ORIGINAL ARTICLE

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Cold-induced photoinhibition affects establishment of *Eucalyptus nitens* (Deane and Maiden) Maiden and *Eucalyptus globulus* Labill

Abstract The effects of cold-induced photoinhibition on *Eucalyptus globulus* and *Eucalyptus nitens* seedlings were assessed between planting and age 23 weeks. The seedlings were subjected to four treatments before planting: non-hardened (NH) *E. globulus* and *E. nitens*, cold-hardened (CH) *E. nitens*, and nutrient-starved (NS) *E. nitens*. Seedlings were planted alongside established 1-year-old *E. nitens* saplings. The experimental site was at 350 m above sea level, which is considered marginal for the establishment of *E. globulus* plantations due to low mean minimum temperatures. Cold and sunny conditions after frost increased photoinhibition in the order: NH *E. globulus* > NH and CH *E. nitens* > NS and established *E. nitens*. As a result there was 20% mortality of NH *E. globulus* seedlings. NS *E. nitens* seedlings were severely photoinhibited and had high anthocyanin levels at planting; levels of photoinhibition decreased after planting, anthocyanin levels remained high and there was no mortality. Carotenoid levels were low in *E. globulus* compared to *E. nitens* treatments. It was concluded that cold-induced photoinhibition is a factor determining the range of environments where *E. globulus* can be successfully planted, and not frost tolerance alone. Anthocyanin was synthesised in response to in-creased photoinhibition. Anthocyanin levels were correlated to the severity of the photoinhibition. Thus, *E. nitens* seedlings nutrient starved in the nursery are pre-conditioned to photoinhibitory conditions experienced soon after planting. This treatment is a useful risk-management tool where cold-induced photoinhibition is likely to occur during seedling establishment.

Keywords Photoinhibition · *Eucalyptus* · Anthocyanin · Carotenoids · Seedlings

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Introduction

Sustained decreases in the efficiency of photosystem II occur in leaves exposed to high levels of irradiance and low temperature (Adams et al. 1994). Low temperature also limits the rate at which Calvin cycle enzymes function (Powles 1984). These changes are manifest in a reduced photosynthetic capacity (Berry and Bjorkman 1980). If light is absorbed in excess under these environmental conditions, the excess energy must be dissipated or the chloroplast membranes sustain oxidative damage (Foyer et al. 1994). Photoinhibition of photosynthesis, whereby a sustained decrease in the efficiency of photosynthetic energy conversion occurs, prevents such damage (Huner et al. 1993). This can be observed as a decrease in the quantum yield of photosynthesis (ϕ) or a decrease in the ratio of variable to maximal chlorophyll fluorescence (F_v/F_m).

This decrease in photosynthetic efficiency is due to active down-regulation of photosynthesis through increased rates of dissipation of thermal energy (Adams and Demmig-Adams 1995). This is realised in the xanthophyll cycle (Adams and Demmig-Adams 1995; Verhoeven et al. 1996) when de-epoxidation of violaxanthin to zeaxanthin (via antheraxanthin) occurs in response to an increase in transthylakoid pH (Yamamoto 1985). Zeaxanthin (and antheraxanthin) are epoxidised to violaxanthin under low light. However, sustained zeaxanthin engagement may result if night-time temperatures are be-

low zero (Adams and Demmig-Adams 1994, 1995). This provides photoprotection to plants under early morning conditions of high irradiance and low temperature.

Evergreen species that grow in cold climates frequently experience high irradiance and low temperature. Cold-induced photoinhibition has been observed in high-altitude coniferous forests (Leverenz and Öquist 1987; Öquist and Huner 1991; Ottander and Öquist 1991; Verhoeven et al. 1996, 1999), alpine plants (Streb et al. 1997; Germino and Smith 1999; Manuel et al. 1999), high-altitude eucalypts (Ball et al. 1991; Holly et al. 1994; Ball et al. 1997), seedlings of *Eucalyptus globulus* Labill. growing in a nursery (Close et al. 1999a), and seedlings of *E. globulus* and *Eucalyptus nitens* (Deane and Maiden) Maiden established in plantations (Close et al. 1999b). Cold-induced photoinhibition can also affect annual crops such as *Brassica napus* L. (Farage and Long 1991) and *Gossypium hirsutum* L. (Königer and Winter 1991) and reduce crop yields.

The establishment and distribution of regenerating tree seedlings has been shown to reflect the degree of cold-induced photoinhibition. For example, *Picea englemannii* Parry ex. Engelm. and *Abies lasiocarpa* (Hook.) Nutt. (Germino and Smith 1999) and *Eucalyptus pauciflora* Sieb ex Spreng (Ball et al. 1991) are restricted to particular microsites near their low-temperature limits of distribution, and this is linked to high levels of incident irradiance. *E. pauciflora* regenerates under the southwestern aspect of established tree canopies where they are protected from exposure to high light and night-time radiation frosts (Ball et al. 1991).

In Tasmania, large areas of eucalypts are planted at high altitudes with low mean annual temperatures (<10°C). High-pressure systems in winter bring clear night skies and the leaf temperature of seedlings can drop below air temperature due to radiation cooling (Jordan and Smith 1995). Cold air stratification near ground level further increases the exposure of leaves to low temperature (Jordan and Smith 1994) ahead of photoinhibitory conditions developing early in the morning. *E. nitens* is the preferred species for planting in these cold environments to which *E. globulus* is poorly adapted (Tibbits 1986). The seedlings are produced in nurseries at low altitudes. In nursery trays, seedlings self shade to a large degree. Thus seedlings are not only exposed to cold-induced photoinhibitory conditions at planting, but also are not acclimated to conditions of high light and low temperature. Damage to seedlings of a range of conifer species has been reported to result from sudden changes in light conditions, the degree of damage being species specific (Gnojek 1992; Leiffers et al. 1993; Spunda et al. 1993; Mohammed and Parker 1999). Adams et al. (1994) hypothesise that this is due to differences in the capacity of the xanthophyll cycle to dissipate energy. Inherently low levels of photosynthesis also pre-dispose immature leaves to cold-induced photoinhibition (Krause et al. 1995; Dodd et al. 1998).

A nursery practice used to harden seedlings prior to transplanting is nutrient starvation (Close et al. 1999b).

This has been shown to maximise survival and growth after planting and to provide a degree of acclimation to low temperature. The treatment induces the synthesis of large amounts of anthocyanins in the leaves (Close et al. 1999b) though the physiological function of these pigments is unclear at present (Barker et al. 1997; Dodd et al. 1998). Cold-hardened *E. nitens* seedlings are also less susceptible to cold-induced photoinhibition than non-hardened seedlings (Warren et al. 1998).

In the experiment described in this paper, four hypotheses were postulated and then tested: (1) that established, fully acclimated 1-year-old *E. nitens* saplings experience less photoinhibition than *E. nitens* seedlings recently planted; (2) that artificially cold-hardened (CH) *E. nitens* and nutrient-starved (NS) *E. nitens* seedlings experience less photoinhibition than non-hardened (NH) *E. nitens* seedlings; (3) that NH *E. nitens* seedlings experience less photoinhibition than NH *E. globulus* seedlings; and (4) that the degree of photoinhibition measured in seedlings will be manifested in terms of seedling growth.

Materials and methods

Site description

A 1-ha trial was established on an ex-pasture site (Watson's Block) approximately 15 km south of Ridgley (43°20′S, 39°80′E) (397150 E, 544250 N AMG reference) in Tasmania at an altitude of 400 m above sea level. One section of the trial (0.25 ha) was planted in 1996 and a second section in 1997 (0.75 ha). The latter area was treated with 2.5 l ha^{-1} glyphosate (Roundup, 450 g l^{-1} active ingredient) and 0.6 l ha^{-1} metsulphuron methyl (Brushoff, 600 g kg^{-1} active ingredient) in June 1996. The former area was treated with glyphosate only. Bare soil was mounded using a plough approximately 2 months prior to planting. The mounds were cultivated with a rotary hoe to create a fine tilth and allow maximum contact between the potting medium and soil. The soil type was lithic eutrudox (or brown ferrosol) with an average soil depth of approximately 50 cm. Minimal weed growth ensured mounds remained bare during the 23-week experimental period. A weather station located 0.5 km west of the site indicated that the mean annual rainfall and temperature are approximately 1390 mm and 9.7°C, respectively.

Plant material

Seedlings were raised from single family seedlots 2078, 2202 and 2972 of North Forest Products (NFP) *E. nitens* and seedlot 2200 of NFP *E. globulus* (ex. NFP, Burnie, Tasmania). Seedlots 2078 and 2202 were germinated in the first week of March 1997 and grown for approximately 1 month in a nursery (Hills, Devonport, Tasmania) before transportation to Hobart. At the nursery, seedlings were fertilised every 10 days with Aquasol (1 g l^{-1}).

In Hobart, one half of the 2078 *E. nitens* seedlings were cold-hardened (CH) for 2.5 months. Each day during this period seedlings were placed in a cool room at 2°C for 14 h overnight and moved outside at approximately 08:00 hours Australian eastern standard time (AEST). The other seedlings (NH *E. nitens* and NH *E. globulus*) were grown under the prevailing diurnal conditions (Table 1). During this 2.5-month period CH and NH *E. nitens* and NH *E. globulus* were irrigated with Hoagland's solution (7.5 g l^{-1}) at 4-week intervals and immediately prior to out-planting.

Seedlots 2202 and 2972 were germinated in March 1996 and raised at the NFP nursery under ambient environmental conditions (Table 1). Seedlings were fertilised every 10 days with Aquasol (1 g l^{-1}) until they attained their planting height. Subsequently, the

Table 1 Seedling seedlot, height at out-planting and mean temperatures during the pre-experimental period. CH Cold hardened, NH non-hardened, NS nutrient starved (planted in 1997), Est. established (planted in 1996), *min.* minimum, *max.* maximum

Treatment	*Eucalyptus nitens* CH	*E. nitens* NH	*E. nitens* NS	*Eucalyptus globulus* NH	*E. nitens* Est.
Seedlot	2078	2078	2972	2200	2202
Height (cm)	9.2	10.5	15.2	16	65.2[a]
Mean daily min. (°C)	2	5	2.5	5	2.4[b]
Mean daily max. (°C)	12	12	9.5	12	9.5[b]

[a] Heights at planting of 1997 seedlings

[b] Temperatures during August and September 1997

2972 seedlings were nutrient starved (NS) in the nursery for approximately 1 year. The 2002 seedlings (referred to as established *E. nitens* saplings) were planted in October 1996 and received 100 g diammonium phosphate fertiliser near the root collar 2 months after planting.

Experimental design

The established *E. nitens* saplings were planted in the 0.25-ha area at between and within row spacings of 3.5 m and 2.5 m, respectively. Nine adjacent saplings were removed from a row within this planting in August 1997. The cleared section of mound was recultivated with a rotary hoe. New seedlings were planted in September 1997. They were arranged in groups of three consisting of one each of CH and NH *E. nitens* and NH *E. globulus* seedlings. A randomly selected seedling was planted in the cleared mounds, opposite an established *E. nitens* sapling in the adjacent row. The other two seedlings in the group were planted 0.6 m either side (in the direction of the row) of the central seedling. Thus there were nine groups of three seedlings from each treatment. These seedlings and the adjacent saplings were used for physiological measurements.

In the 0.75-ha area, CH and NH *E. nitens* and NH *E. globulus* were planted also in September 1997 in the same arrangement. Spacing between rows was also 3.5 m.

These seedlings were planted into three blocks perpendicular to the incline on the site and used for height measurements. Each block consisted of three rows, each row containing 20 seedling groups. NS *E. nitens* seedlings and seedlings of two other nursery treatments (not reported here) were planted similarly in separate groups of three seedlings in an unreplicated block. This block consisted of four rows, each row containing ten seedling groups. Between and within row spacings of this unreplicated block were also 3.5 m and 0.6 m, respectively. No fertilisers, herbicides or insecticides were applied throughout the period of the experiment.

Environment

Air temperature was monitored at heights of 15, 30 and 45 cm above ground as well as at 1.3 m (reference height) at 10-min intervals with thermocouples attached to three-star stakes. Shields comprising two white PVC plumbing pipes of different diameters (50 and 90 mm with 20-mm holes drilled for ventilation), with the smaller pipe secured within the larger pipe, prevented contact of direct radiation with the thermocouples. Values were summed hourly and daily averages were calculated from hourly data. Data was recorded and calculated by a CR10X measurement and data-logging system (Campbell Scientific, Logan, Utah).

Plant measurements

Chlorophyll fluorescence and gas exchange

Pre-dawn maximal photochemical efficiency, F_v/F_m, was assessed using a PAM-2000 fluorometer and 2030-B leaf-clip holder (Heinz Walz, Effeltrich, Germany). Single measurements were made on each leaf of the most recently expanded leaf pair and on a single older leaf from the leaf pair one node back.

Photosynthetic light response curves were obtained for one leaf of the most recently expanded leaf pair, using an open-flow gas analysis system (LCA2, Analytical Development Corporation, Hoddesdon, Hertfordshire, U.K.). A Parkinson PLC-B leaf chamber in conjunction with a lamp comprising four 150-W Wotan xenon quartz globes was used. Incident photon flux densities (PFDs) below 750 μmol m^{-2} s^{-1} were obtained using neutral density filters. PFDs above 750 μmol m^{-2} s^{-1} were obtained by increasing power to the lamp. All gas exchange measurements were made between 11:00 and 14:00 hours AEST.

Each measurement series was replicated on the same leaves of three seedlings/saplings within the physiology plot. Chlorophyll fluorescence and gas exchange measurements were made on the same day on 15 September, 8 and 21 October and 24 November 1997 and 6 January, 4 February and 5 March 1998 (0, 4, 5, 10, 16, 20 and 23 weeks after planting, respectively).

Height

Monthly measurements of height (to 0.5 cm) were made on the 20 seedling groups in the middle row of each of the three blocks containing the CH and NH *E. nitens* and NH *E. globulus* treatments. Height was measured on the two ten-seedling-group inner rows of the four-row block containing NS *E. nitens*. Seedling leaf photodamage and mortality were observed during seedling height measurement. Leaf photodamage was ranked as nil, moderate or severe.

Laboratory measurements

Pigment analysis

The most recently expanded leaf pairs of three randomly selected seedlings of NH *E. globulus*, CH, NS *E. nitens* and established *E. nitens* saplings were sampled at 0, 5, 9 and 11 weeks after planting. Leaves were immediately shielded from light and stored in a cool box (4±1°C) prior to freezing at –20°C within 24 h.

A representative sample of leaf tissue (0.2 g) was cut and ground in liquid nitrogen using a mortar and pestle. Chlorophyll and carotenoids were extracted in 2 ml of 80% aqueous acetone buffered to pH 7.8 with sodium phosphate. The homogenate was collected and combined with three 1.5-ml washings of the mortar and pestle, vortexed and then centrifuged at 2500 r.p.m. for 10 min. The resulting supernatant was diluted fourfold. Absorbance was measured using a UV-visible spectrophotometer (Varian Carey 1E, Sydney, Australia) at 663 and 646 nm for chlorophyll determination (Porra et al. 1989). Absorbance at 470 nm was measured for carotenoid determination (Lichtenthaler 1987).

Anthocyanins were extracted using the same volumes as above with acidified ethanol (to pH 1 with concentrated HCl). The homogenate was then immersed in boiling water for 1.5 min and left to extract for 24 h in the dark at 5°C. Extracts were centrifuged as above before absorbance measurement at 530 and 657 nm. The formula absorbance$_{530}$-0.25×absorbance$_{657}$ was used to correct for chlorophyll and degradation products (Mancinelli et al. 1975).

Artificial frosting

Leaf discs, 8 mm in diameter, were cut with a punch from the most recently expanded leaf pair of three seedlings/saplings per treatment and placed in test tubes. Racks of test tubes were placed in baths containing 30% aqueous ethylene glycol solution and chilled at $4°C\ h^{-1}$. Electrolyte conductivity of 2 ml deionised water containing leaf discs was measured just before planting and at week 7 after planting, using chilling temperatures of $-4°C$ and $-7°C$. At week 17, temperatures of $-2°C$ and $-4°C$ were used. The frost tolerance index was expressed as a ratio of electrolyte conductivity measured after cooling treatment relative to maximum electrolyte conductivity measured after immersion of discs in a 70°C water bath (Raymond et al. 1986).

Statistical analysis

There were no statistically significant effects of block on mean seedling heights. At each sampling date, treatment effects on chlorophyll fluorescence, leaf disk electrolyte conductivity, total chlorophylls, carotenoids, anthocyanins and seedling height were tested using one-way ANOVA and the ANOVA procedure of SAS (SAS Institute 1989). A non-rectangular hyperbolic function was used to describe the light response curves of seedlings:

$$Y = 2\alpha X A_x / \alpha X + \sqrt{(\alpha X + A_x)^2 - 4\theta X A_x}\ ,$$

where Y is the assimilation rate (A), α approximates the efficiency of photosynthesis (ϕ); A_χ gives the light-saturated value of A (A_{max}); θ describes the slope of the curve; and X is PFD (Long and Hällgren 1993). ANOVA (PROC ANOVA in SAS) was used to quantify treatment effects on ϕ and A_{max}. Linear regression analysis (PROC GLM in SAS) was used to compare changes in ϕ and A_{max} with time. In all analyses the least-squares method was used to estimate means and SEs.

Results

Environment

Differences in minimum temperatures measured at 0.15, 0.30, 0.45 and 1.3 m above ground were always within 1°C. There were six frost events (with the lowest −3.3°C) within 3 weeks of planting. No frosts were measured subsequently. During the period of measurement daily minimum and maximum air temperatures rose steadily (Table 2).

Artificial frosting

Before planting, NH *E. globulus* seedlings were least frost tolerant ($P<0.001$), established *E. nitens* saplings and CH *E. nitens* seedlings were most frost tolerant ($P<0.05$) and NH *E. nitens* and NS *E. nitens* had intermediate frost tolerance (Fig. 1).

A significant decrease in the frost tolerance index had occurred by week 7 after planting in established *E. nitens* saplings ($P<0.01$), CH *E. nitens* ($P<0.001$), and NH *E. nitens* ($P<0.001$) seedlings. By comparison NH *E. globulus* ($P<0.01$) and NS ($P<0.01$) *E. nitens* seedlings had a significantly increased frost tolerance index. Comparison of treatments at week 7 showed NS *E. nitens* and NH *E. globulus* seedlings had greater frost tolerance compared

Table 2 Number of frosts and absolute and average minimum and maximum air temperatures measured at 0.3 m in the weeks preceding each set of physiological measurements in the field

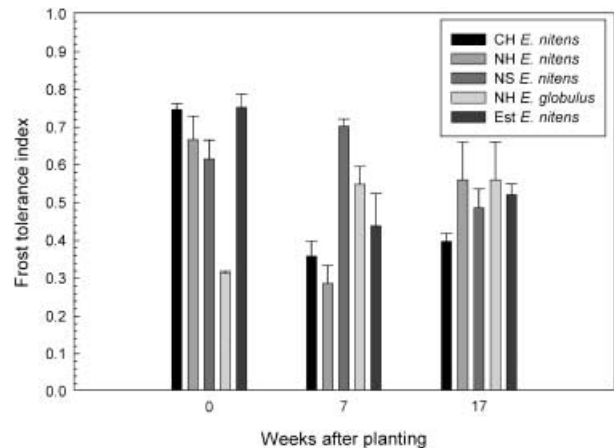| Week	Frosts	Air temperature (°C)			
		Minimum		Maximum	
		Absolute	Average	Absolute	Average
1	1	−0.4	3.7	16.6	14.6
3	5	−3.3	2.1	16.8	14.3
5	0	1.9	3.9	18.8	13.9
9	0	0.4	5.6	22.2	17.5
15	0	1.2	6.2	23.8	17.6
19	0	2.9	8.7	28.1	22.8
23	0	4.3	6.9	28.4	24.6

Fig. 1 Frost tolerance index of four seedling treatments and the established (Est) *E. nitens* saplings at 0, 7 and 17 weeks after planting. At weeks 0 and 7, frost tolerance was assessed at the test temperature of −7°C. At week 17, the test temperature was −4°C. Bars indicate ±SE. *CH* Cold hardened, *NH* non-hardened, *NS* nutrient starved

to other treatments. There were no significant differences in frost tolerance between treatments at week 17 (Fig. 1).

Chlorophyll fluorescence

F_v/F_m of NH *E. globulus* seedlings decreased markedly following planting (Fig. 2). The lowest levels (<0.3) were recorded at weeks 3 and 5 and were associated with severe photodamage, leaf abscission and a moderate level (20%) of seedling mortality. Young, expanding, less hardy leaves had senesced by week 5. F_v/F_m of residual mature leaves and new expanding leaves had maximal values (>0.7) at week 9.

F_v/F_m of CH and NH *E. nitens* seedlings decreased until week 5 and this was associated with moderate photodamage. However, F_v/F_m increased to maximal values at week 9. During this period NH *E. nitens* appeared to have lower F_v/F_m levels than CH *E. nitens* seedlings but differences were not significant. F_v/F_m of both these
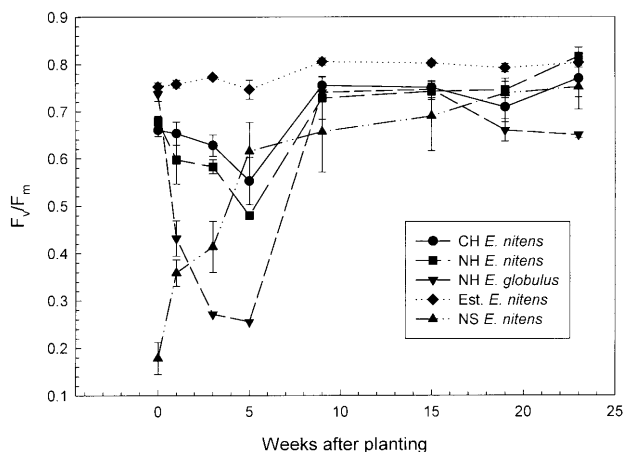
Fig. 2 Changes in photochemical efficiency (F_v/F_m) with time after planting for CH, NH and NS *E. nitens* and NH *E. globulus* seedlings compared to those of Est. *E. nitens* saplings. Bars indicate ±SE. For abbreviations, see Fig. 1

treatments was significantly lower ($P<0.0001$) than that of established *E. nitens* saplings between weeks 1 and 9. F_v/F_m of *E. nitens* saplings was relatively constant and high ($F_v/F_m>0.7$) throughout the experiment and no photodamage was observed.

F_v/F_m levels of NS *E. nitens* seedlings increased steadily from very low (<0.2) to levels still indicative of photoinhibition ($F_v/F_m=0.62$) at week 9. Maximal values of this treatment were not reached until week 19. No leaf photodamage was associated with low F_v/F_m levels. Prior to week 5, F_v/F_m of NS *E. nitens* seedlings was significantly lower ($P<0.0001$) than that of the other *E. nitens* treatments. From week 9 there were no significant differences between *E. nitens* treatments.

Gas exchange

Changes of A_{max} of CH *E. nitens*, NH *E. nitens* and NH *E. globulus* seedlings were similar to each other throughout the experiment (Fig. 3a). Linear regression analysis showed that rates of increase of A_{max} were similar ($P=0.73$) (i.e. no significant differences in slope) but with differing Y intercepts ($P<0.001$), in the order of magnitude: CH *E. nitens* (0.0048)>NH *E. nitens* (0.0025)>NH *E. globulus* (0.0007). These relationships differed significantly ($P<0.001$) from that for established *E. nitens* saplings in which A_{max} remained high and relatively constant (12–17 µmol m^{-2} s^{-1}) throughout the experiment. A_{max} of CH *E. nitens* was greater than that of NH *E. nitens* ($P<0.05$) seedlings at weeks 3 and 5. A_{max} of NH *E. globulus* seedlings decreased after planting and was significantly lower ($P<0.05$) than that of other treatments at weeks 3 and 5. A_{max} of *E. globulus* increased markedly after week 5 and by week 9 was similar to levels in the *E. nitens* seedlings. There were no significant differences in A_{max} between seedling treatments after week 9. A_{max} was similar between seedlings and established *E. nitens* saplings after week 15.
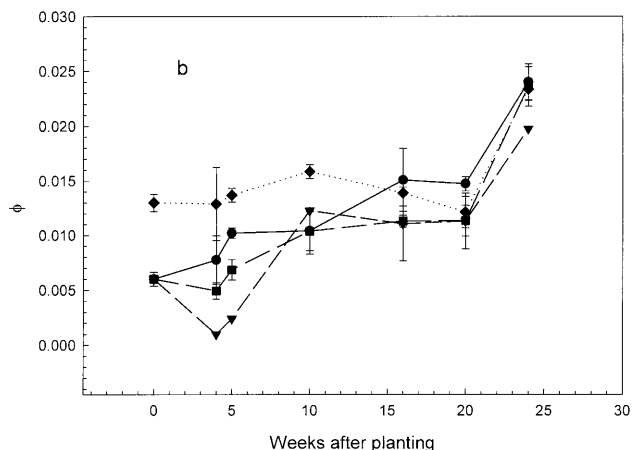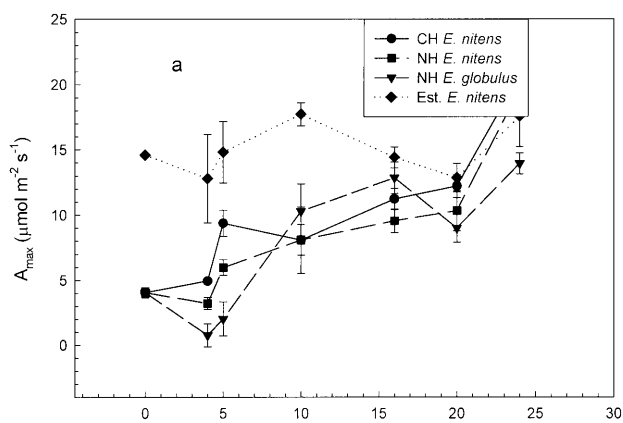

Fig. 3 Changes in maximum photosynthesis (A_{max}) (**a**) and quantum yield, ϕ (**b**) with time after planting for CH and NH *E. nitens* and NH *E. globulus* seedlings compared to those of Est. *E. nitens* saplings. Bars indicate ±SE. For abbreviations, see Fig. 1

The response of ϕ for all treatments followed the same trends as A_{max} except for week 3 when ϕs of CH and NH *E. nitens* seedlings were not significantly different (Fig. 3b). Maximum ϕs (ϕ ranged from 0.019 to 0.024) occurred at week 23.

Pigments

Total chlorophyll was significantly higher ($P<0.001$) in established *E. nitens* saplings compared to the seedling treatments until week 11 (Fig. 4a). NS *E. nitens* had significantly less ($P<0.001$) total chlorophyll at planting and week 5 compared to CH *E. nitens* and NH *E. globulus* seedlings. There was no significant difference between the latter treatments. Total chlorophyll increased between weeks 5 and 11 in all seedling treatments.

Chlorophyll *a/b* ratios of seedling treatments (Fig. 4b) decreased between planting and week 5, but then increased and remained at a high level between weeks 9 and 11 (primarily due to changes in chlorophyll *a* content). There were no significant changes in chlorophyll
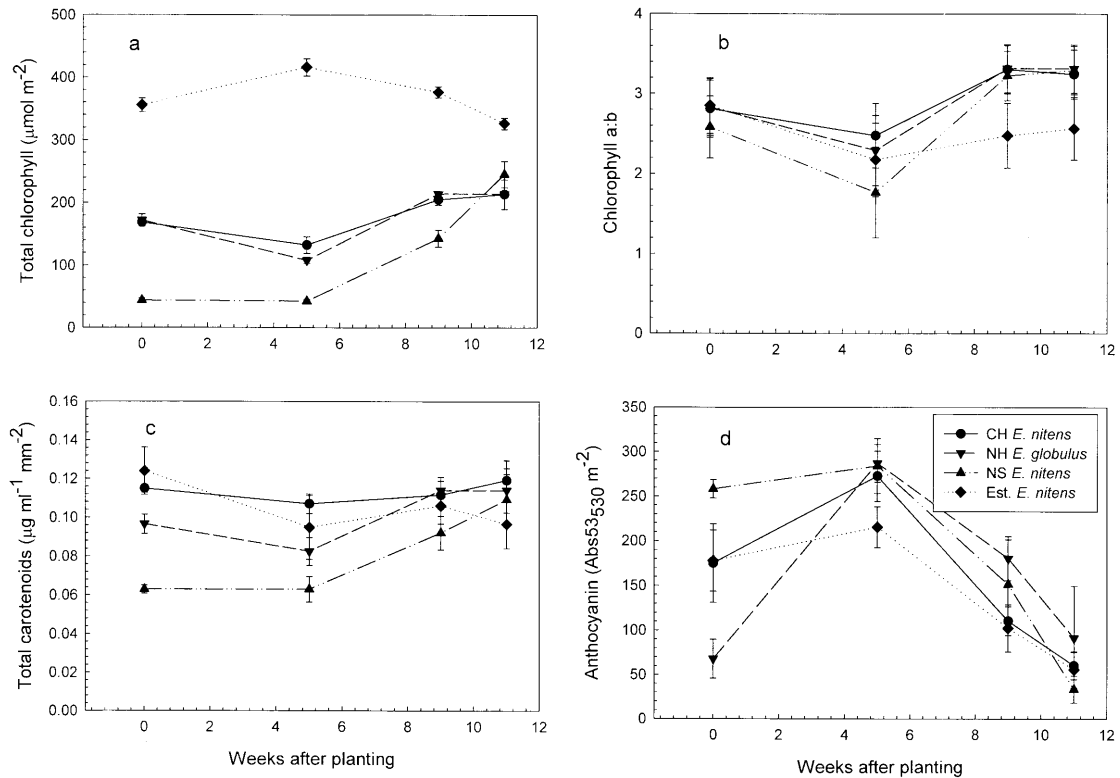
Fig. 4 Variation during seedling establishment of total chlorophyll (**a**), chlorophyll *a/b* ratio (**b**), total carotenoids (**c**), and anthocyanin (**d**) in CH, NH and NS *E. nitens* seedlings compared to those of Est. *E. nitens* saplings. Bars indicate ±SE. For abbreviations, see Fig. 1

a/b ratios of the established *E. nitens* saplings throughout the measurement period. At week 5, NS *E. nitens* had lower chlorophyll *a/b* ratios ($P<0.05$) than other treatments.

The total carotenoid pool of the established *E. nitens* saplings decreased between planting and week 5 but then remained relatively stable thereafter (Fig. 4c). At planting, the established *E. nitens* saplings and CH *E. nitens*, NH *E. nitens*, and NS *E. nitens* had significantly different ($P<0.0001$) carotenoid pool sizes, ranked as follows: established=CH>NH>NS *E. nitens*. The pool size of NS *E. nitens* remained significantly lower than those of the other treatments at week 5 ($P<0.05$). Carotenoid pool sizes had largely converged by week 9.

Leaf anthocyanin levels were significantly higher ($P<0.01$) in NS *E. nitens* and significantly lower ($P<0.01$) in NH *E. globulus* seedlings than levels in CH *E. nitens* seedlings and established *E. nitens* saplings at planting (Fig. 4d). Anthocyanin levels increased significantly between pre-planting and week 5 in NH *E. globulus* and CH *E. nitens* seedlings: in NH *E. globulus* seedlings the increase was >fourfold. After week 5, leaf anthocyanin levels decreased and were not significantly different for any treatment.

Height

The heights of NH *E. globulus* and NS *E. nitens* seedlings were significantly ($P<0.001$) greater than those of CH and NH *E. nitens* at planting (Fig. 5). At age 4 weeks, the CH and NH *E. nitens* seedlings had increased in height ($P<0.001$); the NH *E. globulus* and NS *E. nitens* seedlings showed no increase during this period.

All *E. nitens* seedlings were actively growing by age 8 weeks, although growth of NS *E. nitens* was slower than that of CH and NH *E nitens*. At age 12 weeks and
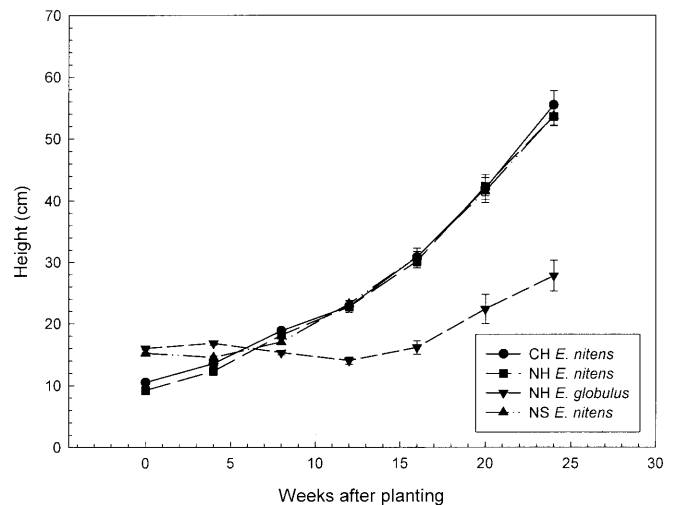

Fig. 5 Height profile (cm) during seedling establishment of CH, NH and NS *E. nitens* and NH *E. globulus* seedlings. Bars indicate ±SE. For abbreviations, see Fig. 1

until the end of the experiment there were no significant differences in heights between treatments of *E. nitens* seedlings. A significant increase in height ($P<0.05$) was not recorded for NH *E. globulus* seedlings until age 16 weeks. Height was significantly lower ($P<0.0001$) in NH *E. globulus* than in *E. nitens* seedling treatments aged 8 weeks until the end of the experiment. The height of established *E. nitens* saplings increased (increment ~ 200 cm) throughout the experiment (data not shown).

Discussion

This work has shown that newly transplanted seedlings, regardless of hardening pre-treatments, are inherently susceptible to cold-induced photoinhibition relative to established, acclimated *E. nitens* saplings. Overall, artificial cold hardening had little effect on the physiology or growth of *E. nitens* seedlings. *E. globulus* seedlings were more susceptible to cold-induced photoinhibition than *E. nitens* seedlings. Reductions in growth were measured in *E. globulus* but not *E. nitens* seedlings. Differences in levels of carotenoids and anthocyanins at planting are proposed as a major factor contributing to species differences in performance. Nutrient starving of seedlings caused severe cold-induced photoinhibition but high initial levels of anthocyanins in this treatment appeared to minimise leaf tissue damage.

Photoinhibition and photodamage

No photoinhibition was detected in 1-year-old *E. nitens* saplings throughout the experimental period. F_v/F_m of these saplings was close to 0.8 at every measurement, a value typical of non-photoinhibited plants of a wide range of species (Björkman and Demmig 1987). A_{max} was similar also to levels reported previously for non-photoinhibited *E. nitens* (Davidson et al. 1995; Battaglia et al. 1996). These results indicate that the low temperatures experienced during the trial period were not stressful for acclimated individuals of *E. nitens*.

Artificial cold hardening induces a suite of biochemical and physiological changes in plants (Guy 1990; Wanner and Junttila 1999) that act to decrease the low temperature limit at which leaf damage occurs (Guy et al. 1987; Gilmour et al. 1988). However, the significant difference in frost tolerance at planting between CH *E. nitens* and NH *E. nitens* seedlings was not linked to any observed differences in rates of physiological processes measured in the field. This indicates that frost damage did not occur in *E. nitens* seedlings. In other studies of seedling establishment (e.g. Ball et al. 1991; Orlander 1993; Holly et al. 1994; Germino and Smith 1999), it has been demonstrated that the combination of high irradiances with low temperatures causing cold-induced photoinhibition, and not simply low temperature damage per se, can affect tree seedling performance and mortality in the field. The observation that there was no effect of nu-

trient starvation on frost tolerance of NS *E. nitens* seedlings was consistent with other studies (Hellergren 1981; Toivenen et al. 1991; Hawkins et al. 1994).

A marked photoinhibitory response immediately after planting was apparent for NH *E. globulus* seedlings and was associated with leaf abscission and seedling mortality. Responses to sudden exposure to cold-induced photoinhibitory conditions and capacities for subsequent acclimation to such conditions vary according to species (Ferrar and Osmond 1986; Logan et al. 1998; Mohammed and Parker 1999). These differences may depend in part on the capacity of the xanthophyll cycle to dissipate energy (Demmig-Adams and Adams 1996). This hypothesis is supported by the observation that levels of total carotenoids in *E. nitens* saplings and CH *E. nitens* were greater than in NH *E. globulus*. Thus *E. globulus* may have a lower inherent capacity for the dissipation of excess energy than *E. nitens*. Similar species differences in photochemical efficiency (F_v/F_m) have been reported for conifers. For example shade-grown *Tsuga canadensis* (L.) Carr. (Mohammed and Parker 1999) sustained greater photoinhibition after exposure to high light conditions relative to *Picea abies* (L.) Karst. (Gnojek 1992; Leiffers et al. 1993) and *Pinus taeda* L. (Zhang et al. 1997). The findings here suggest that sensitivity to cold-induced photoinhibition may be a factor determining the range of environments where *E. globulus* can successfully be planted, in addition to its low frost tolerance (Tibbits 1986). Sensitivity to cold-induced photoinhibition has been suggested to partially explain the natural distribution and habitat niches of *E. pauciflora* (Ball et al. 1991; Warren et al. 1998), *E. nitens* (Warren et al. 1998) and *Ilex aquifolium* (Groom et al. 1991).

Sustained xanthophyll-dependent energy dissipation (i.e. low pre-dawn F_v/F_m) following nights of freezing temperatures has been linked to a reduction of photodamage during the following morning under conditions of high light and low temperature (Adams et al. 1994). This response was induced in *E. nitens* by nutrient starvation which, by decreasing chlorophyll levels and photosynthetic capacity, increases the excess of excitation energy (Jacob 1995) and decreases the ability of the plant to synthesise and repair any damage to photosystem II (Balachandren and Osmond 1994; Godde and Hefer 1994).

Pigments

Total chlorophyll levels for established *E. nitens* saplings were similar to those reported previously for *E. nitens* (Warren et al. 1998) but were low compared to other species (e.g. Demmig-Adams and Adams 1992). Low chlorophyll levels have been proposed as an adaptation to excess irradiance through passive reduction in light absorption (Tardy et al. 1998). Seedlings in all treatments had significantly lower chlorophyll levels than *E. nitens* saplings. This finding was consistent with other investigations of immature vs. mature foliage (Krause et al. 1995;

Dodd et al. 1998). However, low levels of chlorophyll in conjunction with low levels of carotenoids can predispose seedlings to photoinhibition (Krause et al. 1995). The low levels of both types of pigment in the NS *E. nitens* seedlings were consistent with this treatment being most photoinhibited at planting. It was not possible to distinguish the greater susceptibility of *E. globulus* relative to *E. nitens* to photoinhibition on the basis of chlorophyll levels.

The decrease in total and chlorophyll *a/b* levels between planting and 5 weeks after planting in the CH and NH *E. globulus* seedlings, which is indicative of photooxidative stress (MacWilliam and Naylor 1967; Haldimann 1998, 1999), was consistent with the observed decreases in F_v/F_m in these treatments. In NS *E. nitens* seedlings there was no decrease in chlorophyll or F_v/F_m levels, which accords with the lack of visible photodamage in this treatment relative to other seedling treatments.

Plants resistant to cold-induced photoinhibition commonly have higher levels of carotenoids (Demmig-Adams and Adams 1996; Haldimann 1999). Such a relationship was observed in this experiment. The order of magnitude of carotenoid pools at planting was: established *E. nitens* saplings>CH *E. nitens*>NH *E. globulus*>NS *E. nitens* seedlings, and this corresponded to the relative degree of photoinhibition as measured by F_v/F_m. It is notable that NH *E. globulus* had lower levels of carotenoids at planting and week 5 compared to CH *E. nitens*. Thus carotenoid as well as chlorophyll levels were correlated to the degree of photoinhibition experienced. Increased levels of carotenoids have often been reported as a consequence of prolonged exposure to photoinhibition (Adams et al. 1994; García-Plazaola et al. 1999). However, nutrient starvation did not increase levels of carotenoids in NS *E. nitens* seedlings, which remained in the same proportion to chlorophylls as in other treatments.

The presence of anthocyanins in young eucalypt leaves has been reported previously (Sharma and Crowden 1974) but was not correlated with specific environmental conditions. Increased synthesis of anthocyanin has been reported in *Pinus sylvestris* L. seedlings following early frosts (Nozzolillo et al. 1989), similar to the responses of CH *E. nitens* and NH *E. globulus* observed in this trial. Two patterns of anthocyanin levels were apparent after planting. In CH *E. nitens* and NH *E. globulus* anthocyanin levels increased. This increase was associated with decreased total chlorophyll levels and increased photoinhibition in both treatments, and with moderate and severe visible leaf photodamage in CH *E. nitens* and NH *E. globulus*, respectively. In contrast, anthocyanin levels of established *E. nitens* saplings and NS *E. nitens* seedlings remained constant. This was associated with unchanged chlorophyll levels in both treatments, no change in the photoinhibition of established saplings and a marked decrease in the photoinhibition of NS *E. nitens*. No visible leaf tissue damage was observed in either treatment.

This combination of results indicates strongly that anthocyanin synthesis is induced by environmental conditions that result in photoinhibition. Established saplings and NS *E. nitens* seedlings were acclimated to the conditions prevailing between planting and 5 weeks after planting. After week 5 anthocyanin was gradually desynthesised in parallel with the gradual increase in temperatures and photosynthetic rates. This indicates that anthocyanin has a protective function during periods of cold-induced photoinhibition. The same phenomenon has been observed in *P. sylvestris* seedlings after removal to a warm greenhouse (Nozzolillo et al. 1989), though in this case it was not associated with photoinhibition.

Accumulation of anthocyanins in *P. sylvestris* seedlings in response to nutrient starvation at mild temperatures has also been reported previously (Nozzolillo et al. 1989; Toivenon et al. 1991). These, as yet, unexplained observations and the results from this experiment are however consistent with current ecophysiological evidence that indicates a role for anthocyanins in light attenuation (Krol et al. 1995; Barker et al. 1997; Dodd et al. 1998) thereby decreasing cold-induced photoinhibition.

Seedling height

Severe photoinhibition and photodamage which occurred soon after planting were associated with no increase in height of NH *E. globulus* until age 16 weeks. Similarly, impaired growth was observed in *Eucalyptus polyanthemos* Schau. and *Pinus ponderosa* Laws. under conditions of severe depression of the efficiency of photosystem II (Holly et al. 1994; Adams et al. 1994). In contrast, moderate photoinhibition had no effect on CH and NH *E. nitens*, and an increase in height was observed 4 weeks after planting. No growth occurred in NS *E. nitens* until F_v/F_m had recovered from very low levels. However, by age 8 weeks photoinhibition and increases in height were similar to those of other *E. nitens* treatments. This is consistent with the growth of photoinhibited *Pinus banksianna* Lamb. upon transfer to non-photoinhibitory conditions in a greenhouse (Nozzolillo et al. 1989).

In conclusion, it has been shown that cold-induced photoinhibition restricted the establishment of *E. globulus* on a site considered marginal for planting this species. However, comparisons between CH and NH *E. nitens* treatments indicated no benefit of artificial cold hardening in terms of subsequent growth after planting. Hardening seedlings by withholding nutrients negated the effects of cold-induced photoinhibition during establishment. This may have been due to sustained overnight xanthophyll engagement and/or the possible reflection of incident light by anthocyanins. Increased anthocyanin synthesis was measured as a result of cold-induced photoinhibition after planting in the non-NS seedlings, the degree of photoinhibition correlating to the relative increases in anthocyanin levels. In contrast, established *E. nitens* saplings experienced no cold-induced photoinhibition and no increase in anthocyanin levels. Most impor-

tantly, pre-conditioning for photoinhibition through nutrient starving had no long-term effect on growth performance, suggesting this as a practical and useful risk management tool in seedling production for eucalypt plantation forestry in cold environments.

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