoperiod. Woltering (1989) found 1.3% and 13% CO_2 with *Gerbera jasmesonii* in semi-closed and tightly sealed containers respectively during the photoperiod. Righetti et al. (1988) found 20% CO_2 in *Prunus* shoot cultures grown in the light in (airtight) jars. Jackson et al. (1991) also reported 8.5% CO_2 in the dark in airtight vessels containing *Ficus lyrata* and this decreased to 0.2% and 1% at the end of the photoperiod with loose and intermediate sealing of the culture vessels respectively. While cauliflower plantlets were grown *in vitro* in an airtight vessel, Zobayed et al. (1999b) found high concentration of CO_2 in the culture vessel during the photoperiod, which was just below atmospheric in well ventilated vessels. To observe this occurrence, we cultured cauliflower cuttings in a relatively small culture vessel (50 ml) and capped with silicone rubber bung (airtight system) or attached with forced ventilation (flow rate 5 ml min^{-1} throughout the culture period). In the airtight vessel, from days 4 to 7 when the new leaves were unfolded and beginning to photosynthesis, the CO_2 concentration in the culture vessel decreased to nearly compensation point, 40 μmol mol^{-1} (Figure 10a) during the photoperiod. Around day 13, the concentration increased to reach 7200 μmol mol^{-1}. This increase of CO_2 concentration was thought to have been due to the accumulation of ethylene (Figure 10a) in the culture vessel, which impeded photosynthesis (Figure 10a), so that respiratory CO_2 accumulated; this also probably indicated the first

stages of ethylene-induced premature senescence of the tissues. It should be mentioned that cauliflower is known to very sensitive to accumulated ethylene (Zobayed et al., 1999a and b). In case of the forced ventilation system the CO_2 concentration in the culture vessel was just below atmospheric (290 - 300 µmol mol^{-1}) during the first 14 days of culture and was reduced to 270 µmol mol^{-1} at the end of the experiment. No ethylene was accumulated in the culture vessel and the net photosynthetic rate (per plantlet) was gradually increased with time (Figure 10b).

4.3. Relative Humidity

Relative humidity in the culture vessel is an important environmental factor that affects the water relations of cultured tissues (Jeong et al., 1995). Relative humidity is normally high in the culture vessel and may have some deleterious effects on cultured plantlets. Several studies have demonstrated that lowering relative humidity in the culture vessel improved the resistance of tissues to water loss (Wardle et al., 1983; Smith et al., 1990 and 1992). It has also been reported that the growth of *in vitro* grown plantlets may be improved by manipulating the relative humidity inside culture vessels (Wardle et al., 1983; Smith et al., 1990). The improvement in gas exchanges under forced ventilation might have been expected to reduce the relative humidity in the culture vessel which can enhance transpiration. Thus, an improved growth obtained under forced ventilation, may also, at least partially result in the increased transport of minerals from the culture medium. Loss of water from the medium in the vessel under forced ventilation system with high number of air exchanges is generally significant and the medium tends to desiccate when the culture period is longer than one month. This problem can be solved by any of the following three methods: i) supply more volume of medium in the vessel (Kozai and Kubota, 2001); ii) keep the relative humidity of culture room 70-80% during the photoperiod (Fujiwara and Kozai, 1995) or iii) supply humid air in the culture vessel by the forced ventilation system (Zobayed et al., 1999a and b, Armstrong et al., 1997).

Other volatile substances released *in vitro* are ethane, ethanol, methane, acetylene and acetaldehyde (Thomas and Murashige, 1979a and b). Rice (*Oryza sativa* L.) callus culture modified the atmosphere of the culture vessel by producing CO_2, ethylene and ethanol, while utilizing oxygen (Adkins et al., 1990). These changes in the gaseous atmosphere of the culture vessel can suppress the growth of cultured explants and promote necrosis (Adkins, 1992). A well ventilated vessel can overcome all of these problems and improve the growth and quality of the plantlets significantly.

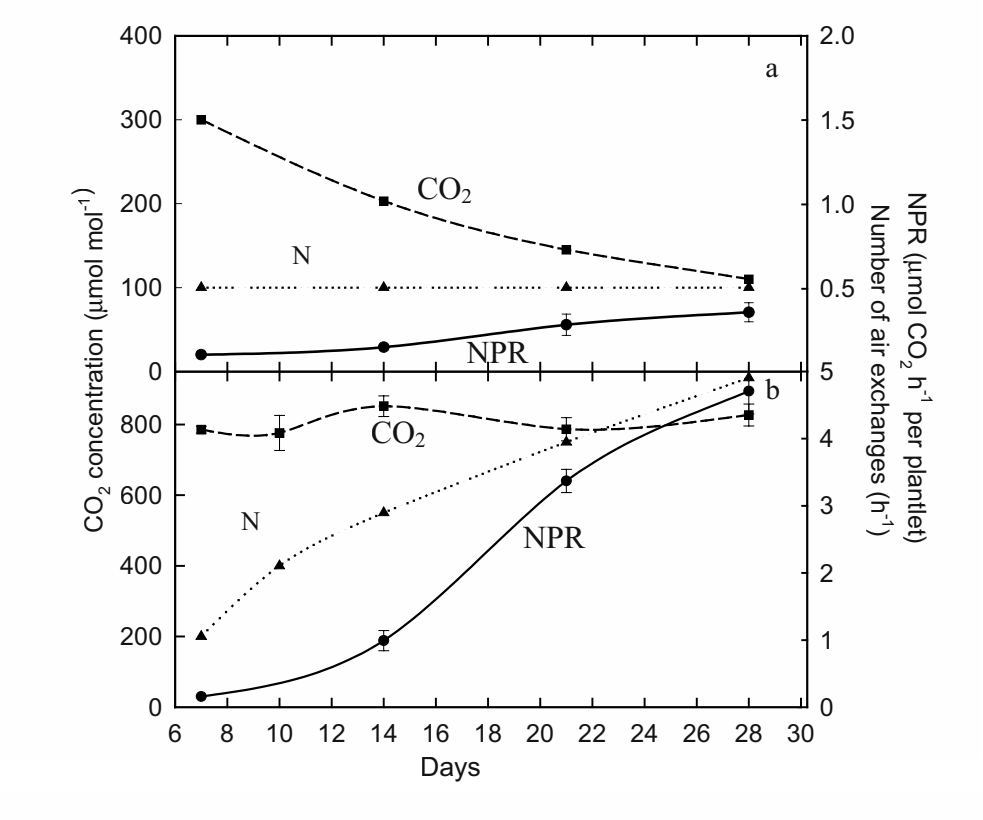

Figure 11. CO_2 concentrations (CO_2), net photosynthetic rates (NPR) and numbers of air exchanges (N) of a culture vessel with Eucalyptus plantlets under (a) natural ventilation and (b) forced ventilation.

5. BENEFICIAL IMPACT OF VENTILATION

During the last decade there has been an increasing awareness that *in vitro* cultures can be benefited from improvements in ventilation. With conventional micropropagation, the exchange of gases between tissues and the outer air is restricted and can often seriously limit the growth and development of plantlets. This inability to grow well in *in vitro* culture has been said to have greatly hampered micropropagation research and its commercial application. Thus, establishing a method for effectively controlling the gaseous environment

in the culture vessel should contribute towards the high production efficiency and value-added product quality, thereby considerably expanding the application of plant micropropagation techniques. A list of micropropagated plant species along with summarize results benefited from different ventilation systems are presented in Table 1. Some of these benefits are discussed below.

a) By using ventilated vessels (natural or forced ventilation), CO_2 depletion during the photoperiod can considerably be reduced, and depending on the plantlet size, CO_2 concentrations can be maintained near to atmospheric concentration. CO_2 concentration could be enriched above atmospheric very easily by increasing concentrations outside the vessels (Kozai et al., 1999) and thereby increasing photosynthesis and giving even higher yield (Zobayed et al., 2000a; Kozai et al., 1999; Kubota and Kozai, 1992). Natural or forced ventilation systems also adequately reduce the accumulation of CO_2 in the dark period. Therefore, it is possible to optimize the growth of *in vitro* plants throughout the culture period by using forced ventilation system. However, the concentrations of different gases including CO_2, water vapour, O_2 and ethylene in the vessel headspace under natural ventilation can be manipulated by number of factors such as the photosynthetic efficiency of the chlorophyllous plants, metabolic activity of the plants, the size and leaf area of the plants, the culture room environment and most importantly the number of air exchanges of the vessel. Thus, the gaseous concentrations in vessels with natural ventilation are often unpredictable and uncontrollable (Figure 11a). In contrast, one of the most important features of the forced ventilation system is that the number of air exchanges can be controlled precisely during the growing cycle and thus almost all the gaseous concentrations including CO_2 and water vapour concentration can be manipulated to maximize the biomass production (Figure 11b). Figure 11, shows different gaseous concentration in the culture vessel containing eucalyptus plantlets. In a natural ventilation system (Figure 11a) the number of air exchange was almost constant (uncontrollable) throughout the culture period and thus with increase of biomass and the photosynthetic activity of plantlets in the culture vessel the CO_2 concentration was depleted during the photoperiod with time which seriously restricted the net photosynthetic rates (Figure 11a) and thus the growth. On the other hand in the forced ventilation system the CO_2 concentration in the culture vessel during the photoperiod was constant throughout the growth period by increasing the flow rate of the ventilation system (thus the number of air exchange) every 3-4 days. As a result the net photosynthetic rates per plantlet increased with time followed by growth.

(b) Using t_{50}'s as indicators (Figure 2 and Armstrong et al., 1997) both forced and natural ventilation systems were found to maintain the vessels free from ethylene, and this also would probably apply to any other potentially toxic gases. Thus the need for the use of ethylene inhibitors, absorbents or antagonists can be eliminated.

(c) By providing appropriate ventilation the adverse effects of accumulated ethylene and unusually high relative humidity such as hyperhydricity of plants can be prevented. The symptoms of hyperhydricity in the relatively airtight vessels include malfunctioning of stomata, chlorophyll deficiency, cell hyperhydricity, hypolignification, reduced deposition of epicuticular waxes and changes in enzymatic activity and protein synthesis (Ziv, 1991a and b). Hyperhydrated plantlets appeared to be `glassy` with thick, translucent, and brittle leaves, showing excessive basal growth and callus formation (Ziv, 1991b) and with little or no root development.

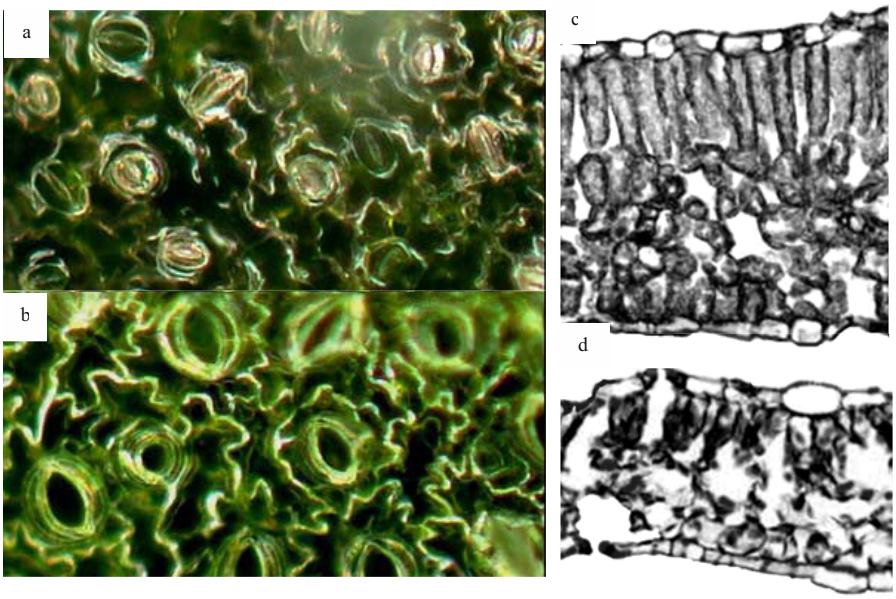

Figure 12. a, b) Stomata on the abaxial surface of the leaf of potato plantlets grown under photoautotrophic conditions with forced ventilation (a) and under photomixotrophic conditions with natural ventilation (b), photographs were taken during the dark period; c, d) Transverse sections of leaves of Eucalyptus plantlets grown under photoautotrophic conditions with forced ventilation (c) and under photomixotrophic conditions with natural ventilation (d).

The physiological abnormalities and morphological disorders mentioned above are the major shortcomings of the conventional micropropagation system. The incorporation of ventilation in the culture vessel especially the forced ventilation system ensure the normality of plants and significantly increases the biomass production and net photosynthetic rate (Kubota and

Kozai, 1992; Zobayed et al., 1999b and c; Hoe and Kozai, 1999), increase the leaf area, shorten the multiplication and growth period. By using ventilation in the culture vessel, i) the occurrences of leaf hyperhydricity in cauliflower (Zobayed et al., 1999a and b), potato (Zobayed et al., 2001a) and eucalyptus (Zobayed et al., 2001b) has been avoided, ii) leaf epinasty in cauliflower (Figure 6b; Zobayed et al., 1999a) and leaf and flower-bud abscission in *Annona* (Armstrong et al., 1997; Zobayed et al., 2002) was prevented, iii) failure of leaves to unfold in potato in low-ventilated system was improved (Zobayed et al., 2001a), iv) regenerated shoot maturation in *Annona* was increased (Zobayed et al., 2002) and carbohydrate accumulation in the plant tissues increased (Wilson et al., 2001). Along with photoautotrophic micropropagation system, forced ventilation ensures *in vitro* acclimatization of micropropagated plants during rooting stage (Kozai et al., 1999) and the *ex vitro* survival percentage increases significantly after transplanting even without any special *ex vitro* acclimatization.

(d) Various anatomical and physiological abnormalities common in the conventional micropropagation system can be prevented by applying ventilation. These abnormalities include the production of malfunctioning stomata, which remain permanently widely open even in the dark and unorganised palisade and mesophyll tissues in leaf. Figure 12 shows the abaxial surface of the leaf taken in the dark period. Stomata on the leaves grown under forced ventilation remained close during the dark period (Figure 12a; Zobayed et al., 1999d, 2000b and 2001c), while in the airtight system (natural ventilation), stomata remained fully open even in the dark period (Figure 12b). This suggested that ventilated plants had functional stomata. Transverse sections of leaves of the plants grown under forced ventilation showed normal, organized palisade and spongy mesophyll layers (Figure 12c), whereas, the airtight treatment showed thin and unorganised palisade and spongy mesophyll layers (Figure 12d).

(e) In conventional airtight system, accumulated ethylene result a low chlorophyll content in leaf; the chlorophyll contents can increase significantly by using forced ventilation and this, together with the CO_2 enrichment, no doubt responsible for the high photosynthetic rate followed by high yield observed in many plant species (Table 1).

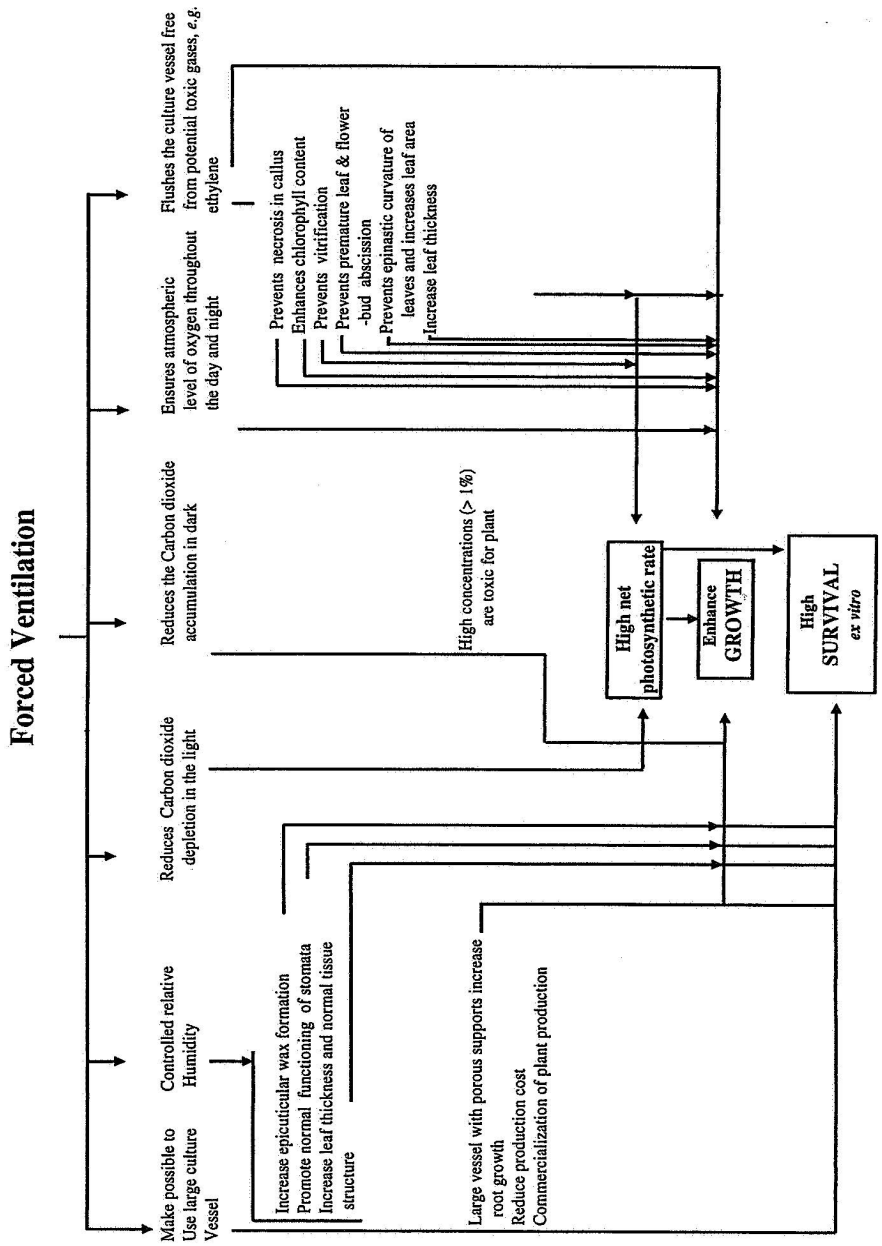

Figure 13. Schematic diagram summarizing the beneficial impact of micropropagated plants under forced ventilation.

(f) With forced ventilation, the relative humidity in the culture vessels can be controlled. The hyperhydricity of plants commonly reported in conventional systems has at least partly been correlated with too high relative humidity in the vessels. Relatively low relative humidity in the culture vessel can also contribute for the wax deposition on the leaf surface and as a result water loss can be controlled during *ex vitro* acclimatization.

g) Due to the forced ventilation, the air movement (velocity) inside the vessel is increased which is known to be beneficial for the cultured plantlets. High air movement can reduce the leaf boundary layer and thus increase the *in vitro* transpiration rate.

h) By enhancing the growth (root and shoot growth), shortening the multiplication/rooting cycle, eradicating the *ex vitro* acclimatization, and thus the production cost should be reduced significantly.

i) By using photoautotrophic culture system with forced ventilation, a considerably large vessel can be employed and hundreds or thousands of plantlets can be cultured without reducing the fresh and dry weight of the plantlets. Thus, the labour cost can be significantly reduced (Kozai et al., 1999) and automation of the micropropagation system becomes easier.

j) In such a scaled-up vessel, plug trays with cells containing the artificial substrate (rockwool, vermiculite and cellulose fibre) can be used to culture the plantlets. Thus each plant can be pulled up from the cell with minimum damage of roots and thus acclimatized *ex vitro* easily.

6. CONCLUSION

Micropropagation is often looked upon as being practical only for propagation of those plants, which are difficult to propagate by conventional propagation methods. This review suggests that by adopting a proper ventilation method, micropropagation technique is indeed applicable to the 'difficult to propagate' species, and equally it may offer economic advantages for some species which are considered relatively 'easy to propagate'. Figure 13 shows the schematic diagram of summarize pathway to improve the productivity and the quality of micropropagated plants under forced ventilation. Both morphological and physiological disorders generally reported in conventional micropropagation system can be improved significantly by using forced ventilation (Figure 13). Therefore, by providing appropriate ventilation in the micropropagated plantlets, high production efficiency and plant quality can be achieved and thereby considerable expansion of the micropropagation technology can be possible.

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Chapter 10

Y. XIAO & T. KOZAI

A COMMERCIALIZED PHOTOAUTOTROPHIC MICROPROPAGATION SYSTEM USING LARGE VESSELS WITH FORCED VENTILATION

Contents

Key words: Calla lily, china fir, CO_2 enrichment, forced ventilation, photoautotrophic micropropagation, production cost, sugar-free.

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 187-204.

1. INTRODUCTION

A photoautotrophic micropropagation system (called a PAM hereafter) that uses a sugar-free culture medium has many advantages over the conventional photomixotrophic micropropagation system (called a PMM hereafter) that utilizes a sugar-containing culture medium (Kozai, 1991). The advantages include the use of large culture vessels with minimum risk of microbial contamination and the enhancement of plantlet growth at a high photosynthetic photon flux (PPF) and a high CO_2 concentration inside the vessel (Fujiwara et al., 1988; Kozai and Iwanami, 1988).

In order to increase CO_2 concentration in the vessel under pathogen-free conditions, both natural and forced ventilation methods have been employed. Putting gas-permeable filter disks on the vessel lid enhances natural ventilation (Aitken-Christie et al., 1995). Forced ventilation can be conducted by supplying CO_2 enriched air with an air pump into the vessel through a gas-permeable filter disk (Kozai et al., 2000).

The forced ventilation rate can be easily controlled during the production process by using an airflow controller, while the natural ventilation rate is difficult to change with passage of days (Aitken-Christie et al., 1995). In addition, it is difficult to obtain a high natural ventilation rate for a large vessel. Thus, for commercial production, forced ventilation is more convenient and practical than natural ventilation in a PAM that uses large vessels.

Furthermore, many reports show that a PAM with forced ventilation enhances the growth of plantlets compared with a PAM with natural ventilation. Fujiwara et al. (1988) developed a 20-L vessel with forced ventilation for enhancing the photoautotrophic growth of strawberry (*Fragaria* x *ananassa* Duch.) plantlets during the rooting and acclimatization stages. Kubota and Kozai (1992) used a 2.6-L vessel containing a multi-cell tray with forced ventilation for photoautotrophic growth of potato (*Solanum tuberosum* L.) plantlets. Heo and Kozai (1999) developed a similar system using a 12.8-L vessel for the photoautotrophic growth of sweet potato (*Ipomoea batatas* (L.) Lam.) plantlets. Heo et al. (2000) developed another type of a 11-L vessel containing air distribution pipes to improve an airflow pattern in the vessel for obtaining the uniform growth of sweet potato plantlets. Zobayed et al. (1999) engineered a 3.5-L vessel with units of forced ventilation and sterile nutrient solution supply for uniform and enhanced growth of sweet potato plantlets. Recent advancement of in research on PAM using large culture vessels with forced ventilation has been summarized by Zobayed et al. (2004).

Commercial application of the PAM may be advantageous to additional ornamental species such as calla lily (*Zantedeschia elliottiana*) and China fir (*Cunninghamia lanceolata* (Lamb.) Hook). However, economic analysis of the PAM has rarely been conducted until recently (Kubota and Kozai, 2001). The objectives of this study were (1) to assess plantlet growth of calla lily and China fir in the PAM

in comparison with that in the PMM, and (2) to assess the possibility of commercialization of the PAM based on the estimation of production cost of calla lily plantlets. The commercial production site was owned and operated by a research institute where the first author was working for, as a production manager.

Calla lily, a herbaceous flowering plant for which there is currently a large demand, is conventionally propagated by tubers, resulting in a limited multiplication rate. The multiplication rate can be improved by a conventional micropropagation system but wide application has been limited by its high production costs, which are mainly due to poor plantlet growth, high percent of biological contamination of the medium, and labor intensive work (Lorenzo et al., 1998).

China fir is a rapid-growing woody plant that is used in the timber, furniture, and ornamental industries. In the recent years, large quantities of China fir plantlets have been produced by PMM. However, China fir, like many other woody plant species, does not easily develop roots *in vitro*. Plant growth regulators have been supplied to the medium to promote *in vitro* rooting of woody plantlets, often without success (Kozai et al., 2000). In addition, sugar-containing medium often causes callus formation at the base of shoots and a low percentage of survival for many plant species during the *ex vitro* acclimatization (Kozai and Zobayed, 2000). This chapter is an extended version of Xiao and Kozai (2004) and Zobayed et al. (2004).

2. PAM (PHOTOAUTOTROPHIC MICROPROPAGATION) SYSTEM

2.1. System configuration

System configuration of PAM is given in Table 1, in comparison with that of PMM (photomixotrophic micropropagation) system. The PAM system consisted of a culture room (floor area: 20 m^2) and culture modules. The culture room equipped with an air conditioner was thermally insulated using 10-cm thick foamed polystyrene walls to reduce the cooling and heating loads of the air conditioner. The culture room was almost airtight. To prevent insects and airborne fungi entering into the room, a small air pump continuously pumped a little amount of fresh air into the room through air filters to keep the inside room air pressure slightly higher than the outside air pressure. Ultraviolet lamps were installed on the ceiling for sterilizing the room air. Nine culture modules can be placed on the floor in the culture room. Each culture module consisted of a 5-shelf unit with or without castors, five culture vessel units, a forced ventilation unit for supplying CO_2 enriched air, and five lighting units (Figure 1 and Figure 2). A 20-cm-high step stool was available for reaching the uppermost vessels.

2.2. Multi-shelf unit

This consisted of a 220-cm high steel frame supporting five shelves (130 cm x 52 cm) each for holding one vessel. Thus, the total culture shelf area is about 3.4 (=1.3 x 0.52 x 5) m^2. The vertical distance between shelves was 40 cm; 2 cm for a thermally insulated panel covered with a white paper underneath for reflection of

light downward, 20 cm for the culture vessel, 5 cm for airflow through the gap between the upper surface of the vessel and the insulation panel to remove the heat generated by the fluorescent lamps, 3 cm for fluorescent lamps and 10 cm for airflow through the gap between the fluorescent lamps and the bottom surface of the upper shelf.

Table 1. Basic specifications of the PAM (photoautotrophic micropropagation) and the PMM (photomixotrophic micropropagation) systems.

Item (Unit)	**PAM**	**PMM**
Vessel volume (L)	120	0.37 (ϕ =7 cm)
Vessel bottom area (cm^2)	5980	38.5
Number of vessels per module	5	500
Ventilation type	Forced	Natural
Vessel ventilation rate (mL s^{-1})	0-60 (controllable)	0.05 (fixed)
Supporting material	Vermiculite	Agar (6 g L^{-1})
Medium sucrose conc. (g L^{-1})	0	30
Nutrient solution	Murashige and Skoog (1962)	
Light source	Cool white fluorescent lamps	
Floor area of culture room (m^2)	20	
No. of modules per culture room	9	
No. of shelves per module	5	
Area per shelf (cm^2)	6760 (130 cm wide x 52 cm deep)	
Height of module (cm)	220	
Room air temperature (C)	22-23 C	
Room relative humidity (%)	70-80	

10. COMMERCIAL APPLICATION

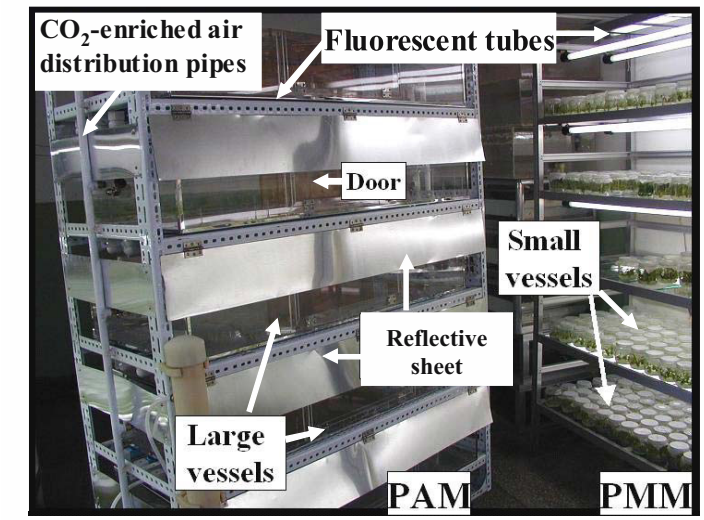

Figure 1. A culture module of the photoautotrophic micropropagation system using large culture vessels (PAM system) in the left and a culture module of the photomixotrophic micropropagation system using small vessels with natural ventilation (PMM system) in the right.

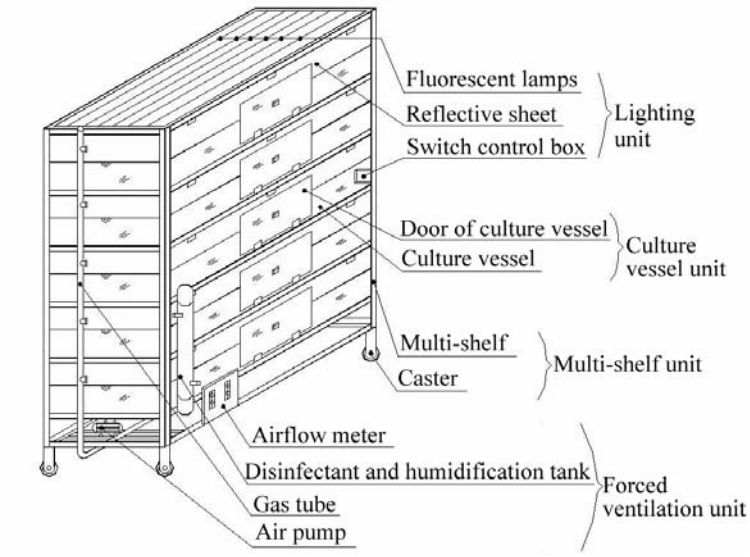

Figure 2. The culture module of photoautotrophic micropropagation system using large culture vessels with forced ventilation.

2.3. Culture vessel unit

This consisted of a plexiglass box (115 cm wide x 52 cm deep x 20 cm high; air volume, 120 L; culture area, about 0.6 m^2) with two air inlets (diameter: 5 mm) and six air outlets (diameter: 20 mm) for forced ventilation. The two inlets for providing air forcedly into the vessel were located on the sidewalls 8 cm from the bottom of vessel. Each air inlet was connected to an air-valve for controlling the number of air exchanges of the vessel, defined as the hourly ventilation rate of the vessel divided by vessel air volume. The six air outlets for discharging the vessel air naturally to the culture room were located at different points of the upper surface of the vessel (Figure 2). Locations of the air outlets are determined by trial and error to obtain a uniform air distribution in the vessel. Gas-permeable microporous filters (diameter: 20 mm, pore diameter: 0.5 μm) were attached to the air outlets to prevent dust and microbes from entering. Three trays (48×36×7 cm) were placed in each vessel. The culture vessel had a door (45 cm wide and 13 cm high) at the front side for accessing the trays.

2.4. Forced ventilation unit for supplying CO_2-enriched air

A forced ventilation unit for supplying CO_2 enriched air consisted of a CO_2 container with gas tubes, pressure gauges, airflow meters, an air pump and valves, an air disinfection and humidification tank, and a CO_2 concentration controller (Figure 3). This unit was used for forced ventilation of all the modules.

Pure CO_2 from the CO_2 container passed through the gas tube with the CO_2 pressure gauge and airflow meter into the disinfection and humidification tank containing 2% $NaClO_3$ (w/v) solution. In addition, the culture room air was sent by the air pump with a microporous filter through a gas tube with an airflow meter into the disinfection and humidification tank in order to dilute pure CO_2 in the container. The pure CO_2 and culture room air were mixed in the gas tube before being sent into the disinfection and humidification tank. Finally, the disinfected CO_2- enriched air was passed through the gas tubes with the airflow meter and valves into the culture vessel through the two air inlets of the vessel. The CO_2 concentration of the mixed air was measured and adjusted by using a CO_2 concentration controller.

2.5. Lighting unit

White fluorescent lamps were used as a light source. Six 36-W fluorescent lamps each with one switch were installed on each shelf to adjust PPF on the shelf at a desired level in a range between 50 and 200 μmol m^{-2} s^{-1}. To increase the uniformity of PPF distribution over the shelf and the ratio of light energy received by the plantlets to the light energy emitted from the lamps, the vessel was surrounded by white reflective sheets. Two sheets (120×13 cm) were installed on the front and back of the vessel, and another two (50×13 cm) were installed on the left and right side of the vessel. The four sheets were connected with the culture shelf by hinges.

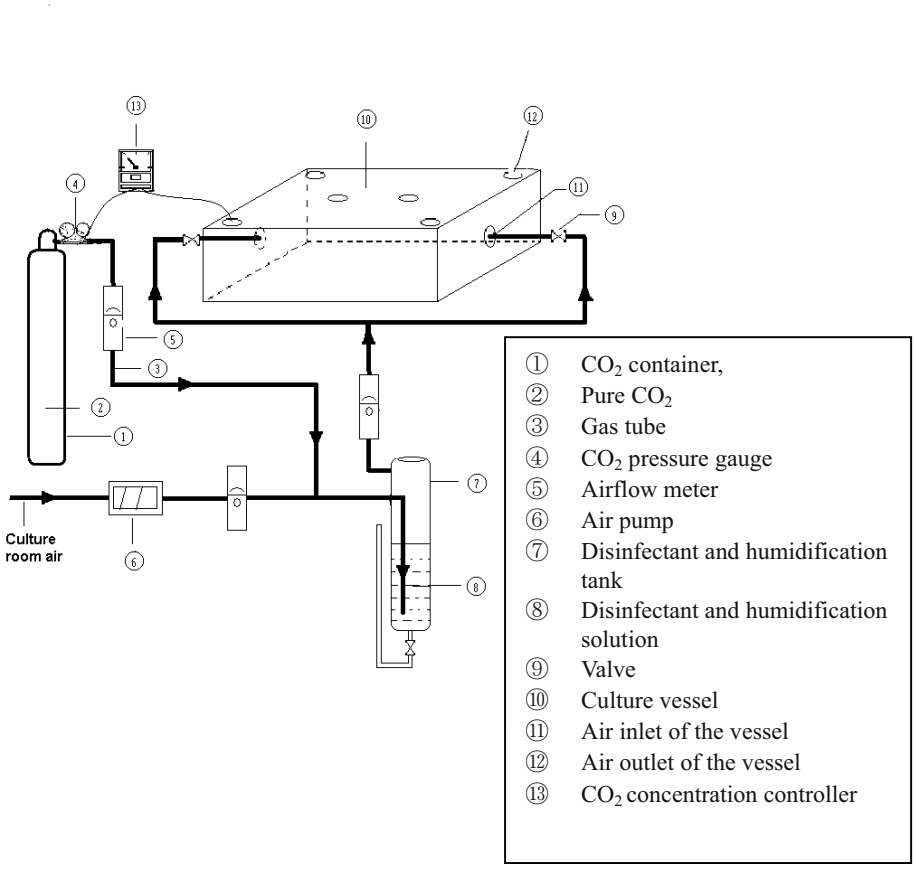

Figure 3. Schematic diagram of a forced ventilation unit for CO_2 enriched air supply.

2.6. Sterilization

The culture vessels were sterilized as follows (Xiao et al., 2000): (1) wash the culture vessel with clean water, (2) wipe the culture vessel with 0.2% sodium dichloroisocyanurate ($C_3O_3N_3Cl_2Na$), a disinfectant, (3) stifle the culture vessel with $KMnO_4$ (5 g m^{-3}), formaldehyde (10 ml m^{-3}) for 10 hours, and (4) spray the culture vessel with 70% ethanol before transplanting. Trays were cleaned with water, and sterilized by dipping them into a disinfectant solution with 0.2% sodium dichloroisocyanurate for twenty minutes. Substrate (vermiculite) in 5-L cloth bags and nutrient solution in 1000-mL bottles were autoclaved at 121 to 123 C for 40 min. The substrate was sprayed with water to get it wet before autoclaving for increasing its thermal conductivity. Sterilized nutrient solution was supplied to the substrate.

A 5-shelf unit of the PMM (photomixotrophic micropropagation) system was identical to the one used in the PAM, although the ventilated vessels, white

reflective sheets and forced ventilation unit were not used.

3. METHODS OF ANALYSIS

3.1. Calla lily plantlet growth

The growth of calla lily plantlets in the PAM was compared with that in the PMM. Experimental conditions are described in Table 2. Twenty plantlets were harvested at random from each of five vessels in the PAM on day 15, and two vessels each containing 10 plantlets were selected at random from each of five culture shelves and harvested on days 15 and 30 in the PMM for destructive measurements of shoot length, number of shoots, leaf area, fresh weight and dry weight. Each culture shelf was considered as a replication in both systems. ANOVA (Analysis of Variance) was run and then treatment means were compared using a least significant difference (LSD) test ($P<0.05$). Percent loss of plantlets *in vitro* due to microbe contamination of the medium was calculated by counting dead or heavily damaged plantlets among all the plantlets.

3.2. China fir plantlet growth

The growth, percent loss *in vitro* and percent *in vitro* rooting of China fir plantlets in the PAM under the presence/absence of NAA (1 mg L^{-1}) in the medium (called PAM-1 and PAM-0, respectively, hereafter) were compared with those in the PMM. Experimental conditions are described in Table 2. Forty plantlets were selected at random from each of 2 vessels in the PAM-0 and the PAM-1, respectively, and 5 vessels each containing 8 plantlets were selected at random from each of 2 culture shelves in the PMM for destructive measurements of shoot length, number of shoots, leaf area, shoot and root fresh weight, and shoot and root dry weight of plantlets. Each culture shelf was considered as a replication. The PMM treatment was considered as a control. ANOVA was conducted for the PAM-0 and the PAM-1, in which the presence/absence of NAA was considered as a factor. Treatment means were compared using a LSD test ($P<0.01$).

3.3. Percent survival during the acclimatization ex vitro

In vitro rooted plantlets were transplanted for acclimatization *ex vitro* in a bamboo-structured plastic greenhouse with a simple shading screen in Sept. 2001 in Kunming, China. Three thousand calla lily plantlets and 2,000 China fir plantlets from the PAM were transplanted with substrate (vermiculite), while 3,000 calla lily plantlets and 700 China fir plantlets from the PMM were transplanted without substrate (agar). Transplanting the plantlets with substrate reduces the amount of labor hour and root damage, and makes it possible to use automatic transplanting instead of manual transplanting. During the *ex vitro* acclimatization, average air temperature was 17 C, the highest and lowest daily average air temperatures were 23 C and 14 C, and average relative humidity during daytime was 76%. PPF was not

measured due to a technical problem. Twelve days after transplanting *ex vitro*, all the dead plantlets were counted in each treatment, and the percent survival *ex vitro* was determined. This experiment was conducted only once. In addition, 4000 *in vitro* rooted calla lily plantlets produced in the PAM were sold without *ex vitro* acclimatization directly to a farmer, just as a trial. Then, the farmer transplanted the plantlets in a bamboo-structured plastic greenhouse without any shading screen (i.e., without *ex vitro* acclimatization). The number of dead plantlets was counted 12 days after transplanting to determine the percent survival *ex vitro*.

3.4. Production cost of calla lily plantlets: A case study

Production cost was divided into the investment (or indirect) cost and the direct production cost. The direct production cost was further divided into *in vitro* multiplication, *in vitro* rooting, and *ex vitro* acclimatization costs (The greenhouse construction cost for *ex vitro* acclimatization was included in the acclimatization cost). *In vitro* multiplication cost was divided into labor, medium, electricity and other costs. The amounts of electric energy consumed for lighting, cooling, air pumping and autoclaving during the experiments were measured separately by watt meters (DD282, Shanghai Sanxing Ammeter Co., Ltd.). Electricity cost per kWh was 8.1 US cents. The costs of labor, electricity, medium, acclimatization and initial investment for *in vitro* rooting and *ex vitro* acclimatization in the PMM and the PAM were recorded and/or calculated based upon their prices as of 2001 in Kunming, China. The number of labor hours, production costs by components, and sales prices were recorded. Costs for supervision, administration, sales and transportation of plantlets to farmers were not included in the calculation of production cost. In this experiment, the cost of *in vitro* multiplication was equal to the cost of *in vitro* rooting in both the PMM and the PAM, because these two processes differed only by the combination of plant growth regulators in the medium only.

4. CALLA LILY PLANTLET GROWTH

Shoot length, leaf area, fresh and dry weight per plantlet on day 15 were 1.8, 1.8, 1.7 and 2.0 times greater in the PAM than in the PMM, respectively (Table 3). The growth on day 15 in the PAM was similar to or greater than the growth on day 30 in the PMM (Table 3). Most of the plantlets on day 15 in the PAM nearly reached the inner surface of the vessel lid (about 15 cm from the medium surface), and their morphology and quality of plantlets seemed suitable for *ex vitro* acclimatization, according to visual observation (Figure 4).

Table 2. Conditions in calla lily and China fir Experiments using the PAM (photoautotrophic micropropagation) and the PMM (photomixotrophic micropropagation) systems

Exp. conditions common to calla lily and China fir	**PAM**					**PMM**
	Day 0-3	**Day 4-5**	**Day 6-9**	**Day 10-12**	**Day 13-15**	**Day 0-15/30**
PPF (μmol m^{-2} s^{-1})	50	50	70	100	100	50
Photoperiod (h)	12	12	14	16	16	14
CO_2 conc. (μmol mol^{-1})	1,500	1,500	1,500	1,500	1,500	400
Vessel ventilation (mL s^{-1})	0	5-8	13-20	25-30	50-60	0.05
RH in vessels (%)	95	95	90-95	80-90	80	95-100

Calla lily	**PAM**	**PMM**
Days of culture	15	30
No. of explants/vessel	1500	10
No. of explants/ treatment	7500	5000
Medium NAA (mg L^{-1})	0	1
Type of explants	Shoots each with one unfolded leaf	
Leaf area, fresh and dry weights per explant	657 mm^2, 243 mg and 13 mg	
Nutrient solution supplied on day 0 per plantlet (mL)	6	6

China fir	**PAM-0**	**PAM-1**	**PMM**
Days of culture	28	28	28
No. of explants/vessel	1200	1200	8
No. of explants/treatment	2400	2400	1600
Medium NAA (mg L^{-1})	0	1	1
Type of explants	Single node leafy cuttings		
Nutrient solution supplied On day 0 per plantlet (mL)	8 (and 4 mL was added on day 18)		8

RH: relative humidity, NAA: α-naphthaleneacetic acid

Table 3. Growth and development of calla lily (Zantedeschia elliottiana) plantlets during in vitro rooting stage in the PAM (photoautotrophic micropropagation) and PMM (photomixotrophic micropropagation) systems.

Treatment code	Shoot length (mm)	Number of leafy shoots	Leaf area (cm^2)	Fresh weight (mg)	Dry weight (mg)
PAM (on day 15)	91.4a	3.7a	12.8a	674a	45a
PMM (on day 15)	51.3c[z]	3.3a	7.3b	395b	23b
PMM (on day 30)	76.3b	3.4a	9.8b	579ab	36a

[z]Mean separation within columns by LSD test at $P < 0.01$ (n=100).

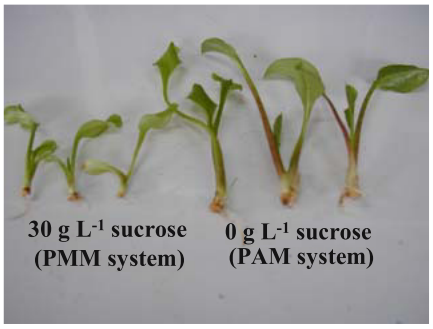

Figure 4. Calla lily (Zantedeschia elliottiana) plantlets on day 15 cultured on sugar-containing medium (PMM system, Left) and sugar-free medium (PAM system, Right) (See also Table 3).

The PAM shortened the period of *in vitro* multiplication as well as rooting by half (from 30 to 15 days), compared with that in the PMM. The greater plantlet growth in the PAM than in the PMM was mainly due to the increased photosynthesis and transpiration under high PPF, high CO_2 concentration, enhanced air movement, and low relative humidity in the vessel (Aitken-Christie et al., 1995). Under such environmental conditions, the plantlets generally develop physiologically and morphologically normal stomata. Low relative humidity enhances transpiration, and thus nutrient uptake. The percent loss of *in vitro* plantlets due to contamination was 0% on day 15 in the PAM, and 5% on day 30 in the PMM (Table 4). Therefore, the monthly production capacity of calla lily plantlets in the PAM is about 3 times (= 30/15 x 67,500/(0.95 x 45,000) higher than that in the PMM (The factor of 0.95 in the above expression comes from the 5% loss of *in vitro* plantlets in the PMM). Percent rooting *in vitro* was 98% in the PAM (day 15) and in the PMM (day 30).

5. CHINA FIR PLANTLET GROWTH

Stem length, number of shoots, leaf area, fresh and dry weight of plantlets on day 28 were 1.7, 2.1, 5.3, 2.5 and 2.9 times greater in the PAM-0 than in the PMM (Table 5). There were no significant differences in shoot growth, number of shoots or leaf area between the PAM-1 and the PAM-0 (Figure 5). The percentages of rooted plantlets *in vitro* on day 28 in the PAM-0, PAM-1, and PMM, were 91%, 93% and 65%, respectively. In the PAM, the presence of NAA in the medium had little effect on the increase in percent *in vitro* rooting, although roots formed 2 to 3 d earlier in the PAM-1 than in the PAM-0.

Table 4. Production performance and sales price of the PAM (photoautotrophic micropropagation) and PMM (photomixotrophic micropropagation) systems.

Calla lily	**PAM (A)**	**PMM (B)**	**A/B ratio**
Percent loss *in vitro*	0% on day 15	5% on day 30	0
Percent rooting *in vitro*	98% on day 15	98% on day 30	1.0
Multiplication or rooting cycle	15 days	30 days	0.5
Percent survival *ex vitro* on day 12	95%	60%	1.6
Price per *in vitro* rooted plantlet	7.23 US cents	6.02 US cents	1.09
Price per *ex vitro* acclimatized plantlet	18.1 US cents	14.5 US cents	1.25
Yearly production capacity of *in vitro* plantlets per module	152,000	52,000	2.92

China fir	**PAM-0 (A)**	**PAM-1**	**PMM (B)**	**A/B ratio**
Percent rooting *in vitro* on day 28	91%	93%	65%	1.4
Percent survival *ex vitro* on day 12	95%	97%	16%	5.9

The higher percent of *in vitro* rooting in the PAM-0 and the PAM-1 than in the PMM was probably due to the absence of sugar in the medium, the use of porous supporting material and enhanced photosynthesis. Sugar in the medium can inhibit adventitious root development in the early stage (Jarvis, 1986). Porous supporting materials such as vermiculite, perlite or mixtures of these materials promoted the *in vitro* rooting of plant species such as sweetpotato (Afreen-Zobayed et al., 1999), coffee (Nguyen et al., 1999) and sugarcane (Xiao et al., 2002). Endogenous phytohormones such as auxin necessary for rooting and carbohydrates must be more produced by plantlets in the PAM than by plantlets in the PMM.

Table 5. Growth and development of China fir (Cunninghamia lanceolata) plantlets on day 28, in the PAM-0, PAM-1 and PMM systems. PAM and PMM denote photoautotrophic and photomixotrophic micropropagation, respectively. PAM-0: PAM with absence of NAA in the medium, PAM-1: PAM with presence of NAA in the medium.

Treatment code	Shoot length (mm)	No. of shoots	Leaf area (cm^2)	Fresh weight (mg)		Dry weight (mg)	
				Shoot	Root	Shoot	Root
PAM-0	77.2a[z]	68a	3.88a	299a	84a	58a	4.7a
PAM-1	73.5a	56a	3.13a	272a	78a	57a	4.1a
PMM	46.1b[z]	33b	7.30b	105b	51b	19b	2.8b

[z]Mean separation within columns and experiment by LSD test at P <0.01 (n=80)

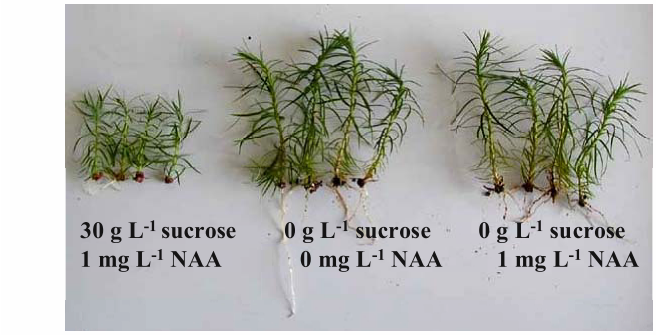

Figure 5. China fir (Cunninghamia lanceolata) on day 28 in the PMM (Left), PAM-0 (middle), and PAM-1 (Right) systems. PAM-0: PAM with absence of NAA in the medium, PAM-1: PAM with presence of NAA in the medium.

6. PERCENT SURVIVAL DURING ACCLIMATIZATION *EX VITRO*

Percent survival of calla lily plantlets during the *ex vitro* acclimatization was 95% in the PAM and 60% in the PMM, and that of China fir was 95% in the PAM-0, 97% in the PAM-1, and 16% in the PMM. The percent survival was about 6 times higher in the PAM-0 and the PAM-1 than in the PMM. The lower percent survival in the PMM was probably due to the malfunction of stomata in leaves (Aitken-Christie et al., 1995) and callus formation at the base of the nodal cuttings. Callus formation has been considered a cause of poor root initiation and limited uptake of nutrient and water (Nguyen, et al., 1999). The higher percent survival in the PAM was probably because the plantlets were already acclimatized and functionally photoautotrophic *in vitro* (Kozai and Zobayed, 2000). The percent survival of calla lily plantlets transplanted in the farmer's greenhouse without shading (i.e., no acclimatization procedure) was 80%, compared with 95% in the greenhouse with a shading screen. Thus, by further improving the *in vitro* acclimatization method in the PAM, the *ex vitro* acclimatization process could be eliminated under moderate weather conditions (Heo and Kozai, 1999). This simplification is one of the advantages of the PAM.

7. PRODUCTION COST OF CALLA LILY PLANTLETS: A CASE STUDY

The investment cost of the module (excluding the culture room) was US$15,180 or CNY126,000 (Chinese Yuan) for the PAM (1 US$ = 8.3 CNY as of 2003), and was 5,807 US$ for the PMM. Since the lifetime of the PAM and PMM was considered to be 10 years, yearly depreciation was 1,518 US$ for the PAM and 581 US$ for the PMM. The yearly production capacity per module of the PAM was about 152,000 plantlets (= 365/18 x 7,500), since one multiplication cycle was 18 days; 15 days for multiplication, 1 day for harvesting and 2 days for cleaning, preparation and transplanting. On the other hand, the yearly production capacity of the PMM was about 52,000 plantlets (= 365/33 x 5,000 x 0.95), since one multiplication cycle was 33 days; 30 days for multiplication, 1 day for harvesting and 2 days for cleaning, preparation and transplanting. Therefore, if the PAM and PMM were operated throughout the year, the yearly depreciation of investment cost per plantlet was 0.010 US$ (= 1,518/152,000) or 1.00 US cent in the PAM, and 0.0112 US$ (= 581/52,000) or 1.12 US cent in the PMM. In other words, the investment cost per plantlet was a little lower in the PAM than in the PMM, as shown in Table 6. The above figures indicate that the investment cost accounted for 18% of the production cost in the PAM and 12% of the production cost in the PMM.

7.1. Production cost per acclimatized plantlet

The production cost per *ex vitro* acclimatized plantlet from the PAM was 59% of that from the PMM (Table 6). The cost for *ex vitro* acclimatization in the PAM and the PMM accounted for 50% and 56% of the production cost, respectively. Thus, an increase in percent survival during *ex vitro* acclimatization significantly decreases

the production cost. Of the total costs *for ex vitro* acclimatization in the PAM, 20% was spent on the construction of the greenhouse, 56% was spent on labor, 19% was spent on supplies such as substrate, electricity, water, fertilizer and pesticide and 5% was spent on other items. On the other hand, in the PMM, 11% was spent on the construction of the greenhouse, 38% was spent on labor, 11% was spent on supplies such as substrate, electricity, water, fertilizer and pesticide and 40% was spent on other items. The lower cost of *ex vitro* acclimatization in the PAM than in the PMM was mainly due to a higher percentage of survival *ex vitro* and less labor. The cost for *in vitro* multiplication, which was equal to that for *in vitro* rooting, in the PAM was 58% of that in the PMM (Table 6). Labor cost in the PAM was less than half of that in the PMM. This result is consistent with the prediction by Kozai et al. (2000). The reduced labor cost in the PAM significantly reduced the cost for *in vitro* multiplication and rooting. Electric energy consumption per plantlet during the *in vitro* multiplication was 27.2 Wh (or 97.9 kJ = 27.2 Wh x 3600 s) in the PAM; 69% for lighting, 24% for cooling, 2% for air pumping and 5% for autoclaving. On the other hand, it was 42.8 Wh in the PMM; 56% for lighting, 21% for cooling, 23% for autoclaving using electricity. Electric energy consumption per plantlet in the PAM, therefore, was 64% of that in the PMM. The lower electricity consumption in the PAM was mainly due to the reduction in multiplication and rooting periods by half, a low electric energy consumption for autoclaving the medium and vessels, and a high percent utilization of light energy by using white reflective sheets. A preliminary experiment showed that PPF was 1.65 times higher on a shelf with the reflective sheets (86 ± 8 μmol m^{-2} s^{-1}) than on a shelf without the reflective sheets (52 ± 12 μmol m^{-2} s^{-1}) when four fluorescent lamps were turned on. Average air temperature outside the culture room was 18 C during the experiment.

7.2. Cost, labor time and electricity consumption for in vitro multiplication or rooting

The electricity consumption for cooling increases with increasing outside air temperature. Thus, the cooling cost would be increased by 50-60% when outside air temperature was around 35 C (Aitken-Christie et al., 1995).

7.3. Sales price of in vitro and ex vitro acclimatized plantlets

The sales price of calla lily plantlets *in vitro* was about 7.23 US cents when produced using the PAM, and was 6.02 US cents when produced using the PMM; i.e., the sales price was 20% higher in the PAM than in the PMM because of the higher quality produced by the PAM. The sales price of *ex vitro* acclimatized plantlets was 18.1 US cents when produced in the PAM, and was 14.5 US cents when produced in the PMM and acclimatized *ex vitro*; i.e., the sales price was 25% higher in the PAM than in the PMM because of the higher quality produced by the PAM. In this experiment, it was not possible to record the expenses for supervision, administration, sales, transportation of plantlets to farmers, etc., and thus to calculate the profit per plantlet. This is because the personnel doing were doing other jobs

concurrently. According to Chu (1992), the supervision cost accounts for 13% of the total production cost in the micropropagation industry. In any case, The PAM could produce higher quality plantlets at a lower cost than the PMM, which shows that the PAM has a commercial advantage over the PMM.

Table 6. A comparison of production cost, labor time and electricity consumption per plantlet of calla lily in the PAM (photoautotrophic micropropagation) and the PMM (photomixotrophic micropropagation) systems.

Cost per *ex vitro* acclimatized plantlet (US cent)	**PAM (A)**	**PMM (B)**	**A/B Ratio**
Investment cost	1.00 (18%)	1.12 (12%)	0.89
in vitro multiplication	0.84 (16%)	1.44 (16%)	0.58
in vitro rooting	0.84 (16%)	1.44 (16%)	0.58
ex vitro acclimatization	2.65 (50%)	5.06 (56%)	0.52
Total	5.33 (100%)	9.06 (100%)	0.59
***In vitro* multiplication cost per plantlet (US cent)**	**PAM (A)**	**PMM (B)**	**A/B Ratio**
Labor cost	0.35 (42%)	0.75 (52%)	0.47
Electricity cost	0.22 (26%)	0.36 (25%)	0.61
Medium cost	0.16 (19%)	0.22 (15%)	0.73
Others	0.11 (13%)	0.11 (8%)	1.00
Total	0.84 (100%)	1.44 (100%)	0.58
***Ex vitro* acclimatization cost per plantlet (US cent)**	**PAM (A)**	**PMM (B)**	**A/B ratio**
Investment for greenhouse	0.53 (20%)	0.56 (11%)	0.95
Labor	1.48 (56%)	1.92 (38%)	0.77
Supplies	0.50 (19%)	0.56 (11%)	0.89
Others	0.13 (5%)	2.02 (40%)	0.06
Total	2.65 (100%)	5.06 (100%)	0.52
Labor time for *in vitro* multiplication per plantlet (s)	**PAM (A)**	**PMM (B)**	**A/B Ratio**
Vessel washing	0.48 (2.4%)	3.84 (9%)	0.13
Harvesting	0.48 (2.4%)	7.68 (18%)	0.06
Medium preparation	0.96 (4.8%)	3.84 (9%)	0.25
Excising/transplanting	17.28(85.6%)	25.92 (60%)	0.67
Others	0.96 (4.8%)	1.92 (4%)	0.50
Total	20.16 (100%)	43.2 (100%)	0.47
Electricity consumption for *in vitro* multiplication per plantlet (Wh)	**PAM (A)**	**PMM (B)**	**A/B Ratio**
Lighting	18.7 (69%)	24.1 (56%)	0.65
Cooling	6.49 (24%)	9.1 (21%)	0.71
Air pumping	0.56 (2%)	-	-
Autoclaving	1.50 (5%)	9.6 (23%)	0.20
Total	27.2 (100%)	42.8 (100%)	0.16

1 US$ = 8.3 CNY (Chinese Yuan), as of 2001. Wh (Watt-hour): 3600 Joule

10. COMMERCIAL APPLICATION

8. CONCLUSIONS

In comparison with plantlets produced by the conventional micropropagation system using small vessels with sugar-containing medium, plantlets produced by the photoautotrophic micropropagation system using large vessels with sugar-free medium resulted in better growth, lower percent loss due to contamination, higher quality, higher percent survival *ex vitro*, and lower production costs. Therefore, the photoautotrophic micropropagation system has advantages over the conventional micropropagation system for commercial production of calla lily plantlets and China fir plantlets with respect to production costs and sales price. This system should be useful for commercial production of micropropagated plantlets of other plant species.

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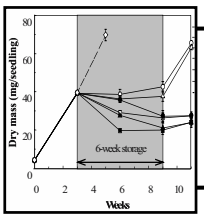
Chapter 11

C. KUBOTA



LOW TEMPERATURE STORAGE OF PLANTS UNDER DIM LIGHT

Contents

Key words: Dim light, light quality, low temperature storage.

1. INTRODUCTION

In the production of quality plantlets by micropropagation, there is a crucial need to store plantlets for a short term if production facilities and labor are to be used efficiently and a flexible production schedule is intended. For this type of storage, not only plant growth but also the plant quality needs to be preserved. For the storage of plantlets in the middle of the multiplication stage, photosynthetic and growth abilities of explants produced from the stored plantlets is an important quality index, while visual quality in addition to the photosynthetic and growth abilities is important for stored plantlets ready to be sold as transplants. Dark storage (storing plantlets under darkness) has been utilized in commercial operations, but there are problems such as rapid deterioration of plantlets during storage, and thus the number of species storable under darkness is limited. The positive effects of dim light in low temperature storage have been shown for seedlings of several species by Heins et al. (1992). Similar effects were observed in photoautotrophically micropropagated plantlets (e.g., Kubota and Kozai, 1994). Through the investigation on optimizing storage environment for photoautotrophically cultured plantlets, the optimum conditions for storing plantlets, and later for seedlings or transplants in general, were shown to be the combinations of low temperature with PPF close to the light compensation point (in case of broccoli (*Brassica oleracea* L. Botrytis Group) plantlets, the combination of 5 C and 2 μmol m^{-2} s^{-1} PPF gave the best result). These findings contributed to discovering a theory to optimize plantlet

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 205-212.

storage environment based on mass balance of the vessels and plantlets under various environmental conditions, a new area of controlled environment in plant production system. In this chapter, factors affecting low temperature storage will be summarized with emphasis on tissue culture and especially applications in photoautotrophic culture conditions. Information on storing seedlings (transplants) is also added when necessary. Principles and applications of low temperature storage of transplants are also discussed in Kubota (2003).

2. FACTORS AFFECTING REGROWTH ABILITY AND VISUAL QUALITY OF PLANTLETS DURING STORAGE

2.1. Temperature, light intensity and photoperiod

Traditionally, harvested fresh horticultural produce are stored under low temperature, since lowering temperature can slow the metabolic processes (e.g., respiration) and thereby prevent undesirable loss of dry mass and associated deteriorations of quality. Post harvest temperature is generally selected to be the lowest possible temperature that does not cause chilling injury. For storage of plantlets, placing vessels under low temperature and dark environment has been practiced in commercial laboratories to slow down the growth to intentionally delay the subculture timings especially for limited plant species that are relatively tolerate to low temperatures. In conventional micropropagation, the medium contains and therefore provides energy during the storage. However, a drawback of sugar-containing medium in a prolong storage period is that it often causes an increased chance of contamination during storage.

According to Reed (1993), storage conditions for *in vitro* genetic conservation of temperate genera are typically 4 or 5 C in darkness. However, positive effects of illumination during storage were also reported. Dorion et al. (1991) successfully stored rose plantlets at 2 to 4 C under 9 to 18 μmol m^{-2} s^{-1} PPF at 8 h photoperiod for 6 months. Baubault et al. (1991) reported that *in vitro Rhododendron* plantlets were successfully stored at 4 C under 30 μmol m^{-2} s^{-1} at 14 h photoperiod for up to 12 months. Reed (1999) also reported the positive effect of illumination on mint plantlets during storage, indicating the recommendation as the combination of 4 C with 12 h photoperiod.

Under photoautotrophic culture conditions, the medium does not provide an energy source to the plantlets during storage, and therefore, maintaining carbon balance during storage by sustaining a minimum amount of photosynthesis is important for success in maintaining photosynthetic and regrowth ability of photoautotrophic plantlets during storage. Among a number of environmental factors affecting plantlet growth and quality deterioration during storage, temperature and light environments are the most important factors to manipulate. The same principle can apply to storage of transplants.

Illumination during storage has been shown in many horticultural species to extend storability of transplants (Heins et al., 1992, 1994; Kubota and Kozai, 1995). Heins et al. (1992) also found that transplants could be stored under higher air

temperature when light was provided during storage than in the dark. Kubota and Kozai (1995) found that the optimum light intensity for storage was the light compensation point of photosynthesis at the storage temperature, when the light was provided continuously (24 h per day), showing that the CO_2 exchange rates of plantlets exhibiting the best storability were maintained at null during storage.

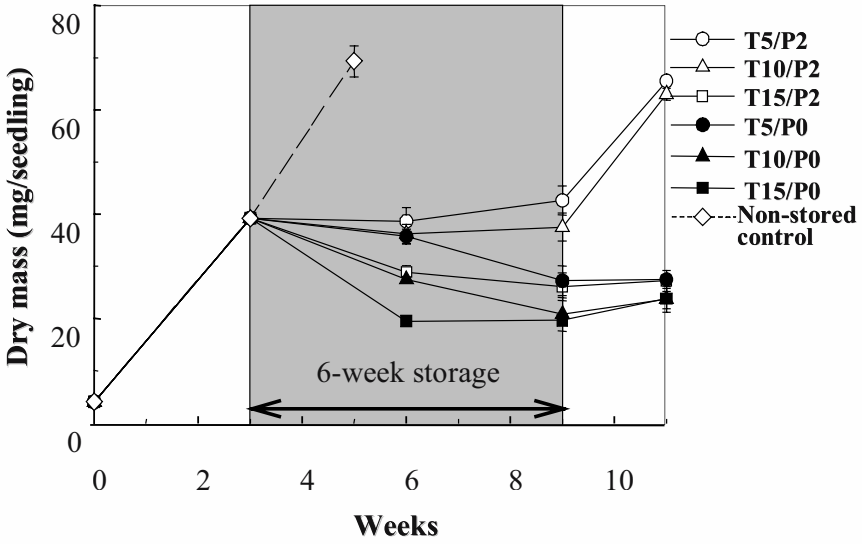


*Figure 1. Dry mass of broccoli plantlets grown in vitro for 3 weeks and stored for 6 weeks under various combinations of air temperature and photosynthetic photon flux (PPF) (after Kubota and Kozai, 1994). Numerical values after letter **T** and **P** denote the storage air temperature (C) and PPF (μmol m^{-2} s^{-1}), respectively. Seedlings were regrown in the same growth conditions (23 C, 160 μmol m^{-2} s^{-1} PPF and 16 h photoperiod) for 2 weeks subsequent to storage. The dry mass increase of non-stored seedlings was shown for comparison. PPF of 2 μmol m^{-2} s^{-1} was the light compensation point at 5 and 10 C of the seedlings examined in the experiment.*

Under continuous lighting conditions, the light compensation point maintains the carbon balance of the plant at null so that the dry mass per plant does not change. For example, dry mass of photoautotrophic broccoli plantlets *in vitro* were maintained unchanged through the 6 week storage under a light compensation point (2 μmol m^{-2} s^{-1} PPF) at 5 and 10 C air temperature and these conditions also maintained regrowth ability of the plantlets (Kubota and Kozai, 1994) (Figure 1). Dry mass of eggplant (*Solanum melongena* L.) seedlings increased linearly with increasing PPF, from 0 to 16 μmol m^{-2} s^{-1} (Kozai et al., 1996), indicating that 5 μmol m^{-2} s^{-1} was the light compensation point that maintained dry mass at the storage temperature (9 C). Kubota et al. (1995) stored photoautotrophically cultured broccoli plantlets at 5, 10 or 15 C under 2 or 5 μmol m^{-2} s^{-1} PPF and showed that lowering air

temperature in conjunction with a PPF either close to or higher than the light compensation point preserved regrowth ability of the plantlets. Higher PPF than the light compensation points, however, caused undesirable shoot elongation and dry mass increase of the plantlets. After 6 weeks of storage, a small difference of 2 and 5 μmol m^{-2} s^{-1} PPF caused considerable differences in plantlet dry mass and quality after storage, suggesting that light environment during storage should be carefully selected for each crop.

Photoperiod is also a factor affecting carbon balance and thereby dry mass of plantlets during storage. Under lighting cycles consisting of photo- and dark periods, the light intensity needs to be increased and carbon gain during the photoperiod needs to compensate the loss of carbon during the dark period. The light intensity providing plants the null daily carbon balance and thus keeping dry mass unchanged is called the "daily light compensation point" where the daily integrated gross photosynthetic rate is balanced with the daily integrated respiration rate. Kubota et al. (2002) stored eggplant plug seedlings at various combinations of light intensity and photoperiod and showed that, as long as the daily PPF is equal to the daily light compensation point (shown to be 430 mmol m^{-2} d^{-1} at 9 C), seedling growth and quality after 4 weeks of storage were not affected by the combinations of PPF and photoperiod. This will allow flexibility in designing the light environment for storage and may make light installation in storage more practical and more realistic in transplant production.

Light compensation points of plant photosynthesis are affected by other environmental conditions that affect photosynthetic and respiration rates (temperature and CO_2 concentration). Kozai et al. (unpublished) showed, using unrooted chrysanthemum cuttings, the relationship between light intensity (PPF), temperature, and CO_2 concentration that give null carbon balance (null net photosynthetic rate) of plants. The relationship can be depicted in a 3-axied graph as a surface, namely "compensation surface", where given values of two environmental variables of the three (air temperature, CO_2 concentration and PPF) determine value of the third environmental variable. This concept was also demonstrated by Fujiwara et al. (2001) in the low temperature storage of tomato (*Lycopersicon esculentum* Mill.) grafted seedlings showing various combinations of PPF and CO_2 concentration that provide null carbon balance for the seedlings at 10 C air temperature. Based on these findings, transplants/plantlets can be theoretically stored at lower PPF when air temperature is lower and/or CO_2 concentration is higher. However, as shown by Fujiwara et al. (1999c and 2001), a lowest threshold of PPF that can maintain normal metabolic activities during storage is likely to exist. For example, tomato grafted seedlings were stored under various combinations of CO_2 concentration and PPF that were determined to be light compensation points at the corresponding CO_2 concentration (2.5, 1.9, 1.3, 0.9, and 0.5 μmol m^{-2} s^{-1} PPF at 0.05, 0.25, 0.50, 0.75, and 1.00% CO_2, respectively and the best storability of the seedlings after 28 days were at 1.9 and 1.3 μmol m^{-2} s^{-1} PPF under 0.25 and 0.50% CO_2, respectively, while significant quality degradation was observed at 0.9 and 0.5 μmol m^{-2} s^{-1} PPF (Fujiwara et al., 2001). Perhaps a certain minimal level of light is necessary to provide gross photosynthesis (energy) and/or

to induce regulatory effects (signals) sufficient for maintaining chlorophyll synthesis and other important metabolic functions (Fujiwara, personal communication).

2.2. Sugar in the medium

When plantlets were cultured photomixotrophically (with sugar in the medium), light compensation points were generally higher than those plantlets culture photoautotrophically, primarily due to a higher dark respiration rates as affected by higher sugar concentrations in the medium and therefore in the plantlet tissue. Kubota and Kozai (1995) reported that 20 – 50% greater dark respiration rates were observed for photomixotrophic broccoli plantlets compared with those for the photoautotrophic plantlets over air temperatures ranging from 3 to 25 C.

Sugar in the medium seems to be able to maintain dry mass of plantlets under a wider range of environmental conditions during low temperature storage. For example, Kubota and Kozai (1995) compared photoautotrophic and photomixotrophic broccoli plantlets stored under the same conditions (5, 10, or 15 C) under darkness or 2 μmol m^{-2} s^{-1} PPF. Photoautotrophic plantlets maintained the dry mass unchanged under illumination at 5 or 10 C, while photomixotrophic plantlets did so at all conditions except for 15 C under darkness. Apparently sugar in the medium compensated for the respiratory loss of carbon and contributed to maintaining the dry mass during the storage. However, chlorophyll concentrations of leaves were maintained at higher levels with illumination during storage and chlorophyll fluorescence parameters indicated the high photosynthetic activities of chlorophyll when stored under light. In fact, the broccoli plantlets stored in darkness either lost their regrowth ability or showed significant leaf damage presumably due to photoinhibition during the subsequent culture period, indicating the necessity of illumination to maintain photosynthetic and regrowth ability of the photomixotrophic plantlets for assuring normal growth after removing them from the storage.

2.3. Light quality

Light quality is one of the environmental factors affecting plant growth and development. However, physiology of plants under different light qualities at low temperature has not been well investigated. Along with findings on optimum environmental conditions for storing transplants, effects of light quality on transplants stored at low temperature have been examined (Fujiwara et al., 1999a; Kubota et al., 1996 and 1997; Wilson et al., 1998a and b). Broccoli plantlets grown in tissue culture vessels exhibited greater stem elongation and decrease in chlorophyll concentration under red and blue light than those under white light after 6 weeks in storage at 5 C (Kubota et al., 1996). However, Wilson et al. (1998a, 1998b) showed that quality of broccoli seedlings was best maintained under red light compared with white or blue light. Fujiwara et al. (1999b) also reported that the quality of harvested culinary herbs was better maintained under red light than white light. The red light source employed in Wilson et al. (1998a and 1998b) and

Fujiwara et al. (1999b) was light emitting diodes, while that in Kubota et al. (1996 and 1997) was fluorescent lamps covered with a spectral filter. Therefore conflicts of the findings with regard to the plant responses to red light during storage may include potential effects of different spectra employed in these experiments.

Light emitting diodes have been considered as a light source for plant production (Bula et al., 1991; Brown et al., 1995). Application of LEDs to low temperature storage may be more feasible than for biomass production, since the light fixture size is smaller and reduction in irradiance at low temperatures is relatively less than for fluorescent lamps. If such lighting systems become available at a reasonable price, introduction of low temperature storage under dim lighting conditions will be facilitated for various horticultural operations including tissue culture propagation facilities.

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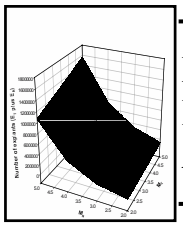
Chapter 12

C. KUBOTA



MODELLING AND SIMULATION FOR PRODUCTION PLANNING IN PHOTO AUTOTROPHIC MICROPROPAGATION

Contents

Key words: Modelling, multiplication rates, photoautotrophic micropropagation, production planning, simulation.

1. INTRODUCTION

The seasonal market regulates transplant production planning. The number of transplants produced should meet the constraints of narrow market windows. Transplant production via micropropagation generally involves many subcultures and transitions of stages. Therefore, production planning to meet the demand becomes sometimes challenging. Rough estimation of production easily creates overproduction or underproduction. Use of modelling and simulation techniques for optimizing production planning is an important area where such techniques can be used effectively. Simulating production based on mathematical models is also effective for decision making to select proper environmental conditions and production methods. Photoautotrophic micropropagation is considered to be well adapted to modelling and simulation of number of plants produced, since plant growth and development rates in photoautotrophic micropropagation are more predictable than conventional photomixotrophic culture and they can be manipulated by controlling environmental conditions.

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 213-224.

Simonton and Thai (1988) and Walker (1991) reported modelling and simulation for production planning in conventional micropropagation. The models were designed for scheduling where the objectives were matching production to demand (Simonton and Thai, 1988) and maximizing profit (Walker, 1991). The simulations were based on known multiplication ratios obtained empirically and they demonstrated the high potential for using such techniques as strategic production tools. In photoautotrophic micropropagation, multiplication ratios can be expressed using environmental independent variables, which makes the simulation more powerful and useful in production planning and scheduling with an aim of accomplishing potential objectives of production such as maximizing production, meeting the production with market demand, minimizing electric energy consumption, and minimizing labor input.

Conventional vegetative propagation (i.e., cutting production) is also recognized as an area where models can contribute to enhanced production efficiency. Vegetative propagation has been a significant mode of transplant production (Hartmann et al., 1997), and the number of species propagated vegetatively has been increasing in commercial transplant production operations. Propagation often begins with *in vitro* micropropagation for establishing virus-free or pathogen-free stock plants, followed by propagation *ex vitro*. Plantlets produced by micropropagation are transferred to sterile soil or to hydroponic systems in an Arthropod-excluding screen house and grown as stock plants for increasing pathogen-free propagules and transplants. An ecological modelling technique successfully simulates the discrete production of propagules and the number of stock plants (plants grown to provide the cuttings that become propagules) accommodated in the production areas, as affected by different propagation methods and environmental conditions (Kubota and Kozai, 1999 and 2001). The model could estimate the numbers of each constituent (i.e., stock plants of different ages, cuttings produced, stock plant bases to be discarded, etc.) for each day after start of the propagation process. It is advantageous to quantitatively understand the propagation process and to simulate the effects of any unexpected modifications in propagation methods during the propagation period. Re-scheduling of production is therefore easily performed with such day-by-day or week-by-week simulation. Models developed for vegetative propagation (such as Kubota and Kozai, 2001) can apply to simulating photoautotrophic micropropagation with little modification.

2. A MODEL FOR PHOTOAUTOTROPHIC MICROPROPAGATION PRODUCTION PLANNING AND ITS GENERIC STRUCTURE

Based on the generic vegetative propagation models reported by Kubota and Kozai (2001), here a model adapted more to the production planning in photoautotrophic micropropagation is described.

The followings are definitions and statements to be used in modelling and simulation of production planning of photoautotrophic micropropagation.

1) *Explants* are propagules used as starting materials in micropropagation.

Propagation is production of propagules and *in vitro propagation* is therefore production of explants.

2) *Plantlets* are plants grown *in vitro* in order to produce explants or to be transplanted into *ex vitro* conditions. Their status of maturity (age and size) is irrelevant to the definition of plantlets.

3) *Multiplication cycle* or *subculture cycle* is the duration of time required for plantlets to grow and produce new explants.

4) Explants are often classified as having different multiplication rates. For example, shoot tip cuttings (terminal shoots with apical buds) generally have greater growth and multiplication rates than nodal cuttings. The bases of plantlets with roots remaining after harvesting the leafy cuttings are sometimes re-used as explants for their higher growth and multiplication rates.

5) A portion of explants can leave the propagation process by being either sold or transferred to the ex-vitro finishing stage. The rest of the harvested explants are used for serial multiplication (subculture).

6) A portion of the plantlets can be stored (e.g., at low temperature) to control production.

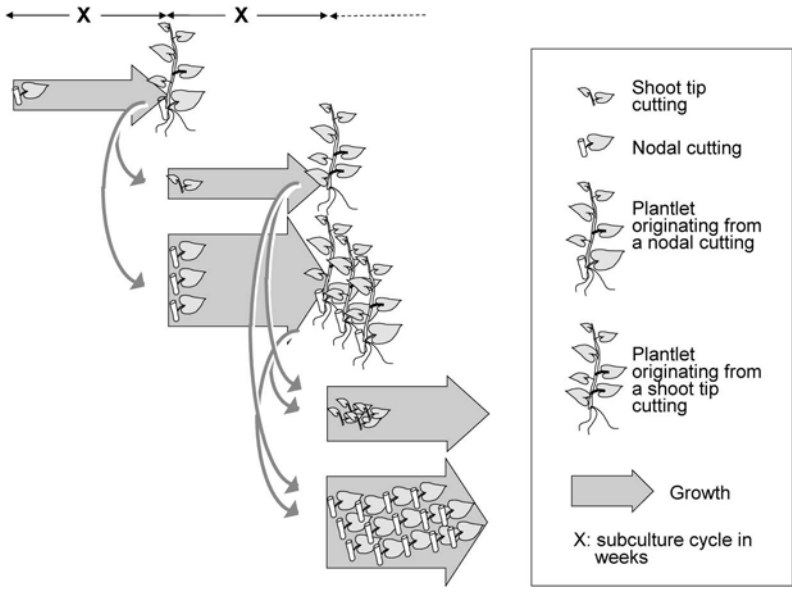


Figure 1. An example process of micropropagation by photoautotrophic culture. Micropropagation starts with a single leafy cutting as explant. Nodal cuttings and shoot tip cuttings are used as explants and produce designated number of explants after X weeks of subculture.

Figure 1 shows a process of photoautotrophic micropropagation, starting with a single nodal leafy cutting as an initial explant. In this example, nodal cuttings and

shoot tip cuttings used as explants are considered to have different multiplication ratios (a nodal cutting produce 3 nodal cuttings and one shoot tip cutting, while a soot tip cutting produce 5 nodal cuttings and one shoot tip cutting for the same duration of subculture).

According to the vegetative propagation model developed by Kubota and Kozai (2001), the dynamics of the number of explants and plantlets in the micropropagation shown in Figure 1 are expressed in a simple matrix notation using an interval of one week.

$$\mathbf{N}(t+1) = \mathbf{M} \cdot \mathbf{N}(t) \tag{1}$$

where $\mathbf{N}(t)$ and $\mathbf{N}(t+1)$ are the population vectors (single column matrices) showing the number of each population constituent (shoot tip cuttings, nodal cuttings, plantlets originating from shoot tip cuttings, and plantlets originating from nodal cuttings) on week t and week $t+1$, respectively. The multiplication matrix (**M**) contains all the multiplication and transition parameters that are identical for each population constituent. The observation interval is weekly and all the numbers shown in the population vectors are those after the scheduled operations (cutting, transferring, etc.) of that week.

The population vector on week t is expressed as follows, considering the four population constituents shown in Figure 1:

$$\mathbf{N}(t) = [E_T(t); \quad E_N(t); \quad \mathbf{P_T}(t); \quad \mathbf{P_N}(t)] \tag{2}$$

where

$$\mathbf{P_T}(t) = [P_{T,1}(t); \quad P_{T,2}(t); \quad \ldots\ldots P_{T,X-1}(t)] \tag{3}$$

$$\mathbf{P_N}(t) = [P_{N,1}(t); \quad P_{N,2}(t); \quad \ldots\ldots P_{N,X-1}(t)] \tag{4}$$

where $E_T(t)$ and $E_N(t)$ are numbers of shoot tip cuttings and nodal cuttings harvested as explants on week t. $\mathbf{P_T}(t)$ and $\mathbf{P_N}(t)$ are population vectors consisting of the numbers of plantlets originating from shoot tips and nodal cuttings, respectively, and they are further classified into plantlets grown for x weeks (x =1, 2,… X-1) of the subculture period. By combining equations (1) and (2), the dynamics of the number of explants and plantlets are now expressed as:

$$\begin{bmatrix} E_T(t+1) \\ E_N(t+1) \\ \mathbf{P_T}(t+1) \\ \mathbf{P_N}(t+1) \end{bmatrix} = \begin{bmatrix} \mathbf{0} & \mathbf{0} & \mathbf{M_{PT,\,ET}} & \mathbf{M_{PN,\,ET}} \\ \mathbf{0} & \mathbf{0} & \mathbf{M_{PT,\,EN}} & \mathbf{M_{PN,\,EN}} \\ \mathbf{M_{ET,\,PT}} & \mathbf{0} & \mathbf{M_{PT,\,PT}} & \mathbf{0} \\ \mathbf{0} & \mathbf{M_{EN,\,PN}} & \mathbf{0} & \mathbf{M_{PN,\,PN}} \end{bmatrix} \cdot \begin{bmatrix} E_T(t) \\ E_N(t) \\ \mathbf{P_T}(t) \\ \mathbf{P_N}(t) \end{bmatrix} \tag{5}$$

where $\mathbf{M_{PT,ET}}$, $\mathbf{M_{PN,ET}}$, $\mathbf{M_{PT,EN}}$, $\mathbf{M_{PN,EN}}$, $\mathbf{M_{ET,PT}}$, $\mathbf{M_{PT,PT}}$, $\mathbf{M_{EN,PN}}$, and $\mathbf{M_{PN,PN}}$ are multiplication submatrices containing multiplication ratios and transition parameters (0 or 1) of each population constituents. Note that all the plantlets produced after X weeks of subculture are to be used for producing explants or moved to successive stages (shipping or storage) after the week of operation and therefore they are not shown in the population vectors of $\mathbf{P_T}(t)$ and $\mathbf{P_N}(t)$. For example, when X=4, $\mathbf{P_T}(t)$=[P_{T1}; P_{T2}; P_{T3}] and $\mathbf{P_N}(t)$=[P_{N1}; P_{N2}; P_{N3}] and the multiplication submatrices are:

$$\left.\begin{aligned}
&\mathbf{M_{PT,ET}} = [0 \quad 0 \quad 1] \\
&\mathbf{M_{PN,ET}} = [0 \quad 0 \quad 1] \\
&\mathbf{M_{PT,EN}} = [0 \quad 0 \quad m_T] \\
&\mathbf{M_{PN,ET}} = [0 \quad 0 \quad m_N] \\
&\mathbf{M_{ET,PT}} = [1; \quad 0; \quad 0] \\
&\mathbf{M_{PT,PT}} = [0 \quad 0 \quad 0; \quad 1 \quad 0 \quad 0; \quad 0 \quad 1 \quad 0] \\
&\mathbf{M_{EN,PN}} = [1; \quad 0; \quad 0] \\
&\mathbf{M_{PN,PN}} = [0 \quad 0 \quad 0; \quad 1 \quad 0 \quad 0; \quad 0 \quad 1 \quad 0]
\end{aligned}\right\} \tag{6}$$

where m_T and m_N are multiplication ratios of shoot tips and nodal cuttings, respectively.

The multiplication ratios of m_T and m_N are affected by *in vitro* environmental conditions in photoautotrophic micropropagation and therefore expressed mathematically using *in vitro* environmental conditions as independent variables. For example,

$$m_T = f(v_1, v_2, v_3 \ldots) \tag{7}$$

$$m_N = g(v_1, v_2, v_3 \ldots) \tag{8}$$

where m_T and m_N are expressed using different equations ($f(v)$ and $g(v)$) having multiple independent variables of environmental conditions (v_1, v_2, v_3…), which could be air temperature, PPF, CO_2 concentration, etc.

After producing sufficient plantlets, a portion of plantlets will be moved to the *ex vitro* finishing stage or to short-term storage. Such an event can be expressed by weighing the population vector after, for example, $P_{T2}(t)$ (or the sum of $P_{T2}(t)$ and $P_{N2}(t)$) reaches a target value.

Using the equations (5-6), production of micropropagation shown in Figure 1 was simulated and dynamic changes in numbers of the population constituents of the equations (2-4) are shown in Figure 2, where X, m_T, and m_N are given as the constants 4, 5, and 3, respectively. The simulation was started using 20 nodal cuttings on week 0. Quantities of the two kinds of explants and plantlets increased with increasing number of weeks of operation. All the explants produced were used for the successive multiplication in this simulation. The total number of nodal cuttings and shoot tip cuttings was 24,560 and 7,120 after 20 weeks of operation.

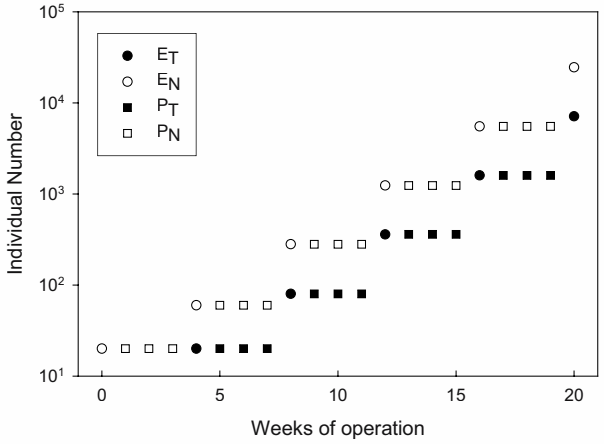


Figure 2. Time courses of numbers of explants (nodal cuttings, E_T; shoot tip cuttings, E_N) and plantlets originating from nodal cuttings (P_T) and shoot tip cuttings (P_N) when micropropagation was started with 20 nodal cuttings on week 0 following the procedure shown in Figure 1. All explants produced in each 4-week subculture were used for generating explants for the successive subculture.

Figure 3 shows the sensitivity of the 20 week production to the multiplication ratios. The production of explants (total number of two types of explants, nodal cutting and shoot tip cutting) after 20 weeks of micropropagation starting with 20 nodal cuttings increased exponentially with increasing multiplication ratios of the explants (m_T and m_N). It is noted that multiplication ratio of nodal cuttings had more pronounced effect on total production of explants than that of shoot tip cuttings. This is because plantlets produced more nodal cuttings than shoot tip cuttings.

3. ENVIRONMENTAL FACTORS AFFECTING MULTIPLICATION RATES IN SWEETPOTATO VEGETATIVE PROPAGATION

Assessing multiplication rates is critical to predict the number of plantlets produced after many cycles of subculture, as shown in Figure 3. In photoautotrophic culture, modelling multiplication ratios using environmental variables as independent

variables is relatively easier than for conventional photomixotrophic culture, since growth and development of photoautotrophic plantlets are more highly regulated by physical environmental conditions. However, little research has been done for numerical analysis of multiplication rates in photoautotrophic culture as affected by environmental conditions. Because of the limited availability of data in photoautotrophic culture, data from the vegetative propagation of sweetpotato using single nodal cutting as propagules under controlled environment are used as alternatives hereafter.

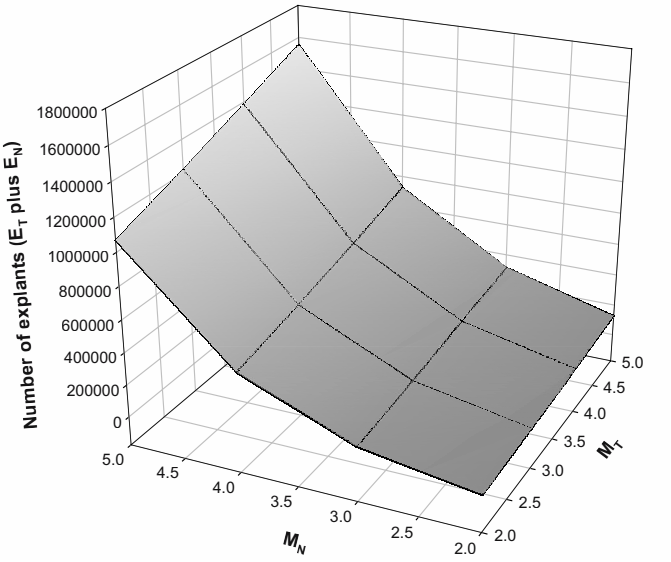


Figure 3. Number of sweetpotato explants produced after 20 weeks of micropropagation using nodal cuttings and shoot tip cuttings as explants, as affected by multiplication ratio of nodal cutting (m_N) and of shoot tip cutting (m_T). Duration of subculture was 4 weeks. Total production of explants (nodal cuttings, N_N, plus shoot tip cuttings, E_T) was shown.

Number of leaves harvestable as explants generally increases linearly after a lag time prior to first leaf emergence:

$$L_P = (T - T_0)\, R_L \tag{9}$$

where L_P is number of leaves usable as explants per plantlet, T is days after planting explants, T_0 is days to start producing leaves usable as an explant, and R_L is the leaf emergence rate (d^{-1}). For explants with n number of leaves, multiplication ratio (m)

is expressed as:

$$m = L_P / n \tag{10}$$

In the equation (9), T_0 and R_L can be expressed as functions of environmental variables. Air temperature, light intensity, photoperiod, and CO_2 concentration are

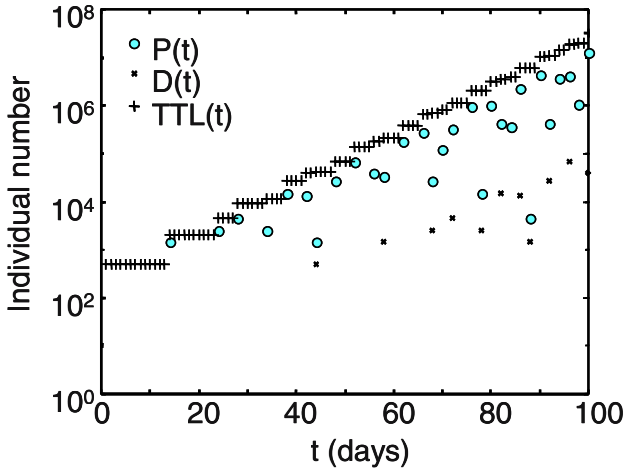


Figure 4. Simulated numbers of cuttings to be produced (P), stock plant bases to be discarded (D), and total number of stock plants maintained in the propagation system (TLL). Matrix transition models with daily observation interval were applied. The propagation began with 500 cuttings on day 0 (Kubota and Kozai, 1999).

such variables to consider. Fujiwara et al. (2003) examined effects of air temperature on leaf development of sweetpotato plants under controlled environment conditions. Leaf emergence rates R_L obtained in Fujiwara et al. (2003) were significantly affected by air temperature and the response was similar to that of a general temperature response curve having optimum temperature to maximize the rate. Lag time T_0 was also affected significantly by air temperature but the sensitivity of T_0 to air temperature was rather small compared to that of R_L. Yamaguchi (1999) examined PPF and photoperiod on such parameters to determine the number of nodal cuttings produced per plantlet. It was noted that factors affecting T_0 and R_L are different: PPF significantly affected R_L but not T_0, while photoperiod affected neither T_0 nor R_L.

Using a similar model, different culture methods can be examined. For example, Aitken-Christie and Jones (1987) showed that, using *Pinus radiata* shoots, a propagation method of maintaining a 'shoot hedge' (shoot clumps maintained like mini-hedges in the same vessel for multiple harvests of shoots) *in vitro* contributed to rapid production of shoots *in vitro*. A simulation was made to assess the use of

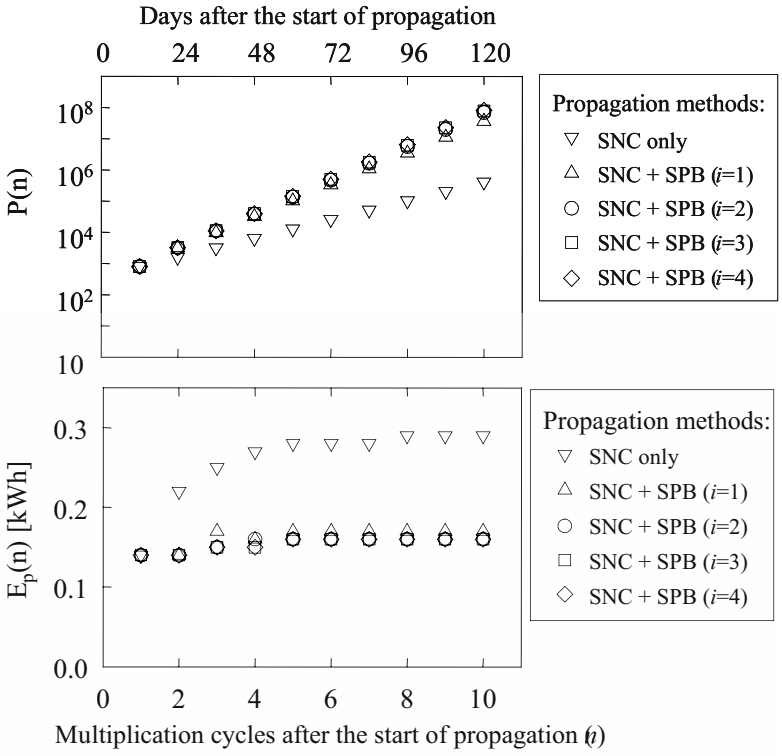


Figure 5. Simulated time courses of total production of harvestable single nodal cuttings (P(n)) and electric energy consumption per propagule (Ep(n), kWh) after many repetitions of multiplication cycles as affected by production method (use of single nodal cuttings (SNC) only vs. use of single nodal cuttings and stock plant bases (SPB) as propagules), and number of repetitive use of the SPB as propagules (i) (after Lok et al., 2002b). The initial number of SNC used as propagules were 400. Multiplication ratios of SNC and SPB were 1.8 and 3.7, respectively. Multiplication cycle was 12 days.

stock plant bases in addition to conventional single nodal cuttings as propagules for vegetative propagation of sweetpotato (Kubota, 2000). Figure 4 shows the simulated results of number of cuttings, total number of stock plants growing in the production system for the successive cutting production, and stock plants bases to be discarded after repeated usage as starting material (propagule) (Kubota, 2000). The graph may look complicated since, in this simulation, stock plants had different subculture duration (10 days) from that of single nodal cuttings (14 days). The stock plant bases were discarded after processing 3 multiplication cycles. Multiplication ratios were 5 for stock plant bases when it was the first or second cycle of repetitious use, or otherwise it was 3 for stock plant bases and nodal

cuttings. The simulated results generally agree with the observation by Aitken-Christie and Jones (1987) in terms of enhanced production by using shoot hedges (or stock plant bases) as propagules. Lok et al. (2002a and, b) also predicted the production of single nodal cuttings of sweetpotato as affected by propagation method (use of stock plant bases and nodal cuttings as propagules versus use of only nodal cuttings as propagules) and planting density. Use of stock plant bases, that are eventually discarded, as propagules in a conventional propagation method increased the production of single nodal cuttings 89 to 197 times after 10 multiplication cycles than that using only single nodal cuttings. This is a result of the greater multiplication ratio of stock plant bases than single nodal cuttings (1.8 to 3.7 times) (Figure 5). The number of single nodal cuttings produced after many repetitions of the multiplication cycle was greatest at planting densities of 59-118 m^{-2}, followed by at 236 m^{-2} and it was the smallest at 473 m^{-2}. Based on such a simulation of production Lok et al. (2002a and b) simulated electric energy consumption per propagule produced. In Lok et al. (2002a), electric consumption per propagule was shown to converge after many repetitions of subcultures and the number of repetitive uses of stock plants did not have significant effects on electric energy consumption (Figure 5). Such analyses of energy and other resource usage in addition to production of explants are important in decision making on selecting propagation method, environmental conditions and other culture conditions. Simulation of important resource usage as well as production will serve as a significant tool for future production planning, scheduling and management of photoautotrophic micropropagation to maximize production efficiency while minimizing use of energy, water, labor and other resources.

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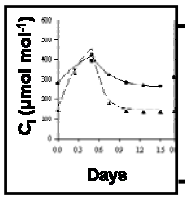
Table 1. List of Symbols

Symbol	Description	Unit
E_N	Number of nodal cuttings harvested as explants	-
E_T	Number of shoot tips harvested as explants	-
L_P	Number of leaves harvestable as explants per plantlet	-
$\mathbf{M}$	Multiplication matrix	-
$\mathbf{M_{EN,PN}}$	Submatrix representing transition from nodal cuttings to plantlets	-
$\mathbf{M_{ET,PT}}$	Submatrix representing transition from shoot tips to plantlets	-
$\mathbf{M_{PN,EN}}$	Submatrix representing multiplication of nodal cuttings	-
$\mathbf{M_{PN,ET}}$	Submatrix representing production of shoot tips from plantlets originating from nodal cuttings	-
$\mathbf{M_{PN,PN}}$	Submatirx representing transition of plantlets originating from nodal cuttings	-
$\mathbf{M_{PT,EN}}$	Submatrix representing multiplication of shoot tips	-
$\mathbf{M_{PT,ET}}$	Submatrix representing production of shoot tips from plantlets origination from shoot tips	-
$\mathbf{M_{PT,PT}}$	Submatrix representing transition of plantlets originating from shoot tips	-
m_N	Multiplication ratio of nodal cuttings	-
m_T	Multiplication ratio of shoot tips	-
n	Number of leaves per explant	-
$\mathbf{N}$	Population vector	-
$\mathbf{P_N}$	Population vector of plantlets originating from nodal cuttings	-
$P_{N,x}$	Number of plantlets originating from nodal cuttings subculture for x weeks	-
$\mathbf{P_T}$	Population vector of plantlets originating from shoot tips	-
$P_{T,x}$	Number of plantlets origination from shoot tips subcultured for x weeks	-
$\boldsymbol{R_L}$	Emergency rate of leaf usable as explant	d^{-1}
T	Days after planting explants	D
T_0	Days to start producing leaves usable as explants	d
T_c	Duration of subculture (single cycle)	week
t	Weeks after start of micropropagation	week
v	Environmental variables	-
X	Subculture duration for explants	week
x	Subculture duration for explants	week

Chapter 13

G. NIU



MODELLING AND SIMULATION IN PHOTOAUTOTROPHIC MICROPROPAGATION

Contents

Key words: CO_2 concentration, computer simulation, light cycle, modelling, photosynthesis.

1. INTRODUCTION

Micropropagation has been commercialized for nearly three decades. Researchers have attempted to apply environmental control, automation, and system analysis in micropropagation to optimize the system. Modelling and simulation can be useful tools to achieve these goals.

A mathematical model is a representation of a real system and usually describes the structure or function of that particular system. Simulation is a means using mathematical equations written in computer code to predict how a plant grows under a given environment. Models in field crop science are generally used for predicting growth and development under given weather, soil environment, and cultivar or genetic conditions. Numerous models have been developed, applied, and disseminated for field crops, and these models and computer simulation have been proved useful for prediction of crop yield and for management of natural resources.

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 225-236.

However, there is limited work on modelling and computer simulation for micropropagation.

In photoautotrophic micropropagation, plantlets are cultured in a small culture vessel, which has a certain ventilation rate to allow air exchanges between the inside and outside air of the vessel. While the environmental conditions in a culture room, such as temperature, photosynthetic photon flux (PPF), and CO_2 concentration are maintained almost constant, the microenvironment inside the culture vessels is usually not directly controlled and monitored. Due to the small volume and semi-closed system of the culture vessels, the gas concentrations, especially CO_2 concentration, changes rapidly with time. Since CO_2 concentration inside the vessel (C_i) is one of the primary environmental factors that influence plantlet growth, prediction of the dynamic changes of C_i under various culture conditions is necessary for optimization of the photoautotrophic micropropagation system and promotion of plantlet growth. It is, however, impractical or time-consuming to experimentally characterize the dynamic changes of C_i under various culture conditions. Mathematical models can be developed to describe the micropropagation system. Approaches used for field crop model development can be employed in micropropagation. This chapter introduces the basic concept and methodology of modelling and simulation in photoautotrophic micropropagation.

2. MODEL DESCRIPTION

The basic components in a culture vessel system include the plantlets in the vessel, the culture vessel itself, and the environment surrounding the vessel. The ultimate goal of modelling the photoautotrophic micropropagation is to predict the "behaviour" of the system under various culture conditions so as to provide appropriate information for optimization of the system. Specific objectives are to develop mathematical models to describe the relationship between the photosynthesis of the plantlet and its environment, to describe the dynamic changes of C_i with time, and to predict the plantlet growth (dry weight increase) over a certain culture period under various culture conditions. C_i depends on photosynthesis and respiration rates of the plantlets, the physical properties of the vessel such as number of air exchanges per hour and air volume of the vessel, and CO_2 concentration outside the vessel. The photosynthetic rate of plantlets depends primarily on C_i, temperature, and PPF. Therefore, photosynthesis must be quantified or modelled in order to simulate C_i and the plantlet growth.

2.1. Net photosynthetic rate

In photoautotrophic micropropagation, plantlets grow through fixing CO_2, the sole carbon source in the headspace of the vessel. The photosynthetic responses of *in vitro* plantlets to C_i and PPF are similar to those of greenhouse- or field-grown crops (Evers, 1982; Kozai et al., 1990; Nakayama et al., 1991). There are a number of photosynthesis models to describe the photosynthetic responses to CO_2

concentration or to PPF at cellular level (Farquhar et al., 1980) or at whole plant level (Charles-Edwards, 1981). It would be practical to develop a whole-plant level model for *in vitro* plantlets, since information regarding photosynthesis at whole-plant level rather than cellular-level can be obtained. In micropropagation, PPF is relatively low and CO_2 concentration inside a vessel frequently decreases to a low level near the CO_2 compensation point during photoperiod (Fujiwara et al., 1987; Infante et al., 1989). Therefore, to accurately predict photosynthesis of *in vitro* plantlets, CO_2 and light compensation points should be considered in the model. Niu et al. (1996a) developed a net photosynthetic rate model for *in vitro* plantlets at whole-plant level as shown in equation (1):

$$P_n = (P_m - R,)\cdot\{1-exp[-f_c\cdot (C_i - C_c)]\}\cdot\{1-exp[-f_l\cdot (I - I_c)]\} \quad (1)$$

where P_n is the net photosynthetic rate (μmol g^{-1} h^{-1}), P_m is maximum gross photosynthetic rate (μmol g^{-1} h^{-1}), R is dark respiration rate (μmol g^{-1} h^{-1}), C_c is CO_2 compensation point (μmol mol^{-1}), f_c is the gradient of the P_n vs. C_i curve at $C_i = C_c$ (mol μmol^{-1}), I is PPF (μmol m^{-2} s^{-1}), f_l is the gradient of the P_n vs. I curve at $I = I_c$ (m^2 s μmol^{-1}), and I_c is the light compensation point (μmol m^{-2} s^{-1}). This equation indicates that P_n decreases because of limitation due to C_i or PPF and the interaction of C_i and PPF. The expressions in the first and the second braces on the right side of the equation represent the effects of C_i and PPF on P_n, respectively. P_m and R are assumed to be temperature-dependent.

$$P_m = a + b\cdot T + c\cdot T^2 \quad (2)$$

$$R = d\cdot exp\ (e\cdot T) \quad (3)$$

where a, b, c, d, and e are dimensionless parameters, T is air temperature. In micropropagation, temperatures are maintained in the range of 22 to 25 C.

2.2. Parameter estimation for net photosynthetic rate model

In-situ measurement is necessary for estimating the parameters without disturbing the environment inside the vessel. Generally, there are two methods to estimate the parameters for the net photosynthesis model. Parameters of equation (1) can be estimated by culturing the plantlets under various combinations of a range of CO_2 concentrations and light intensities. In situ P_n was then estimated using the following equation according to Fujiwara et al. (1987).

$$P_n = k\cdot N\cdot V\cdot (C_o - C_i) \quad (4)$$

where k is conversion factor $\{273/[22.4 \times (273 + 25)]\} = 4.09 \times 10^{-2}$ mol L^{-1} at 25 C), N is number of air exchanges (h^{-1}) of the vessel, V is air volume of the vessel

(L), C_o is CO_2 concentration outside the vessel (μmol mol^{-1}), and P_n is the net photosynthetic rate (μmol h^{-1}). After the measurement, dry weight of the plantlets in the vessel can be determined by destructive measurement. To express P_n on a unit dry weight basis (μmol g^{-1} h^{-1}) as shown in equation (1), both sides of equation (4) were divided by the dry weight of the plantlets per vessel. The C_c and I_c can be obtained from literatures or estimated experimentally. By inputting P_n, C_i, I, C_c, and I_c into equation (1), P_m, R, f_c, and f_l can be estimated. In this method, it is important to be sure that the environment inside the vessel reaches equilibrium or steady state when the measurement is taken. That is, C_i, C_o, PPF, and temperature are not changing with time.

The second method of estimating parameters is based on the time course of C_i during photoperiod and dark period, respectively, through numerical integration by using the differential equation in the following section. In this case, the C_i that is dynamically changing with time will be used for estimation, but C_o, PPF, and temperature will be maintained constant. CO_2 exchange rate between the inside and outside air of the vessel is determined based on the changes in C_i during a short time interval "dt" and the number of air exchanges of the vessel. In both methods, the environment inside the culture vessel is not disturbed while the measurement is taken.

In order to characterize the net photosynthetic curves for any *in vitro* cultured species under various culture conditions, a system was developed by Niu et al. (1998). Measurement of net photosynthetic rate and estimation of parameters using this system was based on the first method as indicated above. More detail information for this system is described in Chapter 6.

2.3. Time course of C_i and growth of in vitro plantlets

As mentioned above, C_i changes rapidly with time in a culture vessel. C_i depends on CO_2 exchange rate of plantlets in the vessel and the physical properties of the vessel. In order to simulate the dynamic changes of C_i, the following assumption may be made: (1) temperatures of air, plantlets, and the vessel are the same, (2) air temperatures and air pressures inside the vessel are the same as those outside the vessel, (3) the diffusivity of CO_2 in the vessel is large enough to achieve uniform spatial distribution of CO_2, (4) CO_2 is neither absorbed nor released by the inner walls of the vessel, (5) PPF is uniform inside the vessel, and (6) CO_2 exchange rate of medium in the vessel is negligibly low. According to the CO_2 balance, mathematical equations were developed for describing the dynamic changes of C_i for a tiny time increment of "dt" and for describing CO_2 exchanges of plantlets for a certain period from t_1 to t_2 (Fujiwara et al., 1987; Fujiwara and Kozai, 1995):

$$V \cdot dC_i = k_1 \cdot M \cdot dt + N \cdot V \cdot (C_o - C_i) \cdot dt \quad (5)$$

$$P = k_2 \cdot \left\{ N \cdot V \cdot \int_{t_1}^{t_2} (C_o(t) - C_i(t)) \cdot dt - V \cdot (C_{i2} - C_{i1}) \right\} \quad (6)$$

where k_1 is a conversion coefficient [(273 +25) × 22.4/275 = 24.45 L mol^{-1} at T of 25 C], M is CO_2 exchange rate of plantlets (μmol h^{-1}), P is the CO_2 exchange from time t_1 to t_2 (μmol), k_2 is a conversion coefficient (4.09×10^{-2} mol L^{-1} at 25 C), t is time (h), $C_o(t)$ is a function expressing the time course of C_o (μmol mol^{-1}), $C_i(t)$ is a function expressing the time course of C_i, C_{i1} is the C_i at t_1, and C_{i2} is the C_i at t = t_2.

For a given lighting cycle in the culture period, M is expressed as follows (Niu et al, 1996a):

$$M = -P_n \cdot W \ (PP < t \leq LC, \ photoperiod) \quad (7)$$

$$M = R \cdot W \ (0 < t \leq PP, \ dark \ period) \quad (8)$$

where W is dry weight of the plantlet (g), PP is photoperiod (h), and LC is the lighting cycle (PP + dark period, h). W is expressed by the following equation:

$$W = W_0 + k_3 \cdot M \quad (9)$$

where W_0 is initial dry weight, and k_3 is conversion factor (30 g mol^{-1}, 1 mol CO_2 converts to 30 g CH_2O).

3. MODEL APPLICATION AND VALIDATION

3.1. Net photosynthetic rate

The parameters of net photosynthesis model for *Cymbidium* were estimated according to the first method (Niu et al., 1996a) and that for potato (*Solanum tuberosum* L. cv. Benimaru) plantlets were estimated using the second method (Niu and Kozai, 1997). Temperatures were 25 C in both cases. The same set of parameters for potato plantlets was used for simulation under various conditions (Niu et al., 1996b and 1997). C_c for potato plantlets was determined to be 70 μmol mol^{-1} by measuring the CO_2 concentration in a sealed vessel containing potato plantlets cultured photoautotrophically. A value of 10 μmol m^{-2} s^{-1} was given to I_c according to Nobel (1991) and Nobel et al. (1993).

Using the net photosynthetic model, the photosynthetic response curves (P_n vs. C_i or P_n vs. PPF) were modelled under given conditions. Figure 1 shows the simulated photosynthetic response to C_i and PPF of potato plantlets, respectively. The parameters for this simulation were estimated according to the time course of C_i in the vessel culturing potato plantlets without sugar in the medium (Niu and Kozai,

1997). Simulated P_n increases with C_i over a range of C_i before it became saturated at a C_i around 600 μmol mol^{-1}. The CO_2 saturation point for potato plantlets was relatively low compared with those of other plantlets, such as *Cymbidium* plantlets *in vitro* (Kozai et al., 1990; Niu et al., 1996a). The response of P_n to PPF was similar to its response to C_i. Potato plantlets had a light saturation point around 1000 μmol m^{-2} s^{-1}, which was relatively high compared to those of *Rubus* (Donnelly and Vidaver, 1984), *Betula* (Smith et al., 1986) and *Actinidia* (Infante et al., 1989). Increasing C_i under high PPF will greatly enhance the growth of potato plantlet grown photoautotrophically. Since artificial light sources are usually used for micropropagation, there is limitation in increasing PPF. High PPF does not increase electricity cost in most cases, because the increase in electricity consumption for lighting and cooling due to high PPF can be compensated by the decrease in electricity consumption by shortening the culture period, resulting from increased growth rate (refer to Chapter 16, this book).

3.2. Effects of vessel property (N, V) and CO_2 enrichment on C_i

Since it is impractical to continuously monitor C_i inside a closed culture vessel under various conditions, models can be used to simulate the time courses of C_i under given conditions, provided that the photosynthetic response curves and the physical property of the vessel (volume and number of air exchange) are known. Figure 2 shows the simulated time course of C_i as affected by the number of air exchanges per hour (N), the air volume (V) of the vessel, and CO_2 concentration outside the vessel (C_o) when culturing a small *Cymbidium* plantlet (dry weight 7.0 g, at the beginning of stage III). Figure 2A shows the results of diurnal changes of C_i when C_o is at 400, 1000, or 3000 μmol mol^{-1}, while the other culture conditions remain unchanged, with a number of air exchanges per hour of the vessel of 0.1 h^{-1}.

C_i increases with time during dark period and starts to decrease rapidly at the onset of photoperiod (photoperiod is from 8:00 h to 24:00 h) until it reaches a steady state level. When the number of air exchanges per hour is low (e.g., N = 0.1 h^{-1}), the steady state C_i during photoperiod is lower than the ambient level, even though C_o is 2000 μmol mol^{-1}.

Figure 2B shows the diurnal changes of C_i when N is at 0.1, 1.0, or 3.0 h^{-1}, while C_o stays the same (400 μmol mol^{-1}). At low N without CO_2 enrichment outside the vessel, the steady state C_i during photoperiod is about 130 μmol mol^{-1}, which is slightly above the CO_2 compensation point. When N is increased to 3.0 h^{-1}, the steady state C_i is about the same as that when C_o is 2000 μmol mol^{-1} and N is 0.1 h^{-1} (Figure 2A).

Air volume of the vessel, or more accurately, air volume per plantlet, is another factor influencing C_i and plantlet growth as shown in Figure 2C. When the air volume per plantlet is 10 cm^3, C_i changes rapidly and the steady state C_i during photoperiod is only slightly higher than that when N is 0.1 h^{-1} in Figure 2B. As the plantlets grow and biomass increases, the changes in C_i become more rapidly. The steady state C_i during photoperiod decreases with time during the culture period (Niu and Kozai, 1997).

3.3. Effect of lighting cycle on C_i and growth

The models can be verified by comparing the simulated time course of C_i with the measured time course of C_i and comparing the simulated and measured dry weights during the culture period. The following example shows how time course of C_i and dry weight of plantlets were simulated and validated using the above models.

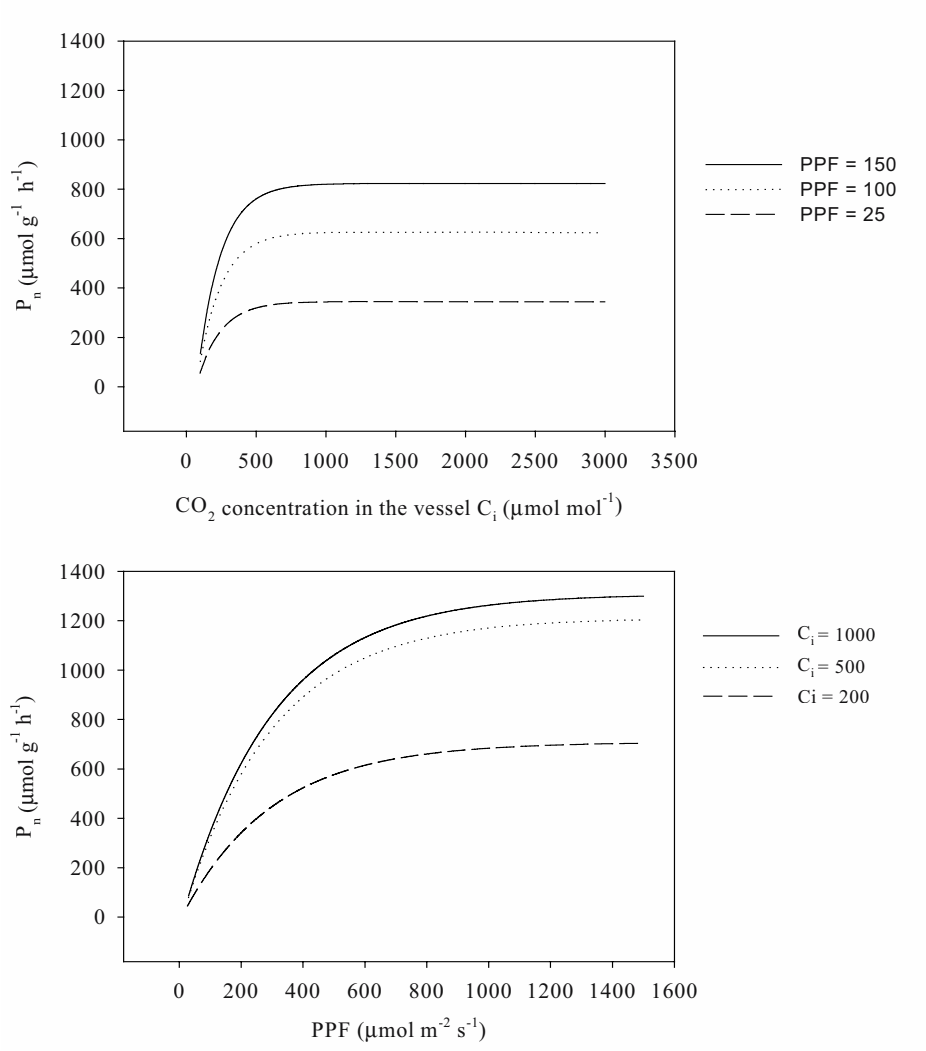


Figure 1. Simulated net photosynthetic response of potato plantlets to CO_2 concentration inside the vessel (Ci) and photosynthetic photon flux (PPF) (Niu and Kozai, 1997).

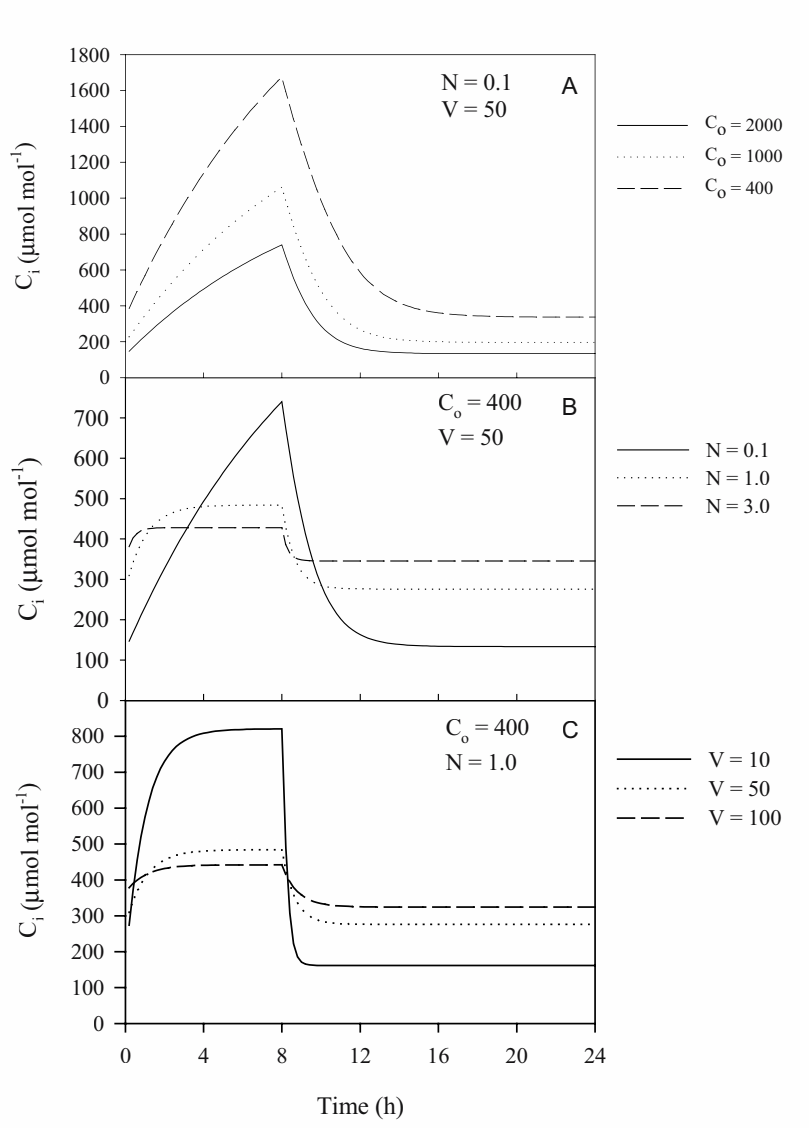


Figure 2. Simulated time courses of CO_2 concentration inside the vessel (C_i) as affected by CO_2 concentration outside the vessel (C_o, Figure 2A), number of air exchanges (N, Figure 2B), and air volume per plantlet (V, Figure 2C). Dark period: 0 to 8 h; photoperiod: 8 to 24 h (Niu et al., 1996a).

In conventional micropropagation, a lighting cycle (16 photoperiod + 8 h dark period) of 24 h is usually used. If the ratio of photoperiod to dark period remains unchanged with different lighting cycles, the photosynthetically active radiation received by the cultures and the electricity consumption per day are approximately the same. However, the growth of the cultures may be different because C_i is affected by lighting cycle.

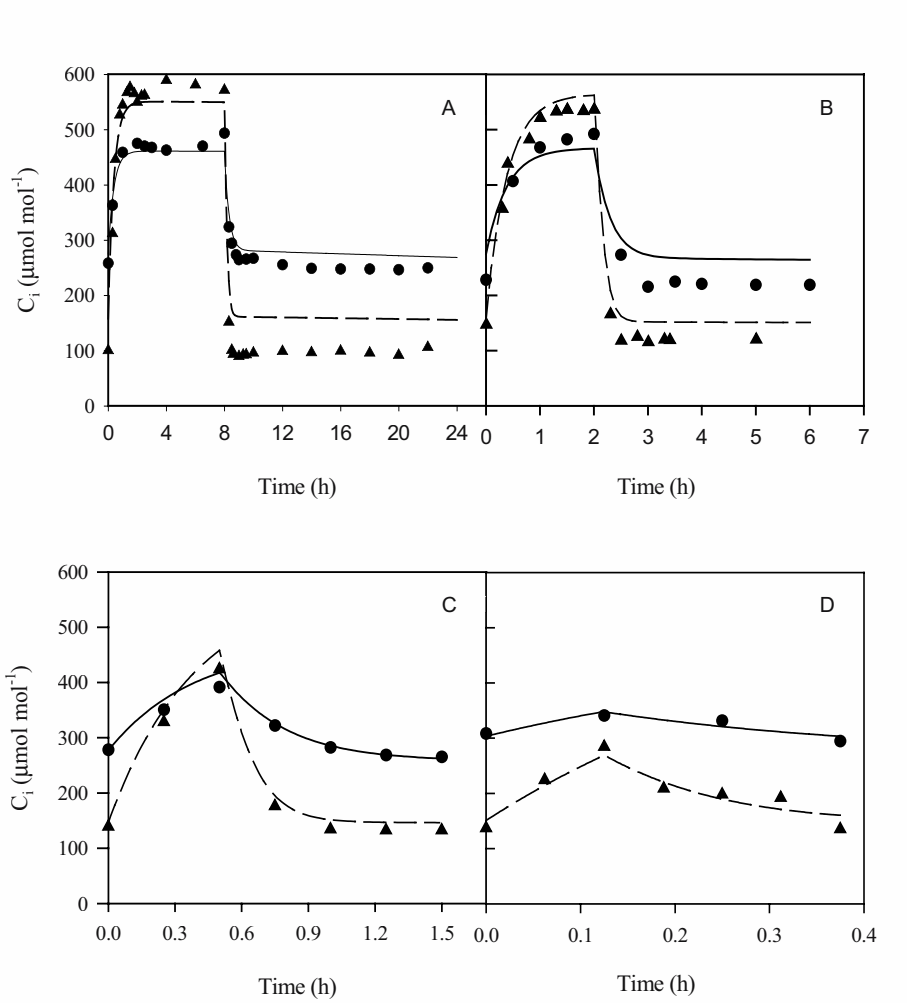


Figure 3. Simulated time courses of CO_2 concentration inside the vessel (C_i) as affected by lighting cycles (24, 6, 1.5, and 0.375 h, with same ratio of photoperiod to dark period). Solid and dash lines represent simulated time course of C_i on days 7 and 21, respectively. Circle and triangle symbols represent measured C_i on days 7 and 21, respectively (Niu et al., 1997).

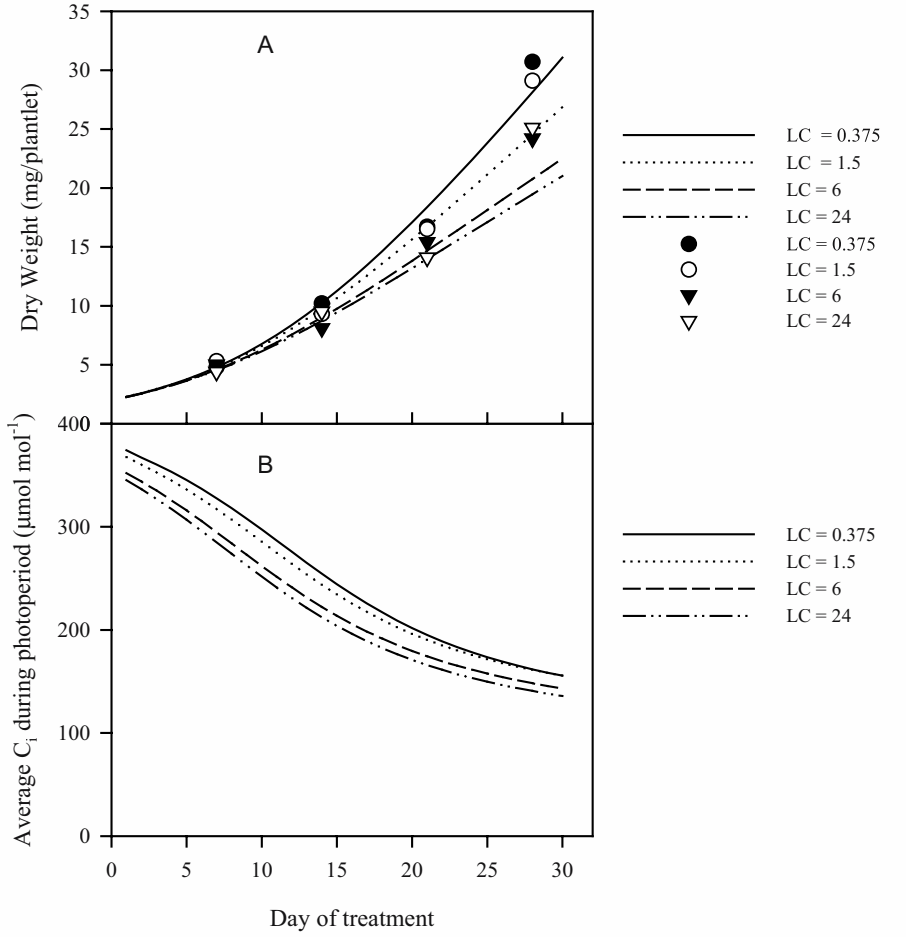


Figure 4. Simulated (lines) and weekly measured (symbols) dry weight of potato plantlets (A) and simulated average CO_2 concentration in the vessel during photoperiod (B) under various lighting cycles over the course of the whole experimental period.

Potato (*Solanum tuberosum* L. cv. Benimaru) plantlets were cultured under four lighting cycles with same ratio of photo/dark period (16 h/8 h, 4 h/2 h, 1 h/0.5 h, and 0.25 h/0.125 h) photoautotrophically *in vitro* for 28 days under ambient CO_2 conditions (Tateno, 1991; Niu et al., 1997). The number of air exchanges per hour was 2.6 h^{-1} and PPF was 100 µmol m^{-2} s^{-1}. The diurnal changes of C_i in each treatment was determined on days 7, 14, 21, and 28 using a gas chromatograph at

various times during the days. Dry weight of plantlets was determined weekly by destructive measurement. The models and parameter values used in Niu and Kozai (1997) were adopted for simulating the time courses of C_i and dry weight accumulation of the plantlets. Simulated and measured results of the diurnal changes of C_i on days 7 and 21 are shown in Figure 3 (for other simulated results, refer to Niu et al., 1997). The simulated results of C_i during photoperiod overestimated when the lighting cycles were 24 and 6 h. This indicates the actual photosynthetic rate of the plantlets was lower than that used for simulation. In other treatments, simulated C_i generally agreed with measured ones.

The simulated and weekly measured dry weights of the plantlets are shown in Figure 4A. Simulated dry weights were within the 95% confidence intervals of the measured values in all treatments (Niu et al., 1997; the confidence intervals are not shown in the figures). Shorter lighting cycle led to a higher dry weight of the plantlets. The effect of lighting cycle on the accumulation of dry weight was more apparent in the late period of the culture. The differences in dry weight of the plantlets among different lighting cycles may be attributed to the differences in C_i during the photoperiod. The simulated average C_i during photoperiod was higher when lighting cycle was shorter throughout the treatment period as shown in Figure 4B. This is because photoperiods in the lighting cycles 1.5 h and 0.375 h were so short that C_i did not reach a steady state before the next dark period started.

4. CONCLUDING REMARKS

Modelling and simulation in micropropagation have shown their usefulness in characterizing the dynamic changes of CO_2 concentration inside the vessel under various culture conditions. The effects of culture conditions (vessel ventilation rate, air volume, light intensity, lighting cycle, CO_2 concentration outside the vessel, etc.) on growth of *in vitro* plantlets can be predicted using the above models. Simulation results provide information on how far the actual environment is from the optimal ones in order to maximize the growth (net photosynthetic rate or dry weight) of the plantlets. It should be noticed that the optimal conditions might not lead to the lowest production costs. Compromise should be made in determining the environmental conditions such as CO_2 concentration, light levels, number of plantlets in a culture vessel, etc. in commercial micropropagation for considering both optimal growth and the production cost. The photosynthetic model needs to be calibrated for various species at different physiological stages and under various culture conditions in order to more accurately predict the plantlet growth.

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Chapter 14

C. KUBOTA & T. KOZAI

FREQUENTLY ASKED QUESTIONS

1. Costs for increased light intensity or PPF and cooling load

Cost of increased light intensity and methods of its cost reduction are described in Chapters 4 and 16. In short, increased light intensity in photoautotrophic micropropagation can be achieved by improving the current lighting system of fluorescent lamps by using reflective sheets inside the shelf and transparent plastic caps as a closure.

Cost for cooling is about 25% of cost for lighting when a recently developed air conditioner is used in a culture room with thermally insulated walls. More discussion is described in Chapter 16.

Costs, it is important to keep the culture room airtight. For more information, see Chapter 16 and 17, Kozai, 1991; Jeong et al., 1993 and Zobayed *et al.,* 2000.

2. How can I measure and control CO_2 concentration?

CO_2 concentration in the culture room can be measured and controlled using an infrared CO_2 analyzer/controller (IRGA) with a solenoid valve, liquid CO_2 container and connecting tubes. An IRGA is widely used for CO_2 enrichment in the greenhouse and it can be introduced into a tissue culture room. The cost of an infrared CO_2 controller is about 1,000 US$. IRGA can be used in a forced ventilated vessel for controlling CO_2 concentration inside the vessel. The diurnal changes in CO_2 concentration can be recorded by an analogue or digital recorder connected to the IRGA.

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For measurement of CO_2 concentration in a small culture vessel, a gas chromatograph is used which requires a small amount (a quarter milliliter) of sample gas for measurement. Sample gas is taken from the vessel using a syringe. The gas chromatograph system costs 5,000-10,000 US$. For commercial application of CO_2 enrichment in the culture room, a consultation with a specialist is recommended. For detailed information on measurement and control of *in vitro* environmental factors, see Kozai and Smith (1995).

3. *Cost for gas permeable filter*

Cost of the gas permeable filter varies by countries and/or type of material. In North America, European countries and Japan, price of a gas permeable filter disk (20 mm in diameter) which can be reused (autoclaved) about 20 times is 10 US cents. In China, a relatively low grade filter is widely used at a cost of 1 US cent/filter. In Vietnam, an inexpensive paper filter disk is used commercially. In Thailand and India, relatively high quality and low price filter disks are going to be commercialized. Make one to three holes (10 mm in diameter) on the cap or sidewalls of the vessel. Then, seal each hole with a filter disk using a glue that will survive autoclaving.

4. *What is the allowable maximum CO_2 concentration for humans? Is CO_2 toxic?*

Average CO_2 concentration of the outside air is 380-400 μmol mol^{-1} (or ppm). In the CO_2 enriched greenhouse, its concentration is usually kept at about 1,000 μmol mol^{-1}. In a public place such as a theater and a department store with crowded people, it is often required by environmental regulations to keep CO_2 concentration at 5,000 μmol mol^{-1} or lower, because in such places its concentration can exceed 10,000 μmol mol^{-1} easily if no ventilation is provided.

CO_2 (carbon dioxide) is not toxic at all if its concentration in the air is lower than 5,000-10,000 μmol mol^{-1} (ppm). However, CO (carbon monoxide) is very toxic at a concentration of 1 μmol mol^{-1}.

5. *Effect of increased PPF on the air temperature in the vessel.*

Air temperatures in the vessel can be increased by about 1 C or 2 C at most under increased PPF in photoautotrophic micropropagation. Numerical data on the increase in air temperature as function of PPF is given in Chapter 4. In case that the temperature of 1 C is problematic, the set point of air temperature for the culture room should be lowered by 1 C. Increase in

electricity cost for cooling due to the change in setpoint by 1-2 C is almost negligible.

6. *When is the earliest possible stage for starting photoautotrophic micropropagation?*

Photoautotrophic micropropagation can be introduced once the cultures developed photosynthetic organs (chlorophyllous leaves with developed gas exchange apparatus (stomata)). Multiplication ratio in photoautotrophic micropropagation is comparable to that in conventional micropropagation (heterotrophic or photomixotrophic), when considering the type and number of usable explants as multiplication ratio.

7. *Use of natural light economical and/or beneficial?*

Use of natural light with or without artificial light increases the electricity consumption and thus electricity cost for cooling during the photoperiod. Thus, reduction in electricity cost for lighting by use of natural light does not necessarily reduce the total electricity cost for lighting and cooling. When, the air temperature outside the culture room with glass windows for use of natural light is lower than the setpoint (20-25 C) of air temperature in the culture room during the dark period, heating is necessary. Under such a condition, condensation occurs frequently on the inside surface of glass windows, which may spread pathogens in the culture room.

In addition, light intensity and air temperature in the culture room tend to be variable with time when natural light is used, resulting in the unpredictable changes in environment, growth, and development of plants *in vitro*. More discussion is given in Chapter 16. The authors do not recommend the use of natural light in photoautotrophic micropropagation.

8. *Can multiplication rate be higher under photoautotrophic than under heterotrophic and photomixotrophic conditions?*

Plants with one or two shoots produce more unfolded leaves under photoautotrophic than under heterotrophic and photomixotrophic conditions. Nodal cuttings, each with one or two leaves, are usually used as explants in photoautotrophic micropropagation. Plants with multiple shoots, under photoautotrophic conditions, can produce more large shoots which can be used as explants than under heterotrophic and photomixotrophic conditions. In this case, the number of shoots per plant would be the same and can be increased by use of plant growth regulators under the three trophic conditions.

But multiplication rate or the number of harvestable shoots as explants per plant is higher under photoautotrophic condition. For more discussion, see Chapter 3.

9. What type of explant is suitable?

For photoautotrophic micropropagation, use of chlorophyllous or leafy explants is essential. Somatic embryos with green-colored cotyledons at late cotyledonary stage have photosynthetic ability to grow photoautotrophically. In many cases, leaf area of 25 mm^2 (5 x 5 mm) is large enough for photoautotrophic growth of explants provided that the environment is favorably controlled for promoting photosynthesis. For more information, see Miyashita et al. (1996).

10. Pathogen-free vs. aseptic plants

In micropropagation, generally, we need disease-free plants, not aseptic plants. However, in conventional micropropagation, asepsis is necessary to avoid microbial contamination of the sugar-containing medium. In photoautotrophic micropropagation, asepsis is not necessarily required to avoid microbial contamination of sugar-free medium because microorganism does not grow quickly in the medium containing only inorganic nutrients. In fact, inoculation of microorganism on the medium for symbiosis, to promote the growth and development of cultures would become an important technique in future photoautotrophic micropropagation. For more discussion, see Kozai and Smith (1995) and Herman (1996).

11. Can we exclude autoclaving or other sterilization processes in photoautotrophic micropropagation?

No. The vessels and medium must be sterilized to exclude pathogens. Once it is achieved, some extent of microbial growth can be acceptable as long as it is a non-pathogenic microbe.

12. What are the advantages of photoautotrophic micropropagation?

1) promotion of growth and photosynthesis, 2) smooth transition to *ex vitro* environment with high survival percentage, 3) Elimination of morphological and physiological disorders, 4) Little loss of plantlets due to

microbial contamination of the culture medium, 5) flexibility in the design of the vessel (use of larger vessels), 6) easier automatic environmental control and automation. See also Chapter 3.

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Chapter 15

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PLANT SPECIES SUCCESSFULLY MICROPROPAGATED PHOTOAUTOTROPHICALLY

Contents

Key words: Forced ventilation, natural ventilation, nutrient medium, photoautotrophic micropropagation, supporting material.

1. INTRODUCTION

The concept of photoautotrophic micropropagation is derived from the research that revealed relatively high photosynthetic ability of chlorophyllous cultures such as leafy explants, cotyledonary stage somatic embryos and plantlets *in vitro*. While studying the environmental conditions of tissue culture vessels containing leafy green plantlets of *Caiathea, Spatihphyllum, Phirodendron imbe Nepenthes, Dracaena, Cymbidium, Limonium*, *Syngonium*, *Cordyline, Ficus lyrata,* Fujiwara et al. (1987) found that *in vitro* plantlets could not fully achieve their photosynthetic ability during the light period because the CO_2 concentration in the closed vessels were too low in most of the light periods. Therefore, they concluded that the tissue cultured plantlets can be grown photoautotrophically (to be able to photosynthesise) during and after the multiplication stage by improving the CO_2 and light environments in the culture vessels. 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* x *ananassa*) plants have been grown successfully under photoautotrophic conditions by Kozai and Sekimoto (1988). Carnation (*Dianthus*

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caryophyllus L.) (Kozai and Iwanami, 1988) and tobacco (*Nicotiana tabacum* L.) plants (Pospisilova et al., 1988) were also grown photoautotrophically in the same year (1988). The concept of growing plantlets in a large vessel under photoautotrophic conditions with forced ventilation system was first successfully implemented by Fujiwara et al*.,* (1988). The vessel, volume of 19 L (58 cm long, 28 cm wide and 12 cm high) with an attached air pump for the forced ventilation, was used to enhance the photoautotrophic growth of strawberry (*Fragaria* x *ananassa* Duch.) explants and/or plants during the rooting and acclimatization stages. This was an aseptic micro-hydroponic system with a nutrient solution control system. Since then the trend of developing protocols for the *in vitro* growth of plants under photoautotrophic conditions started and is still continuing and so far nearly 50 different plant species have been reported to grow successfully under photoautotrophic conditions. Among these potato plant has been studied most extensively by many authors (Kozai et al., 1988; Takazawa and Kozai, 1992; Kozai et al., 1992 and 1995b; Tanaka et al., 1992; Miyashita et al., 1997; Kitaya et al., 1995c and 1997a; Fujiwara et al., 1995; Hayashi et al., 1995; Miyashita et al., 1995 and 1996; Roche et al., 1996; Niu. and Kozai, 1997; Niu et al., 1997; Zobayed et al., 1999a; Kim et al., 1999; Xiao et al., 2000; Pruski et al., 2002) followed by sweetpotato (Nagatome et al., 2000, Kozai et al., 1996; Ohyama and Kozai, 1997; Niu et al., 1998; Afreen et al., 1999; Zobayed et al., 1999; Afreen et al., 2000; Zobayed et al., 2000; Heo and Kozai, 1999; Wilson et al., 2000; Kubota et al., 2002).

Studies of *in vitro* plant growth under photoautotrophic conditions and the conventional photomixotrophic conditions (control) covered a wide range of area including growth, morphology and development, physiology and anatomy, biochemistry and molecular studies. More specifically, biomass accumulation of root, shoot and leaf, *ex vitro* growth and survival, tuber yield, CO_2 and ethylene concentrations, profile of CO_2 concentrations in the vessel, multiplication coefficient, low temperature storage, changes in nutrient composition in the medium, nutrient uptake, development of photosynthetic tissues, dark respiration, net photosynthetic rates and plotting the photosynthesis curve, carbon isotope composition, stress response, chlorophyll fluorescence, photosynthetic capacity, photoinhibition, carbohydrate status, chlorophyll and xanthophyll contents, ultrastructure of chloroplast, thylakoid membrane proteins, abscisic acid content, enzyme activities (RuBPC, PEPC), anatomical features of leaves, stomatal density, transpiration, leaf water potential, relative water content, wax content, gene expression and many other aspects have been studied under those conditions. The plant species successfully micropropagated photoautotrophically are listed in the following table. The scientific names along with the common names, the main controlled factors and the measured factors for each of those studies are also presented.

2. LIST OF PLANT SPECIES SUCCESSFULLY MICROPROPAGATED PHOTOAUTOTROPHICALLY

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Acacia mangium	acacia	type of supporting materials, sucrose conc.	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C. and Kozai, T. (1999)
Ananas comosus	pineapple	no. of air exchanges, sucrose conc.	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C. and Kozai, T. (1999)
Brassica campestris L.		seeds/leafy explants, PPF	growth, CO_2 conc. in the vessel, photosynthesis	Kozai, T., Ohde, N. and Kubota, C. (1991)
Brassica oleracea L.	broccoli	PPF during low temp. storage	growth suppression during low temp. storage	Kubota, C. and Kozai, T. (1994)
Brassica oleracea L.	broccoli	PPF and temp. during low temp. storage	growth suppression during low temp. storage, chlorophyll fluorescence	Kubota, C. and Kozai, T. (1995)
Brassica oleracea L.	broccoli	light quality during low temp. storage	growth, CO_2-photosynthesis curve, chlorophyll content	Kubota, C., Rajapakse, N. and Young, R. (1996)
Brassica oleracea L.	broccoli	light quality during low temp. storage	growth, carbohydrate status, chlorophyll content	Kubota, C., Rajapakse, N. and Young, R. (1997)
Brassica oleracea L.	cauliflower	types of ventilation	growth, ethylene conc. in the vessel, chlorophyll content	Zobayed, S.M.A., Armstrong, J. and Armstrong, W. (1999)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Brassica oleracea L.	kale	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Caiathea	-	CO_2 conc.	CO_2 conc., photosynthesis curve	Fujiwara, K., Kozai, T., and Watanabe, I. (1987)
Caladium bicolor	-	CO_2 conc.	*in vitro* and *ex vitro* growth	Doi, M., Oda, H., Ogasawara, N. and Asahira, T. (1992)
Chrysanthemum morifolium	chrysanthemum	CO_2 conc., no. of air exchanges	ultrastructure of chloroplast	Cristea, V., Dalla Vecchia, F. Craciun, C., and La Rocca, N. (1998)
Chrysanthemum morifolium	chrysanthemum	CO_2 conc.	growth, photosynthesis, chlorophyll conc., enzyme activities (RuBPC, PEPC)	Cristea, V., Dalla Vecchia, F. and La Rocca, N. (1999)
Chrysanthemum morifolium	chrysanthemum	O_2 conc.	growth, photosynthesis	Tanaka, F., Watanabe, Y. and Shimada, N. (1991)
Citrus macrophylla L.	citrus	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Coffea arabusta	coffee	sucrose conc., type of supporting material, no. of air exchanges	growth, callus formation, photosynthesis, CO_2 conc. in the vessel	Nguyen, Q., Kozai, T. and Nguyen, U. (1999a)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Coffea arabusta	coffee	PPF, CO_2 conc.	PPF, CO_2 conc., photosynthesis	Nguyen, Q., Kozai, T., Niu, G. and Nguyen, U. (1999b)
Coffea arabusta	coffee	PPF, CO_2 conc.	growth, photosynthesis	Nguyen, Q., Kozai, T. and Heo, J. (2000)
Coffea arabusta	coffee	different stage embryos, PPF, CO_2 conc.	photosynthetic ability, growth, NPR	Afreen F., Zobayed, S.M.A. and Kozai, T. (2002a)
Coffea arabusta	coffee	designing bioreactor, PPF, CO_2 conc.	photosynthetic ability, growth, NPR, survival	Afreen F., Zobayed, S.M.A. and Kozai, T. (2002b)
Cordyline		CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T., and Watanabe, I. (1987)
Cucumus melo L.	melon	PPF, CO_2 conc.	growth, rooting, photosynthesis	Adelberg, J., Fujiwara, K., Kirdmanee, C. and Kozai, T. (1999)
Cunninghamia lanceolata	-	forced and natural ventilation, CO_2 conc.	growth, morphology, % survival *ex vitro*, production cost	Xiao, Y. and Kozai, T. (2004)
Cymbidium	cymbidium	CO_2, PPF	growth, CO_2 conc. in the vessel	Kozai, T., Oki, H. and Fujiwara, K., (1987)
Cymbidium	cymbidium	CO_2, PPF	CO_2 conc.-PPF-photosynthesis curve	Kozai, T., Oki, H. and Fujiwara, K., (1990)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Cymbidium	cymbidium	no. of air exchanges, sucrose conc.	growth, stomatal density, chlorophyll content, photosynthesis	Kirdmanee, C., Kubota, C., Jeong, B-R., and Kozai, T. (1992)
Cymbidium	cymbidium	PPF, CO_2 conc.	CO_2 conc. in the vessel, photosynthesis	Niu, G., Kozai, T. and Kitaya, Y. (1995)
Cymbidium	cymbidium	PPF, temp., CO_2 conc., no. of air exchanges	CO_2 conc. in the vessel, photosynthesis	Niu, G., Kozai, T. and Kitaya, Y. (1996)
Cymbidium	cymbidium	PPF, CO_2 conc., sucrose conc.	growth, photosynthesis	Heo, J., Kubota, C., and Kozai, T. (1996)
Cymbidium	cymbidium	CO_2 conc., light quality	growth, SPAD	Tanaka, M., Takamura, T., Watanabe, H., Endo, M., Yangagi, T. and Okamoto, K. (1998)
Daucus carota L.	carrot	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Dendrobium	dendrobium	CO_2 conc.	growth	Mitra, A., Dey, S. and Sawarkar, S.K. (1998)
Dianthus caryophyllus L.	carnation	CO_2 and sucrose conc.	growth, CO_2 conc.-photosynthesis curve	Kozai, T. and Iwanami,Y. (1988)
Dianthus caryophyllus L.	carnation	medium strength & composition	growth, CO_2 conc. in the vessel,	Kozai, T., Kubota, C. and Watanabe, I. (1990a)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Dracaena	-	CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T., and Watanabe, I.(1987)
Eucalyptus camaldulensis	eucalyptus	type of supporting materials, CO_2 conc.	growth, photosynthesis, transpiration, leaf water potential, % rooting	Kirdmanee, C., Kitaya, Y. and Kozai, T. (1995c)
Eucalyptus camaldulensis	eucalyptus	type of supporting materials, CO_2 conc.	growth, photosynthesis, transpiration, % survival *ex vitro*	Kirdmanee, C., Kitaya, Y. and Kozai, T. (1995b)
Eucalyptus camaldulensis	eucalyptus	type of supporting materials, CO_2 conc.	anatomical features of leaves, % survival *ex vitro*	Kirdmanee, C., Kitaya, Y. and Kozai, T. (1995a)
Eucalyptus camaldulensis	eucalyptus	natural ventilation and forced ventilation (with CO_2-enriched air); designing vessel	growth, CO_2 and ethylene conc., photosynthesis, % survival *ex vitro*	Zobayed, S.M.A., Afreen-Zobayed, F., Kubota, C. and Kozai, T. (2000)
Eucalyptus camaldulensis	eucalyptus	natural ventilation and forced ventilation (with CO_2-enriched air)	physiology of plants includes, stomata, photosynthesis, leaf anatomy	Zobayed, S.M.A, Afreen F. and Kozai, T. (2001)
Eucalyptus tereticornis Smith.	eucalyptus	CO_2 conc., sucrose conc., PPF, supporting material	growth, photosynthesis, multiplication coefficient	Khan, P.S.S.V., Kozai, T., Nguyen, Q., Kubota, C. and Dhawan, V. (2002)
Festuca arundinacea Schreb.	tall fescue (turfgrass)	CO_2 conc., sucrose conc.	growth, % survival *ex vitro*	Seko, Y. and Kozai, T. (1996)
Fragaria x ananassa	strawberry	forced and natural ventilation	growth, photosynthesis	Fujiwara, K., Kozai, T. and Watanabe, I. (1988)
Fragaria x ananassa	strawberry	no. of air exchanges, PPF	growth, CO_2 conc. inside the vessel, chlorophyll content	Kozai, T. and Sekimoto, K. (1988)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Fragaria x ananassa	strawberry	sucrose conc.	growth, changes in nutrient composition in the medium, net photosynthetic rate	Kozai, T., Iwabuchi, K., Watanabe, K. and Watanabe, I. (1991a)
Fragaria x ananassa	strawberry	medium composition and strength	growth, photosynthesis	Yang, C-S., Kozai, T. and Fujiwara, K. (1995)
Fragaria x ananassa	strawberry	supporting material, no. of air exchanges, pH	growth	Jeong, B.R., Im, M.Y. and Hwang, S.J. (1999)
Garcinia mangostana	mangosteen	no. of air exchanges, sucrose conc.	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C., and Kozai, T. (1999)
Gardenia jasminoides Ellis	gardenia	PPF, no. of air exchanges	growth, carbon isotope composition	Serret, M.D., Trillas, M.I., Matas, J. and Araus, J.L. (1997)
Gardenia jasminoides Ellis	gardenia	PPF, sucrose conc.	anatomy, development of photosynthetic tissues, photosynthesis, dark respiration	Serret, M.D. and Trillas, M.I. (2000)
Gardenia jasminoides Ellis	gardenia	PPF, micropropagation stages, tube cap closure, sucrose conc.	chlorophyll fluorescence, photosynthetic capacity, photoinhibition, photosynthesis	Serret, M.D., Trillas, M.I., Matas, J. and Araus, J.L. (2001)
Gynura pseudo-china		no. of air exchanges, sucrose conc.	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C., and Kozai, T. (1999)
Ipomoea aquatica	pak-bung	sucrose conc.	growth, photosynthetic potential of hairy roots	Nagatome, H., Tsutsumi, M., Kino-Oka, M. and Taya, M. (2000)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Ipomoea batatas L.	sweet potato	temp.	growth, CO_2 conc. in the vessel, photosynthesis	Kozai, T., Kitaya, Y., Kubota, C., Kobayashi, R. and Watanabe, S. (1996)
Ipomoea batatas L.	sweet potato	PPF, CO_2 conc.	vertical profile of CO_2 conc. in the vessel	Ohyama, K. and Kozai, T. (1997)
Ipomoea batatas L.	sweet potato	PPF, CO_2 conc.	PPF-CO_2 conc.-photosynthesis curve	Niu, G., Kozai, T. and Kubota, C. (1998)
Ipomoea batatas L.	sweet potato	type of supporting material	growth, rooting, photosynthesis, % survival	Afreen, F., Zobayed, S.M.A., Kubota, C. and Kozai, T. (1999)
Ipomoea batatas L.	sweet potato	forced and natural ventilation, stagnant and non-stagnant nutrient solution, PPF, CO_2 conc.	growth, photosynthesis, % dry mass	Zobayed, S.M.A., Kubota, C. and Kozai, T. (1999)
Ipomoea batatas L.	sweet potato	type of supporting material	growth, photosynthesis, % survival and growth *ex vitro*	Afreen, F., Zobayed, S.M.A., Kubota, C., Kozai, T. and Hasegawa, O. (2000)
Ipomoea batatas L.	sweet potato	forced and natural ventilation,	water control, stomatal movement, wax deposition, % survival	Zobayed, S.M.A., Afreen, F., Kubota, C. and Kozai, T. (2000)
Ipomoea batatas L.	sweet potato	forced and natural ventilation	growth, photosynthesis	Heo, J. and Kozai, T. (1999)
Ipomoea batatas L.	sweet potato	sucrose conc.	growth, carbohydrate status	Wilson, S., Heo, J. , Kubota, C. and Kozai, T. (2001)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Ipomoea batatas L.	sweet potato	sucrose conc.	growth	Heo, J. , Wilson, S. and Kozai, T. (2001)
Ipomoea batatas L.	sweet potato	sucrose conc., PPF, CO_2 conc.	growth, dry weight, photosynthesis, carbon balance	Kubota, C., Ezawa, M., Kozai, T. and Wilson, S. (2002)
Ipsea malabarica (Reichb. f.) J. D. Hook	malabar (daffodil orchid)	sucrose conc.	*in vitro* conservation	Martin, K.P. and Pradeep, A.K. (2003)
Lactuca sativa L.	lettuce	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Lagerstroemia speciosa Pers.		medium composition, capping systems, no. of air exchanges	shoot multiplication and root induction, CO_2 and ethylene conc., growth, % *ex vitro* survival	Zobayed, S.M.A. (2000)
Lagerstroemia thorellii Gagnep.	-	medium composition, capping systems, no. of air exchanges	shoot multiplication and root induction, CO_2 and ethylene conc., growth, % *ex vitro* survival	Zobayed, S.M.A. (2000)
Lilium asiatic hybrid 'Mona' bulblets	-	CO_2 conc., sucrose conc.	growth and net photosynthesis	Lian, M.L., Murthy, H.N. and Kee-Yoeup, P. 2003
Limonium Hybrid	statice	CO_2 conc., sucrose conc.	growth, CO_2 conc. in the vessel	Kozai, T., Iwanami Y. and Fujiwara, K. (1987)
Limonium spp.		no. of air exchanges, PPF, CO_2 conc. H_2PO_4 conc.	growth, chlorophyll conc.	Lee, E.J. and Jeong, B.R. (1999)
Limonium latifolium	statice	natural/forced ventilation	growth, % survival, costs	Xiao, Y., Zhao, J. and Kozai, T. (2000)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Limonium spp.	statice	PPF, CO_2 conc., sucrose conc.	growth, % shoot dry matter, chlorophyll and sugar content of leaves, stomata, photosynthesis, % survival of plantlets *ex vitro*	Lian, M.L., Murthy, H.N. and Paek, K.Y. (2002)
Lycopersicon esculentum Mill.	tomato	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Lycopersicon esculentum Mill.	tomato	PPF, CO_2 conc.	PPF-CO_2 conc.-photosynthesis curve	Niu, G., Kozai, T. and Kubota, C. (1998)
Lycopersicon esculentum Mill.	tomato	medium composition, PPF, CO_2 conc.	growth, dry weight, fungal growth	Kubota, C. and Tadokoro, N. (1999)
Lycopersicon esculentum Mill.	tomato	sucrose conc.	growth, carbohydrate status	Wilson, S., Kubota, C. and Kozai, T. (2000)
Lycopersicon esculentum Mill.	tomato	sucrose conc., PPF, CO_2 conc.	growth, dry weight, photosynthesis, carbon balance	Kubota, C., Ezawa, M., Kozai, T. and Wilson, S. (2002)
Malus pumila hybrid MM 106 paradisiaca x Northern Spy	apple	sucrose conc., PPF, CO_2 conc.	growth, dry weight	Morini, S. and Melai, M. (2003)
Mentha piperita	-	no. of air exchanges, sucrose conc.	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C. and Kozai, T. (1999)
Mentha rotundifolia	apple mint	photoperiod, PPF, DIF	growth, stem length	Jeong, B.R., Kozai, T. and Watanabe, K. (1996)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Mokara	-	CO_2 conc., PPF	growth, nocturnal acidity, chlorophyll conc., head space gaseous composition of culture vessel, nitrogen uptake	Hew, C.S., Hin, S.E., Yong, J.W.H., Gouk, S.S. and Tanaka, M. (1995)
Musa	banana	CO_2 conc.	photosynthesis, enzyme activities, gene expression	Regev, I., Gepstein, S., Duvdevani, A., Magdar, D. and Khayat, E. (1997)
Musa spp.	banana	PPF, no. of air exchanges	growth	Nguyen, Q., Kozai, T. and Heo, J. (2000)
Nepenthes		CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T. and Watanabe, I.(1987)
Nicotiana tabacum L.	tobacco	PPF, CO_2 conc., cap, sucrose conc.	growth, CO_2 conc. in the vessel	Kozai, T., Takazawa, A. and Watanabe, I. (1990)
Nicotiana tabacum L.	tobacco	no. of air exchanges, CO_2 conc.	growth	Ticha, I. (1996)
Nicotiana tabacum L.	tobacco	sucrose conc.	leaf water potentials, relative water content, photosynthesis	Pospisilova, J., Solarova, J., Catsky, J., Ondrej, M. and Opatrny, Z. (1988)
Nicotiana tabacum L. cv Samsun	tobacco	PPF and sucrose conc.	stress response, chlorophyll and xanthophylls contents, thylakoid membrane proteins, abscisic acid content	Hofman, P., Haisel, D., Komenda, J., Vagner, M., Ticha, I., Schafer, C. and Capkova, V. (2002)
Nicotiana tabacum L.	tobacco	PPF and sucrose conc.	photosynthetic parameters and protective systems against excess excitation energy	Kadlecek, P., Rank, B. and Ticha I. (2003)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Paulownia fortunei	paulownia	photoperiod, no. of air exchanges	growth	Nguyen, Q., Kozai, T. and Heo, J. (2000)
Paulownia fortunei	paulownia	CO_2 conc., medium type, PPF	growth, stomata, moisture loss	Khan, P.S.S.V., Kozai, T., Nguyen, Q., Kubota, C. and Dhawan, V. (2003)
Phirodendron Imbe		CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T., and Watanabe, I.(1987)
Pinus radiate	radiata pine	medium composition, sucrose conc.	growth, chlorophyll fluorescence	Aitken-Christie, J., Davies, H., Kubota, C. and Fujiwara, K. (1992)
Pleioblastus pygmaea	sasa	sucrose conc., supporting materials	growth, photosynthesis	Watanabe, Y., Sawa, Y., Nagaoka, N. and Kozai, T. (2000)
Pogostemon cablin		no. of air exchanges, sucrose conc., explant size	growth	Ermayanti, T., Imelda, M., Tajuddin, T., Kubota, C. and Kozai, T. (1999)
Raphanus sativus L.	radish	CO_2 conc.	growth	Tisserat, B., Herman, C., Silman, R. and Bothast, R.J. (1997)
Rehmannia glutinosa	-	CO_2 conc., no. of air exchanges, PPF.	growth, chlorophyll conc. *ex vitro* survival, stomatal resistance, transpiration rate	Seon, J.H., Cui, C.H., Paek, K.Y., Yang, C.S., Gao, W.Y., Park, C.H. and Sung, S.N. (1999)

Scientific Name	Common name	Main controlled factors	Main measured factors	Authors
Rehmannia glutinosa	-	CO_2 conc., no. of air exchanges, PPF.	photosynthesis, stomatal conductance, transpiration, chlorophyll fluorescence, chlorophyll conc., carbohydrate conc., survival rate	Seon, J.H., Cui, Y.Y., Kozai, T. and Paek, K.Y. (2000)
Rehmannia glutinosa	-	no. of air exchanges, sucrose conc., photo and dark period temperature, PPF	growth, total sugar, reducing sugar, starch content, photosynthesis, *ex vitro* survival	Cui, Y-Y., Hahn, E-J., Kozai, T. and Paek, K.Y. (2000)
Rhododendron		medium strength	growth, photosynthesis	Valero-Aracama, C., Zobayed, S.M.A. and Kozai, T. (2000)
Rosa hybrida Hort.	rose	no. of air exchanges	growth, CO_2 conc. in the vessel, photosynthesis	Hayashi, M., Lee H-C. and Kozai, T. (1993)
Rosa hybrida Hort.	rose	PPF, no. of air exchanges, fibrous supporting material	growth, *ex vitro* survival	Horan, I., Walker, S., Roberts, A.V., Mottley, J. and Simpkins, I. (1995)
Rosa hybrida Hort.	rose	CO_2 conc., supporting material, PPF	growth, carbohydrate conc., enzyme activities, photosynthesis-PPF curve	Genoud-Gourichon, C., Sallanon, H. and Coudret, A. (1996)
Rubus idaeus L.	red raspberry	CO_2 conc., RH	growth, % water loss, stomatal aperture, *ex vitro* growth	Deng, R. and Donnelly, D.J. (1993a)
Rubus idaeus L.	red raspberry	CO_2 conc.	growth, photosynthesis	Deng, R. and Donnelly, D.J. (1993b)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Saccharum spp.	sugarcane	CO_2 conc., light level, hormone conc.	shoot growth, dry weight of shoot	Erturk, H. and Walker, P.N. (2000a)
Saccharum spp.	sugarcane	rooting period, explant size, medium type	growth	Erturk, H. and Walker, P.N. (2000b)
Saccharum spp.	sugarcane	light level, supporting medium	growth	Xiao Y., Lok Y. H. and Kozai T. (2002)
Saccharum spp.	sugarcane	herbicide	shoot growth	Erturk, H. and Walker, P.N. (2003)
Solanum tuberosum L.	potato	PPF, CO_2 conc.	growth, CO_2 conc. in the vessel, photosynthesis, respiration	Kozai, T., Koyama, Y. and Watanabe, I. (1988)
Solanum tuberosum L.	potato	vessel type	growth, CO_2 conc. in the vessel	Takazawa, A. and Kozai, T. (1992)
Solanum tuberosum L.	potato	lighting direction, sucrose conc.	growth, CO_2 conc. in the vessel, photosynthesis	Kozai, T., Kino, S., Jeong, B.R., Hayashi, M., Kinowaki, M., Ochiai, M. and Mori, K. (1992)
Solanum tuberosum L.	potato	relative humidity	transpiration, photosynthesis, leaf resistance	Tanaka, K., Fujiwara, K. and Kozai, T. (1992)
Solanum tuberosum L.	potato	% red light	growth, chlorophyll content, photosynthesis	Miyashita, Y., Kimura, T., Kitaya, Y. and Kozai, T. (1997)
Solanum tuberosum L.	potato	PPF, lighting direction	growth, shoot length, CO_2 conc. in the vessel	Kitaya, Y., Fukuda, O., Kozai, T. and Kirdmanee, C. (1995a)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Solanum tuberosum L.	potato	medium volume and strength	growth, changes in medium composition, CO_2 conc. in the vessel, photosynthesis	Kozai, T., Jeong, B.R., Kubota, C. and Murai, Y. (1995b)
Solanum tuberosum L.	potato	sucrose conc.	growth, sugar uptake, carbon balance	Fujiwara, K., Kira, S. and Kozai, T. (1995)
Solanum tuberosum L.	potato	lighting cycle	growth, carbon balance, CO_2 conc. in the vessel	Hayashi, M., Fujiwara, K., Kozai, T., Tateno, M. and Kitaya, Y. (1995)
Solanum tuberosum L.	potato	co-culture with mushroom	growth, CO_2 conc. in the vessel	Kitaya, Y., Sakami, K. and Kozai, T. (1995c)
Solanum tuberosum L.	potato	red, far-red	growth, shoot length, photosynthesis	Miyashita, Y., Kitaya, Y., Kozai, T. and Kimura, T. (1995)
Solanum tuberosum L.	potato	explant leaf area, fresh weight, stem length	growth	Miyashita, Y., Kitaya, Y., Kubota, C. and Kozai, T. (1996)
Solanum tuberosum L.	potato	CO_2 conc., PPF, supporting material	growth	Roche, T.D., Long, R.D., Sayegh, A.J. and Hennerty, M.J. (1996)
Solanum tuberosum L.	potato	PPF, no. of air exchanges,	growth, CO_2 conc. in the vessel, photosynthesis	Niu, G. and Kozai, T. (1997)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Solanum tuberosum L.	potato	lighting cycle, sucrose conc.	growth, CO_2 conc. in the vessel, photosynthetic characteristics	Niu, G., Kozai, T., Hayashi, M. and Tateno, M. (1997)
Solanum tuberosum L.	potato	natural and forced ventilation, sucrose conc.	stomatal characteristics, transpiration, wax content	Zobayed, S.M.A., Afreen, F., Kubota, C. and Kozai, T. (1999)
Solanum tuberosum L.	potato	CO_2 conc., PPF, lighting direction	growth, tuber yield	Kim, H.S., Lee, E.M., Lee, M.A., Woo, I.S., Moon, C.S., Lee, Y.B. and Kim, S.Y. (1999)
Solanum tuberosum L.	potato	natural and forced ventilation	growth, % survival, costs	Xiao, Y., Zhao, J. and Kozai, T. (2000)
Solanum tuberosum L.	potato cv. russet burbank	CO_2 conc., sucrose conc.	shoot growth	Pruski, K., Astatkie, T., Mirza, M. and Nowak, J. (2002)
Spatihphyllum		CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T., and Watanabe, I. (1987)
Syngonium		CO_2 conc.	photosynthesis-CO_2 conc. curve	Fujiwara, K., Kozai, T. and Watanabe, I. (1987)
Vitis rupestris Scheele	grapevine	no. of air exchanges	growth, photosynthesis-CO_2 conc. curve, daily balance of CO_2 exchanges	Galzy, R. and Compan, D. (1992)

Scientific Name	*Common name*	*Main controlled factors*	*Main measured factors*	*Authors*
Vitis vinifera L.	grapevine	no. of air exchanges	growth, photosynthesis-CO_2 conc. curve, daily balance of CO_2 exchanges	Galzy, R. and Compan, D. (1992)
Vitis vinifera L.	grapevine	CO_2 conc.	growth, morphology	Fournioux, J.C. and Bessis, R. (1993)
Wrightia tomentosa	-	CO_2 conc., growth regulator, sucrose	growth	Vyas, S. and Purohit, S.D. (2003)
Zantedeschia elliottiana	-	forced and natural ventilation, CO_2 conc.	growth, morphology, % survival *ex vitro*, production cost	Xiao, Y. and Kozai, T. (2004)
Zoysia japonica	zoysiagrass	CO_2, sucrose conc.	growth, % survival *ex vitro*	Seko, Y. and Kozai, T. (1996)

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Chapter 16

T. KOZAI

RECONSIDERATION OF CONVENTIONAL MICROPROPAGATION SYSTEMS

Contents

Key words: Closed system, conventional micropropagation, environmental pollution, open system, photoautotrophic micropropagation.

1. INTRODUCTION

Much effort has been made so far to reduce production costs and culture periods by improving culture methods in terms of compositions of plant growth regulators and nutrient components, types of supporting materials, equipments, culture vessels with lids, and culture room environment.

However, production costs are still high and culture periods required for initiation, multiplication, shoot growth, rooting and acclimatization are still long in conventional micropropagation system. In addition, in many cases, physio-morphological quality of plant is still not high enough for wider commercialization. A main reason for high production costs is that we have not paid much attention on the energy and material efficiencies in micropropagation systems. Furthermore, we have paid little attention on the environmental friendliness of micropropagation systems or release of pollutants to the outside environment from micropropagation systems.

In this chapter, conventional or current micropropagation systems are discussed from viewpoints of maximum use of resources for efficient production and minimum release of pollutants for environmental conservation. A concept of a

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 267-274.

closed micropropagation system as one type of closed plant production system is introduced to produce high quality plants and to reduce the production costs by improving the energy and material efficiencies and environmental friendliness of micropropagation systems.

2. RESOURCES, PRODUCTS AND BY-PRODUCTS AS POLLUTANTS

In any production system, we need some resources for production. Resources include labor, time, structures (buildings, etc.), equipments, supplies, energy and information. Generally, in any production system, some by-products are obtained, in addition to the targeted product(s). When these by-products are useless, or harmful for the producer, they are released as wastes or environmental pollutants from the production system to its environment, with or without additional costs. Environmental pollutants include thermal or heat energy, noise and any kind of deleterious materials.

Recently, the producer is increasingly required to make the by-products non-polluting or inert before releasing them to the environment, resulting in an increase in production costs.

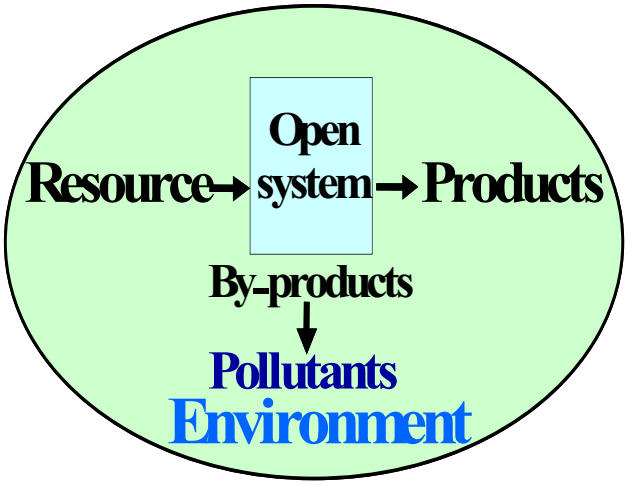


Figure 1. The current micropropagation system is a typical open or one-way production system.

3. OPEN VS. CLOSED PRODUCTION SYSTEMS

A production system releasing a significant amount of pollutants is called an open production system or a one-way production system (Figure 1). In the following

description, conventional micropropagation systems are considered as typical open production systems. On the other hand, a production system releasing a negligible amount of pollutants is called a closed production system (Figure 2). In a closed production system, all by-products are considered to have some economic value and are recycled as secondary products to be used in the closed production system or in other production systems. A closed production system releasing no pollutants is called a zero emission production system.

4. NECESSARY MATERIALS IN CONVENTIONAL MICROPROPAGATION SYSTEMS

Material resources needed in conventional micropropagation systems include the followings: culture medium (consisting of water, sugar, agar or other gelling agents, inorganic nutrients, plant growth regulators, other organic substances such as vitamins and amino acids), explants, plantlets, vessels, vessel lids, water needed for washing vessels, vessel lids and plants, sterile papers, aluminium foils, small tools such as forceps, scalpels, razor blades, needles and scissors, alcohol for sterilization and lamps. Electricity and/or fuel are needed as energy resource mainly for autoclaving, lighting, cooling and heating. In addition to the material and energy resources, labor and time resources are necessary.

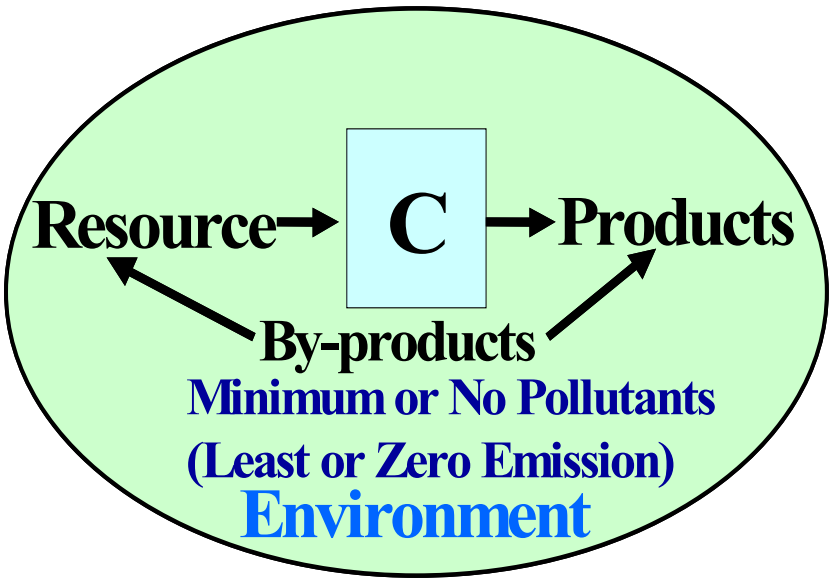


Figure 2. Closed production system (C).

High percentages of resources and their derivatives (by-products) are wasted (Table 1). Nearly 100% of water, papers and agar are wasted and thus pollute the environment. Almost 100% of electric energy and fuel energy consumed are converted into heat energy and released to the environment as thermal pollutants. It has been estimated that in a culture medium to be disposed of after one culture period, 30% of organic nutrients, 60% of sugar added at the beginning of culture period remained unused. Sugar absorbed by cultures are mostly synthesized into carbohydrates but a significant percentage of sugar is converted into CO_2 as a result of respiratory activity of cultures, and then released to the environment. Plastic vessels and lids can be re-used 15-30 times before damaged. Glass vessels can be used more repeatedly than plastic ones, but need more energy for transportation due to their heavy weights and fragile characteristics. Explants are excised from regenerated plants. However, weight percentage of plant parts which can be used as explants over the weight of regenerated plants is less than 50%. In addition, a small percentage of plants are lost due to microbial contamination of culture medium. In summary, most of the material and energy resources are neither efficiently used, nor incorporated into products (i.e., marketable plants), but end up as pollutants.

Table 1. Estimated percents of resources wasted per operation

Water (polluted)	99%
Agar	100%
Sugar	60%
Inorganic nutrients	30%
Plants (dead and unused)	50%
Papers (non-sterile)	100%
Heat	100%
CO_2	100%
Vessels (damaged)	1%
Vessel caps (damaged)	50%
Lamps (broken)	0.1%

5. LABOR REQUIRED IN CONVENTIONAL MICROPROPAGATION SYSTEMS

Labor required in conventional micropropagation systems includes 1) medium preparation, dispensing and discarding, 2) vessel washing, handling and transportation, 3) autoclaving, 4) excising and transplanting plants and explants, 5) capping and uncapping, 6) removal and discarding of dead and contaminated plants, 7) acclimatization, 8) equipments and room sterilization, 9) recording jobs and labeling on culture vessels, and 10) supervision.

The cost for labors mentioned above accounts for 40-60% of the total production cost. On the other hand, the labor cost per hour has been increasing in most countries where micropropagation is an important industry. Thus, reduction of labor cost is essential to reduce the total production cost.

6. CHARACTERISTICS OF CONVENTIONAL AND FUTURE MICROPROPAGATION SYSTEMS

Based upon the discussion above, it can be said that conventional micropropagation systems consume large amounts of supplies, electricity and fuel, release large amounts of pollutants to the environments, and are labor intensive. Much time is also required to grow cultures, producing a relatively high percentage of physiologically poor plants.

Thus, in order to make the future micropropagation systems more efficient and profitable, we need to use a minimum amount of resources, to produce little or no environmental pollutants and to produce plants with high physiological quality and with a minimum loss of regenerated plants. In order to minimize the amount of environmental pollutants, the consumption of resources must be minimized by efficient use of resources. Then, the costs for resources and for the handling and processing of pollutants will reduce naturally.

7. KEY CONCEPTS OF FUTURE MICROPROPAGATION SYSTEMS

Key concepts for minimizing amounts of resources and pollutants in future micropropagation systems are: 1) closed plant production systems, and 2) photoautotrophic (photosynthesis-dependent) micropropagation systems for growing plants using sugar-free culture medium. Use of porous or fibrous root supporting materials, which can be used repeatedly, or transplanted with transplants, is also important in future micropropagation systems. Definition and characteristics of photoautotrophic micropropagation systems and the use of porous supporting materials have been described elsewhere. Those of closed plant production systems are described in the following section.

8. DEFINITION AND CHARACTERISTICS OF CLOSED PLANT PRODUCTION SYSTEMS

Closed plant production systems include closed micropropagation systems, closed plant propagation systems and closed transplant production systems. Closed plant production systems are defined as and characterized by: 1) use of optically opaque and thermally well-insulated walls with heat transmission coefficient of less than 0.01-0.1 W m^{-2} K^{-1} (thermal insulating panel which is 15-20 cm thick), 2) minimized and controlled ventilation through air filters with higher air pressure in the culture room compared with outside air pressure, and 3) efficient use of electricity for lighting and cooling. Closed plant production systems are equipped with a CO_2 enrichment system in most cases for promoting photosynthesis of plants.

Advantages of closed plant production systems include: 1) environmental factors such as air temperature, relative humidity, CO_2 concentration, PPF, photoperiod, light quality and air current speed can be adequately controlled to control the transplant growth and to produce value-added transplants, 2) prevention of insects, pests and pathogens from entering the culture room is easily accomplished, 3) plants with high physiological and morphological qualities can be produced, 4) a comfortable working environment can be provided throughout the year, 5) transpired water from plants and evaporated water from the substrate can be collected at the cooling panel of air conditioner and can be recycled, 6) carbon dioxide gas produced by plants due to dark respiration is accumulated during the dark period, which can be used for photosynthesis of plants during the photoperiod.

9. SOME CONSIDERATIONS FOR REDUCING THE CONSUMPTION OF ENERGY RESOURCE

In a closed micropropagation system or a closed culture room, the exchanges of heat and radiative energy through the walls are minimal because thermally insulated walls are used. During the photoperiod, the lamps generate much heat, and thus cooling is necessary even during the winter in any places in the world. When the outside air temperature is higher than 25 C during the dark period, a minimum amount of cooling may be necessary to keep the air temperature of the culture room at around 25 C (during the summer in tropical, subtropical and temperate regions). However, greater cooling is necessary under such conditions if the culture room is not thermally well insulated. On the other hand, in cold climate regions where minimum outside air temperatures are regularly below zero, a heating, or air conditioning system with a minimum heating capacity, may be required if thermal transmission coefficient of the walls is higher than 0.1 W m^{-2} K^{-1}. In hot climate regions, the heating system is not required even during the dark period in the winter if the walls are thermally well insulated. This is because a small but sufficient amount of heat is generated from equipments such as fans and pumps and from the plants.

16. Reconsideration of Conventional Micropropagation Systems

In the closed micropropagation system, the cooling load of air conditioners during the photoperiod accounts for 95-99% of the total cooling load throughout the year. The rest (1-5%) is the cooling load due to heat transmission through the walls, warm air infiltration and the heat generated by equipment such as fans installed in the culture room. Thus, the cooling load of air conditioners during the dark period is negligibly small compared with that during the photoperiod. In a closed culture room equipped with a recently developed air conditioner, the electric consumption of air conditioner accounts for about 20-25% of the total electric consumption and that of lamps accounts for 75-80%. The percentage of electric consumption due to air conditioning will be higher than 25% when the thermal insulation of the walls is low, air infiltration is too much and/or the energy performance (Coefficient Of Performance, C.O.P.) of air conditioners is low.

Again, the use of natural or solar light for reducing electric consumption for lighting increases the electric consumption for cooling, the condensation of water at the inside surface of glass walls in the winter, and the heating load during the dark period in the winter. Furthermore, light intensity and air temperature fluctuate with time and their spatial variations in the culture room become significant. Therefore, the use of natural light for reduction in electricity cost cannot be practical in most cases.

On the other hand, use of solar energy for heating water for washing, autoclaving and other purposes is practical for reducing energy costs in micropropagation. Temperature of well or city water ranges between 15 and 30 C in most places and this can be raised to 50-70 C using a solar energy collector with a black-colored double layer panel through which the water flows. If water of 100 C is required then instead of heating the water of 25 C directly using fuel, if it is pre-heated to 70 C using the solar energy collector and then heated to 100 C using fuel, the fuel consumption is reduced by 67% [=100 (70-25)/(100-25)].

In many cases, electric energy is used to obtain hot water and steam (110-115 C) for autoclaving. Then, the consumption of electric energy for autoclaving is considered to account for about 20% of the total electricity costs. However, hot water/steam can be obtained by using fuel other than electricity, and the cost of fuel is being about 30% of the cost of electricity. An autoclave driven with fuel instead of electricity is commercially available although it is not currently widely used. Its appearance, function and price are almost the same as those driven with electricity. If both fuel and solar energy are used for autoclaving instead of electricity, energy cost for autoclaving is reduced by 90% [100 – 0.3 x (100 – 67)].

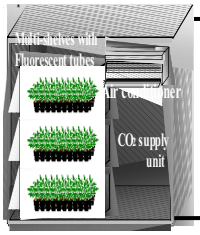
It is also noted that autoclaving is not the only method for sterilization. In most cases, 70-80 C of water for 1-2 hours has the same effect as pressurized steam at 115 C for 15 min in terms of sterilization. This water can be obtained using a solar heater alone.

Current micropropagation systems have contributed greatly to the development of the micropropagation industry. However, we are regularly requested to provide improvements to the systems for further development of the industry. In this chapter, current micropropagation systems are reconsidered mainly from the viewpoint of environmental control engineering aiming at the production of high quality transplants with minimum use of resources and minimum release of pollutants. The

two concepts discussed in this chapter for the improvement of current micropropagation systems are 'closed plant production system' and 'photoautotrophic micropropagation'. It is hoped that these two concepts will contribute to the initiation of discussion on the improvement of current micropropagation systems for producing high quality plants with minimum resources and environmental pollution.

Chapter 17

T. KOZAI



CLOSED SYSTEMS WITH LAMPS FOR HIGH QUALITY TRANSPLANT PRODUCTION AT LOW COSTS USING MINIMUM RESOURCES

Contents

T. Kozai et al. (eds.), Photoautotrophic (sugar-free medium) micropropagation as a new propagation and transplant production system, 275-311.

Key words: Artificial light, closed system, CO_2 concentration, ethylene, quality transplant, transplant production.

1. INTRODUCTION

A 'closed plant production system' or simply a 'closed system' has been commercialized in Japan since 2002, largely based upon the research at Chiba University, for production of tomato, cucumber, eggplant, lettuce and spinach transplants from seeds. Commercial productions of herbs, leafy vegetables, bedding plants such as pansy, medicinal plants, and orchid plants are now under trial in Japan.

The closed system is defined as a warehouse-like structure covered with opaque thermal insulators, in which ventilation is kept at a minimum, and lamps are used as the sole light source for plant growth.

Advantages of the closed system over a greenhouse for producing high quality transplants include: 1) rapid and efficient growth of transplants mainly resulting from a considerably higher light utilization efficiency (2-3 times) of transplants due to optimized growth conditions, 2) the significantly higher quality transplants produced under uniformly controlled environments in the protected area free from pest, insects/pathogens and the disturbance of outside weather, 3) the higher (about 10 times) productivity per floor area per year, mainly due to the use of multi-layered shelves (e.g., 4-5 shelves) with the ratio of planting area to floor area of 1.2-1.5, a high planting density per tray area (1,500 transplants m^{-2}), a high percentage of salable transplants (>90%), higher sales price due to their higher quality and uniformity of transplants (10-20% over greenhouse), and shorter production period (30-70% of greenhouse), 4) the drastically higher utilization efficiencies of water, CO_2, (about 15 times for water and 2 times for CO_2) and fertilizers mainly due to the minimized ventilation and recycling use of dehumidified water by air conditioners, resulting in little waste water to the outside, 5) virtually no requirement of heating

cost even in the winter because of its thermally insulated structure 6) the lower labor cost (50% or less) due to the smaller floor area, the worker-friendly shelves, comfortable working environments), and 7) the easier control of plant developments such as stem elongation, flower bud initiation, bolting, root formation (Kozai et al., 1998; Kozai, 1998; Kozai et al., 1999; Kozai et al., 2000a, b and c; Chun and Kozai, 2001, Kozai et al., 2004).

High electricity cost and initial investment are often mentioned as a disadvantage of the closed system. However, electricity cost for transplant production could be reduced considerably by using thermally insulated walls and multi-shelves, and advanced lighting and air conditioning systems (Kozai, et al., 2004). The electricity cost for lighting and cooling per transplant was found to be roughly 0.5 to 1.0 US cent, which accounts for 1-5% of the sales prices of tomato, eggplant, pansy, and sweetpotato transplants in Japan.

Since only about 10% of greenhouse floor area is required to produce the same amount of transplants, initial cost per annual plant production in closed systems is lower than that in greenhouses. By using a closed system with a floor area of 150 m^2 with 60 shelves having 960 plug trays in total, about 10 million transplants can be produced annually.

In this chapter, the definition, concept, theoretical backgrounds, methods, materials, applications, and advantages of the closed system using lamps over a greenhouse using sunlight are described from biological, engineering and economic points of view.

2. GENERAL FEATURES OF HIGH QUALITY TRANSPLANTS

General features of high quality transplants are given in Table 1. However, it is often difficult to produce transplants having such features in the greenhouse under variable weather conditions and limited resources. Problems often encountered in transplant production using the greenhouse are listed in Table 2.

To produce high quality transplants having features listed in Table 1 and to avoid problems listed in Table 2, we need intensive labor and a greenhouse heavily equipped with a variety of environmental control units, both of which are costly. Aims of this chapter is propose an alternative method, called the closed transplant production system, for producing high quality transplants at a low cost.

Table 1. General features of high quality transplants.

No.	Item
1	Genetically superior and uniform.
2	Physiologically and morphologically normal and uniform with a compact plant form without elongated shoots, and with normal color.
3	Developmental stages of flower buds, leaves and roots are uniform and as planned.
4	Tolerant to high and low temperatures, strong wind, variable solar

	radiation, and dry and wet soil and air conditions.
5	Tolerant to and uninfected by pathogens and insect pests, requiring less agrochemicals during cultivation.
6	Low labor and other costs for handling, transportation and transplanting, leading to low production costs.
7	No or little physical damages of aerial and root parts at transplanting
8	High ability of rapid growth after transplanting, leading to high quality and high yield at harvest when cultivated under variable cultivation conditions.

Table 2. Problems often encountered in transplant production using the greenhouse.

No.	Item
1	Non-uniform transplant growth due to the non-uniform environmental conditions.
2	Season, weather and human-skill dependent growth rate due to the sensitiveness of growth rate to environmental conditions.
3	Difficult to standardize the details of production technology. High-level expertise and labor-intensive work needed for high quality transplant production.
4	A greenhouse with large air volume (high ridge) and equipped with an environmental control system needed for precise environmental control under variable solar radiation, wind speed and temperatures outside the greenhouse.
5	High initial investment and operation costs for a greenhouse with large air volume (high ridge) and equipped with an environmental control system.
6	Possible damages of crops by insect pests, fungi and bacteria throughout the year. Difficult to reduce the costs for preventing these damages.
7	Difficult to avoid the stable employment of workers and a low operation rate of the greenhouse due to the varying demands of transplants with season.
8	To meet the high demand of transplants in early fall (September), the transplant production needs to be started under hot climate (August), when the air temperature in the greenhouse is still too high to produce high quality transplants.
9	A possible mismatch of supply and demand due to the unstable transplant productivity under variable weather and unpredictable demands, which results in high production costs.

3. DISADVANTAGES OF USE OF THE SUN AS LIGHT SOURCE IN TRANSPLANT PRODUCTION AND ADVANTAGES OF USE OF LAMPS AS LIGHT SOURCE IN TRANSPLANT PRODUCTION

Most people think that use of solar radiation or natural light from the sun is more economical than use of artificial light from lamps for plant production, because solar

radiation is free of charge. This is generally true. However, this free solar radiation is often disadvantageous over artificial light in transplant production using the greenhouse because we have to invest a lot for controlling the greenhouse environment to use the solar radiation efficiently. Disadvantages of use of solar radiation in transplant production are given in Table 3.

On the other hand, use of artificial light is sometimes advantageous over natural light in transplant production, and it is worth to compare the advantages of artificial light with the disadvantages of natural light. Rationale and advantages of use of lamps as light source in transplant production are listed in Table 4.

Table 3. Disadvantages of the sun as light source in transplant production.

No.	Item
1	Only about 50% of solar radiation energy (waveband: 300-3000 nm) is photosynthetically active (waveband: 400-700 nm). Radiation with a wavelength longer than 800 nm has a thermal effect only. Thus, air and leaf temperatures tend to be high under solar radiation due to the heating action of thermal radiation.
2	Difficult to control the light intensity within a range suitable for transplant production. The light intensity is too low in the morning and evening or on cloudy and rainy days, while it is often too high around noon on clear days.
3	Seasonal changes of daylength often affect photomorphogenesis, development and growth of transplants.
4	Seasonal and diurnal changes of light quality (red/far-red ratio, blue/red ratio, etc.) caused mainly by the changes in solar altitude and atmospheric transmittance affect flower bud development, shoot elongation, germination, rooting, photosynthesis, transpiration, etc.
5	Difficult to control the light quality independent of light intensity.
6	Light intensity at plant level is often reduced by surrounding and greenhouse structures. Direction and orientation of direct solar light beam is determined by the solar position.
7	Rapid changes in light intensity with time cause rapid changes in temperature and relative humidity in the greenhouse, which is unfavorable for high quality transplant production. To lessen the rapid changes in air temperature and relative humidity, a tall greenhouse (6-10 m high) with large air mass is needed for growing short transplants with about 0.2 m high (Figure 1).

Figure 1. In order to lessen the rapid changes with time in air temperature and relative humidity, a tall greenhouse (6-10 m high) with large air mass is needed for growing short transplants with only about 0.2 m high, which is space inefficient and costly.

Table 4. Rationale and advantages of use of lamps as light source for transplant production.

No.	Item
1	Optimum light intensity is relatively low (photosynthetic photon flux of 150 - 300 μmol m^{-2} s^{-1}), which can be obtained by 5 or 6 fluorescent tubes (40 W each) installed about 40 cm above the shelf.
2	Optimum light intensity, which depends on growth stage, planting density and plant species, can easily be provided.
3	Light and dark periods can be controlled precisely. Lighting cycle (light period plus dark period) needs not to be 24 hours.
4	Lamps which emit photosynthetically active radiation (400–700 nm) only or phytochrome sensitive active radiation (e.g., 700–800 nm) only can be used.
5	Intensity and light period for photomorphogenesis can be set independently of those for photosynthesis.
6	Planting density of transplants is relatively high (1000-3000 m^{-2}) and production period of transplants is relatively short (2-4 weeks). Electricity cost per transplant is relatively low (0.3–1.0 US cent per transplant) because it is proportional to a product of light intensity and production period, divided by the planting density.
7	Sales price of transplants is relatively high, especially in case of fruit vegetables (20-100 US cents), and the electricity cost accounts for 5% or less of the production cost.

4. CLOSED PLANT PRODUCTION SYSTEM

4.1. Definition

A 'closed plant production system' or simply called 'closed system' in this chapter is defined as a warehouse-like structure, 1) covered with opaque thermal insulators, 2) in which ventilation is minimized, and 3) which uses lamps as the sole light source for plant growth (Table 5). In addition, multi-layered shelves each with lamps are used whenever possible to increase a production capacity per floor area.

Table 5. Definition of the closed plant production system.

No.	Item
1	A warehouse-like structure constructed with opaque thermally insulated walls, minimizing the heat transmission through walls.
2	Minimized or controlled heat and mass energy exchanges by ventilation or air infiltration. (A heat pump (or an air conditioner) is used instead of a ventilation fan even when the set point of room air temperature is higher than the outside air temperature.)
3	Lamps are the sole light source for plant growth and development.
4	Multi-layered shelves each with lamps are used whenever possible.

On the other hand, a greenhouse can be called an 'open plant production system' or simply 'open system', in the sense that: 1) it is covered with transparent materials such as glass sheets for use of sunlight, and 2) it requires ventilation especially in summer, and thus heat energy, radiation energy, CO_2 and water exchanges between inside and outside the greenhouse are considerable and are not well controlled.

4.2. Main Components

Main components of a typical closed transplant production system (Figure 2) are: 1) a warehouse-like structure covered with opaque thermal insulators, 2) air conditioners for home use, which are mostly used for cooling and dehumidification, and sometimes for heating, 3) fans for internal circulation of air, 4) a CO_2 supply unit for promoting plant photosynthesis, 5) multi-shelves having lamps and their fixtures, installed in the warehouse-like structure, and 6) an environmental control unit. Fluorescent lamps are usually used as a light source for plant growth.

Figure 2. Schematic diagram showing four of the components of the closed transplant production system (left) and the inside view of a closed system commercially used for tomato transplant production in Shizuoka, Japan (right). Fans for internal circulation of air, usually installed at the back-wall of each shelf, are not shown in the above diagram. Each unit of the commercial type closed system has a floor area of 22 m^2 (6.3 m long, 3.6 m wide), 7 sets of four 4-layered shelves (4 shelves at one side and 3 shelves in the other side). Each shelf with six 40 W fluorescent tubes is 0.75 m wide and 1.5 m long. Then, one unit of the closed system holds 112 (= 7 x 4 x 4) cell trays (30 cm wide x 60 cm long). Since 200 tomato transplants are grown in one tray, 22,400 transplants are produced in one batch, totaling 400,000 transplants per year (one cycle is 20 days, 18 batches per year).

4.3. Characteristics of main components of the closed system

All the main components of the closed system are mass-produced for home use or for industrial use, except for a CO_2 supply unit. Therefore, in most countries, 1) a thirty to sixty percent discount is common for bulk purchases due to the significant reduction in costs for packaging, labor, shipping, transportation, etc., 2) significant technical advancement and price reduction have been achieved year by year, which are forced by strong competition among manufacturers, and importantly, 3) recycling and/or reuse systems for fluorescent tubes, air conditioners and thermal insulators have been established by laws for environmental conservation and resource saving in most countries (Table 6). A CO_2 supply unit is widely used for CO_2 enrichment in the greenhouse, and its cost is about US$1,000.

These main components of the closed system can be used without any modification for constructing the closed system. And each component has a rich variety of types, sizes, capacities etc. Therefore, we are able to construct various types of closed systems. These characteristics significantly reduce the investments for research and development of the closed system.

When the room air temperature is higher than its set point, air conditioners are operated for cooling and air is not ventilated because of the reasons listed in Table 7.

Table 6. Advantages of use of mass-produced electric or industrial products as main components of the closed system.

No.	Item
1	Cost performance of the product has been improved every year due to severe competitions among the manufacturers.
2	Recycling and reuse systems for the products have been established socially, and thus the products are environmentally friendly.
3	In case of malfunction or a decrease in performance, the part(s) or the product itself can by replaced by a newly released part or a product with a higher cost performance.
4	Easy access to the parts of the products at a low price.
5	A considerable (60-80%) discount percentage is possible for bulk purchase.
6	Virtually maintenance free or easy maintenance. Free repair and replacement are guaranteed for one year or so in case of malfunction of the products.

Table 7. Reasons for use of air conditioners (heat pump) instead of ventilation fans even when outside air temperature is lower than the set point of room air temperature.

No.	Reason
1	Evapotranspirated water is lost to the outside by ventilation (More than 95% of irrigated water is evapotranspirated).
2	Respired CO_2 by plants and enriched CO_2 are lost to the outside by ventilation.
3	Dusts, pathogens, and insects can enter to the closed system by ventilation.
4	Relative humidity and air temperature in the closed system are affected by the relative humidity and air temperature outside the system, if ventilated. Then, the accuracy of environment control is decreased
5	Electricity cost for cooling accounts only for 15% or less of that for lighting
6	Ventilation fans with air filters are necessary for ventilation.

4.4. Components unnecessary in the closed system but necessary in the greenhouse

Components which are unnecessary in the closed system, but are often or definitely necessary in the greenhouse are: 1) thermal screens, 2) shading screens, 3) roof and side ventilators or fan ventilators, 4) oil or gas heaters, 5) benches, beds or bags containing substrate, 6) transparent covering materials such as glass sheets and plastic films, and, in some areas, and 7) an evaporative cooling system and/or a supplemental lighting system using high pressure sodium and/or metal halide lamps (Table 8). Most of these components have been developed and used only for the

greenhouse industry, except for transparent glass sheets. Thus, investment for research and development of the greenhouse components are costly and limited.

Components common to both closed system and greenhouse for transplant production are cell trays, an irrigation unit and an environmental control unit. However, control algorithms of these units for the closed system are much more simpler than those for the greenhouse. On the other hand, a CO_2 supply unit is indispensable for the closed system, but dispensable for the greenhouse.

Table 8. Equipment and facilities necessary in the closed system and the greenhouse, respectively, for transplant production.

Item	Closed system	Greenhouse (Open system)
Basic structure	A warehouse like structure covered with opaque thermal insulators with $1/10^{th}$ of greenhouse floor area.	A greenhouse structure covered with transparent glass sheets or plastic film.
Basic facility	Multi-layered shelves with fluorescent lamps.	Bench or beds.
Basic environmental control equipment	Air conditioners (or heat pumps), air mixing fans for internal circulation of air, CO_2 enrichment system, nutrient solution supply system, and timer clock.	Heating system, thermal screen system, shading screen system, natural or forced ventilation system, and timer clock.
Optional environmental control equipment	None.	CO_2 enrichment system, evaporative cooling system, nutrient solution supply system, mesh screen for preventing insects from entering, air mixing fans, computerized environmental control system, and alarm system for strong wind, snow, rain and human invasion.

4.5. General features of the closed system in comparison with those of the greenhouse

The features of the closed system in comparison with the greenhouse are shown in Table 9 from viewpoints of transplant production, and in Table 10 from viewpoints of resource saving and environmental conservation.

Table 9. General features of the closed transplant production system in comparison with the greenhouse.

No.	Item
1	Annual productivity of transplants is about 10-fold (See Table 11). Transplants are produced as scheduled. A risk of physical damage of transplants is minimized because they are produced in a warehouse like structure.
2	Production period is shortened by about 30% and high quality transplant is almost always produced due to the optimized environment regardless of the weather and season.
3	Operation rate of the system and, thus, annual production capacity are high, because of the shortened production period regardless of the weather and season.
4	Favorable environments, which cannot be achieved in nature or in the greenhouse, can easily be realized (e.g., horizontal air current speed within the transplant canopy of 50 cm s^{-1}, relative humidity of 60% during dark period, etc.) (Table 12).
5	The system is of resource saving, environmentally friendly, space-saving, and labor-saving (Consumptions are 1/20 for irrigation water, 1/2 for CO_2 enrichment, 2/3 for fertilizer, 1/10 for pesticide, and 1/10 for floor area. Labor hour is halved and there is no nutrient-containing water wasted to the outside.
6	High cost performance and easy recycling of main components of the system (air conditioners, fluorescent tubes, air mixing fans, thermal insulating boards, multi-layered shelves, CO_2 controller, etc.). No pollutants are released to the outside.
7	High discount percentages of the main components by bulk purchase.
8	Cost for cooling accounts only for about 15% of electricity cost even in summer. Electricity cost for lighting and cooling per transplant is 0.5-1.0 US cent and accounts for less than 5% of production cost.
9	Low initial investment cost per transplant.
10	Low operation cost per transplant. No or little heating cost even in winter at high latitudes. Relative humidity during the photoperiod is naturally kept at 60-70%.
11	No hard work under comfortable working environments.
12	Mental stress is lightened and environmental management work is reduced because no attention is necessary for the effect of outside weather on the transplant growth.
13	Suited for production of scions and stocks for (robotic) grafting because seedlings grow uniformly.
14	Suited for nursing and acclimatization of grafted transplants.
15	No sudden rise of room air temperature even around noon on clear days in summer in case of electricity failure due to the thermally insulated structure of the system.

16	No sudden drop of room air temperature even at night in winter in case of electricity failure due to the thermally insulated structure of the system.
17	Light utilization efficiency in the closed system is over 2 times that in the greenhouse because of the optimized environment.
18	Cost for CO_2 enrichment per transplant is negligibly small because of the air tightness of the closed system.
19	An environmental controller is simple in algorithm and less expensive because considerations on weather are unnecessary.
20	Disinfection of the closed system is relatively easy.

Table 10. Features of the closed system with respect to resource saving, environmental friendliness, labor saving, space saving and cost effectiveness in comparison with those of the greenhouse.

No.	Feature	Description
1	Resource saving	More than 95% of evapotranspiratred water is collected at the cooling panel (or evaporator) of air conditioner, and is reused for irrigation. Thus, net amount of water required for irrigation is 5% or less of evapotranspirated water.
2	Environmentally friendly, Resource saving	No waste water containing chemical fertilizer application is released to the outside. Thus, the amount of chemical fertilizers can be reduced by 30-40%, resulting in resource saving and environmental conservation.
3	Resource saving	Eighty to ninety percent of CO_2 supplied to the closed system is absorbed by photosynthesis of plants when CO_2 concentration is kept at 700 ～ 1000 μmol mol^{-1}(ppm). It is about 50% when supplied to the greenhouse.
4	Environmentally friendly, Resource saving	Insect pests can rarely enter the closed system due to its structural characteristics. Thus, the amount of pesticide used in the closed system is less than 10%. The pesticide supplied to the closed system is not released to the outside due to its structural characteristics.
5	Resource saving	Since walls are covered with thermal insulators (thickness: 10 cm), even in winter at cold regions, room temperature during photoperiod can be kept at 25-30 C by heat generated from lamps, so that no other heating source is necessary, although heating is necessary during dark period. Even during dark period in winter at cold regions, heating load of the closed system is about $1/10^{th}$ of that of the greenhouse. Ventilation cost of the closed system is zero because

		of no ventilation facility in the closed system. Electricity cost for lighting and cooling is only 1-5% of the production cost.
6	Resource saving	Shading screen, ventilators/ventilation fans, thermal screen, heating system, and supplemental lighting system are unnecessary, which are often necessary in the greenhouse.
7	Space saving, Resource saving	Floor area of the closed system is about 10% of the greenhouse floor area. Thus, the closed system needs less material and energy for construction. The closed system can be built at shaded place next to the tall buildings, waste land such as desert, on the roof of the building, etc.
8	Labor saving, Comfortable working space	Less labor for transportation and handling due to the $1/10^{th}$ of working floor area. Comfortable working environment regardless of the outside weather (Table 11). Automatic irrigation is easy because timing and amount of irrigation is not affected by outside weather.
9	Safety	Outer structure is physically rigid so that the transplants are safer with respect to strong wind and other physical disasters. It is relatively easy to keep commercial secrets of products.
10	Low cost	Annual rate of operation can be high in the closed system because the system can be operated throughout the year, so that depreciation per transplant can be lowered if marketing and production planning are reasonable.

Table 11. Reasons for labor saving characteristics of the closed system in comparison with the greenhouse.

No.	Reason
1	Floor area and thus working area of the closed system is $1/10^{th}$ of that of the greenhouse.
2	Easy irrigation and fertilization, because of no influence of the outside weather on the growth of transplants in the closed system.
3	A low risk of invasion of insect pests to the closed system, and much less application of pesticide.

4	No need to pay attention to the outside weather to control the environment in the system.
5	Comfortable working environment throughout the year.
6	Little heavy or hard work.
7	Raising of plug transplants to the pot transplants are unnecessary in most cases.

Table 12. Examples of environmental control which is easy to realize technically at a low cost in case of the closed system but is difficult to realize technically and economically in case of the greenhouse.

No.	Items
1	Provide 24-h light period and change the set points of light intensity and air temperature every day.
2	Change light and dark periods each time independently each other.
3	Control flower bud growth, shoot and hypocotyls elongation, bolting, photosynthetic activity by modifying the light quality of lamps.
4	Maintain the horizontal air current speed within the transplant canopy at 30 cm s^{-1} or higher.
5	Maintain the relative humidity within the densely populated transplant canopy at 85% or lower during light period.
6	Maintain the air temperature in dark period higher than that in light period.
7	Maintain the relative humidity during the dark period at 80% or lower.
8	Control the CO_2 concentration during the light period at 1000 μmol mol^{-1} with CO_2 utilization efficiency higher than 85%.
9	Provide uniform environments over and within the transplant plug trays.

4.6. Initial investment cost is comparable to that of the greenhouse

As will be explained more in detail in a later section, an increase in yearly productivity per floor area of the closed system is about 10 times that of the greenhouse for many kinds of transplants, if the number of transplants to produce is the same. Then, floor area of the closed system can be reduced to 10% of the greenhouse floor area to obtain the same yearly production of transplants.

It is quite possible that the initial investment of the closed system is roughly equal to or lower than that for constructing a well-equipped greenhouse for transplant production that has about 7 times the floor area of closed system, at least, in Japan and in other countries with similar social, economic and technical conditions. In Japan, the construction cost of an aluminum-structured glasshouse is currently about 200 US$ m^{-2} and the equipment cost for heaters, thermal and shading

screens, ventilators, etc. is also about 200 US$ m^{-2}. Thus, a well-equipped glass house costs about 400 US$ m^{-2}.

On the other hand, the construction cost of the closed system having the same production capacity as the greenhouse is roughly equal to or lower than 2,800 (= 400 x 7) US$. In many other countries, the construction costs of both the closed system and the greenhouse are less expensive than in Japan.

4.7. Reduction in costs for transportation and labor is significant

Labor cost can be lowered in the closed system than in the greenhouse, because its working floor area is about 15% of the greenhouse floor area, which reduces a daily total distance of walking by workers in the closed system to roughly 15% of that in the greenhouse. In addition, more comfortable working environment is, in general, provided by the closed system than by the greenhouse. Initial investment and operation costs for handling and transportation of cell trays (often called 'plug trays') can also be lower in the closed system than in the greenhouse due to the smaller floor area of the closed system.

4.8. Uniformity and precise control of microenvironment over the trays are achieved

A typical air flow pattern in the closed system is shown in Figure 3. Air is sucked in at the back wall of each shelf by several micro fans (3-4 W each). Thus, air temperature, relative humidity and CO_2 of outgoing air from the back wall of each shelf are the same over the trays as well as shelves. Air flows horizontally over cell trays on each shelf at a horizontal air current speed of 0.1-0.5 m s^{-1}. Generally, a higher horizontal air current speed is applied as transplants grow and/or as PPP increases.

Each shelf is 60-65 cm wide, which is about 5 cm longer than the length of cell trays. Then, the microenvironment along the air flow over the cell trays does not change significantly. Furthermore, air flow rate and PPF on each shelf can be controlled and kept, respectively, at the same levels over the shelves, so that temperature rise along with the air flow over the cell trays is less than 1 C. In short, the structure of the multi-shelves guarantees a uniform distribution of aerial environment over shelves and within the transplant canopy. This uniformity of the microenvironment is difficult to achieve in the greenhouse.

In the closed system, air temperature, CO_2 concentration and relative humidity as well as PPF can be controlled as desired even in summer. On the other hand, in the greenhouse, air temperature and PPF are often too high and CO_2 concentration is often too low on a clear day. If roof and side ventilators are opened fully to lower the air temperature in the greenhouse, pest insects may enter the greenhouse. PPF and thus air temperature and relative humidity change rapidly and frequently within a day. Therefore, accurate control of greenhouse environment is difficult to achieve.

4.9. Growth and development of transplants and their uniformity are enhanced

When air moves slowly and unevenly over the transplant canopy caused by natural convection, as often observed in the greenhouse, the microenvironment over the canopy is significantly different from that within the canopy, and air movement or diffusion of CO_2 and water vapor are restricted within the canopy, compared with above the canopy. As a result, CO_2 concentration during the photoperiod is about 50 μmol mo1^{-1} lower within the canopy than over the canopy, and relative humidity is generally 10-20% higher within the canopy than over the canopy. Under such conditions, photosynthesis and transpiration of transplants are often restricted.

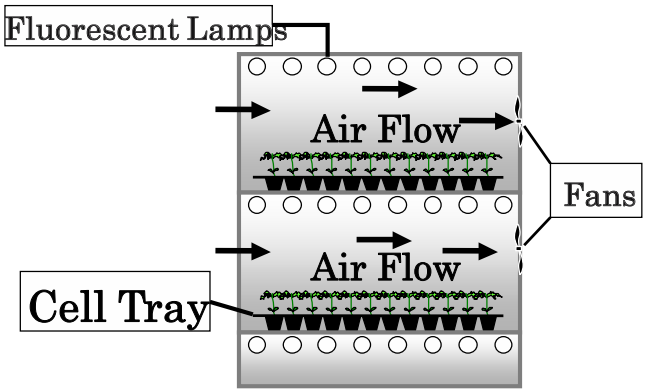


Figure 3. Cross section of multi-shelves (only two shelves are shown) showing the direction of air flow and positions of micro fans. This air flow guarantees the uniformity of microenvironments over the trays and within the transplant canopy.

Moderate and uniform horizontal airflow, caused by horizontal forced convection, over and within the transplant canopy in the closed system improves the microenvironment within the transplant canopy considerably: 1) increase in CO_2 concentration and reduction in relative humidity within the canopy, 2) increases in CO_2 and water vapor exchange coefficients in the canopy and 3) increase in light penetration to the lower part of the canopy due to the fluttering of leaves by moving air. As a result, photosynthesis and transpiration of transplants are enhanced and their growth is promoted. In addition, uniformity of microenvironments over and within the canopy promotes uniform growth of transplants.

Also, a combination of reduced relative humidity and fluttering of leaves within the canopy makes the transplants compact in shape and vigorous. Development of plants is significantly influenced by temperature, photoperiod, and light quality. These environmental factors can be controlled precisely in the closed system, so that development of flower buds, bolting, stem elongation of transplants can be controlled easily in the closed system (Chun et al., 2000; Chun and Kozai, 2001;

Kubota and Chun, 2001; Ohyama et al., 2001; Taiyo Kogyo Co., personal communication). Some examples are shown below.

5. VALUE-ADDED TRANSPLANTS PRODUCED IN THE CLOSED SYSTEM

Using environmental control function of the closed system, value-added transplants can be produced relatively easily, as shown in Table 13.

Table 13. Examples of value-added transplants produced in the closed system, which are often difficult to produce in the greenhouse.

No.	Items
1	Number of nodes below the first flower cluster of tomato plants can be set at about 8 in summer.
2	Enhanced flower bud differentiation and growth of pansy and strawberry transplants in summer by providing relatively low temperatures.
3	Retarded bolting of oriental spinach varieties in summer by providing a light period shorter than the critical photoperiod (11 h/day)
4	Increased number of runner plants obtained from strawberry mother plants by CO_2 enrichment and a photoperiod of 16 h/d.
5	Production of virus-free sweetpotato transplants using single node leafy cuttings as explants.
6	Uniform growth of cucumber and tomato seedlings used for grafting as scions and stock plants throughout the year.
7	Enhanced nursing and acclimatization of grafted cucumber, watermelon, eggplant and tomato transplants.
8	Production of vigorous Chinese cabbage, broccoli and cabbage transplants with short but thick hypocotyls.
9	Enhanced or controlled flower bud differentiation and bolting of statice (*Limonium latifolium*) and *Eustoma russellianum* Don. Transplants.
10	Year round production of herb, lettuce and chicory transplants fro hydroponic culture

5.1. Some examples of enhanced growth of transplants

In this section, some examples of transplants produced in the closed system are shown. All photographs shown in this section were taken in Japan during 2000 – 2003.

5.1.1. Tomato (*Lycopersicon esculentum* Mill.)

Tomato seedlings, 14 days after sowing, grown in the closed system in 128-, 200- and 288-cell trays were not significantly different from each other (Figure 4). In the greenhouse, the growth of tomato transplants grown on 200- and 288-cell trays is restricted due to the high planting density. Thus, we can double the planting density

in the closed system with no retarded growth compared with the greenhouse. Development of the first flower bud and its growth were enhanced by optimal control of temperature, PPF and photoperiod in the closed system, which would result in earlier harvest of tomato fruits in the greenhouse (Ohyama et al., 2003).

Figure 4. Tomato seedlings grown in the closed system in 128-, 200- and 288-cell trays. There are no significant differences in growth among the treatments. The standard number of cells is 128 or 72 per tray when using the greenhouse for transplant production. Thus, the transplanting density per tray can be at least 1.5 (=200/128) to 2.2 (=288/128) times higher in the closed system than in the greenhouse.

5.1.2. Spinach (Spinacia oleracea)

Spinach transplants were more vigorous when grown for 14 days after sowing on 288-cell trays in the closed system than when grown on 144-cell trays in the greenhouse (Figure 6). Namely, planting density can be doubled in the closed system compared with that in the greenhouse. Bolting of spinach could be inhibited during transplant production by short photoperiod treatment in the closed system, which resulted in delayed bolting when they were subsequently grown under long photoperiod in the greenhouse (Chun et al., 2000). It is essential that bolting in spinach plants at the time of harvest is delayed in order to keep their economic value high.

5.1.3. Sweetpotato (Ipomoea batatas L. (Lam.))

A single node cutting each with one unfolded leaf of sweetpotato could be grown to a transplant within 14 days. In the greenhouse, it takes at least 20 days, and normally 25 days (Figure 8, Lok et al., 2002). In the closed system, virus-free transplants can be produced easily, which increases the economic values of vegetatively propagated transplants, such as sweetpotato transplants.

Figure 5. Tomato seedlings grown in the closed system 20 days after sowing (DAS). The growth is uniform over the tray.

Greenhouse 144 cells/tray 17 DAS | **Greenhouse 144 cells/tray 13 DAS** | **Closed system 288 cells/tray 12 DAS**

Figure 6. Spinach transplants grown for 12 days using the 288-cell trays in the closed system are greater, greener and more vigorous than those grown for 13 or 17 days using 144-cell trays in the greenhouse.

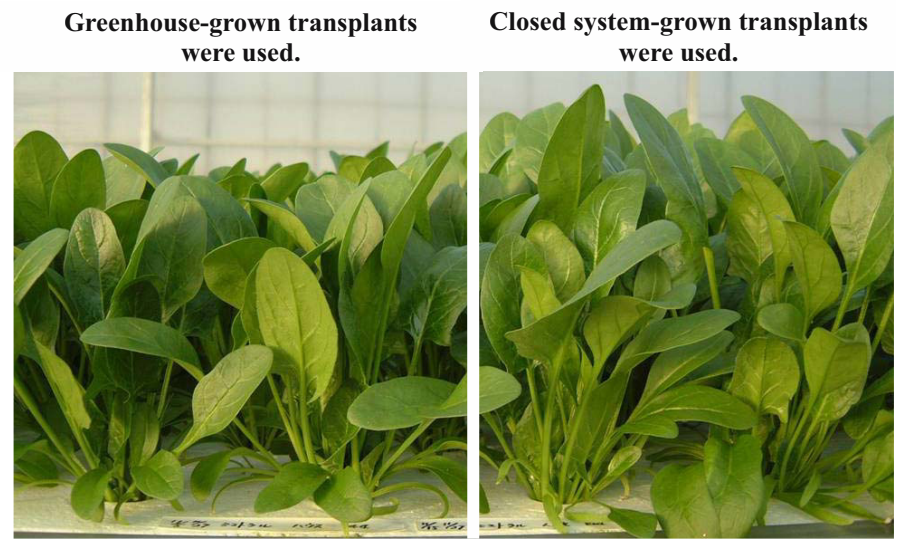


Figure 7. Spinach plants at harvest, grown using a hydroponic system in the greenhouse. The growth of spinach plants using closed system-grown transplants was greater than those using greenhouse-grown transplants.

Figure 8. Sweetpotato single node cutting with one leaf (Left, Day 0) and the transplant with 5 unfolded leaves 14 days after transplanting the single node cutting in the closed system (Right). The single node cutting with one leaf can be wilted easily in the greenhouse and it takes 3-4 weeks to grow to the transplant like the one shown above.

Figure 9. Pansy transplants 29 days after sowing (DAS) in the closed system (Left) and in the greenhouse (Right). Transplants grown in the closed system are more vigorous and uniform in growth compared with those grown in the greenhouse.

Figure 10. Potted pansy plants 64 days after sowing (DAS) ready for shipping. The plants in Left were grown for the first 29 days in the closed system and then grown for 35 (= 64 – 29) days in the greenhouse. Many plants in Left are flowered due to the enhanced flower bud growth compared with those in Right grown for 64 days in the greenhouse.

5.1.4. Pansy (*Viola x wittrockiana* Gams.)

Pansy transplants were more uniform and vigorous with higher percent of salable transplants when grown in the closed system than in the greenhouse (Figure 9). Growth of pansy transplants was significantly greater when grown in the closed system than in the greenhouse (Omura et al., 2000). Pansy transplants grown in the closed system and moved to a greenhouse flowered earlier than those grown from the beginning in the greenhouse (Figure 10).

5.1.5. Eggplant (*Solanum melongena* L.) *and Chinese cabbage* (*Brasicca campestris* L. (pekinensis group))

Vigorous and compact transplants of eggplant and Chinese cabbage could be produced successfully in the closed system with an electricity cost of about 1 US cent per transplant (Ohyama et al., 2001).

5.1.6. Grafted transplants

In order to produce high quality grafted transplants, it is essential to obtain rootstocks and scions in uniform size and shape. It is also essential to control the environment precisely for a few days after grafting to promote a successful union of grafted parts. The closed system is suitable to realize such environmental conditions. We could produce high quality grafted transplants of cucumber, tomato and eggplant (Figures 11, 12 and 13).

5.1.7. Lettuce and cabbage transplants for field cultivation

Transplants for field cultivations such as lettuce and cabbage plants are often transplanted by using transplanting machine. For transplanting with a high percent of success, transplants are required to be uniform in growth, vigorous and compact in shape. Transplants grown in the closed system show such characteristics to be suited for automatic transplanting machine, as shown in Figures 14, 15, 16 and 17.

6. PRODUCTIVITY PER FLOOR AREA IS TEN TIMES THAT OF THE GREENHOUSE

Increase in yearly productivity per floor area of the closed system is about 10 times that of a standard greenhouse for many kinds of transplants, as explained below and Table 14.

1) The ratio of planting area to floor area is usually 0.8 in the greenhouse, while it is 2.0 to 3.0 in the closed system with use of the multi-shelves (4 or 5 shelves). Thus, planting area per floor area of the closed system is about 2-3 times that of the greenhouse. On the other hand, use of multi-shelves is not practical in the greenhouse using solar light because of obstruction of solar light by the multi-layered shelves.

Figure 11. Uniform growth of cucumber (cv. encore 10) seedlings to be used as scions for grafting (Day 7).

Scion：encore 10, stock： Hikari Power G

Figure 12. Grafted cucumber transplant 19 days after sowing, nursed in the closed system (Left). Seedlings used as scions (Upper Right) and those used as root stock plants (Lower Right) were grown in the closed system. Percent success of grafting is over 90%.

Figure 13. Grafted tomato transplants 20 days after sowing.

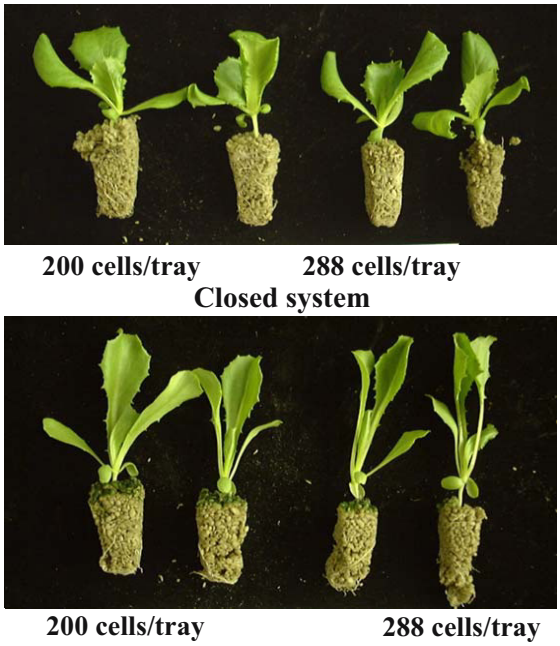


Figure 14. Crisp head lettuce (cv. Cisco) transplants grown for 16 days after sowing in the closed system using 200- and 288-cell trays show wider leaves and shorter hypocotyls than those grown for 16 days after sowing in the greenhouse.

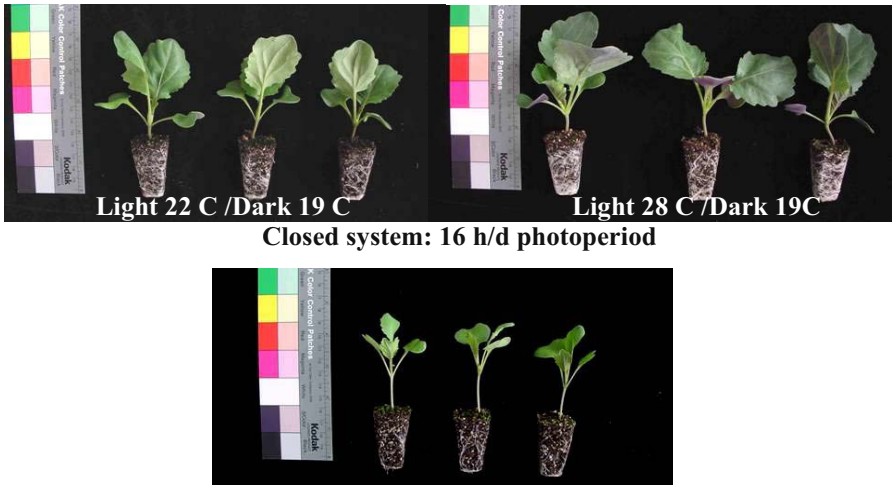


Figure 15. Cabbage (cv. Kinkei 201) transplants grown for 14 days after sowing under a photoperiod of 16 h d^{-1} are larger and are characterized by wider leaves and shorter hypocotyls than those grown for 14 days after sowing in the greenhouse during October.

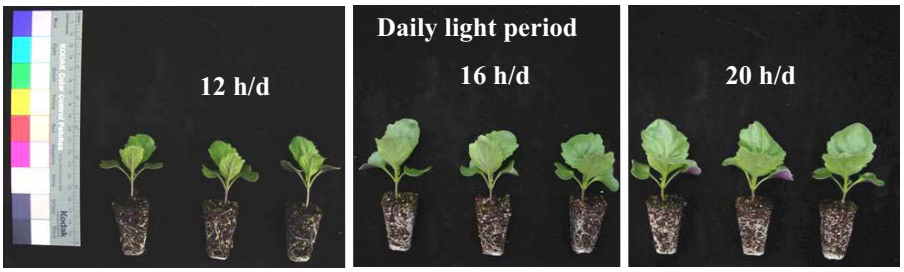


Figure 16. Growth of Cabbage (cv. Kinkei 201) transplants grown for 13 days in the closed system are greater with increasing the light period from 12 h d^{-1} to 20 h d^{-1}.

Figure 17. Cabbage (cv. Kinkei 201) transplants grown for 13 days after sowing in the closed system using the 128-, 200—and 288-cel trays. There were not much differences in growth among the treatments.

2) The planting density per tray area can be about 2 times, as described previously. The physical and physiological reasons for this statement will be given in a later section.
3) The production period can be reduced by about 30%, mainly due to the promotion of photosynthesis by CO_2 enrichment at 800-1,000 μmol mol^{-1}, and control of horizontal air current over and within the transplant canopy under an optimal combination of temperature, PPF and soil-plant water relations.
4) Annual operation rate of the closed system is increased by 20% due to the shortened production period even in summer and inter.
5) Percentage of salable plants and/or sales price can increase by about 10% due to their high quality and uniform growth.

Thus, the total increase in yearly productivity per floor is higher than 7-11 folds (=2.5 x 2.0 x 1.1 x 1.3 x 1.2x1.1), with the average of 10.

7. COSTS FOR HEATING, COOLING, VENTILATION AND CO_2 ENRICHMENT

7.1. Heating cost is a few percent of that of a greenhouse even in northern countries

Heating cost of the closed system is a few percent of that of a greenhouse during the winter even in northern countries, because all the walls/roofs of the closed system are covered with thermal insulators, such as formed polystyrene sheets, which are about 15 cm thick. The heat transmission coefficient of 10-cm thick insulated walls (0.28 W m^{-2} K^{-1}) is about 1/15th of that of a greenhouse with a double-layer thermal screen (4 W m^{-2} K^{-1}). Furthermore, the wall and roof areas of the closed system are only about 1/7th of those of the greenhouse. Since the heating load is proportional to

the heat transmission coefficient multiplied by the wall and roof areas, the heating load of the closed system is about 1/98th (=1/14 x 1/7) of the greenhouse. If the thickness of thermal insulators is 15 cm, which corresponds to a heat transmission coefficient is 0.2 W m^{-2} K^{-1}, this value is reduced to 1/140th (=1/20 x 1/7).

Table 14. Annual production capacity of the closed system in comparison with that of the greenhouse.

No.	Item	Ratio to the greenhouse	Accumulated ratio	Remark
1	Increase in ratio of production area to floor area by use of multi-layered shelves	2-3 (=1.6/0.8-2.4/0.8)	2-3	closed system: 1.6-2.4, greenhouse: 0.8
2	Increase in planting density by providing a higher air current speed within the transplant canopy	2	4-6 (=2x2-2x3)	
3	Increase in percent marketable transplants by providing the favorable environments	1.1	4.4-6.6	
4	Reduction in production period by providing the favorable environments	1.3	5.7-8.6	
5	Increase in annual operation rate	1.2	6.8-10.3	
6	Increase in sales price due to the high quality	1.1	7.5-11.3 (Average: 10)	

When the lamps are turned on, the air in the closed system needs to be cooled by an air conditioner even when the outside temperature is below –30 C. Thus, lighting can be done at night to reduce cooling and/or heating costs when the air temperature at night is low. Also, off-peak electricity at night can be used with a reduced electricity cost. In Japan, nighttime (from 10 p.m. to 8 a.m.) discount of electricity price is roughly 50%.

During the dark period, heat generated by fans and other equipment in the closed system is often enough for keeping the air temperature at a set point in the closed

system when outside temperature is around –5 C or higher. Even when the outside temperature is –30 C, only a little heating is necessary during dark period, and an air conditioner driven by electricity can be used as a heater.

7.2. Cooling load and electricity consumption do not change significantly throughout the year

Cooling load of the closed system is almost equal to the heat generated by lamps, fans, etc. in the closed system, because heat entering into the closed system from outside is negligibly small due to its thermally insulated structure. In other words, outside temperature does not affect the cooling load of the closed system. Then, electricity consumption for cooling the closed system is heat generated by lamps etc. divided by the C.O.P. for cooling of the air conditioner. C.O.P. for cooling is defined as the cooling capacity divided by electricity consumption of an air conditioner, both in a unit of W (Watt). The C.O.P. of a recent air conditioner for home use is 4-5 during summer (25 C inside and 35 C outside the closed system), and is 8-10 during winter (25 C inside and –5 C outside) in Tokyo area, Japan. The C.O.P. for cooling of the closed system increases as the air temperature difference increases (when outside temperature is higher than inside temperature) (Ohyama and Kozai, 2002, Figure 18).

Then, during winter, the cooling cost of a closed system should be lower than the heating cost of a greenhouse in northern and temperate countries, because total wall area and heat transmission coefficient of the closed system are 1/7th and 1/15th, respectively, of those of the greenhouse.

Since electricity consumption for lighting of the closed system is almost constant throughout the year, the electricity consumption for lighting, cooling and other equipment does not change significantly throughout the year.

7.3. Cooling cost accounts for 15% or lower of the costs for lamp lighting and cooling

In the case that the C.O.P. is 4 in summer, electricity consumption for cooling accounts for about 20% of electricity consumption for lamp lighting and cooling. Electricity consumptions or lighting and cooling account for, respectively, 75-80 and 15-20% of total electricity consumption of the closed system (Ohyama et al., 1998, Ohyama et al., 2000a). Costs for fans, heating, and others account for the rest of 5-7%.

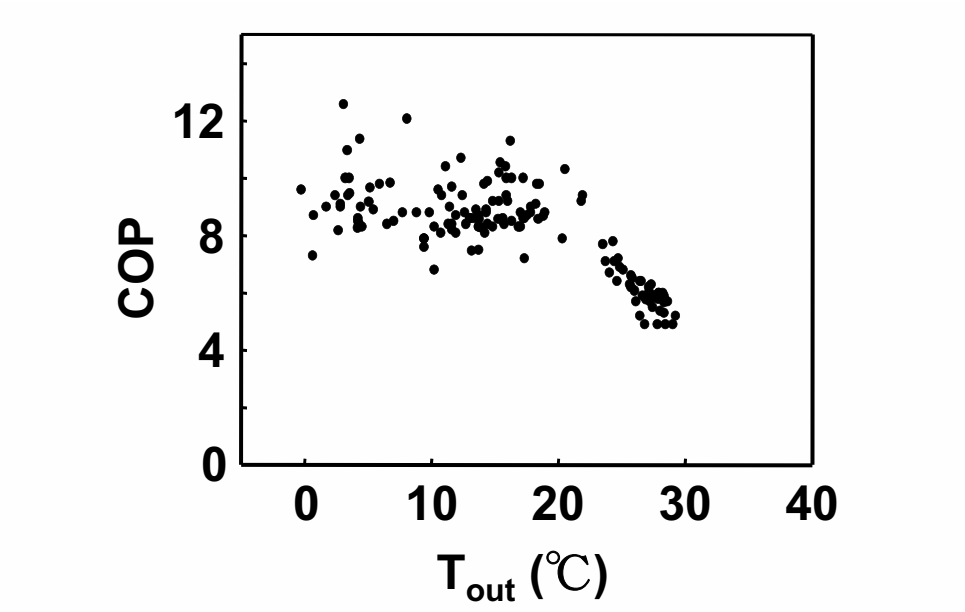


Figure 18. C.O.P. (Coefficient of performance of the closed system with a refrigerator) for cooling is as high as 8-10 when air temperatures inside and outside the closed system are 25 C and 0-20 C, respectively (Ohyama et al., 2002).

In the case that the C.O.P. is 9 in winter, electricity consumption for cooling accounts only for about 10% of the total electricity consumption of the closed system.

7.4. Electricity consumption for lighting is three times that for supplemental lighting

In northern countries such as the Netherlands, 50-100 μmol m^{-2} s^{-1} of supplemental lighting in a greenhouse for growing crops is currently common in winter (Mercelis et al., 2002), because the supplemental lighting significantly promotes the growth of seedlings, leafy vegetables and other greenhouse crops.

When supplemental lighting of 70 μmol m^{-2} s^{-1} was given during winter in New Hampshire, USA, for 11 h d^{-1} using high pressure sodium lamps, daily integral of PPF in a greenhouse was increased by 45% (from 6.18 to 8.96 mol m^{-2} d^{-1}), resulting in an increase in daily average of PPF from 156 to 226 μmol m^{-2} s^{-1} (Donnelly and Fisher, 2002). This means that electricity consumption for lamp lighting at PPF of 226 μmol m^{-2} s^{-1} in the closed system is only 3.2 (=226/70) times that for supplemental lighting in the greenhouse during winter. The daily average PPF of 226 μmol m^{-2} s^{-1} can be provided by six 40 W fluorescent lamps easily if the lamps are placed 40 cm above the plants with photoperiod of 11 h d^{-1}. The daily average PPF

of 250 μmol m^{-2} s^{-1} is achieved if the photoperiod is set at 16 h d^{-1} instead of 11 h d^{-1} with the same set-up of fluorescent tubes. The daily-integrated PPF over 16 h is 16.4 mol m^{-2} d^{-1}, which is equal to the daily integral of PPF on a clear day in September in Tokyo. By doing so in the closed system, no heating cost is required and no condensation occurs on the inside surfaces of wall s during winter in the closed system.

Electric energy consumption for lighting is relatively small, because: 1) PPF (photosynthetic photon flux) of 200-300 μmol m^{-2} s^{-1} is high enough for production of transplants with LAI (leaf area index) of 2-4, 2) the transplant production period is 15-30 days, 3) planting density is 400-1000 plants m^{-2}, and 4) transplants are placed 20-30 cm below fluorescent lamps with reflectors, which results in a high ratio of light energy received by cell trays to light energy emitted by lamps. The short distance of 20-30 cm can be achieved because the outer surface temperature of fluorescent tubes is about 40 C when the surrounding air temperature is 25 C. Surface temperatures of high pressure sodium and metal halide lamps are about 100 C, so that they cannot to be placed close to the plants.

7.5. Electricity cost is 1-5% of sales price of transplants

The total electricity consumption per transplant is roughly 400 kJ or 120 Wh when 200-cell trays are used, and it costs about 1 US cent under current conditions in Japan, about 0.8 US cent in the USA and about 0.5 US cent in Canada. The cost will be half if 400-cell trays are used, because the lighting cost per tray is constant. On the other hand, the sales price of transplants is typically 20 US cents to 1 US dollar per transplant. Thus, in Japan, the electricity cost currently accounts for 1 to 5% of the sales price of transplants. This percentage can be further reduced by, for example, using a co-generation system and an advanced lighting system.

7.6. Relative humidity is always 70% during photoperiod

When lamps are turned on and an air conditioner is also turned on for cooling, dehumidification and collection of condensed water at cooling panels of air conditioner occurs. Thus, relative humidity in the closed system naturally tends to be about 70% during the photoperiod when the closed system is filled with transplants.

During the dark period, the relative humidity in the closed system tends to be about 90% or higher as it is in the greenhouse at night if air is not dehumidified by an air conditioner. This high relative humidity in the closed system during the dark period can be reduced to 70-80% relative humidity at minimal electricity cost by operating the air conditioner intermittently using its dehumidification mode that requires minimum electricity.

When heat-generating equipment such as an air pump and water pump is operated during the dark period, the air conditioner is naturally operated to keep the air temperature at a set point, resulting in a reduction in relative humidity.

7.7. PAR utilization efficiency is about 2 times higher than that in the greenhouse

In the closed system, PAR (photosynthetically active radiation) utilization efficiency, defined as the percent of chemical energy fixed by plant photosynthesis over PAR energy emitted from fluorescent tubes, is about 2 times higher that that in the greenhouse, when LAI (leaf area index) of the canopy is about 2.5 (Ohyama et al., unpublished). The reasons for the high efficiencies of the closed system is that PPF is controlled in a range 0-300 μmol m^{-2} s^{-1} depending upon the growth stage and CO_2 concentration is kept at 800-1,100 μmol mol^{-1} with an air horizontal current speed of 0.1-0.5 m s^{-1}.

Since about 25% of electric energy is converted into PAR energy by fluorescent lamps, electric energy conversion efficiency at the end of transplant production period is about 2.8% and its average efficiency over the production period is about 0.3%. For comparison, the percent conversion from electrical energy to PAR energy is about 30-35% for high-pressure sodium lamps, and about 27-32% for high-pressure metal halide lamps, and about 10-15% for LED (light emitting diode) lamps.

7.8. Ventilation cost is negligibly small

In the closed system, ventilation is minimized in order to: 1) minimize the release of water and CO_2 supplied into the closed system to the outside of the closed system, 2) minimize the environmental disturbance by the weather, and 3) protect insects and/or pathogens from entering into the closed system. In most commercialized closed systems, no ventilation unit is installed, so that its initial investment cost is zero.

In a closed system developed for research purposes, a small ventilation unit is sometimes installed to keep the air pressure inside the closed system relative to the air pressure outside the closed system slightly positive. This positive air pressure is required to prevent insects and dusts from entering through the leakage of the closed system.

Increasing the ventilation rate can reduce the cooling cost of an air conditioner in case that the actual and set point of air temperatures are higher inside than outside the closed system. However, this small decrease in cooling cost does not compensate for the advantages of minimum ventilation.

The number of air exchanges per hour of the closed system, which is defined as hourly ventilation rate divided by the air volume of the closed system, needs to be kept at 0.01-0.02 h^{-1}. In case that the closed system is completely airtight, accumulation of ethylene gas in the closed system may cause a physiological damage to the transplants. In this case, a little ventilation is required to avoid the ethylene accumulation in the closed system.

7.9. CO_2 cost is negligibly small

Cost for CO_2 enrichment is negligible due to the minimum ventilation. Nearly 90% of CO_2 supplied to the closed system is fixed by plant photosynthesis (Figure 19).

The remaining 10% is released to the outside of the closed system. The same applies for CO_2 produced by respiration of microorganisms, if they are present. CO_2 produced by respiration of plants and microorganisms is mostly accumulated in the system during the dark period and reaches 1500 μmol mol^{-1} or higher at the end of dark period (Yoshinaga et al., 2000). This accumulated CO_2 is reused as a carbon source for photosynthesis during the following photoperiod.

The price of liquid CO_2 in a container is 14-15 US cents per kg in Japan, and tens of thousands of transplants can be produced using 1kg of CO_2 in the closed system. Thus, cost of CO_2 per transplant is negligibly small compared with other operation costs.

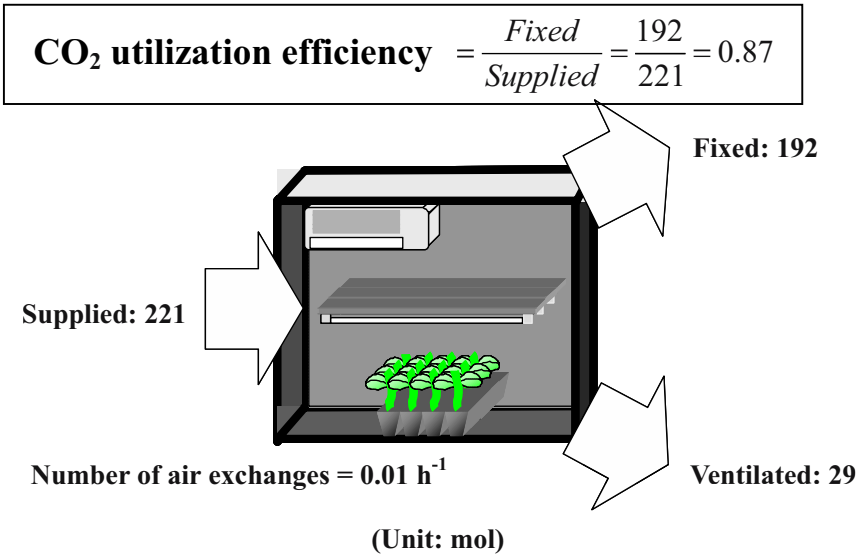


Figure 19. CO_2 utilization efficiency of the closed system, which is defined as the weight ratio of fixed CO_2 by plant photosynthesis to supplied CO_2 during the transplant production (Yoshinaga et al., 2000).

7.10. Amount of water required for irrigation is a few percent of that for the greenhouse

In the closed system, only a few percent of irrigated water is discharged as water vapor to the outside along with the restricted infiltrated air (Ohyama et al., 2000b). Another few percent of irrigated water is stored in transplants as they grow and/or in substrate filled in cell trays. The rest of approximately 95-98% of evapotranspirated water is condensed at the cooling panel of an air conditioner and is collected as drained water, which is reused as irrigation water in the following transplant production periods (Figure 20). In other words, if evapotranspirated water is not reused for irrigation, as in the greenhouse, an amount of water required for irrigation

would be 20 to 50 times that of the closed system (Figure 20).

Closed systems can use two kinds of irrigation systems. One is a closed water circulation system, in which water is circulated from a tank via cell trays to the tank again for collection and reuse of drained water. The other is a one-way irrigation system in which water supplied from a tank to cell trays, and all of it is evapotranspirated from the cell trays with transplants, resulting in no water drainage from cell trays. The latter system is simpler and preferable compared with the former system.

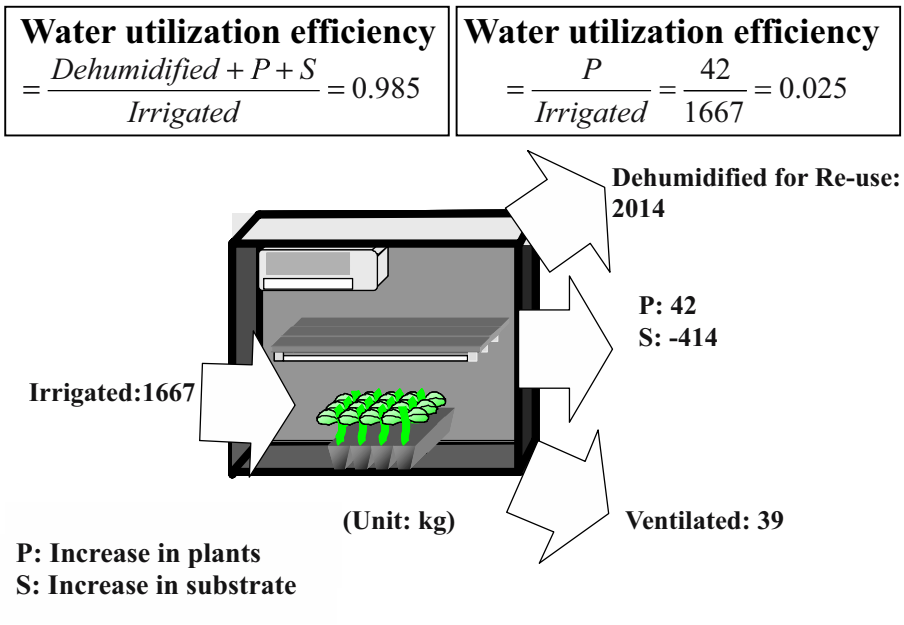


Figure 20. Water utilization efficiency of the closed system, which is defined as the weight ratio of water captured in plants or substrate to the supplied water during the transplant production (Ohyama et al., 2000b). Left: Water collected at the cooling panel (evaporator) is reused for irrigation. Right: Water collected at the cooling panel (evaporator) is not reused for irrigation.

7.11. Disinfection of the closed system is easy

The evapotranspirated water is basically aseptic and water collected at the cooling panels (or an evaporator) of an air conditioner is also virtually aseptic if no microorganisms exist in the water collection system and if the water collection system consists of tubes and a tank only (Ohyama et al., 2000b).

In one of our closed systems, the substrate (either in bags or cell trays) is disinfected by submerging it in hot water (60 C in winter and 70 C in summer) in a water tank overnight, heated by a solar heater installed on the roof of the closed system. Water collected at the cooling panels is sterilized either by heating it in the

hot water from a solar collector, by irradiating it with ultraviolet radiation lamps, and/or by supplying ozone gas to the water. A small hole for minimum ventilation is covered with a filter to prevent insects, dust and microorganisms from entering the closed system. Thus, disinfection of the closed system is relatively easy and inexpensive.

7.12. The environmental control unit is simpler than that in greenhouse

An environmental control unit including an irrigation sub-unit is much simpler for the closed system than for the greenhouse, because the environment in the closed system is not disturbed by the weather outside the closed system. On the other hand, the greenhouse environment is considerably disturbed by the weather, especially by solar radiation. Also, the set point of each environmental factor in the closed system can be basically predetermined once the seeds are sown or cuttings are transplanted.

Furthermore, in the closed system, the amount of water required for irrigation is predictable when environmental set points are determined for each growth stage of transplants, because the air temperature, relative humidity and horizontal air current speed are controlled precisely at the set points. The same applies for CO_2 enrichment. This makes the control algorithms of an irrigation unit for the closed system much simpler than the one for the greenhouse. On the other hand, in the greenhouse, a sophisticated control unit is necessary if irrigation is to be conducted according to the changes in solar radiation.

7.13. Production management is easier in the closed system

It is easier to predict growth and development of transplants under precisely controlled environments in the closed system than in the greenhouse. This also makes it easier manage transplant production, i.e., to meet the customer's demand easier. Also, computer simulations using mathematical models can be practically used in production management. The costs for electricity, labor, etc. can be also predictable (Kubota and Kozai, 2001).

7.14. The closed system is an environmentally friendly system

The closed system is an environmentally friendly system in the sense that: 1) It needs only a few percent of water that is consumed for irrigation in the greenhouse and it does not release waste water containing fertilizers, pesticide and/or fungicide, etc. to the outside of the closed system, 2) It also does not need any pesticides or insecticides, except for an emergency, and needs much less fertilizers than the greenhouse does, because of no drained water from the closed system. 3) The main components of the closed system are mass produced for home use or industrial use, so that their recycling and reuse systems have been already established, 4) The floor area of the closed system is only 10% of the greenhouse floor area for the same transplant production capacity, so that material resources and labor for constructing the production system can be saved significantly, 5) Nearly 90% of CO_2 supplied for

promoting photosynthesis of plants in the closed system is fixed by plants, so that its contribution to the increase in atmospheric CO_2 concentration is negligible. On the other hand, in the greenhouse, roughly 50% of CO_2 supplied is released to the outside due to ventilation (Yoshinaga et al., 2000), 6) Heating load is negligibly small, so that oil or natural gas is not required for heating in the closed system, and 7) Percentages of salable plants are higher in the closed system than in the greenhouse, so that amount of wasted plants is less in the closed system than in the greenhouse. In greenhouses with supplemental lighting, light pollution at night, meaning the loss of light emitted from lamps to the outside, has become a problem in northern countries where supplemental lighting are common in winter. In the near future, a 'zero emission plant production system' meaning no emission of wastes, resulting in minimum use of resources, will be realized by advancing the concept and methodology of the closed system. These advantages of the closed system can more than overcompensate for its use of electricity for lighting and cooling.

8. CONCLUSIONS

It can be concluded that quality and productivity of transplants are definitely higher and the growth period of transplants can be shortened by approximately 30% when produced in the closed system using lamps than in the greenhouse using sunlight. The closed system is energy and material efficient especially with respect to the amounts of water required for irrigation and energy required for heating in winter. In addition, the closed system is an environmentally friendly system for plant production in the sense that it does not release polluted water that contains fertilizers to the outside and that it seldom requires pesticide and fungicide. Initial and operation costs of the closed system per annual production of transplants can be lower than or comparable to those of the greenhouse, and it has been commercialized in 2002 in Japan and is expected to be commercialized in other countries in the near future.

9. ACKNOWLEDGEMENT

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Chapter 18

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CONCLUDING REMARKS

Micropropagation is often looked upon as being practical only for propagation of those plants, which are difficult to propagate by conventional propagation methods. This book suggests that by adopting the photoautotrophic micropropagation method, micropropagation technique is indeed applicable to the 'difficult to propagate' species, and equally it may offer economic advantages for some species which are considered relatively 'easy to propagate'.

In conventional, heterotrophic and photomixotrophic micropropagation, an essential part of the technology is the culture medium formulation (combinations and strength of plant growth regulators, vitamins, amino acids, sugar and other organic substances). Thus, inter-relational effects of the medium components on the growth and development of cultures are always somewhat subtle and only skilful experts can operate the micropropagation process. Moreover, environmental conditions of the plant microclimate cannot be controlled directly in the conventional system of plant tissue culture as it can be in photoautotrophic system. This type of conventional micropropagation system does not meet a requirement for commercialization on a large scale. This is partly because current technology of conventional micropropagation is based upon the plant tissue culture technology mainly developed for research purposes, which is, in principle, labor intensive and is not optimized for a large-scale production.

On the contrary, in photoautotrophic micropropagation, inorganic macro- and micro-nutrients only are basically supplied in the culture medium. Thus, effects of medium components on the growth and development of cultures and the cause-effect relationships are much simpler to understand than in conventional micropropagation. One of the most important advantages of photoautotrophic micropropagation is that its technology can be built based upon general plant physiology and ecology dealing with photosynthesis, respiration, transpiration, etc. It is important to note that photoautotrophic micropropagation is a kind of vegetative propagation under disease-free conditions. Then, it is natural that control of physical environmental factors in the culture room is more important in photoautotrophic micropropagation than in conventional micropropagation.

There is a huge demand of genetically superior and disease free transplants in horticultural, agricultural, forestry and environmental conservation industries. Thus, development of innovative vegetative transplant production systems including a micropropagation system is important in order to produce a large number of high

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quality transplants at a low cost in a short time with minimum use of resource and minimum environmental pollution. In order to establish such a transplant production system, it is essential to integrate recent technological and scientific developments based upon a unified concept and methodology of photoautotrophic micropropagation.

Current advanced greenhouse technology for plant propagation and transplant production has been well developed for a large-scale production. Thus, for the development of a large-scale photoautotrophic micropropagation and transplant production system, technologies of plug seedling production, hydroponic culture, greenhouse environment control, and greenhouse crop management can be applied with careful consideration of disease protection. The current photoautotrophic micropropagation system can be improved further for a larger scale production by incorporating recent technologies of computers, robotics, energy-saving, recycling, environmental conservation, ecological engineering, etc. At the same time, photoautotrophic micropropagation is suitable also for a small-scaled operation.

The production system for high quality transplants should be simple and robust and should have a high adaptability to a diversity and specificity of plants and social and economic situations, but it should still be based upon a unified concept integrating different technologies in a systematic and simple way. Photoautotrophic micropropagation technology is based upon such a concept and technologies upon which a future system should be based.

Subject Index