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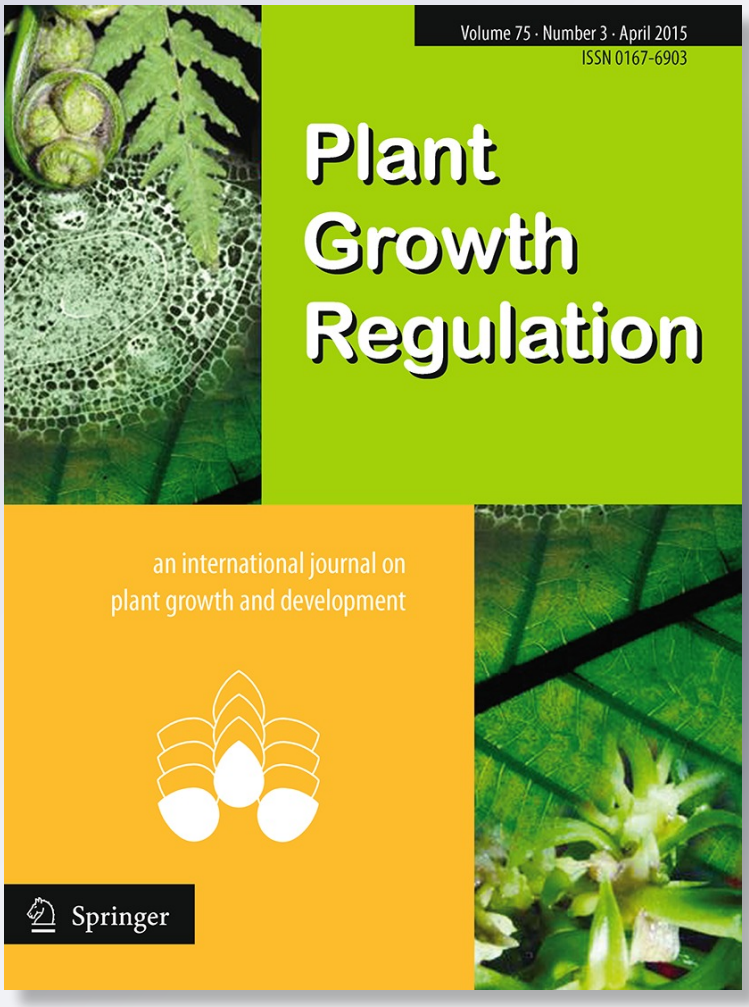

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ORIGINAL PAPER

Influence of in vitro growth conditions on the photosynthesis and survival of *Castanea sativa* plantlets during ex vitro transfer

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Abstract Adequate in vitro micro-environments are crucial to induce life compatible leaf development. Key morphological and physiological traits are needed to allow ex vitro survival. We study, how in vitro light and ventilation affect physiological performance and survival of ex vitro *Castanea sativa* plantlets. In vitro treatments consisted of two irradiances of 50 and 150 μmol m^{-2} s^{-1} in ventilated vessels (VL_{50} and VL_{150}, respectively), compared to traditional cultures at 50 μmol m^{-2} s^{-1} in non-ventilated vessels (NVL_{50}). After the exposure to each condition a photoinhibitory treatment (PhT) was also applied to study whether the above in vitro conditions exerted photoprotection and facilitated the recovery of *C. sativa* during sudden ex vitro transfer. During rooting, a decrease in net photosynthesis (Psat), electron transport rate (ETRII) and maximal efficiency of PSII (F_v/F_m) were observed. Transpiration rates (E) decreased, concomitantly with a rise in water use efficiency (WUE), mainly in microplants originating from ventilated treatments (V). Throughout this stage, the PhT was lethal for all in vitro treatments. During acclimation, the number and leaf size increased principally in plantlets originating from V treatments. These microplants were also able to recover their ETR and F_v/F_m. Initially, the PhT produced a drastic drop in F_v/F_m of plantlets in all treatments however they did show a tendency to recover. Transferring plantlets to the greenhouse produced a decrease in the Psat in all treatments; however, over time Psat increased reaching values of 3.2 and 5.3 μmol CO_2 m^{-2} s^{-1} in microplants originating from VL_{50} and VL_{150}, respectively. Transpiration rate were similar in all treatments and remained at levels of about 0.9 mmol H_2O m^{-2} s^{-1}; thus, WUE increased significantly, reaching values of almost 3.8 μmol CO_2/mmol H_2O in microplants originating from VL_{150}. After the PhT, all of the plantlet's recovery capacity increased concomitantly with their dynamic heat dissipation and their de-epoxidation capacity. Our results suggest that managing in vitro conditions can improve plantlets photosynthetic performance in early stages after ex vitro transfer, playing a key role in the ameliorating the transfer stress.

Keywords Acclimation · Ex vitro transfer · Fluorescence · Photosynthesis · Photoinhibition

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Introduction

The success of micropropagation systems can be effectively measured by the percentage of plantlets that survived when they are transferred from in vitro culture vessels to greenhouse or field conditions (Kirdmanee et al. 1995). However, the combined effects of nutritional and environmental stresses during this transfer account for a high percentage of plant mortality (Pospísilová et al. 1999, 2009; Swain et al. 2010). In vitro cultured plants must withstand the transition from heterotrophic or mixotrophic conditions to the fully autotrophic conditions present in greenhouses (Kozai et al. 1992; Valero-Aracama et al. 2006). Additionally, they must cope with drastic environmental changes characterized by lower air humidity and higher irradiance (Osório et al. 2010). Microplants often have limited water loss control (Semorádová et al. 2002) and their roots and root-stem connections exhibit limited hydraulic conductivity (Fila et al. 1998). Besides, the poor microplant's photosynthetic capacity could limit them to reach a positive carbon balance (Preece and Sutter 1991). In greenhouses microplants commonly are exposed to much greater irradiance than that in in vitro culture rooms, which induce a low photochemical light use capacity and limited photoprotective mechanisms (Álvarez et al. 2012; Sáez et al. 2012a), which are reflected in their high susceptibility to photoinhibition (Osório et al. 2010; Sáez et al. 2013).

The transfer from in vitro to ex vitro conditions includes two stages. The first, called acclimation consists of transferring plantlets from culture vessels to containers with substrate. This is critical because plants have to adjust very fast to these contrasting environmental conditions to ensure their survival and growth under different environmental conditions (Debergh and Zimmerman 1991; Preece and Sutter 1991). The second stage is the post-acclimation, when plantlets become photoautotrophic; this stage is less variable and has more established characteristics. Several treatments have been evaluated to prepare plantlets for ex vitro transfer (Van Huylenbroeck and Debergh 1996; Van Huylenbroeck et al. 1998). These treatments included variations in light intensity, reductions in air humidity and increase in CO_2 concentrations (Pospísilova et al. 2000; Carvalho et al. 2001; Lian et al. 2002; Mosaleeyanon et al. 2004; Deccetti et al. 2008). Despite these studies, transfer from in vitro to ex vitro continues to be the main bottleneck for the micropropagation of many species (Hazarika 2006). The success of ex vitro transfer is highly dependent on the morphological and/or physiological characteristics acquired during in vitro growth (Fila et al. 2006; Serret and Trillas 2000). Especially, modifying the in vitro environmental conditions to gradual and soft environmental gradients more similar to ex vitro conditions such us greenhouse (e.g. increases in light and vapor pressure deficit) can improve acclimation and survival during ex vitrotransfer (Premkumar et al. 2003). Thus, *Castanea sativa* leaves formed in in vitro cultures differ anatomically and physiologically from those developed in nurseries (Sáez et al. 2012a). However, managing the environmental conditions in vitro to reach more similar ex vitro environments can induce the development of more similar leaves to nursery seedlings (Sáez et al. 2012b). In order to explore the impacts of managing the microenvironmental conditions during in vitro cultures on ex vitro plants, we evaluated whether ventilation and increased light during in vitro culture improve a suit of anatomical and photosynthetic traits, and plant performance as survival and growth.

Materials and methods

Plant material and culture conditions

Castanea sativa microshoots were grown on half-strength MS medium (Murashigue and Skoog 1962) supplemented with 0.22 μM 6-benzyladenine (BA), 0.024 μM indolebutyric acid (IBA) and 30 g L^{-1} sucrose, and gelled with 7 g L^{-1} agar at a pH of 6.2. Microshoots were put in 120 mL vessels containing 30 mL of the medium, under three different treatments, two photosynthetic photon flux density (PPFD) levels of 50 and 150 μmol m^{-2} s^{-1} in ventilated vessels (VL_{50} and VL_{150}, respectively). The vessels were ventilated by perforating a hole in magenta caps and covering them with membrane filter disks 2 cm in diameter (Micropore 3 M^{TM}, Chile) (Sáez et al. 2012 b), compared with traditional culture conditions (third treatment: PPFD: 50 μmol m^{-2} s^{-1} and non-ventilated vessels; NVL_{50}) closed with conventional magenta caps (Sigma Aldrich, St. Luis, MO, USA). In vitro cultures were exposed to a 16 h photoperiod at 24 ± 2 °C and 60 % relative humidity in a growth chamber. The microshoots were subcultivated every 45 days and after 8 months, rooting was induced by the immersion of the base of microshoots in 2.45 μM IBA for 1 min. Microshoots rooting took place ex vitro along the acclimation stage according to Vieitez et al. (2007) being transplanted to 15 cm^3 plastic pots with a sterilized mixture of peat and perlite 3:1 until adventitious root appears, c.a 18 days. Once rooted, microplants were transplanted to 45 cm^3 pots filled with 1 cm of sand and a mixture of organic soil and perlite 3:1. Plants were exposed for 20 days in the same growth chamber and environmental conditions, with the exception of PPFD maintained at 50 μmol m^{-2} s^{-1} for all treatments, used for in vitro culture and root induction. Subsequently, microplants were transferred to a polycarbonate greenhouse

under natural light and irrigated with micro-sprayer three times a day. Greenhouse trials were conducted in the month of June (winter), reaching air temperatures in average during the experiment that range from 3 ± 1 to 23 ± 2 °C and mean midday temperature of 17 ± 5 °C. Midday PPFD varied between 90 and 900 μmol m^{-2} s^{-1}.

Net photosynthesis and water use efficiency

Light response curves of net CO_2 assimilation (from 0 to 1,500 μmol m^{-2} s^{-1}) were measured using an infrared gas analyzer (GFS-3000 Walz, Effeltrich, Germany). Net photosynthesis at light saturation (Psat) was obtained using Photosynthesis Assistant 1.1 software (Dundee Scientific, United Kingdom). The instantaneous water use efficiency (WUE) was calculated as the ratio Psat and transpiratory rate (E). Leaf chamber was maintained at CO_2 concentration of 380 μg g^{-1} with an air flow rate of 200 cm^{-3} · min^{-1} and 75 % of air relative humidity. In order to avoid the effects of measuring temperature on photosynthetic responses, air temperature was maintained between 15 and 20 °C. Leaves were photographed inside the leaf chamber and their leaf area was measured with Sigma Scan Pro 5.0 software (SPSS, Chicago, IL, USA). When necessary, gas exchange values were adjusted for leaf chamber area/measured leaf area ratio.

Stomatal density and morphology

Stomatal density and morphology were analyzed with a scanning electron microscope (SEM: Jeol JSM6380 nLV, Tokio, Japan). Fresh leaf samples from expanded leaves were collected and sections of 1 mm^2 were immediately fixed at 4 % glutaraldehyde and post-fixed at 1 % osmium tetroxide. Stomatal density (N° mm^{-2}), length (μm), width (μm) and percentage of open stomata were determined using Image J software (Wayne Rasband/NIH, Bethesda, MD, USA).

Chlorophyll fluorescence

Using a pulse-amplitude modulated fluorimeter (FMS 2, Hansatech Instrument, Kingś Lyn, UK) chlorophyll fluorescence was measured in topmost fully expanded leaves of explants and dark adapted for 30 min. According to Rosenqvist and van Kooten (2003), minimal fluorescence (F_o) was determined by applying a weak modulated light (6 μmol m^{-2} s^{-1}) and maximal fluorescence (F_m) was induced by a short saturating pulse (0.8 s and 9,000 μmol m^{-2} s^{-1}). The maximal efficiency of PSII (F_v/F_m) was calculated as the ratio F_m-F_0/F_m. All fluorescence data were recorded under steady state conditions after 10 min at PPFD of 600 μmol m^{-2} s^{-1} (F_s). The maximal fluorescence in light ($F_{m'}$) was obtained after equal saturating pulse as for F_m determination. Minimal fluorescence in light ($F_{o'}$) was determined by turning off the actinic light and immediately applying a 2 s far red pulse. Electron transport rate through photosystem II (PSII) [ETR(II)] was calculated as: ETR(II) = 0.84 (ΦPSII) (PPFD) 0.5, where ΦPSII is the effective quantum yield of PSII (Genty et al. 1989). Factor 0.5 assumes that the efficiency and light absorption is equal in both photosystems. Factor 0.84 is the mean value of absorbance for green leaves (Demmig-Adams et al. 1987). Non-photochemical quenching was evaluated as $NPQ = (F_m–F_{m'})/F'_m$ (Bilger and Björkman 1990).

Photoinhibition treatment

During ex vitro transfer, five randomly selected plants from each treatment were subjected to a photoinhibitory conditions (PhT). Plants were transferred to a homemade photoinhibition chamber consisting of a horizontal freezer with a glass upper door and a heat water filter. Plants were exposed to a PPFD of 1,000 μmol m^{-2} s^{-1} for 2 h at 18 ± 2 °C. The light was provided by two 450-watt metal-halide lamps. The air temperature in the chamber was monitored each 5 min with a thermocouple located in the shade but near to the leaves. The F_v/F_m recovery from PhT was measured under darkness at room temperature. NPQ components, including both fast relaxing (NPQ_f) and slow relaxing components (NPQ_s), were calculated from 6 h dark relaxation kinetics of F_v/F_m according to Coopman et al. (2008).

Xanthophyll pigments and de-epoxidation capacity

Violaxanthin, antheraxanthin and zeaxanthin (VAZ) were determined immediately before and after the PhT. For this, leaf discs (3.86 mm in diameter) were collected, and instantaneously frozen in liquid nitrogen and stored at −80 °C. Three discs (ca 30 mg fresh weight) were powdered in liquid nitrogen. A spatula tip of $CaCO_3$ was added before extracting with 1 mL 100 % HPLC-grade acetone at 4 °C under dim light. Pigments were separated and quantified by reversed-phase HPLC as described (García-Plazaola and Becerril 1999). The instrument system consisted of a quaternary pump with an automatic degassing system and an autosampler. Signals from a diode array detector were integrated and analyzed using Agilent ChemStation B.04.01 software (Agilent Technologies, Waldbronn, Germany). Chromatography was carried out using a Spherisorb ODS-1 reversed phase column (5 μm particle size; 4.6 × 250 mm, Atlantil Hilit, Waters, Ireland) and a Nova-pack C-18 (4 μm; 3.9 × 20 mm) guard column (Waters, Ireland). The mobile phase consisted of two

components: solvent A, acetonitrile: methanol: Tris Buffer (0.1 M, pH 8.0) (42:1:7) and solvent B, methanol: ethyl acetate (34:16). Pigments were eluted using a linear gradient from 100 % A to 100 % B within the first 12 min, followed by an isocratic elution with 100 % B for the next 6 min. Absorbance was monitored at 445 nm. Retention times and response factors of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) were determined by injecting pure standards purchased from DHI (Hoershholm, Denmark). The de-epoxidation state was calculated as: DEPS = (Z + 0.5A)/(VAZ).

Statistics

Measurements were made during the following stages: in the last in vitro subculture (Iv), rooting (Rs), acclimation (As) and during the greenhouse period. Until rooting measurement were done in persistent in vitro formed leaves. Subsequent measurements were performed on newly formed leaves that were marked with silicone rings. Survival was assessed at the end of each stage, being calculated from the total number of plants that were transferred in each stage. All experiments were arranged in a completely randomized design. One way ANOVA was used to test differences among in vitro treatments within each stage. Assumptions for parametric statistic were checked and when appropriate, variables were transformed to follow the former assumptions (Quinn and Keough 2006). Generalized Lineal Models (GLM) and deviance analyses were used to compare survival, assuming a Binomial error distribution (McCullagh and Nelder 1989). Differences among means were established using LSD -test ($P < 0.05$). Each mean was calculated from at least three replicates. Statistical analysis was performed with InfoStat/L software (FCA-UNC, Argentina).

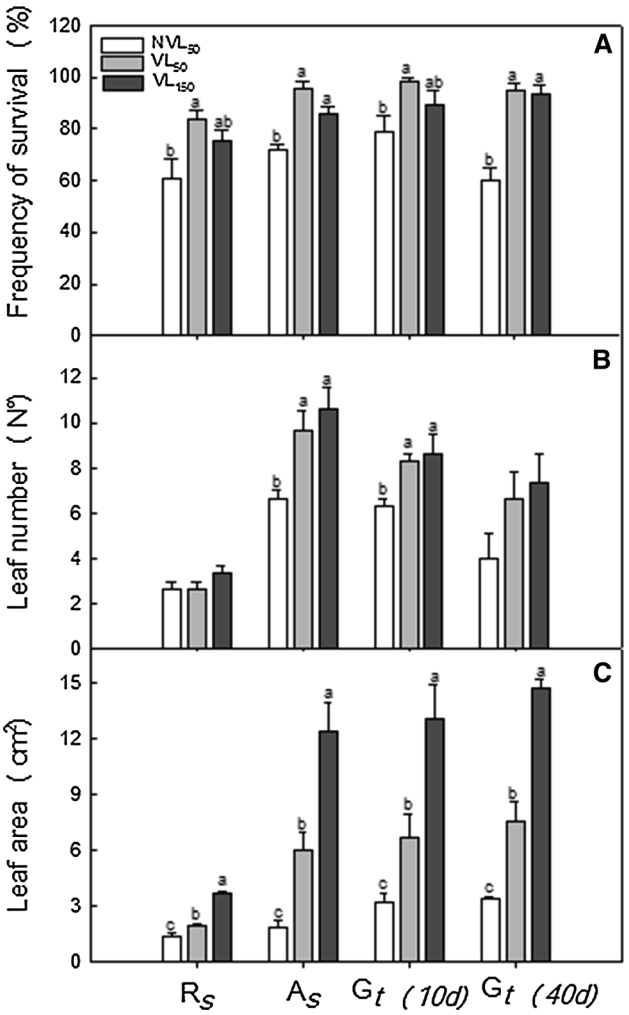

Fig. 1 Effects of in vitro treatments on frequency of survival (**a**), leaf number/plant (**b**) and foliar area (**c**) of micropropagated plants of *C. sativa* during their ex vitro transfer: rooting (R_s), acclimation (A_s), 10 ($G_{t\ (10d)}$) and 40 ($G_{t\ (40d)}$) days after greenhouse transfer. Bars show mean ± SE. Different letters indicate significant differences among treatments according to the LSD test ($P < 0.05$)

Results

Survival and growth

Survival varied among treatments and different transfer stages (Fig. 1a; $P < 0.05$). The lowest survival was observed in the traditional culture (NVL_{50}) in all measured stages being nearly 60 %. (Fig. 1a). Ventilated treatments (VL_{50}) showed the highest survival during acclimation as well as in the greenhouse, reaching about 90 % at the end of the greenhouse transfer, similar to that observed in VL_{150}. No significant differences were found in the leaf number during R_s (Fig. 1b). However, plants in VL_{150} showed the greatest foliar area, in all transfer stages (Fig. 1c). The leaf number significantly increased during acclimation. Subsequently, the greenhouse transfer exerted for all treatments an important defoliation. At the end of the evaluation period ($G_{t(40d)}$), leaf number and area remained unchanged showed no significant increases with respect to ($G_{t(10d)}$). Finally, after 40 days after transfer to greenhouse ($G_{t(40d)}$), the leaf number increased in acclimatized plants, compared to the microplants that grown under in vitro culture, showed an increase of over 100 % in leaf number and a fivefold in leaf area.

Net photosynthesis and water use efficiency

During the in vitro culture and the ex vitro transfer, leaves developed in VL_{150} maintained the highest Psat (Fig. 2a). During the ex vitro transfer, VL_{50} always presented higher Psat than the traditional culture (NVL_{50}). In the first

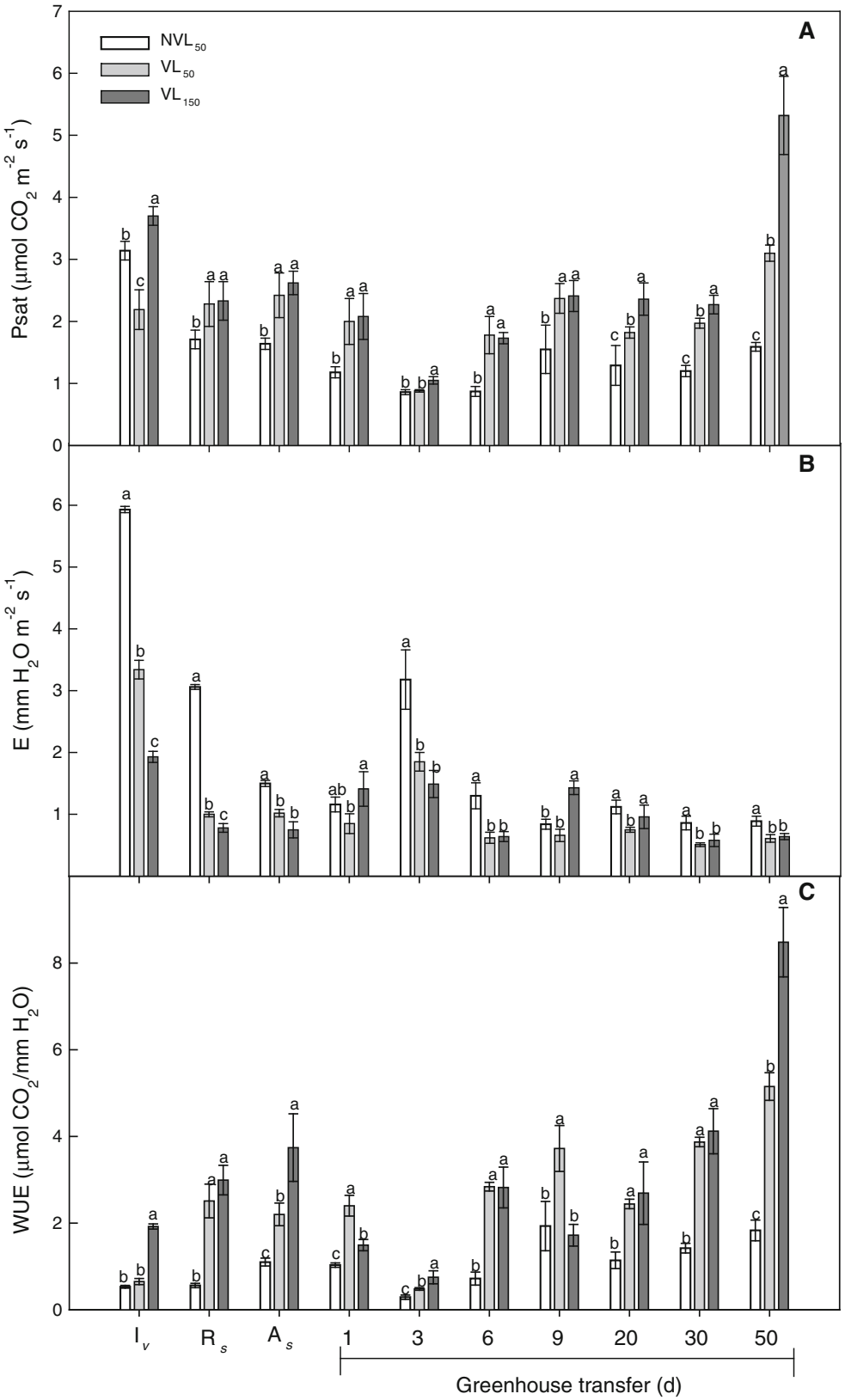

Fig. 2 Effects of in vitro treatments on net photosynthesis (**a**), transpiration rate (**b**) and WUE (**c**) of *C. sativa* microplants rooting and subsequent transfer to greenhouse conditions. Measurements were made during the last in vitro subculture (I_v), rooting (R_s), acclimation (A_s) and greenhouse transfer. Each *bar* represents mean ± SE (n = 3). *Different letters* indicate significant differences among treatments according to the LSD test ($P < 0.05$)

Table 1 Stomatal characteristics during ex vitro transfer of *C. sativa* microplants cultured under different in vitro reatments

	Transfer stage	In vitrotreatment		
		NVL_{50}	VL_{50}	VL_{150}
Density (N° mm^{-2})	In vitro culture	380.5 ± 27.0 b	315.1 ± 32.5 bc	540.7 ± 82.9 a
	Rooting	146.3 ± 4.8 c	192.3 ± 23.9 b	516.5 ± 30.5 a
	Acclimation	243.7 ± 12.4 a	221.2 ± 22.3 a	180.0 ± 4.4 b
	Greenhouse 10 days	178.6 ± 12.6 b	138.4 ± 11.3 c	228.9 ± 14.2 a
	Greenhouse 40 days	213.4 ± 13.1 a	140.0 ± 8.1 b	210.4 ± 7.2 a
	P value	0.0019	0.0162	0.0057
Open stomata (%)	In vitro culture	90.7 ± 10.2 a	64.8 ± 5.8 b	18.6 ± 2.3 c
	Rooting	79.1 ± 5.4 a	70.1 ± 3.6 b	23.5 ± 1.7 c
	Acclimation	72.4 ± 3.9 a	66.3 ± 5.1 b	34.1 ± 4.0 c
	Greenhouse 10 days	78.3 ± 3.0 a	45.4 ± 4.2 b	27.9 ± 3.0 c
	Greenhouse 40 days	51.6 ± 2.4 ab	60.0 ± 2.0 a	69.4 ± 3.3 a
	P value	0.0063	0.0005	0.0071
Width (μm)	In vitro culture	20.8 ± 0.8 a	17.5 ± 0.5 b	10.0 ± 0.8 c
	Rooting	19.2 ± 0.6 a	13.3 ± 0.4 b	18.2 ± 0.3 a
	Acclimation	18.8 ± 0.5 b	18.8 ± 0.5 b	21.3 ± 0.4 a
	Greenhouse 10 days	13.7 ± 1.1 c	20.7 ± 0.8 a	17.4 ± 0.6 b
	Greenhouse 40 days	16.0 ± 0.3 b	19.0 ± 0.9 a	19.0 ± 0.5 a
	P value	<0.0001	0.0002	<0.0001
Length (μm)	In vitro culture	20.4 ± 0.9 a	20.6 ± 0.6 a	14.2 ± 1.4 b
	Rooting	27.7 ± 0.8 a	24.7 ± 0.4 b	23.9 ± 0.9 a
	Acclimation	24.3 ± 0.9 b	24.2 ± 0.9 b	29.7 ± 0.4 a
	Greenhouse 10 days	17.7 ± 1.5 c	24.7 ± 0.9 a	21.5 ± 0.6 b
	Greenhouse 40 days	21.4 ± 0.5 b	24.5 ± 0.4 a	26.0 ± 0.4 a
	P value	<0.0001	0.0098	<0.0001

NVL_{50}, microshoots cultured in non-ventilated vessels at 50 μmol photons m^{-2} s^{-1}; VL_{50}, microshoots cultured in ventilated vessels at 50 μmol photons m^{-2} s^{-1} and VL_{150}, microshoots cultured in ventilated vessels at 150 μmol photons m^{-2} s^{-1}. Mean ± SE (n = 20) with different letters within a row are significantly different among treatments ($P < 0.05$)

transfer stage (R_s), plants from NVL_{50} and VL_{150} showed a decrease in Psat, while VL_{50} was able to maintain its Psat, it is likely because this treatment experienced a lower environmental change. Despite the high Psat of VL_{150}, likely that the decrease Psat during this stage (R_s) could be attributed to the lower light intensity, in addition to the other changes associated with the transition from heterotrophic to autotrophic conditions. New leaves produced during and after acclimation (A_s) maintained or increased their Psat. In the first days of greenhouse transfer, a slight decrease in Psat was observed; this was more drastic in the third day, where all treatments decreased their Psat to about 0.9 μmol CO_2 m^{-2} s^{-1} (Fig. 2a). Within six to nine days, all plants increased their Psat. Thus, VL_{150} increased its Psat from 3.7 μmol CO_2 m^{-2} s^{-1} at the beginning of the study to 5.3 μmol CO_2 m^{-2} s^{-1} at the end, and VL_{50} from 2.1 to 3.2 μmol CO_2 m^{-2} s^{-1}. In contrast, Psat values of NVL_{50} at greenhouse were lower than that observed in vitro.

Regarding water loss control measured as transpiration rate (E), during the firsts days of the ex vitrotransfer, E was reduced in all treatments (Fig. 2b). However, NVL_{50} maintained E values higher than ventilated treatments. At the end of the acclimation stage (A_s), treatments exhibited more similar transpiration rate. Following the greenhouse transfer of NVL_{50}, E showed an increase on the third day, which coincided with the decline in Psat, then E became stable after the sixth day and remained low until the end of the evaluation period. WUE increased as the ex vitro transfer took place (Fig. 2c). The lowest values were determined during the first days of greenhouse transfer, where decreases in Psat and increases in E occurred. WUE increased reaching 3.8 μmol CO_2/mmol H_2O or even higher values in ventilated treatments after 30 days in the greenhouse.

Stomatal density and morphology

Stomatal density and anatomy showed significant differences among treatments and ex vitrotransfer stages (Table 1; Fig. 3). A decrease in stomatal density was observed during rooting, with slight changes in the percentage of stomatal opening. The lowest stomatal density was observed in the new leaves formed during the acclimation stage of plants originating from VL_{150}, which was concomitant with the lowest percentage of stomatal

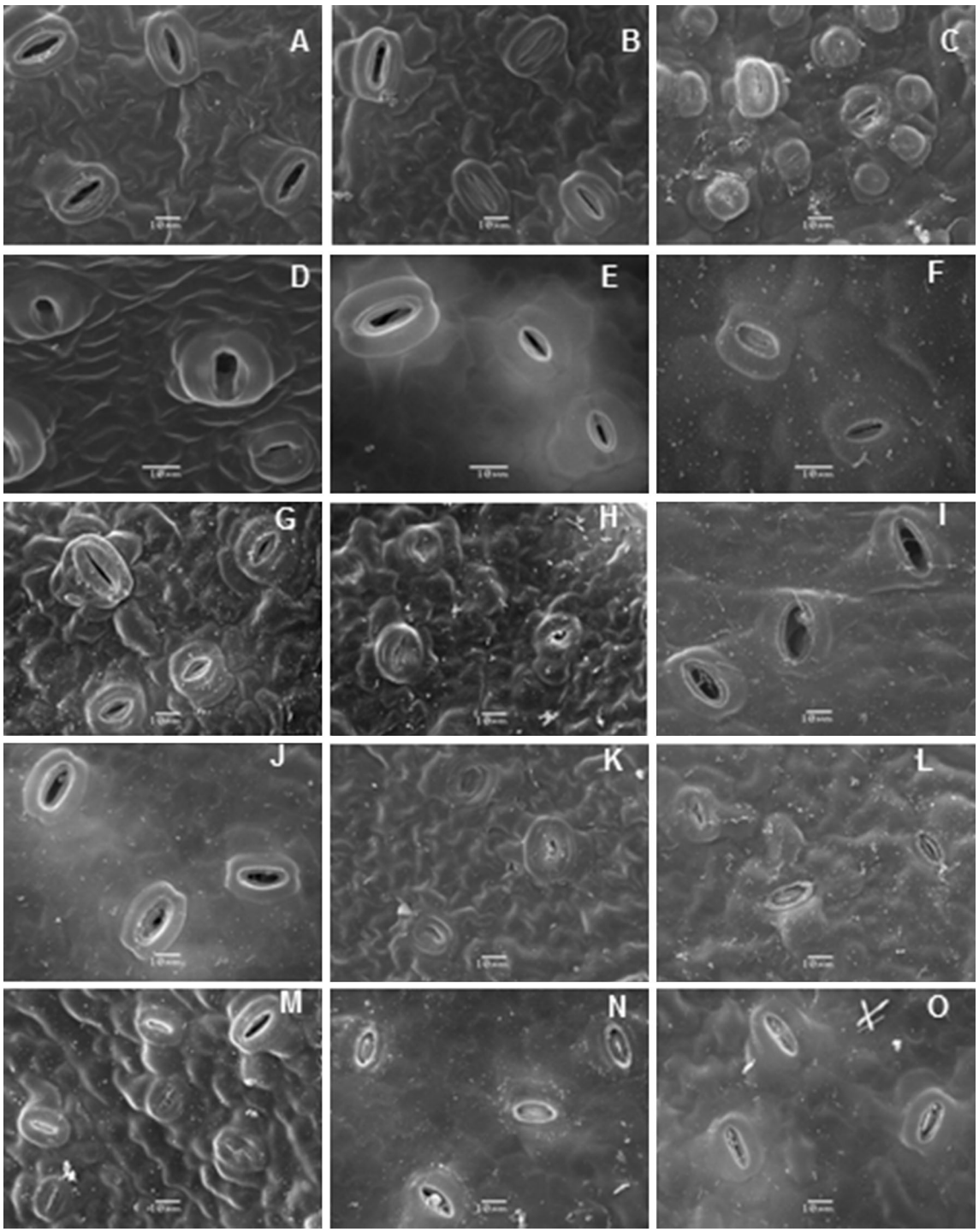

Fig. 3 Stomata of *C. sativa* microplants cultured under different in vitro treatments: NVL_{50} (*left*), VL_{50} (*middle*) and VL_{150} (*right*). Samples were taken in the last in vitro subculture (**a**–**c**), rooting (**d**–**f**), acclimation (**g**–**i**), 10 (**j**–**l**) and forty (**m**–**o**) days after the transfer to the greenhouse. *Bars* indicate 10 μm

opening, but contrary to the increase in their size. Low light treatments (NVL_{50} and VL_{50}) maintained stable their percentages of stomatal opening and size. Conversely, stomatal size differed significantly among treatments, being smaller in NVL_{50} at the end of the evaluation period. Regarding the stomatal shape, the width/length proportion varied among treatments, but only in non ventilated treatment varied among stages. In this treatment (NVL_{50}) the stomata were reducing their proportion during ex vitro-transfer (from 1.1 to 0.7) becoming more elliptical at the

end of the transference stage. In treatments ventilated by contrast, this proportion was always close to 0.8, which reflects a more elliptical shape during all phases and is consistent with a greater ability to control water loss. Additionally, it was possible to observe that wax deposition increased according to the progress of the ex vitrotransfer (Fig. 3).

Light energy partitioning

The electron transport rate (ETRII) dropped during the first and second transfer stages. The largest decreases were observed in all treatments during rooting and 6 days after the greenhouse transfer (Fig. 4a). During rooting, ETRII decreased in average for all treatments from ca. 40 to 15 µmol electrons m^{-2} s^{-1} and was fully recovered at the end of the acclimation stage. In general, ETRII in ventilated treatments surpassed the in vitro values. In each treatment, the highest values were observed during the first day in the greenhouse, where ventilated treatments reach highest ETRII ca. 50 µmol electrons m^{-2} s^{-1}. A fall of ETRII was observed on the third and sixth day in the greenhouse, and then it increased from day 9 ahead in all treatments; although the rates achieved in NVL_{50} never exceeded those observed in vitro. On the contrary, ventilated treatments showed ETRs equal or even greater than in vitro. A similar trend was observed in the maximal efficiency of PSII (F_v/F_m), it decreased slightly during rooting and acclimation in all treatments, values were lower than 0.6 in VL_{50} followed by NVL_{50} with 0.64 and VL_{150} which decreased only to 0.77 (Fig. 4b). A complete recovery was determined at the end of acclimation, except in NVL_{50} where despite the relatively normal value (0.7) showed a high variability. During the greenhouse transfer, F_v/F_m tended to stabilize within 9 days in the ventilated treatments, however in non-ventilated treatments F_v/F_m decreased to the lowest values observed in this study (0.58) after 30 days of transfer to greenhouse and later tended to recover. Regarding non-photochemical quenching (NPQ), a significant increase was observed from the beginning of the ex vitro transfer (Fig. 4c). Ventilated treatments reached two and threefold higher values than those observed during the in vitro culture. A decline in NPQ, which coincided with the trends observed in the ETR and F_v/F_m was determined in NVL_{50}, but this was recovered by the end of the ex vitro transfer.

F_v/F_m recovery after photoinhibition

The decline of F_v/F_m post-PhT was lower and with a faster recovery with the exposition to ex vitroconditions (Fig. 5). In vitro culture and rooting stages were the most susceptible stages to PhT (Fig. 5a, b). In these stages, F_v/F_m decreased to values close to 0.2 without full recovery within the evaluation period, being the most sensitive treatment VL_{50}. During acclimation the fall of F_v/F_m post-PhT was similar (Fig. 5c), but after 25 h F_v/F_m had recovered to nearly 75 %. At this stage, the most vulnerable treatment corresponded to VL_{150}. A drastic change was observed 10 days after transferring the plantlets to the greenhouse (Fig. 5d); reaching F_v/F_m values close to 0.2, but the recovery was fast. One hour after the PhT 50 % F_v/F_m had been recovered and after 50 h all of the treatments showed values close to 0.7. Similar values were observed 40 days after the transfer to the greenhouse (Fig. 5e), but with decreased drop in F_v/F_m (nearly 0.3). At this stage, all treatments reached values close to 0.7 within 10 h of recovery.

Effect of photoinhibition treatment on NPQ and its components

NPQ and its components were evaluated post-PhT (Fig. 6). During the in vitro culture, NPQ was mainly determined by a slow relaxing component (NPQ_s 84 %) in all treatments (Fig. 6a). During rooting (Fig. 6b), a change was only observed in VL_{50} where nearly 51 % of NPQ was determined by NPQ_s. The other in vitro treatments maintained values close to 80 %. Thus, during the first transfer stage, the highest proportion of NPQ was determined by NPQ_s and only a small portion was determined by a fast kinetics dissipation of excess absorbed energy (NPQ_f). This proportion began to change throughout the second transfer stage and became completely opposite at the end of the greenhouse transfer. During the acclimation, the slow relaxing component accounted for 65, 50 and 21 % of NPQ in NVL_{50}, VL_{50} and VL_{150}, respectively (Fig. 6c). This last treatment showed a slower kinetic recovery of F_v/F_m (Fig. 5c), concomitant with its low NPQ_f proportion. Ten days after transfer to the greenhouse, photoinhibited plants from all treatments displayed a greater proportion of the fast relaxing component (Fig. 6d). Thus, during this stage more than 60 % of NPQ was determined by NPQ_f. The same trend was observed after 40 days in the greenhouse (Fig. 6e), where over 85 % of NPQ corresponded to NPQ_f.

Xanthophylls pigments and de-epoxidation capacity

Like the increase in the recovery of F_v/F_m and NPQ_f proportion post-PhT, the ability to de-epoxidate violaxanthin (V) to zeaxanthin (Z) increased over time in ex vitro conditions (Fig. 7). During the in vitro culture, the microshoots grown under different treatments showed differences in the xanthophylls per unit of chlorophyll. The ventilated treatments had similar sizes, which were almost half of that observed in NVL_{50} (Fig. 7a). Post- PhT none of

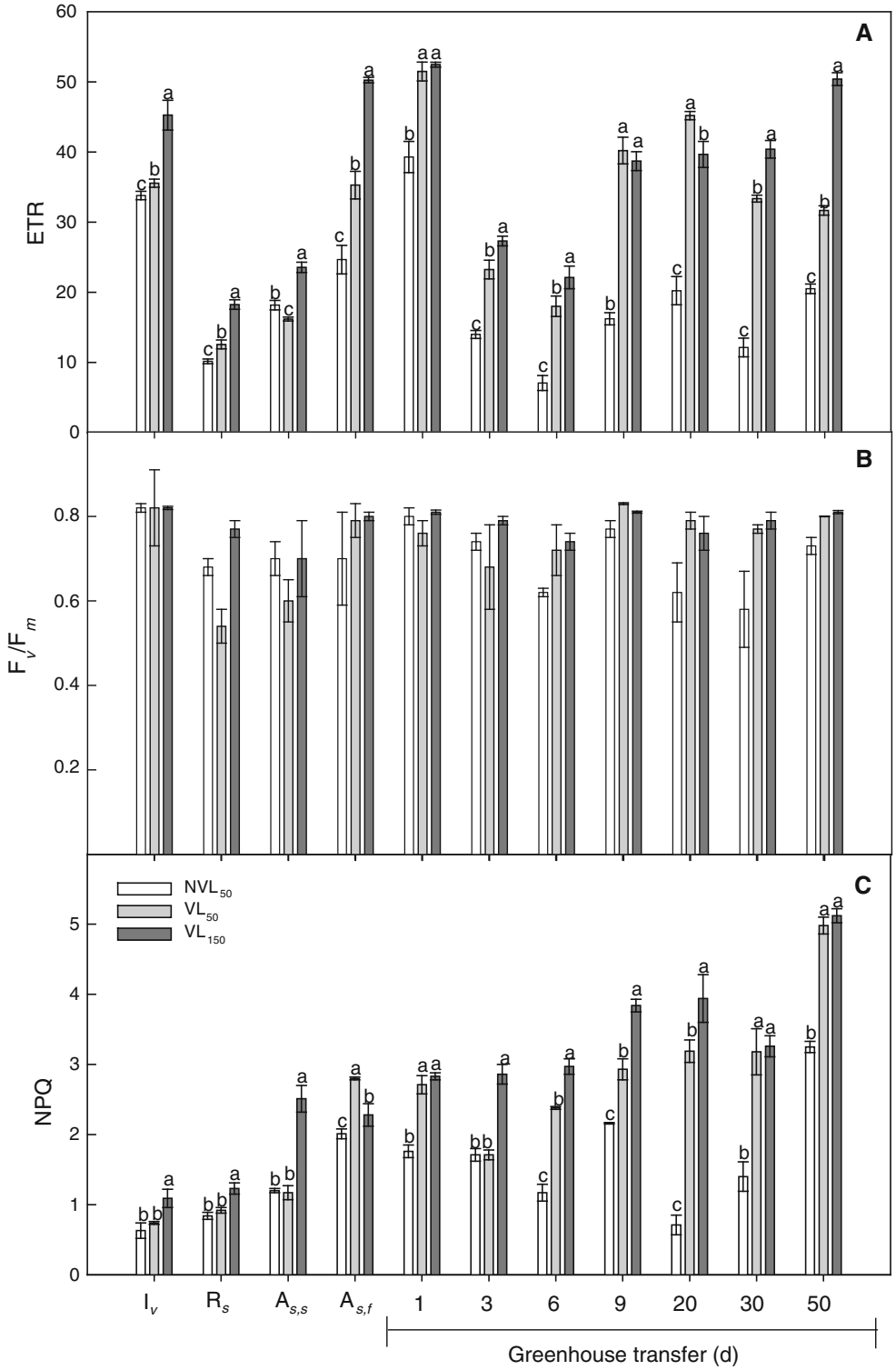

Fig. 4 Effects of in vitro treatments on ETR (**a**), maximal efficiency of PSII (**b**) and non- photochemical quenching (**c**) of *C. sativa* microplants rooting and subsequent transfer to the greenhouse. Measurements were made during the last in vitro subculture (I_v), rooting (R_s), during the beginning ($A_{s,\ s}$) and the end ($A_{s,\ f}$) of acclimation stage and the greenhouse transfer. Each *bar* represents the mean ± SE (n = 5). *Different letters* indicate significant differences among treatments according to the LSD test ($P < 0.05$)

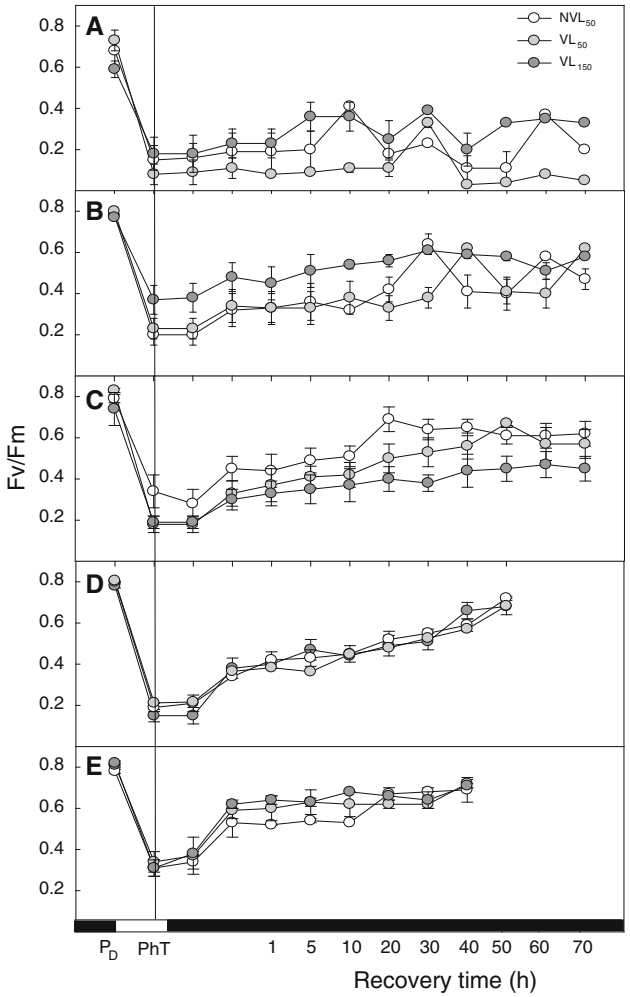

Fig. 5 Effect of in vitro treatments on *C. sativa* microplants' maximal efficiency recovery kinetics of PSII (F_v/F_m) subjected to a photoinhibition treatment (PhT). Plants were subjected to PhT in the last in vitro subculture (**a**), rooting (**b**), acclimation (**c**), 10 (**d**) and 40 (**e**) days after transfer to the greenhouse. The *black bar* at the bottom of the graph represents dark periods, pre-darkened leaves (P_D) and recovery time (h). The white section of the *bar* represents the PhT. Three individual plants (n = 3) were measured in each stage, points show mean ± SE

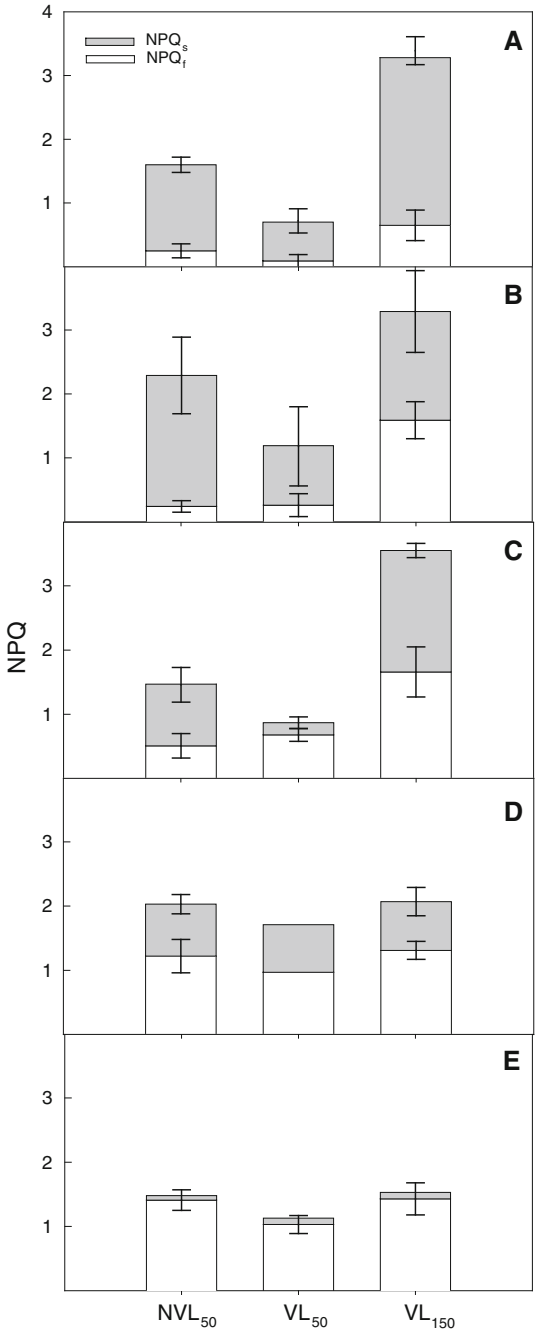

Fig. 6 Effects of in vitro treatments on NPQ components, slow relaxing component (NPQ_s) and fast relaxing component (NPQ_f) of *C. sativa* microplants subjected to a photoinhibition treatment (PhT). The PhT was applied in the last in vitro subculture (**a**), rooting (**b**), acclimation (**c**), 10 (**d**) and 40 (**e**) days after the transfer to the greenhouse. Three individual plants (n = 3) were measured in each stage. *Bars* show mean ± SE

the treatments achieved a complete de-epoxidation, showing similar levels of antheraxanthin (A) and a static pool of de-epoxidable V (Fig. 7b). During rooting, differences were found among treatments (Fig. 7c, d). NVL_{50} showed undetectable amounts of xanthophylls, VL_{50} showed no de-epoxidation capability and VL_{150} was the only treatment which showed conversion capability, deploying 41 % of the de-epoxidate state. A noticeable change was observed during the acclimation (Fig. 7e, f), associated with other changes detected, including a faster recovery of F_v/F_m post PhT and an increased proportion of NPQ_f (Figs. 5 and 6, respectively). During this stage, all treatments showed similar proportions of VAZ in pre-dark adapted leaves and de-epoxidation capacity. The same was observed during the transfer to the greenhouse (Fig. 7h, j), where an active conversion of V to A and Z was observed post-PhT, reaching a DEPS of 47 % in NVL_{50} and VL_{150} and 36 % in

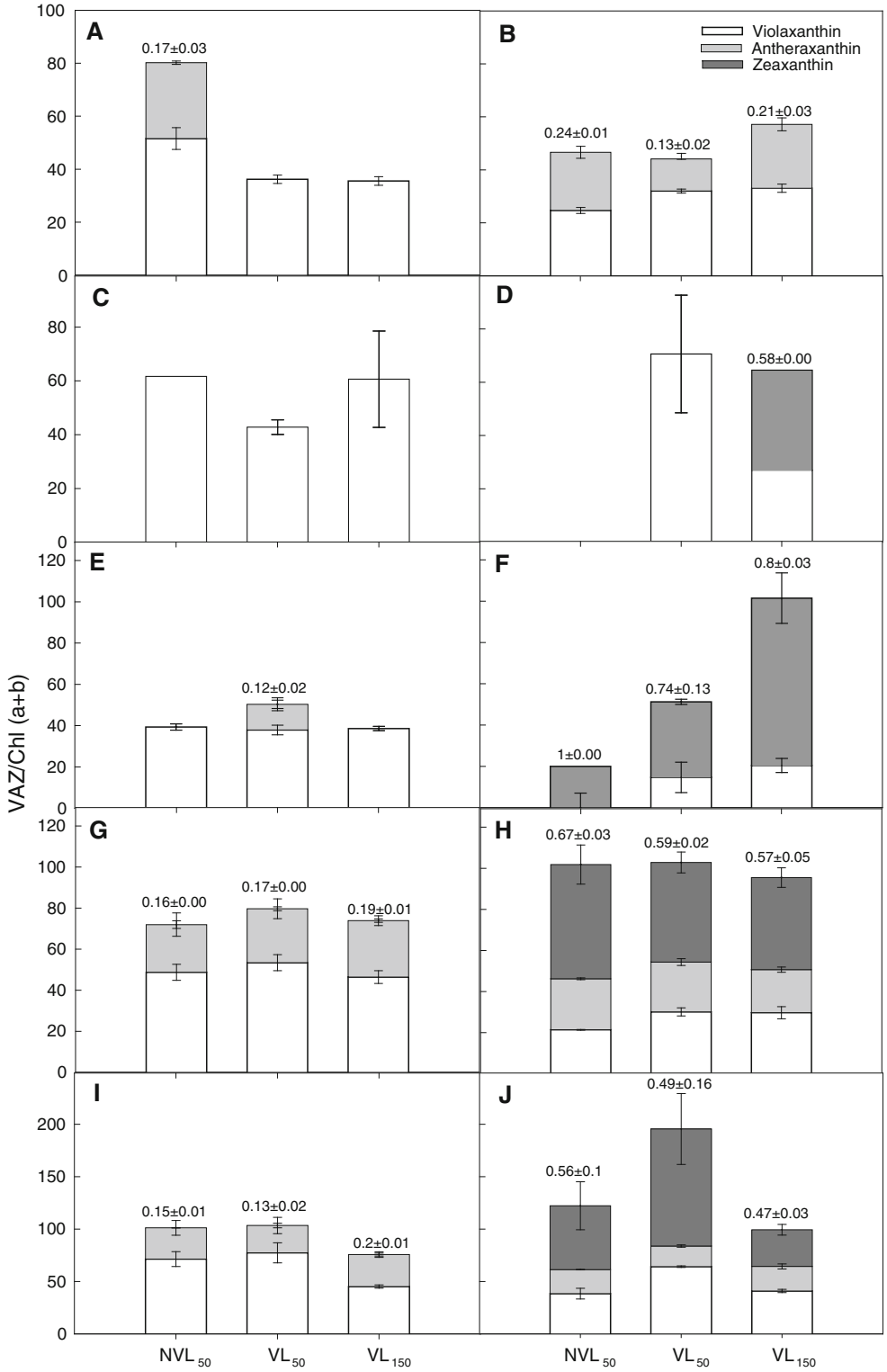

Fig. 7 Effects of in vitro treatments on *C. sativa* microplants' xanthophylls pigments (violaxanthin, antheraxanthin, zeaxanthin) in pre-darkened leaves (*left*) and de-expoxidation capacity after photoinhibition treatment (*right*) in the last in vitro subculture (**a**, **b**), root induction (**c**, **d**), acclimation (**e**, **f**), 10 (**g**, **h**) and 40 (**i**, **j**) days after the transfer to the greenhouse. Three individual plants (n = 3) were measured in each stage, bars show mean ± SE. De-epoxidation state (numbers on *top* of the *bars*) was calculated as Z + 0.5A/VAZ

VL_{150}. Additionally, in the greenhouse all treatments had a slightly de-epoxidized pool (around 15 %) in the pre-dark state, possibly as a static pool, which could play a structural role.

Discussion

The effect of in vitrotreatments on the behavior of *C. sativa* microplants during their ex vitro transfer was studied in two transfer stages. First, transfer from in vitroculture vessels to pots with substrate in growth chamber (rooting and acclimation); and second, the transfer from growth chamber to greenhouse (post-acclimation). Both transfer stages were accompanied by sudden changes in environment conditions. The first was the transition from the culture medium supplemented with nutrients and sucrose in very humid sterile vessels into sterile soil, lower air humidity and no additional external carbon source. The second transfer was to a greenhouse environment in non-sterile soil in addition to a drastic increase in irradiance (ca. tenfold higher than in vitro culture).

The differences observed in light saturated net photosynthesis, transpiration rates and WUE during the in vitro culture between ventilated microplants gradually disappeared during the transfer. Thus, at the end of this stage, plants coming from VL_{50} and VL_{150} reached values of WUE (around 4.0 μmol CO_2/mm H_2O,) becoming more similar to those reported previously in nursery grown plants (Sáez et al. 2012b). However, in this stage and at the end of the second transfer stage, parameters associated with light energy partitioning (maximal efficiency of PSII, ETR and non-photochemical quenching), remained different among treatments. These differences were mainly with respect to traditional culture (NVL_{50}, Fig. 4) and are consistent with survival (Fig. 1a), which had the same trend that the proliferation rate in vitro between ventilated and not ventilated treatments, where the first had significantly higher number of new microshoots formation (Sáez et al. 2012b).

In the first transfer stage, specifically the rooting stage, a decrease in Psat compared to in vitro occurred (Fig. 2a). The greatest decreases found in NVL_{50} and VL_{150} coincided with the high mortality observed in these treatments, mainly in NVL_{50}, which reach about 40 % of mortality (Fig. 1a). These results indicate that plants could not be able to adjust to the autotrophic growth conditions, at least during the first days after the transfer (Swain et al. 2010). Additionally, this confirms the high stress level for non-ventilated plants during the transition from mixotrophic to autotrophic conditions and the susceptibility of these plants to environmental changes. In this sense, limited ventilation (NVL_{50}) results in a high relative humidity, which can cause numerous malformations at anatomical level (Majada et al. 2001; Apóstolo et al. 2005; Joshi et al. 2006), restricting water loss control when subjected to ex vitro transfer. In addition, changes in the photon flux density (as the passages of 150–50 μmol m^{-2} s^{-1}) induced a light limitation of photosynthesis (Coopman et al. 2008). During acclimation, a slight increase in Psat was observed in microplants originating from ventilated treatments (VL_{50} and VL_{150}), concomitant with a significantly higher survival (about 90 %). During this stage, we observed a significant increase in plant leaf number (Fig. 1b), which were also significantly larger in size (Fig. 1c). This was most likely because their growth was not limited by the culture vessels and because in ex vitro conditions the higher light levels and CO_2 favored the overall plant growth (Estrada-Luna et al. 2001). Additionally, the faster growing rates and survival during the acclimation (e.g. larger leaf area development) observed in these treatments, compared to the traditional culture (NVL_{50}) likely are associated with their higher photosynthetic capacity (Kirdmanee et al. 1995). Indeed, low ex vitro survival have been correlated with low leaf expansion. Conversely, high survival has been found in cultures with elongated leaves and morphological similarities to acclimatized plants produced ex vitro (Valero-Aracama et al. 2006).

Similar to Psat, transpiration rates (E) also decreased during the initial ex vitro transfer (Fig. 2b). This decrease was drastic in microplants originating from ventilated treatments, reflecting the high stomatal density and water loss control, which corresponds to our previous experiments (Sáez et al. 2012b) and data in the literature (Majada et al. 1998; Decetti et al. 2008). Despite studies of in vitro culture plants that indicate low ability to regulate water loss (Majada et al. 2001; Apóstolo et al. 2005; Joshi et al. 2006; Sáez et al. 2012a), microplants originating from traditional cultures decreased their E significantly during the ex vitro transfer. This reflects some ability to water loss control; although E values were higher than in nursery grown plants and in those grown in ventilated vessels (Sáez et al. 2012b). Transpiration rates measured after 4 weeks in the greenhouse were similar in all treatments, but at this time Psat significantly differed among them, being higher in those treatments with higher survival rate. Thus, it is likely that in *C. sativa* microplants, the photosynthetic capacity is the main cause of poor ex vitro stablishment, rather than water loss control. This is in agreement with Valero-Aracama et al. (2006) who affirmed that although high transpiration rates may exacerbate the stressful transition to ex vitro conditions, other physiological limitations, including poor photosynthetic capacity or limited carbohydrate reserves during acclimation, had a greater impact on ex vitro plant survival.

During last period of ex vitro conditions, transpiration rate was similar in all treatments, but stomatal anatomy

differed among treatments (Fig. 3). In agreement with Brutti et al. (2002), we found greater stomatal density during the in vitro culture compared to the rooting stage (Table 1). Although a decrease in stomatal density after transfer has been observed in several species (Johansson et al. 1992; Apóstolo and Llorente 2000; Brutti et al. 2002), such changes depend on the species (Seon et al. 2000; Pospisilová et al. 2009) and, according to our results, on the transfer stage. The decrease in stomatal density was sometimes compensated by an increase in stomatal size (Pospisilová et al. 2009) and changes in stomata shape, from a ring to an elliptical, reflecting the greater functional stomatal apparatus (Tichá et al. 1998). These reflect the great dynamic of the stomatal apparatus (Brutti et al. 2002), concomitant with a higher water loss control. These changes, along with others found in Psat, but mainly in E, caused considerable increases in WUE after the ex vitro transfer (Fig. 2c), reaching values as high as those observed in nursery grown plants (Sáez et al. 2012b). Thus, as reported by Estrada-Luna et al. (2001), micropropagated plants became more efficient in water usage as they become acclimated. Levels of Psat, E and WUE reached at the end of the greenhouse transfer of VL_{150}, and to a lesser degree of VL_{50} plants, were almost the same as those observed in nursery grown plants, indicating that this acclimation improve their carbon metabolism (Rodríguez et al. 2008).

On the other hand, ETRs showed less significant differences between ventilated treatments; although ETRs were always higher in plants cultured at higher levels of irradiance (VL_{150}). ETRs significantly decreased during rooting and were recovered by the end of acclimation in the growth chamber, showing a peak during the early days in the greenhouse (Fig. 4a). Subsequently, ETRs tended to increase at the end of the greenhouse transfer in microplants originating from ventilated treatments. According to Kadlecek et al. (2001), the transient peak in ETRs immediately after the transfer from the growth chamber to the greenhouse may be interpreted as stress reactions to the considerable increase in irradiance. It has been proven that electron transport through PSII is inhibited when photoinhibition is induced by excessive irradiance (Powles 1984). The term photoinhibition describes the irreversible decline in photosynthetic activity when light energy exceeds the use capacity in photosynthesis. The first indication that not all of the absorbed photons are used in photosynthesis is a reduction in the maximum photochemical efficiency, measured as the F_v/F_m (Kumar and Kumar 2003). As shown in Fig. 5b, all treatments decrease F_v/F_m after being transferred from culture vessels to the soil. However, during this stage, the plants were not subjected to increased light intensities. Thus, given that the photosynthetic apparatus is one of the most sensitive structures to damage under stress conditions (Walters 2005), it is likely that the decrease in F_v/F_m during this stage is due to additional environmental changes imposed by the ex vitro transfer.

In greenhouse conditions, photoinhibitory impairment might occur as a result of sudden changes in temperature and light intensity (Powles 1984; Bolhar-Nordenkamp et al. 1989; Ögren and Evans 1992). To avoid or minimize photoinhibition, photosynthetic organisms have evolved constitutive characteristics, such as the presence of carotenoids to detoxify singlet oxygen or trap chlorophyll in triplet states (Horton and Ruban 2005). These, combined with long and short-term photoprotection mechanisms, help handle and dissipate excess absorbed energy. Non-photochemical quenching (NPQ) constitutes the fastest short-term photo protective strategy and involves the dissipation of excess light energy at the antenna level of PSII (Müller et al. 2001). During the in vitro culture, microshoots of *C. sativa* exhibited low values of NPQ, compared to nursery-grown plants (Sáez et al. 2012a), and only microshoots cultured in VL_{150} reached higher values than others in vitro treatment; however, these continued to be low compared to nursery-grown plants (Sáez et al. 2012b). During the ex vitro transfer, NPQ increased in all treatments (Fig. 4c). In microplants originating from ventilated treatments, NPQ reached levels as high as those found in nursery grown plants. This increase indicates that ex vitro conditions promoted an increase in thermal dissipation capacity and hence an increase in plants' ability to grow under higher light conditions (Osorio et al. 2010). In order to study if the increase in NPQ determined a greater capacity to withstand a sudden increase in light, we subjected microplants to a photoinhibition treatment (PhT). VL_{150} microplants were less susceptible to PhT during the in vitro culture and rooting, and exhibited a higher NPQ than in other treatments. However, none of the treatments' showed recovery of F_v/F_m within the evaluation period (Fig. 5), and in these cases, NPQ was mainly determined by the slow relaxing component (NPQ_s) (Fig. 6a, b). This component has been associated with prolonged exposure to light stress, sustained down-regulation and photodamage (Horton and Ruban 2005). Chronic photoinhibition usually describes a situation in which core PSII proteins were damage and repair is needed to recover full photochemical capacity, in fact photosynthetic efficiency remains low after hours of darkness (Björkman and Demmig 1987). During the ex vitro transfer, plants became less susceptible to PhT and declines in F_v/F_m were lower (Fig. 5d, e). This was most likely associated to changes in the NPQ components, which became more dependent on the fast relaxing component (NPQ_f) and accounted for more than 90 % of the NPQ at the end of transfer (Fig. 6e). NPQ_f reflects heat dissipation mediated by the activation of the xanthophyll cycle (Demmig et al. 1987). This indicates that although the light intensity used in the PhT could be

saturating, during the ex vitro transfer microplants are able to dissipate excess absorbed energy with minimal photodamage, showing an active violaxanthin to zeaxanthin conversion at the end of greenhouse transfer (Fig. 7). The de-epoxidation capacity found in vitro and at the beginning of the ex vitro transfer (Fig. 7a, b) indicates that microplants were unable to reach an effective amount of de-epoxidation after the PhT. Rather, plants maintained a pool of slightly de-epoxidated xanthophylls, concomitant with low F_v/F_m recovery and a high NPQ_s component, except in VL_{150} microplants. It is likely that by this, VL_{150} microplants are more resistant to ex vitro conditions, and therefore, have higher survival. Finally, the long-term effects of in vitro treatments on ex vitro performance were explicated in our experiments. If in vitro plantlets are cultured in ventilated vessels and maintained under a light higher light intensity (150 μmol m^{-2} s^{-1}; normally used 50 μmol m^{-2} s^{-1}), higher photosynthetic capacity and WUE can be reached in greenhouse. Additionally, plants are able to adjust to a sudden light increase. Higher light acclimation correlated with higher proliferation rate during in vitro culture, and growth during the ex vitro transfer. In fact, our results show that higher environmental gradients than commonly used during in vitro culture is necessary to reduce the stress of transfer and the acquisition of faster autotrophic behavior. Overall, the higher availability of key growth dependent factors, such us light and DPV allows a more successful micropropagation system reflected in greater survival at greenhouse conditions.

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