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Auxins or Sugars: What Makes the Difference in the Adventitious Rooting of Stored Carnation Cuttings?

María Ángeles Agulló-Antón · José Sánchez-Bravo · Manuel Acosta · Uwe Druege

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Abstract Cold storage of cuttings is frequently applied in the vegetative propagation of ornamental plants. *Dianthus caryophyllus* was used to study the limiting influences of auxin and sugars on adventitious root formation (ARF) in cuttings stored at 5°C. Carbohydrate levels during storage were modulated by exposing cuttings to low light or darkness. The resulting cuttings were treated (or not) with auxin and planted, and then ARF was evaluated. Carbohydrate levels in the cuttings were monitored and the influence of light treatment on indole-3-acetic acid (IAA) and zeatin (Z) in the basal stem was investigated. Dark storage for up to 4 weeks increased the percentage of early rooted cuttings and the final number and length of adventitious roots, despite decreased sugar levels in the stem base. Light during cold storage greatly enhanced sugar levels, particularly in the stem base where the Z/IAA ratio was higher and ARF was lower than observed in the corresponding dark-stored cuttings. Sugar levels in nonstored and dark-stored cuttings increased during the rooting period, and auxin application enhanced the accumulation of sugars in the stem base of nonstored cuttings. Auxin stimulated ARF most strongly in nonstored, less so in light-stored, and only marginally in dark-stored cuttings. A model of auxin-sugar interactions in ARF in carnation is proposed: cold storage brings forward root induction and sink establishment, both of which are promoted by the accumulation of auxin but not of sugars, whereas high levels of sugars and probably also of cytokinins act as inhibitors. Subsequent root differentiation and growth depend on current photosynthesis.

Keywords Root development · Light · Dark exposure · Temperature · Carbohydrates · Source-sink · Plant hormones · Signaling

M. Á. Agulló-Antón · J. Sánchez-Bravo · M. Acosta
Departamento de Biología Vegetal (Fisiología Vegetal), Facultad de Biología, Universidad de Murcia, 30100 Espinardo, Murcia, Spain

U. Druege (✉)
Department Plant Propagation, Leibniz-Institute of Vegetable and Ornamental Crops (IGZ), Kuehnhaeuser Strasse 101, 99189 Erfurt-Kuehnhausen, Germany
e-mail: druege@erfurt.igzev.de

Introduction

De novo root formation in nonroot plant organs, commonly known as adventitious root formation (ARF), is a complex physiological process that is influenced by different endogenous and environmental factors. ARF can be particularly stimulated in stems when excised from donor plants, and there is a substantial body of evidence that auxins contribute to root initiation (Jarvis 1986; Blakesley 1994; Ludwig-Müller and others 2005; Osterc and others 2009). Since their discovery, the exogenous auxin treatment of cuttings has become routine in agricultural practice to ensure and enhance rooting (Hartmann and others 2002). Once initiated, ARF is an energy-requiring process that needs a supply of carbohydrates to the root generation region (Okoro and Grace 1976; Haissig 1984; Veierskov 1988; Li and Leung 2000; Ahkami and others 2009). However, even though the application of sugars to the rooting medium has been seen to increase subsequent root formation (Li and Leung 2000; Takahashi and others 2003), the carbohydrate levels needed at the beginning of rooting to control the speed or the intensity of ARF are a matter of debate (Druege and others 2000, 2004).

Both factors, auxins and carbohydrates, seem to be interrelated. Thus, several authors have reported that the stimulation of ARF in cuttings by indole-3-acetic acid (IAA) coincided with increased sugar availability at the site of root primordia development (Nanda and Jain 1972). It has been found that auxin stimulates the mobilization of carbohydrates in leaves and the upper stem and increases the translocation of assimilates toward the rooting zone (Altman and Wareing 1975; Veierskov and Andersen 1982; Haissig 1986). Auxins may also influence carbohydrate utilization. It has been demonstrated that exogenous auxin applied to the rooting zone activates sugar metabolism to release energy and to provide carbon skeletons for the synthesis of other essential compounds such as proteins (Haissig 1974).

Before being planted for rooting, excised cuttings are commonly stored in darkness at low temperatures, which permits cutting producers to regulate market supply during surplus production or peak demand without significantly varying propagation and production schedules (Garrido and others 1996; Druege and others 2000). A typical response to such storage conditions is a depletion of carbohydrates, which may impair subsequent root formation (Druege and others 2004). Studying ARF in leafy stem cuttings of *Pelargonium* under the influence of dark cold storage, Rapaka and others (2005) demonstrated that the intensity of ARF depended on the strength of the carbohydrate source in leaves, reflected by the leaf sucrose level as a function of the interplay between the initial level at the time of planting and current photosynthesis. However, the results of a recent study of ARF in *Petunia* shoot tip cuttings suggest that the early establishment of a carbohydrate sink at the site of root regeneration is a key metabolic event in ARF (Ahkami and others 2009).

Dianthus caryophyllus L., commonly known as carnation or clove pink, is widely produced for ornamental plant markets. Carnation plants are propagated vegetatively by rooting leafy shoot tip cuttings under greenhouse conditions and natural photoperiod, which allows large-scale multiplication of plants while maintaining their genetic characteristics. Therefore, carnation cuttings have been adopted as a suitable model for studying adventitious rooting in leafy shoot tip cuttings (Garrido and others 2002; Acosta and others 2009).

Considering the relationships discussed above, the present study was carried out to throw light on the interrelationship between auxin and carbohydrates in the ARF of cold-stored carnation cuttings. Furthermore, we aimed to evaluate the carbohydrate source versus carbohydrate sink limitation of ARF under such conditions. Because under ex vitro conditions the application of sugars includes the risk of side effects and artifacts due to inadequate distribution within the cutting, the magnitude of the carbohydrate source was modulated by application of light during cold storage and compared to dark storage. A continuous supply of low light, even below a photosynthetic photon flux density (PPFD) of 5 μmol m^{-2} s^{-1}, can substantially increase carbohydrate levels in plant tissues when exposed to low temperatures, which is the result of net photosynthesis (Kubota and others 1997). Therefore, in experiment 1 (modulation of carbohydrate source), cuttings were either used immediately or after storing at low temperature for different periods in darkness or under low light (PPFD = 10–15 μmol m^{-2} s^{-1}). Changes in carbohydrate levels were analyzed during storage and ARF was rated after cuttings had been planted and exposed to light for rooting. In experiment 2 (modulation of carbohydrate source versus carbohydrate sink), cuttings were first stored for different periods under both light conditions and then treated with or without exogenous auxin before planting. The course of carbohydrate levels during storage and during subsequent rooting in light was analyzed and rooting parameters were determined. In addition to auxin, cytokinin is involved in the regulation of sink-source relations and leaf senescence (Werner and others 2008). Both hormones also act as important controllers of meristematic activity (Muller and Sheen 2008), and current evidence confirms a crosstalk between them in the establishment of a normal root (Moubayidin and others 2009; Werner and Schmülling 2009; Perilli and others 2010). To evaluate the direct influence of the light treatment during storage on auxins and cytokinins, which act as important controllers of meristematic activity, the cytokinin/auxin ratio in the stem base of cuttings was analyzed after exposure to cold storage in darkness or low light.

Materials and Methods

Plant Material and Chemicals

Carnation (*Dianthus caryophyllus* L., cv. Master) cuttings were provided by Barberet & Blanc (Puerto Lumbreras, Murcia, Spain). Shoot tip cuttings 15–18 cm in length, with three or four nodes and one to two pairs of mature leaves were used. Auxins [indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA)] were from Duchefa (Haarlem, The Netherlands), whereas the enzymes used for carbohydrate metabolism were from Roche Diagnostics GmbH (Mannheim, Germany). Other reagents were of analytical grade.

Experiment 1: Modulation of the Carbohydrate Source

Homogeneous samples of cuttings were placed in plastic bags that ensured a relative humidity (RH) close to 100%

and vertically stored in a cold chamber (5 ± 2°C) either in darkness or under continuous low light (fluorescence tubes, "LSK 65-1 neutral white," NARVA Lichtquellen GmbH + Co. KG Brand, Erbisdorf, Germany) at a PPFD of 10–15 μmol m^{-2} s^{-1}, measured at the top of the cuttings. The concentrations of glucose, fructose, sucrose, and starch in the mature leaves and in the basal stem (0.5 cm) were determined weekly during the storage. Control (nonstored) cuttings and cuttings stored for up to 4 weeks in darkness or low light were planted and the speed and intensity of ARF was determined as described below.

Experiment 2: Modulation of the Carbohydrate Sink Versus Carbohydrate Source

Nonstored cuttings, cuttings stored in darkness for 3 weeks, and cuttings stored in low light for the same time (same storage conditions as in experiment 1) were hydrated, with or without auxin treatment, and planted for rooting. The concentrations of starch, sucrose, glucose, and fructose were determined before the hydration step and at different times during the rooting process in the mature leaves and in the basal stem. Rooting was studied as specified below.

Rooting Conditions and Evaluation of the Rooting Response

Before planting, cuttings were hydrated by submerging the basal 3 cm for 20 h in an aqueous fungicide solution (1 g l^{-1} benomyl), without or with an auxin treatment (1.5 μM IBA and 1.0 μM NAA). Hydration conditions were 17°C, RH 60%, 12 h of tenuous light (PPFD = 1–3 μmol m^{-2} s^{-1}), and 8 h of darkness. After hydration, cuttings were planted in trays with perlite with a transparent plastic cover and introduced into a controlled chamber with a 10:14 (light:dark) photoperiod (average PPFD = 100 μmol m^{-2} s^{-1} at plant level), air temperature of 22/20°C (day/night), and RH of 85/60% (day/night) outside the plastic cover. Cuttings were watered once a day.

The percentage of rooted cuttings, the mean number of roots per rooted cutting, the mean root length, and the total root length per cutting were determined at different times during the rooting process (4 replications of 6 cuttings per treatment on each occasion). Roots of each cutting were counted and assigned to different classes of root length (intervals of 1 cm). To calculate root length, the midvalues of the respective class were used. For example, roots measuring 2–3 cm were assigned to a value of 2.5 cm. The rooting parameters were calculated for each replication as follows:

$$\%\ \text{rooted cuttings} = N \times 100/6$$

$$\text{Root number per rooted cutting} = \Sigma n_i/N$$

$$\text{Mean root length} = \sum \left[\sum (n_{Lx}L_x)\right]_i / \sum n_i$$

$$\begin{aligned}&\text{Total root length per cutting}\\ &= (\%\ \text{rooted cuttings}\\ &\quad \times\ \text{root number per rooted cutting}\\ &\quad \times\ \text{mean root length})/100\end{aligned}$$

where N is the number of rooted cuttings, n is the number of roots, i is each of the 6 cuttings of each replication, L_x is the mean length of each length interval, and n_x is the number of roots in each length interval. Final data were the mean values of the parameters calculated for all the replications.

Measurement of Carbohydrate Levels

Samples from leaves (central part of the lowest pair of leaves that were fully developed) and the stem base (0.5 cm) were collected at different time points (in each case from 8 individual cuttings) and immediately frozen in liquid nitrogen and stored at −80°C until analysis. Sugars (glucose, fructose, and sucrose) and starch were determined by enzymatic assays according to Trethewey and others (1998) and Hajirezaei and others (2000) with minor modifications. Frozen plant samples were crushed in a micromill (Retsch MM301, Retsch GmbH, Haan, Germany) and sugars were extracted in 80% ethanol at 80°C (Druege and others 2000). After centrifugation (15,000 rpm), the supernatant was reduced to dryness in a Speed-Vac (Savant SPD11V, Thermo Scientific, Karlsruhe, Germany) and resolved in autoclaved ultrapure water (UPW, TKA GmbH, Niederelbert, Germany). Concentrations of glucose, fructose, and sucrose were measured successively in microplates via NADH-specific extinction at 340 nm after addition of glucose-6-phosphate dehydrogenase, hexokinase, phosphoglucose isomerase, and invertase. After washing the pellets with 80% ethanol and water, starch was fragmented by KOH (0.2 N) and then digested by amyloglucosidase. Released glucose was measured as described.

Measurement of Plant Hormone Levels as Affected by Light During Storage

Cuttings were cold-stored in complete darkness or under low light as described above, and after 3 weeks the levels of IAA and zeatin (Z) were analyzed in the basal stem (0.5 cm). Zeatin and IAA were extracted following the procedure described in Albacete and others (2008) with some modifications. For this, 500 mg of fresh plant material (consisting of 0.5-cm basal stems from 20 cuttings)

were homogenized in liquid nitrogen and extracted in 2.5 ml of extraction mixture (methanol:water, 80:20 [v:v]). After 2 min of vortexing followed by 10 min of extraction at 0°C, the solids were separated by centrifugation (20,000×*g*, 15 min) and re-extracted in an additional 2.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridges (Waters, Milford, MA, USA) to remove interfering lipids and some plant pigments before being evaporated to dryness. The residue was dissolved in 1.5 m of methanol:water, 20:80 (v:v).

The analyses were carried out on an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermostated μ-well plate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass spectrometer using an electrospray (ESI) interface. Prior to injection, samples and standards with known concentrations of each hormone (0.01, 0.05, 0.1, and 0.5 ppm) were filtered through 13-mm-diameter Millex filters with a 0.22-µm-pore-size nylon membrane (Millipore, Bedford, MA, USA). Twenty microliters of each standard or sample was injected onto a Supelco Discovery C18 HPLC column (5 µm, 100 × 2.1 mm, Supelco, Bellefonte, PA, USA), maintained at 40°C, and eluted at a flow rate of 0.1 ml min^{-1}. Mobile phase A (water:acetic acid, 99.5:0.5) and mobile phase B (acetonitrile:acetic acid, 99.5:0.5) were used for the chromatographic separation. The elution program maintained 5% B for 5 min, followed by a linear gradient from 5 to 60% B in 15 min, another linear gradient from 60 to 100% B in 10 min, and finally maintained 100% B for another 10 min. The column was equilibrated with the starting composition of the mobile phase (5% B) for 20 min before each analytical run. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V and a scan speed of 26,000 (*m*/*z*)/s from 50 to 400 *m*/*z*. The nebulizer gas (He) pressure was set to 30 psi, whereas the drying gas was set to a flow of 8 l min^{-1} at a temperature of 350°C.

The chromatogram of each hormone from both standards and samples was obtained and the peak area was quantified using the Data Analysis program for LC/MSD Trap ver. 3.2 (Bruker Daltonik GmbH, Germany). For quantification of Z and IAA, calibration curves were constructed for each analyzed component (0.05, 0.25, 0.5, 0.75, and 1 mg l^{-1}). Validation was carried out by obtaining calibration curves corrected with internal standards: [$^{2}H_5$]*trans*-zeatin (D-Z) (Olchemin Ltd, Olomouc, Czech Republic), and [$^{13}C_6$]indole-3-acetic acid (^{13}C-IAA) (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Recovery values were over 35% for IAA and 60% for Z. The measurements were performed with three analytical replications.

Statistical Analysis

Means and standard errors (SE) were calculated. Statistical analysis of data was performed using the Statistica 8.0 software (StatSoft, Inc., Tulsa, OK). The differences between the data groups were analyzed by *t* test ($p \leq 0.05$) when only two groups were compared. In the other cases, data were subjected to analysis of variance (ANOVA) and the mean values were compared by a Newman–Keuls test ($p \leq 0.05$).

Results

Modulation of Source Activity

The first experiment was carried out to test how the modulation of the carbohydrate source by illuminating cuttings during cold storage influences carbohydrate availability in source (leaf) and sink (basal stem) tissues and the subsequent rooting of carnation cuttings under moderate light conditions. Whereas starch levels in leaves of the first node and in the basal stem of cuttings almost invariably remained below the detection limit of 0.5 µmol g^{-1} FM, the sugar levels were much higher and responded to the experimental conditions. The influence of storage duration and light conditions on the concentrations of individual sugars and the resulting sum of total soluble sugars (TSS) in leaves and basal stems are illustrated in Figs. 1 and 2, respectively. With regard to the leaves, nonstored cuttings revealed very low sucrose levels compared with the glucose and fructose levels, which were both similar. Concentrations of glucose and particularly sucrose increased after 14 days of dark storage, which determined a similar response of TSS. However, except for sucrose, this increase was significant only at 21 days of storage (DOS), when it amounted to +5.3 µmol g^{-1} FW of TSS compared to the initial level (Fig. 1). The application of light during storage strongly enhanced sugar levels in leaves, beginning at 14 DOS. After 4 weeks, sugar levels in light-treated cuttings had reached more than 300% of the initial total sugar levels and were more than double the respective levels of the dark-stored cuttings.

Basal stems contained much higher sugar levels than the leaves, with glucose constituting the biggest sugar fraction. After 4 weeks of dark storage, glucose and TSS had decreased by about 50% of initial levels, which amounted to a final loss of TSS of about 26 µmol g^{-1} FW (Fig. 2). As in the leaves, the application of light during storage strongly enhanced levels of all individual and total sugars. However, in contrast with the leaves, a significant light effect was already observed in basal stems after 7 DOS and was generally more pronounced, particularly in the case of

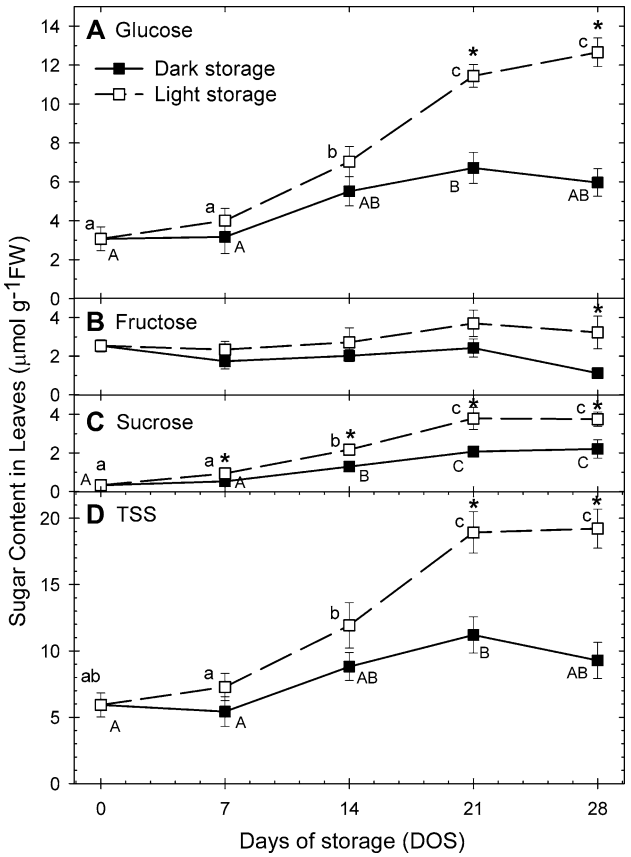

Fig. 1 Influence of duration and light conditions of storage on sugar levels in the leaves of carnation cuttings: **a** glucose, **b** fructose, **c** sucrose, and **d** total soluble sugars. Mean values ± SE of eight replicates are represented. Different letters indicate a significant effect ($p \leq 0.05$) of the storage period for the same storage condition in dark- (*A*, *B*, *C*) or light- (*a*, *b*, *c*) stored cuttings. *Asterisks* indicate a significant effect of light ($p \leq 0.05$) for the specified day of storage

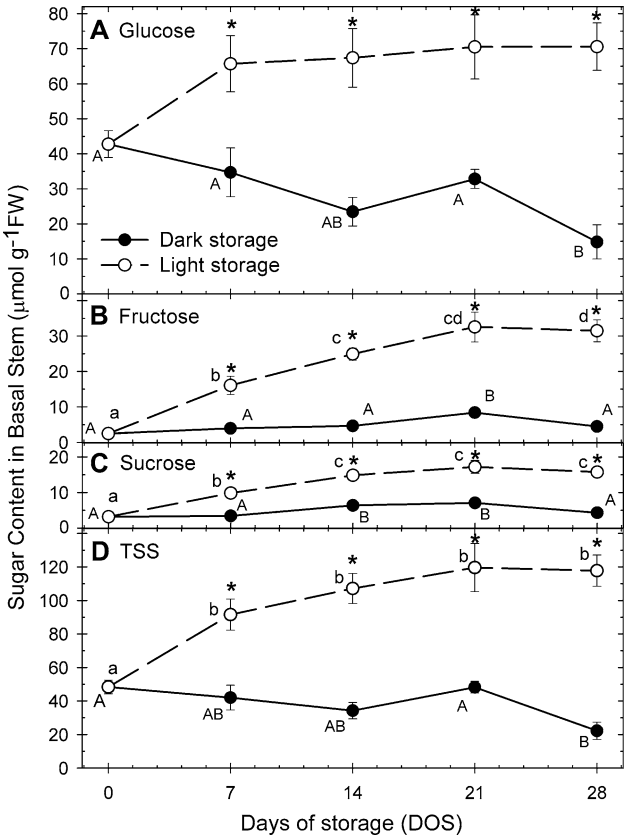

Fig. 2 Influence of duration and light conditions of storage on sugar levels in the basal stem of carnation cuttings: **a** glucose, **b** fructose, **c** sucrose, and **d** total soluble sugars. Mean values ± SE of eight replicates are represented. *Different letters* indicate a significant effect ($p \leq 0.05$) of the storage period for the same storage condition in dark- (*A*, *B*, *C*) or light- (*a*, *b*, *c*) stored cuttings. *Asterisks* indicate a significant effect of light ($p \leq 0.05$) for the specified day of storage

glucose, followed by fructose. Between 2 and 4 weeks of storage, basal stems of light-stored cuttings contained between 248 and 530% of the TSS levels determined in the respective tissue of dark-stored cuttings.

The rooting of cuttings was rated 10 and 18 days after planting and exposure to light to determine the earliness and final intensity of ARF (Table 1). The percentage of rooted cuttings was calculated to assess the frequency and stability of ARF among individual cuttings. Furthermore, mean root number per rooted cutting and mean root length were determined to characterize the intensity of ARF per rooted cutting and root growth. Calculation of the mean total root length per inserted cutting provided a general parameter that described the overall rooting response of the particular treatment. With both light conditions, storage for longer than 3 weeks brought forward and intensified ARF, as reflected by the increased percentage of early rooted cuttings, the higher final number of roots per rooted cutting, and the higher average total root length per inserted cutting. However, despite significantly higher sugar levels, storage in the light did not improve rooting compared to dark storage. There was even a negative influence of light. Thus, dark storage for 3 weeks resulted in a significant increase in the percentage of early rooted cuttings and of total root length after 10 and 18 days of rooting. In contrast, storage in the light needed one additional week to significantly increase the percentage of early rooted cuttings, whereas total root length after 10 days was not significantly affected.

Modulation of Source Versus Sink Activity

Application of low light dramatically enhanced the carbohydrates in cutting tissues while maximum levels were attained after 3 weeks of storage already. However, the rooting response to the increased carbohydrate levels was imperceptible or even negative. Considering that auxins have a stimulatory effect on sink activity and regulate ARF, the question arises as to how the application of

Table 1 Influence of duration and light conditions of storage on rooting of carnation cuttings

Storage condition	Weeks in storage	% Rooted cuttings		Mean root number/ rooted cutting		Mean root length (cm)		Total root length/ cutting (cm)	
		10 days rooting	18 days rooting	10 days rooting	18 days rooting	10 days rooting	18 days rooting	10 days rooting	18 days rooting
Dark	0	12.5 ± 4.2 A	95.8 ± 4.2	2.7 ± 0.9	12.1 ± 0.9 AB	0.4 ± 0.1	0.8 ± 0.1	0.2 ± 0.1 A	9.6 ± 0.9 A
	1	37.5 ± 10.5 AB	100.0 ± 0.0	4.9 ± 1.8	10.5 ± 0.7 A	0.5 ± 0.0	0.8 ± 0.1	0.8 ± 0.3 AB	8.6 ± 1.4 A
	2	25.0 ± 10.8 AC	95.8 ± 4.2	2.1 ± 0.7	14.4 ± 0.3 B	0.4 ± 0.1	0.9 ± 0.1	0.3 ± 0.1 A	12.8 ± 0.9 AB
	3	62.5 ± 4.2 B	100.0 ± 0.0	4.0 ± 0.8	14.7 ± 0.4 B	0.6 ± 0.0	1.0 ± 0.0	1.4 ± 0.3 B	14.9 ± 0.6 B
	4	54.2 ± 8.0 BC	100.0 ± 0.0	4.3 ± 1.0	19.9 ± 1.5 C	0.5 ± 0.0	1.0 ± 0.1	1.2 ± 0.2 B	19.7 ± 2.2 C
Light	0	12.5 ± 4.2 a	95.8 ± 4.2	2.7 ± 0.9	12.1 ± 0.9 a	0.4 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	9.6 ± 0.9 a
	1	20.8 ± 8.0 a	100.0 ± 0.0	2.2 ± 0.6	10.3 ± 0.5 a	1.5 ± 1.2	0.8 ± 0.0	1.4 ± 1.2	8.5 ± 0.5 a
	2	25.0 ± 4.8 a	100.0 ± 0.0	2.4 ± 0.7	14.7 ± 0.8 a	0.5 ± 0.0	0.9 ± 0.0	0.3 ± 0.1	13.9 ± 1.1 b
	3	41.7 ± 14.4 ab	100.0 ± 0.0	3.3 ± 0.9	13.5 ± 1.0 a	0.5 ± 0.0	1.0 ± 0.1	0.7 ± 0.2	14.1 ± 1.3 b
	4	66.7 ± 11.8 b	95.8 ± 4.2	4.3 ± 0.9	19.0 ± 1.8 b	0.5 ± 0.0	0.9 ± 0.1	1.4 ± 0.3	17.2 ± 1.8 b

Mean values ± SE of four replicates (each consisting of 6 cuttings) are presented for 10 and 18 days of rooting. Different letters indicate a significant effect ($p \leq 0.05$) of the storage period on rooting of dark- (A, B, C) or light- (a, b, c) stored cuttings

auxins influences subsequent carbohydrate dynamics and the rooting response to the different storage conditions. To detect the possible interdependency between sugars and auxin in controlling ARF in carnation, cuttings were either planted with no storage or stored for 3 weeks in darkness or light and subsequently hydrated in a solution containing auxins or in an auxin-free medium before being transferred to the rooting chamber. Carbohydrate levels were measured at the time of insertion and at different intervals during rooting before assessing ARF.

As in the first experiment, starch in most of leaf and stem tissues was below the detection limit so that the data could not be statistically analyzed. The time course of levels of individual sugars and of TSS in leaf and basal stems in response to storage and auxin treatment is illustrated in Figs. 3 and 4. According to the results of experiment 1, cuttings stored in the light revealed the highest leaf sugar levels before hydration (day −1) and, in this respect, were followed by the dark-stored cuttings (Fig. 3). The leaves of nonstored cuttings contained the lowest concentrations of glucose.

Without auxin application, the nonstored cuttings showed the steepest rise in leaf sugar content during rooting, with the increase at day 6 reaching about 700% of initial TSS level. In dark-stored cuttings, the increase of the initially higher leaf sugar concentrations was much weaker or even absent. As a consequence, from day 3 onward, nonstored cuttings contained the same sugar levels as dark-stored cuttings. Also, in the light-stored cuttings, the change of leaf sugars during rooting was small and no substantial increase was observed before day 6 of rooting. Nevertheless, sugar levels in the leaves of those cuttings continued to be higher than in the nonstored or dark-stored counterparts (Fig. 3).

Post-storage auxin application via the stem base increased sugar levels in leaf tissues during rooting, although there was a lag period of at least 3 days before this increase became evident. Furthermore, it appears that (1) leaf sugars in dark-stored cuttings were particularly responsive to auxin, and (2) auxin application did not enhance leaf sugar levels when other factors had already resulted in a high level. Thus, light-stored cuttings responded to auxin only on day 3, when a strong increase in leaf glucose and TSS was observed, whereas no such responses were observed at day 6 when sugar levels were already high even without auxin application.

According to the results of experiment 1, cuttings stored in light showed the highest basal stem sugar levels before hydration. Glucose levels, which represented the major sugar fraction in basal stems, showed a transient decrease in all cuttings during hydration and the first day of rooting, after which they increased, determining a similar response in TSS. Fructose and sucrose did not show such a transient decrease. Instead, the levels slightly increased until day 6 in nonstored and dark-stored cuttings. In the case of light-stored cuttings, the transient decrease in glucose and TSS levels observed until day 1 of rooting was stronger and the recovery thereafter was less pronounced than in the other treatments. In addition, these cuttings did not show increased fructose and sucrose levels during rooting. Consequently, the highest sugar levels observed in the light-stored cuttings at the beginning of the experiment ended in similar TSS levels for both light-stored and nonstored cuttings at day 6.

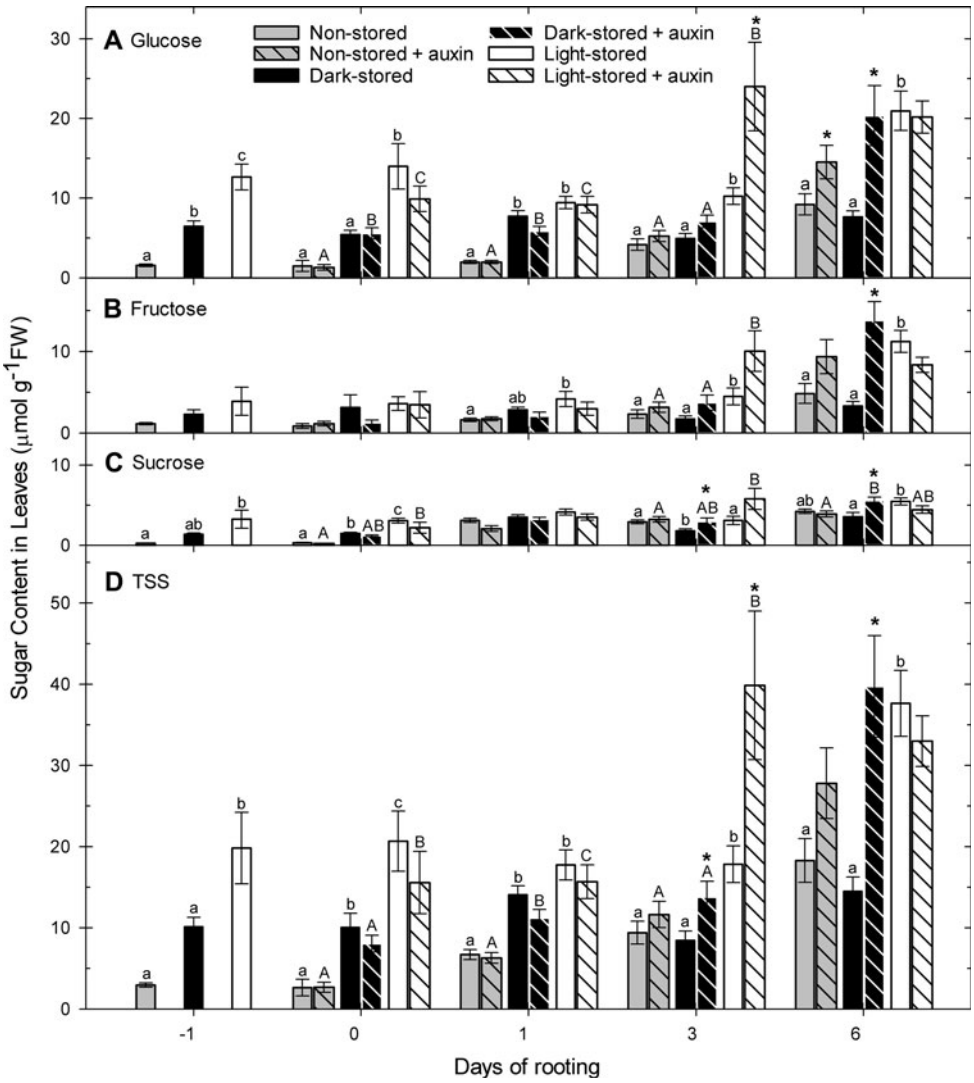

Fig. 3 Influence of storage treatment (nonstored or stored in darkness or light for 3 weeks) and auxin application on sugar levels (**a** glucose, **b** fructose, **c** sucrose, and **d** total soluble sugars) in leaves of carnation cuttings during rooting. Mean values ± SE of eight replicates are represented. *Different letters* indicate a significant effect ($p \leq 0.05$) of the storage treatment (combination of storage duration and light conditions) for the specified auxin treatment and day of rooting. *Asterisks* indicate a significant effect of auxin application ($p \leq 0.05$) for the specified storage treatment and day of rooting

Auxin application to the stem base raised sugar levels in the same tissues during rooting. However, in contrast to the leaves, the sugar response in basal stems was faster but almost exclusively restricted to nonstored cuttings. In these tissues, auxin application counteracted the transient sugar depletion and enhanced subsequent sugar accumulation in the stem base. On day 1 of rooting, the levels of all individual sugars and TSS in nonstored cuttings were enhanced following auxin treatment. During the later rooting period, glucose was the most auxin-responsive sugar, determining a similar trend for TSS. No significant effect of auxin application was found for the sugars in the basal stems of dark-stored cuttings, even though glucose and TSS levels tended to increase at day 6. The only effect of auxin on sugars in the basal stems of light-stored cuttings was a slight increase of sucrose on day 3. Unlike in leaves, the effect of auxin on basal stem sugars did not appear to depend on the sugar level because tissues with both a low (dark-stored cuttings) and high (light-stored cuttings) sugar content failed to respond to auxin application.

Similar to the first experiment, storage in darkness for 3 weeks improved the earliness and final intensity of subsequent rooting, as reflected by the higher number of roots determined after 10 days and the total root length per cutting determined at the end of the rooting period. However, this was true only for cuttings not treated with auxin (Table 2). Confirming the results of experiment 1, storage in light did not further improve the rooting response. Furthermore, the results strengthened the slightly negative influence of light versus dark storage observed in the first experiment. Thus, application of light during storage reduced the number of roots per rooted cutting at day 10 and the mean and total root length at day 18 of rooting. As a result, the rooting of light-stored cuttings was similar to that of nonstored cuttings.

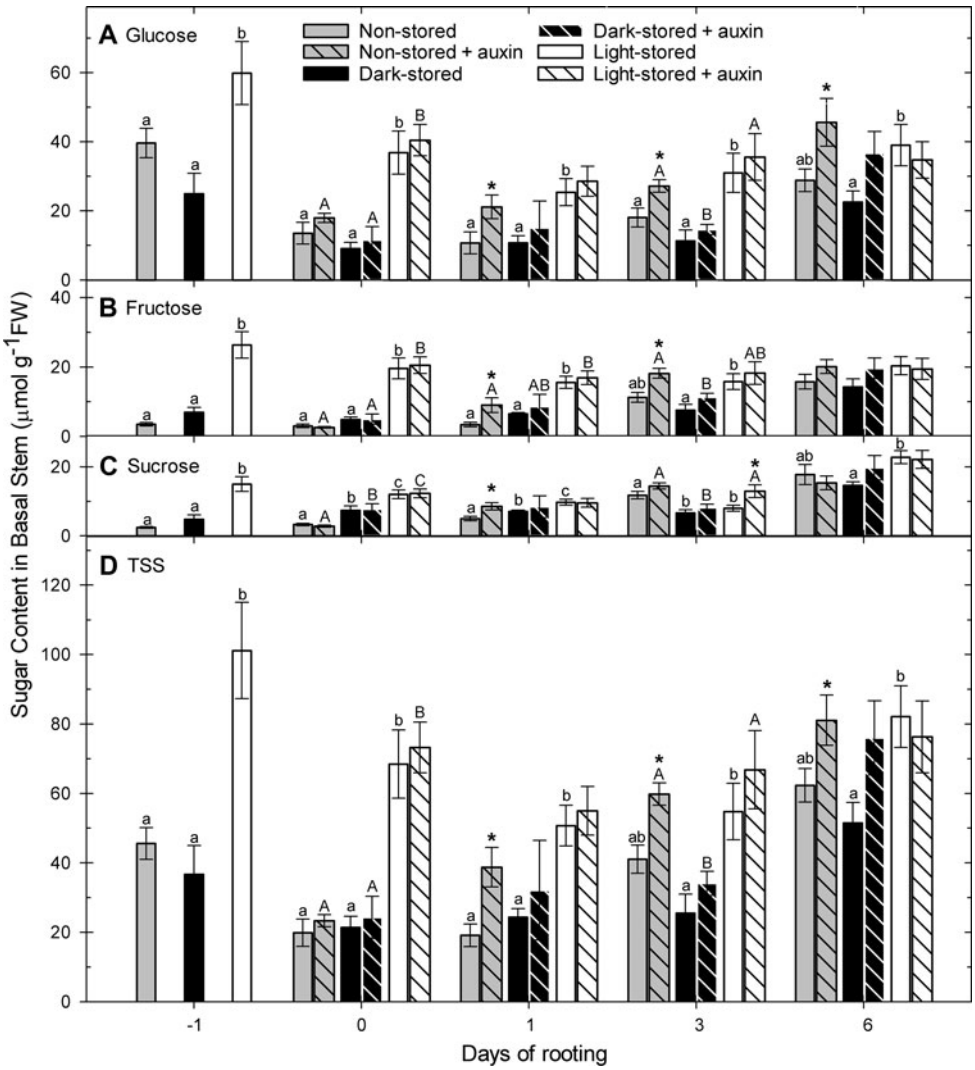

Fig. 4 Influence of storage treatment (nonstored or stored in darkness or light for 3 weeks) and auxin application on sugar levels (**a** glucose, **b** fructose, **c** sucrose, and **d** total soluble sugars) in basal stem of carnation cuttings during rooting. Mean values ± SE of eight replicates are represented. *Different letters* indicate a significant effect ($p \leq 0.05$) of the storage treatment (combination of storage duration and light conditions) for the specified auxin treatment and day of rooting. *Asterisks* indicate a significant effect of auxin application ($p \leq 0.05$) for the specified storage treatment and day of rooting

Auxin application improved ARF. However, this influence of auxin was most consistent in nonstored cuttings and for cuttings stored in light, enhancing the percentage of early rooted cuttings, the number of roots after 10 and 18 days, and the mean and total root length observed at the end of the rooting period. The values of final total root number and total root length illustrate that the most pronounced auxin response was obtained in the nonstored cuttings. Even though the mean number and total length of roots formed by dark-stored cuttings by day 18 were also enhanced by auxin, the magnitude of these effects was less than that observed for the other treatments. As a result of auxin application, nonstored and dark-stored cuttings revealed the same rooting intensity, whereas final root number and total root length were lower for the cuttings stored in light. In accordance with the above observations, the distribution of root lengths pointed to the strongest auxin response in nonstored cuttings, which showed a higher number of roots measuring up to 3 cm after auxin application (Fig. 5). With light-stored cuttings, auxin significantly enhanced only the number of roots measuring between 1 and 2 cm. The application of auxin to dark-stored cuttings only marginally increased the number of roots measuring between 2 and 3 cm.

Light Response of Cytokinin/Auxin Ratio in the Stem Base

The endogenous level of the cytokinin Z and of free IAA was measured by LC-MS in 3-week stored cuttings. Table 3 gives the differences in the stem base hormonal levels of light-stored and dark-stored cuttings. As can be seen, illumination of cuttings during cold storage reduced IAA levels, enhanced cytokinin levels, and increased the cytokinin/auxin ratio in the basal stems when compared to the values for same tissues of dark-stored cuttings.

Table 2 Influence of storage conditions and post-storage auxin treatment on rooting of carnation cuttings

Storage	Auxin	% Rooted cuttings		Mean root number/ rooted cutting		Mean root length (cm)		Total root length/ cutting (cm)	
		10 days rooting	18 days rooting	10 days rooting	18 days rooting	10 days rooting	18 days rooting	10 days rooting	18 days rooting
Nonstored	No auxin	37.5 ± 8.0	91.7 ± 4.8	1.6 ± 0.2 a	9.8 ± 0.6	0.5 ± 0.0	0.7 ± 0.1 ab	0.3 ± 0.1	6.9 ± 0.5 a
	Auxin	79.2 ± 4.2*	100.0 ± 0.0	5.3 ± 0.8*	19.7 ± 1.1 A*	0.5 ± 0.0	1.0 ± 0.0*	2.1 ± 0.4*	19.2 ± 4.1 A*
3 weeks dark-stored	No auxin	33.3 ± 15.2	100.0 ± 0.0	4.1 ± 0.6 b	13.4 ± 0.2	0.4 ± 0.1	0.8 ± 0.0 a	0.7 ± 0.4	11.4 ± 0.7 b
	Auxin	75.0 ± 10.8	100.0 ± 0.0	4.6 ± 0.6	18.4 ± 1.3 A*	0.5 ± 0.0	1.0 ± 0.1	1.8 ± 0.4	17.7 ± 0.7 A*
3 weeks light-stored	No auxin	45.8 ± 12.5	100.0 ± 0.0	2.0 ± 0.5 a	10.2 ± 1.4	0.5 ± 0.0	0.6 ± 0.0 b	0.5 ± 0.2	6.2 ± 1.0 a
	Auxin	83.3 ± 6.8*	100.0 ± 0.0	4.7 ± 0.7*	14.8 ± 0.9 B*	0.5 ± 0.0	0.8 ± 0.1*	2.1 ± 0.4*	12.3 ± 1.5 B*

Mean values ± SE of four replicates (each consisting of 6 cuttings) are presented for 10 and 18 days of rooting. Different letters indicate a significant effect ($p \leq 0.05$) of the storage treatment (combination of storage duration and light conditions) for the specified auxin treatment and day of rooting. Asterisks indicate a significant effect of auxin application ($p \leq 0.05$) for the specified storage treatment and day of rooting

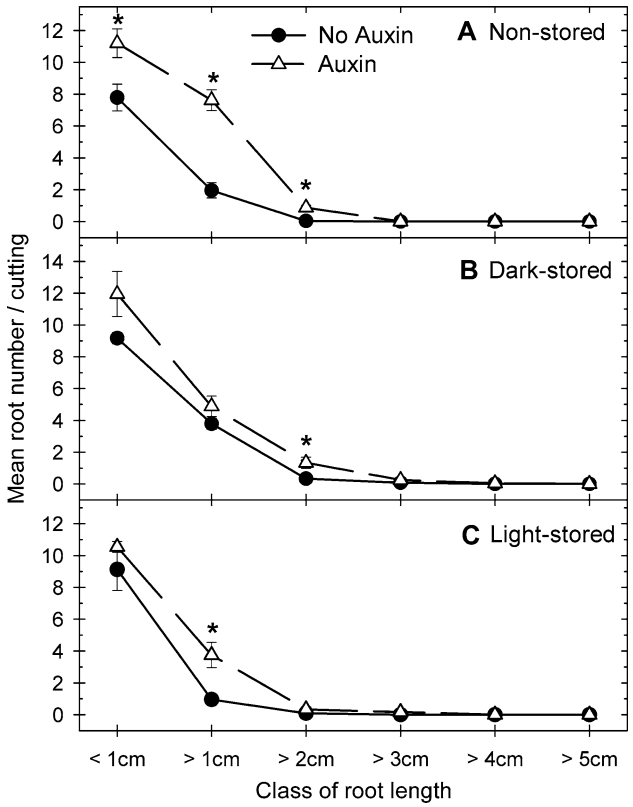

Fig. 5 Influence of auxin application on root length distribution in **a** nonstored, **b** 3-week dark-stored, and **c** 3-week light-stored carnation cuttings after 18 days of rooting. Mean values ± SE of four replicates (each consisting of 6 cuttings) are represented. *Asterisks* indicate a significant effect of auxin application ($p \leq 0.05$) for the specified storage treatment and class of root length

Discussion

A surprising result of the carbohydrate analysis was the very low starch content in the carnation tissues, in almost all cases remaining below the detection limit of

Table 3 Influence of storage illumination conditions on the hormonal levels in basal stem of carnation cuttings

Storage	IAA (ng g^{-1} FW)	Zeatin (ng g^{-1} FW)	Zeatin/IAA
3 weeks dark-stored	10.9 ± 0.6	219.6 ± 2.7	20
3 weeks light-stored	6.0 ± 0.1	309.1 ± 0.6	51

Mean values ± SE of three analytical replicates are represented

0.5 μmol g^{-1} FM, which was based on the small sample size of about 60 mg and a critical extinction difference of 0.028 determined as the triplicate of the standard deviation (3*s) of the four blank values per each microplate. In only one of 72 leaf samples of experiment 1, starch could be detected at a level of 1.2 μmol g^{-1} FM. Regarding experiment 2, starch was detected in only 10 of 216 leaf samples and 2 of 216 stem samples at a similar level without showing homogeneity within a specific data group. Parallel analysis of known *Petunia* samples with higher starch contents within the line of carnation samples confirmed the function of the assay. Considering the trace starch levels compared to the sugars, the latter of which greatly reflected the higher carbohydrate availability under the influence of light, no effort was made to improve the sensitivity of the assay.

One explanation for the trace amounts of starch is loss of starch during the short cold and dark transport of cuttings from the production site to the institute where the experiments were carried out. This explanation was supported by a control experiment. When cuttings were excised from donor plants of 'Master' at 4 h after sunrise, starch could be detected only in leaves at a low level of 0.9 ± 0.1 μmol g^{-1} FM ($n = 10$) but decreased to a level below the detection limit after subsequent transport simulation (data not shown). Considering the low starch level in

the young shoots when excised from the donor plants and that starch remained almost absent in experiments 1 and 2, even when light promoted accumulation of sugars, it appears that starch does not provide a major carbohydrate reserve fraction in young shoots (subsequent cuttings) of the respective carnation cultivar.

Considering the initial carbohydrate status of carnation cuttings at the start of the experiments, the very much higher sugar (especially glucose) levels in the basal stems than in leaves are in line with results obtained with chrysanthemum and pelargonium cuttings. It suggests that the stem tissue acts as a carbohydrate store (Druege and others 2000; Rapaka and others 2005) which may also serve to satisfy carbohydrate demands in other tissues of the cutting when darkness impedes photosynthetic activity.

The results suggest that after separation from the mother plant, carnation cuttings maintain substantial metabolic activity, even when exposed to low temperatures. A typical response to cold storage in darkness is a depletion of carbohydrates in the cutting, although the magnitude and even direction of the response may differ among different carbohydrates and different cutting tissues and depends on the preconditioning of the donor plant (Druege and others 2000). Interestingly, the leaves of carnation cuttings increased their sugar level during dark storage. Starch hydrolysis in darkness may provoke increased sugar levels in leaves (Dennis and Blakeley 2001; Smith and others 2005). However, the near absence of starch during the experiments does not support an important role of this process. The metabolism of other sugars, such as pinitol and myo-inositol, both of which have been described in carnation (Ichimura and others 1998), may have increased the levels of the primary sugars glucose and sucrose. Inositol-linked processes particularly under the influence of stress are only poorly understood (Loewus and Murthy 2000). Nevertheless, the carbohydrates measured were sufficient to characterize the higher carbohydrate and particular sugar availability in response to light, which was the primary objective of the present study. The retranslocation of sugars within the cutting may also have contributed to the increase in leaf sugars as a result of changes in the source-sink relationships, which are strongly responsive to environmental conditions (Geiger and others 1996; Roitsch 1999). Considering the kinetics of sugars and other compounds during low-temperature storage of spinach plants, Toledo and others (2003) suggested a retranslocation of carbohydrates due to changes in the relative sink strengths of the particular organs. In the present study, the slight increase in sugar concentration in leaves (Fig. 1d) was associated with a general trend for decreased carbohydrates in basal stems (Fig. 2d), which was most pronounced for glucose (Fig. 2a). Thus, the higher sugar reserve observed in the basal stem may have supplied the leaves with the necessary carbon to maintain their metabolic activity and, possibly, may have contributed to their tolerance to chilling stress. In this context, the significant increase of sucrose as the main transport fraction in leaves (Fig. 1c) and the basal stem (Fig. 2c) beginning after 2 weeks of dark storage may reflect current carbon retranslocation.

It has already been shown that low-intensity illumination during cold storage can improve the carbohydrate status of intact young plants, for example, of *Brassica* and *Spinacia* (Kubota and others 1997; Toledo and others 2003). To our knowledge, this is the first study providing evidence that sugar availability in leafy stem cuttings during cold storage can be dramatically enhanced by the application of low light. This indicates the ability of carnation cuttings to photosynthesize even at 5°C and a PPFD below 15 μmol m^{-2} s^{-1}. The observation that the light-induced increase in carbohydrate concentration was most pronounced in the basal stem of cuttings, where it reached an almost steady level after the first week, while the increase in leaves was postponed and less pronounced points to the rapid retranslocation of current photosynthates and a high carbohydrate sink activity of the stem base under the light conditions.

The observed increase of carbohydrate levels in response to auxin application was most pronounced in the stem base of nonstored cuttings, the site of auxin application, which is in line with results obtained with other plant species (Nanda and Jain 1972; Veierskov and Andersen 1982). The early response observed after the first day of rooting already suggests that auxin stimulated the establishment of the new carbohydrate sink (Veierskov and Andersen 1982). The metabolic responses of *Petunia* shoot tip cuttings indicated that the early sink establishment at the site of root regeneration is a metabolic key event in ARF and involves invertases (Ahkami and others 2009), which can be stimulated by auxin (Roitsch and others 2003). The observation that auxin application did not stimulate sugar accumulation in cold-stored cuttings reflects the modified physiological status of these cuttings, which may be related to a different auxin status at the time of auxin application (Garrido and others 2003; Oliveros-Valenzuela and others 2008).

The results of the rooting tests in this study confirm our previous findings that a dark cold storage period could reduce the optimum rooting period in certain cultivars (Garrido and others 1998). The observations that the storage-mediated promotion of rooting occurred despite lower carbohydrate levels in the stem base of dark-stored cuttings (Fig. 2) and that rooting was not further improved but rather impaired by application of light during storage (Table 1), which dramatically enhanced sugar availability in the cutting tissues, strongly support the conclusion that the strength of the carbohydrate source

did not limit ARF in carnation when under the influence of cold storage. In contrast, the data corroborate the presence of sufficient carbohydrates to maintain the postharvest viability of cuttings even after storage for long periods in darkness.

The generally accelerated and more intense ARF observed in the stored cuttings (in darkness or light) than in nonstored cuttings suggests that certain changes ("maturation"), possibly related to the presence of rooting induction factors, occur during the storage of cuttings. The fact that the treatment of cuttings with auxins enhanced root formation confirms the important role of auxins in carnation ARF. In various plant species it has been observed that higher auxin concentrations are required during the induction phase of root formation (de Klerk and others 1999; Ludwig-Müller and others 2005; Ludwig-Müller 2009). In the present study, auxin application particularly increased the number of roots measuring up to 2 cm in length, whereas the number of longer roots was only marginally affected (Fig. 5). This indicates that auxin enhanced the formation of new root primordial rather than the later growth of already formed roots, which underlines its effect on the induction and initiation of root formation. The weak rooting response of dark-stored cuttings to auxin application may result from the different internal auxin status discussed above, but it may also reflect the importance of carbohydrate availability during the first hours of rooting under light conditions, when hormonal requirements are already satisfied.

As regards the inhibitory effect of light during cold storage on subsequent ARF (Table 2), it must be borne in mind that not only the leaves but also the stem bases were exposed to light, which may have particular effects on rooting (Eliasson 1978). The levels of IAA and Z in the basal stem of carnation cuttings measured in the present study (Table 3) suggest that the reduced ARF of light-stored cuttings was, at least in part, mediated by a lower auxin level and higher cytokinin/auxin ratio in the stem base, providing unfavorable conditions for the initiation of adventitious roots (Blakesley 1994; Ludwig-Müller 2009). That auxin and light can act antagonistically on ARF was demonstrated in *Eucalyptus* (Fett-Neto and others 2001). Although peroxidase-catalyzed or direct-light-mediated oxidation of IAA have been described in vitro, the relevance of such reactions to in vivo processes is questionable (Normanly and others 2004) so that the observed lower level of free IAA in the stem base of light-stored cuttings may be more likely the consequence of altered transport (Naqvi and Gordon 1967) or changes in hormone metabolism, such as conjugation (Normanly and others 2004). In accordance with our results, the concentration of free and conjugated IAA in the internodes of pea seedlings was reduced by application of white light compared with complete darkness, whereas free IAA was most responsive (Sorce and others 2008). The application of white light to maize seedlings stimulated the metabolism of applied ^{14}C-IAA, particularly in mesocotyl tissues where a high proportion was converted to indole-3-acetyl-aspartate and the resulting reduced pool of free IAA was considered a possible reason for the observed reduced growth (Zelená 2000). Cytokinin-auxin crosstalk plays a key role in controlling meristem activity, with auxin being responsible for cell proliferation and cytokinin for cell differentiation (Moubayidin and others 2009). Studies with cytokinin-overproducing *ipt*-GUS-expressing *Arabidopsis* indicate an important role for aerial parts and light in cytokinin production and the cytokinin-mediated inhibition of ARF (Guo and Hu 2008). Furthermore, a recent study with shoot tip cuttings from transgenic *Populus* plants revealed a cytokinin type-B response regulator as a negative regulator of ARF, which reinforces the inhibitory role of cytokinin action in ARF (Ramírez-Carvajal and others 2009).

The observation that the rooting of light-stored cuttings was more responsive to auxin application than the rooting of dark-stored cuttings can be explained by compensation of the lower auxin level and the higher endogenous cytokinin/auxin ratio (Table 3). However, bearing in mind, on the one hand, the very high sugar levels in the light-stored cuttings, which strongly exceeded those in nonstored cuttings at the time of auxin application (see day −1 in Fig. 4), and, on the other hand, the lowest number and total length of roots formed after auxin application in the same cuttings (Table 2), it appears that there exists an inhibitory influence of supraoptimal sugar levels on auxin-induced ARF, which was first discussed by Hansen and Eriksen (1974). Application of sugars during ARF in vitro showed that the sugar response depends on the plant genotype, the type and concentration of sugar, the light conditions, and the particular phase of rooting (Takahashi and others 2003; Corrêa and others 2005). Furthermore, increasing the sucrose concentrations shifted the dose-response curve of auxin so that higher auxin concentrations had to be applied to produce the maximum number of roots (Calamar and de Klerk 2002). In accordance with these observations, studies on *Arabidopsis* revealed interactions between sugar and auxin response pathways. Thus, the auxin-induced stimulation of lateral root growth was attenuated in a transgenic line overexpressing the glucose sensor HKL1, a hexokinase-like protein (Karve and Moore 2009). The expression of BT2, a BTB protein that suppresses sugar signaling but enhances auxin signaling, was repressed by sugars but enhanced under darkness (Mandadi and others 2009). In addition, transcriptome data from *Arabidopsis* seedlings showed that the induction of almost 61% of IAA-upregulated genes and suppression of 44% of IAA-downregulated genes were lost when glucose was applied, even though

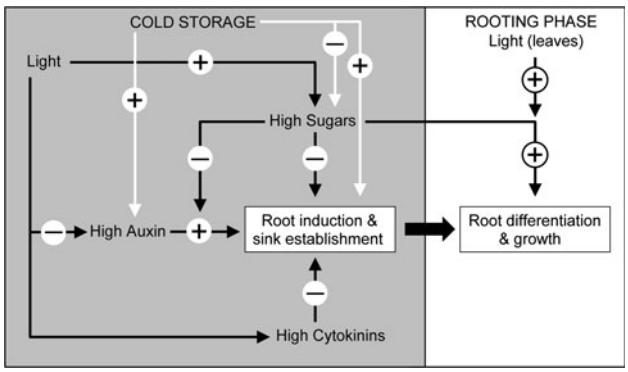

Fig. 6 Schematic presentation of proposed interactions of auxins, cytokinins, and sugars on ARF in carnation cuttings under the influence of cold storage, as based on the results presented in Tables 1, 2, and 3 and Figs. 1, 2, 3, 4, and 5. *Arrows* marked with a *plus* or *minus* sign indicate promotional versus inhibitory influences, respectively

glucose could not affect these genes when applied alone in the absence of auxin (Mishra and others 2009).

Based on the processes discussed above, we propose a model of auxin-sugar interaction in ARF of carnation cuttings under the influence of storage, which is illustrated in Fig. 6. Cold storage (white arrows) reduces carbohydrate levels but brings forward root initiation via inductive processes and the establishment of a new sink in the stem base, both of which are stimulated by auxin accumulation. During this phase, the reduced carbohydrate levels are not critical to the carbohydrate sink of the early processes of ARF (early sink limitation). The application of light during cold storage impairs root induction and sink establishment by reducing the auxin/cytokinin ratio and permitting the accumulation of supraoptimal sugar levels which reduce the auxin response. The increase in carbohydrate demand during the subsequent differentiation and growth of adventitious roots is covered by current photosynthesis if light conditions and other environmental factors allow for sufficiently high photosynthetic activity (source limitation).

The present study has provided new insights into the interactions between auxin and carbohydrates in their regulation of ARF in shoot tip cuttings under the influence of cold storage. The results as a whole point toward a predominant role for auxins in limiting ARF in stored carnation cuttings when post-storage light conditions allow for sufficient photosynthesis to meet the carbon demand of the developing roots. A challenge in the future is to unravel the underlying complex regulatory processes at the molecular level. From a practical point of view, the results reinforce the benefit of auxin application to ARF in carnation, but, in contrast to the storage of young plants of a variety of species, the application of light during storage of cuttings seems to have an inhibitory effect.

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