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The effect of photoautotrophy on photosynthesis and photoinhibition of gardenia plantlets during micropropagation

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Abstract

We studied the relationships between the degree of photoautotrophy, photosynthetic capacity, and extent of photoinhibition of *Gardenia jasminoides* Ellis plantlets *in vitro*. Two successive micropropagation stages (shoot multiplication and root induction), and three culture conditions [tube cap closure, photosynthetic photon flux density (PPFD), and sucrose concentration] which may influence the development of photoautotrophy *in vitro* were assayed. The ratios of variable chlorophyll fluorescence to either maximal (F_v/F_m) or ground (F_v/F_0) values were low, irrespective of the culture stage or growing conditions. Incomplete development of the photosynthetic apparatus and permanent photoinhibition may be involved. However, F_v/F_m and F_v/F_0 increased from shoot multiplication to root induction owing to a decrease in F_0 and an increase in F_m. This suggests that photoinhibition decreases later during micropropagation, when the photoautotrophy of plantlets is more advanced. The low sucrose content and high PPFD increased the photoinhibition of plantlets, whereas growth in tubes with permeable caps showed the opposite effect. The only culture factor with a significant (positive) effect on maximum photosynthetic rate (P_{max}) was PPFD. At shoot multiplication net photosynthetic rate (P_N) was positively correlated with the half time of the increase from F_0 to F_m ($t_{1/2}$). Such association may be mainly due to a common response of both traits to higher PPFD in culture. Within each culture stage, no relationship was observed between P_N and the degree of photoautotrophy, which was positively correlated with F_v/F_m and F_v/F_0 during root induction. During shoot multiplication, these correlations were not significant, or were even negative. Hence during the last stage of micropropagation, plantlets with a higher degree of photoautotrophy are less photoinhibited, whereas they do not follow this pattern at the earlier stage.

Additional key words: chlorophyll fluorescence; *Gardenia jasminoides*; *in vitro* culture; irradiance; photosynthetic pigments; respiration; root induction; saccharose; shoot multiplication; vapour pressure deficit.

Introduction

The photoautotrophic growth of plantlets *in vitro* can be enhanced if adequate environmental conditions are provided (Kozai *et al.* 1990, 1997, Kozai 1991). By lowering the sucrose content in the medium and by increasing PPFD and/or CO_2 concentration, photosynthetic characteristics of plantlets *in vitro* (Solárová 1989, Kozai 1991, Kubota and Kozai 1992, Hdider and Desjardins 1994, Kozai *et al.* 1997, Serret and Trillas 2000) as well as the relative contribution of photosynthesis to the total plantlet biomass accumulation (Serret *et al.* 1996, 1997) can be improved. However, the link (if any) between the degree of photoautotrophy and the photosynthetic capacity of a plantlet *in vitro* is not sufficiently known.

Enhancement of photoautotrophic growth may be necessary to avoid potential photoinhibition of plantlets *in vitro* (Dubé and Vidaver 1992, Hdider and Desjardins 1994). However, this may not always be the case. For example, increased PPFD during micropropagation may enhance photoautotrophy of plantlets. However, absorption of excessive photons by leaves is regarded as a potential source of damage *in vitro* (Aitken-Christie *et al.* 1992), leading to higher photoinhibition and then to lower contribution of photosynthates to the total carbon gain of plantlets (Serret *et al.* 1996).

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Abbreviations: Car – carotenoids; Chl – chlorophyll; F_m – maximum chlorophyll fluorescence; F_0 – ground fluorescence emission, when all reaction centres are open and the photochemical quenching is minimal; F_v – variable chlorophyll fluorescence ($F_m - F_0$); P_{max} – maximum photosynthetic rate; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; R_D – dark respiration rate; RC – reaction centre; $t_{1/2}$ – half-time of the increase from F_0 to F_m; VPD – vapour pressure deficit.

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Fast-chlorophyll (Chl) fluorescence-derived parameters, such as F_v/F_m and F_v/F_0, are used to study photoinhibitory impairment (Ögren and Öquist 1985, Lichtenthaler and Rinderle 1988) as well as the organisation and functioning of photosynthetic apparatus (Genty *et al.* 1989). These parameters are the ratios of variable Chl fluorescence ($F_v = F_m - F_0$) to either maximal (F_m) or ground (F_0) Chl fluorescence. F_v/F_m is used to evaluate the photosystem 2 (PS2) in the dark-adapted state with fully open PS2 reaction centres (RCs), and it is highly correlated with the quantum yield of net photosynthesis in intact leaves (Björkman and Demmig 1987, Demmig and Björkman 1987). For this reason, it has been widely used as fast indicator of photoinhibition, even when only the evaluation of the photochemical and non-photochemical fluorescence quenching would provide the final proof of photoinhibition (Lichtenthaler and Burkart 1999). On the other hand, the ratio F_v/F_0 is a reliable indicator of the potential photosynthetic capacity of leaves. It has also been proposed as an indicator of the state and effectiveness of electron transport chain (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996, Yordanov *et al.* 1997, Georgieva and Lichtenthaler 1999). Any factor affecting the uptake of excitation energy by open PS2 RCs also modifies these Chl fluorescence ratios (Lichtenthaler and Rinderle 1988, Genty *et al.* 1989, Schreiber and Bilger 1993).

Increases in F_0 should be distinguished from decreases in F_m, even if both changes are caused by photoinhibition (Baker and Horton 1987, Pearcy and Sims 1994). The former is associated with photodamage, whereas the latter is linked to photoprotection (Osmond *et al.* 1987, Kamaluddin and Grace 1992, Araus and Hogan 1994, Georgieva and Lichtenthaler 1999). As shown by Rintamaki *et al.* (1994), inhibition of D1-protein of the PS2 RC increases F_0. On the other hand, a decline in F_m may point to an increase in non-photochemical quenching (Bolhàr-Nordenkampf *et al.* 1989). Processes other than photoinhibition, such as acclimation and adaptation to PPFD (Araus and Hogan 1994, Yordanov *et al.* 1997) or development of the photosynthetic apparatus both *ex vitro* (Babani and Lichtenthaler 1996 and references herein) and *in vitro* (Serret *et al.* 1996) may also affect these Chl fluorescence parameters.

We describe the photosynthetic capacity and fast Chl fluorescence responses of gardenia plantlets, at two successive stages of culture *in vitro*. We tested whether the degree of photoautotrophy of these mixotrophic plantlets alters the photoinhibitory impairment during micropropagation. The association between the degree of photoautotrophy and the photosynthetic capacity of plantlets *in vitro* was also studied. At each micro-propagation stage, varying degrees of photoautotrophy were attained by changing the type of tube closure, the sucrose content in the medium, and PPFD. Photoautotrophy was assessed as the difference be-tween the stable carbon isotope composition of the sucrose used as heterotrophic carbon source and that of leaflets (Serret *et al.* 1996, 1997, see also Yakir *et al.* 1991).

Materials and methods

Plants and growth conditions: Micropropagated gardenia (*Gardenia jasminoides* Ellis) plantlets were derived from 2 cm-long shoot tips of plants grown in the greenhouse. Plantlets were aseptically grown in *Pyrex* tubes (52 cm^3). During shoot multiplication, explants of 74±6 mg, consisting of 2-3 leaflets and with a minimum shoot length of 1.5 cm, were cultured in 13 cm^3 of medium of Murashige and Skoog (1962) with 8.0 kg m^{-3} of agar (*Difco*) and various sucrose concentrations (3.0, 0.5, and 0 %, m/v). The vitamins used in the culture were thiamine HCl (50 g m^{-3}), m-inositol (5 kg m^{-3}), Ca-pantothenate (0.5 kg m^{-3}), and glycine (0.2 kg m^{-3}). The growth regulator used in this phase was N^6-benzyladenine (1.0 g m^{-3}). pH was adjusted to 5.5. After four weeks of culture, root formation *in vitro* was induced by transfer to a medium containing the same macroelements at half the concentration, plus 2.0 g m^{-3} indol-3-yl-acetic acid at pH 5.0. Thus, explants of about 171±15 mg, derived from plantlets grown during the shoot multiplication stage, were sub-cultured for a further four weeks. During root induction, sucrose content was maintained the same as during shoot multiplication. Plantlets were placed in a growth chamber (*E-15*, *Conviron*, Winnipeg, Manitoba, Canada), under a 12-h photoperiod at constant temperature (20.0 ± 2.0 °C). Irradiation was provided by fluorescent cool white tubes (*F72T12/CW/VHO* 160 W, *Sylvania*, München, Germany) supplemented with incandescent bulbs (*Sylvania*). Photosynthetic photon flux density (PPFD, wavelength 400-700 nm) was adjusted by the use of a shadow mesh. For each sucrose concen-tration in the culture media and during both culture stages, two PPFD were assayed: 50±5 µmol m^{-2} s^{-1} (low PPFD) and 110±10 µmol m^{-2} s^{-1} (high PPFD) inside the culture tubes, at the level of culture medium. The CO_2 concen-tration inside the growth chamber was 750 µmol mol^{-1} during both stages. The amount of CO_2 (quality C-45, purity > 99.995 %) injected into the chamber was con-trolled by an infrared gas analyser (*APBA-250E, Horiba Instruments*, Irvine, USA). Control culture tubes were loosely closed with aluminium caps (*Sero-Tap*, 24-26 mm, *Selecta*, Abrera, Spain), provided with an inner spring that prevented tight closure, thus allowing gas exchange with the surrounding atmosphere. Thereafter, half the tubes were loosely closed and the other half was tightly sealed with paraffin film (*Parafilm*, *American Nat. Can*, Greenwhich, USA) to avoid gas exchange. The half time for CO_2 retention (t_{50}), measured following Jackson *et al.* (1994), was more than 30 times higher in the tight-closed than in the loosely-closed tubes (Serret *et al.* 1997).

Environmental conditions inside the tubes: PPFD inside the tubes was measured as stated previously (Serret *et al.* 1997). Relative humidity and temperature were also monitored inside the tubes, by introducing a small head with *Vaisala* sensors (*Humicap* and *Pt100*, respectively, *Vaisala*, Helsinki, Finland) through the tube cap, and vapour pressure deficit (VPD) was further calculated. The CO_2 concentration inside the tube during the dark and light periods was measured by a portable gas analyser (*LI-6200 Portable Photosynthesis System, LI-COR*, Lincoln, USA), after correction for air dilution in the gas exchange system (Serret *et al.* 1996, 1997). Measurements were recorded at the end of the dark period and after 1.5, 4.0, and 6.0 h of irradiation.

Photosynthesis and respiration: Maximum net photosynthetic (P_{max}) and dark respiration (R_D) rates of leaflets were measured polarographically using a Clark-type oxygen electrode (*Rank Brothers,* Bottisham, UK) at 25 °C as reported by Serret and Trillas (2000). Gardenia leaflets (3-7 mg fresh mass) were transversely cut into 1 mm-thick slices with a scalpel in 0.2 mM $CaCl_2$, further incubated for 30 min in Petri dishes, with the same buffer used later in the O_2 electrode. For photosynthetic measurements, the reaction chamber was filled with 4 cm^3 of 10 mM TES [N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, *Sigma*] in 0.2 mM $CaCl_2$, pH 7.2. The photosynthetic capacity was measured in saturating CO_2 (provided by 0.020 cm^3 of 1.0 M $KHCO_3$ in the reaction chamber) and PPFD (approximately 1 000 μmol m^{-2} s^{-1}). R_D was measured in the dark in 4 cm^3 of 20 mM MES (2-N-morpholino-ethanesulfonic acid, *Sigma*) in 0.2 mM $CaCl_2$, pH 6.5. For each growing condition, about 6-10 measurements of P_{max} and R_D were performed. Rates were expressed per dry mass or total Chl units.

Chl fluorescence: Attached leaflets were used for the determination of parameters F_0 and F_m, and F_v (defined as $F_m - F_0$) and the half-time [ms] of the increase from F_0 to F_m ($t_{1/2}$). The ratios F_v/F_m and F_v/F_0 were then calculated. Measurements were performed after 4 weeks of either shoot multiplication or root induction, using a portable fluorimeter (*Plant Stress Meter, Biomonitor AB S.C.I.*, Umeå, Sweden; Bolhàr-Nordenkampf *et al.* 1989). PPFD, runtime, and dark adaptation period for all measurements were 200 μmol m^{-2} s^{-1}, 5 s and 30 min, respectively. The F_v/F_m measured at 400 μmol m^{-2} s^{-1} did not differ significantly from the value obtained at 200 μmol m^{-2} s^{-1}, indicating that a further increase in the PPFD did not modify F_m. For each growth condition assayed, measurements were performed on the adaxial side of 13-20 intact leaflets, at two times: just before the irradiation, and 6 h later.

Photosynthetic pigments: The leaflets used in Chl fluorescence determinations were subsequently macerated with a small amount of 80 % (v/v) cold acetone. Contents of Chl *a* and *b* and total carotenoids were calculated spectrophotometrically according to the equations of Lichtenthaler (1987), as described in Serret *et al.* (1996).

Degree of photoautotrophy: Photoautotrophy in all the culture conditions studied was ranked from the difference between the carbon isotope composition ($\delta^{13}C$, ‰) of sucrose (sugar cane sucrose) used as carbon source and that of leaflets [$\delta^{13}C$(sucrose) – $\delta^{13}C$(leaflets)] as reported in Serret *et al.* (1996, 1997). Highest differences were assigned to the most autotrophic leaflets. The plantlets used were the same as those for Chl fluorescence and gas exchange measurements. These results are available in Serret *et al.* (1997) and so they were here merely used to study the correlations with photosynthesis and Chl fluorescence.

Results and discussion

Changes in the ratio of variable to ground Chl fluorescence (F_v/F_0) outsized those in the ratio of variable to maximum Chl fluorescence (F_v/F_m) in response to the changing growing conditions or to the micropropagation stages (Tables 1 and 2). Indeed, the ratio F_v/F_m, is relatively inert and slow in response, whereas F_v/F_0 is much more sensitive to changes in photosynthetic quantum conversion capacity (Babani and Lichtenthaler 1996). Given the distinct formulation of both ratios, their correlation is expressed by a hyperbolic function, where F_v/F_m shows lower sensitivity than F_v/F_0 as the values of both ratios increase (Babani and Lichtenthaler 1996).

Effect of micropropagation stage on Chl fluorescence: The culture stage had a strong effect on Chl fluorescence. For a given growing condition, the ratios F_v/F_m and F_v/F_0 and $t_{1/2}$ increased, whereas F_m and F_0 significantly decreased ($p < 0.05$) from shoot multiplication to root induction (compare Tables 1 and 2). After 6 h of irradiation leaflets tended to show slightly (but not significantly) lower values of F_v/F_m and F_v/F_0 and higher $t_{1/2}$ than immediately before the irradiation. A progressive increase in the ratio F_v/F_m throughout the *in vitro* stages was already reported in gardenia and other plant species during micropropagation (Serret *et al.* 1996, Rival *et al.* 1997). The lower values of

Table 1. Effects of sucrose concentration, PPFD, tube closure and time of measurement during the light/darkness photoperiod on the chlorophyll fluorescence parameters F_v/F_m, F_v/F_0, F_m, F_0, and $t_{1/2}$ measured on gardenia leaflets developed during shoot multiplication. Values are means ± SE of 13-20 leaflets. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Treatment		F_v/F_m	F_v/F_0	$t_{1/2}$	F_m	F_0
Sucrose [%]	3.0	0.706±0.007	2.55±0.06	37.27±1.31	1.23±0.05	0.34±0.02
	0.5	0.662±0.007	2.18±0.06	33.60±1.33	1.45±0.05	0.47±0.02
	0.0	0.619±0.008	1.96±0.07	38.62±1.52	1.37±0.05	0.46±0.02
	significance	***	***	*	**	***
PPFD [μmol m^{-2} s^{-1}]	110	0.630±0.006	1.99±0.05	47.22±1.16	1.19±0.04	0.41±0.01
	50	0.694±0.006	2.47±0.05	25.78±1.10	1.51±0.04	0.44±0.01
	significance	***	***	***	***	n.s.
CO_2 (tube closing)	loose	0.678±0.006	2.35±0.05	33.52±1.08	1.43±0.04	0.44±0.01
	tight	0.646±0.006	2.11±0.05	39.48±1.18	1.27±0.04	0.41±0.01
	significance	***	**	***	*	n.s.
Photoperiod	dark	0.667±0.006	2.30±0.05	31.08±1.12	1.38±0.04	0.42±0.01
	light	0.657±0.006	2.16±0.05	41.91±1.14	1.32±0.04	0.43±0.01
	significance	n.s.	n.s.	***	n.s.	n.s.
Sucrose × PPFD		***	***	n.s.	***	n.s.
Sucrose × CO_2		n.s.	n.s.	*	n.s.	n.s.
Sucrose × Time		**	*	n.s.	n.s.	n.s.
PPFD × CO_2		n.s.	*	n.s.	n.s.	n.s.
PPFD × Time		n.s.	n.s.	n.s.	***	**
CO2 × Time		*	n.s.	n.s.	n.s.	n.s.
Sucrose × PPFD × CO_2		n.s.	n.s.	n.s.	*	n.s.
Sucrose × PPFD × Time		n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × CO_2 × Time		**	n.s.	n.s.	n.s.	n.s.
PPFD × CO_2 × Time		**	**	n.s.	n.s.	n.s.
Sucrose × PPFD × CO_2 × Time		n.s.	n.s.	n.s.	n.s.	n.s.

F_v/F_m and F_v/F_0 during shoot multiplication compared with root induction were associated with a higher F_0 (Tables 1 and 2), in agreement with previous results on gardenia in similar experimental conditions (Serret *et al.* 1996).

The ratio F_v/F_m typically ranges between 0.75 and 0.85 for non-stressed leaves *ex vitro* (Demmig and Björkman 1987, Bolhàr-Nordenkampf *et al.* 1989). Here it was in general lower, even for leaflets growing during root induction in *a priori* non-photoinhibitory conditions such as low PPFD and tubes with permeable caps (Tables 1 and 2). Low F_v/F_m during micropropagation was reported in oil palm (Rival *et al.* 1997). Here, F_v/F_0 was also low, irrespective of the treatment, suggesting (in absence of stress) low values for these ratios in leaflets *in vitro*. The low and steady values of F_v/F_0 and F_v/F_m along the photoperiod may result from incomplete development of the photosynthetic apparatus. In addition, the lower F_0 during root induction compared with shoot multiplication is not necessarily due to a lesser photoinhibitory impairment. On the contrary, it may result from changes in the structure of the photosynthetic apparatus. In such context, several alternatives should be considered.

A decrease in F_0 may be due to acclimation or adaptation to higher irradiance, and it is probably associated with smaller antenna (Araus and Hogan 1994, Yordanov *et al.* 1997). In smaller antenna, the path for quanta to the RC is shorter, which involves lower energy losses and thus lower F_0 and higher F_v/F_0 (Yordanov *et al.* 1997). In addition, $t_{1/2}$ during root induction is higher than during shoot multiplication (Tables 1 and 2), as reported already in Serret *et al.* (1996). This suggests adaptation to higher PPFD where smaller antenna size and a larger plastoquinone pool are common responses (Anderson *et al.* 1988). However, other evidence does not support this model of acclimation to irradiance. Thus, F_v/F_m hardly changes in response to an increase in F_0 associated with shade acclimation (Araus and Hogan 1994). Moreover, the decrease in Chl *a/b* and the increase in Chl/Car from shoot multiplication to root induction (Table 3) do not agree with progressive acclimation to higher PPFD (Pearcy and Sims 1994). These changes seem to be rather linked to a progressive development of the photosynthetic apparatus (Lee *et al.* 1985).

Thus, another developmental model for comparison would be greening. The no-sucrose treatment gave low F_v/F_m and F_v/F_0 (Tables 1 and 2) and a low Chl content during shoot multiplication (Table 3), which would reflect a delay in the greening process. Low F_v/F_m and F_v/F_0 ratios were reported for *ex vitro* greening leaves in absence of stress (Babani and Lichtenthaler 1996). Moreover, F_v/F_m and F_v/F_0 as well as F_0 and F_m increase during greening, especially at the beginning, tending to a plateau (Babani

Table 2. Effects of sucrose concentration, PPFD, tube closure, and time of measurement during the light/darkness photoperiod on chlorophyll fluorescence parameters F_v/F_m, F_v/F_0, F_m, F_0, and $t_{1/2}$ of gardenia leaflets developed during root induction. Values are means ± SE of 13-20 leaflets. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Treatment		F_v/F_m	F_v/F_0	$t_{1/2}$	F_m	F_0
Sucrose [%]	3.0	0.751±0.004	3.18±0.05	118.90±2.80	0.44±0.01	0.11±0.00
	0.5	0.740±0.004	3.02±0.05	99.90±2.77	0.52±0.01	0.14±0.00
	0.0	0.687±0.005	2.23±0.06	162.05±3.43	0.37±0.01	0.11±0.00
	significance	***	***	***	***	***
PPFD [μmol m^{-2} s^{-1}]	110	0.712±0.004	2.58±0.05	140.21±2.48	0.43±0.01	0.12±0.00
	50	0.740±0.003	3.04±0.04	113.70±2.36	0.46±0.01	0.12±0.00
	significance	***	***	***	n.s.	n.s.
CO_2 (tube closing)	loose	0.768±0.003	3.42±0.04	112.92±2.26	0.52±0.01	0.12±0.00
	tight	0.684±0.004	2.21±0.05	140.98±2.61	0.37±0.01	0.11±0.00
	significance	***	***	***	***	n.s.
Photoperiod	dark	0.733±0.004	2.89±0.05	121.40±2.40	0.44±0.01	0.11±0.00
	light	0.719±0.004	2.73±0.05	132.51±2.43	0.45±0.01	0.12±0.00
	significance	n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × PPFD		***	***	n.s.	n.s.	*
Sucrose × CO_2		***	***	***	***	**
Sucrose × Time		n.s.	n.s.	n.s.	n.s.	n.s.
PPFD × CO_2		***	***	*	**	n.s.
PPFD × Time		n.s.	n.s.	n.s.	n.s.	n.s.
CO2 × Time		n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × PPFD × CO_2		***	***	n.s.	***	n.s.
Sucrose × PPFD × Time		n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × CO_2 × Time		n.s.	n.s.	n.s.	n.s.	n.s.
PPFD × CO_2 × Time		n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × PPFD × CO_2 × Time		n.s.	n.s.	n.s.	n.s.	n.s.

and Lichtenthaler 1996). However, both Chl fluorescence ratios increased from shoot multiplication to root induction in our study, whereas F_0 and F_m decreased. On the other hand, during the development of photosynthetic apparatus associated with leaf greening, the total Chl content and the ratio Chl/Car usually increase, whereas the ratio Chl *a/b* decreases (Babani and Lichtenthaler 1996, and references herein). In our leaflets, the changes in Chl/Car and Chl *a/b* from shoot multiplication to root induction agreed with a greening process, and even the Chl content increased somewhat (Table 3), which agrees with that reported in other plantlets *in vitro* (Rival *et al.* 1997).

Taken as a whole, the pattern of changes in fluorescence and pigments during micropropagation does not match those typical of greening or acclimation to higher irradiance, suggesting (at least in part) photoinhibition. Thus, leaflets from root induction may be less photoinhibited (show higher F_v/F_m and F_v/F_0) than those from the earlier stage, and their photoinhibition may be associated with photoprotective (*i.e.*, decrease in F_m) mechanisms rather than with photodestruction (increase in F_0) (Kamaluddin and Grace 1992, Araus and Hogan 1994, Pearcy and Sims 1994). In contrast, the lower F_v/F_m and F_v/F_0 ratios measured during shoot multiplication, in spite of the higher F_m values, were caused by the high values of F_0. Hence, higher F_m has been associated with low non-photochemical quenching and reflects either active use of energy in the Calvin cycle (Georgieva and Yordanov 1994) or the lack of photoprotective (*i.e.*, non-photochemical quenching) mechanisms during this early stage of micropropagation, which is more probable.

Effect of culture conditions on Chl fluorescence: The three growing factors assayed (tube closure, PPFD, and sucrose concentration) significantly ($p < 0.05$) affected F_v/F_m, F_v/F_0, $t_{1/2}$, and F_m during the two micropropagation stages, whereas F_0 was only modified by the sucrose concentration (Tables 1 and 2). Higher sucrose concentration increased the ratios F_v/F_m and F_v/F_0, whereas the effect on $t_{1/2}$, F_m, and F_0 was less clear. An increase in PPFD decreased F_v/F_m, F_v/F_0, and F_m and increased $t_{1/2}$. The same pattern of variation was observed in tightly sealed tubes and in those with the permeable cap. Nevertheless, the response to these culture conditions showed significant ($p < 0.05$) interactions, especially between sucrose and PPFD during shoot multiplication (Table 1). During root induction, there were also significant interactions between sucrose and both PPFD and tube closure, and between PPFD and tube closure (Table 2).

Within a given culture stage, leaflets growing under low sucrose concentration, and to a lower extent under high PPFD, were the most photoinhibited. These culture conditions stimulate photoautotrophy of plantlets *in vitro* (see Serret *et al.* 1996, 1997 for gardenia). The decrease in F_v/F_0 and F_v/F_m associated with low sucrose concentration was not due to a decrease in F_m (this parameter showed the opposite pattern) but to an increase in F_0, whereas high PPFD decreased F_m, with no changes in F_0. As pointed out above, a decline in these fluorescence ratios caused by an increase in F_0 suggests inactivation of PS2 RCs by photodamage, whereas if it were due to a decrease in F_m, it would result from an increase in photoprotection (Kamaluddin and Grace 1992, Araus and Hogan 1994, Pearcy and Sims 1994, Georgieva and Lichtenthaler 1999). In addition, low sucrose concentration (mostly during shoot multiplication) decreased total Chl content as well as the Chl *a/b* and Chl/Car ratios (Table 3), suggesting lower synthesis and/or more degradation of Chl (Šesták 1985). In this regard, recent results in gardenia grown *in vitro* show that sucrose induces chloroplast formation (Serret and Trillas 2000). In other species, sucrose enhanced Chl content and no photoinhibition during micropropagation was found (Synková 1997, Tichá *et al.* 1998). In addition, the larger Chl *a/b* ratio at high sucrose agrees with smaller light-harvesting complexes (see Lawlor 1993), which might decrease photoinhibition of leaflets. However, other studies do not support this hypothesis (Capellades *et al.* 1990, Aitken-Christie *et al.* 1992, Hdider and Desjardins 1994, Van Huylenbroeck and Deberg 1996), even when the concentrations of sucrose they assayed were extremely high, ranging between 3 and 6 %. High concentration of sucrose in the medium can undermine plantlet growth (Wolf *et al.* 1998).

Table 3. Effects of sucrose concentration, PPFD, and tube closure on total chlorophyll (*a*+*b*) content [g kg^{-1}(f.m.)], and the Chl *a/b* and Chl/Car ratios of gardenia leaflets developed during shoot multiplication and root induction. Values are means ± SE of 5-6 samples, each one containing 1-2 leaflets. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Treatment		Shoot multiplication			Root induction		
		Chl	Chl *a/b*	Chl/Car	Chl	Chl *a/b*	Chl/Car
Sucrose [%]	3.0	2.00±0.13	3.06±0.07	4.91±0.11	2.10±0.13	2.80±0.04	4.79±0.07
	0.5	1.47±0.13	3.02±0.07	4.50±0.11	1.88±0.12	2.60±0.04	4.72±0.06
	0.0	1.41±0.18	2.61±0.10	4.12±0.16	1.88±0.17	2.68±0.06	4.69±0.09
	significance	*	n.s.	**	n.s.	**	n.s.
PPFD [μmol m^{-2} s^{-1}]	110	1.36±0.12	2.93±0.07	4.43±0.10	1.73±0.10	2.65±0.04	4.60±0.06
	50	1.90±0.11	2.86±0.06	4.59±0.01	2.17±0.11	2.73±0.04	4.86±0.06
	significance	*	n.s.	n.s.	n.s.	n.s.	*
CO_2 (tube closing)	loose	1.89±0.13	3.04±0.06	4.56±0.09	2.08±0.10	2.71±0.04	4.76±0.05
	tight	1.37±0.11	2.75±0.07	4.46±0.11	1.82±0.12	2.67±0.05	4.70±0.07
	significance	n.s.	*	n.s.	n.s.	n.s.	n.s.
Sucrose × PPFD		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Sucrose × CO_2		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
PPFD × CO_2		n.s.	n.s.	n.s.	n.s.	*	n.s.
Sucrose × PPFD × CO_2		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

The increase in PPFD during shoot multiplication had significant negative effect on Chl content or pigment ratios (Table 3) and it tended to decrease Chl content and increase Chl/Car during root induction. Results suggest that leaflets have acquired plasticity to acclimate to higher PPFD (Pearcy and Sims 1994) already at shoot multiplication. Therefore, low or zero concentrations of sucrose in the medium, rather than a higher PPFD, may induce photodamage. Serret and Trillas (2000) observed that the gardenia leaflets showing the most altered chloroplast ultrastructure were those cultured at low sucrose concentration combined with low PPFD. Moreover, the interaction for F_v/F_m and F_v/F_0 between sucrose and PPFD was highly significant at both growing stages. This agrees with the fact that plantlets growing under low sucrose concentration are less sensitive to photoinhibition at increasing PPFD. In this regard, Genoud *et al.* (1999) reported that an increase in PPFD has a positive effect on the F_v/F_m ratio in plantlets cultured in sucrose-free medium. In contrast, Tichá *et al.* (1998) suggested that an elevated PPFD is beneficial in photomixotrophic (*i.e.*, with a little sucrose in the medium) tobacco cultures, whereas it causes photoinhibition in fully photoautotrophic (*i.e.*, sugar-free) cultures.

Lower cap permeability to gas diffusion can have an overall negative effect on the culture conditions, since it decreases photoautotrophy (Serret *et al.* 1997) and increases photoinhibition (Tables 1 and 2). During most of the irradiation period, the CO_2 concentration inside the tightly closed tubes (Table 4) was close to the CO_2 compensation point in C_3 plants (Lawlor 1993). Our results agree with those reporting that low CO_2 concentration inside tightly closed vessels can be attained after a few hours of irradiation (Solárová 1989, Kozai *et al.* 1990, Kozai 1991). Reduced CO_2-availability inside tightly closed tubes may affect electron transport by limiting the consumption of assimilatory power (Peterson 1990) and thus enhance the photoinhibitory impairment of plantlets. High PPFD can further enhance the photoinhibitory effect, not only directly *via* the increase in incoming energy, but also indirectly, since CO_2 concentration inside the tube decreases faster and the temperature inside the tube is about 5 °C higher than that of the growth chamber temperature (Table 4). In contrast, the slight increase in VPD probably has a minor influence. As reported for *ex vitro* plants, within the *in vitro* tightly closed tubes, combination of high irradiance and temperature plus a low CO_2 concentration may have a synergistic effect on photoinhibition (Powles 1984, Ludlow 1987, Peterson 1990, Faria *et al.* 1996). Other factors may favour the growth within ventilated tubes, such as an increase in amount of water (and thus accompanying nutrients) entering the plantlets (due to a higher VPD, Table 4) and reduced ethylene accumulation (Righetti *et al.* 1990, Murphy *et al.* 1998). Tube closure inhibited Chl content (Table 3). Other studies conclude that higher CO_2 availability markedly stimulates the Chl content and growth of plantlets *in vitro* (Righetti 1996, Solárová and Pospíšilová 1997, Mitra *et al.* 1998), especially when it is combined with low or free-sucrose media (Seko and Nishimura 1996, Tichá 1996).

Photosynthesis, respiration, and degree of photoautotrophy: $P_{\max}$, evaluated as O_2 release at PPFD and CO_2 saturation, did not increase in the late stages of the *in vitro* culture when expressed on a dry mass basis. Rather, $P_{\max}$ tended to decrease (Table 5). $P_{\max}$ (on fresh mass basis) decreases from shoot multiplication to root induction also in other plants (Sallanon *et al.* 1995, 1997, Rival *et al.* 1997). For most culture conditions, R_{D} calculated per unit dry mass was doubled during shoot multiplication with respect to root induction. On the other hand, free-sucrose medium lowered R_{D}, suggesting limitation by substrate availability, in agreement with previous studies on *in vitro* cultures (Galzy and Compan 1992, Hdider and Dejardins 1994).

Nevertheless, gardenia plantlets show more developed photoautotrophy at root induction than at shoot multiplication (Serret *et al.* 1996, 1997), which is expected to modify $P_{\max}$. The tendency of $P_{\max}$ and R_{D} to decrease between shoot multiplication and root induction may be due to a progressive increase in the amount of inert (mostly support) structures in the leaflets developed at late stages of micropropagation (Nguyen and Kozai 1998).

The sucrose concentration and tube closure had no significant effect on $P_{\max}$, although slightly higher rates were observed in plantlets cultured both in sucrose-free medium and with loose caps. PPFD was the only culture condition with a significant (positive) effect on $P_{\max}$ per unit dry mass, which agrees with previous results obtained during shoot multiplication in gardenia (Serret and Trillas 2000). Other culture conditions also failed to modify $P_{\max}$

Table 4. Effect of tube closure, PPFD [µmol m^{-2} s^{-1}] inside tubes, and photoperiod on temperature, water vapour pressure deficit, and CO_2 concentration inside the tubes *in vitro*. Each value of temperature and water vapour deficit is the mean ± SE of 3-6 measurements, whereas those for CO_2 concentration represent the mean ± SE of 4-5 tubes. For details see Materials and methods.

Tube cap	PPFD	Temperature [°C]			Vapour Pressure Deficit [Pa]			CO_2 [µmol mol^{-1}]								
		chamber	Δt^a ($t_{in} - t_{out}$) inside tubes		chamber	inside tubes		chamber	shoot multiplication – light period				root induction – light period			
			lower leaves	upper leaves		lower leaves	upper leaves		0 h	1.5 h	4 h	6 h	0 h	1.5 h	4 h	6 h
Loose	0	20.0 ± 0.1	-0.0 ± 0.1	0.0 ± 0.2	935 ± 55	50.6 ± 5.1	52.0 ± 2.0	—	—	—	—	—	—	—	—	—
	50	20.1 ± 0.1	1.9 ± 0.4	2.4 ± 0.3	964 ± 81	78.6 ± 8.8	97.2 ± 9.0	380	5600 ± 1119	673 ± 400	131 ± 34	43 ± 5	7531 ± 1169	2820 ± 796	1355 ± 445	80 ± 3
	110	20.7 ± 0.2	5.8 ± 0.5	6.5 ± 0.3	1049 ± 47	207.2 ± 8.4	245.7 ± 9.2	380	7267 ± 2250	360 ± 149	132 ± 35	61 ± 8	5492 ± 1226	695 ± 545	197 ± 34	63 ± 6
Tight	0	20.0 ± 0.1	-0.1 ± 0.2	0.0 ± 0.1	935 ± 55	58.0 ± 2.0	77.8 ± 11.2	—	—	—	—	—	—	—	—	—
	50	20.1 ± 0.1	0.5 ± 0.4	2.0 ± 0.3	964 ± 81	130.6 ± 17.8	180.5 ± 35.8	380	1271 ± 153	339 ± 58	327 ± 89	288 ± 39	1013 ± 57	559 ± 49	391 ± 56	321 ± 33
	110	20.7 ± 0.2	4.5 ± 0.5	6.0 ± 0.4	1049 ± 47	235.8 ± 26.1	290.1 ± 22.6	380	1154 ± 56	276 ± 35	236 ± 43	248 ± 26	1152 ± 53	309 ± 29	305 ± 37	299 ± 33

Table 5. Effects of sucrose concentration, PPFD, and tube closure on maximum photosynthetic rate (P_{max}) and dark respiration rate (R_D) [μmol(O_2) kg^{-1}(d.m.) s^{-1}] of gardenia leaflets developed during shoot multiplication and root induction. Values are means ± SE of 6-10 samples, each sample consisting of at least two leaflets. n.s., no significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Treatment		Shoot multiplication		Root induction	
		R_D	P_{max}	R_D	P_{max}
Sucrose [%]	3.0	29.23±1.40	111.59±10.50	15.17±0.75	92.49±9.84
	0.5	35.66±1.47	133.60±11.87	14.58±0.69	78.58±9.84
	0.0	20.96±1.88	154.18±25.17	18.52±0.79	173.04±19.27
	significance	***	n.s.	n.s.	n.s.
PPFD [μmol m^{-2} s^{-1}]	110	30.05±1.28	182.43±10.99	16.93±0.59	146.93±9.84
	50	27.18±1.24	83.81±10.28	15.25±0.62	82.48±8.76
	significance	n.s.	***	n.s.	**
CO_2 (tube closing)	loose	33.21±1.18	142.19±9.87	16.26±0.57	116.94±8.76
	tight	24.02±1.37	124.06±11.55	15.91±0.64	112.47±9.84
	significance	**	n.s.	n.s.	n.s.
Sucrose × PPFD		n.s.	n.s.	n.s.	*
Sucrose × CO_2		**	n.s.	n.s.	n.s.
PPFD × CO_2		***	*	n.s.	n.s.
Sucrose × PPFD × CO_2		n.s.	n.s.	**	n.s.

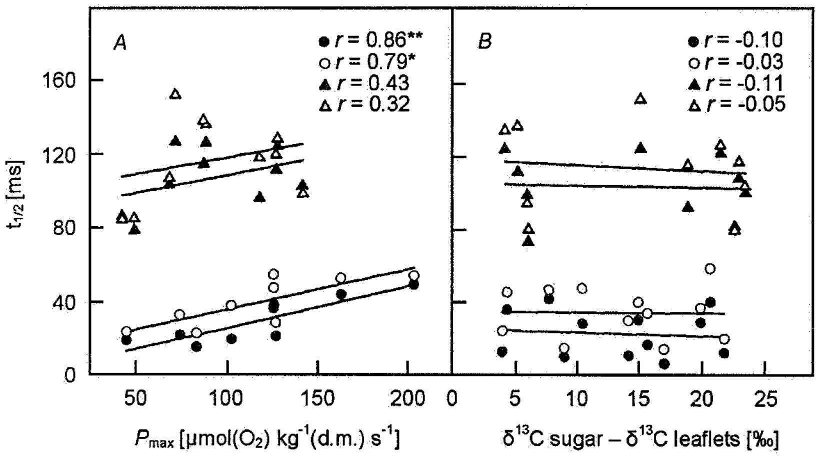

Fig. 1. *A*: Relationship between P_{max} and the half time required for fluorescence to increase from F_0 to F_m ($t_{1/2}$). *B*: Relationship between the degree of photoautotrophy and $t_{1/2}$. Within each culture stage, the culture conditions were plotted together, each point representing the mean value for a given combination of PPFD, sucrose, and tube closure. *Open and closed symbols* correspond to $t_{1/2}$ measurements performed just before the beginning of the irradiation (dark) and after 6 h of photoperiod (light), respectively. *Circles* and *triangles* correspond to measurements performed during shoot multiplication and root induction, respectively. For details see Materials and methods.

in *Rosa hybrida* (Genoud-Gourichon *et al.* 1996), whereas the sucrose content enhanced this rate in tobacco (Tichá *et al.* 1998) and coffee (Nguyen *et al.* 1999).

For each culture stage, the mean P_{max} and R_D values for each culture condition were plotted together (*i.e.*, 12 combinations) and linked to the degree of photoautotrophy attained in the same type of leaflets (as reported in Serret *et al.* 1997). No significant correlation was observed between P_N rates expressed either per unit dry mass or per Chl content and the degree of photoautotrophy at any stage. Moreover, no correlation was observed between R_D (either per unit dry mass or Chl content) and the degree of photoautotrophy for any of the two growing stages. Previous studies suggest that instantaneous or even daily patterns of P_{max} only provide a poor estimate of the overall (*i.e.*, integrated in time) contribution of photosynthesis to the carbon balance of a plantlet *in vitro* (Kozai *et al.* 1990, Kozai 1991, Jeong *et al.* 1993, Hdider and Desjardins 1994).

Photosynthesis and Chl fluorescence: We studied the correlation between P_{max} and Chl fluorescence parameters in the same type of leaflets within each culture stage. P_{max}

expressed either per unit dry mass or on the basis of Chl content did not correlate ($p < 0.05$) with either F_v/F_0, F_v/F_m, F_m, or F_0 within each culture stage. Babani and Lichtenthaler (1996) reported a strong positive linear correlation between P_{max} (expressed per unit leaf area) and F_v/F_0 only at a given threshold in the degree of greening of etiolated barley leaves, whereas no correlation with F_v/F_m was observed. On the other hand, P_{max} per unit dry mass was positively correlated ($p < 0.05$) with $t_{1/2}$ during shoot multiplication (Fig. 1). Both parameters were also positively related during root induction, but no statistical significance was attained. The positive correlation between P_{max} and $t_{1/2}$ may be mostly due a common response of both traits to higher PPFD during culture. Thus as PPFD increased, not only P_{max} (Table 5) but also $t_{1/2}$ (Tables 1 and 2) increased. Indeed, PPFD was the only factor with a significant effect on P_{max} per unit dry mass during both stages *in vitro*, and it also had the largest effect on $t_{1/2}$ during shoot multiplication. The parameter $t_{1/2}$ is a function of the rate of photochemical reactions and the pool size of electron acceptors on the reducing site of PS2, including the plastoquinone pool (Öquist and Wass 1988, Bolhàr-Nordenkampf *et al.* 1989). An increase in $t_{1/2}$ is expected during acclimation to higher irradiance (Araus and Hogan 1994 and references herein).

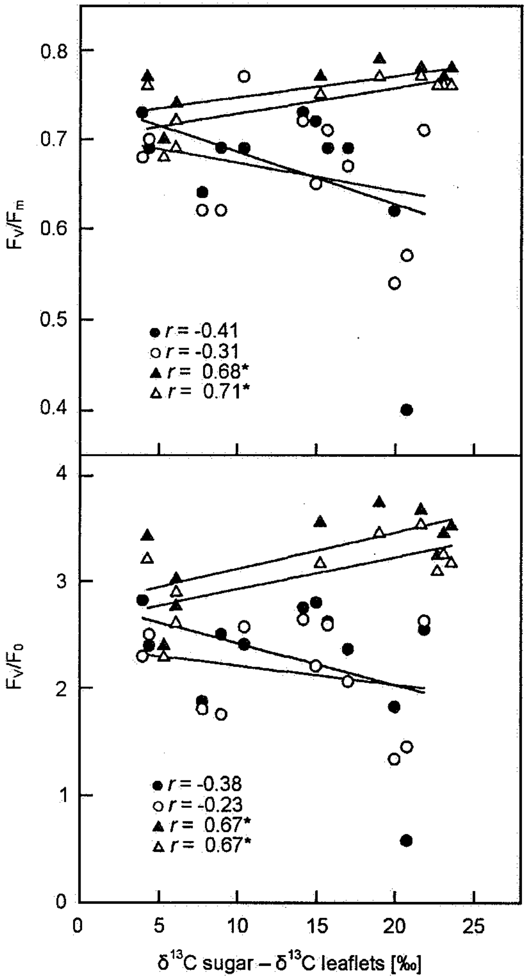

Fig. 2. Relationships between the degree of photoautotrophy and (F_v/F_m) or (F_v/F_0). *Open and closed symbols* represent F_v/F_m and F_v/F_0 measurements just before the beginning of irradiation (dark) and after 6 h of photoperiod (light), respectively. *Circles and triangles* correspond to measurements performed during shoot multiplication and root induction, respectively. For details see Materials and methods.

Degree of photoautotrophy and Chl fluorescence: No correlation between the degree of photoautotrophy and $t_{1/2}$ was found at any of the two micropropagation stages (Fig. 1), further supporting the lack of similarities between the developmental pattern of acclimation to irradiance and that of photoautotrophy. During root induction, the degree of photoautotrophy was positively correlated ($p < 0.05$) with the ratios F_v/F_m and F_v/F_0 (Fig. 2). However, such relationship tended to be negative during shoot multiplication, although not significantly. The positive correlations between photoautotrophy and F_v/F_m and F_v/F_0 during root induction seem to disagree with the pattern deduced from Table 2, according to which the plantlets showing the highest photoinhibition are those cultured under photoautotrophy-inducing conditions (low sucrose and high PPFD). However, there is no such contradiction. Thus, whereas each point plotted in the figures represents a particular combination of PPFD, sucrose, and tube closure, the values in the table represent the means of all leaflets sharing a given treatment. Moreover, no interaction between sucrose, PPFD, and cap permeability was found during shoot multiplication (Table 1), whereas these culture factors showed a highly significant interaction during root induction (Table 2).

In conclusion, the above results suggest that during the late stage of micropropagation, plantlets with a higher degree of photoautotrophy are less photoinhibited, whereas the opposite pattern seems to occur at the earlier stage. Results on chloroplast development of gardenia during shoot multiplication may agree with the negative effect of photoautotrophy-inducing culture conditions (Serret and Trillas 2000). Photoautotrophy may be beneficial, *i.e.*, enhance the photosynthetic performance and the further acclimation to *ex vitro* conditions (Serret *et al.* 2001) only at the late stage of the culture *in vitro*.

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