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BRIEF COMMUNICATION

Effect of irradiance during acclimatization on content of proline and phytohormones in micropropagated *Ulmus minor*

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Abstract

This study aimed to investigate the effects of irradiance on plant growth and content of proline and phytohormones during *ex vitro* acclimatization of micropropagated *Ulmus minor* plants. *In vitro* rooted plants were acclimatized to *ex vitro* conditions in a climate chamber with two irradiances, 200 μmol m^{-2} s^{-1} (high irradiance, HI) and 100 μmol m^{-2} s^{-1} (low irradiance, LI) for 40 d. Immediately after the *ex vitro* transfer, the plants experienced a water deficit [wilting leaves with the reduced relative water content (RWC)], but following the experiment, the recovery of the RWC was more pronounced in the HI treatment. Also, the content of proline, ABA, and JA-Ile were higher in HI treatment. Growth analyses revealed that HI improved growth and biomass production.

Additional key words: abscisic acid, growth analysis, jasmonic acid, net assimilation rate, relative water content.

Plant micropropagation has been successfully applied to agriculture and forestry for large scale production of important and economically valuable species. However, the commercial use of micropropagation is limited in many species due to the poor plantlet survival rates during the acclimatization (*e.g.* Pospíšilová *et al.* 2009). The main causes for plantlet mortality are related to an abnormal morphology, anatomy, and physiology of tissue cultured plantlets. In particular, the deficient functioning the overall water housekeeping system (*e.g.*, poor stomatal control and cuticular abnormalities) associated with the highly differential vapour pressure between *in vitro* and *ex vitro* conditions can induce water deficit and plant dehydration during *ex vitro* acclimatization (Pospíšilová *et al.* 1999). Moreover, the use of irradiance higher than that used under *in vitro* conditions can result in an increased stress and consequently growth reduction (Dias *et al.* 2011, 2013). Therefore, an acclimatization period is required for a progressive adaptation to the new growing environment. Among the factors that can be manipulated during acclimatization, irradiance seems to be one of the most critical (Estrada-Luna *et al*. 2001, Carvalho and Amâncio 2002, Osório *et al.* 2010).

Plants can alter their metabolism to overcome stress effects and the amino acid proline occurs often in higher plants and plays a significant role in plant adaptation to a broad range of stresses (Ashraf *et al.* 2007). Besides its importance as an osmolyte for osmotic adjustment, proline can protect cells against oxidative stress (Ashraf *et al.* 2007). Several phytohormones, such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and jasmonyl-isoleucine (JA-Ile) are critical for plant growth and development and play an important role in integrating several stress signals and controlling downstream stress responses (Corcuera *et al.* 2012).

Research dealing with the physiological performance and the antioxidant responses of *U. minor* during *ex vitro* acclimatization were already analysed in previous studies (Dias *et al.* 2011, 2013). However, up to the knowledge of the authors, information concerning the role of proline and phytohormones in plant protection during *ex vitro* acclimatization at different irradiances is unknown. Thus, the aim of this work was to elucidate: *1*) the effects of irradiance on content of proline and phytohormones

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Abbreviations: ABA - abscisic acid; DM - dry mass; FM - fresh mass; HI - high irradiance; JA - jasmonic acid; JA-Ile - jasmonyl-isoleucine; LAR - leaf area ratio; LDM - leaf dry mass; LI - low irradiance; NAR - net assimilation rate; RGR - relative growth rate; RWC - relative water content; SA - salicylic acid; SLA - specific leaf area.

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during acclimatization of micropropagated *U. minor* plants, and *2*) the role of proline and phytohormones on plant protection/recovery and further growth.

Micropropagated *in vitro* grown plants of *Ulmus minor* Mill. were kept in closed transparent glass vessels in a growth chamber at temperature of 22 ± 2 ºC, a 16-h photoperiod, and irradiance of 50 ± 10 µmol m^{-2} s^{-1}. Rooted plants of a shoot length of approx. 8 cm with at least 2 roots (5 - 10 cm long) and 7 - 9 leaves were transplanted to 400 cm^3 pots containing a mixture of peat and *Perlite* (3:2, v:v) saturated with water. Plants were acclimatized as described in detail by Dias *et al.* (2011). Irradiation was provided by eight cool white fluorescent lamps (*Osram*) and irradiance at the plant level was 200 ± 20 µmol m^{-2} s^{-1} for the high irradiance (HI) treatment and 100 ± 20 µmol m^{-2} s^{-1} for the low irradiance (LI) treatment. These irradiances were achieved by changing the distance of the plants from the lamps. Relative humidity (RH) of 98 % was maintained inside the growth chamber for the first 7 d of acclimatization and gradually decreased by 7 % every week to 65 %. the plants were watered weekly with Hoagland's solution (*H2395*, *Sigma*, München, Germany).

the plants were harvested at day 0 (*in vitro* plants), 7, 14, 25, and 40 in the middle of the photoperiod. The height of shoots and the length of roots were measured. The leaf area was calculated. The fresh mass (FM) and dry mass (DM) of individual organs were determined. DM was obtained after drying at 60 ºC until constant mass. These data were used to calculate: plant total DM, specific leaf area (SLA = leaf area/leaf mass), leaf area ratio (LAR = leaf area/plant mass), relative growth rate (RGR = increase in biomass per gram of biomass and day), net assimilation rate (NAR = the increase in biomass per leaf area and day), and shoot to root DM ratio. Further, the relative water content [RWC = (FM - DM)/(WSM - DM) × 100] was determined, where water saturated mass (WSM) was determined after leaf tissue discs floating on water at 5 ºC for 180 min.

Proline content was quantified at day 0, 14, and 40 as described by Hamid *et al.* (2003). Briefly, the leaf samples were extracted with sulphosalicylic acid. The extracts were maintained at 100 ºC for 1 h with the addition of glacial acetic acid and acid ninhydrin. After adding toluene, the absorbance was read at 520 nm and the proline content was determined from a standard curve. The content of phytohormones was determined at day 0 and 40. The hormone extraction was carried out as described in Durgbanshi *et al.* (2005). Briefly, the leaf tissue (0.4 g) was extracted in ultrapure water. Before extraction, the samples were spiked with deuterated standards of every compound. After centrifugation, the pH of the supernatant was adjusted to 3.0 and partitioned twice against diethyl ether. The organic layer was recovered and evaporated. The dry residue was resuspended, filtered and injected in an HPLC system (*Watersalliance 2695*, *Waters Corp*., Milford, USA). The hormones were detected according to their specific transitions using a multiresidue mass spectrometric method (*Quattro LC Triple Qquadrupole*, *Micromass*, Manchester, UK).

Each acclimatization treatment was performed twice. All determinations were obtained with randomly-chosen plants. The data were analysed by *t*-test (RGR, LAR, and NAR) as well as one-way analysis of variance (*ANOVA*) (RWC, shoot growth, plant total DM, shoot/root ratio, SLA, LDM, proline, and phytohormones) using the *Sigma Stat* program (*Windows*, *v. 3.1*). Comparisons between means were evaluated by Post Hoc test (Tukey's multiple comparison test) at a significance level set to 0.05.

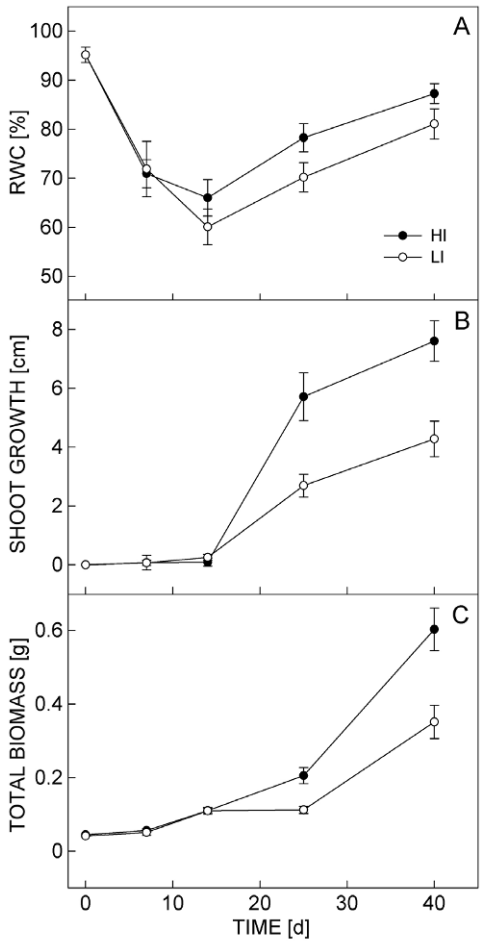

Fig. 1. RWC (*A*), shoot growth (*B*), and dry mass of the whole plant (*C*) of micropropagated *U. minor* during acclimatization at irradiance of 200 (HI) and 100 (LI) µmol m^{-2} s^{-1}. Means ± SD, *n* = 12 for RWC, 20 for shoot growth, and 10 for biomass.

Immediately after *ex vitro* transfer, plants became wilted (visual observation). The RWC decreased by 31 % in the HI and 37 % in the LI treatment from day 0 to day 14. However, after this period, a significant increase was observed for both treatments (Fig. 1*A*). Plantlets acclimatized at HI showed a better recovery of RWC than those at LI (up to day 14). The shoot growth was low during the first 14 d and independent of the treatments (Fig. 1*B*). After this period, shoot growth increased significantly under both irradiances. At the end of the

experiment, plants grown under HI were considerably higher than plants under LI. Plants acclimatized at HI showed a significant increase of plant total DM over the acclimatization period (Fig. 1*C*). In the case of LI, this parameter also increased, but not significantly between day 14 and 25. The RGR and NAR were significantly higher in plants under HI than those under LI (Table 1). However, LAR and SLA were significantly higher in plants grown under LI than those grown under HI (Table 1). On the contrary, the highest LDM was observed in plants under HI (Table 1). The highest shoot/root ratio was in *in vitro* plants and at the end of the experiment, plants acclimatized at HI showed a higher shoot/root ration than those at LI (Table 1). Plants acclimatized at HI showed the highest content of ABA and JA-Ile whereas those grown *in vitro* showed the lowest content of these phytohormones (Table 1). No significant differences were observed between JA content at HI and LI. A similar SA content was observed in all treatments. *In vitro* plants showed the highest proline content and it decreased significantly after *ex vitro* transfer. Plants acclimatized under HI always showed a higher proline content than those grown at LI (Table 1).

Immediately after *ex vitro* transfer, *U. minor* plantlets dehydrated quickly, became wilted and experienced water deficit. This might be due to a deficient functionality of the stomata, characteristic of *in vitro* grown plants (Pospíšilová *et al.* 1999, 2009). However, an improvement of the water control mechanisms was observed after day 14: plantlets slowly recovered from dehydration and at the end of the experiment, the RWC was above 80 %. Similar results were observed in *Capsicum annuum* during acclimatization (Estrada-Luna *et al.* 2001). In general, HI treatment promoted a better recovery of the RWC.

Under *in vitro* conditions, plantlets are exposed to different stresses leading to several anatomical, morphological, and physiological malformations. To enhance plant tolerance under such stress conditions, some species increased the production of different types of compatible organic solutes, such as proline (Ashraf *et al.* 2007). *In vitro* plantlets showed the highest proline production which could be a mechanism to overcome the *in vitro* stress conditions and a protection against oxidative stress, as observed previously in *U. minor* plantlets grown *in vitro* under the same experimental conditions (Dias *et al*. 2011). Curiously, the imposition of other stresses associated with *ex vitro* transfer reduced proline production. However, plantlets acclimatized at HI showed a higher content of this amino acid. This higher content of proline may protect cells from dehydration (Ashraf *et al.* 2007) causing better RWC recovery.

In general, acclimatization induced a rise of JA-Ile, JA, and ABA production and HI treatment has a greater effect on JA-Ile and ABA production. These results confirm the important role of JA-Ile, the most biological active form of jasmonates, in plant stress protection (Fonseca *et al*. 2009). *U. minor* plantlets grown under HI showed improved RWC recovery and higher growth/ biomass compared to those under LI. HI treatment also induced higher ABA accumulation in leaves compared to LI treatment. This explain the lower water loss found in HI treatment, since ABA accumulation in leaves is described to be involved in stomata opening regulation (Pospíšilová *et al.* 2000) and so in reduction of the transpiration rate (E). Our results also corroborate with the data published by Dias *et al.* (2013): *U. minor* plantlets acclimatized under HI had a lower E and a higher net photosynthetic rate compared to those grown under LI. Irradiance did not affect JA production. Similarly to other species (Munné-Bosch *et al.* 2008, Corcuera *et al.* 2012), SA seems not to play a crucial role in *U. minor* stress protection.

HI treatment promoted a remarkable rise in plant growth and biomass production and a higher investment of biomass to the root system. On the other hand, plants grown under LI showed higher SLA reflecting higher

Table 1. RGR, LAR, NAR, SLA, LDM, shoot/root ratio, and ABA, SA, JA, JA-Ile, and proline content measured in *U. minor* plants grown at 200 μmol $m^{-2}s^{-1}$ (HI) and 100 μmol $m^{-2}s^{-1}$ (LI) after 40 d. Results are means ± SD of twelve replications for RGR, LAR, NAR, SLA, LDM, and shoot/root, six replications for proline content, and four replications for ABA, SA, JA, and JA-Ile content. Different letters indicate significant differences between treatments at a significance level lesser than 0.05. The proline content was also measured after 14 d and the values are following: HI - 0.57±0.04^{b}, LI - 0.45±0.02^{c}

Parameters	*In vitro*	HI	LI
RGR [g g^{-1} d^{-1}]	-	0.023±0.002^{a}	0.019±0.003^{b}
LAR [cm^2 g^{-1}(DM)]	-	33.4 ±1.20^{a}	68.2 ±3.3^{b}
NAR [g m^{-2} d^{-1}]	-	3.2 ±0.03^{a}	1.9 ±0.7^{b}
SLA [cm^2 g^{-1}(DM)]	429.3 ±57.0^{a}	424.1 ±46.9^{a}	527.9 ±20.8^{b}
LDM [mg]	9.1 ±1.50^{a}	19.1 ±1.20^{b}	13.1 ±1.0^{c}
Shoot/root ratio	1.86±0.28^{a}	0.52 ±0.04^{b}	0.65 ±0.053^{c}
ABA [ng g^{-1}(FM)]	89.0 ±3.40^{a}	241.6 ±62.0^{b}	158.9 ±2.9^{c}
JA [ng g^{-1}(FM)]	59.9 ±6.30^{a}	89.8 ±20.5^{b}	79.7 ±5.1^{ab}
JA-Ile [ng g^{-1}(FM)]	0.4 ±0.06^{a}	96.6 ±1.00^{b}	64.7 ±6.8^{c}
SA [ng g^{-1}(FM)]	107.9 ±20.0^{a}	138.1 ±30.9^{a}	122.9 ±20.6^{a}
Proline [μmol g^{-1}(FM)]		0.21 ±0.02^{d}	0.14 ±0.02^{e}

leaf areas without the corresponding increase in mass. RGR is the product of NAR and LAR (Poorter and Remkes 1990). NAR is determined by the ratio of carbon gained through photosynthesis and carbon lost through respiration. LAR reflects the ratio of leaf area produced per unit of plant DM. According to our results, HI promotes a higher RGR than LI. These results may reflect the higher growth at HI. NAR variations follow the same trend as the RGR suggesting that NAR is the main contributor of RGR as also observed by Carvalho and Amâncio (2002) and Senevirathna *et al.* (2003) for other species. Contrarily to RGR and NAR, LAR was higher at LI. These results confirm that LI promotes a higher investment in leaf area (as also suggested by the greater SLA).

In conclusion, the results demonstrate that: *1*) irradiance affects growth and content of proline and phytohormones, *2*) proline plays an important role in *U. minor* stress protection under *in vitro* conditions, and *3*) the higher content of proline, ABA, and JA-Ile at HI treatment promotes a better recovery of RWC and therefore improves plant growth. Plant phytohormones and proline play an important role in plant recovery from the stresses imposed during acclimatization and consequently affect plant growth.

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