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Importance of in vitro pretreatment for ex vitro acclimatization and growth

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Abstract

The influence of in vitro pretreatment on the physiology and growth of tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets during ex vitro transfer and acclimatization was studied. Nodal cuttings were cultured on solid Murashige-Skoog medium with 3% sucrose or without sucrose, and in low (60 μmol m^{-2} s^{-1}) or high (200 μmol m^{-2} s^{-1}) photosynthetic photon flux densities. 35 days old plantlets were transferred to soil. After a 20-days-period of acclimatization in the greenhouse the plants were transferred to the open air. The distinctive effects of the different in vitro culture conditions on pigments and photosynthetic parameters disappeared during greenhouse acclimatization. Both the transfers were accompanied by a transient increase in starch content and the transfer to open air by a transient decrease in maximum photochemical efficiency of Photosystem II. In the open air, photosynthetic capacity and starch content started to increase with only small effects of the different pretreatments. Pretreatment had a pronounced effect on growth under these conditions. Growth was highest in plants originally grown with 3% sucrose under high irradiance, and lowest in plants originally grown without sucrose under high irradiance (plantlets suffering from photoinhibition in vitro). Thus, photomixotrophic in vitro culture at elevated photon flux density is most suitable for later ex vitro development of the plants.

Keywords: Acclimatization; Chlorophyll fluorescence; Growth; In vitro/ex vitro transfer; Photosynthetic apparatus; Tobacco (*Nicotiana tabacum* L. cv Samsun)

1. Introduction

Transfer and acclimatization to the ex vitro environment is the final but frequently most hazardous step in a successful micropropagation system [1]. The specific in vitro environment, with artificial medium usually supplied with sugar(s), the growth of plantlets in small air-tight vessels with high air humidity, low gas exchange and thus a CO_2-shortage during almost the whole photoperiod, ethylene production and relatively low photosynthetic photon flux density (PFD), induces disturbances in plant development and photosynthetic performance [2,3]. After the transfer from in vitro to ex vitro, plants have to correct the abnormalities and to acclimatize to the new environments in the greenhouse and the field [4]. We could demonstrate in earlier experiments that tobacco plantlets cultured in vitro show considerable differences in pigment content, photosynthetic performance and growth depending on the presence of sugars and the PFD [5,6]. As compared to the photoautotrophically grown plantlets the photomixotrophically ones showed an increase in the chlorophyll (chl) a + b content, the photosynthetic capacity at CO_2 and light saturation, the leaf area development and dry matter accumulation. An impairment of photosynthetic function (photoinhibition, [7]) could be detected if the plantlets were grown photoautotrophically at the higher PFD. It was manifested as a reduction in chl and carotenoid content, a high ratio of xanthophyll cycle

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pigments to chl and a loss in photochemical efficiency accompanied by partial loss of RC II proteins [6].

The aim of the present investigation was to clarify whether the different growth conditions during in vitro pretreatment affect: (a) the short-term responses to the abrupt changes during transfer; and (b) the long-term development under ex vitro conditions. To maintain a higher water status of the plants at the beginning of acclimatization [8], a two-step acclimatization process was applied in our experiments.

2. Materials and methods

2.1. Plant material

Tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets were cultured in vitro on solidified Murashige and Skoog basal medium (M 5519, Sigma) with 0.7% (w/v) agar (A 1296, plant cell culture tested, Sigma) at day/night temperatures of 25/18 °C and a 16-h photoperiod with 3% sucrose (3%) or without sucrose (0%), and under low (LL: 60 μmol m^{-2} s^{-1}) or high (HL: 200 μmol m^{-2} s^{-1}) PFDs. According to these conditions the different plants were abbreviated as 3% HL, 3% LL, 0% HL and 0% LL. These descriptions were used throughout, even though during ex vitro culture plants were exposed to the same conditions. The cultivation vessels were covered with a CO_2-permeable transparent film (Suncaps, Sigma) and enhanced CO_2 concentration ($\pm$ 700 μmol mol^{-1}) for photosynthesis was achieved by putting beakers containing 2 M carbonate/bicarbonate buffer into the cultivation chamber [6,9].

2.2. Transfer to ex vitro environment

At day 35 in vitro plantlets were transferred to pots (10 cm in diameter) filled with a 1 cm layer of sand and a mixture of soil and perlite (3:1). The agar medium was carefully washed out of the roots. The potted plantlets were transferred to a transparent plastic 'tent' in the greenhouse to reduce water loss. Shadings were used to reduce irradiance during the first days of acclimatization. Starting with day 10 after the transfer to the greenhouse, the 'tent' was stepwise opened and the shading of the 'tent' reduced. At day 17 the 'tent' was removed completely. For all the plants the irradiance in the greenhouse varied from 30 to 90 μmol m^{-2} s^{-1} and the temperature from 18 to 26 °C. After a 20 day acclimatization period in the greenhouse, the plants were carefully transferred into a bed of soil in open air. There the irradiance varied between 200 and 1400 μmol m^{-2} s^{-1} and the temperature between 24 and 31 °C (measured at 15:00 h.).

2.3. Measurements of photosynthesis and fluorescence

Net oxygen evolution rates were measured in the interval from 09:00 to 15:00 h with a Clark type gas-phase leaf-disc oxygen electrode (LD2/2, Hansatech, King's Lynn, UK) [6,10] at 25 C in CO_2-enriched air (2 M bicarbonate buffer), and at PFD of 700 μmol m^{-2} s^{-1}. PFD was provided by a Björkman lamp (LS2, Hansatech, King's Lynn, UK) equipped with neutral density filters. Incident PFD (400–700 nm) was measured with a LI-189 quantum-meter (LI-COR, Lincoln, Nebraska) equipped with Quantum Sensor LI-190SA.

Chl a fluorescence emission from the upper surface of the leaves was measured in modulated light with a pulse amplitude modulation fluorometer (PAM, Walz, Effeltrich, Germany) simultaneously with O_2 evolution: the tip of the fiber optic of the PAM was inserted at an angle of 45 in the top water jacket of the LD2 chamber. The minimum chl fluorescence yield in dark (F_0) was elicited with a dim red (650 nm) light modulated at 1.6 kHz. The maximum chl fluorescence yield in dark (F_m) and light (F'_m) were induced by a flash of intense white light from the light source KL 1500 (Schott, Germany). The maximum photochemical efficiency of PS II was calculated in 30-min-dark-adapted leaves as $(F_m - F_0)/F_m = F_v/F_m$.

2.4. Biochemical analyses

Chls a + b and carotenoids were extracted from leaf discs (6 cm^2) with acetone and analysed by HPLC as described in [6].

Carbohydrate analyses were done with leaf samples (10 cm^2) taken together with the samples for photosynthesis measurements. The samples were frozen in liquid nitrogen and stored at -20 °C. Starch, sucrose, glucose and fructose were extracted and measured using enzymatic and spectrophotometric methods as described by [11].

Dry matter was determined after 24 h at 80 °C. Leaf area was determined by scanning leaf images and applying a computer program.

2.5. Statistical analyses

Analysis of variance (program Number Cruncher Statistical System) was applied. Each result is the mean of at least three measurements.

3. Results

3.1. Transitional acclimatization period in the greenhouse (days 35–55)

In the first stage of the ex vitro acclimatization in the

greenhouse, most of the significant differences found after the application of different in vitro culture conditions gradually disappeared, e.g. in contents of soluble carbohydrates (glucose, fructose, sucrose; Fig. 1(A)) and starch (Fig. 1(B)), photosynthetic capacity (Fig. 2(A)) and maximum photochemical efficiency of PS II in dark adapted leaves (F_v/F_m, Fig. 2(B)) and in pigment contents (Figs. 3 and 4). The changes were most pronounced for 0% HL plantlets which at the beginning had a considerably reduced chl content, chl a/b ratio (Fig. 3) and D_1/LHC II ratio. An 65% increase in proportion of D_1/LHC II proteins of 0% HL plants was detected during 10 days acclimatization in the greenhouse (data not given). The highest deepoxidation state of the xanthophyll cycle pigments normalized to chl a + b content (DESC) was found in HL plants, especially in the 0% HL ones (Table 1). DESC decreased very quickly immediately after transfer with exception of the 0%HL plants where it was enhanced by 21%. Slightly higher values were maintained for the HL plants during the whole acclimatization in the greenhouse (Table 1).

In vitro pretreatment had a significant influence on plant growth in this first stage of acclimatization. By the end of in vitro culture all plantlets had developed 6–7 leaves. The formation of new leaves during ex vitro growth in the greenhouse was different for the different pretreatments (Fig. 5(A)). Total leaf area (Fig. 5(B)) and total plant dry matter (Fig. 5(C)) were significantly lower in 0% plants than in 3% plants, indicating a lasting effect of the different sucrose availability during the in vitro phase. At the end of growth in the greenhouse, 0% HL plants had significantly lower leaf numbers than the other plants (Fig. 5(A)), showing that this pretreatment had been the most unfavourable for ex vitro development. The ratio of leaf dry matter to leaf

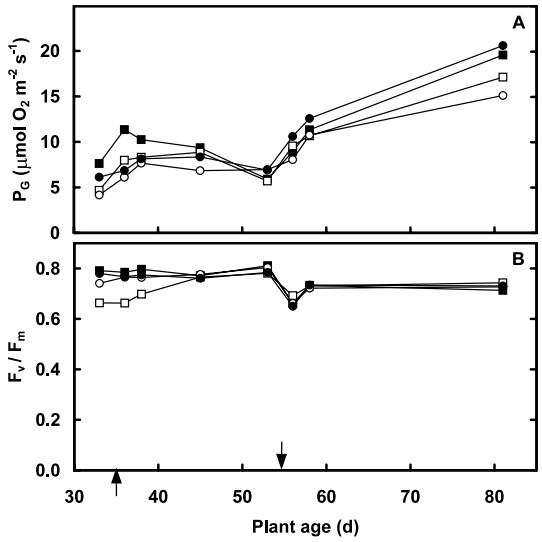

Fig. 2. Photosynthetic capacity, i.e. gross photosynthetic rate at light and carbon dioxide saturation (A) and maximum photochemical efficiency, F_v/F_m, in dark adapted leaves (B) in in vitro plantlets (day 33), during transfer of the plants from in vitro to the greenhouse (days 35–55) and from the greenhouse to open air (days 55–81). Days of transfer were marked by arrows. For explanation of the plant symbols see Fig. 1. Mean S.E. in: (A) 0.76; and (B) 0.01.

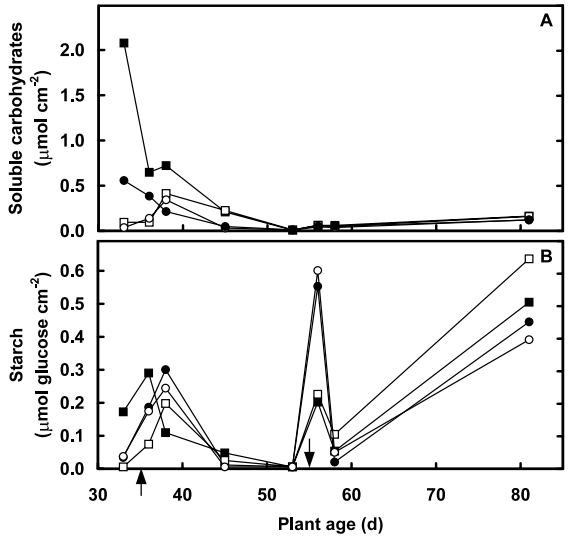

Fig. 1. Contents of soluble carbohydrates (glucose, fructose and sucrose (A)) and starch (B) in in vitro plantlets (day 33), during acclimatization of the plants in the greenhouse (days 35–55) and in open air (days 55–81). Days of transfer were marked by arrows. Plants were originally cultured in vitro in media with 3% sucrose in high light, HL (200 μmol m^{-2} s^{-1}; ■) and low light, LL (60 μmol m^{-2} s^{-1}; ●), or in absence of sucrose (0%) in HL (□) and LL (○). Mean S.E. in (A) 0.07; and (B) 0.08.

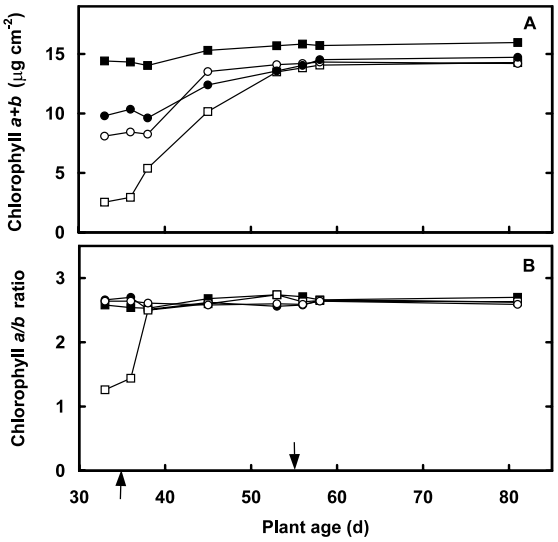

Fig. 3. Chlorophyll a + b content (A) and chlorophyll a/b ratio (B) in in vitro plantlets (day 33), during transfer of the plants from in vitro to the greenhouse (days 35–55) and from the greenhouse to open air (days 55–81). Days of transfer were marked by arrows. For explanation of the plant symbols see Fig. 1. Mean S.E. in: (A) 0.87; and (B) 0.07.

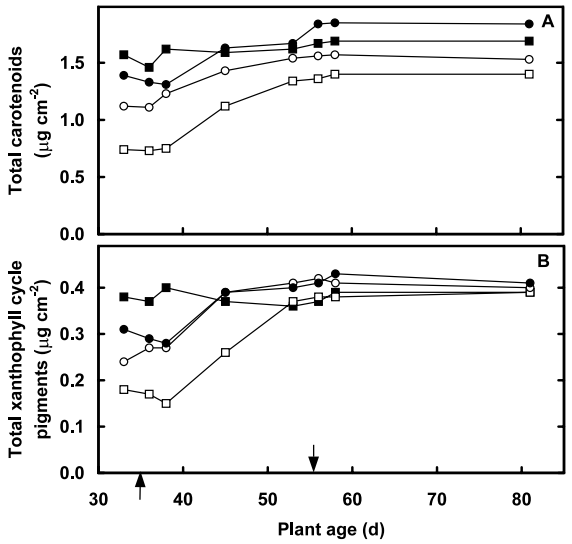

Fig. 4. Total carotenoids pool (A) and total xanthophyll cycle pigments pool (B) in in vitro plantlets (day 33), during transfer of the plants from in vitro to the greenhouse (days 35–55) and from the greenhouse to open air (days 55–81). Days of transfer were marked by arrows. For explanation of the plant symbols see Fig. 1. Mean S.E. in: (A) 0.24; and (B) 0.45.

area decreased in 3% HL plants during acclimatization in the greenhouse while it remained more or less unchanged in the other plants (Fig. 5(D)).

3.2. Transfer and acclimatization to open air (days 55–81)

On day 55, plants were transferred from the greenhouse to a bed of soil in open air. All the plants survived to the end of the transfer experiment. In this period where all the plants were growing under the same, but several times higher PFD, most of the photosynthetic parameters developed in parallel with no significant differences among the plants with different in vitro pretreatment. A very small increase in soluble carbohydrates (Fig. 1(A)) and a pronounced increase in starch content (Fig. 1(B)) were found. Photosynthetic capacity (Fig. 2(A)) rose considerably, with the highest increase in originally photomixotrophically grown plants (3% HL and 3% LL). At the end of in vitro culture, photosynthetic capacity was between 4 and 8 μmol m^{-2} s^{-1} whereas in ex vitro plants 46 days after transfer much higher values of P_G, between 15 and 21 μmol m^{-2} s^{-1}, were reached (Fig. 2(A)). F_v/F_m after a transient decrease, dropped to a lower level than it was in the greenhouse (Fig. 2(B)). Stabilized levels of chl a + b contents (Fig. 3(A)), chl a/b ratio (Fig. 3(B)), total carotenoids (Fig. 4(A)) and xanthophyll cycle pigments (Fig. 4(B)) were reached in all the plants during acclimatization in open air. HL plants showed a decrease in DESC immediately after transfer to open air whereas the opposite was true for the LL plants. The differences in DESC between 3% HL and 3% LL plants were higher than between 0% HL and 0% LL ones, respectively (Table 1).

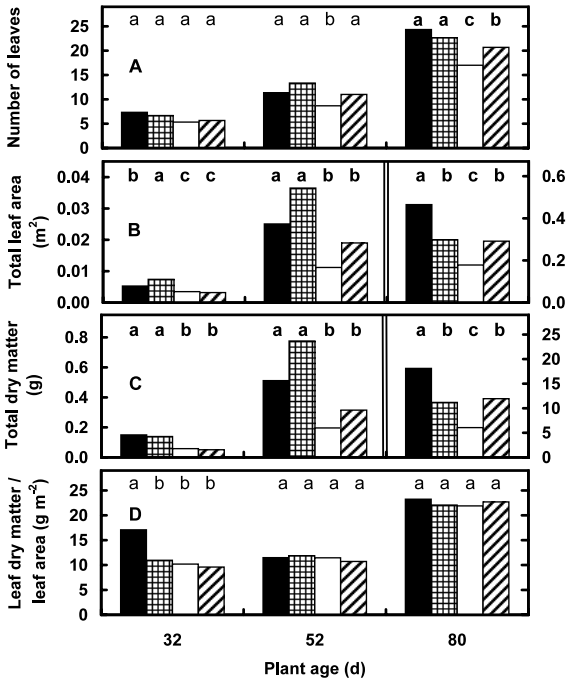

Fig. 5. Number of leaves developed per plant (A), plant total leaf area (B), plant total dry matter (C) and the ratio of leaf dry matter to leaf area (D) in in vitro plantlets (day 32), in plants after acclimatization in the greenhouse (day 52) and in plants after acclimatization in open air (day 80). Plants originally cultured in vitro on MS medium with 3% sucrose in HL (full columns) and LL (crosshatched), and in absence of sucrose (0%) in HL (open) and LL (hatched, downwards). Different letters above the columns indicate significant differences at $P = 0.05$ and 0.01 (in bold), respectively.

Table 1
Deepoxidation state of the xanthophyll cycle pigments normalized to chl a + b content DESC = (Z + 0.5 A)/(chl a + b) at the end of in vitro culture (day 33) and during acclimatization after transfer to the greenhouse (days 35–55) and to open air (days 55–81)[a]

Plants	DESC			
	3% HL	3% LL	0% HL	0% LL
Day 33	28.70	5.50	30.80	9.40
Day 36	20.25	5.31	37.29	7.70
Day 38	9.62	6.23	9.28	6.66
Day 45	7.52	6.85	6.40	5.92
Day 53	7.65	5.53	6.67	5.32
Day 56	7.26	6.77	6.15	6.34
Day 58	6.68	6.20	5.33	5.94
Day 81	7.21	5.77	5.96	5.28

[a] Tobacco plantlets were cultured for 35 days under in vitro conditions with 3% sucrose or without (0%) sucrose in the medium, and under low (LL = 60 μmol m^{-2} s^{-1}) or high irradiance (HL = 200 μmol m^{-2} s^{-1}).

On the other hand, growth of the 0% HL plants which suffer in vitro from photoinhibition was significantly delayed: the plants were smallest in height (not shown), developed a smaller number of leaves (Fig. 5(A)), the smallest total leaf area (Fig. 5(B)) and accumulated the smallest amount of dry mass (Fig. 5(C)). The best growth was observed in plants grown before ex vitro transfer photomixotrophically under high irradiance (3% HL). During the 4 weeks of growth in open air under natural irradiance, the ratio leaf dry matter to leaf area increased approximately twice in all the plants under study (Fig. 5(D)).

3.3. Transient stress after transfer

Transplantation from the greenhouse to outdoors (and to a certain extent also the transplantation from in vitro to the greenhouse) was accompanied by a transient peak, e.g. in starch content (Fig. 1(B)) and in dark respiration rate (data not shown). This was also obvious in the fluorescence parameter F_v/F_m (Fig. 2(B)) during the transfer from the greenhouse to open air.

4. Discussion

This study was on the effect of in vitro pretreatment with high or low irradiance, and presence or absence of sucrose in the medium, on the acclimatization of tobacco plants to ex vitro environment. A two-stage acclimatization was performed: first transfer from in vitro to pots with soil in the greenhouse, and the second one from the greenhouse to a bed of soil in open air. Both the transfers were accompanied with sudden changes in the plant environment: the first one was the transition from the very humid sterile cultivation vessels into septic soil, drier air, lower CO_2 concentration and a changed irradiance. The main change in the second transfer was the dramatic increase in irradiance.

During the first acclimatization stage the originally distinct differences among the in vitro plantlets in contents of soluble carbohydrates and starch (Fig. 1), in P_G (Fig. 2(A)), in F_v/F_m (Fig. 2(B)), in pigment contents (Figs. 3 and 4) and in relative content of PS II (data not given) gradually disappeared due to transplantation of all the plants to the same environment in the greenhouse. During the second acclimatization stage, after some rapid transient peaks, photosynthetic parameters either slowly or considerably increased or stabilized to a certain level. A slow increase was observed in soluble carbohydrates (Fig. 1(A)). A considerable increase in starch content (Fig. 1(B)) and photosynthetic capacity (Fig. 2(A)) as well as more or less stabilized levels of the maximum photochemical efficiency of PS II (Fig. 2(B)), chl a + b content and chl a/b ratio (Fig. 3), the total carotenoids content (Fig. 4(A)) and the xanthophyll cycle pigments content (Fig. 4(B)) were found. This corresponds to a similar experiment with S*pathiphyllum floribundum* plantlets grown under in vitro conditions with 3 and 6% sucrose in the medium, where the differences in net photosynthetic rate, chl, starch, sucrose and glucose contents and F_v/F_m disappeared during the 15 days of ex vitro acclimatization and then an increase in net photosynthetic rate, starch content and F_v/F_m, and stabilized level of sucrose content were found [12].

The most interesting short-term effect during transfer of the plants to ex vitro conditions were the transient peaks in several characteristics immediately after transfer from in vitro, and more expressed after transfer from the greenhouse to open air. We interpret this peaks as stress reactions to the abrupt changes: the first transfer as the shock concerned with the removal of the root system from the agar medium and transplanting the plantlets into soil. Probably, the root hairs may be damaged during this procedure and the absorption of water and nutrients by the roots could be transiently impaired. The second transfer was a shock reaction mainly to the abrupt and considerable increase in irradiance.

The most pronounced peaks were found in starch content. An explanation could be as follows: immediately after transfer growth was interrupted (measured as plant height, number of leaves and sizes of selected leaves; data not shown). Nevertheless, P_G was still high or even increased after transfer, i.e. new photosynthates were produced (Fig. 2(A)) and this surplus in assimilates was directed to an increased starch synthesis (Fig. 1(B)). A similar peak in starch content after transfer was described by [12] but connected with a decrease in P_N; the reason for the difference could be the different procedure used for the transfer (unrooted shoots in the case of [12]). After the transplantation from the greenhouse to a bed of soil, the starch peak in our experiments was three times higher in the originally LL plantlets than in the HL ones. It seems that the HL plantlets were used to grow under higher irradiance during their in vitro pretreatment therefore the shock reaction to the abrupt change in irradiance was lower than in the LL plants. Another peak was found in this transfer: a transient decrease in F_v/F_m followed by an increase but to a lower level corresponding to the dramatic increase in irradiance in open air (Fig. 2(B)). A decrease in F_v/F_m is well known if plant environment changed to a more stressful one, e.g. when irradiance was increased [12–16]. On the other hand, an immediate increase in F_v/F_m was observed when irradiance increased from 21 to 105 μmol m^{-2} s^{-1} [17].

The long-term effect of in vitro pretreatment on acclimatization to ex vitro environment was proved in our experiments. It can be stressed that mostly growth parameters were affected. If the in vitro grown plantlets

were cultured photoautotrophically without sucrose in HL, very low levels of soluble carbohydrates and starch reserves were found (Fig. 1) and a poorer photosynthetic apparatus (smaller leaf area) was developed (Fig. 5). As was shown earlier, photoautotrophic HL plantlets suffered from photoinhibition under irradiances which were still very low for ex vitro growth [6]. The low soluble carbohydrate and starch contents were a 'handicap' which affected significantly the whole plant development. The effect was manifested, e.g. in reduced number of leaves, leaf area and plant dry matter (Fig. 5(A–C)). On the other hand, the presence of 3% sucrose in the medium showed a stimulating effect (Fig. 5(A–C)). Leaf area at the beginning of the acclimatization phase was found to be an important characteristics for achieving a high leaf area at the end of acclimatization [18]. The importance of higher starch or sucrose reserve levels for acclimatization was stressed also by [12,19–21]. On the other hand, avocado plants 4 months after transplantation grew better with lower sucrose pretreatment than those from the higher sucrose concentration: stems grew longer, more leaves and a larger leaf area were produced [22].

Survival rates 46 days after transfer in our originally photomixotrophically (3% sucrose) and photoautotrophically (0% sucrose) grown plants under HL and LL were 100%. A 100% survival for all transplantation treatments was also found in grapevine plants by [20]. Survival rates in other plant species were described to be lower, e.g., for avocado plants grown in a smaller sucrose concentration than in a large one; with proceeding ex vitro growth (2 and 6 months) survival rates were 100 and 70%, and 90 and 70%, respectively. The higher survival rates corresponded to the higher sucrose concentration [22].

In conclusion, only small effects of the different in vitro pretreatments on pigments and photosynthetic parameters during acclimatization were found. On the other hand, we could show in our experiments that in vitro conditions prior to acclimatization were important for ex-vitro growth of micropropagated plants which is in accordance with [16,23,24].

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