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Photosynthetic parameters of *Ulmus minor* plantlets affected by irradiance during acclimatization

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Abstract

In order to set up large-scale acclimatization protocols of micropropagated plants, an in-depth knowledge of their physiological responses during *in vitro* to *ex vitro* transfer is required. This work describes the photosynthetic performance of *Ulmus minor* micropropagated plants during acclimatization at high irradiance (HI; 200 ± 20 μmol m^{-2} s^{-1} or low irradiance (LI; 100 ± 20 μmol m^{-2} s^{-1}). During this experiment, leaf pigment content, chlorophyll *a* fluorescence, gas exchange, stomata morphology, the activity of the Calvin cycle enzymes, and content of saccharides were measured in persistent and new leaves. The results indicated that HI induces a higher photosynthetic performance compared to LI. Therefore, plants acclimatized under HI are likely to survive better after field transfer.

Additional key words: Calvin cycle enzymes, chlorophyll, elm, fluorescence, micropropagation, net photosynthetic rate, stomata, transpiration rate.

Introduction

The advantages of tissue culture for the rapid production of high-quality plants are widely recognized (Park 2002). Large-scale micropropagation is particularly useful in commercial plantation forestry (Park 2002, Merkel and Nairn 2005). Nowadays, improvement programs can be found for *Eucalyptus* species (*e.g.* Pinto *et al.* 2008, 2011), *Pinus* and *Picea* species (Park 2002), and *Olea maderensis* (Brito *et al.* 2009). However, for the large-scale acclimatization of micropropagated species, processes that are particularly sensitive during this stage (*e.g.* plant-water relations and photosynthesis) must be well understood in order to maximize survival rates.

*In vitr*o plants are grown in a culture medium with a large concentration of sugars, under low irradiance, high air humidity and low CO_2 concentrations. Once transferred to *ex vitro* conditions, the plants are very susceptible to several stresses since they have not developed adequate anatomical, morphological, and physiological features required to survive in the new environment (Pospíšilová *et al.* 1999, Pinto *et al.* 2011). During *in vitro* culture, plantlets usually show low or undetectable net photosynthetic rate (*e.g.* Fuentes *et al.* 2005, Osório *et al.* 2005, Rybczyński *et al.* 2007). The low amount and activity of RuBisCO seems to be one of the major constraints in *in vitro* culture (Hdider and Desjardins 1995). Fila *et al.* (2006) also point to the low CO_2 mesophyll conductance of *in vitro* plants as an important limitation for their photosynthesis. In addition, the deficient functioning of the overall water house-keeping system (*e.g.* poor stomatal control and cuticular abnormalities) also contributes to the problems occurring after *ex vitro* transfer (Pospíšilová *et al.* 1999, Hazarika 2006).

Ulmus spesies belong to Europe´s noble hardwoods.

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Abbreviations: Car - carotenoids; Chl - chlorophyll; DM - dry mass; c_i/c_a - ratio of intercellular to atmospheric CO_2 concentration; E - transpiration rate; F_v/F_m - variable to maximum chlorophyll fluorescence (maximal efficiency of PSII); g_s - stomatal conductance; HI - high irradiance; LI - low irradiance; P_N - net photosynthetic rate; PSII - photosystem 2; qN - non-photochemical quenching; qP - photochemical quenching; RH - relative humidity; RuBisCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; sFBPase - stromal fructose-1,6-bisphosphatase; Φ_{PSII} - effective quantum efficiency of PSII.
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However, the elm population has been in sharp decline since the beginning of the 20th century due to Dutch elm disease (Dunn 2000). There have been efforts at germplasm preservation and breeding programs involving biotechnological strategies (Harvengt *et al.* 2004), and research dealing with *in vitro* micropropagation of *U. minor* has been successfully conducted (Conde *et al.* 2004, 2008, Dias *et al*. 2011). However, plant performance during acclimatization was only evaluated through the survival rate under a single irradiance (Conde *et al.* 2008). However, there have been no tests to ascertain whether plant performance might improve using a higher irradiance during acclimatization.

The goal of this study is to fill this knowledge gap. In micropropagated *U. minor* plants, leaf pigments, chlorophyll *a* fluorescence, gas exchange parameters, stomata morphology, Calvin cycle enzymes, soluble sugars, and starch were studied during acclimatization under two irradiances. To our knowledge, this is the first comprehensive report that covers such a wide range of physiological parameters by taking into account two factors: irradiance and leaf origin.

Materials and methods

Explants were obtained from a micropropagated *in vitro* culture collection of *Ulmus minor* Mill. *In vitro* cultures were kept in closed transparent glass vessels in a growth chamber at a temperature of 22 ± 2 ºC, a 16-h photoperiod and a photosynthetic photon flux density (PPFD) of 50 ± 10 µmol m^{-2} s^{-1}. Rooted plantlets of a height about 8 cm with at least 2 roots (5 - 10 cm long) and 7 to 9 open leaves were transplanted to 400-cm^3 pots containing an autoclaved mixture of peat and *Perlite* (3:2, v:v). Plants were acclimatized as described by Conde *et al.* (2008). Irradiation was provided by eight cool white fluorescent lamps (*Osram*, Munich, Germany) and PPDF at the plant level was either 200 ± 20 µmol m^{-2} s^{-1} (high irradiance, HI) or 100 ± 20 µmol m^{-2} s^{-1} (low irradiance, LI). These irradiances were achieved by changing the distance of plants from the light source. The LI corresponded to irradiance used by Conde *et al.* (2008). According to a curve showing the relation of net photosynthetic rate to irradiance (data not shown) photosynthesis saturation was achieved at approximately 200 µmol $m^{-2}s^{-1}$. Therefore, a higher irradiance was not used in the acclimatization treatment to avoid possible photoinhibition. A relative humidity (RH) of 98 % was maintained inside the growth chamber for the first 7 d of acclimatization and decreased by 7 % every week to 5 %. Plants were watered weekly with Hoagland's solution (*Sigma*, Munich, Germany).

All parameters were measured at day 0 in *in vitro* leaves (immediately after the removal of the lid) and 7, 14, 25 and 40 d after *ex vitro* transfer in persistent leaves (those formed under *in vitro* conditions) and in the new leaves (formed after *ex vitro* transfer).

Pigments were extracted from the leaf disc in a cold acetone/50 mM Tris buffer (pH 7.8; 80:20, v:v) and centrifuged at 2 800 *g* for 5 min according to Sims and Gamon (2002). Absorbances at 470, 537, 647 and 663 nm were determined with a *Thermo Fisher Scientific* (Waltham, USA) spectrophotometer (*Genesys 10-uv S*). The contents of chlorophyll (Chl) *a*, (Chl *b*), and carotenoids (Cars) were calculated using the Sims and Gamon formulae (2002).

Chlorophyll *a* fluorescence parameters were measured on the adaxial side of the leaf using a pulse amplitude modulation system (*FMS 2*, *Hansatech Instruments*, Norfolk, England). Minimal fluorescence (F_0) was measured in 30 min dark-adapted leaves by applying a weak modulated light, and maximal fluorescence (F_m) was measured after applying a 0.7 s saturating pulse of white light (> 1 500 µmol m^{-2} s^{-1}) to the same leaves. In light-adapted leaves, steady-state fluorescence (F_s), maximal fluorescence (F'_m) after 0.7 s saturating pulse (>1 500 mol m^{-2} s^{-1}) and minimal fluorescence (F'_0) measured when actinic light was turned off, were determined. Definitions of fluorescence parameters (F_v/F_m, qP and Φ_{PSII}) were used as described by Van Kooten and Snel (1990) and non-photochemical quenching (qN) was calculated according to Walters and Horton (1991).

Leaf gas exchange measurements were performed using a portable infrared gas analyser (*LCpro+*, *ADC*, Hoddesdon, UK), operating in open mode under growth chamber conditions. Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and the ratio of intercellular to atmospheric CO_2 concentration (c_i/c_a) were estimated using the equations developed by Von Caemmerer and Farquhar (1981). Measurements were always performed in the middle of the daily photoperiod at growth temperature (24 ± 2 ºC) and atmospheric CO_2 concentration.

For scanning electron microscopy, leaf samples were fixed in 2.5 % (v/v) glutaraldehyde in a 1.25 % (m/v) piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPES) buffer (pH 7.4) and washed in PIPES in accordance with Pinto *et al.* (2011). Then, the tissues were fixed in 1 % (m/v) osmium tetroxide and dehydrated through a graded ethanol series. Dehydrated samples were put through a critical point dryer (*CPD 030*, Baltec, Cheshire, UK) using CO_2 as transition agent. Samples were coated with gold using a metalizer (*FFC-1100*, *Jeol*, Tokyo**,** Japan) at 1 100 - 1 200 V, 5 mA. Samples were observed in a scanning electronic microscope (*S4100*, *Hitachi*, Tokyo, Japan) at 20 kV.

For extraction of Calvin cycle enzymes, leaf samples were ground to a powder in a mortar with liquid nitrogen as described by Dias and Brüggemann (2010). Ribulose-1.5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) activity was measured as described by Lilley and Walker (1974). This assay followed NADPH oxidation measured spectrophotometrically at 340 nm.

Total activity was achieved after incubation in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min. Stromal fructose-1.6-bisphosphatase (sFBPase, EC 3.1.3.11) was assayed as described by Dias and Brüggemann (2007). This assay was coupled to NADP reduction measured spectrophotometrically at 340 nm.

Soluble sugars were extracted from frozen leaf discs with 80 % (v/v) of ethanol at 80 ºC for 20 min as described by Correia *et al.* (2005). Glucose, fructose, and sucrose were quantified using a spectrometric enzyme-coupled assay described by Jones *et al.* (1977) and starch was quantified in accordance with Stitt *et al.* (1978).

Each acclimatization treatment was repeated twice. All determinations were obtained with randomly-chosen plants. For each parameter, we considered 6 replications per treatment for pigments, Calvin cycle enzymes, sugars, and 8 replications per treatment for Chl *a* fluorescence and gas-exchange parameters. A two-way analysis of variance (*ANOVA*) was performed using the *Sigma Stat* program for *Windows* (*v. 3.1*) to determine the effects of irradiance, days of acclimatization, and their interaction. Differences between the means were compared using a Holm-Sidak test at a probability level of 0.05.

Results

After 40 d of acclimatization of *U. minor* plants under HI and LI, the survival rates were 88.9 and 86.3 %, respectively. Morphologically, both plants looked similar, with well-developed leaves and no necrosis or chlorosis. New leaves appeared after day 14 and reached full expansion after 25 d of acclimatization.

The content of Chl *a* increased significantly in persistent leaves under both treatments from day 0 to day 40 (Tables 1, 2). However, Chl *a* content under LI was significantly higher than under HI after day 7. The Chl *a* in new leaves increased significantly in both treatments, and the highest content was under LI ($P < 0.05$). A similar pattern was observed for Chl *b* in persistent leaves (Tables 1, 2). However, in new leaves, the Chl *b* increase was similar under HI and LI. The content of Cars increased significantly from day 0 to day 14 in persistent leaves and decreased significantly from day 25 to day 40 in both treatments (Tables 1, 2). After day 0, higher Cars content in persistent leaves was always under HI than under LI. Concerning the new leaves, a similar pattern of Cars decrease was observed between days 25 and 40 for both treatments (Tables 1, 2).

Irrespective of the treatment, maximal efficiency of photosystem II (PSII) measured as F_v/F_m increased in the persistent leaves significantly from day 0 to the end of the experiment (Tables 1, 2). There were differences in the new leaves under LI and HI: F_v/F_m of the former increased significantly from day 25 to day 40, whereas in the latter F_v/F_m remained constant. Persistent leaves under HI and LI showed a significant increase of effective quantum efficiency of PSII (Φ_{PSII}) from day 0 to day 14 and stabilized thereafter (Tables 1, 2). However, the values reported under HI were significantly higher than those under LI. Irrespective of treatment, the Φ_{PSII} increased significantly in the new leaves (Tables 1, 2) and new leaves developed under HI always showed significantly higher Φ_{PSII} than those under LI. The photochemical quenching (qP) in persistent leaves increased significantly from day 0 to day 7 and from day 25 to the end of the experiment (Tables 1, 2). Plants grown under HI showed a significantly higher qP than those grown under LI. Only new leaves under HI showed a significant increase in qP from day 25 to day 40. Non-photochmical quenching (qN) in persistent leaves under HI decreased significantly from day 0 to day 25 and stabilized thereafter. A similar pattern was also found for persistent leaves under LI, although this was less pronounced. A significant decrease of qN in new leaves was only observed under LI (Tables 1, 2).

U. minor plantlets showed measurable net photosynthetic rate (P_N) at day 0 (Table 1). Persistent leaves grown under HI showed a significant increase of P_N in the first 7 d of acclimatization and remained stable by day 25. After this period, a second increase was observed. However, in persistent leaves grown under LI, no significant changes were observed in P_N during the first 7 d of acclimatization, and by the end of the experiment, P_N values were similar to day 0. At the end of the experiment, 1.7 times higher P_N was observed in persistent leaves under HI than under LI. Also the new leaves under HI showed a significantly higher P_N than those under LI (Tables 1, 2). The ratio of internal to external CO_2 concentration (c_i/c_a) in persistent leaves at day 0 was approximately 1.0. After being transferred to *ex vitro* conditions, the persistent leaves under HI showed a gradual decrease of c_i/c_a during the whole period of acclimatization, whereas those under LI showed only a significant decrease until day 14 and stabilized thereafter. Between days 25 and 40, c_i/c_a ratio in new leaves decreased under both HI and LI, however, it was higher under LI than under HI (Tables 1, 2).

The highest values of stomatal conductance (g_s) and transpiration rate (E) in persistent leaves were observed at day 0. The both parameters decreased from day 0 to day 25. After this period, g_s and E increased significantly in persistent leaves under LI but remained constant under HI, while g_s and E in new leaves increased significantly. However, significantly higher g_s and E were always under HI than under LI (Tables 1, 2). *In vitro* leaves presented fully open stomata, in contrast with acclimatized leaves that presented reduced stomata aperture. Furthermore, the thickness of the cuticle increased under *ex vitro* conditions (Fig. 1).

Persistent leaves grown under HI showed a significant increase in maximal RuBisCO activity during the first 7 d of acclimatization and the activity stabilized thereafter.

Table 1. Pigment content (Chl *a*, *b* and Cars) [mg g $^{-1}$(DM)], Chl *a* fluorescence parameters (F_v/F_m, Φ_{PSII}, q_P, q_{NP}), P_N [µmol(CO_2) m^{-2} s^{-1}], g_s [mol m^{-2} s^{-1}], E [mmol(H_2O) m^{-2} s^{-1}], enzyme activities (RuBisCO and sFBPase) [µmol m^{-2} s^{-1}], and sugar content (glucose, fructose, sucrose, and starch) [µmol mg^{-1}(DM)] in persistent leaves and new leaves of *U. minor* plants during acclimatization at two irradiances (HI and LI). Values are means ± SD n = 6 - 8.

Parameter		Persistent leaves					New leaves	
		0 d	7 d	14 d	25 d	40 d	25 d	40 d
Chl *a*	HI	1.34±0.10	2.86±0.16	4.01±0.05	4.17±0.05	4.18±0.05	5.54±0.09	5.92±0.07
	LI	1.34±0.10	3.04±0.14	4.34±0.09	4.57±0.05	4.87±0.11	5.89±0.09	6.50±0.05
Chl *b*	HI	0.69±0.13	1.36±0.09	2.05±0.03	2.33±0.04	2.42±0.08	2.78±0.07	3.06±0.15
	LI	0.69±0.13	1.61±0.09	2.58±0.08	2.70±0.17	2.71±0.04	2.84±0.09	3.10±0.04
Cars	HI	0.52±0.05	1.51±0.18	2.20±0.10	2.07±0.03	1.65±0.13	2.21±0.11	1.10±0.11
	LI	0.52±0.05	1.80±0.09	2.45±0.17	2.49±0.07	2.29±0.11	2.00±0.09	0.98±0.05
F_v/F_m	HI	0.84±0.03	0.89±0.04	0.88±0.04	0.91±0.01	0.91±0.01	0.90±0.01	0.89±0.01
	LI	0.84±0.04	0.89±0.01	0.88±0.01	0.90±0.01	0.91±0.01	0.89±0,01	0.90±0.01
Φ_{PSII}	HI	0.12±0.02	0.17±0.03	0.31±0.03	0.28±0.02	0.32±0.03	0.23±0.01	0.33±0.02
	LI	0.12±0.02	0.15±0.02	0.19±0.02	0.17±0.01	0.19±0.02	0.14±0.01	0.21±0.01
qP	HI	0.20±0.03	0.34±0.01	0.36±0.01	0.38±0.02	0.48±0.03	0.34±0.02	0.45±0.0
	LI	0.19±0.03	0.25±0.03	0.28±0.03	0.32±0.2	0.37±0.04	0.27±0.03	0.28±0.01
qN	HI	1.34±0.14	1.00±0.11	0.85±0.04	0.46±0.03	0.49±0.02	0.78±0.03	0.73±0.02
	LI	1.34±0.14	1.01±0.08	1.00±0.08	0.72±0.02	0.56±0.02	0.81±0.03	0.65±0.03
P_N	HI	1.77±0.54	2.77±0.72	3.50±0.34	3.35±0.25	4.18±0.75	3.15±0.37	8.08±0.66
	LI	1.77±0.54	2.34±0.43	1.32±0.31	0.88±0.20	2.50±0.57	1.77±0.37	4.56±0.49
g_s	HI	0.71±0.11	0.28±0.08	0.17±0.05	0.08±0.01	0.08±0.01	0.10±0.02	0.16±0.01
	LI	0.71±0.11	0.19±0.04	0.07±0.05	0.05±0.02	0.09±0.01	0.06±0.01	0.09±0.01
E	HI	4.54±0.27	2.36±0.21	1.91±0.28	1.09±0.16	1.09±0.10	1.26±0.19	2.26±0.09
	LI	4.54±0.27	1.96±0.09	0.96±0.09	0.60±0.08	1.44±0.20	0.80±0.12	1.42±0.08
c_i/c_a	HI	0.99±0.02	0.95±0.02	0.89±0.03	0.85±0.02	0.74±0.04	0.85±0.03	0.76±0.01
	LI	0.99±0.02	0.94±0.02	0.90±0.02	0.92±0.04	0.87±0.02	0.88±0.02	0.82±0.01
RuBisCO	HI	3.98±0.28	6.28±0.37	6.17±0.59	6.34±0.53	7.06±0.66	7.46±1.05	12.4±1.71
	LI	3.98±0.28	3.98±0.17	4.39±0.47	4.07±0.53	5.38±0.50	5.58±0.38	6.57±0.36
sFBPase	HI	5.20±0.72	7.32±0.87	7.63±0.66	6.67±0.68	8.80±0.98	8.59±1.20	11.6±1.10
	LI	5.20±0.72	5.04±0.56	4.45±0.85	4.48±0.57	4.70±0.74	4.22±0.46	8.13±1.18
Glucose	HI	12.3±1.02	17.3±10.9	5.57±0.50	8.60±0.55	7.51±1.20	8.19±1.06	4.49±0.50
	LI	12.3±1.02	4.66±0.55	4.60±0.81	6.75±0.56	6.57±0.65	8.44±0.93	4.14±0.45
Fructose	HI	8.39±1.81	14.6±1.50	4.63±0.59	7.07±0.83	8.79±0.96	7.35±0.56	5.72±0.39
	LI	8.39±1.81	3.61±0.70	3.53±0.96	7.43±1.25	8.00±0.82	10.1±0.80	6.43±0.57
Sucrose	HI	127 ±9.51	106 ±9.17	50.0±3.15	59.8±2.16	83.8±3.89	36.6±0.56	24.4±1.29
	LI	127 ±9.51	31.9±4.65	30.7±2.13	64.2±9.08	70.6±2.03	61.5±8.10	28.9±3.26
Starch	HI	2.76±0.41	2.91±0.14	1.61±0.16	1.85±0.33	2.12±0.24	1.61±0.28	1.15±0.12
	LI	2.76±0.41	3.69±0.24	1.93±0.29	2.27±0.29	2.90±0.35	1.89±0.50	1.05±0.13

Moreover, RuBisCO activity in persistent leaves was always significantly higher under HI than under LI. In persistent leaves grown under LI, a significant increase of RuBisCO activity was only found after day 25. At the end of the experiment, persistent leaves grown under HI and LI showed 1.8 and 1.3 times higher RuBisCO activity than at day 0, respectively. RuBisCO activity increased in the new leaves grown under HI (Tables 1, 2). The sFBPase activity in persistent leaves grown under HI increased significantly during the first 7 d and stabilized thereafter (Table 1). This parameter was significantly lower in persistent leaves grown under LI remaining constant during the whole experiment. In new leaves, irrespective of treatment, a similar pattern of sFBPase activity increase was observed. However, new leaves under HI showed a significantly higher sFBPase activity than those under LI.

Glucose and fructose contents in persistent leaves under HI increased from day 0 to day 7 and decreased from day 7 to day 14 (Tables 1, 2). In contrast, contents of both sugars under LI decreased from day 0 to day 7 but at the end of the experiment, fructose content reached similar values to those observed at day 0. Concerning new leaves, there was a similar decrease in glucose concentrations in plants under HI and LI (Table 2). Fructose content decreased more in leaves of plants grown under HI than under LI (Table 1). Sucrose content in persistent leaves of HI decreased from day 0 to day 14 and increased thereafter (Tables 1, 2). In persistent leaves, the decrease of sucrose from day 0 to day 7 was

higher under LI than HI. The content of sucrose in new leaves decreased significantly and at end of the experiment, it was similar under both treatments (Tables 1, 2). Starch content increased from day 0 to day 7 in persistent leaves under HI (Tables 1, 2), while under LI, no significant variations were observed during the same period. After a significant decrease from day 7 to day 14, starch content increased significantly until day 40 in persistent leaves under both HI and LI. In new leaves, starch content decreased significantly and no significant differences were observed between treatments (Table 2).

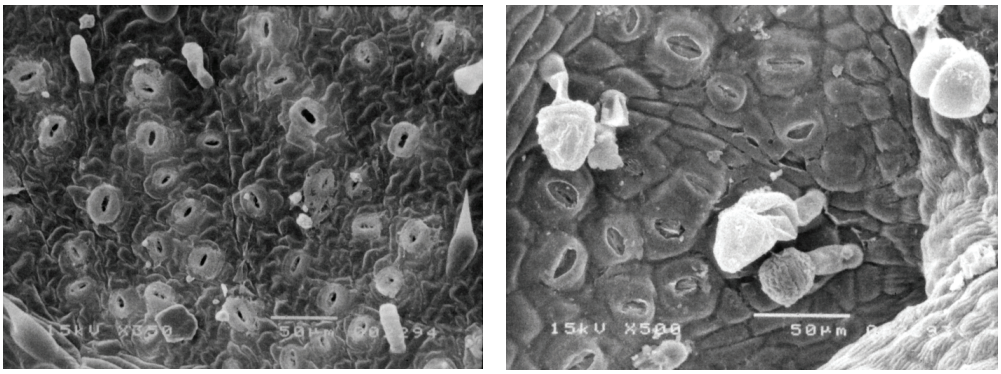

Fig. 1. Scanning electron microscope micrographs of *Ulmus minor* abaxial leaf side: *in vitro* leaf showing opened stomata (*left*); *ex vitro* acclimatized leaf showing closed stomata (*right*). *Bar*: 50 µm.

Discussion

In vitro conditions are often regarded as promoting heterotrophy (Hazarika 2006). However, the results reported in this work showed that micropropagated *U. minor* plants have a positive P_N at day 0. This suggests that plants under *in vitro* conditions may already perform some photosynthesis, supporting a mixotrophic nutrition. Low P_N was reported for several micropropagated species under *in vitro* conditions (*e.g.* Osório *et al.* 2005, Faisal and Anis 2009, Pospíšilová *et al*. 2009). According to Grout (1988), *in vitro* plants can be classified in two groups based on their photosynthetic response: the first group includes plants with leaves that are photosynthetically competent and able to adapt to autotrophic conditions; the second group includes plants with *in vitro* formed leaves that accumulate reserves to be consumed during the first days after *ex vitro* transfer (until new leaves appear) and which never become fully autotrophic. As suggested by our results, the different irradiance during acclimatization (HI and LI) induce different photosynthetic competence in *U. minor* persistent leaves. Persistent leaves under HI showed an enhancement of P_N directly after *ex vitro* transfer, assigning a switch to an autotrophic metabolism. In contrast, LI was less efficient in stimulating P_N, forcing these leaves to use the available reserves (mainly due to sucrose, glucose and fructose consumption) to fulfil all the energy demands. For these leaves, the increase of P_N at the end of the experiment may be explained by the re-establishment of the photosynthetic capacity up to complete autotrophy due to the formation of new leaves and the translocation of the assimilates from these leaves to the persistent ones (*e.g.* Amâncio *et al.* 1999, Van Huylenbroeck *et al.* 2000). This hypothesis is also supported by the high survival rate reported under LI treatment. New leaves showed a diffe-rent behaviour. The high P_N indicated that these leaves act as normal autotrophic leaves. Moreover, the reduced sugar content found in these leaves, as compared to persistent leaves, confirms that most of the sugars produced are exported to persistent leaves and new growing structures. Thus, new leaves act as a source of energy to other organs, contributing to plant growth. This observation is in agreement with the results obtained in *Calatea louisae* and *Spathiphyllum floribundum* (Van Huylenbroeck *et al.* 2000). As described for persistent leaves, HI also induced a higher P_N in these leaves than LI.

During acclimatization, stomatal control of water loss is critical for plant survival. In some species, *in vitro* leaves have stomata that were abnormally and continuously open (Hazarika 2006). *In vitro* leaves of *U. minor* showed fully open stomata, which corroborated the highest E values measured at day 0. However, after this critical initial period, the stomata were able to adapt to *ex vitro* conditions and functioned similarly as stomata formed in the new leaves. Also in micropropagated *Nicotiana tabacum* (Pospíšilová *et al.* 2009) and *Capsicum annuum* (Estrada-Luna *et al.* 2001), leaf stomata developed *in vitro* were functional after *ex vitro* transfer. Moreover, the formation of a wax cuticle in acclimatized *U. minor* plants may also contribute to the reduction of water loss similarly as mentioned by Pospíšilová *et al.* (1999).

Stomatal limitation did not affect the P_N of persistent leaves under HI. This may be explained by a probable increase in the mesophyll conductance to CO_2 diffusion,

Table 2. Two-way *ANOVA* for the content of Chl *a*, Chl *b*, and Car, F_v/F_m, Φ_{PSII}, q_P, q_{NP}, P_N, E, g_s, c_i/c_a, activity of RuBisCO and sFBPase, and content of glucose, fructose, sucrose and starch. The *F*-values and the respective probability level (*P*) for the effects of days (d), irradiance (I) and their interaction are shown for the different parameters studied.

	Variables	Persistent leaves		New leaves	
		F	*P*	*F*	*P*
Chl *a*	d	1872.07	<0.001	190.44	<0.001
	I	130.25	<0.001	169.28	<0.001
	d×I	17.14	<0.001	10.14	0.006
Chl *b*	d	657.13	<0.001	38.35	<0.001
	I	104.23	<0.001	1.55	0.231
	d×I	9.56	<0.001	0.03	0.865
Car	d	432.88	<0.001	602.39	<0.001
	I	102.15	<0.001	14.75	0.001
	d×I	11.29	<0.001	1.06	0.319
F_v/F_m	d	43.08	<0.001	0.06	0.810
	I	0.00	0.990	0.00	0.983
	d×I	0.79	0.540	9.84	0.006
Φ_{PSII}	d	52.45	<0.001	212.95	<0.001
	I	116.03	<0.001	265.39	<0.001
	d×I	13.66	<0.001	6.27	0.024
q_P	d	97.45	<0.001	46.16	<0.001
	I	65.59	<0.001	155.45	<0.001
	d×I	6.11	<0.001	24.23	<0.001
q_{NP}	d	217.93	<0.001	56.30	<0.001
	I	38.72	<0.001	3.65	0.080
	d×I	11.43	<0.001	19.61	<0.001
P_N	d	14.66	<0.001	236.91	<0.001
	I	96.67	<0.001	95.52	<0.001
	d×I	13.36	<0.001	18.25	0.001
E	d	200.36	<0.001	157.33	<0.001
	I	12.55	0.001	89.22	<0.001
	d×I	5.11	0.002	14.93	0.002
g_s	d	369.14	<0.001	65.96	<0.001
	I	40.11	<0.001	91.12	<0.001
	d×I	17.76	<0.001	5.52	0.035
c_i/c_a	d	98.63	<0.001	103.83	<0.001
	I	40.67	<0.001	32.19	<0.001
	d×I	18.82	<0.001	3.24	0.087
RuBisCO	d	29.95	<0.001	62.88	<0.001
	I	14.94	<0.001	106.25	<0.001
	d×I	10.16	<0.001	27.69	<0.001
sFBPase	d	2.49	0.058	55.67	<0.001
	I	98.99	<0.001	71.68	<0.001
	d×I	7.26	<0.001	1.02	0.328
Glucose	d	134.99	<0.001	130.70	<0.001
	I	205.37	<0.001	0.02	0.903
	d×I	106.17	<0.001	0.75	0.399
Frcutose	d	34.97	<0.001	119.01	<0.001
	I	57.05	<0.001	56.41	<0.001
	d×I	49.81	<0.001	7.98	0.012
Sucrose	d	190.84	<0.001	121.95	<0.001
	I	100.09	<0.001	50.88	<0.001
	d×I	53.26	<0.001	29.21	<0.001
Starch	d	39.42	<0.001	23.53	<0.001
	I	29.19	<0.001	0.40	0.535
	d×I	3.95	0.028	2.04	0.173

which compensates the reduction of g_s after *ex vitro* transfer, as reported Fila *et al.* (2006) in *Vitis vinifera* plantlets after *ex vitro* transfer. In plants grown under LI, besides stomatal limitations on photosynthesis, non-stomatal limitations (*e.g.* decreased activity of the Calvin cycle enzymes) can also occur. In persistent leaves under LI, RuBisCO activity was comparable to that of *in vitro* plants. Considering the low RuBisCO activity of *in vitro* plants (Hdider and Desjardins 1995, Carvalho *et al.* 2005) and the fact that *U. minor* plants were acclimatized under light-limiting photosynthesis, the results obtained in this study indicate that the use of LI poorly promotes RuBisCO activity. It seems unlikely that ribulose-1,5-bis-phosphate (RuBP) regeneration is limited by reduced sFBPase activity, since, according to the stoichiometry of the Calvin cycle (1 sFBPase to 3 CO_2) (Brüggemann *et al.* 1994), the latter enzyme activity still largely exceeded the P_N.

Acclimatization had a marked effect on leaf pigment content. According to Amâncio *et al.* (1999), high sugar concentration in the *in vitro* culture medium may inhibit chlorophyll synthesis. Thus, the observed increase in Chl *a* and Chl *b* after *ex vitro* transfer may be a response to autotrophic conditions. Similar results were reported after *ex vitro* transfer of *Calatea louisae* (Van Huylenbroeck *et al.* 2000), *Ceratonia siliqua* (Osório *et al.* 2005), and *Rauvolfia tetraphylla* (Faisal and Anis 2009). The low content of Chl *a* and Chl *b* under HI conditions suggests a low investment in light-harvesting pigments under this treatment (Osório *et al.* 2005). Cars play key roles in protecting Chl against photoinhibition (Choudhury and Behera 2001). The increase in Car content directly after *ex vitro* transfer may reflect a protection of the photosynthetic machinery from photo-oxidative damage, as reported for other micropropagated species (Van Huylenbroeck *et al.* 2000, Faisal and Anis 2009).

Chlorophyll fluorescence analysis revealed that the very low qP found at day 0 indicates that a high proportion of the primary quinone electron acceptor of PSII is in a reduced state and that a high fraction of the absorbed light energy is dissipated through qN which prevents photooxidative damage of the photosynthetic membranes. The F_v/F_m values were similar to those obtained for other micropropagated species (*e.g.* Carvalho *et al.* 2001, Osório *et al.* 2010) and are within the range of values of nonstressed plants (0.75 - 0.85) (Schreiber *et al.* 1995). As the acclimatization process proceeds, both treatments promote an increase of photochemical efficiency. In persistent leaves under HI, the high values of qP induce an increase of Φ_{PSII}, resulting in a higher photosynthetic ability as compared to LI. This result is also in accordance with the higher P_N values reported under HI than under LI. Under LI conditions, the low qP and Φ_{PSII} indicates a limitation of the Calvin cycle, *e.g.* a non-stomatal limitation of photosynthesis by diminished capacity of the use of NADPH and ATP (Dias and Brüggemann 2010). New leaves revealed similar photosynthetic efficiency to persistent leaves.

In conclusion, the results reported in this study showed that persistent and new leaves are photo-

synthetically competent and able to adapt to *ex vitro* conditions after a short initial period of acclimatization. HI has a strong positive effect on P_N and photosynthetic efficiency, promoting higher growth in these plants. LI reduced photosynthetic efficiency in persistent leaves mainly due to an impairment of the Calvin cycle (*e.g.* RuBisCO activity). The results obtained can be used as a starting point for the establishment of acclimatization protocols for other micropropagated woody species for large-scale commercial production.

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