

Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions

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Abstract

The growth and water relations of *Paulownia fortunei* in photoautotrophic cultures (nutrient medium lacking sucrose and growth regulator) with CO₂ enrichment (PWAH) or without CO₂ enrichment (PWAL) were compared with those in photomixotrophic shoot (PWC; 30 g dm⁻³ sucrose and 0.3 mg dm⁻³ N⁶-benzyladenine) and root cultures (PWR; 0.3 mg dm⁻³ indole-3-butyric acid). The photoautotrophic and photomixotrophic cultures were incubated under photosynthetic photon flux 125 and 60 µmol m⁻² s⁻¹, respectively. 100 % sprouting and significantly higher number of shoots (1.6) were obtained with PWAH as compared to PWAL and PWC. PWAH and PWAL stimulated spontaneous rooting from the cut end of axillary shoots. In PWAH, 84 % of shoots rooted with an average of 5.9 roots per shoot and 4.0 cm of root length in 21 d. Rooting of photomixotrophic shoot cultures were stimulated by an auxin treatment. In this case, 98.3 % of shoots were rooted with an average of 4.6 roots per shoot and 1.9 cm length. A microscopic observation on leaf abaxial surface prints from photomixotrophic shoot and root cultures showed widely open (6 - 8 µm) spherical stomata (12 - 14 µm) and from photoautotrophic cultures elliptical stomata (10 - 12 µm) with narrow openings (3 - 4 µm). Leaves from photomixotrophic cultures had higher stomatal index as compared to photoautotrophic cultures. The rate of moisture loss from detached leaves was not varying significantly in different cultures.

Additional key words: CO₂ enrichment, micropropagation, stomata, water loss.

Introduction

Photoautotrophic micropropagation is a tissue culture technique whereby a chlorophyllous explant is placed in environmental conditions that induce it to photosynthesize and thus grow and multiply. This requires cultivation of the micropropagated material under conditions of carbon dioxide enrichment and high PPF. Photoautotrophy reduces or removes the requirement for the addition of sucrose or any of the sugar to the medium

(Kozai 1991). Photoautotrophic micropropagation offers a number of advantages including non-axenic culture of plants, reducing the problems experienced in contamination and overcomes problems associated with acclimatization of plants. It has the other possibilities of the production of superior quality plants that are easier to wean, grow faster and mature earlier (Long 1997). The stomata from plantlets in the photoautotrophic cultures

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Abbreviations: BA - N⁶-benzyladenine; IBA - indole-3-butyric acid, PPF - photosynthetic photon flux density; PWAH - photoautotrophic cultures with CO₂ enrichment; PWAL - photoautotrophic cultures without CO₂ enrichment; PWC - photomixotrophic shoot cultures; PWR - photomixotrophic root cultures; SI - stomatal index.

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appeared to function normally, closing in darkness and opening in the light. The rate of transpiration was lower in the photoautotrophic cultures than in the photomixotrophic cultures (Zobayed *et al.* 1999). A better understanding of the stomatal characteristics and water relations of photoautotrophic cultures might bring new solutions to the problems raised by photomixotrophic micropropagation. Photoautotrophic micropropagation has already been reported for many woody species like *Gardenia jasmonides* (Serret *et al.* 1997), *Garcinia mangosteen* (Kubota *et al.* 1998), *Acacia mangium* (Imelda *et al.* 1998), *Coffea arabusta* (Nguyen *et al.* 1999), *Eucalyptus camadulensis* (Zobayed *et al.* 2000) and *Myrtus communis* (Lucchesini *et al.* 2001).

Materials and methods

Single node leafy cuttings measuring 1 - 2 cm with two leaves and two pre-existing axillary meristems from photomixotrophically grown *Paulownia fortunei* Hemsley cultures were inoculated at a density of four explants per 370 cm³ box-type polycarbonate vessel (similar to the Magenta vessel). The nutrient medium consisted of Murashige and Skoog (1962, MS) mineral salts used for the photoautotrophic cultures (PWAL and PWAH). No growth regulators, vitamins and sucrose were added to the MS formulation. MS medium supplemented with sucrose (30 g dm⁻³) and BA (0.3 mg dm⁻³) was used for photomixotrophic shoot cultures (PWC). Half strength MS medium supplemented with sucrose (20 g dm⁻³) and IBA (0.3 mg dm⁻³) was used for rooting of photomixotrophic cultures (PWR) (Table 1). Agar (8 g dm⁻³) was used as the gelling agent in all cultures. The pH of the medium was adjusted to 5.7 before adding agar. The medium was dispensed (70 cm³) aliquots and autoclaved at 120 °C and 1.06 kg cm⁻² for 20 min.

Two gas permeable filter membranes (*Milli Seal*, *Millipore*, Tokyo, Japan) with pore size of 0.5 µm were used to cover a pair of holes (10 mm diameter) of a clear lid in photoautotrophic culture vessels. Photomixotrophic culture vessels were remained sealed without any filter membranes. The number of air exchanges per hour (vessel ventilation rate divided by air volume of the vessel, N) of the vessel without membrane filter was 0.2 h⁻¹ and vessel with two-membrane filters was 3.5 h⁻¹, according to the method used by Kozai *et al.* (1986). CO₂ enriched air (1400 - 1500 µmol mol⁻¹) and CO₂ non-enriched air (400 to 450 µmol mol⁻¹) was pumped to outside of the culture vessel for 21 d. All the cultures were incubated at air temperature 22 ± 2 °C under cool-white fluorescent lamps and a 16-h photoperiod in the growth room. Photosynthetic photon flux (PPF) measured on the empty shelf was 60 µmol m⁻² s⁻¹ for photomixotrophic cultures and 125 µmol m⁻² s⁻¹ for photo-

Paulownia fortunei Hemsley belongs to the family *Scrophulariaceae* is commonly hailed as a 'Miracle tree'. Its plantation is getting momentum on waste and marginal lands in various parts of India and many other countries for useful timber (Singh and Arora 1999). So far there has been no report on photoautotrophic micropropagation in this species.

The present study aimed to induce photoautotrophy in shoot cultures of *P. fortunei* that had been still cultured only photomixotrophically. The growth and water relations of *Paulownia fortunei* of photoautotrophic cultures were compared those of photomixotrophic cultures.

autotrophic cultures (Table 1). The relative humidity in the growth room varied between 80 - 85 %. The above conditions for photomixotrophic culture were close to those in a conventional micropropagation system.

Table 1. Concentration of growth regulators [mg dm⁻³] and sucrose [g dm⁻³], PPF [µmol m⁻² s⁻¹] and CO₂ concentration [µmol mol⁻¹] in different cultural conditions.

	BA	IBA	Sucrose	PPF	CO ₂ conc.
PWC	0.3	0	30	60	425
PWR	0	0.3	20	60	425
PWAL	0	0	0	125	425
PWAH	0	0	0	125	1450

The growth response of the explants was measured in terms of percentage of nodal segments sprouting, number of shoots per nodal segment, shoot height, the number of new nodal segments generated per active explant and multiplication coefficient after 21-d culture period. The multiplication coefficient is defined as the proportion of explants forming axillary shoots × the mean number of new nodal segments per explant forming shoots. Rooting response was examined in terms of rooting percentage, number of roots per shoot and root length.

Detached leaves from shoots/plants of PWC, PWR, PWAL, and PWAH cultures were used to study stomatal characteristics and water loss in *P. fortunei*. Stomatal distribution was studied from quick fix leaf imprints (Sampson 1961). A thin layer of quick fix (an adhesive mark) was applied uniformly on the abaxial surface of the leaves. After 5 min, the dried membrane was carefully peeled off. The size of stomata and aperture, number of stomata (NS) and epidermal cells (NE) was determined from leaf imprints at 40× magnification of light microscope. Stomatal index (SI) was calculated using

following formula:

$$SI = NS \times 100 / (NS + NE)$$

where NS = number of stomata in the microscopic field, NE = number of epidermal cells in the microscopic field.

For water loss experiments, detached leaves were allowed to dry in air of ambient laboratory conditions (relative humidity approximately 55 % and temperature 21 °C). Leaves from photomixotrophic and photoautotrophic cultures were weighed immediately after excision and every 30 min thereafter for 2 h. The leaves were then oven-dried at 70 °C for 24 h, and reweighed to determine the dry mass. The per cent of water loss as calculated using the following formula:

$$WL = (FM_0 - DM) - (FM_t - DM) / (FM_t - DM) \times 100$$

where WL = water loss [%], FM_0 = fresh mass at time zero, FM_t = fresh mass after time t, DM = dry mass.

For growth experiments, there were four treatments with five jars per treatment. Twenty explants were cultured per treatment and all treatments were repeated with three replicates. For stomatal characteristics, three leaf imprints were taken from each leaf and stomatal counts were made at three different locations on each imprint. Each treatment had 15 replicates in water loss experiments. The effect of different treatments on growth and water loss experiments was quantified in terms of mean and standard deviation obtained from various replications and subjected to analysis of variance (ANOVA). The *t*-test was performed at $P = 0.01$ and 0.05 .

Results and discussion

Growth characteristics: The pre-existing axillary meristems of nodal segments proliferated into 2 - 3 shoot buds after one week of incubation under the photomixotrophic (PWC) as well as the photoautotrophic cultural treatments (PWAH and PWAL). After 21 d, nodal explants cultured under PWAH had higher percentage of sprouting and significantly more number of shoots (1.6) when compared to PWAL and PWC. The developed shoots were also vigorous with green, expanded leaves under high PPF and CO₂ enrichment, which may be due to photoautotrophic conditions as reported by Kirdmanee *et al.* (1995) in *Eucalyptus camadulensis*. Of the three cultural conditions tested,

PWC was superior to PWAL and PWAH for shoot height, the number of nodal segments generated per active explant and the multiplication coefficient (Table 2). However, the leaves are much smaller and shoots were least vigorous in photomixotrophic treatments as compared to that of photoautotrophic treatments. The shoots with small leaves in photomixotrophic cultures may be attributed to low PPF as reported in *Certonia siliqua* (Vinterhalter *et al.* 2001).

The clearest difference between the photoautotrophic and photomixotrophic cultures was the development of roots from axillary shoots (Fig. 1). With in 21 d, in PWAH, 84 % of shoots were rooted with an average of

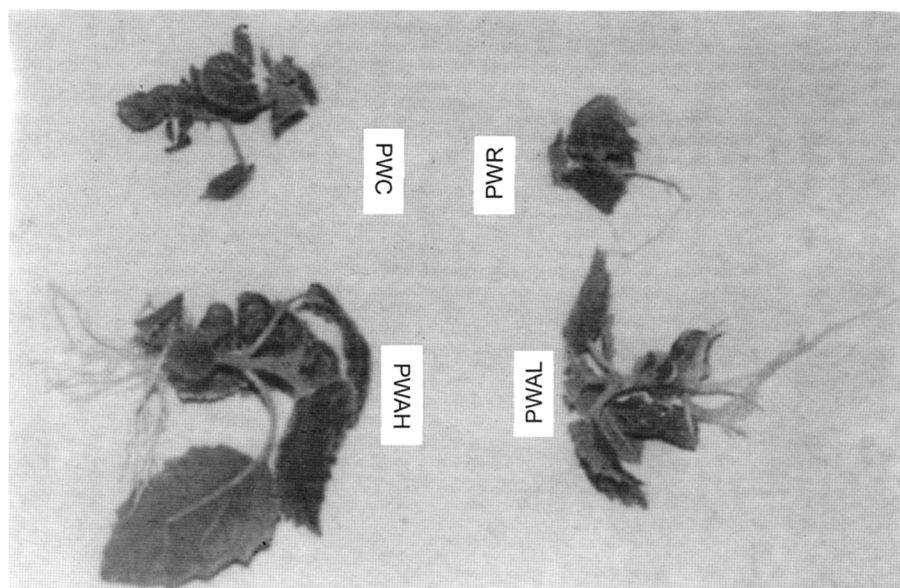


Fig. 1. Growth of *P. fortunei* in photomixotrophic cultures (PWC and PWR) and photoautotrophic cultures (PWAL and PWAH). For treatment codes, refer to Table 1.

Table 2. Growth characteristics of *P. fortunei* grown under photomixotrophic and photoautotrophic cultural conditions. For treatment codes, refer to Table 1. PWR was measured after transfer to rooting medium. Data were collected after 21-d incubation. Means \pm SD, $n = 3$, * - significantly different from PWC treatment at $P < 0.01$, ME - multiplication coefficient.

	Bud break	Shoot number	Shoot length [cm]	Axillary buds [shoot ⁻¹]	ME	Rooting [%]	Root number	Root length [cm]
PWC	98.3 \pm 3.7	1.0 \pm 0.1	2.4 \pm 0.2	2.1 \pm 0.0	2.7	callus	callus	
PWR	-	-	-	-	-	98.3 \pm 3.7	4.6 \pm 0.3	1.9 \pm 0.1
PWAH	100.0	1.6 \pm 0.3*	1.6 \pm 0.4*	1.8 \pm 0.3*	1.8	84.9 \pm 9.1	5.9 \pm 0.4*	4.0 \pm 0.6*
PWAL	91.6 \pm 5.8	1.2 \pm 0.2	1.3 \pm 0.2*	1.5 \pm 0.2*	1.3	78.3 \pm 15.1	3.5 \pm 0.4*	3.3 \pm 0.3*

5.9 roots per shoot and 4.0 cm length and in PWAL, 78 % of shoots rooted with an average of 3.5 roots per shoot with an average root length of 3.3 cm. Development of shoot and root in a single step is highly advantageous in commercial micropropagation in terms of reducing one stage and subsequently the production cost of the finished plantlet. On the other hand none of axillary shoots grown under photomixotrophic cultural conditions were unable to produce roots and the response was restricted to the development of green-compact basal callus. Therefore, shoots from photomixotrophic cultures were excised and transferred onto half strength MS medium containing 20 g dm⁻³ sucrose and 0.3 mg dm⁻³ indole-3-butyric acid (IBA) for rooting for further 21 d (PWR). In PWR, 98.3 % of shoots rooted with an average of 4.6 roots per shoot and 1.9 cm length. Thus rooting of photomixotrophic shoot cultures need a separate rooting stage as reported in number of other species (*e.g.* Bergmann and Moon 1997, Sha Valli Khan *et al.* 1999). The formation of callus at axillary shoot base of

photomixotrophic cultures may be related to the presence of plant growth regulator and sucrose in the culture medium. Nguyen *et al.* (1999) also observed callus induction at shoot base of *Coffea arabusta* cultured *in vitro* on agar medium containing 20 g dm⁻³ sucrose under PPF of 75 μ mol m⁻² s⁻¹ and 10-h photoperiod. There was no callus production in photoautotrophic cultures.

The axillary shoots produced significantly more number of elongated roots in photoautotrophic cultures as compared to photomixotrophic root cultures. The main adventitious root also gave rise to fine laterals for those plantlets grown in the photoautotrophic cultures (Fig. 1). The number of laterals was higher in high PWAH than PWAL. In the PWR, the main adventitious root did not produce laterals that might have been related to poor shoot growth of the plantlets. Probably the production of laterals is greatly influenced by culture conditions like the lower relative humidity, higher CO₂ concentration and higher PPF (Heo and Kozai 1999). The presence of

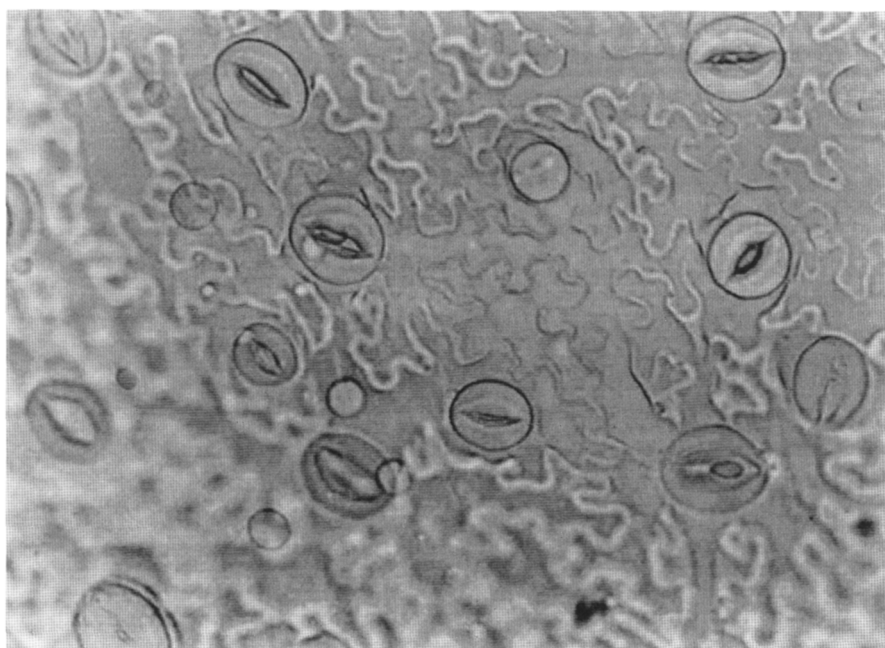


Fig. 2. Photomicrograph of PWAH leaves at a magnification of 400 \times .

lateral roots could have resulted in a higher nutrient absorption, as the laterals are more permeable than the main adventitious root (Zobayed *et al.* 1999). Lucchesini *et al.* (2001) also observed plantlet growth and root formation in photoautotrophic culture of *Myrtus communis*. The vigorous growth of shoots and lateral root development are additional advantages of the photoautotrophic cultures.

Stomatal characteristics: Microscopic observations on leaf adaxial surfaces of photomixotrophic and photoautotrophic cultures showed no stomata. In comparison, leaf abaxial surface prints from photomixotrophic shoot and root cultures showed the presence of stomata. Leaves from PWC and PWR had widely open (6 - 8 μm pore width), spherical stomata (diameter of 12 - 14 μm). In contrast, leaf abaxial surface prints from PWAL and PWAH showed elliptical stomata (length of 10 - 12 μm) with narrow openings (3 - 4 μm pore width) (Fig. 2). The rounded shape is usually considered to be associated with abnormal *in vitro* stomatal function and elliptical shape is characteristic of *in vivo* stomata endowed with normal function. This agrees with previous results in conventional micropropagated plantlets of *Syzygium alternifolium* (Sha Valli Khan *et al.* 1999). Leaves from PWC and PWR had higher stomatal index 46.6 and 47.8, respectively as compared to PWAL (38.3) and PWAH (28.3). Findings of the present investigation confirm previous results reported in *Eucalyptus camadulensis* (Zobayed *et al.* 2000) and *Nicotiana tabacum* L. (Voleniková and Tichá 2001). However, the higher stomatal density in the photomixotrophic cultures may be due to high relative humidity. The presence of sugar in the medium and accumulated ethylene in the sealed vessels may also play a role and led to the development of abnormal stomata together with higher stomatal index subsequently reduces the chances of survival upon transplantation.

Water loss experiment: Leaves from photomixotrophic cultures shrivelled whereas those from photoautotrophic cultures showed slight wilting after 30 min. It clearly

demonstrates the inability of photomixotrophic cultures resist to desiccation. Similar results were reported for grape (Fila *et al.* 1998). The percent of moisture loss from leaves of photomixotrophic cultures was not varying significantly than that of photoautotrophic cultures. This is true at each of 30, 60, 90 and 120-min intervals of air-drying (Table 3). The rate of water loss in photomixotrophic and photoautotrophic conditions correlates with the findings of Zobayed *et al.* (2000). Higher PPF during photoautotrophic micropropagation is associated with substantially higher rates of transpiration (Zobayed *et al.* 1999). This in turn may result in plantlets *in vitro* expressing more strongly developed root system, thick cuticle, epicuticular waxes and a better stomatal functioning (Serret *et al.* 1996). Such characteristics would further improve acclimation to *ex vitro* conditions.

Table 3. Percentage of water loss from detached leaves of photomixotrophic and photoautotrophic cultures of *P. fortunei*. For treatment codes, refer to Table 1. Data was collected after 21-d incubation. Means \pm SD, $n = 3$, * - significantly different from PWC treatment at $P < 0.01$.

	Time [min]			
	30	60	90	120
PWC	48.8 \pm 6.8	69.0 \pm 6.1	74.8 \pm 18.3	83.0 \pm 3.7
PWR	36.0 \pm 6.2	57.1 \pm 8.3*	70.5 \pm 8.4*	77.9 \pm 7.4*
PWAH	42.9 \pm 8.8	58.4 \pm 10.8	71.7 \pm 10.0*	75.8 \pm 9.7*
PWAL	33.7 \pm 4.4*	54.6 \pm 6.1*	64.1 \pm 7.0*	73.1 \pm 7.2*

Conclusions: In the present study, the photoautotrophic cultures of *P. fortunei* were obtained for the first time with satisfactory shoot growth and spontaneous rooting under high PPF and high CO_2 . Results also showed the leaf wilting and the consequent desiccation of leaves grown under photomixotrophic conditions. This phenomenon might be associated with high stomatal density and insufficient functionality of stomata in photomixotrophic cultures as compared to photoautotrophic cultures.

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