

Responses of Broccoli Seedlings to Light Quality during Low-temperature Storage in Vitro: II. Sugar Content and Photosynthetic Efficiency

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Abstract. Broccoli (*Brassica oleracea* L. Botrytis group 'Green Duke') seeds were cultured photoautotrophically (without sugar) or photomixotrophically (with sugar) in vitro for 3 weeks at 23 °C and 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux (PPF). In vitro seedlings were stored for 0, 4, 8, or 12 weeks at 5 °C in darkness or under 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of white (400–800 nm), blue (400–500 nm), or red (600–700 nm) light. Photosynthetic ability and soluble sugar contents were determined after removal from storage. Photomixotrophic seedlings contained approximately five times more soluble sugars than did photoautotrophic seedlings. Dark storage reduced soluble sugars in both photoautotrophic and photomixotrophic plants, but photosynthetic ability was maintained for up to 8 weeks in the latter whereas it decreased in the former. Illumination in storage increased leaf soluble sugars in both photoautotrophic and photomixotrophic seedlings. Soluble sugars in stems decreased during storage regardless of illumination, but remained higher in illuminated seedlings. Red light was more effective in increasing or maintaining leaf and stem soluble sugars than was white or blue light. Regardless of media composition or illumination, storage for more than 8 weeks resulted in dramatic losses in quality and recovery, as well as photosynthetic ability. Seedlings stored for 12 weeks completely lost their photosynthetic ability regardless of media composition or illumination. The results suggest that carbohydrate, supplied in the media or through illumination, is essential for maintenance of photosynthetic ability during low-temperature storage for up to 4 or 8 weeks.

In vitro culture is often associated with mass production at a competitive price. Since labor accounts for 60% to 80% of the in vitro production costs (Donnan, 1986), measures to distribute

labor costs are necessary. One such method is the use of storage systems to hold plantlets until market availability. Dark storage is more commercially feasible than light storage, but reduces plant quality and regrowth potential (Koranski et al., 1989). In the past, low temperature has been used to reduce respiration (Pritchard et al., 1991) and low light has been used to maintain photosynthetic ability during storage (Kubota and Kozai, 1994). In more

sophisticated storage systems, specific wavelengths of light optimized plant growth (van Lieburg et al., 1990). Techniques used for storage of greenhouse plants have been applied to storage of plants in tissue culture (Kubota et al., 1996).

The spectral distribution of light affects many aspects of growth and development of cultures such as shoot initiation (Schneider-Moldrickx, 1983), fresh mass (Wengerodt and Augsten, 1984), bud development (Kadkade and Seibert, 1977), morphology (Felker et al., 1995), polysaccharide accumulation (Szasz and Barsi, 1971), and stem elongation (Appelgren, 1991). In the past, conventional light sources served as artificial light sources for plant growth. However, when conventional light sources are used for in vitro plants during storage, overheating becomes a problem. Light-emitting diodes (LEDs) provide an alternative without heat buildup (van Lieburg et al., 1990).

In this study, we investigated the influence of illumination at the light compensation point (5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) by different light spectra (provided from white or blue fluorescent tubes or red LEDs) on the carbohydrate status and

photosynthetic ability of broccoli seedlings cultured in vitro either photoautotrophically or photomixotrophically and stored at 5 °C for up to 12 weeks.

Materials and Methods

Plant material and culture conditions. Culture methods and conditions have been described previously (Wilson et al., 1998). Briefly, '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) for 3 weeks in Murashige and Skoog (1962) liquid media (10 mL per seedling) supplemented with vitamins (Gamborg, 1970).

Storage treatments. Three weeks after initiation of culture, vessels were stored 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 (LED), and 20-W blue fluorescent tubes (General Electric F20T12-B) for 0 (control), 4, 8, or 12 weeks at 5 °C in darkness or under 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux (PPF). Preliminary experiments indicated that 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF was sufficient to achieve the light compensation point during storage at 5 °C (Wilson et al., 1998). Spectral distributions of the three light sources in storage have been reported (Wilson et al., 1998).

Net photosynthetic rates. Upon removal from storage at 0, 4, 8, and 12 weeks of storage, net photosynthetic rates (NPR) of seedlings were measured to evaluate photosynthetic ability of seedlings immediately after removal from storage. Three vessels from each treatment were placed in a growth chamber with 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF from cool-white fluorescent tubes at 22 °C. The NPR was measured at 1500 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂. The CO₂ equilibration period was 30 min darkness followed by 1 h light of 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF. The following equation, as described by Fujiwara et al. (1987), was used to estimate NPR:

$$\text{NPR} = k V N (C_{\text{out}} - C_{\text{in}}) / L$$

where k is the conversion factor (4.1×10^{-2} mol·L⁻¹), V is volume of the vessel (0.375 L), N is the number of air exchanges (3.22 h^{-1}), C_{in} and C_{out} are CO₂ concentrations inside and outside of the vessel, and L is the total leaf area per vessel (cm²).

Concentrations of CO₂ inside and outside of three representative vessels per treatment were measured weekly during storage using a gas chromatograph with a thermal conductivity detector (8A; Shimadzu Co., Kyoto, Japan).

Carbohydrate analysis. Following NPR measurements, leaves and stems were frozen in liquid N₂ and freeze-dried for dry mass measurements. To obtain sufficient tissue for carbohydrate analysis, two seedlings were pooled to generate a sample. Leaves and stems were separately ground, and soluble sugars from 50 mg of leaf tissue and 25 mg of stem

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tissue were extracted overnight with 12 methanol : 5 chloroform : 3 water (by volume) as described by Miller and Langhans (1989). Sucrose, glucose, and fructose were separated and detected using an HPLC with a refractive index detector (Waters Associates, Milford, Mass.) and a Bio-Rad HPX-87C column (Bio-Rad, Richmond, Calif.) maintained at 85 °C. Quantification of individual sugars was based on an internal standard of mannitol (1 mg).

Experimental design and statistical analysis. Vessels (photoautotrophic and photomixotrophic) were arranged in a 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 with four seedlings was considered a replication. Three vessels from each treatment were used for laboratory measurements. Data were analyzed by analysis of variance and treatment differences were separated using least square means at $P = 0.05$. Treatment effects and interactions are shown in Table 1.

Results

Soluble sugars. Before storage, photomixotrophic seedlings had approximately three and five times more leaf and stem total soluble sugars (TSS), respectively, than did photoautotrophic seedlings (Fig. 1). Both leaf and stem TSS decreased more in darkness than in any light treatment. In photoautotrophic seedlings, leaf and stem TSS decreased rapidly during 4 weeks of dark storage and remained very low; however, in photomixotrophic seedlings, leaf and stem TSS decreased gradually to 20% to 25% of the original level during 12 weeks of dark storage.

Light in storage increased both leaf and stem TSS in photoautotrophic seedlings. However, in photomixotrophic seedlings stored under red or white light, stem TSS decreased $\approx 50\%$ during 4 weeks, then remained relatively constant; in blue light, TSS continued to decrease during 12 weeks of storage. During 12 weeks of storage, leaf and stem TSS were highest under red light in photoautotrophic or photomixotrophic seedlings, except that red or white light produced equal TSS in photomixotrophic leaves at 12 weeks.

The changes in individual sugars during storage were significantly affected by light (Fig. 2). Leaf sucrose of dark-stored photoautotrophic seedlings dropped to nondetectable levels within 4 weeks (Fig. 2A), while that in photomixotrophic seedlings remained unchanged for 8 weeks and dropped about 50% when stored for 12 weeks (Fig. 2B). In photoautotrophic seedlings, light slightly increased leaf sucrose concentrations compared to dark storage (Fig. 2A), but in photomixotrophic seedlings, blue or white light did not (Fig. 2B). Red light-stored photoautotrophic and photomixotrophic seedlings had higher leaf sucrose levels than did those in any other treatment at the end of 12-week storage. Leaf sucrose concentrations of seedlings stored in white light were not significantly different from that of

those stored in blue light, regardless of media composition.

Leaf glucose and fructose decreased during dark storage (Figs. 2 C–F). Regardless of media composition, seedlings stored in light maintained higher leaf glucose and fructose concentrations than did dark-stored seedlings. In photoautotrophic seedlings red, blue, and white light gradually increased the leaf glucose and fructose concentrations during storage. However, at the end of the 12-week storage period, leaves of red light-stored photoautotrophic seedlings had more glucose and fructose than did white- or blue light-stored seedlings. In photomixotrophic seedlings, leaf glucose and fructose increased rapidly under red light between 4 and 8 weeks, but leveled off thereafter. Blue and white light gradually increased leaf glucose during storage (Fig. 2D). At the end of 12 weeks, leaf glucose of red- and white light-stored photomixotrophic seedlings was greater than that of blue light-stored seedlings. Leaf fructose of photomixotrophic seed-

lings stored in blue and white light decreased slightly during the first 4 weeks but increased gradually thereafter (Fig. 2F). After 12 weeks of storage, leaf fructose levels were similar under all light treatments.

In darkness, stem sucrose of photoautotrophic seedlings dropped to undetectable levels within 4 weeks (Fig. 3A), whereas that of photomixotrophic seedlings decreased $\approx 70\%$ within 4 weeks and gradually decreased thereafter (Fig. 3B). Light significantly increased stem sucrose in photoautotrophic seedlings, with red light being most effective (Fig. 3A). In photomixotrophic seedlings, stem sucrose decreased $\approx 50\%$ to 60% within 4 weeks regardless of the light quality and continued to decrease slowly under white or blue light. Under red light stem sucrose of photomixotrophic seedlings increased slightly between 4 and 8 weeks but remained unchanged thereafter (Fig. 3B).

Stem glucose and fructose decreased during dark storage (Figs. 3 C–F). Under photo-

Table 1. Abbreviated analysis of variance for the main effects and interactions of sucrose, light quality, and time on soluble sugar levels and net photosynthetic rate (NPR) of broccoli seedlings.

Treatment	Significance of F value								
	Leaf total sugars	Stem total sugars	Leaf sucrose	Stem sucrose	Leaf glucose	Stem glucose	Leaf fructose	Stem fructose	NPR
Sucrose (S)	**	**	**	**	**	**	**	**	**
Light qual. (L)	**	**	**	**	**	**	**	**	NS
Week (W)	**	*	NS	**	**	*	**	NS	**
S × L	NS	NS	**	**	*	NS	NS	*	NS
S × W	NS	NS	**	NS	NS	NS	NS	NS	NS
L × W	**	**	**	**	*	*	*	*	*
S × L × W	*	NS	*	NS	**	NS	*	NS	NS

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

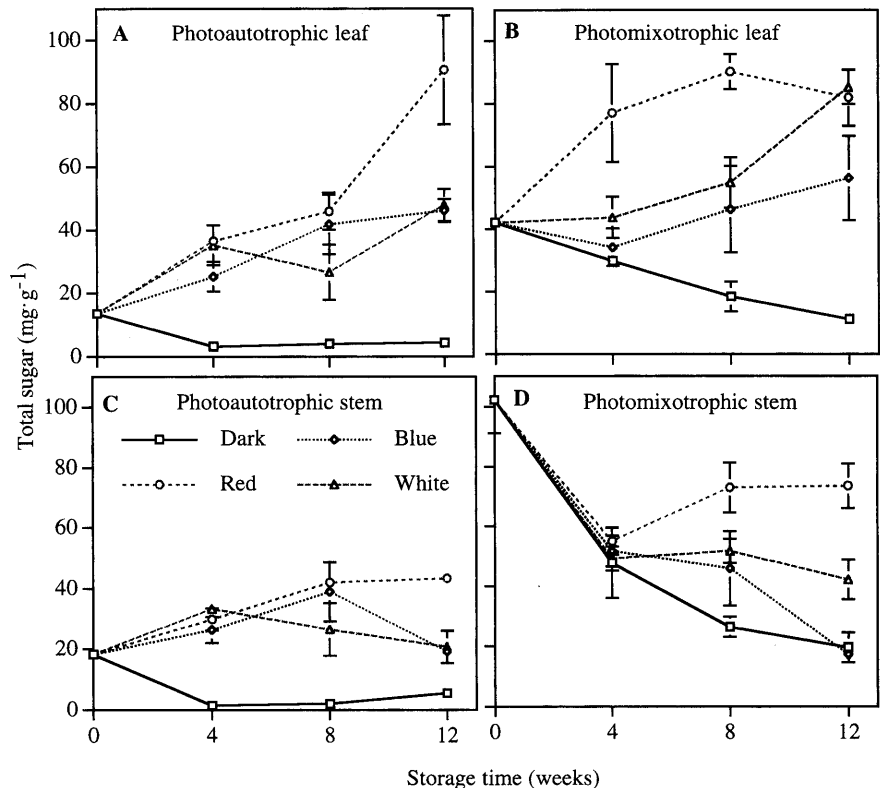


Fig. 1. Effects of light during storage on leaf and stem total soluble sugar concentration in photoautotrophic and photomixotrophic broccoli seedlings. Means \pm SE are shown.

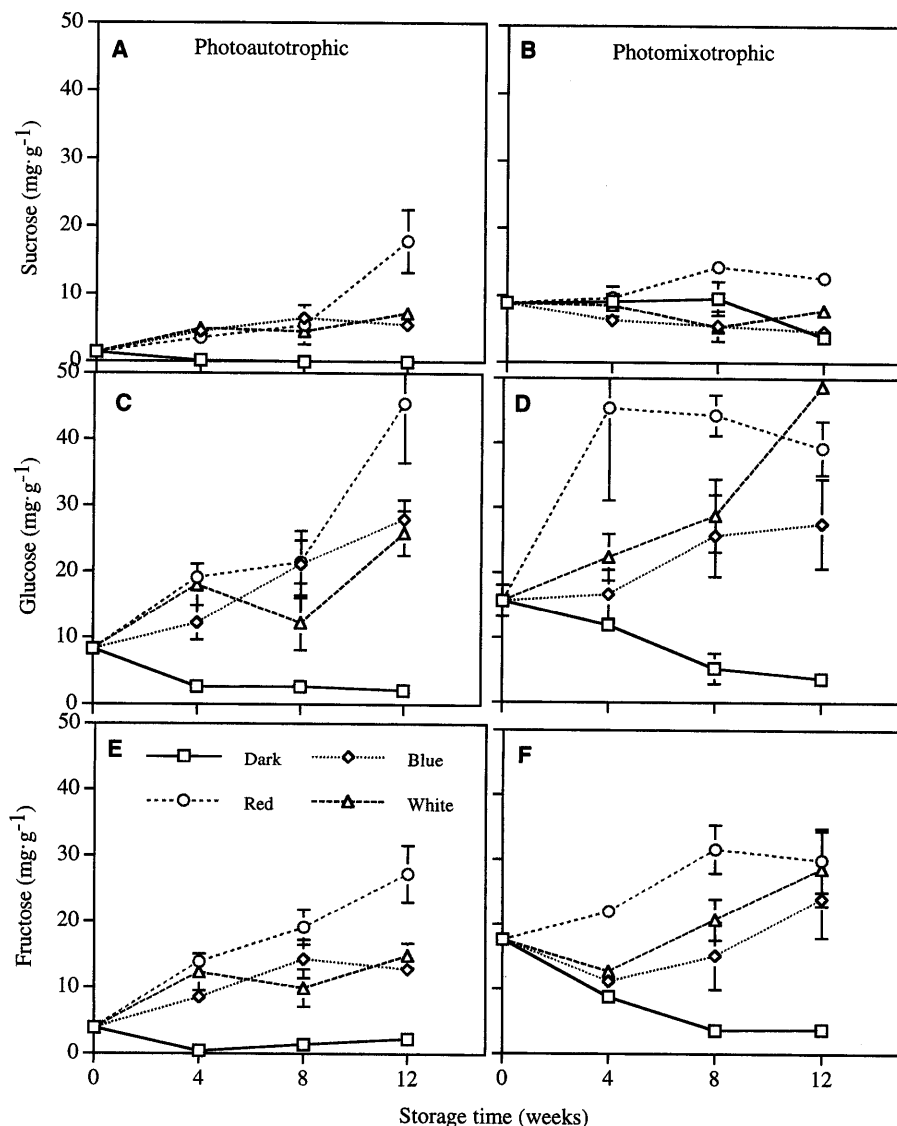


Fig. 2. Effect of light during storage on leaf soluble sugars in photoautotrophic and photomixotrophic broccoli seedlings. Means \pm SE are shown.

autotrophic conditions, stem glucose and fructose levels were relatively unchanged in the light (Figs. 3 C and E). In photomixotrophic seedlings, stem glucose and fructose decreased during 4 weeks of storage regardless of light in storage (Figs. 3 D and F), but red or white light maintained the levels thereafter, while blue light did not.

Photosynthetic ability. The levels of CO_2 in dark-stored photoautotrophic vessels were higher than ambient levels outside the vessel ($\approx 650 \mu\text{mol}\cdot\text{mol}^{-1}$) during the first 4 weeks of storage; thereafter, they were not significantly different from ambient (Fig. 4A). The levels in vessels stored in light were lower than ambient, regardless of light quality, and lowest under red light. Photomixotrophic cultures stored in darkness accumulated more CO_2 after 1 week of storage than did photoautotrophic cultures (Fig. 4B). Unlike photoautotrophic seedlings, photomixotrophic seedlings stored in blue or white light continued to produce CO_2 throughout 12 weeks of storage. However, CO_2 accumulation decreased after 4 weeks, with the exception of white light-

stored photomixotrophic seedlings. Levels of CO_2 in photomixotrophic cultures stored under red light were higher than ambient after 1 week of storage but lower than ambient thereafter. Under blue light, CO_2 levels in photomixotrophic cultures were appreciably higher than ambient in early storage but decreased to levels equivalent to ambient by week 12; levels under white light increased during storage of photomixotrophic seedlings.

Prior to storage, media composition did not significantly affect the NPR of seedlings (Fig. 5). The NPR of photoautotrophic and photomixotrophic seedlings was reduced after 4 weeks of storage, regardless of light quality. After 8 weeks of storage under red light, the NPR remained constant but decreased by week 12, regardless of media composition. After 12 weeks of storage, NPR of both photoautotrophic and photomixotrophic seedlings had fallen considerably relative to nonstored seedlings.

Discussion

Concentrations of CO_2 were high during the first 4 weeks in dark-stored photoau-

totrophic cultures, but maintained close to ambient thereafter. Rapid decline of plants and severely reduced TSS concentrations during this time indicated that insufficient substrate was available for respiration. Similarly, Kubota et al. (1997) showed that 6 weeks of dark storage reduced TSS in photoautotrophic seedlings by 60% to 80%.

In dark-stored cultures, the higher than ambient CO_2 concentrations indicated that photomixotrophic seedlings had higher respiration rates than photoautotrophic seedlings. The respiration rate declined during storage, probably due to loss of respiratory substrate. The availability of sugars probably played a key role in maintaining plantlet life in dark storage.

When light was supplied to photoautotrophic seedlings during storage, CO_2 concentrations inside the vessels were lower than ambient. This indicates that photosynthesis was occurring during storage, regardless of light quality, although red light was more effective than blue or white light. Light also stimulated photosynthesis in photomixotrophic seedlings. However, under blue light, vessels had higher CO_2 levels than ambient, suggesting that respiration was greater than photosynthesis during early storage but declined later. Seedlings stored under white light were at their CO_2 compensation point during early storage, but respiration increased later. Seedlings stored in red light generally had higher leaf and stem TSS concentrations than those stored in white or blue light after 12 weeks, which is consistent with increased leaf dry mass (Wilson et al., 1998) and higher rates of photosynthesis under red light. Similarly, Szasz and Barsi (1971) and Warrington and Mitchell (1976) have shown that red light increases soluble sugar and starch concentrations in leaf tissue. In contrast, Kubota et al. (1997) reported that blue light produces higher glucose and fructose concentrations in stems and leaves after 6 weeks than does white or red light.

For short-term storage, providing sucrose in the media is more commercially feasible than providing light. If seedlings are intended for longer storage (e.g., up to 8 weeks), light becomes essential, as the sugar supply in the media becomes depleted. However, photosynthetic ability of both photoautotrophic or photomixotrophic seedlings and regrowth potential (Wilson et al., 1998) was lost, regardless of light treatment, as storage duration increased to 12 weeks. Maintaining photosynthetic ability and carbohydrate reserves plays a significant role during storage of micropropagated seedlings. Future investigations will determine if decreasing storage temperature from 5 $^{\circ}\text{C}$ to 1 $^{\circ}\text{C}$ will better maintain plantlet quality during storage.

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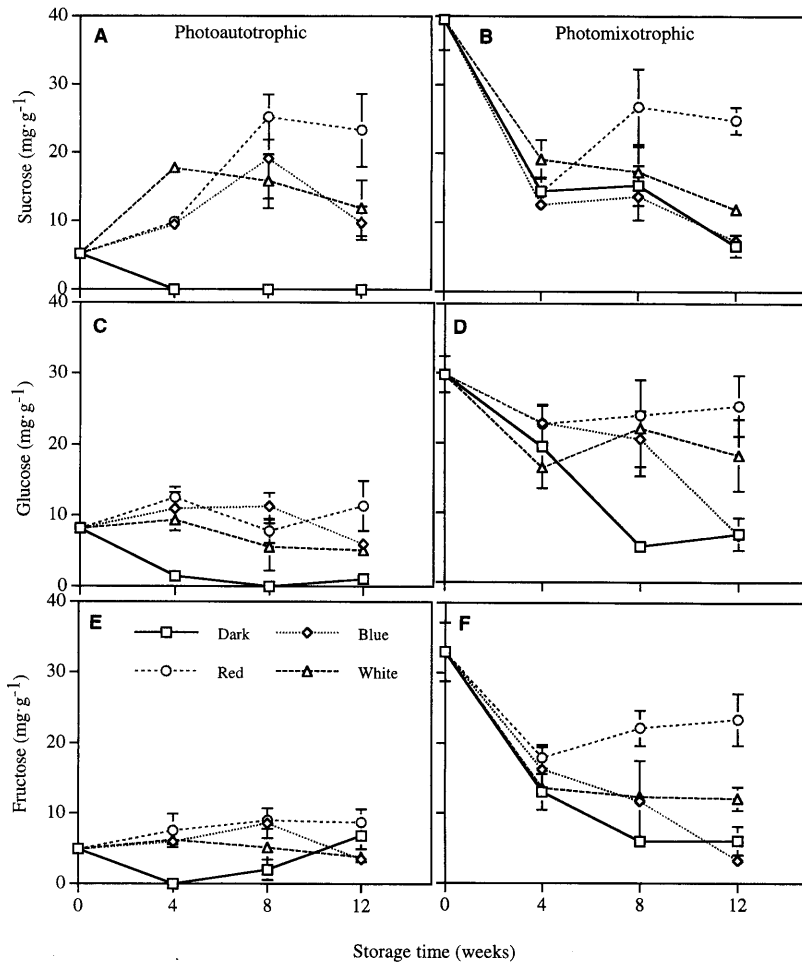


Fig. 3. Effect of light during storage on stem soluble sugars in photoautotrophic and photomixotrophic broccoli seedlings. Means \pm SE are shown.

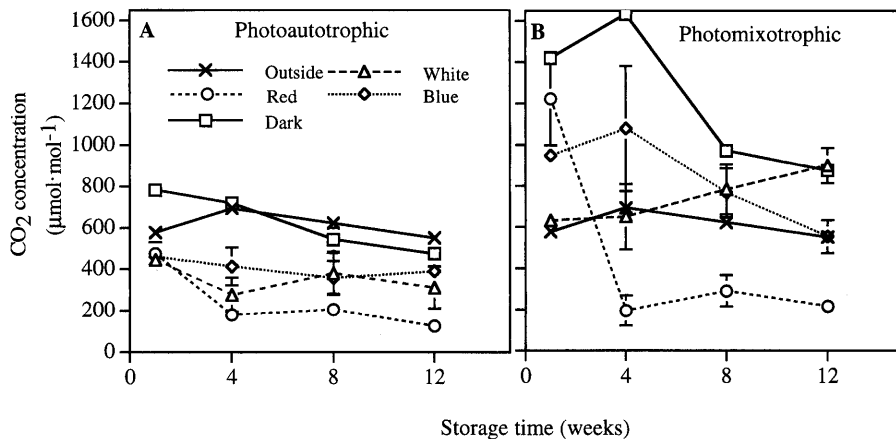


Fig. 4. Effect of light on CO₂ concentrations inside and outside vessels during storage of photoautotrophic and photomixotrophic broccoli seedlings. Means \pm SE are shown.

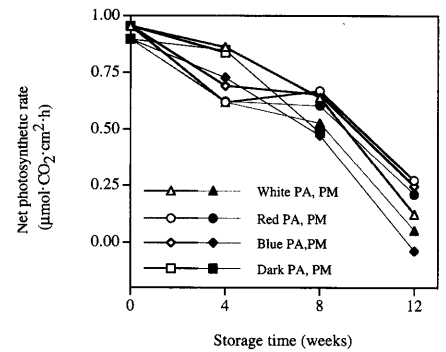


Fig. 5. Effect of light during storage on net photosynthetic rate of photoautotrophic (open symbols) and photomixotrophic (closed symbols) broccoli seedlings. Net photosynthetic rates were determined at 1500 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂.

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