



RESEARCH PAPER

Insights on the development, kinetics, and variation of photoinhibition using chlorophyll fluorescence imaging of a chilled, variegated leaf

Sander W. Hogewoning* and Jeremy Harbinson

Wageningen University, Department of Plant Sciences, Horticultural Production Chains Group, Wageningen, The Netherlands

Received 26 May 2006; Revised 19 September 2006; Accepted 26 September 2006

Abstract

The effect of chilling on photosystem II (PSII) efficiency was studied in the variegated leaves of *Calathea makoyana*, in order to gain insight into the causes of chilling-induced photoinhibition. Additionally, a relationship was revealed between (chilling) stress and variation in photosynthesis. Chilling treatments (5 °C and 10 °C) were performed for different durations (1–7 d) under a moderate irradiance (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The individual leaves were divided into a shaded zone and two illuminated, chilled zones. The leaf tip and sometimes the leaf base were not chilled. Measurements of the dark-adapted F_v/F_m were made on the different leaf zones at the end of the chilling treatment, and then for several days thereafter to monitor recovery. Chilling up to 7 d in the dark did not affect PSII efficiency and visual appearance, whereas chilling in the light caused severe photoinhibition, sometimes followed by leaf necrosis. Photoinhibition increased with the duration of the chilling period, whereas, remarkably, chilling temperature had no effect. In the unchilled leaf tip, photoinhibition also occurred, whereas in the unchilled leaf base it did not. Whatever the leaf zone, photoinhibition became permanent if the mean value dropped below 0.4, although chlorosis and necrosis were associated solely with chilled illuminated tissue. Starch accumulated in the unchilled leaf tip, in contrast to the adjacent chilled irradiated zone. This suggests that photoinhibition was due to a secondary effect in the unchilled leaf tip (sink limitation), whereas it was a direct effect of

chilling and irradiance in the chilled illuminated zones. The PSII efficiency and its coefficient of variation showed a unique negative linearity across all leaf zones and different tissue types. The slope of this curve was steeper for chilled leaves than it was for healthy, non-stressed leaves, suggesting that the coefficient of variation may be an important tool for assessing stress in leaves.

Key words: *Calathea makoyana*, chilling injury, chlorophyll fluorescence imaging, down-regulation, F_v/F_m , heterogeneity, photoinhibition, photosystem II, PSII efficiency, starch accumulation.

Introduction

Many plants of tropical origin suffer from various physiological disorders when exposed to low temperatures (Lyons, 1973; Levitt, 1980). The temperatures that provoke these disorders vary depending on the species or variety, the duration of exposure, and the physiological process under consideration. It seems unlikely that there is only one physiological basis for chilling sensitivity, rather that different syndromes will be important under different conditions of chilling. Photosynthesis is susceptible to chilling, especially when low temperatures are combined with exposure to physiologically significant levels of irradiance (e.g. Powles *et al.*, 1983; Allen and Ort, 2001), although the effects of dark chilling on photosynthesis, including photosystem II (PSII) efficiency, are well known (e.g. Bodner and Larcher,

* To whom correspondence should be addressed. E-mail: sander.hogewoning@wur.nl

Abbreviations: F_v/F_m , ratio of variable to maximum fluorescence—the relative quantum efficiency for electron transport by photosystem II if all photosystem II reaction centres are open; F_m , maximum fluorescence yield in dark-adapted tissue; F'_m , maximum fluorescence yield in light-adapted tissue; F_o , minimum fluorescence yield; F_s , fluorescence in steady-state; PSI, photosystem I; PSII, photosystem II; Q_a , primary quinone acceptor of photosystem II; Φ_{PSII} , the relative quantum yield of photosystem II electron transport.

1989; Tjiskens *et al.*, 1994; Allen and Ort, 2001). In general, chilling in the light can produce damage to both PSI (e.g. Havaux and Davaud, 1994; Terashima *et al.*, 1998) and PSII, and, therefore, a loss of the photochemical efficiency measured under strictly light-limiting conditions (i.e. the maximum quantum yield for electron transport). In most cases, chilling in the dark produces a loss of photosynthetic capacity without any loss of light-limited efficiency, at least in the short term (e.g. Kingston Smith *et al.*, 1997; Allen *et al.*, 2000). Nonetheless, the quantum yield for PSII electron transport measured by chlorophyll fluorescence (Φ_{PSII}) has been reported as being sensitive to dark chilling to the extent that it has been used as a general indicator of chilling injury (e.g. van Kooten *et al.*, 1992), in much the same way as electrolyte leakage. When damage does occur to the electron transport abilities of PSI and PSII *in vivo*, it is not often known to what extent this damage is a primary event, or a result of an overall restriction of cell metabolism and assimilate transport due to low temperatures. This is an important question as strategies based on improving the low temperature competence of photosynthesis by means of protecting PSI and PSII from primary damage would be of questionable value if the damage to PSI and PSII were to be secondary in nature.

Damage of some kind is a common response to stress. However, the spatial pattern and variability of this damage within apparently homogeneous tissue types, and recovery from it, have not often been subjected to a detailed examination. There is a suggestion that the variability of the Φ_{PSII} /irradiance response at moderate irradiances is increased when damage occurs (Kingston Smith *et al.*, 1997; Fig. 2), although this has not yet been subject to systematic investigation. Use can be made of changes in variation; for example, Schouten *et al.* (2004) have developed models to predict the shelf-life of ripening fruits that use changes in both the mean values and the variance of colour measurements. A more complete understanding of how stresses producing photoinhibition affect not only the mean value of Φ_{PSII} but also its variance is important because measurements of Φ_{PSII} are widely used as a means with which to assess stress, ripening, senescence, etc.

The experimental subject for this study was *Calathea makoyana*, a tropical herbaceous monocotyledon in the family Marantaceae; it is a shade-demanding C_3 plant. The Marantaceae contains many species with leaves that are naturally variegated. *Calathea makoyana* leaves have distinct markings, due principally to the distribution of chlorophyll, which results in lobed, dark green regions extending from the mid vein and sometimes merging with the dark green margin of the leaf. Within interlobate regions, chlorophyll is concentrated around the lateral and commissural veins, producing a fine, dark green network with pale green tissue in between (Fig. 3).

Fluorescence imaging technology allows the photosynthesis of this complex type of leaf to be conveniently

explored for the first time. During cultivation, leaves of *C. makoyana* exposed to light stress display variation in the degree of chlorosis from one type of leaf region to another. One aim of this study was to determine if the regions of these leaves differed in their sensitivity to photoinhibition. Chlorophyll fluorescence imaging was also used to explore how chilling of leaves of this species in either light or darkness affected the efficiency and variance of PSII electron transport. The application of different treatments (chilled in light, chilled in the dark, unchilled leaf tip and base) on a single leaf by dividing the leaf into different zones allowed exploration of whether the chilling treatment of a leaf zone affects neighbouring leaf zones. This method enabled us to reveal if chilling-induced photoinhibition is either a direct effect of low temperature, possibly in combination with light, or due to secondary processes (or a combination of both). Additionally, an investigation was carried out to determine if variation in photosynthesis over a leaf can be useful as tool to assess severity of stress effects. We consider that the patterns of damage and recovery observed in a subject with such an overtly heterogeneous leaf organization will have implications for the interpretation of images of PSII efficiency (and related parameters) in general.

Materials and methods

Plant material

Plants of *C. makoyana* were grown in a peat-based compost in pots in a greenhouse. At least 2 weeks prior to the experimentation, some plants were transferred to a growth cabinet. In the cabinet, plants were grown under an irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) provided by warm white fluorescent tubes, with a 16 h photoperiod. Temperatures were maintained at 25 °C during the day and at 20 °C during the night, and the relative humidity was 80%. The plants were watered via a dripping system with a 0.5 g l^{-1} solution of fertilizer (10+20+20 NPK) daily. Under these conditions, growth was vigorous for a plant of this type.

Chilling treatments

All experiments were performed on attached, healthy, and fully expanded leaves. Prior to each treatment, a dark-adapted F_v/F_m control image (method described below) was made to verify the leaf's health. Any leaves with an F_v/F_m image which by visual assessment had many pixels with a value <0.75 , or which had anything worse than small blemishes in the F_v/F_m image, were rejected. The leaf was chilled by pressing it against the surface of a nickel-plated brass block which had internal channels through which cold water was circulated. The leaf was maintained in contact with the block by means of a sheet of transparent bubble-plastic packing material, which also served to insulate the leaf-block combination. A sheet of transparent glass was placed on the bubble-plastic in order to hold the leaf firmly in contact with the chilling block without damage. As a control, a leaf was pressed on the chilling block for 7 d and not chilled. Furthermore, to verify that the loss of F_v/F_m was not altered by fixing the leaf to the block, a whole plant was chilled to 10 °C in a temperature-controlled room for 2 d. Leaves of this plant were held in a horizontal position with two leaves exposed to the same irradiance as the leaves chilled on the block, and two leaves shaded by black plastic. The effects of these treatments on F_v/F_m

were the same as observed in leaves chilled on the metal block. The irradiance for leaves chilled on the metal block was provided by means of a bank of twelve 55 W lamps (Philips type PL-L 840/4p TL) and the irradiance was $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at the leaf surface with a 16 h photoperiod.

Leaves were placed on the chilling block in such a way that the leaf tip was irradiated but remained unchilled. A strip of black plastic in the middle of the leaf provided a transverse chilled but unirradiated zone, which was flanked by illuminated chilled zones. In some of the treatments, the base of the leaf was chilled and in some not (Fig. 1A, B); this depended on leaf-size. By this method, the different effects of temperature/light combinations on any chilling-induced loss of F_v/F_m and its ability to recover could be examined. The chilling treatments were performed for periods of 1, 2, 3, 4, and 7 d, at 5 °C and 10 °C, and each treatment was repeated at least twice. The temperature of the room in which the chilling treatments were applied was 23 °C. The leaf tip temperature was measured using both thermocouples attached to the leaf and a Fluke 80 T-IR infrared temperature probe at 5 mm distance from the leaf. The greater part of the leaf tip remained at room temperature, with only a 5 mm wide strip adjacent to the chilling block having a lower temperature. This area was excluded for the data presented herein.

At the end of each treatment, chlorophyll fluorescence images of the leaf were made after a period of 30 min dark adaptation to examine reduction of the F_v/F_m measured. Plants were then returned to the growth cabinet. Further measurements of F_v/F_m were made at intervals over the following days to record the speed and degree of recovery of F_v/F_m over the leaf. Recovery measurements were continued until the degree of recovery of F_v/F_m stabilized.

Chlorophyll fluorescence imaging

Fluorescence images of leaves were made with two different imaging systems which had different properties. One series of images was produced using a PSI Fluorcam 700MF chlorophyll fluorescence imaging system (PSI, Brno, Czech Republic); Fluorcam v. 3.5 software was used to control the imaging system and to process the images. To obtain the Φ_{PSII} images, fluorescence images produced during measuring light flashes were digitized. Those images captured in the absence of any actinic light (i.e. 'darkness'; the average irradiance produced by the measuring light flashes was $<0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$, an intensity too low to produce any significant perturbation of the photosynthetic machinery) were averaged to produce an image of the F_o relative fluorescence yield, and those images made during the saturating light pulse ($2500 \mu\text{mol m}^{-2} \text{s}^{-1}$) were averaged to produce an image of the F_m relative fluorescence yield. From the two images, the computer calculated the F_v/F_m image pixel by pixel using the expression $(F_m - F_o)/F_m$. Additionally, a series of steady-state Φ_{PSII} images in response to a step-wise increase of irradiance ($30\text{--}260 \mu\text{mol m}^{-2} \text{s}^{-1}$) was made of a healthy leaf of the same species, in order to compare Φ_{PSII} heterogeneity over the leaf between chilled and non-chilled (non-stressed) leaves (three repetitions). The Φ_{PSII} images were calculated using $(F'_m - F_s)/F'_m$. The Fluorcam camera was used to image large areas of leaf. Two images were sufficient to record the entire area of the *C. makoyana* leaves and, to produce an image of the entire leaf, these two images were stitched together using bitmap manipulation software.

A second series of images was made using a laboratory-built imaging system, based upon that described by Genty and Meyer (1995) (Vanacker *et al.*, 1998). This system produced images with the same number of pixels as the Fluorcam (512×512), but the camera was fitted with a 135 mm close-focusing objective (Vivatar). This objective allowed semi-macro images of the leaf to be made (area imaged $\sim 4 \text{ cm} \times 4 \text{ cm}$) and thus allowed smaller features and phenomena on the leaf to be resolved. To gain insight into the Φ_{PSII} distribution all over the leaf, ~ 10 images were made with this system and assembled into

a mosaic (not shown). To estimate the noise floor in this system, the fluorescence from a fluorescent reference disc present in each image was used to produce pseudo- Φ_{PSII} values whose coefficient of variation (Smith, 2003) will be the noise floor of the imaging system. The fluorescent reference discs are used to normalize the fluorescence recorded in the F_o and F_m images, so they have identical mean greyscale values in the final images. To produce the pseudo- Φ_{PSII} images, the same block of pixels was copied from each of the matching F_o and F_m images, and these subsamples were converted to two numerical arrays using ImageJ (<http://rsb.info.nih.gov/ij/>). Using Octave (www.octave.org), the array corresponding to the F_m image was then increasingly offset by successively adding constants to each array element, and a pseudo- Φ_{PSII} array was calculated using the F_o and offset F_m arrays. The mean and the coefficient of variation of these arrays were then calculated.

Carbohydrate and chlorophyll analysis

The effect of chilling and irradiance on the levels of glucose, fructose, sucrose, and starch in the various tissue regions of the leaf in the chilled zone and the unchilled adjacent leaf tip was investigated as follows. Leaves were placed on the chilling block in such a way that approximately half the leaf area towards the leaf tip remained unchilled, and the entire leaf surface was illuminated (Fig. 1C). All other conditions were similar to those of the experiments described above. The leaves were chilled for 4 d at 5 °C. Prior to the chilling treatment, leaf discs of 9 mm in diameter were cut from the unchilled tip and the chilled base, on one side of the main vein. Directly after the chilling treatment (at the end of the 8 h dark period), discs were cut in a similar way on the other side of the main vein. The discs were cut from the green lobate regions and the interlobate areas where the chlorophyll was largely confined to regions around the lateral and commissural veins and where the chlorophyll concentration per unit area of leaf is therefore lower, and analysed separately. The leaf tissue samples, of which each contained five discs, resulting in a total of 20 discs cut on each side of the main vein, were weighed and stored at -20 °C after having been frozen in liquid nitrogen.

Carbohydrate concentrations were determined using an HPAEC-PAD (Dionex Corporation, Sunnyvale, CA, USA) with a Dionex CarboPac PA 10 column (2 mm diameter) and a Dionex PED-2 detector, by first extracting the soluble carbohydrates, and then enzymatically hydrolysing starch.

Chlorophyll content was analysed separately for the green lobate leaf regions and the interlobate regions from three healthy, fully expanded leaves. Chlorophyll was extracted from shredded leaf discs in dimethylformamide (in the dark, for 12 d at 5 °C; five leaf discs per tissue type per leaf, measurements per leaf in duplicate). The chlorophyll content was analysed spectrophotometrically and calculated as in Wellburn (1994).

Results

Effects of chilling treatments on PSII photochemical yield and its speed and degree of recovery

The effect of the chilling treatments on F_v/F_m was clearly different for the leaves or leaf zones chilled in the dark and those chilled in the light. Leaves or leaf zones chilled in the dark did not show any reduction in mean F_v/F_m , regardless of chilling temperature or chilling duration, whereas when chilled in light they showed an obvious reduction in mean F_v/F_m . Even chilling at 10 °C for a period as short as 1 d resulted in a loss of mean F_v/F_m in the illuminated chilled zones (Figs 2, 4A). In Fig. 2, it is clearly visible that even

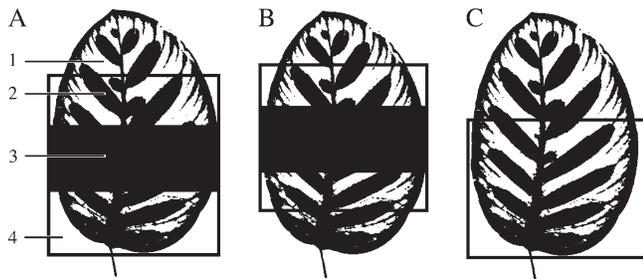


Fig. 1. Schematic diagram of *Calathea makoyana* leaves on the chilling block, two with a shaded zone and an unchilled leaf tip (A, B), of which one has a chilled leaf base (A), and one has an unchilled leaf base (B). (C) A leaf with a large unchilled tip and no shaded zone. The numbers represent an interlobate leaf region (1), a lobate leaf region (2), the shaded zone on the leaf (3), and the metal chilling block (4).

after a chilling treatment of 10 °C for 7 d, the shaded zone was, and remained, unaffected, whereas the chilled, illuminated zones suffered a conspicuous loss of mean F_v/F_m .

A control leaf which was pressed on the chilling block for 7 d and illuminated without being chilled showed a slight reduction of mean F_v/F_m directly after the treatment (<4% less than the mean value before the treatment, whereas the smallest loss of mean F_v/F_m measured after a 1 d chilling treatment at 10 °C was 16%), and after 1 d of recovery mean F_v/F_m was completely restored. Combined with the results obtained for leaves chilled in air (see Materials and methods), this indicates that the considerable and sometimes persistent reduction of mean F_v/F_m of chilled illuminated leaf zones cannot be attributed to mechanical or physical stress of attaching the leaf to the chilling block.

The severity of loss of mean F_v/F_m and the speed and degree of recovery of the leaf zones chilled in light were dependent upon the duration of the chilling treatment. The longer its duration, then the lower were the mean values of F_v/F_m measured 30 min after the treatment (Figs 2, 4A), and the slower was the speed of recovery (Figs 2, 5). If the chilling period was ≤ 2 d, the decrease of mean F_v/F_m was fully reversible, with a recovery half-time of <1 d (Fig. 5). For leaves chilled for a longer period, mean F_v/F_m did not fully recover after every treatment. Photoinhibition ceased to be always fully reversible if the mean F_v/F_m had dropped below a value centred at ~ 0.4 (Fig. 5). In Figs 4A and 5, it is clearly visible that the effect of chilling temperature is negligible (at least for the two temperatures used) compared with the effect of the duration of the treatment.

Photodamage and necrosis after chilling in the light

In the chilled leaf zones that did not fully recover from chilling-induced photoinhibition, visual symptoms of injury developed during the days after rewarming. For a chilling period of 3 d or 4 d, these symptoms were limited to reduced chlorophyll concentration (bleaching) and the occasional

development of small necrotic lesions in the interlobate (Fig. 1, zone 1) leaf regions, where chlorophyll is largely restricted to cells adjacent to the lateral and commissural veins. Leaves chilled for 7 d developed a more general chlorosis, and sometimes developed large necrotic spots or necrosis of larger parts of the chilled illuminated leaf zones during the first week after the treatment (Fig. 3).

For leaves chilled for ≥ 3 d, and which therefore displayed permanent damage to PSII, the degree of recovery was more complete for the dark green lobate (Fig. 1, zone 2) regions on the leaf than for the interlobate (Fig. 1, zone 1) regions (Fig. 2).

Effect of chilling on the unchilled leaf tip

Chilling of the leaf also resulted in changes in the illuminated unchilled leaf tip. Whereas the chilling treatments caused no loss of mean F_v/F_m on the unchilled zone in the leaf base (treatments as in Fig. 1B, data not shown), the unchilled leaf tip showed a considerable loss of mean F_v/F_m . Preliminary experiments on leaves of other *Calathea* species (*C. insignis*, *C. rufibarba*, and *C. zebrina*) showed the same pattern of response (data not shown). The loss of mean F_v/F_m in the irradiated but unchilled leaf tip region was smaller than it was in the adjacent irradiated and chilled zone (Figs 2, 4), though sometimes nearly as severe. The relationship between the temperature and duration of chilling and the loss of mean F_v/F_m was less consistent than it was for the chilled illuminated leaf zones. Recovery became slower and less complete as the initial loss of mean F_v/F_m increased, and the recovery kinetics curve followed a similar pattern to that of zones chilled in the light (compare Figs 5, 6).

In the unchilled leaf tip of leaves chilled for ≥ 3 d, a brownish coloration occasionally became apparent during the first 2 weeks after the treatment, in the pale green interveinal regions within the interlobate regions of the leaf. This coloration was persistent. As the tip remained turgid and otherwise normal, the coloration was not due to necrosis, and was possibly due to anthocyanin formation. A reduced chlorophyll concentration (bleaching) in the pale green interveinal portions in the interlobate regions was not observed in the leaf tip region, though it was typical when interlobate regions were chilled in the light (Fig. 3). Necrosis never developed in the unchilled tip region, which is in marked contrast to the chilled irradiated zones.

Heterogeneity in PSII efficiency after chilling

The chilling-induced decrease of F_v/F_m was heterogeneous, with a consistently greater loss of F_v/F_m in the interlobate leaf zones, where the average chlorophyll concentration per unit area of leaf is lower (Figs 2, 7B). No systematic differences in F_v/F_m heterogeneity between chilled illuminated leaf zones and the unchilled leaf tip were observed. However, the frequency distributions of F_v/F_m obtained with the two imaging systems differed substantially. The

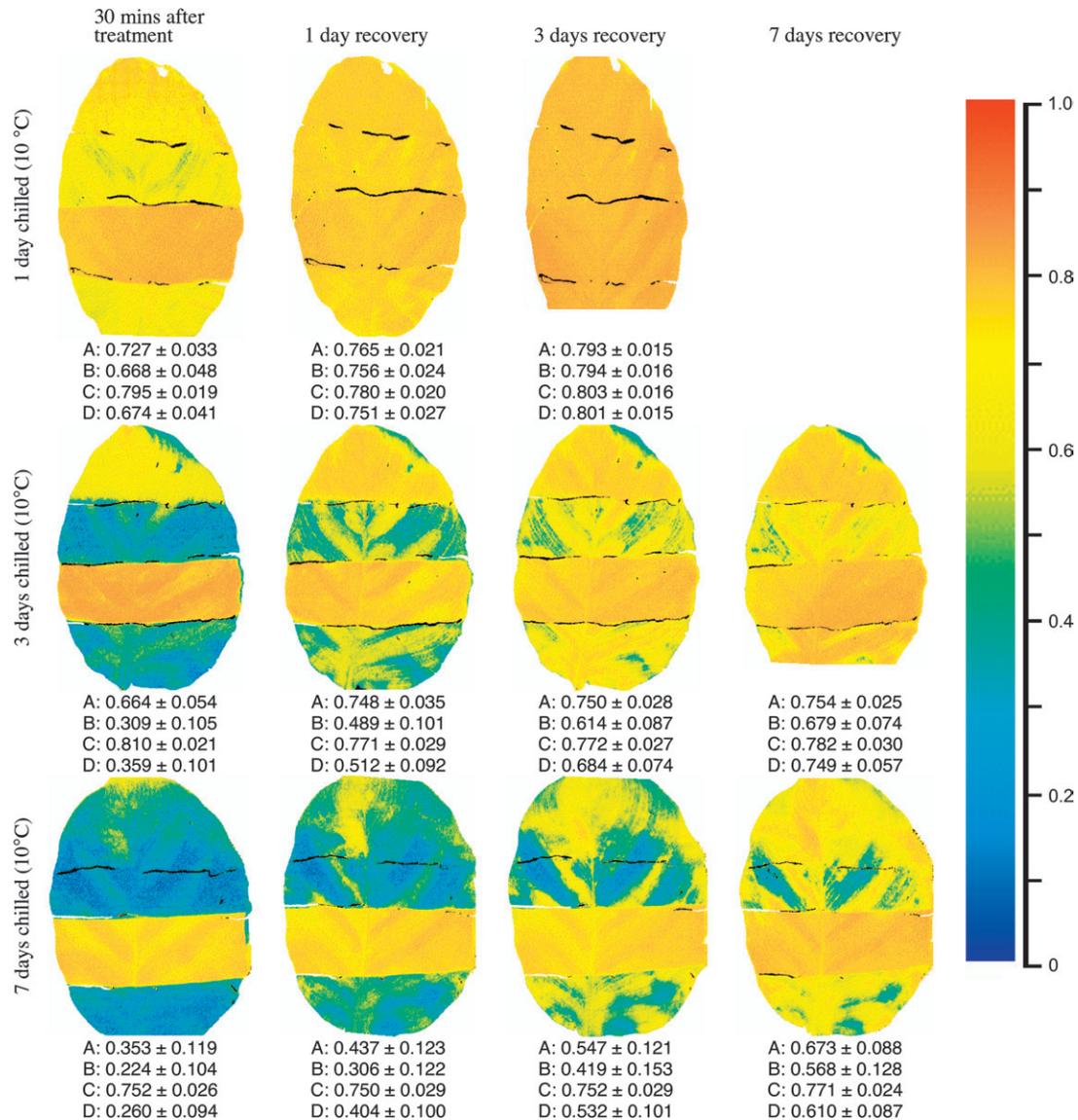


Fig. 2. Images (F_v/F_m) of three *Calathea makoyana* leaves, chilled at 10 °C for 1, 3, and 7 d, respectively; images were taken 30 min after the chilling treatment and after 1, 3, and 7 d recovery. Numbers below the images represent the corresponding mean $F_v/F_m \pm$ SD for the unchilled leaf tip (A), the upper chilled zone (B), the dark chilled zone (C), and the chilled leaf base (D). The leaves were chilled as shown schematically in Fig. 1A. For the calculation of the mean $F_v/F_m \pm$ SD of the leaf chilled for 3 d, the upper right corner of the leaf tip has not been taken into account, as the tissue was damaged prior to the chilling treatment. The (false) colour scale corresponds to the F_v/F_m values over the images.

frequency distributions in the F_v/F_m images recorded with the PSI Fluorcam 700MF chlorophyll fluorescence imaging system (images as in Fig. 2) remained unimodal, regardless of the temperature and duration of chilling (distributions of images with a low mean F_v/F_m did have some noise, see Fig. 7A, row 2). The frequency distributions were negatively skewed (i.e. to lower values of F_v/F_m) until leaves had recovered to almost the values of F_v/F_m found in the control leaf. Analysis of the more detailed images obtained from the laboratory-built imaging system revealed a bimodal frequency distribution for leaf zones which had experienced a considerable loss of mean F_v/F_m (Fig. 7B,

row 2). The bimodal distribution can be accounted for by the distribution of F_v/F_m in the interlobate and lobate regions of the leaves (Fig. 7B, row 2; compare entire image distribution with the distributions from the interlobate and lobate regions of the image). The variation was consistently higher in the F_v/F_m distributions of the interlobate regions than in those of the lobate regions (Fig. 7B). For these more detailed measurements, it is clear that if variation of the F_v/F_m distributions of the two different tissue regions decreases during recovery, the ‘two peaks’ overlap increasingly, until finally displaying a unimodal distribution (Fig. 7B, row 3). For the individual distributions of the

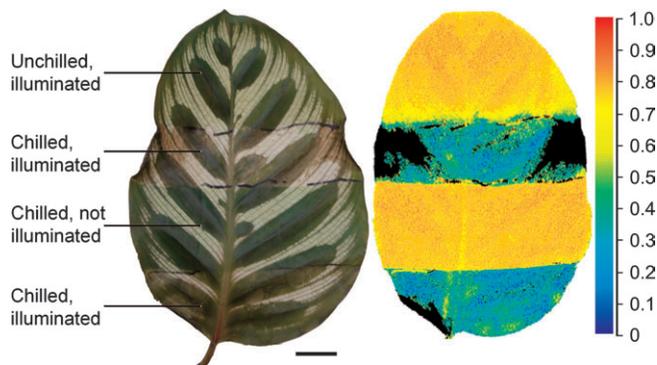


Fig. 3. Digital photo of a *Calathea makoyana* leaf (left), taken after a 2 d recovery period following a chilling treatment for 7 d at 5 °C, as shown in Fig. 1A. The two zones which were chilled in light show obvious chlorosis all over the leaf zones and necrosis at the sides of the leaf zones. An F_v/F_m image (right) of this leaf was taken at the same time, to illustrate that the zone chilled in the dark is unaffected. The leaf tip has already recovered from its initial, moderate loss of F_v/F_m . The (false) colour scale corresponds to the F_v/F_m values over the image. Bar=2 cm.

interlobate and lobate regions of the leaf, no clear trend in the skewness ratio was observed (Fig. 7B). The negative skewness in the distributions of F_v/F_m from images taken with the PSI Fluorcam during recovery (Fig. 7A, row 3) can be accounted for by the greater variation of F_v/F_m in the interlobate leaf region, as can be resolved in the F_v/F_m distributions from images taken with the laboratory-built system (Fig. 7B, row 3).

If the mean value of F_v/F_m remained above 0.5, then its standard deviation was negatively correlated with mean F_v/F_m (Fig. 8, inset). However, if the mean value of F_v/F_m fell below 0.5, then its standard deviation was positively correlated with mean F_v/F_m (Fig. 8, inset). This rather complex relationship between mean F_v/F_m and its standard deviation is greatly simplified if mean F_v/F_m is compared with its coefficient of variation [the standard deviation divided by the mean, which is also the reciprocal of the signal:noise ratio, with 'noise' in this instance being the fluctuation of a signal about its mean value (Smith, 2003)]. This relationship shows a clear linear negative correlation (Fig. 8, circles). The relationship was similar for the chilled leaf zones and the unchilled leaf tip. The steady-state Φ_{PSII} measurements on the healthy, non-stressed leaves show a similar linear correlation; however, the slope is less steep (Fig. 8, squares). This phenomenon can also be seen in both the green lobate regions and the interlobate areas, when they are analysed separately (Fig. 9B, C). This was done using F_v/F_m images taken with the laboratory-built system and manually selecting the interlobate and lobate tissue regions from the chilled illuminated zones and the unchilled leaf tip zone for analysis. The selection and analysis was done using ImageJ. The coefficient of variation is higher for a leaf area containing all tissue types (Fig. 9A) than it is for the separately analysed interlobate and lobate tissue areas of the close-up images (Fig. 9B, C).

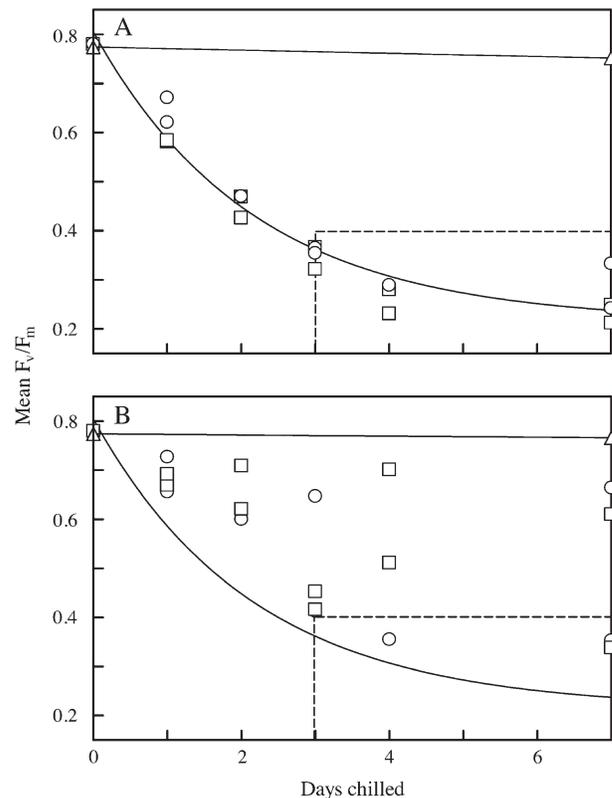


Fig. 4. The effect of duration and degree of chilling on mean F_v/F_m of the chilled illuminated leaf zone (A) and the unchilled leaf tip (B) 30 min after the treatment. Squares correspond to 5 °C treatments, circles to 10 °C, and triangles to 23 °C (control). Each data point refers to a different leaf. The monoexponential (i.e. first order) decay curve from (A) was fitted through combined data sets. The curve is projected on (B), to emphasize that the loss of F_v/F_m in the tip area can be considerable, however never as great as in the chilled zones. The area within the dashed lines is associated with necrosis if it concerns chilled illuminated zones. These data were obtained from images made using the Fluorcam imaging system.

This is to be expected. The variation for all data was above the system noise level (Fig. 9, squares).

Carbohydrate content of the different leaf zones after chilling and chlorophyll content of the different leaf regions

For the chilled leaf region, the glucose, fructose, and sucrose levels increased at most only slightly after the chilling treatment. In the unchilled tip region, the glucose and fructose content was found to have more than doubled and that of sucrose slightly increased after the chilling treatment. The starch level showed a striking increase in the unchilled tip, whereas the increase in the chilled illuminated part of the leaf was at most only slight. In the green lobate regions of the unchilled leaf tip, the starch concentration increased by a factor of 15, and in the interlobate areas the increase was only 7-fold (Table 1).

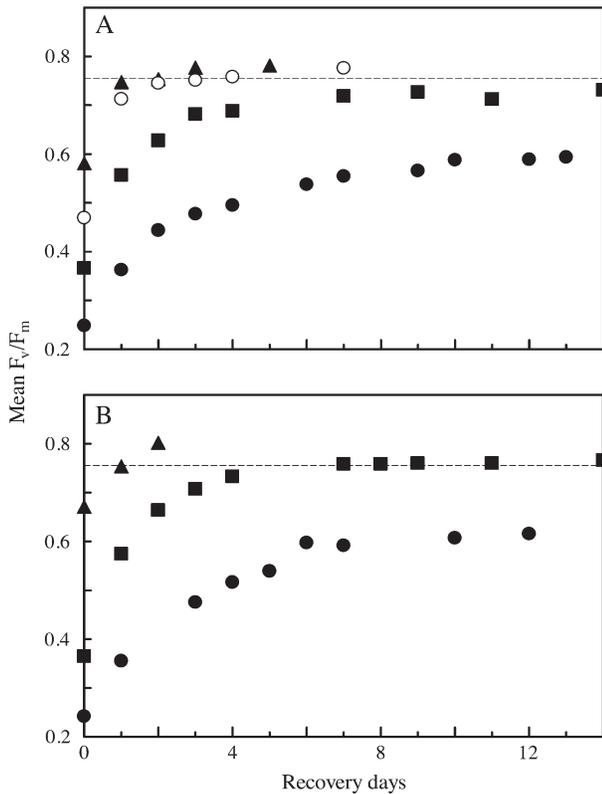


Fig. 5. Recovery of mean F_v/F_m over chilled illuminated leaf zones after chilling treatments at 5 °C (A) and 10 °C (B). Triangles, recovery after a 1 d chilling treatment; open circles, 2 d; squares, 3 d; filled circles, 7 d. The recovery curves shown are consistent with the repetitions (not shown). The dashed lines indicate the F_v/F_m level from which a leaf is considered as completely recovered. These data were obtained from images made using the Fluorcam imaging system.

The total chlorophyll content was 2.7 times greater for the lobate leaf regions (0.90 g m^{-2}) than it was for the interlobate regions (0.33 g m^{-2}).

Discussion

Chilling in the absence of light

Chilling of the *C. makoyana* leaves in darkness results in neither loss of mean F_v/F_m nor any tissue death, even after 7 d of chilling at 5 °C. This indicates that, though *C. makoyana* is a tropical plant, it is fundamentally not very susceptible to chilling injury, in contrast to, for example, *Saintpaulia ionantha* (Bodner and Larcher, 1987), *Phaseolus vulgaris* (Asare-Boamah and Fletcher, 1986), or a tropical cultivar of soya (van Heerden *et al.*, 2004). In *S. ionantha*, 3 °C chilling for as little as 6 h is sufficient to produce severe necrosis and a loss of Φ_{PSII} (Bodner and Larcher, 1989). The absence of any apparent damage to PSII during the dark chilling treatment is significant as it allows the effects of low temperatures alone to be ruled out in causing any injury in the long-term chilling treatments used in this study.

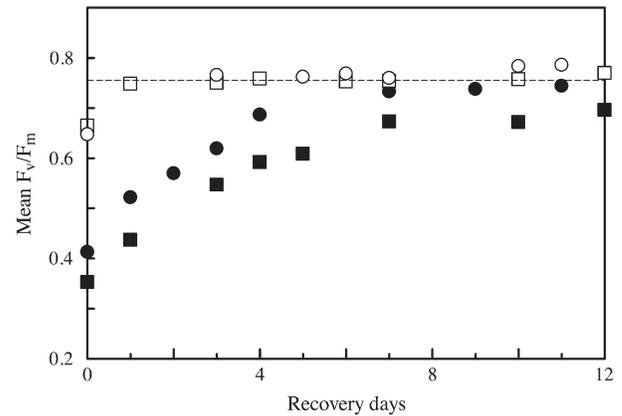


Fig. 6. Recovery of mean F_v/F_m over unchilled illuminated leaf tips after chilling treatments at 10 °C. Circles, recovery after a 3 d chilling treatment; squares, 7 d. Two repetitions of each treatment have been chosen to illustrate that although the initial loss of F_v/F_m after a treatment is very inconsistent in the leaf tip, the speed and degree of recovery after a certain loss of F_v/F_m are similar to those of chilled illuminated tissue (see Fig. 5). The dashed line indicates the F_v/F_m level at which a leaf is considered to be completely recovered. These data were obtained from images made using the Fluorcam imaging system.

Photoinhibition and necrosis due to chilling in the light

In contrast to chilling in the dark, chilling in the light produces a serious reduction of F_v/F_m , implying damage to PSII. The phenomenon of chilling tolerance of F_v/F_m while in the dark and chilling susceptibility of F_v/F_m in the light has been observed in earlier studies, for example on intact cucumber (*Cucumis sativus* L.) leaves (Peeler and Naylor, 1988), but in these reports the chilling was of shorter duration and on less warmth-demanding plants. In contrast to other reports on the effect of temperature on photoinhibition (e.g. Hetherington *et al.*, 1989), in *C. makoyana* the average development of photoinhibition (and post-damage recovery kinetics and capacity) appears to be independent of temperature at the two temperatures used (i.e. 5 °C and 10 °C; Fig. 4A). Although such temperature independence is unusual, it is what theoretically might be expected for a process limited solely by photochemistry. This implies that the physiological component of photoinhibition development, which would be expected to result in temperature sensitivity, has been eliminated at the temperatures used in these experiments. In other regards, it is difficult to compare quantitatively the results obtained with *C. makoyana* with those obtained from other species owing to the different irradiances and temperatures used. In chilled spinach (Somersalo and Krause, 1990) and mango leaves (Sukhvilub *et al.*, 2000), the half-time for the loss of PSII activity was within hours. Even allowing for the lower irradiance used in this investigation, the half-time of 2 d for the loss of F_v/F_m (a drop from ~ 0.8 to 0.5; the decay tends to reach a minimum at an F_v/F_m of ~ 0.2) obtained with the *C. makoyana* leaves (Fig. 4A) indicates a much slower development of photoinhibition.

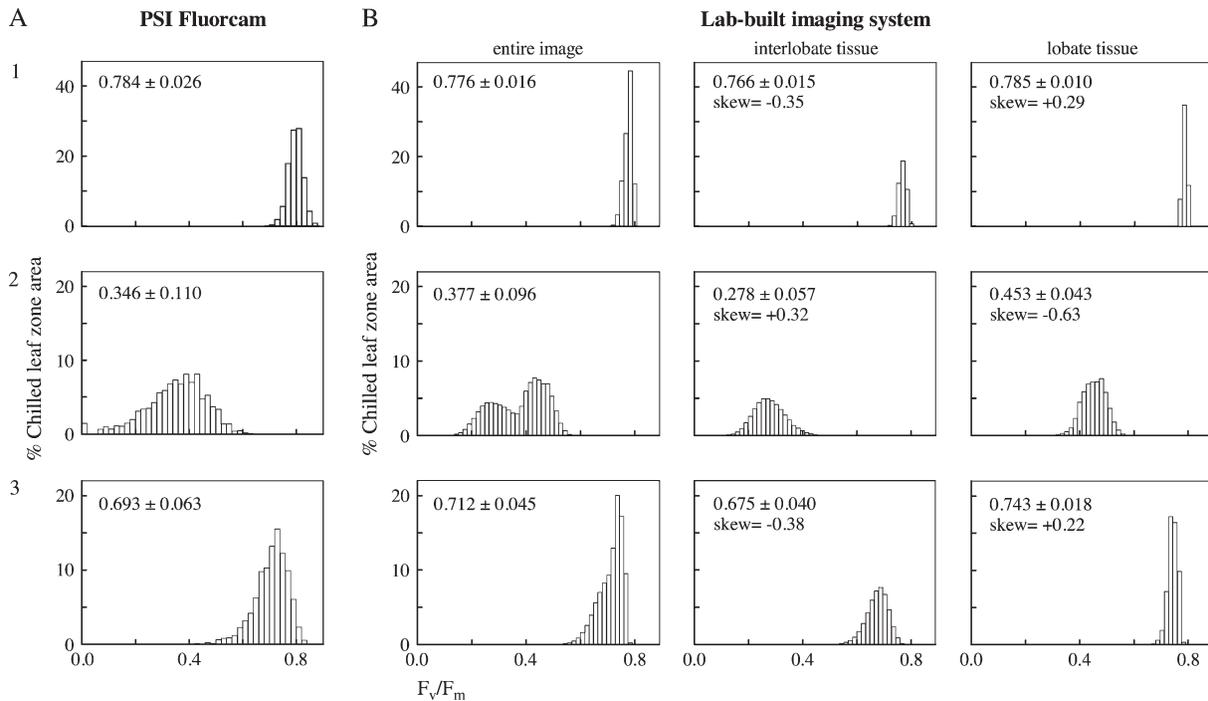


Fig. 7. Frequency distributions of F_v/F_m images from the chilled zones of two leaves, taken before the treatment (row 1), 30 min after a chilling treatment at 10 °C for 3 d (row 2), and after 3 d of recovery (row 3). Mean $F_v/F_m \pm SD$ are indicated. One series of images from one leaf has been taken with the PSI Fluorcam system (A), the other series with the laboratory-built system (B). For the second series (B), the frequency distributions are also given for the interlobate and lobate tissue regions separately, as a percentage of the whole chilled leaf zone area. In these distributions, the skewness ratio is indicated.

In comparison with other reports of recovery from photoinhibition, which usually describe rapid (within hours) and complete recoveries, even from F_v/F_m as low as 0.2 (Somersalo and Krause, 1989; Aguilera *et al.*, 1999; Sukhvirul *et al.*, 2000), the recovery of *C. makoyana* was both slow and became increasingly irreversible once Φ_{PSII} fell below 0.4 (or after at least 3 d of chilling). The slowing of the rate of recovery as the damage increased is also unusual, though shade-adapted *Shorea* seedlings recovered according to a pattern similar to that displayed by *C. makoyana* (Bungard *et al.*, 2000).

Whereas photoinhibition could easily be measured directly after the chilling treatments, visible damage (chlorosis, necrosis) developed during the days after the chilling treatments. Bleaching of chlorophyll as observed in the chilled irradiated zones, especially in the interlobate leaf regions of the *C. makoyana* leaf (Fig. 3), is a well-known effect of chilling in combination with irradiance (e.g. Kratch and Wise, 2000). Necrosis developed in the chilled irradiated leaf zones (Fig. 3), whereas it did not in dark-chilled leaf zones and the leaf tip, which could also develop levels of photoinhibition nearly as severe as found in the chilled leaf regions (Fig. 4). This implies, therefore, that the necrosis was a consequence of chilling in the light and not of chilling, or irradiation leading to photoinhibition, alone. This association of necrosis with chilling in the light on plant leaves, in the absence of chilling damage

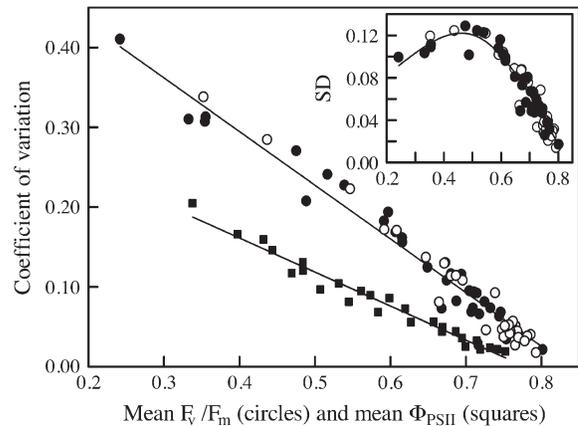


Fig. 8. The relationship between the mean Φ_{PSII} (dark-adapted for circles, steady-state for squares) and their corresponding coefficient of variation and standard deviation (inset). Data were calculated from PSI Fluorcam system images. Circles indicate data from a series of images from leaves chilled for 1, 2, 3, and 7 d at 10 °C, directly after chilling and during their recovery period. Closed circles correspond to data from the chilled zones, open circles to data from the unchilled leaf tip area. Squares indicate data from three healthy, non-stressed leaves, imaged over a range of increasing light intensities.

when treated in the dark, has not been observed in earlier studies. This proves that light-driven processes alone can cause, or at least, initiate damage to, and even total destruction of, leaf tissue under chilling temperatures.

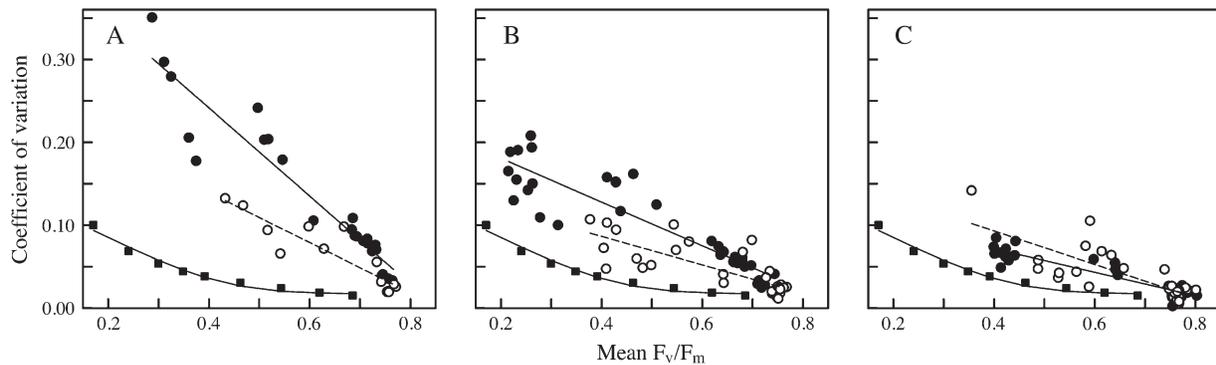


Fig. 9. The relationship between the F_v/F_m mean values and their corresponding coefficient of variation, for both tissue types (A), and the interlobate (B) and lobate (C) tissue regions separately. The leaf was chilled for 3 d at 5 °C as shown in Fig. 1A, and the F_v/F_m images were taken with the laboratory-built imaging system. Data were calculated from images taken 30 min after chilling and during the recovery period. (B) and (C) contain more data points than (A), as several areas had to be analysed per image in order to obtain the data from the two different tissue regions separately. Filled circles (solid lines) correspond to data from the chilled illuminated zones and open circles (dashed lines) to data from the unchilled illuminated leaf tip area. The squares (solid curve) indicate the typical signal:noise ratio developed by the imaging system.

PSII distribution patterns after chilling

The different leaf regions had differing sensitivities to photoinhibition. The greater loss of F_v/F_m in the interlobate leaf regions (where chlorophyll is largely restricted to cells adjacent to the lateral and commissural veins), compared with the chlorophyll-rich lobate regions, may be due to the relatively larger irradiation per unit thylakoid in these areas. Additionally, due to the lower absorption of light in the regions between the commissural veins, the light may be scattered into the chlorophyll-rich tissues immediately surrounding the commissural veins, thus increasing the effective irradiance in the commissural vein tissue and therefore the degree of photoinhibition. The different sensitivity to photoinhibition of leaf regions of *C. makoyana* with different chlorophyll contents and distribution patterns has consequences for the distribution of F_v/F_m within the images. Where the Fluorcam images of photoinhibited leaves show a unimodal distribution (Fig. 7A), the close-up images from the laboratory-built imager show a bimodal distribution (Fig. 7B, row 2), as a result of being able to resolve the two main types of photosynthetic tissue in these leaves adequately. This implies that, in general, a unimodal distribution of Φ_{PSII} values cannot be assumed to reflect a uniformity of tissue or photosynthetic response with that tissue; variability may be present but may not have been resolved.

A difficult question is the underlying cause of the linear relationship between the mean F_v/F_m and its coefficient of variation. The coefficient of variation exceeds that of either the measuring system (Fig. 9) or that produced when Φ_{PSII} in a non-stressed leaf is caused to decrease by increasing the irradiance (Fig. 8). This implies that the increased coefficient of variation is due to the stress, not the instrumentation or the normal behaviour of the leaf. The increase in the coefficient of variation implies that the lower the mean value of F_v/F_m , the relatively more variation there is in F_v/F_m ; this suggests that stress responses are 'noisy'

Table 1. Ratios of increase for glucose, fructose, sucrose, and starch content per mg fresh weight in *Calathea makoyana* leaf discs after a chilling treatment for 4 d at 5 °C (as in Fig. 1C)

The means are the averages of two repetitions.

Leaf disc samples	Glucose	Fructose	Sucrose	Starch
Unchilled tip, lobate	2.1	2.3	1.7	14.5
Unchilled tip, interlobate	3.0	3.4	1.8	6.8
Chilled zone, lobate	1.6	1.3	1.1	1.0
Chilled zone, interlobate	1.3	1.3	1.0	1.6

and possibly during photoinhibition a positive feedback process is acting to increase the relative variability of F_v/F_m , but it is not clear which. As the coefficient of variation is higher for the chilled and thus stressed leaves, than it is for the healthy non-stressed leaves, the coefficient of variation of Φ_{PSII} could be an important tool for revealing the stress history in a leaf.

Differences in the causes of photoinhibition

Although both the illuminated chilled zones and the unchilled leaf tip develop photoinhibition, the underlying causes appear to be different. In contrast to the chilled zone, the carbohydrate content, especially starch, increased significantly in the leaf tip area during the chilling treatment of the leaf (Table 1). As the unchilled base (treatments as shown in Fig. 1B) did not suffer any loss of F_v/F_m , it is likely that transport of assimilates produced in the leaf tip during the chilling treatment was limited by the chilled leaf zone, and, therefore, carbohydrates accumulated in the leaf tip. Assimilate transport can be inhibited strongly under moderate chilling temperatures, but can recover within a few days after rewarming (e.g. Minchin and Thorpe, 1983; Minchin *et al.*, 1983; Sowinski *et al.*, 1998). Goldschmidt and Hubert (1992) showed a decline in the maximum photosynthetic capacity in starch storers, as well as in a

starchless mutant (which only stored hexoses) after preventing assimilate export by girdling leaves of several plant species. Both carbohydrate accumulation and the lack of a direct sink for assimilates produced have been associated widely with inhibition of photosynthesis (e.g. Rufty *et al.*, 1988; Stitt, 1991; de Groot *et al.*, 2003).

If feedback regulation of photosynthetic capacity by sink limitation is responsible for the loss of F_v/F_m in the unchilled leaf tip, the direct reason for the photoinhibition of PSII, although having different underlying causes, may be the same as in the chilled irradiated zones: a more reduced Q_A pool developing in response to a limitation of electron transport even at moderate irradiances. On the one hand, this could be produced by low temperatures, on the other it could have been produced by down-regulation in response to sink limitation of photosynthetic metabolism (Harbinson, 1994, Fig. 16.6).

It is noteworthy that the degree of photoinhibition that developed in the unchilled illuminated leaf tip could be nearly as severe as that which developed in the chilled illuminated zones of the leaf. The presumption of different mechanisms causing photoinhibition for the chilled illuminated zones (a direct effect of cold on the photosynthetic machinery) and the leaf tip (secondary effect), has certain implications for the interpretation of photoinhibition data and breeding strategies to avoid photoinhibition-related damage to photosynthetic tissues. These data imply that a photosynthetic system that is intrinsically capable of functioning at low temperatures will still develop photoinhibition if the other functions required for sustained photosynthetic activity, such as assimilate transport, are sensitive to low temperatures.

References

- Aguilera C, Stirling CM, Long SP. 1999. Genotypic variation within *Zea mays* for susceptibility to and rate of recovery from chill-induced photoinhibition of photosynthesis. *Physiologia Plantarum* **106**, 429–436.
- Allen DJ, Ort DR. 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science* **6**, 36–42.
- Allen DJ, Ratner K, Giller YE, Gussakovsky EE, Shahak Y, Ort DR. 2000. An overnight chill induces a delayed inhibition of photosynthesis at midday in mango (*Mangifera indica* L.). *Journal of Experimental Botany* **51**, 1893–1902.
- Asare-Boamah NK, Fletcher RA. 1986. Protection of bean seedlings against heat and chilling injury by triadimefon. *Physiologia Plantarum* **67**, 353–358.
- Bodner M, Larcher W. 1987. Chilling susceptibility of different organs and tissues of *Saintpaulia ionantha* and *Coffea arabica*. *Angewandte Botanik* **61**, 225–242.
- Bodner M, Larcher W. 1989. Chilling susceptibility of wild *Saintpaulia* species of different altitudinal origin. *Angewandte Botanik* **63**, 501–512.
- Bungard RA, Press MC, Scholes JD. 2000. The influence of nitrogen on rainforest dipterocarp seedlings exposed to a large increase in irradiance. *Plant, Cell and Environment* **23**, 1183–1194.
- de Groot CC, van den Boogaard R, Marcelis LFM, Harbinson J, Lambers H. 2003. Contrasting effects of N and P deprivation on the regulation of photosynthesis in tomato plants in relation to feedback limitation. *Journal of Experimental Botany* **54**, 1957–1967.
- Genty B, Meyer S. 1995. Quantitative mapping of leaf photosynthesis using chlorophyll fluorescence imaging. *Australian Journal of Plant Physiology* **22**, 277–284.
- Goldschmidt EE, Huber SC. 1992. Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose and hexose sugars. *Plant Physiology* **99**, 1443–1448.
- Harbinson J. 1994. The responses of thylakoid electron transport and light utilization efficiency to sink limitation of photosynthesis. In: Baker NR, Bowyer JR, eds. *Photoinhibition of photosynthesis: from molecular mechanisms to the field*. Oxford: BIOS Scientific Publishers, 273–296.
- Havaux M, Davaud A. 1994. Photoinhibition of photosynthesis in chilled potato leaves is not correlated with a loss of photosystem II activity. Preferential inactivation of photosystem-I. *Photosynthesis Research* **40**, 75–92.
- Hetherington SE, He J, Smillie RM. 1989. Photoinhibition at low temperature in chilling-sensitive and chilling-resistant plants. *Plant Physiology* **90**, 1609–1615.
- Kingston Smith AH, Harbinson J, Williams J, Foyer CH. 1997. Effect of chilling on carbon assimilation, enzyme activation, and photosynthetic electron transport in the absence of photoinhibition in maize leaves. *Plant Physiology* **114**, 1039–1046.
- Kratsch HA, Wise RR. 2000. The ultrastructure of chilling stress. *Plant, Cell and Environment* **23**, 337–350.
- Levitt J. 1980. *Responses of plants to environmental stresses*, 2nd edn, Vol. 1. *Chilling, freezing, and high temperature stresses*. New York: Academic Press.
- Lyons JM. 1973. Chilling injury in plants. *Annual Review of Plant Physiology* **24**, 445–466.
- Minchin PEH, Lang A, Thorpe MR. 1983. Dynamics of cold-induced inhibition of phloem transport. *Journal of Experimental Botany* **34**, 156–162.
- Minchin PEH, Thorpe MR. 1983. A rate of cooling response in phloem translocation. *Journal of Experimental Botany* **34**, 529–536.
- Peeler TC, Naylor AW. 1988. A comparison of the effects of chilling on leaf gas exchange in pea (*Pisum sativum* L.) and cucumber (*Cucumis sativus* L.). *Plant Physiology* **86**, 143–146.
- Powles SB, Berry JA, Bjorkman O. 1983. Interaction between light and chilling temperature on the inhibition of photosynthesis in chilling-sensitive plants. *Plant, Cell and Environment* **6**, 117–123.
- Rufty TW, Huber SC, Volk RJ. 1988. Alterations in leaf carbohydrate-metabolism in response to nitrogen stress. *Plant Physiology* **88**, 725–730.
- Schouten RE, Jongbloed G, Tijkskens LMM, van Kooten O. 2004. Batch variability and cultivar keeping quality of cucumber. *Postharvest Biology and Technology* **32**, 299–310.
- Smith SW. 2003. *Digital signal processing. A practical guide for engineers and scientists*. Amsterdam: Newnes.
- Somersalo S, Krause GH. 1989. Photoinhibition at chilling temperature—fluorescence characteristics of unhardened and cold-acclimated spinach leaves. *Planta* **177**, 409–416.
- Somersalo S, Krause GH. 1990. Reversible photoinhibition of unhardened and cold-acclimated spinach leaves at chilling temperatures. *Planta* **180**, 181–187.
- Sowinski P, Richner W, Soldati A, Stamp P. 1998. Assimilate transport in maize (*Zea mays* L.) seedlings at vertical low temperature gradients in the root zone. *Journal of Experimental Botany* **49**, 747–752.

- Stitt M.** 1991. Rising CO₂ levels and their potential significance for carbon flow in photosynthetic cells. *Plant, Cell and Environment* **14**, 741–762.
- Sukhvibul N, Whiley AW, Smith MK, Hetherington SE.** 2000. Susceptibility of mango (*Mangifera indica* L.) to cold-induced photoinhibition and recovery at different temperatures. *Australian Journal of Agricultural Research* **51**, 503–513.
- Terashima I, Noguchi K, Itoh-Nemoto T, Park YM, Kubo A, Tanaka K.** 1998. The cause of PSI photoinhibition at low temperatures in leaves of *Cucumis sativus*, a chilling-sensitive plant. *Physiologia Plantarum* **103**, 295–303.
- Tijskens LMM, Otma EC, van Kooten O.** 1994. Photosystem II quantum yield as a measure of radical scavengers in chilling injury in cucumber fruits and bell peppers—a static, dynamic and a statistical model. *Planta* **194**, 478–486.
- Vanacker H, Harbinson J, Ruijsch J, Carver TLW, Foyer CH.** 1998. Antioxidant defences of the apoplast. *Protoplasma* **205**, 129–140.
- van Heerden PDR, Viljoen MM, De Villiers MF, Kruger GHJ.** 2004. Limitation of photosynthetic carbon metabolism by dark chilling in temperate and tropical soybean genotypes. *Plant Physiology and Biochemistry* **42**, 117–124.
- van Kooten O, Mensink MGJ, Otma EC, van Schaik ACR, Schouten SP.** 1992. Chilling damage of dark stored cucumbers (*Cucumis sativus* L.) affects the maximum quantum yield of photosystem 2. In: Murata N, ed. *Progress in photosynthesis research*. Dordrecht: Kluwer Academic, 161–164.
- Wellburn AR.** 1994. The spectral determination of chlorophyll *a* and chlorophyll *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology* **144**, 307–313.