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The effect of *in vitro* culture conditions on the pattern of photoinhibition during acclimation of gardenia plantlets to *ex vitro* conditions

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Abstract

We tested the effect of growing conditions during micropropagation on the fast kinetics of chlorophyll (Chl) fluorescence of *Gardenia jasminoides* Ellis plantlets during a 4-week acclimation to *ex vitro*. We studied whether photoautotrophic growing *in vitro* produced plantlets with less photoinhibition impairment during acclimation. Of the growing conditions stimulating photoautotrophy *in vitro*, only loose tube caps had a positive effect, whereas low sucrose or sucrose-free content in the medium and high PPFD showed a negative effect. Thus, plantlets cultured with 3 % (m/v) of sucrose were subsequently less photoinhibited throughout acclimation than those cultured with low sucrose (0.5 %) or sucrose-free media. Moreover, at the end of acclimation the former plantlets showed F_v/F_m and F_v/F_0 ratios typical of unstressed *ex vitro* plants as well as a higher Chl content and ratio of Chls to carotenoids. Plantlets cultured at a photosynthetic photon fluence density (PPFD) of 50 µmol m^{-2} s^{-1} also showed a better performance at the end of acclimation than those cultured at a higher (110 µmol m^{-2} s^{-1}) PPFD. Thus except in the case of loose-tube closure, gardenia plantlets cultured *in vitro* under conventional sucrose concentration and PPFD are the least photoinhibited during acclimation. Nevertheless, significant interactions between the *in vitro* growing factors were observed at the end of acclimation.

Additional key words: carotenoids; chlorophylls; CO_2; fluorescence induction; *Gardenia jasminoides*; irradiance; micropropagation; photosynthetic pigments; saccharose.

Introduction

In the past, *in vitro* plantlets were considered to have a low photosynthetic ability to provide a positive carbon balance and therefore to require sugar as a carbon and energy source for their hetero- or mixotrophic growth (Grout and Aston 1978). More recent research, however, has revealed that *in vitro* plantlets may substantially improve their degree of photoautotophy, provided they are under growing conditions which favour photosynthesis (Kozai 1991a,b, Kozai *et al.* 1997, Seko and Nishimura 1996, Serret *et al.* 1996, 1997).

The photosynthetic ability of plants *in vitro* may favour further acclimation to *ex vitro* conditions (Kozai *et al.* 1990, Kozai 1991a,b, Murphy *et al.* 1998). For example, for different species an increase in CO_2 availability within culture vessels led not only to a significant improvement in growth *in vitro* but also to improved rates of survival and growth following transfer *ex vitro* (Genoud-Gourichon *et al.* 1996, Solárová and Pospíšilová 1997, Murphy *et al.* 1998). Thus, it has been postulated (but not demonstrated) that enhancement of *in vitro* photoautotrophic growth is necessary to avoid (or at least alleviate) the potential photoinhibition of plantlets during acclimation (Dubé and Vidaver 1992, Hdider and Desjardins 1994). However, results of other authors do not confirm that a more photoautotrophic plantlet is more successful in overcoming transplantation shock during acclimation (Debergh *et al.* 1992, Pospíšilová *et al.* 1997). Indeed, whereas Capellades *et al.* (1990a,b) showed that the least photoautotrophic plants of *Rosa multiflora* give the best results during acclimation, opposite results were reported by Genoud *et al.* (1996) working with *Rosa hybrida.*

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Abbreviations: Chl, chlorophyll; F_m, maximum chlorophyll fluorescence; F_0, ground fluorescence emission, when all reaction centres are open and photochemical quenching is minimal; F_v, variable chlorophyll fluorescence ($F_m - F_0$); $/F_v/F_m$, the ratio of variable to maximum chlorophyll fluorescence; F_v/F_0, the ratio of variable to ground chlorophyll fluorescence; PPFD, photosynthetic photon flux density; PS, photosystem; $t_{1/2}$, half-time of the increase from F_0 to F_m.

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The study of transient changes in Chl fluorescence of dark-adapted leaves over a period of time (Kautsky curve) provides information essential to the understanding of how photoinhibition impairment as a response to stress affects photosynthetic performance (see, *e.g.*, Trillas *et al.* 1995 and Pospíšilová *et al.* 1999 for studies on acclimation to *ex vitro*). Various fast-Chl fluorescence derived ratios, such as F_v/F_m and F_v/F_0, have been formulated. They are the ratios of variable Chl fluorescence ($F_v = F_m - F_0$) to either maximal (F_m) or ground (F_0) fluorescence. Any factor affecting the efficiency in capturing excitation energy by open photosystem 2 (PS2) reaction centres also modifies these ratios (Lichtenthaler and Rinderle 1988, Genty *et al.* 1989, Schreiber and Bilger 1993). F_v/F_m allows the evaluation of the photochemical efficiency of PS2 in the dark-adapted state with fully open PS2 reaction centres, and it is highly correlated with the quantum yield of net photosynthetic rate in intact leaves (Björkman and Demmig 1987, Demmig and Björkman 1987). As PS2 is particularly susceptible to photoinhibition (Powles 1984), the ratio F_v/F_m is widely used as an indicator in leaves. In contrast, the ratio F_v/F_0 provides a good assessment of potential photosynthetic capacity of leaves and of electron transport chain state and effectiveness (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996, Yordanov *et al.* 1997, Georgieva and Lichtenthaler 1999). In examining changes in the ratios F_v/F_m and F_v/F_0 it is essential to distinguish increases in F_0 from decreases in F_m. An increase in F_0 is characteristic of the destruction of PS2 reaction centres or the impairment of excitation energy transfer from the antenna to the reaction centres (Baker and Horton 1987, Björkman 1987, Bolhàr-Nordenkampf *et al.* 1989). Rintamäki *et al.* (1994) showed that the inhibition of D1-protein in the PS2 reaction centre leads to an increase in F_0. A decline in F_m may in turn indicate an increase in non-photochemical quenching (Baker and Horton 1987, Björkman 1987, Bolhàr-Nordenkampf *et al.* 1989).

This paper reports the effect of *in vitro* growing conditions on the pattern of the fast-Chl fluorescence impairment in gardenia during *ex vitro* acclimation. We tested whether the *in vitro* growing conditions, which induce more photoautotrophic plantlets, alleviate photoinhibitory impairment and therefore favour faster acclimation to *ex vitro* conditions.

Materials and methods

Plants and growth conditions: Micropropagated gardenia (*Gardenia jasminoides* Ellis) plantlets were cultivated during two successive culture stages (shoot multiplication and root induction) *in vitro* as reported by Serret *et al.* (1996, 1997). Three sucrose concentrations in the agar medium (3.0, 0.5, and 0 %, m/v), two PPFD (50±5 and 110±10 μmol m^{-2} s^{-1} at the top of the culture tubes) in the growth chamber (*E-15*, *Conviron*, Winnipeg, Manitoba, Canada), and two kinds of tube closure (tight and loose caps, the former having a half time for CO_2 retention more than 30 times higher than the second, Serret *et al.* 1997), were assayed during both stages. Temperature and CO_2 concentration inside the growth chamber were kept constant, around 20 °C and 750 μmol mol^{-1}, respectively, through a 12/12 h light/darkness period.

Acclimation took place in the same growth chamber as above over a period of 4 weeks. Plantlets from the root induction stage were transplanted to 55 cm^3 pots covered with transparent plastic film to prevent dehydration, and were then returned to the growth chamber under a 12-h photoperiod. The substrate was a mixture of peat : perlite (1 : 1, m/m). Pots were watered daily to field capacity. The plastic cover was gradually opened to provide a final relative humidity of 60 %. The PPFD was 65±3 μmol m^{-2} s^{-1} during the first week and afterwards it was progressively increased to 125±4 μmol m^{-2} s^{-1}. Temperature at plant level was kept constant (23±2 °C) throughout the light/darkness period with a 12-h photoperiod. The CO_2 concentration inside the growth chamber was 350 μmol mol^{-1}.

Chl fluorescence: Absolute values of the 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 ($t_{1/2}$) were determined on attached leaflets using a portable fluorimeter (Plant Stress Meter, *Biomonitor AB S.C.I.*, Umeå, Sweden; Bolhàr-Nordenkampf *et al.* 1989). We also calculated the ratios F_v/F_m and F_v/F_0. Measurements were taken on the last leaflet (fully) developed during micropropagation, on days 0, 1, 3, 7, 14, and 28 of acclimation. The PPFD, runtime, and dark adaptation period for all the measurements were 200 μmol m^{-2} s^{-1}, 5 s, and 30 min, respectively. Preliminary measurements of F_v/F_m done with a PPFD of 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 measuring irradiance does not change F_m. For each *in vitro* growth condition, measure-ments were taken each day on the adaxial side of 7-20 intact leaflets. These leaflets were evaluated twice during the photoperiod: first at the end of the dark period, and the second time after 6 h of irradiation.

Photosynthetic pigments: Leaflets used for Chl fluorescence determinations during acclimation were sampled on day 28. They were subsequently macerated with a small amount of 80 % (v/v) cold acetone and the Chl *a* and *b* and total carotenoid concentrations were determined spectrophotometrically according to the equations of Lichtenthaler (1987), as reported in Serret *et al.* (1996). Chl (*a*+*b*) content was expressed on a fresh mass basis. For each growth condition 5-6 samples (each containing 1-2 leaflets) were analysed. At the end of acclimation we also measured the Chl (*a*+*b*) content on a leaf area basis on the same kind of leaflets as those used for Chl fluorescence. We used a portable Chl meter (*SPAD-502, Soil-Plant Analysis Development Section, Minolta Camera Co.*, Osaka, Japan). For each growth condition about 10 leaflets were sampled.

Results and discussion

Pattern of photoinhibition during acclimation: Regardless of the previous *in vitro* growing conditions, all leaflets showed a fairly similar pattern in Chl fluorescence parameters during acclimation to *ex vitro* conditions, with most changes taking place during the second half (weeks three and four) of the period. Thus, F_v/F_m (Table 1), F_v/F_0, F_m, and to a lesser extent $t_{1/2}$, increased, whereas F_0 decreased (Fig. 1).

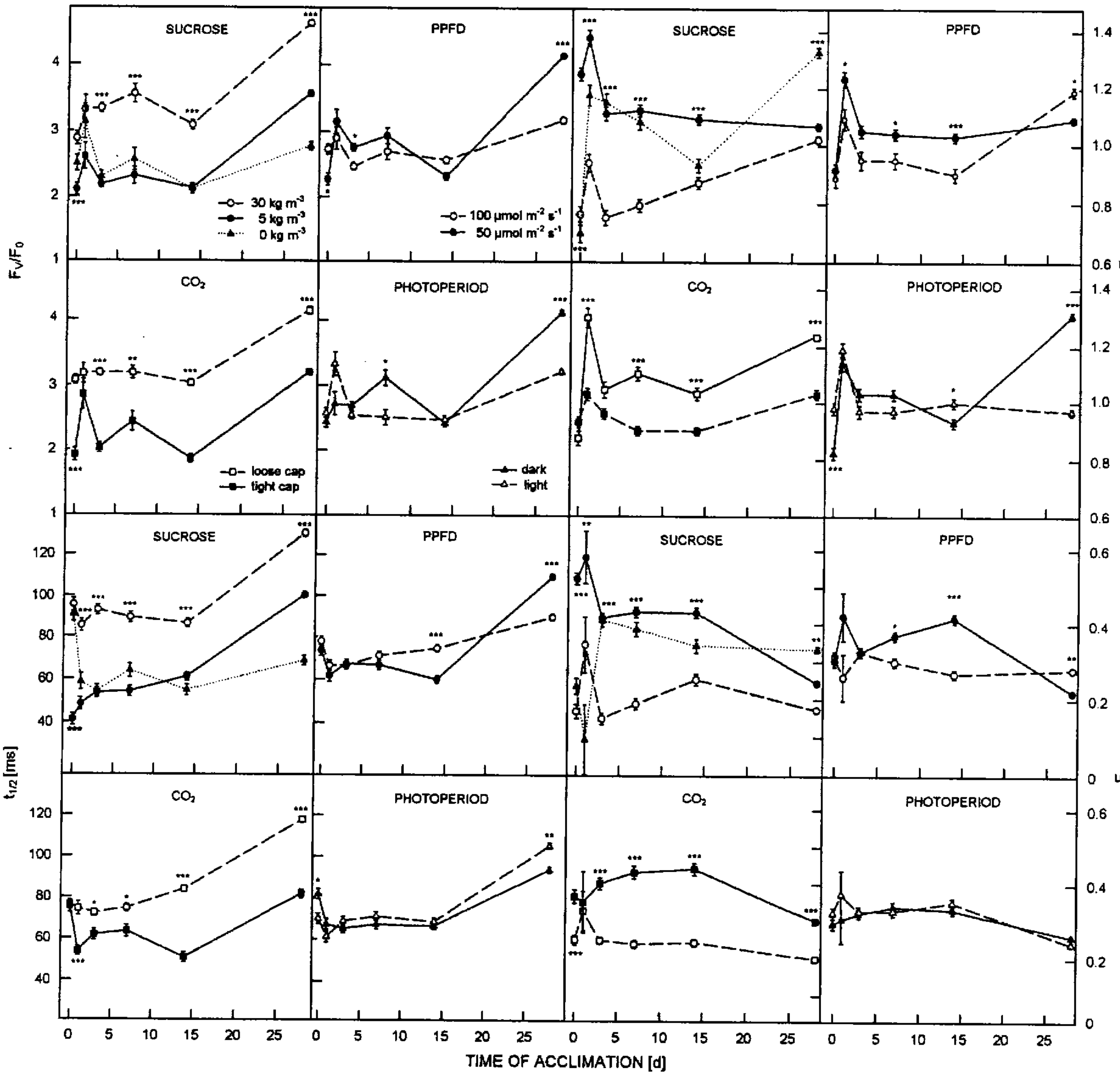

Fig. 1. Effect of sucrose concentration, PPFD, and tube closure during *in vitro* shoot multiplication and root induction on the Chl fluorescence parameters F_v/F_0, F_m, F_0, and $t_{1/2}$ measured on gardenia leaflets during acclimation to *ex vitro* conditions. Measurements were performed at the end of the dark period (dark) and after a 6 h of photoperiod (light), respectively. For more details see Materials and methods. Means ± SE of 7-20 leaflets. Levels of significance: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

The pattern of changes observed in Chl fluorescence parameters is typical of progressive acclimation to *ex vitro* conditions, with the initial photoinhibitory impairment being gradually alleviated (Jorba 1994, Trillas *et al.* 1995). Similar patterns of change may be a response of *ex vitro* plants to higher PPFD (Araus and Hogan 1994). Thus, in our study, while PPFD was progressively increased during acclimation, the fluorescence trait $t_{1/2}$, a simple indicator of the pool size of the electron acceptors on the reducing side of PS2 (Bolhàr-Nordenkampf *et al.* 1989) also increased. On the other hand, an increase in F_v/F_m and F_v/F_0 caused by a decline in F_0 would indicate lesser inactivation of PS2 reaction centres by photodamage, whereas when due to an increase in F_m, it would be the consequence of a decreased plant requirement for photoprotective (non-photochemical quenching) mechanisms (Kamaluddin and Grace 1992, Araus and Hogan 1994, Pearcy and Sims 1994, Georgieva and Lichtenthaler 1999).

Light/darkness-driven changes in fluorescence parameters were only significant at the end of acclimation to *ex vitro* conditions, when F_v/F_m, F_v/F_0, and F_m showed a transient decrease during the light period, whereas $t_{1/2}$ increased, and F_0 showed no changes. The short-term reversible changes in F_v/F_m and F_v/F_0 are associated with a photoprotective mechanism (decrease in F_m), rather than with a permanent photoinhibitory impairment associated with an increase in F_0 (Kamaluddin and Grace 1992, Araus and Hogan 1994, Pearcy and Sims 1994). During earlier acclimation, the steady (and lower) values of the ratios F_v/F_m and F_v/F_0 throughout the dark-light period might be the consequence of an incomplete development of the photoprotective mechanisms and/or due to permanent photoinhibitory impairment. Nevertheless, only relaxation experiments which determine the non-photochemical quenching of Chl fluorescence (q_N) and its three components, of which the photoinhibitory quench (q_I) is one, would be the final proof of photoinhibition (Lichtenthaler and Burkart 1999).

Table 1. Effects of sucrose concentration, PPFD, and tube closure during *in vitro* shoot multiplication and root induction on the Chl fluorescence ratio F_v/F_m, measured on gardenia leaflets during acclimation to *ex vitro* conditions. Measurements were performed at the end of the dark period (dark) and after a 6 h of photoperiod (light), respectively. For more details see Materials and methods. Means± SE of 7-20 leaflets. n.s., not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Growing conditions *in vitro*		Time of acclimation [d] 0	1	3	7	14	28
Sucrose [kg m^{-3}]	30	0.754±0.009	0.742±0.007	0.777±0.008	0.769±0.008	0.739±0.007	0.829±0.002
	5	0.601±0.008	0.650±0.007	0.642±0.007	0.630±0.008	0.637±0.006	0.768±0.002
	0	0.637±0.010	0.667±0.009	0.631±0.010	0.651±0.010	0.638±0.009	0.736±0.003
	significance	***	***	***	***	***	***
PPFD [μmol m^{-1}s^{-2}]	100	0.656±0.007	0.697±0.006	0.666±0.007	0.693±0.007	0.701±0.006	0.759±0.002
	50	0.672±0.007	0.675±0.006	0.700±0.007	0.673±0.007	0.641±0.006	0.797±0.002
	significance	n.s.	n.s.	*	n.s.	***	***
CO_2	loose tube cap	0.738±0.006	0.751±0.005	0.745±0.006	0.746±0.006	0.745±0.005	0.808±0.001
	tight tube cap	0.590±0.008	0.622±0.008	0.622±0.009	0.620±0.009	0.597±0.008	0.748±0.002
	significance	***	***	***	***	***	***
Photoperiod time	dark	0.656±0.007	0.699±0.006	0.676±0.007	0.684±0.007	0.671±0.006	0.801±0.002
	light	0.672±0.007	0.674±0.006	0.690±0.007	0.682±0.007	0.672±0.006	0.755±0.002
	significance	n.s.	n.s.	n.s.	n.s.	n.s.	***
Interactions	sucrose×PPFD	n.s.	n.s.	***	***	n.s.	n.s.
	sucrose×CO_2	***	*	***	***	***	***
	sucrose×time	n.s.	n.s.	n.s.	n.s.	n.s.	***
	PPFD×CO_2	***	*	***	n.s.	***	***
	CO_2×time	n.s.	n.s.	*	n.s.	n.s.	n.s.
	PPFD×time	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Effect of *in vitro* culture conditions: Significant differences in the absolute values of these Chl fluores-cence parameters were observed throughout acclimation, depending on the previous *in vitro* growing conditions. Sucrose content in the medium and the kind of tube closure were the *in vitro* factors which had the greatest effect on Chl fluorescence during acclimation, whereas PPFD had the least effect. Thus, higher sucrose in the medium and loosely closed tubes *in vitro* produced leaflets with significantly higher F_v/F_m, F_v/F_0, and $t_{1/2}$, and lower F_0, throughout the acclimation to *ex vitro* conditions, until day 28. F_m showed a different pattern depending on the treatment. It decreased for leaflets grown at higher sucrose concentrations and increased in those from loosely closed tubes. PPFD treatment *in vitro* had no effect on Chl fluorescence traits at the beginning of acclimation, although on day 28 low-PPFD leaflets resembled those grown in loosely closed tubes.

Chl content per unit fresh mass at the end of acclimation (day 28) was significantly higher in plants previously cultured in media with a high sucrose content (Table 2). The same positive effect of high sucrose concentration was observed on Chl content per unit leaf area. The other two *in vitro* growing factors (PPFD and tube closure) had no significant effect on Chl content either per fresh mass or per unit leaf area during acclimation. Leaflets in high sucrose media also showed a higher Chl (a+b) to carotenoids (x+c) ratio. Other significant effects due to *in vitro* culture conditions were not observed either in the ratio (a+b)/(x+c) or in the Chl a/b ratio.

The improved photosynthetic performance of some of the plantlets during acclimation should be a consequence of their previous *in vitro* development. Therefore, plantlets less photoinhibited during micropropagation, due to the *in vitro* growing conditions, might perform better during acclimation (see Van Huylenbroeck and Deberg 1996, Genoud-Gourichon *et al.* 1999). Thus, the cultivation of gardenia plantlets in loosely sealed tubes diminished photoinhibition and increased photoautotrophy during micropropagation (Serret *et al.* 1997). Alternatively, the addition of some sucrose to the medium also alleviated photoinhibition and led to an increase in total Chl as well as in the ratio (a+b)/(x+c) of gardenia plantlets *in vitro*, even when these growing conditions induced less photoautotrophical development (Serret *et al.* 1996, 1997). Moreover, recent results show that the *in vitro* gardenia leaflets which had the most altered chloroplast ultrastructure were those cultured with low sucrose concentrations combined with low PPFD (Serret and Trillas 2000). For other species very low or zero sucrose content in the medium also affected photosynthesis negatively during acclimation to *ex vitro* conditions, inducing more photoinhibition (Capellades *et al.* 1990b, 1991, Desjardins 1995, Synková 1997). Alternatively, media with sucrose concentrations above 3 % may also induce more photoinhibition and lower Chl content during acclimation (Van Huylenbroeck and Debergh 1996 and references herein), even when growth is enhanced (Van Huylenbroeck and Debergh 1996, Voráčková *et al.* 1998).

Table 2. Effects of sucrose concentration, PPFD, and tube closure during *in vitro* shoot multiplication and root induction on chlorophyll, Chl (a+b) content on fresh mass (FM) basis, and the ratios Chl a/b and Chl/carotenoids [(a+b)/(x+c)] measured on gardenia leaflets after 4 weeks of acclimation to *ex vitro* conditions. Means ± SE of 5-6 samples, each containing 1-2 leaflets. Chl (a+b) content on leaf area basis [relative] on the same day as measured with a portable device are also shown. Means ± SE of about 10 leaflets and 3 measurements per leaflet. For more details see Materials and methods. N.s., not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

		Chl (a+b) [g kg^{-1}(FM)]	Chl a/b	(a+b)/(x+c)	Chl (a+b) [relative]
Sucrose [kg m^{-3}]	30	1.36±0.09	2.72±0.04	5.13±0.07	57.06±1.22
	5	1.09±0.05	2.81±0.04	4.89±0.08	37.55±1.17
	0	0.75±0.06	2.79±0.05	4.35±0.09	35.10±1.50
	significance	*	n.s.	*	***
PPFD [μmol m^{-2} s^{-1}]	100	0.98±0.08	2.79±0.03	4.82±0.06	39.52±1.02
	50	1.15±0.08	2.76±0.03	4.76±0.07	46.95±1.06
	significance	n.s.	n.s.	n.s.	**
CO_2	loose tube cap	1.27±0.07	2.73±0.05	4.69±0.09	47.45±0.87
	tight tube cap	0.86±0.11	2.82±0.03	4.90±0.05	39.02±1.40
	significance	n.s.	n.s.	n.s.	**
Interactions	sucrose×PPFD	n.s.	n.s.	n.s.	n.s.
	sucrose×CO_2	n.s.	n.s.	n.s.	*
	PPFD×CO_2	n.s.	n.s.	n.s.	**

Nevertheless, significant interactions between the *in vitro* growing factors were observed at the end of acclimation (Table 1). The two factors showing the strongest interactions were sucrose and tube closure, followed by the interactions of PPFD with the other two growing conditions. These results are consistent with the finding that plantlets grown in low sucrose medium *in vitro* showed reduced susceptibility to photoinhibition during acclimation when cultured in loosely closed tubes or under high PPFD. In this regard a positive effect of higher CO_2 availability and PPFD (combined with a sucrose-free medium) during *in vitro* culture on further growth during acclimation of plantlets to *ex vitro* was reported for *Rosa hybrida* (Genoud-Gourichon *et al.* 1996). In order to be expressed during acclimation, the interactions between growing factors must already have been present during micropropagation. Thus significant interactions between a higher PPFD and a lower sucrose concentration in the medium in plantets during micropropagation have also been reported, improving Chl fluorescence response (Serret *et al.* 1996) and the development of chloroplast ultrastructure (Serret and Trillas 2000).

Duration of the acclimation period: Values of F_v/F_m typically range between 0.75-0.85 for non-stressed *ex-vitro* leaves (Bolhàr-Nordenkampf *et al.* 1989). In our experiment, on day 28 leaflets from high sucrose medium and loosely sealed tubes showed F_v/F_m values of more than 0.80, which indicates the absence of photoinhibitory impairment, whereas for the other leaflets, with values around 0.75, some degree of stress was still present. Nevertheless, whatever *in vitro* treatment was considered, this ratio at the end of the acclimation was higher than that reported *in vitro* (Serret *et al.* 1996). A higher F_v/F_m ratio after acclimation than existed during in *vitro* culture has also been reported for other species (Trillas *et al.* 1995, Rival *et al.* 1997). An acclimation to *ex vitro* conditions of 3-4 weeks is a common procedure in gardenia cultivation, and is comparable to that used for acclimation *ex vitro* in many other herbaceous plants (*e.g.*, Van Huylenbroeck and Debergh 1996, Synková 1997, Pospíšilová *et al.* 1999). Indeed, Van Huylenbroeck and Debergh (1996), working with *Spathiphyllum* concluded that it is of secondary importance during further acclimation to *ex vitro* conditions whether plantlets are photosynthetically active at transplantation. Thus, no differences in F_v/F_m or other Chl fluorescence parameters between plantlets grown under different *in vitro* conditions are present after several weeks of acclimation to *ex vitro* conditions (Van Huylenbroeck and Debergh 1996).

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