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Responses of Broccoli Seedlings to Light Quality during Low-temperature Storage in Vitro: I. Morphology and Survival

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Abstract. **Broccoli (*Brassica oleracea* L. Botrytis group 'Green Duke') seeds were cultured in vitro photoautotrophically (without sugar in the medium) or photomixotrophically (with sugar in the medium) for 3 weeks at 23 °C and 150 μmol·m^{-2}·s^{-1} photosynthetic photon flux (*PPF*). Vessels were then stored at 5 °C under 1.6, 4.1, or 8.6 μmol·m^{-2}·s^{-1} of white (400–800 nm), red (600–700 nm), or blue (400–500 nm) light. Concentrations of CO_2 inside the vessels were monitored until equilibrium was reached. Light compensation point was reached at 3.5 μmol·m^{-2}·s^{-1} for photoautotrophic seedlings and at 6.5 μmol·m^{-2}·s^{-1} for photomixotrophic seedlings. Therefore, in the long-term storage experiment, seedlings were stored for 4, 8, or 12 weeks at 5 °C in darkness or under 5 μmol·m^{-2}·s^{-1} (average light compensation point) of white, red, or blue light. Illumination during storage was necessary to maintain dry mass, leaf area, and regrowth potentials of in vitro seedlings. All seedlings stored in darkness were of poor quality and died when transferred to the greenhouse. Red light during storage increased seedling dry mass and chlorophyll content and improved overall appearance, whereas blue light decreased chlorophyll content and increased stem elongation. The addition of 2% sucrose to media increased dry mass and leaf area and maintained overall seedling quality during illuminated storage. However, plantlets stored for more than 4 weeks did not survive poststorage greenhouse conditions, regardless of light treatment.**

In vitro propagation has worldwide applications for horticulture (i.e., fast production, greater uniformity, phenotypic improvement, disease elimination, facilitated shipping, and full-year production). However, conventional in vitro propagation systems are challenged by high production costs and low profits. Storage of in vitro–cultured plants reduces production costs by offering versatility in managing labor to meet flexible market demands (John et al., 1993).

Low temperature storage in darkness is used as a holding method for numerous bedding plant species (Lange et al., 1991) and as a method to extend the subculture time of some tissue-cultured plants (Mullin and Schlegel, 1976). However, such storage reduces plant quality, chlorophyll and carbohydrate reserves (Rajapakse et al., 1996), and rooting ability (Paton and Schwabe, 1987).

Low temperature in conjunction with fluorescent lighting at intensities as low as 2 μmol·m^{-2}·s^{-1} maintains photosynthetic ability and dry mass of seedlings during storage (Kubota et al., 1995). In addition, sucrose is commonly provided in the medium to provide a respiratory substrate during in vitro storage (Kubota and Kozai, 1995). Alternatively, photoautotrophic micropropagation (without sugar in the medium) reduces production costs, facilitates growth, and reduces biological contamination (Kozai, 1991).

Light quality is another important factor affecting plant storage (Economou and Read, 1987; John et al., 1993; Kubota et al., 1996). Blue light reduces shoot elongation (Norton et al., 1988), and red light promotes shoot formation and chlorophyll synthesis (Kasperbauer and Peaslee, 1973). Recently, as an alternative to fluorescent lamps, which require much space and generate heat, light-emitting diodes (LEDs) have been used to examine plant responses to relatively narrow wavelengths of light. LEDs provide a radiation source with improved electrical and photosynthetic efficiency (Miyashita et al., 1995; Tennessen et al., 1994). However, effects of LED in low-temperature in vitro plant storage have not been fully investigated.

Effects of low temperature and light quality (provided by fluorescent tubes) have been reported for photoautotrophic in vitro broccoli seedlings stored for 6 weeks (Kubota et al., 1996). In the current research, we investigated the light compensation points for photoautotrophic and photomixotrophic in vitro broccoli seedlings and the influence of light quality (provided by fluorescent tubes or LEDs at the light compensation point) on morphology and regrowth potential of in vitro broccoli seedlings stored at 5 °C for up to 12 weeks.

Materials and Methods

Plant material and culture conditions. 'Green Duke' broccoli seeds were surface-disinfected with 1% sodium hypochlorite solution for 10 min and germinated in vitro photoautotrophically (no sugar in the medium) or photomixotrophically (2% sucrose in the medium) in Murashige and Skoog (1962) liquid media (10 mL per seedling) supplemented with vitamins (Gamborg, 1970). Four seedlings were cultured in each 375 mL GA-7 vessel (Magenta, Chicago). To obtain uniform growth, three seeds were placed on each Sorbarod cellulose support plug (Sorbarod; Baumgartner Papiers SA, Switzerland), and the seedlings were thinned to one per plug when the cotyledons were fully expanded. Two holes (10 mm in diameter) in opposite sides of the vessel were covered with 0.5-m membrane filter disks (Milli-Seal; Millipore K.K., Tokyo) to provide ≈3.2 air exchanges per hour (Kozai et al., 1986). Culture room temperature was 23 ± 2 °C during the 16-h photoperiod and 19 ± 1 °C during the 8-h dark period; photosynthetic photon flux (*PPF*) was 150 ± 20 μmol·m^{-2}·s^{-1} on the culture shelf.

Determination of light compensation point. Three weeks after culture initiation, the seedlings were transferred to smaller GA-7-3 vessels (Magenta) that did not have membrane filters. The vessels were capped with Magenta lids and the closure was sealed with sculpting clay and Parafilm to prevent gas from escaping. Vessels were stored at 5 °C in separate low temperature incubators (Precision 815; Precision Scientific, Chicago) equipped with overhead lighting from 15-W cool-white fluorescent tubes (General Electric F15T12-CW), red light-emitting diodes, or 20-W blue fluorescent tubes (General Electric F20T12-B) at 1.6, 4.1, or 8.6 μmol·m^{-2}·s^{-1} *PPF*. Light intensities within the incubator were adjusted by application of mesh screening or positioning of vessels. Light intensity was measured on the culture shelf in an empty vessel with lid. Spectral distributions of light sources are shown in Fig. 1. A 0.5-mL gas sample was taken from the head space of each vessel at 12-h intervals during storage, and CO_2 concentration inside the vessels was measured using a gas chromatograph with a thermal conductivity detector (8A; Shimazu Co., Kyoto, Japan). Gas sampling was terminated after 3 d when CO_2 concentrations inside the vessels reached a steady state.

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Storage treatments. In a separate experiment, vessels containing in vitro seedlings were stored for 4, 8, or 12 weeks at 5 °C in darkness or under 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white, red, or blue light (average light compensation point selected from previous experiment). At the start of storage, the air diffusive filters of the vessel were covered with plastic tape to minimize the number of air exchanges and to avoid excessive water loss.

After 0 (control), 4, 8, or 12 weeks of storage, seedlings were destructively harvested, and leaf and stem dry mass, leaf area, and chlorophyll concentrations were measured. Leaf area was measured using a LI-3100 area meter (LI-COR, Lincoln, Nebr.). Chlorophyll was extracted from 0.3 g of the most fully expanded leaf and chlorophyll concentrations were determined as described by Moran (1982). Leaves and stems were frozen in liquid N_2 and freeze-dried for dry mass analysis.

Acclimatization to greenhouse. Upon removal from storage at 4, 8, or 12 weeks, plant height and leaf number were recorded. Seedlings were transferred to soilless media (Metro Mix 360; The Scotts Co., Marysville, Ohio) in 804 grow packs and placed under mist for 4 weeks. Seedlings were then transferred to 4-L pots. After an additional week under the mist, seedlings were grown in a greenhouse until flowering. Plant height and leaf number were recorded biweekly until flower maturity. At the mature flower stage, leaf, stem, and flower dry masses were determined.

Experimental design and statistical analysis. All vessels were arranged in completely randomized design under each light treatment. Five photoautotrophic and five photomixotrophic vessels were harvested from each light treatment after 0, 4, 8, or 12 weeks of storage. Each vessel containing four seedlings was considered a replication. From each treatment, three vessels were used for laboratory measurements and two vessels were used for greenhouse evaluations. Data were analyzed by analysis of variance. Treatment differences were separated using least square means at $P = 0.05$. Treatment effects and interactions are shown in Table 1.

Results

Light compensation point. At 5 °C, light compensation point was reached at 3.5 and 6.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in photoautotrophic and photomixotrophic seedlings, respectively (data not shown). Therefore, 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* (average) was chosen for long-term storage at 5 °C because both photoautotrophic and photomixotrophic cultures were stored in the same incubator.

Dry mass. Light quality, storage time, and media composition affected seedling dry mass. Dark storage reduced leaf dry mass regardless of media composition (Fig. 2 A and B). Red light increased leaf dry mass of seedlings during storage, regardless of media composition, whereas both white and blue light maintained leaf dry mass in photoautotrophic seedlings during storage. However, blue light reduced leaf dry mass of photomixotrophic seedlings. Stem dry mass of photoautotrophic seedlings remained relatively unchanged during dark storage, but increased slightly in photomixotrophic seedlings (Fig. 2 C and D). Light during storage increased stem dry mass of both photoautotrophic and photomixotrophic seedlings; however, the increase was greater in the latter.

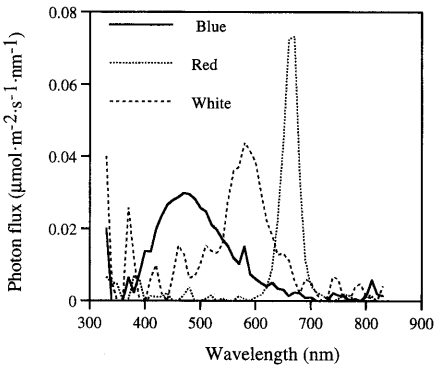

Fig. 1. Spectral photon flux distribution of storage light sources.

Visual quality. Regardless of storage time, photomixotrophic seedlings were of greater visual quality than photoautotrophic seedlings (Fig. 3). After 4 (photoautotrophic) or 8 weeks (photomixotrophic) of dark storage, seedling quality dramatically declined (Fig. 3 B and E). Regardless of light quality or media composition, seedling appearance was poor after 8 weeks of storage (Fig. 3 D and E). Plantlets stored under blue light appeared more leggy and chlorotic than did those stored under white or red light. After 12 weeks, photomixotrophic seedlings stored under red light were shorter, greener, and had larger leaves than those stored under white or blue light (Fig. 3G).

Stem length, leaf area, and chlorophyll. Regardless of light quality, stem length of photoautotrophic and photomixotrophic seedlings increased during lighted storage (Fig. 4 A and B). Under photoautotrophic conditions,

Table 1. Abbreviated analysis of variance for the main effects and interactions of sucrose, light quality, and time on the growth of broccoli seedlings.

	Significance of F value				
Treatment	Leaf dry mass	Stem dry mass	Stem length	Leaf area	Total chlorophyll
Sucrose (S)	**	**	*	**	**
Light quality (L)	**	**	**	**	**
Week (W)	*	**	*	**	**
S × L	**	NS	*	**	NS
S × W	NS	**	NS	**	NS
L × W	**	NS	**	**	**
S × L × W	*	NS	*	**	*

NS, *, **Nonsignificant or significant at $\alpha = 0.05$ and 0.01, respectively.

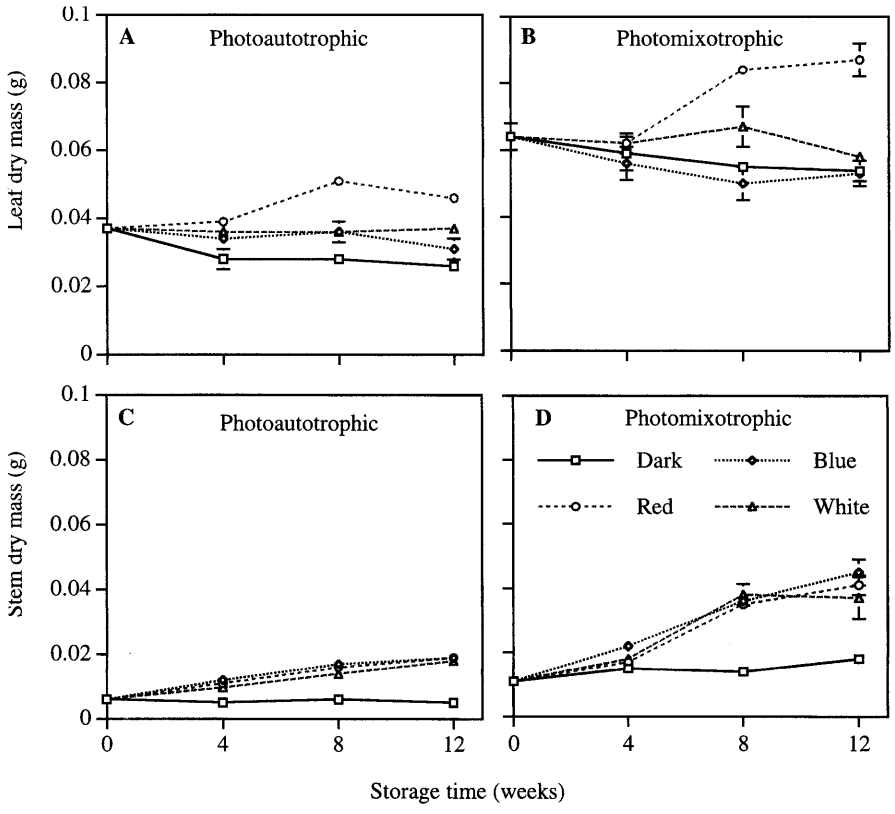

Fig. 2. Change in leaf and stem dry mass of broccoli seedlings grown photoautotrophically or photomixotrophically during storage in dark or light. Means ± SE are shown.

blue light produced the tallest seedlings after 12 weeks of storage. Under photomixotrophic conditions, blue light produced the tallest seedlings after 8 weeks of storage and red light produced the shortest seedlings after 12 weeks of storage. After 12 weeks, the stem length of plants stored under blue or red light was unchanged, while that of plantlets stored under white light had increased. The presence of sucrose in the media did not significantly affect initial stem length. In dark storage, stem length of photoautotrophic seedlings increased for 4 weeks, but seedlings died between 4–8 weeks of storage; stem length of photomixotrophic seedlings increased for 4 weeks and then remained constant, but seedlings died between 8–12 weeks. Leaf area remained relatively unchanged under blue or white light, but increased during storage under red light (Fig. 4 C and D). The increase, however, was greater under photomixotrophic conditions. Media composition did not affect initial leaf area. Leaf area of photoautotrophic seedlings stored in darkness decreased during 8 weeks, and these seedlings died 4–8 weeks into storage; leaf area of photomixotrophic seedlings remained relatively unchanged for 8 weeks but seedlings died between 8–12 weeks. Both photoautotrophic and photomixotrophic seedlings survived 12 weeks of storage when light was provided. During storage, light quality did not affect chlorophyll concentrations of photoautotrophic seedlings. Chlorophyll concentrations of photomixotrophic seedlings remained relatively unchanged during storage under red light, but decreased under both white and blue light (Fig. 4 E and F). Addition of sucrose to the media did not affect initial chlorophyll concentrations significantly, although photomixotrophic seedlings appeared darker green than photoautotrophic seedlings.

Acclimatization to greenhouse. Regardless of the media composition or light in storage, seedlings stored for 8 or 12 weeks did not survive upon transfer from the mist to the greenhouse (these seedlings only survived 4 weeks under the mist). Seedlings stored in light for 4 weeks recovered well in the greenhouse (Fig. 5). Dark-stored photoautotrophic seedlings died after 1 week in the greenhouse, but photomixotrophic seedlings grew normally. However, the recovery of photomixotrophic seedlings stored in darkness was slightly slower than that of those stored in light. Light quality did not affect recovery of photoautotrophic or photomixotrophic plants.

Discussion

Influence of PPF. At 5 °C, 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* is sufficient to maintain seedlings close to their light compensation point. Storage at (or near) zero carbon balance is useful for maintaining plants in commercial micropropagation or quality in nursery plug production, where balancing market demands with labor availability or greenhouse space for acclimatization is sometimes difficult. Storage of seedlings under higher *PPF* than the light compensation points results in undesirable shoot elongation and dry mass increase (Kubota et al., 1995). Illumination at the light compensation point or providing sugar in the media during storage is necessary to preserve seedling quality. Photoautotrophic seedlings had reduced leaf dry mass, leaf area, and poor overall quality after 4 weeks of dark storage. The addition of sucrose to the medium extended dark storage potential to 8 weeks without excessive quality loss, yet the plants did not survive in the greenhouse upon removal from storage, probably because of low carbohydrate reserves. Carbohydrate reserves at harvest influence the quality and poststorage growth potential of plants (Kunneman and Albers, 1992; Paton and Schwabe, 1987; Wilson et al., 1998). The poor responses of seedlings to dark storage were consistent with the observations of Kubota and Kozai (1994) who showed that light intensities as low as 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* are necessary to preserve photosynthetic and regrowth abilities and dry mass of seedlings during low-temperature storage.

Influence of light quality. During storage, the quality of photomixotrophic broccoli seedlings was best maintained under red light, which reduced stem elongation and enhanced chlorophyll content, leaf area, and dry matter accumulation into leaves in comparison with other light treatments. Red light increases carbohydrate synthesis (Szasz and Barsi, 1971), decreases adaxial stomatal aperture (Lu et al., 1993), and increases cytokinin and gibberellin levels in plants (Wareing and Thompson, 1976). These physiological factors may have contributed to the favorable effect of red light on seedling growth during storage. However, Kubota et al. (1996) reported that the quality of broccoli seedlings was best maintained under white light at 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The differences between their observations and ours may be attributed to differences in light source. Kubota et al. (1996) used broader spectra, red acetate filters over fluorescent tubes as their light source, while in the current research we used red LED array with a narrower (600–700 nm) band width. Seedlings responded differently to light quality in storage, but these developmental responses were not significantly different once plants reached maturity in the greenhouse. The poststorage developmental responses were consistent with those of Kubota et al. (1996) who determined that light quality during storage does not affect the regrowth potential of stored plants significantly. Similarly, Decoteau and Friend (1991) reported that end-of-day red or far-red light treatments affected tomato transplant growth but not subsequent fruit production when plants were transferred to the field.

Influence of storage time. Seedling quality greatly diminished between 4 and 8 weeks of storage, depending on media composition and light source. Regardless of light quality or media composition, seedlings stored for 8 or 12 weeks did not perform well in the green-

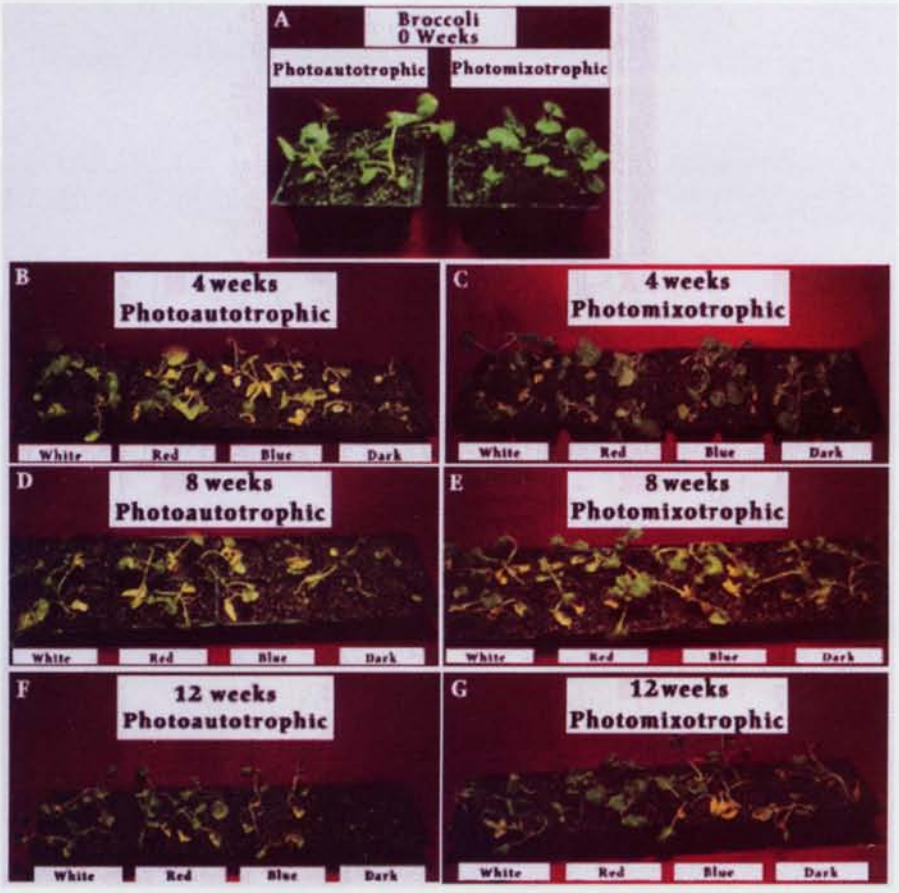

Fig. 3. Effect of light during storage on seedling quality. Photoautotrophic and photomixotrophic broccoli seedlings were stored at 5 °C and 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seedlings were removed from storage at 4, 8, and 12 weeks and transferred from in vitro vessels to soilless media.

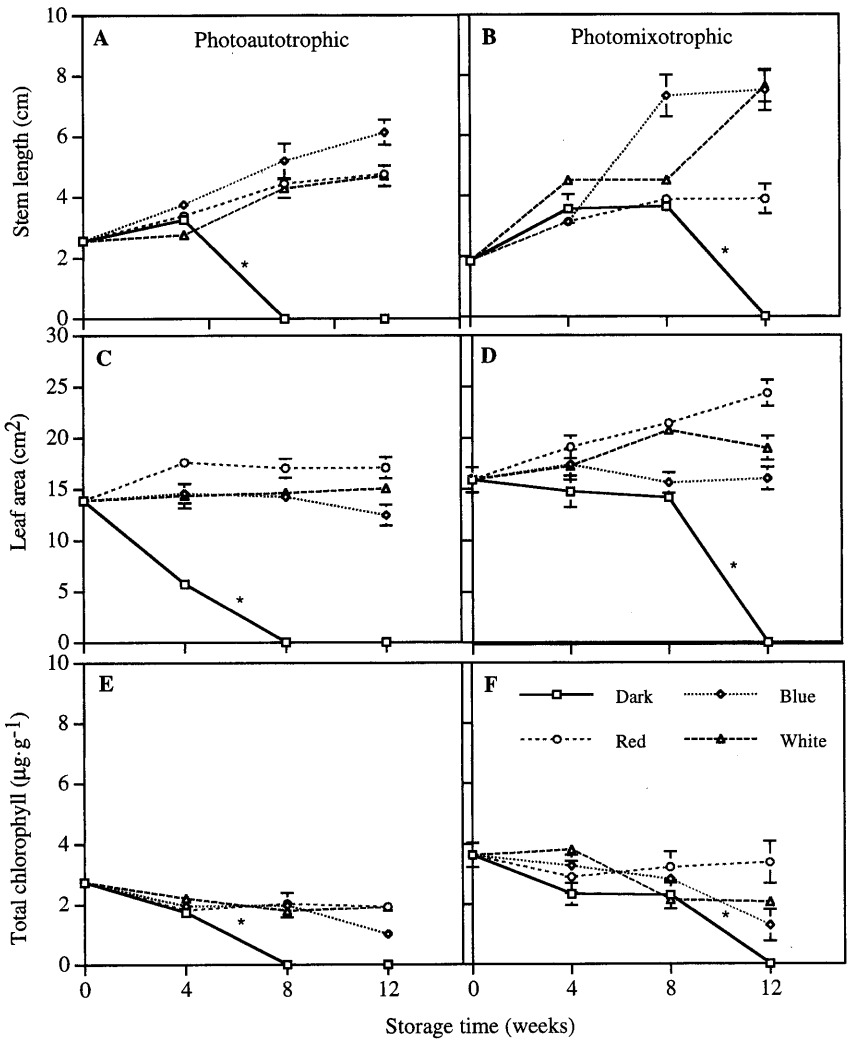

Fig. 4. Change in stem length, leaf area, and chlorophyll concentration of seedlings grown photoautotrophically or photomixotrophically during storage in dark or light. Means ± SE are shown. *The declining values of dark-stored seedlings indicate that they were dying.

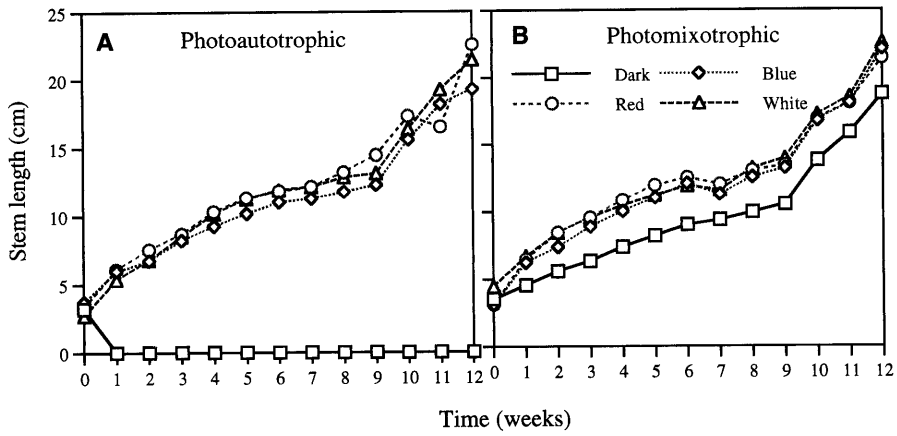

Fig. 5. Stem length of plants during acclimatization in greenhouse for 12 weeks. In vitro seedlings were stored photoautotrophically or photomixotrophically for 4 weeks prior to transfer to the greenhouse. Each value is the mean of eight plants (six plants for photoautotrophic plants stored under blue light, since two plants died during the recovery period in the greenhouse).

house. During low illumination storage at 5 °C, there appears to be a critical point when plants lose their ability to recover. Kubota et al. (1996) reported positive photosynthetic and regrowth potentials of photoautotrophic seedlings stored for 6 weeks. In our experiments, seedlings survived in vitro storage for up to 12 weeks and in vivo acclimatization under intermittent misting in the greenhouse for up to 5 weeks, but they never recovered once removed from the mist. When transferred to the greenhouse, in vitro seedlings sometimes suffer from reduced photosynthesis [due to decreased ribulose bisphosphate carboxylase (RubPcase), low light, and inadequate gas exchange] and lack of well-developed epicuticular wax and fully functional roots and stomata (Preece and Sutter, 1991). Therefore, seedlings stored for 8 or 12 weeks may need to be acclimatized more carefully to greenhouse conditions.

Conclusion

A successful storage system intended for extending intervals between subculturing or meeting market demands of in vitro seedlings must minimize growth and maintain the full developmental and functional potential of the plants on removal from storage. Our results show that dark storage for 4 weeks reduced in vitro seedling quality and regrowth potential, but the reduction was greater when plants were cultured photoautotrophically. Illumination during storage was necessary to maintain seedling quality and regrowth potential after 4 weeks of storage. Red light provided better quality seedlings than did white or blue light. Regardless of the media composition or light during storage, seedlings lost their regrowth potential after 8 weeks of storage; nevertheless, providing light and sucrose in the media maintained seedling quality during the storage period. This suggests that providing light and sucrose in the media is beneficial during storage, but plants lose their ability to recover from storage stress. Future research is needed to investigate methods to improve recovery in the greenhouse after long-term storage.

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