Spatial patterning of pigmentation in evergreen leaves in response to freezing stress

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ABSTRACT

Evergreen leaves of temperate climate plants are often subject to frosts. Changes in carbon gain patterns arise from freezing-related tissue damage, and from interactions between light and temperature stress. We examined relationships between spatial patterns in freezing and concentrations of chlorophyll. Spatial patterns in pigmentation in leaves that had or had not been exposed to naturally occurring frosts were determined by conventional extraction techniques combined with high-resolution hyperspectral imaging of reflectance from intact leaves. Predictive indices were developed to relate reflectance to chlorophyll content and chlorophyll *a/b* ratios within intact leaves. Leaves exposed to frosts had lower chlorophyll contents and more variable *a/b* ratios than protected leaves. In frost-affected leaves, chlorophyll content was highest near leaf centres and decreased toward leaf tips and margins. Decline in chlorophyll content was associated with shifts in chlorophyll *a/b* ratios and increases in red pigmentation due to anthocyanin, with effects being greater on leaf sides exposed directly to the sun. These altered pigmentation patterns were consistent with patterns in freezing. The present results illustrate the fine scale of spatial variation in leaf response to freezing, and raise important questions about impacts of freezing on photosynthetic function in over-wintering evergreens.

Key-words: *Eucalyptus pauciflora*; CASI; chlorophyll content; chlorophyll *a/b* ratio; frost; hyperspectral imaging.

INTRODUCTION

Abiotic factors that impose stress on plants are likely to have spatially variable impacts. Temperature stresses and water stresses in particular will be influenced by leaf size and shape and may differ in magnitude over the surface of the leaf. Despite a growing understanding of how these factors vary over leaf surfaces, and of variation in leaf stomatal responses in particular, the impact of stresses on physiological parameters have generally been considered at either the cell level or averaged across the whole leaf

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(Terashima 1992; Siebke & Weis 1995; Lawson & Weyers 1999). Recent work has used imaging of room temperature fluorescence from chlorophyll *a* to assess pigment distribution and photosynthetic processes (e.g. Siebke & Weis 1995; Buschmann & Lichtenthaler 1998). However, this technology is generally restricted in the distance over which it can operate. The development of new reflectance imaging technologies provides an opportunity to examine whether physiological impacts of abiotic stresses are expressed in a spatially variable fashion within leaves and canopies, and to make links between these measurements and conventional remote sensing measurements (Pinar & Curran 1996; Blackburn 1998a; Jago, Cutler & Curran 1999; Richardson, Berlyn & Gregoire 2001).

Frost effects on leaves are spatially variable. In a recent study of freezing patterns of leaves of snow gum, *Eucalyptus pauciflora*, Ball *et al*. (2002) demonstrated that freezing initiates in the leaf mid-vein and spreads rapidly through the leaf. Cellular water freezes more rapidly at the leaf tip and margins than near the petiole and centre of the leaves. This rapid freeze occurs because the leaf margin is thinner, contains less water than the centre and is exposed to greater boundary layer differentials. The tip and leaf margin also cool more rapidly once most of the cellular water has frozen, leading to steep gradients in leaf temperature across the leaf surface (up to 4 °C; Ball *et al*. 2002).

As winter approaches, leaves of *E. pauciflora* and other frost-tolerant species acclimate to cold temperatures, making them more resistant to low night temperatures. Winter high light-acclimated leaves have lower photosynthetic rates and lower chlorophyll contents (Gilmore & Ball 2000). However, leaves that face early season frosts prior to acclimation exhibit considerable cellular damage, as evidenced by electrolyte leakage at freezing temperatures (Ball *et al*. 2002). Temperature differences between leaf centre and margin during such early season frost events could lead to considerable variation in freezing stress. The pattern of freezing is reflected in higher incidence of necrosis at the tips and margins of frost-damaged leaves. Such damaged leaves also have different patterns of pigmentation around the margins of their leaves, with increased red pigmentation (anthocyanins, e.g. Gamon & Surfus 1999) occurring in a spatial pattern consistent with that of freezing.

Because freezing or chilling is associated with chlorosis (loss of chlorophylls) (Ottander, Campbell & Öquist 1995), spatial patterning of freezing damage is likely to be associated with changes in the spatial distribution of photosynthetic pigments. Chlorosis occurs as a result of photo-oxidative damage, especially under the combination of low temperature and high light conditions. In some species, not only does the total chlorophyll content change, but the chlorophyll *a/b* ratio is also affected by temperature (Ottander *et al*. 1995; Haldimann 1998, 1999). The relative ratio of chlorophyll *a/b* changes with leaf stress and growth conditions. In leaves grown at low light, the relative proportion of chlorophyll *a* decreases due to increased size of the photosystem antennae complexes — chlorophyll *b* is prominent in outer regions of the antenna (Anderson, Chow & Goodchild 1988).

Previous studies have used reflectance spectra to predict concentration of a range of pigments in leaf tissues (e.g. Chappelle, Kim & McMurtrey 1992; Curran *et al*. 1992; Peñuelas, Baret & Filella 1995; Gitelson & Merzlyak 1996; Pinar & Curran 1996; Blackburn 1998b; Datt 1999; Jago *et al*. 1999; Sims & Gamon 2002). Predicting pigment concentrations of leaves using reflectance makes important links between leaf-level processes and plant- or stand-level characteristics, and thus has been the goal of many studies with direct application to remote sensing. As yet, none of these methods have been spatially explicit at the leaf level and often these studies rely on averages over numerous leaves, so as to mimic canopy-level reflectance (Datt 1999), or to enhance reflectance signals (Curran 1989). We were specifically interested in determining whether a high-resolution hyperspectral imaging system (CASI, Itres, Canada) could detect changes in physiology at the leaf level as evidenced by patterns of within-leaf variation in pigmentation.

Eucalyptus pauciflora (snow gum), is among the most cold-tolerant eucalypts. None-the-less, low temperatures are associated with decreased chlorophyll content, photoinhibition, and decreased growth rates in subsequent seasons (Ball *et al*. 1997; Blennow *et al*. 1998; Gilmore & Ball 2000). We examined the impact of freezing stress on the distribution of photosynthetic pigments in *Eucalyptus pauciflora* leaves. Using reflectance spectra, we developed indices to predict variation in chlorophyll content over the leaf surface. Given known patterns of freezing and our observations of reddening over the surface of the leaf, we expected corresponding changes in chlorophyll content. Previous studies have examined distribution of chlorophylls *a* and *b* separately or in total averaged over the surface of the leaf. We asked whether changes in total chlorophyll content were associated with shifts in the ratio of these chlorophylls, and whether the pattern of change was spatially dependent.

METHODS

Seeds of *Eucalyptus pauciflora* Sieb. ex Spreng. were collected from three trees growing on the floor of the Orroral Valley at an elevation of 850 m in New South Wales, Australia. The seeds were cold stratified under moist conditions at 3 °C for 4 weeks before germination on sand flats in a mist house. Similar seedlings of average size were transferred to individual containers filled with soil and grown either under glasshouse conditions, or in a pasture subject to frequent frosts (elevation 700 m) at Bungendore, New South Wales (Ball *et al*. 1997). The pots were 400 mm deep and were made of 155 mm i.d. PVC storm water pipe fitted with a shade cloth bottom.

We collected five snow-gum (*Eucalyptus pauciflora*) leaves that had experienced severe early season frosts and showed signs of frost-induced damage from field-grown seedlings in July 1998 (mid-winter). In December, 1998 (early summer) an additional 11 leaves were collected from these same plants. Approximately half of the latter leaves had over-wintered, the remaining leaves had been initiated and developed during spring and early summer. Furthermore, in December we collected another five leaves that had over-wintered on glasshouse plants protected from winter frosts. To seal vessels, we dipped the petioles of all leaves in silicon gel immediately after removal from the plant. Leaves collected in the field were kept cool and moist overnight before scanning. Leaves were collected from *E. pauciflora* seedlings of uniform age. Some variation in leaf form arose because individuals varied in the timing of the transition from juvenile (dichotomous venation) to adult (parallel venation) foliage.

Each leaf was scanned using a CASI high-resolution hyperspectral imaging system. The CASI uses a push-broom imaging system that collects spatially explicit reflectance spectra at wavelengths ranging from 380 to 930 nm in 1.8 nm steps. Single leaves were placed on a black background and illuminated using halogen light sources fitted with heat filters. Leaves were moved at constant speed in front of the CASI sensor to acquire an image of the entire leaf with spatial resolution of 0.2 mm. A full reflectance spectrum (144 bands ± 2.4 nm) was recorded for each pixel. To determine relative reflectance, we measured total incoming light as reflectance from a photographic white standard.

To develop relationships between reflectance and pigment content we used the CASI to scan the 16 leaves collected in December 1998. Following the scan we removed two ~88 mm² discs from each leaf, bar four leaves that showed very little spatial variation. Only one disc was taken from each of these less-variable leaves. A second CASI scan was taken to determine precisely which portion of the leaf was used for pigment analysis. Leaf discs were removed from the lamina avoiding the mid-vein and attempting to select homogeneously coloured sections. Where possible, one disc was taken from close to the centre of the lamina and one closer to the margin. Leaf discs were frozen in liquid nitrogen and stored at –80 °C until use. Chlorophyll content and *a/b* ratio were determined spectrophotometrically using the equations of Porra, Thomson & Kriedmann (1989) for leaf tissue extracted with 80% aqueous acetone.

Spectral image analysis was conducted using ENVI software (Research Systems Inc, Boulder CO, USA) run-

ning in Linux on a PC. Reflectance values for each image were standardized to incoming light by dividing spectra for each pixel by the spectrum for the white standard at the same position. By matching the images before and after the leaf discs were removed, we were able to calculate average reflectance spectra for each of the collected leaf discs.

Regression analyses were conducted to assess the correlation between the average reflectance spectra from the sun-exposed surface and chlorophyll content of the leaf discs. The sun-exposed leaf surface was selected because pigmentation patterns were more pronounced on this side. Regressions between measured pigment concentration and selected published indices (Blackburn 1998b; Datt 1999) were calculated. To determine the best possible regression equations for our data, simple linear and stepwise multiple regressions were used. For the latter analysis, we standardized the data relative to near-maximum reflectance by dividing all values by reflectance at 770 nm. Reflectance in this region is on the near-infra-red plateau, beyond the red-edge in *E. pauciflora* and standardizing has been shown to minimize radiation interaction and interference of internal leaf structures. Standardizing using the 770 nm reflectance improved the fit of all regressions. One extreme outlier was excluded from regression fitting exercise.

The best-fit equations were used to examine spatial patterning of chlorophyll content and chlorophyll a/b ratio across the sun-exposed surface of the study leaves. For the five mid-winter leaves, five summer collected leaves that had never experienced frost and five summer collected leaves that had been through frost, transects of chlorophyll content were taken from the scanned images across the lamina at approximately the mid-point of the leaf, perpendicular to the mid-vein. Chlorophyll content and a/b ratio values were subsampled from each margin and the centre of the leaf, to either side of the mid-vein ($n = 4$ values per transect). These data were analysed using ANOVA with type of leaf, and position on leaf as main effects. Side (left or right) was initially included in the model as a factor nested within position, but was not significant and was excluded from the final model.

Because redness was visibly higher in mid-winter leaves, we calculated a simple index of anthocyanin content. The index, $R_{Red(650)}/R_{Green(550)}$, was modified from the broadband index used by Gamon & Sufus ($R_{red(600–700)}/R_{green(500–600)}$; Gamon & Surfus 1999). This index was tightly correlated with anthocyanin content in *Pseudotsuga menziesii* ($r^2 = 0.92$, Gamon & Surfus 1999; see also Sims & Gamon 2002). As anthocyanin content was not measured directly, red-index values were not compared to pigment content and were not analysed statistically.

RESULTS

Patterns of pigmentation

Frost-damaged leaves collected in mid-winter showed greater variation in pigmentation than leaves that had not experienced frost. In a typical leaf, increased red pigmentation was apparent around the leaf margin and tip (Fig. 1a). RGB images created using reflectance at three wavelengths (688, 550 and 509 nm for red, green and blue, respectively) illustrated the same pattern (Fig. 1b), and also demonstrated that variation in pigmentation was more apparent on the sun-exposed surface of the frost-damaged leaves (mid-winter).

Representative reflectance spectra from non-frosted and frost-damaged leaves revealed differences in the wavelengths indicative of chlorophyll content, especially those in the 630–730 nm range (illustrated by a representative leaf, Fig. 1c). In this range, chlorophyll *a* and *b* have absorbance features, thus the extent to which reflectance is depressed is indicative of chlorophyll content. Red-coloured pixels on the margin of the frost-damaged leaf had higher reflectance in the 637–700 nm range than did the greener more central pixels of the same leaf. Note that the red-edge or steep incline in reflectance in the 700 nm range was shifted to lower (bluer) wavelengths in these red pixels than in the greener pixels. A green pixel from the never-frosted leaf showed a similar reflectance in the chlorophyll absorbing regions to that of the green pixel from the frost-damaged leaf.

Predicting chlorophyll concentration using regression analyses

Application of published indices for chlorophyll *a* and *b* content to our data produced generally weak correlations (r^2 ranging from 0 to 0.50, Table 1). Blackburn's PSSR indices were calculated using reflectance at bands closest to those used by Blackburn (1998b). The predicted PSSR index for chlorophyll *a* was significantly correlated with measured chlorophyll *a* content, whereas the PSSR index for chlorophyll *b* produced no significant correlation. Blackburn's PSND indices [calculated with alternative bands corresponding most closely to those selected by Blackburn (1998b)] performed better for both chlorophyll *a* and *b*, resulting in significant correlations with r^2 values between 0.13 and 0.4. Datt's (1999) index for chlorophyll content developed on 21 species of *Eucalyptus* performed better for all measures of chlorophyll content. There was no indication of a non-linear relationship nor did transforming the data improve the fit for any of these relationships.

Regressions between reflectance at single bands and measured chlorophyll content (from leaf discs) were calculated using average reflectance spectra for the sun-exposed side of the leaf discs (Table 2, Fig. 2). Multiple regressions including bands known to correspond to absorption peaks did not significantly improve the fit above single band regressions. Therefore, regressions based upon best correlations of single bands and chlorophyll measures were used. (See Curran for discussion of complications associated with application of multiple regression in this context, Curran 1989; Curran *et al.* 1992). Notably, the bands predicting chlorophyll *a*, *b* and total chlorophyll content were
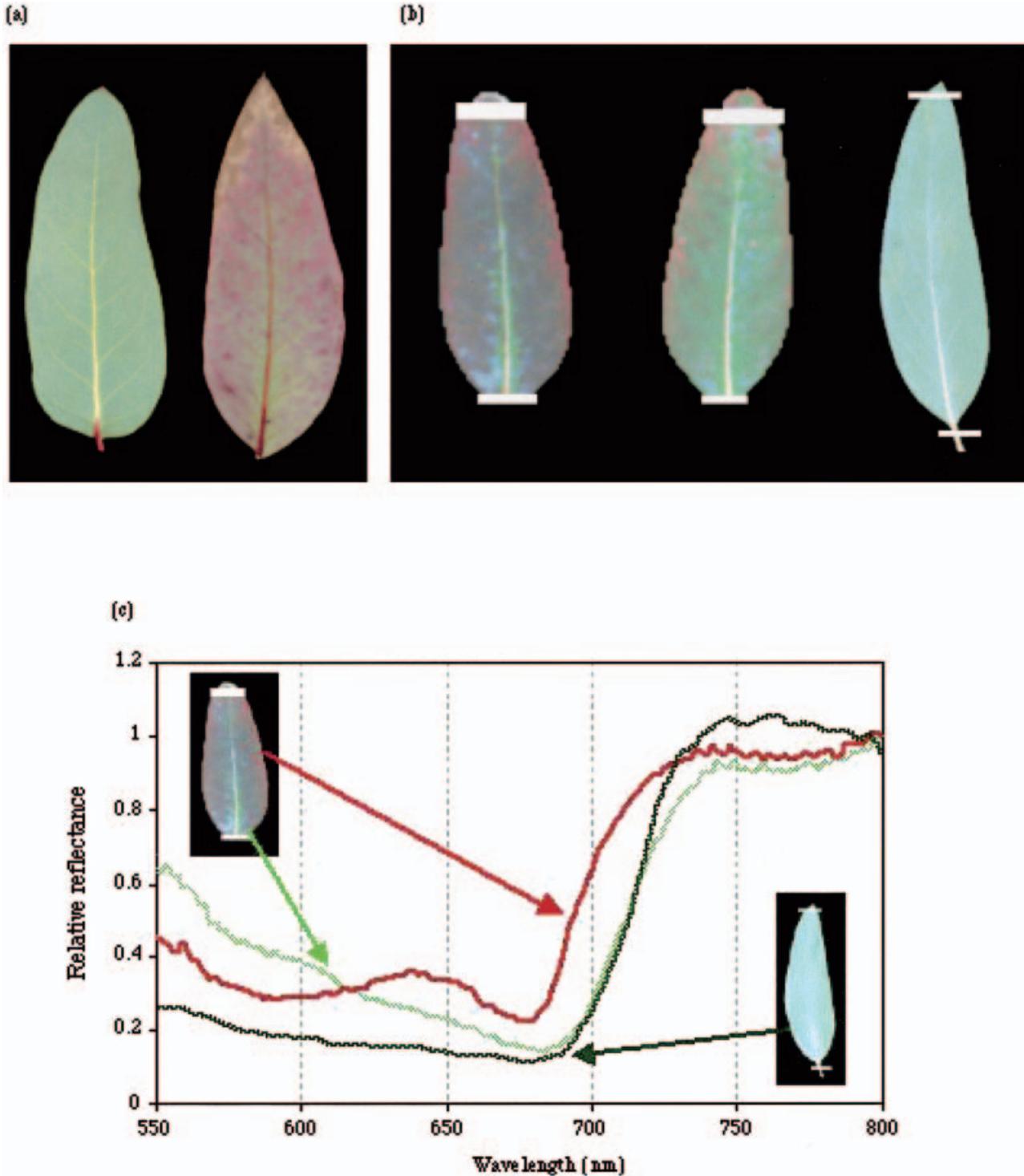
Figure 1. (a) A photograph of representative *Eucalyptus pauciflora* leaves collected summer (left) or in mid-winter (right). Summer-collected leaves had not been subjected to frost. (b) RGB images of sun-exposed (left) and shaded (centre) surfaces of a mid-winter leaf and the sun-exposed surface (right) of a never-frosted leaf. RGB images were composed to mimic appearance of leaves: 688, 550 and 509 nm for red, green and blue, respectively. (c) Reflectance spectra from frost-damaged (left) and never-frosted leaves (right) of *E. pauciflora*. Reflectance spectra (550–800 nm) for representative pixels are shown. Pixels are identified on RGB images.

identical or very close to one another (726 and 731 nm), whereas the chlorophyll *a/b* ratio, which yielded the tightest fit to the regression equation, was correlated with reflectance at 696 nm. Regressions were conducted on both mass (mg mg^{-1}) and area bases. Area-based measures of chlorophyll resulted in better fits to regression models, although both approaches yielded highly significant relationships at the same wavelengths.

Table 1. Relationships between measured chlorophyll content and published predictive relationships[a]

Index	Formula	Chlorophyll *a* (μmol m^{-2})		Chlorophyll *b* (μmol m^{-2})		Total chlorophyll (μmol m^{-2})	
		r^2(adj)	P	r^2(adj)	P	r^2(adj)	P
PSSRa1	R800/R682	0.18	0.015				
PSSRa2	R800/R673	0.18	0.015				
PSSRb1	R800/R635			0.00	0.301		
PSSRb2	R800/R651			0.01	0.254		
PSNDa1	(R800 − R673)/(R800 + R673)	0.40	≤ 0.001				
PSNDa2	(R800 − R682)/(R800 + R682)	0.40	≤ 0.001				
PSNDb1	(R800 − R635)/(R800 + R635)			0.13	0.034		
PSNDb2	(R800 − R651)/(R800 + R651)			0.16	0.020		
Datt	(R849 − R712)/(R849 − R681)	0.51	≤ 0.001			0.47	≤ 0.001

[a]All indices follow Blackburn (1998b) except the final (Datt 1999).

Spatial variation in chlorophyll content and chlorophyll *a/b* ratio

The regression equations above were used to predict chlorophyll content from reflectance at the pixel level for the entire sun-exposed surface of each of the study leaves. Total chlorophyll content was higher in young leaves and over-wintered leaves than in the frost-damaged leaves (Fig. 3). In frost-damaged leaves there was also considerable spatial patterning of chlorophyll content over the surface of the lamina, with marked decreases in chlorophyll content approaching the margins and tips of the leaves (Fig. 3b & c).

An ANOVA comparing total chlorophyll content at leaf centre and margin of the 15 study leaves revealed significant differences among leaf types (mid-winter, recovering and never-frosted) and between positions. The leaf margins had lower chlorophyll content than did the centres, and the summer-collected leaves had significantly higher chlorophyll content than the mid-winter-collected leaves (Table 3a, Fig. 4). When chlorophylls *a* and *b* were examined individually, a corresponding pattern of decline in winter leaves and at the margins was also apparent (data not shown).

Although both chlorophyll *a* and *b* declined toward leaf margins, the proportional decreases differed, leading to differences in spatial patterning in chlorophyll *a/b* ratio (Fig. 5). In young leaves that had never experienced frost, chlorophyll *a/b* ratio was nearly constant (~ 3.5 : 1) over the surface of the leaf, showing declines only along veins (Fig. 5a & d). In leaves scanned in mid-winter, the variation from margin to centre was marked (Fig. 5b & e), with

depressed *a/b* ratios apparent at margins (ratios of 1 : 1), the leaf tip, and in all areas distal to major veins. Notably, leaves that had experienced frost in the preceding winter but were scanned in summer, still showed indication of variation in chlorophyll *a/b* ratio; the ratio was slightly reduced along leaf margins, but there appears to have been recovery relative to mid-winter leaves (*a/b* ratio of 3 : 1 relative to 3.5 in mid-winter and recovering leaves, respectively, Fig. 5c & f).

An ANOVA of the chlorophyll a/b ratio indicated significant differences between leaf margin and centre and among leaf types. The spatial patterning was pronounced only in mid-winter leaves resulting in a highly significant interaction term (Table 3b, Fig. 6). On average, young leaves had higher chlorophyll *a/b* ratios than leaves that had experienced frost in the preceding winter.

Visual examination of the anthocyanin index indicated that patterns of anthocyanin pigmentation correspond to patterns of chlorophyll distribution. Anthocyanins were higher in mid-winter leaves than in never-frosted and recovering leaves, and in mid-winter leaf pigmentation was higher on the leaf margin than the centre (illustrated for typical leaves in Fig. 7).

DISCUSSION

Spatial variation in pigment content

Using hyperspectral imaging, we were able to map significant spatial patterning in pigment distribution over leaf surfaces in response to frost damage. The results demonstrated that spatial distribution of chlorophyll following

Dependent	d.f.	r^2(adj)	P	Equation
Chl*a*	25	0.855	≤ 0.0001	Chl*a* = −3066(R726/R770) + 2994
Chl*b*	25	0.839	≤ 0.0001	Chl*b* = −1090(R731/R770) + 1088
Total Chl	25	0.844	≤ 0.0001	Total Chl = −3729(R726/R770) + 3660
Chl *a/b*	25	0.904	≤ 0.0001	Chl *a/b* = −4.580(R696/R770) + 4.767

Table 2. Regression equations for predicting chlorophyll content (μmol m^{-2}) from reflectance
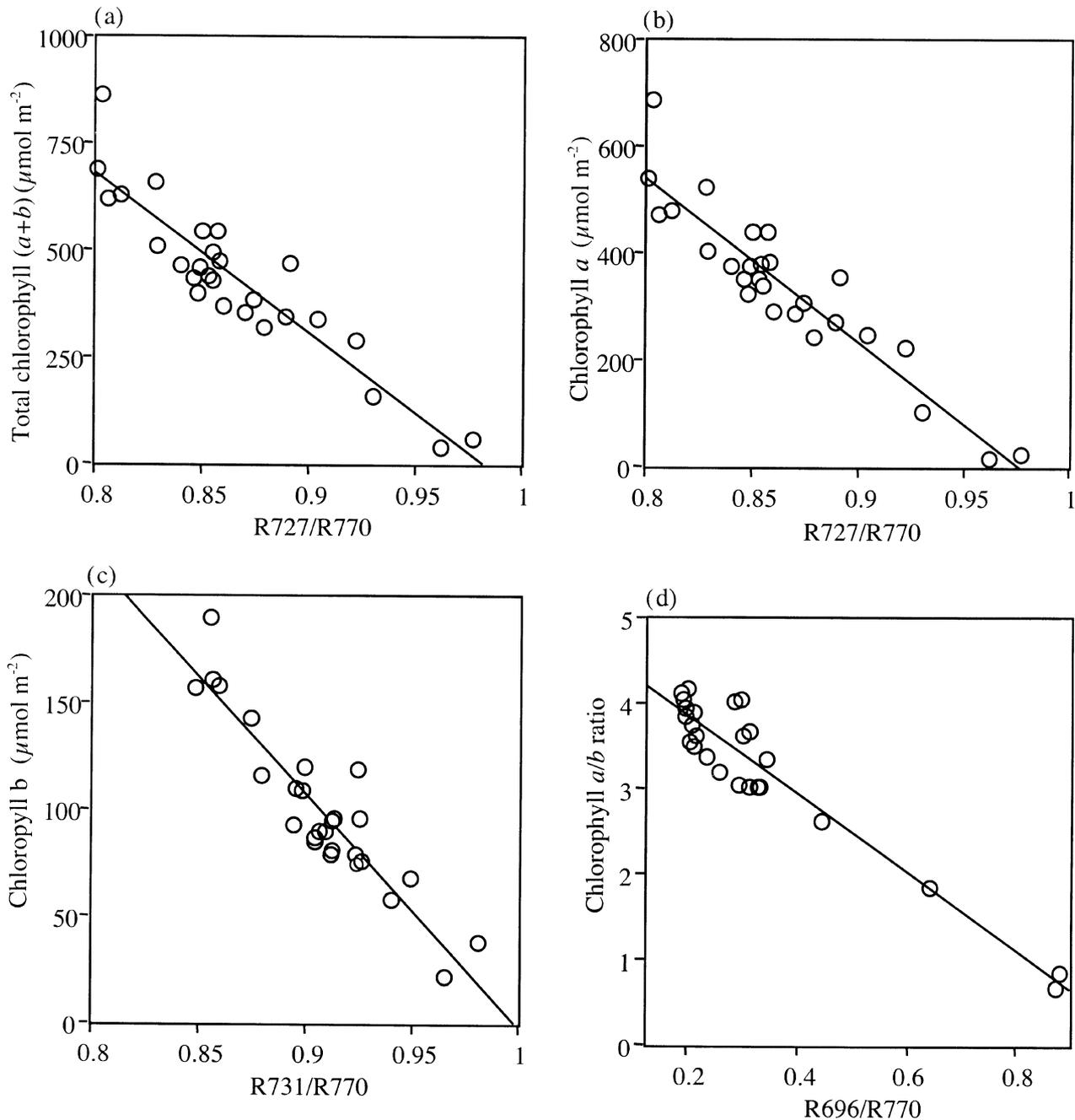
Figure 2. Correlations between chlorophyll content and reflectance. (a) Total chlorophyll ($a + b$); (b) chlorophyll a; (c) chlorophyll b; and (d) chlorophyll a/b ratio.

frost was neither uniform nor random, but directly reflected the recently described pattern of the freezing process in leaves (Ball *et al.* 2002). Moreover, as in other studies of responses to low temperatures in eucalypts (Gilmore & Ball 2000; Matsubara *et al.* 2002) and other temperate evergreens (Ottander & Öquist 1991; Ottander *et al.* 1995; Haldimann 1998, 1999), the results indicate that frost effects on spatial patterning of chlorophyll are reversible.

Total chlorophyll content declined in response to frost damage in a spatially predictable fashion. The decline was greatest along the leaf margin where freezing damage was also most pronounced. Previous studies have demonstrated chlorosis in leaves in response to frost (Ottander *et al.* 1995; Haldimann 1998, 1999). Our results build on those reports, showing that spatial variation in the decline in chlorophyll content follows a similar pattern to the spatial variation of freezing in individual leaves. In the present study, spatial patterning in chlorophyll content was particularly marked with regard to predicted chlorophyll a/b ratios and anthocyanin pigmentation. Frost events depress photosystem II (PSII) function, leading to decreased photosynthetic efficiency and capacity in temperate evergreens (Huner *et al.*
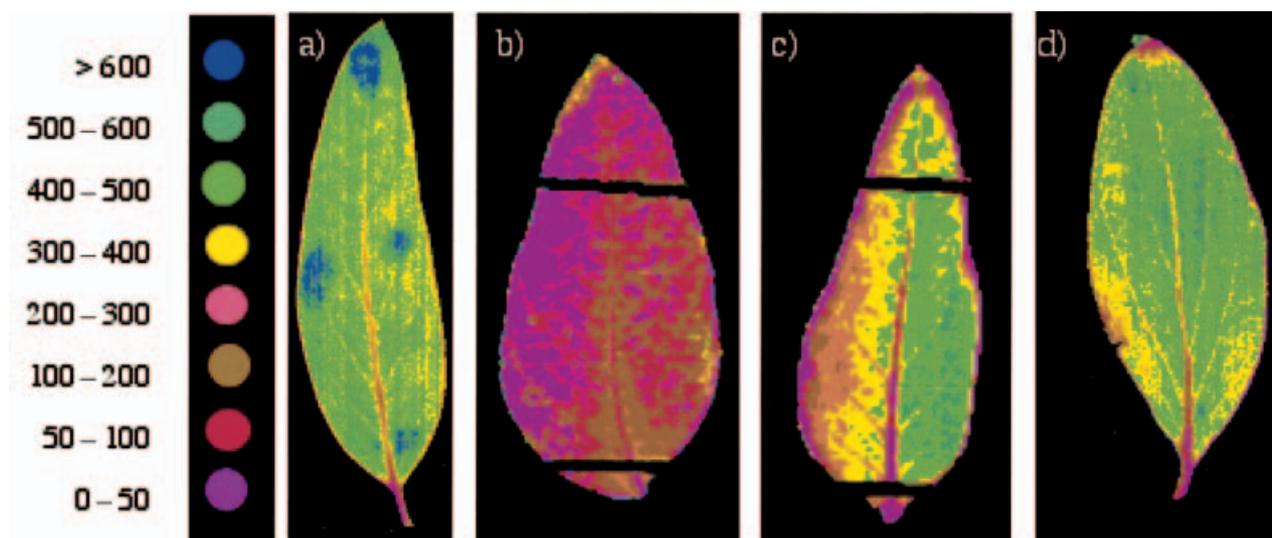
Figure 3. Spatial variation in total chlorophyll content (μmol m^{-2}) as estimated from regression indices for representative leaves that (a) had never been subject to frost; (b, c) were collected mid-winter following frost damage, or (d) were recovering from frost damage. Blue spots in (a) are reflections off underlying adhesive used to attach leaf to background.

1993), including snow gum (Ball, Hodges & Laughlin 1991; Ball *et al.* 1997; Roden, Egerton & Ball 1999; Egerton *et al.* 2000) and other eucalypts (Close *et al.* 2002; Matsubara *et al.* 2002). In previous studies, chlorosis in over-wintering leaves was associated with down-regulation of PSII and reduction in light harvesting (Ottander *et al.* 1995; Matsubara *et al.* 2002). Chlorophyll *b* occurs in the outer antennae of the light-harvesting complex whereas chlorophyll *a* occurs in the outer and inner antennae as well as the reaction cores (Anderson *et al.* 1988). As such, low-temperature-induced reduction in light harvesting could produce an increase in the chlorophyll *a/b* ratio, especially in leaves acclimated to high light (Ottander *et al.* 1995; Havaux & Kloppstech 2001; Matsubara *et al.* 2002). In the present study, however, both chlorophyll *a* and *b* declined along a transect running from mid-rib to margin, but chlorophyll *a* content declined more markedly, leading to a decrease in the *a/b* ratio. This response may be related to strong accumulation of anthocyanin pigments on the sunlit surface of over-wintering leaves.

Anthocyanins are localized in vacuoles of epidermal or subepidermal cells (Chalker-Scott 1999; Havaux & Kloppstech 2001). In other temperate eucalypts, the seasonal appearance of anthocyanins has been related to the occurrence and severity of freeze-induced photo-inhibition (Close *et al.* 2002). Red anthocyanin pigments protect PSII when leaves are exposed to low temperatures, apparently via reduction in the amount of light absorbed by underlying chloroplasts (Gould *et al.* 1995; Havaux & Kloppstech 2001; Feild, Lee & Holbrook 2001; Pietrini, Iannelli & Massacci 2002). In shade-grown leaves, the presence of extensive antennae pigments decreases the chlorophyll *a/b* ratio (Anderson *et al.* 1988). Thus, a relative increase in chlorophyll *b*-containing antennae pigments could reflect an effectively shaded leaf interior. Indeed, Pietrini *et al.* (2002) reported a 13% higher concentration of chlorophyll *b*, leading to a proportionally lower chlorophyll *a/b* in leaves of an anthocyanin-accumulating than an anthocyanin-deficient maize genotype (but see Havaux & Kloppstech 2001).

Table 3. Results of analyses of variance testing effects of leaf type (mid-winter, recovering or never-frosted), and position on leaf (margin or centre) on (a) total chlorophyll content and (b) chlorophyll *a/b* ratio

Source	d.f.	SS	MS	*F*-ratio	*P*
(a) Total chlorophyll content					
Leaf type	2	602455	301228	19.385	≤ 0.0001
Position on leaf	1	1183720	1183720	76.177	≤ 0.0001
Type × position	2	96554	48277	3.1068	0.0528
Error	54	839110	15539		
Total	59	2721839			
(b) Chlorophyll *a/b* ratio					
Leaf type	2	294.6	147.3	41.8	≤ 0.0001
Position on leaf	1	32.7	32.7	9.3	0.0036
Type × position	2	83.2	41.6	11.8	≤ 0.0001
Error	54	190.3	3.5		
Total	59	600.8			
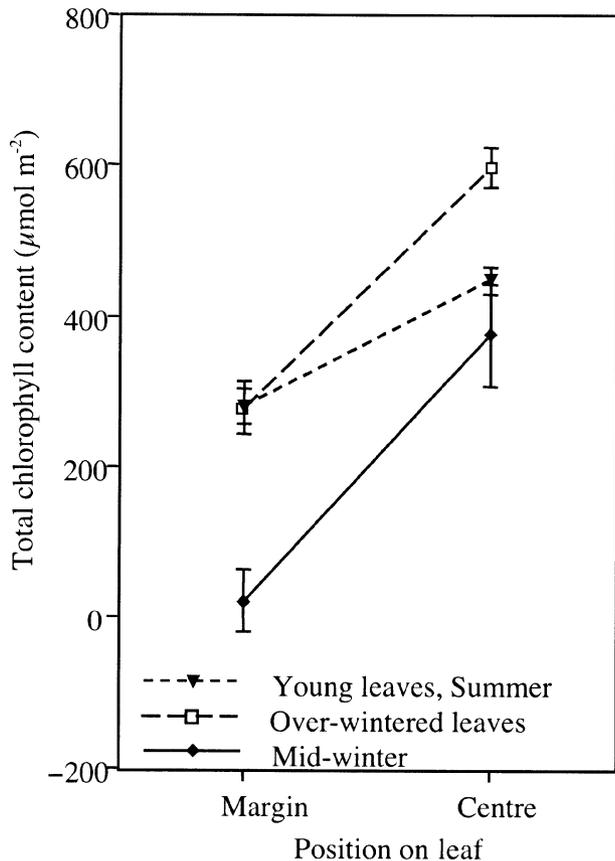
Figure 4. Mean (± SE) total chlorophyll content for margin and leaf centre pixels for leaves that had never frosted, were collected in mid-winter following frost damage, or were recovering from frost.

Predictive indices

Our results suggest that a reliable reflectance index of chlorophyll *a/b* ratio would be a good indicator of stress in these plants. The pattern of chlorophyll *a/b* ratio across the leaf recovers in subsequent seasons suggesting that *a/b* ratio may be highly sensitive to otherwise invisible changes in plant status. We know that chlorosis and red pigmentation are common in evergreen species (Ottander *et al.* 1995), including many exposed to cold winter or night temperatures. Thus, a reflectance-based index of *a/b* ratio could have broad applicability for detecting plant stress.

To some extent the interpretation of our results, particularly the *a/b* ratio results, is limited by the logistical aspects of using leaf discs to develop predictive indices. Our spectrophotometric assessment of chlorophyll *a/b* ratio involved leaf extracts containing other pigments, including anthocyanins. To the extent that anthocyanins and chlorophyll *a/b* ratio are correlated (compare Figs 5 & 7), our index may be either a direct predictor of *a/b* ratio or may be indirectly correlated via a correlation with anthocyanin content. Whether direct or indirect, the correlation itself is robust.

The concentrations of chlorophylls *a* and *b* were strongly correlated with features in the reflectance spectra at 726 and 731 nm, respectively, whereas the chlorophyll *a/b* ratio was best predicted by reflectance at 696 nm. These wavelengths are beyond the absorption spectra of both chlorophylls (Lichtenthaler 1987) and anthocyanins (Pietrini *et al.* 2002), but are within the spectral region where fluorescence from chlorophyll *a* is affected by variation in photosystem structure associated with differences in relative proportions of chlorophylls *a* and *b* (Gilmore, Itoh & Govindjee 2000, Siebke, unpublished). Recently, Katharina Siebke (unpublished) used the same CASI spectrographic imager as in the present study to record room temperature chlorophyll *a* fluorescence spectra from barley wild type and a mutant that lacked chlorophyll *b* (*chlorina f2*). The fluorescence spectra of the wild type showed minor and major peaks centred at 685 and 740 nm and separated by a trough with a minimum near 696 nm. The fluorescence spectra of the *chlorina f2* mutant lacked the trough (Siebke, unpublished). Using a streak camera spectrograph, Gilmore *et al.* (2000) reported a similar fluorescence spectra from the barley wild type, and also found that the fluorescence spectrum from 685 to 740 nm was sensitive to variation in the chlorophyll composition of *chlorina f2* and *f104* mutants (Gilmore *et al.* 2000). Thus, the strong correlations identified in the present study between reflectance features at 696, 726 and 731 nm and the chlorophyll *a/b* ratio, and the concentrations of chlorophylls *a* and *b*, respectively, probably occur because of subtle changes in the fluorescence spectra. We propose that fluorescence characteristics may enable remote measurement of chlorophylls when their absorption features are obscured by protective pigments.

The indices also assume homogeneous distribution of pigments between leaf surfaces; however, our data indicated that differentiation in pigmentation increased with exposure to direct light (Fig. 1). If we could examine the patterns of chlorophyll *a/b* ratio for the exposed and shaded surfaces of the leaf independently we might find an even more pronounced gradient of *a/b* ratio from centre to margin on the exposed side and a reduced decline on the shaded side of the leaf. Finally, the size of the leaf discs is likely to have strongly influenced the error in our predictive equations. Although the leaf discs were less than 1 cm^2 and were sampled from visually homogeneous regions of the leaf, even these small discs represented ~1800 pixels in the reflectance image. The scale of spatial variation demonstrated here makes very clear that a leaf cannot be treated as a uniform chlorophyll source.

The indices developed here to predict chlorophyll contents are likely to be quite species specific. In contrast to studies whose objective is to develop general indices of chlorophyll content for remote sensing or multiple species applications, we were specifically interested in developing the best fitting equations to predict chlorophyll content relative to frost damage. Previous studies have produced
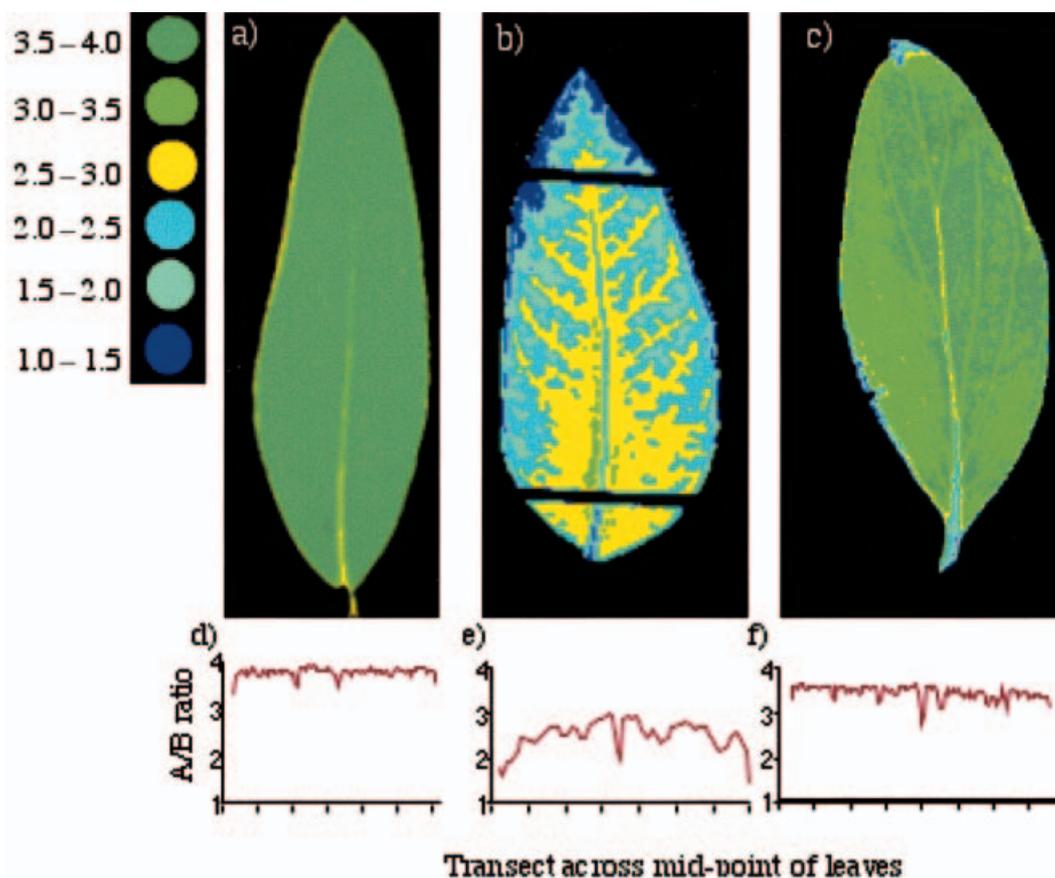
Figure 5. Spatial variation in chlorophyll *a/b* ratio as estimated from regression indices for representative leaves that (a) had never been subject to frost; (b) were collected mid-winter following frost damage; or (c) were recovering from frost damage. (d–f) transects of chlorophyll *a/b* ratio taken across leaf surface taken at approximately the centre of the leaf, perpendicular to the mid-vein.

indices for chlorophyll content using reflectance bands at lower wavelengths (but see Sims & Gamon 2002). The failure of these bands to effectively predict chlorophyll content in *E. pauciflora* may reflect particular aspects of the leaf surface or the complicating influence of other pigments such as anthocyanins. Alternatively, reflectance in the 685 nm region may be insensitive to moderate or high chlorophyll content because of saturation effects and effects of chlorophyll fluorescence on the reflectance signal (Gitelson, Buschmann & Lichtenthaler 2000). Indices that rely on bands on the edge of the chlorophyll absorption and fluorescence feature region may be more effective predictors of chlorophyll content (e.g. Gitelson & Merzlyak 1996; Gitelson, Buschmann & Lichtenthaler 1998; Datt 1999). Notably, the *Eucalyptus*-based index (Datt 1999) performed much better on our leaves than indices generated for a range of temperate deciduous species (Blackburn 1998b).

Other studies have used the position of the red-edge feature of chlorophyll reflectance as an indicator of chlorophyll content (see References in, Gitelson & Merzlyak 1996; but see, Richardson *et al*. 2001; Sims & Gamon 2002). The bands we used for determination of chlorophyll *a* and *b* (and total) are at the extreme reported range of the red-edge, and generally fell beyond the deflection point of the red-edge feature for our leaves (between ~680 and 720 nm). Variation in location of the red-edge can depend on species and condition of the plants (Blackburn 1998b). Rock, Hoshizaki & Miller (1988) found that pollution led to an increase in *a/b* ratio associated with a shift in the red-edge toward blue wavelengths. The index of chlorophyll *a/b* ratio developed here was located much closer to the red-edge than were the other indices of pigment content and therefore is likely to be indicative of relative shifts in the feature. However, in the present study, shift of the red-edge toward bluer wavelengths was associated with a decrease of the chlorophyll *a/b* ratio, a shift in the opposite direction to that found by Rock *et al*. (1988). Our results suggest that the red-edge position may be correlated with a range of shifts in pigment content and thus may not be the most reliable predictor of total chlorophyll content.

CONCLUSIONS

Spatial patterning of stress response at the leaf level has previously been shown using chlorophyll fluorescence. Like the frost effects demonstrated here, effects of heat
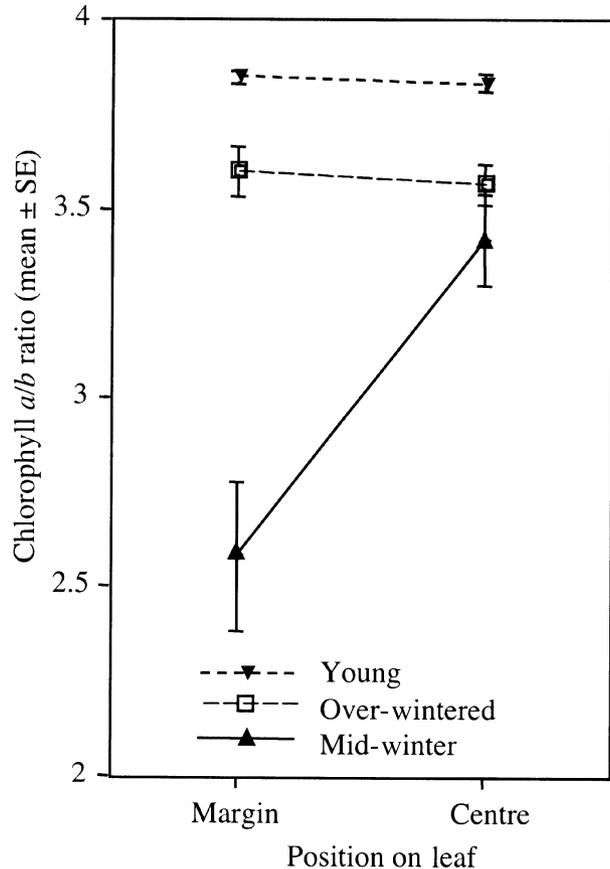
Figure 6. Mean ($\pm$ SE) chlorophyll *a/b* ratio for margin and leaf centre pixels for leaves that had never frosted, were collected in mid-winter following frost damage, or were recovering from frost.

and drought stress on fluorescence were most pronounced at leaf margins (Lang *et al*. 1996). To the best of our knowledge, this is the first time spatially explicit reflectance imaging has been used to assess leaf-level variation in physiology. The patterns of pigment distribution in response to freezing are consistent with previous work on low temperature, chilling or frost effects, but the extent of spatial patterning within a single leaf is perhaps more marked than one might have predicted. Clearly, spatial variation in the freezing process itself has significant impacts on leaf, and therefore whole canopy, physiology.

The results also raise several interesting questions regarding this spatial patterning: Is it consistent between sides of the leaf? Is it likely to be more pronounced in leaves that receive early season frosts (as in the present study) than in leaves that have acclimated to cold? What is the function of the antennae chlorophylls in a condition of cold-induced chlorosis if the reaction-centre core has been damaged? Are these pigments being stored, or are they actively functioning to dissipate excess light energy? What is the specific role of the anthocyanins in frost protection, and how best can reflectance from anthocyanins be unmixed from that of the chlorophylls? and of course, how general is this pattern across the wide range of evergreen species that are regularly exposed to subfreezing temperatures?

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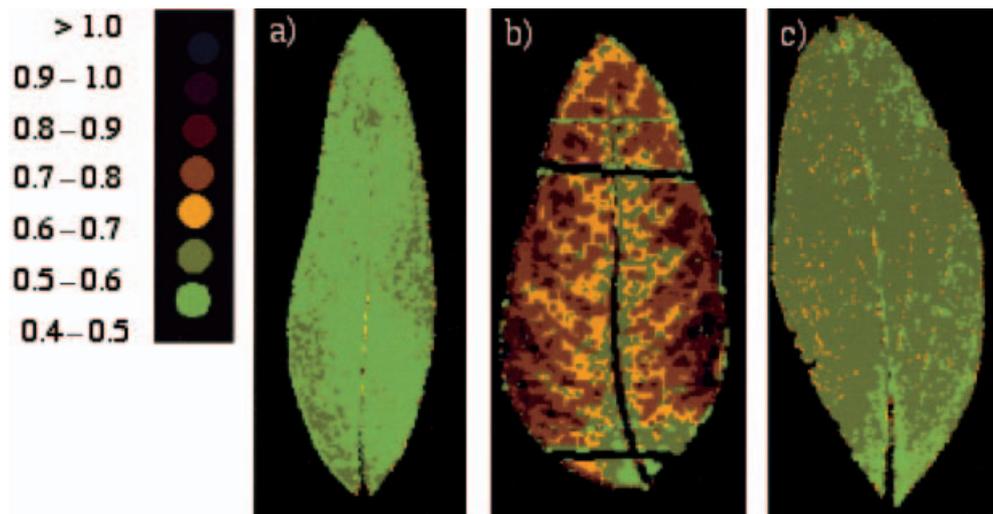

Figure 7. Spatial variation in anthocyanins as estimated from $R_{Red(650)}/R_{Green(550)}$, modified from the broadband index used by Gamon & Sufus ($R_{red(600–700)}/R_{green(500–600)}$, Gamon & Surfus 1999), for representative leaves that (a) had never been subject to frost; (b) were collected in mid-winter following frost damage; or (c) were recovering from frost damage.

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