Ultraweak light emission, free radicals, chilling and light sensitivity

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Ultraweak light emission (UWLE) was measured from leaves of various chilling-sensitive (*Lycopersicon esculentum, Cucumis sativus* and *Phaseolus vulgaris*) and -tolerant (*Pisum sativum* and *Vicia faba*) plants after exposure to low (4–7°C) temperature in the light. UWLE increased upon chilling treatment combined with illumination with 200 μmol m^{-2} s^{-1} PAR in all plants, by about 30% in tolerant and by more than 100% in sensitive plants. It increased more when applied together with 400 μmol m^{-2} s^{-1} PAR: by 90–100% and by 250–280% in chilling-tolerant and -sensitive plants, respectively. Free radical production was detected by spin-trapping EPR spectroscopy in thylakoid membranes isolated from the chilling-treated *Lycopersicon esculentum* and *Vicia faba* leaves. After 12 h chilling at 200 μmol m^{-2} s^{-1} PAR, free radical production was approximately 3 times greater in the former than in the latter species. The same ratio was approximately 6 if chilling was carried out at 400 μmol m^{-2} s^{-1} PAR, indicating the role of photooxidative stress in chilling injury.
Our results also confirm that the stress-induced increase in UWLE is an indicator of free radical production and offers the possibility of using UWLE for monitoring the effect of chilling on temperature-sensitive plants in an early stage.

Key words – Chilling sensitivity, free radicals, electron paramagnetic resonance, light sensitivity, spin trapping, ultraweak light emission

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Introduction

In many plants, prolonged exposure of plants to low (4–7°C) temperature results in injury. Due to its significant impact on agricultural production (Boyer 1982), the mechanism of chilling stress has been extensively studied (for reviews see Lyons 1973, Öquist et al. 1987, Wise 1995). Plants, depending on their genetic origin (biotype) and general physiological status, differ in chilling sensitivity. The effect of low temperature is most severe in plants of tropical or subtropical origin and it is less pronounced or even absent in chilling-tolerant biotypes (Hetherington et al. 1989). Thus, the comparison of plants tolerant and sensitive to chilling provides a good experimental system for the identification of factors contributing to the resulting injury. Plants are

particularly prone to damage when exposed to a combination of low temperature and high irradiance.

Several authors suggest that chilling injury is associated with free radical reactions. Chilling-sensitive plants have less active oxygen scavenging enzymes (Jahnke et al. 1991) but the remaining activity can be stimulated by cold-hardening, a treatment that increases chilling tolerance (Huner et al. 1993). Externally added antioxidants (Wise and Naylor 1987b) or genetic overexpression of the internal radical-scavenging enzymes (Gupta et al. 1993) also lead to increased chilling tolerance. Moreover, pre-treatment of dark-grown seedlings with low concentrations of added or in situ generated free radicals stimulate chilling tolerance (Prasad et al. 1994). Hodgson and Raison (1991a) found that the rate of superoxide reduction by photosystem (PS) I in the Mehler reaction

(Asada et al. 1974) increased in thylakoids during chilling. The extent of this increase was greater in thylakoids isolated from chilling-sensitive plants than in those from chilling-tolerant spinach (Hodgson and Raison 1991a).

The aim of the present work was to investigate to what extent reactive oxygen species contribute to chilling injury by comparing the ultraweak light emission (UWLE) of chilling-sensitive and -tolerant plants. UWLE is an apparently spontaneous photoemission arising from biological samples in the absence of external (light or chemical) excitation (for reviews see Cadenas 1984, Abeles 1986). In plants, unlike fluorescence which originates in PSII, UWLE is not directly related to photosynthetic activity. It is generally accepted that UWLE of leaves originates in chloroplasts as chlorophyll chemiluminescence stimulated by free radical reactions (for review see Hideg 1993). The measurement of UWLE has been utilized to illustrate the role of free radicals in UV stress (Panagopoulos et al. 1990) and paraquat resistance (Hideg and Inaba 1991).

Besides measuring UWLE, which provides an indication of oxidative stress, we have also attempted to give a direct estimate of chilling-induced free radical production using spin-trapping EPR spectroscopy (reviewed by Janzen 1971 and Evans 1979). We used this technique for measuring free radical production in the thylakoids isolated from the chilling-exposed leaves. Here, we compare electron transport impairment, represented as a decrease in F_v/F_m, and free radical production, which was characterized using UWLE and spin-trapping EPR spectroscopy in chilling-sensitive and -tolerant plants upon exposure to low, non-freezing temperature.

Abbreviations – cps, counts s^{-1}; DMPO, 5,5-dimethyl-1-pyrrolin-1-oxide; EPR, electron paramagnetic resonance; F_o, initial fluorescence; F_m, maximal fluorescence; F_v, variable fluorescence, defined as F_m-F_o; PAR, photosynthetically active radiation; PS, photosystem; UWLE, ultraweak light emission

Materials and methods

Tomato (*Lycopersicon esculentum* Mill. cv. Sverana) and cucumber (*Cucumis sativus* L. cv. Ventura) plants were purchased 4 weeks old and kept in the greenhouse until use. Pea (*Pisum sativum* L. cv. Weitor), bean (*Phaseolus vulgaris* L. cv. Stella) and broad bean (*Vicia faba* L. cv. Hangdown-Major) were grown from seeds in the greenhouse at 20°C and 60% humidity, under 300–400 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400–700 nm) provided by Osram Power Star HQI-T 400W/D lamps (Germany) for 16 h daily.

Chilling treatment was performed on 6- to 7-week-old plants in a growth chamber kept at 7°C under various light conditions using the above Osram sources: dark, low (200–250 µmol m^{-2} s^{-1} PAR) or moderate (400–450 µmol m^{-2} s^{-1} PAR) irradiation. Plants were transferred to the cold chamber around mid-day. Duration of the chilling treatment is indicated in figure legends and in the text.

Fluorescence characteristics (F_o and F_m) at ambient temperature were measured with a pulse amplitude modulation chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) on detached leaves that were kept in the dark at room temperature for 15 min prior to measurement. The value of F_v is defined as F_m-F_o, where F_o is the initial and F_m the maximal fluorescence.

UWLE was determined from the adaxial side of detached leaves mounted on a metal plate on wet tissue paper. To remove delayed fluorescence from photosynthesis, leaves were dark-adapted for 3 h at room temperature in a light-tight sample chamber before measurement. Light emission was measured with a cooled (Products for Research Inc., Danvers, MA, USA) red-sensitive photomultiplier tube (Hamamatsu 928, Hamamatsu Photonics K. K., Electron Tube Division, Shizuoka-ken, Japan) operated in photon counting mode together with a DM1B computer of a SPEX fluorimeter (Spectroscopy Laboratory Coordinator, DM1B, Spex Industries, Inc., NJ, USA). Background counts were monitored periodically during the measurement and UWLE is given as counts s^{-1} (cps) above background. The typical background count was 5 cps, measured with the shutter in front of the photomultiplier in the open position, while having the empty leaf mounting plate in the sample chamber.

For estimation of free radical production, thylakoid membranes were isolated from plant leaves immediately after the chilling treatment according to Takahashi and Asada (1982), but in the presence of the spin trap DMPO. The thylakoid preparations containing the spin trap were frozen immediately and kept at −70°C until EPR measurements. This technique is commonly used in medical studies on free radical production. Electron spin resonance spectra of the DMPO radical adduct were measured, after thawing the samples, on 15-ml aliquots with a Bruker ECS-106 (Bruker Analytische Messtechnik GmbH, Germany) spectrometer utilizing the ECS-106 data acquisition program. X-band spectra were recorded at room temperature at 9.43 GHz microwave frequency, 16 mW microwave power and 100 kHz modulation frequency, gain 2×10^4, as described earlier (Hideg and Vass 1993). The concentration of the trapped free radicals is proportional to the area under the EPR absorption function, i.e. to the double integral of the detected EPR signal.

Results

Figure 1 characterizes the chilling sensitivity of the plants used in this study. Plants were kept at 7°C for 12 h under different light conditions, low light (200–250 µmol m^{-2} s^{-1}) or moderate light (400–450 µmol m^{-2} s^{-1}, the same as the illumination during growth). Fluorescence parameters F_o and F_m were measured at the end of the chilling treatment, after a 15-min dark-adaptation period at room temperature. The ratio of variable to maximum fluorescence (F_v/F_m) is a measure of photosyn-
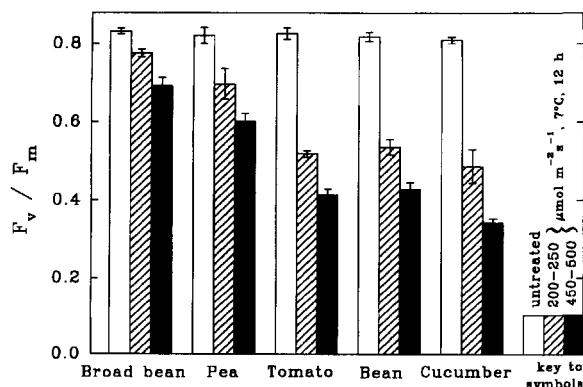
Fig. 1. Changes in variable fluorescence from leaves of chilling-tolerant and -sensitive plants measured at ambient temperature. Each column represents the average of 5 measurements.

Fig. 2. UWLE measured from leaves of chilling-tolerant and -sensitive plants before chilling (labeled as untreated) and after 12 h at 7°C at either 200–250 or 400–450 μmol m^{-2} s^{-1} illumination. All samples were dark-adapted for 3 h before UWLE measurement. UWLE intensity is given as counts s^{-1} above background. Each column represents the average of 3 measurements.

thetic efficiency (Björkman and Demmig 1987). In our experiments F_v/F_m was above 0.8 in all untreated leaves, approaching 0.83, the typical value obtained in healthy plants (Björkman and Demmig 1987). As is shown in Fig. 1, this decreased to 0.70–0.75 in chilling-tolerant plants and to ca 0.5 in -susceptible plants, as a result of exposure to chilling at low light (200–250 μmol m^{-2} s^{-1}). Higher irradiance during chilling resulted in a larger $F_v/$ F_m decrease in both biotypes. This decrease was not caused by low temperature only, since exposure to 7°C in the dark did not markedly affect F_v/F_m, either in susceptible or in tolerant leaves (data not shown), in accordance with the data of Hetherington et al. (1989). This illustrates that while cucumber, tomato and bean (*Phaseolus vulgaris*) are sensitive to chilling, pea and broad bean (*Vicia faba*) are more tolerant.

Figure 2 shows UWLE from the same samples. Dark-adapted plants showed 10–20 cps UWLE, even without the preceding chilling treatment. This light emission was similar in all untreated plants and showed no correlation with their chilling sensitivity. However, the UWLE measured after the chilling treatment showed a different pattern: the more sensitive plants responded with increased UWLE to the treatment. When chilling was carried out at 200–250 μmol m^{-2} s^{-1}, the UWLE of the two tolerant plants was practically unaffected. In contrast, low temperature pre-treatment at the same low irradiance caused a 2- to 3-fold increase in the UWLE of chilling-sensitive plants. All plants were affected when the irradiation was higher (400–450 μmol m^{-2} s^{-1}) during chilling. In this case, tolerant plants also showed an increased UWLE but their response was smaller than that of sensitive ones. Figure 2 demonstrates that the increased UWLE is a common after-effect of chilling stress on plants, but it is more pronounced in chilling-sensitive than in-tolerant plants.

It is generally accepted that UWLE is an indicator of free radical production (Cadenas 1984). Thus, the increased UWLE of chilling-sensitive plants indicates in-
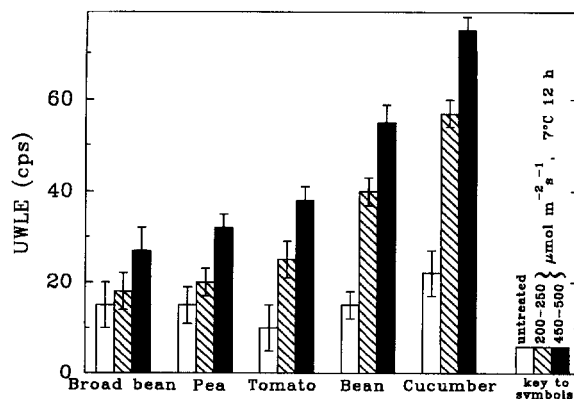
creased concentrations of free radicals. Using spin-trapping EPR spectroscopy we investigated whether these radicals can be detected directly. We used DMPO as a spin trap, a diamagnetic (EPR silent) compound, which is converted into a stable nitroxide radical upon reacting with free radicals (Janzen and I-Ping Liu 1973). Besides numerous applications in medical biochemistry, the technique has been utilized in studies of the stress-induced free radical production of thylakoid membranes (Harbour and Bolton 1975, Bowyer and Camillieri 1985, Price et al. 1989, Härtel et al. 1992, Hideg et al. 1994). However, because the application of in situ spin trapping and even the use of EPR spectroscopy for leaf samples are associated with experimental difficulties, free radical production can only be estimated from the analysis of samples isolated immediately after chilling and mixed with the spin trap. This technique has been applied for the analysis of UV-B irradiated leaves (Hideg and Vass 1996).

Two species with differing chilling sensitivity were compared, chilling-sensitive tomato and chilling-tolerant broad bean. Figure 3 shows that with this trapping protocol free radicals can be detected in tomato but not in broad bean thylakoids as a consequence of prior chilling of the plants. The observed EPR spectrum is characteristic of the hydroxyl radical adduct of DMPO (Janzen and I-Ping Liu 1973). However, the complexity of the experimental system calls for caution with conclusions regarding the chemical nature of the radical species involved (see Discussion).

The data show the extent of co-variation of chilling damage (measured as F_v/F_m loss) and UWLE. We investigated this relation in detail by comparing three parameters: the electron transport impairment (loss of F_v/F_m) and free radical production (UWLE and spin-trapping EPR spectroscopy) in a sensitive (tomato) and a tolerant
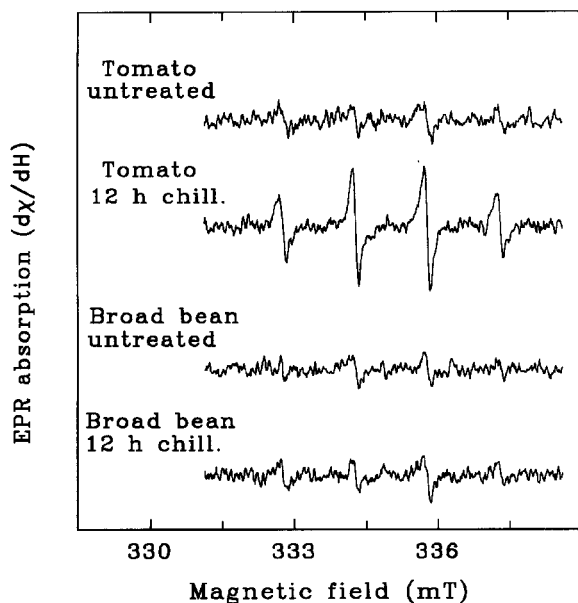
Fig. 3. EPR absorption spectra of the radical adduct of DMPO formed in tomato and broad bean (*Vicia faba*) thylakoids isolated from untreated or chilling-treated (12 h, 7°C, 200–250 µmol m^{-2} s^{-1}) plant leaves.
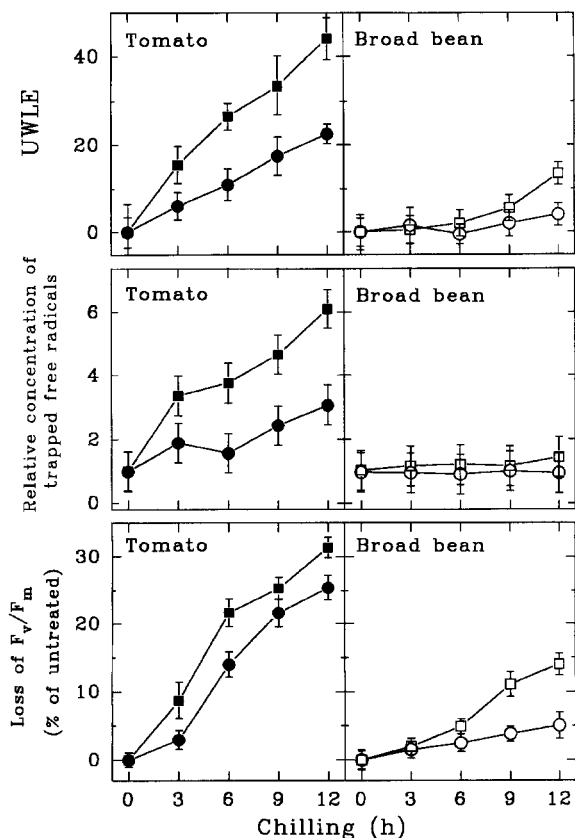

Fig. 4. A comparison of UWLE, free radical production and decrease in variable fluorescence in tomato (closed symbols) and in broad bean (open symbols) upon exposure to chilling at 200–250 µmol m^{-2} s^{-1} (circles) or at 400–450 µmol m^{-2} s^{-1} (squares) illumination. Each point represents the average of 3 measurements. UWLE is given as count per second above the intensity measured from untreated leaves. The concentration of the trapped free radicals was estimated using spin-trapping EPR spectra of isolated thylakoids (see Materials and methods for details). The decrease in fluorescence is given as the percentage loss of F_v/F_m compared to the F_v/F_m of untreated leaves.

(broad bean) plant after various chilling conditions. These three experimental parameters were well correlated (Fig. 4). All three parameters could be increased by increasing either the duration of the preceding chilling treatment or the irradiation during chilling. These changes were more pronounced in the chilling-sensitive than in the tolerant plant. In the latter samples, 3–4 h of treatment had no marked effect on any of the parameters studied.

Discussion

Plants with different chilling tolerance were used in this study. In accordance with Hetherington et al. (1989), we found that broad bean (*Vicia faba*) and pea plants showed chilling-tolerance, while bean (*Phaseolus vulgaris*), tomato and cucumber were more sensitive to chilling, as judged from the loss of variable fluorescence upon treatment.

Without the chilling treatment, approximately the same level of UWLE was detected from the dark-adapted leaves of all plants. The emission was markedly enhanced when the leaves were exposed to low temperature before the UWLE measurement. The observed UWLE was correlated with the extent of chilling injury, characterized by the loss of variable PSII fluorescence: both the increase in chilling time and the increase in irradiance during chilling resulted in increased UWLE (at ambient temperature) after dark incubation. However, this correlation was not absolute, because fluorescence

and UWLE give information about different sub-processes of chilling injury: functional impairment of PSII and subsequent oxidative stress, respectively.

Previous studies have demonstrated that UWLE from isolated plant organelles and leaves is associated with the presence of free radicals, whose reactions can lead to the excitation of pigments in the dark, either directly (Augusto and Cilento 1974) or indirectly via oxygen radicals and/or membrane lipid peroxidation products (Cadenas 1984, Salim-Hanna et al. 1987). The increase of oxidative damage, measured as accumulation of lipid breakdown derivatives has been reported as a characteristic symptom of chilling injury in the light (Van Hasselt 1974, Wise and Naylor 1987a,b, Hodgson and Raison 1991b). In this way, the observed UWLE increase reflects increased oxidative stress, more likely the accu-

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mulation of its terminal products (general membrane decay and lipid peroxidation products) than that of its initiators (free radicals). According to our UWLE data, this process is characteristic of all plants exposed to chilling, but it is more prominent in chilling-sensitive than in -tolerant plants.

This model is supported by the direct detection of free radicals by spin-trapping EPR spectroscopy, in samples isolated from chilling-exposed tomato plants at much higher levels than in those from the chilling-tolerant bean. It should be noted that although these samples were from the thylakoid-enriched pellet fraction of the leaf suspension, they were not purified further and thus may also contain mitochondria and microsomes, which are also potential free radical sources. However, the enhancement of radical production by illumination during chilling demonstrates the role of light-dependent processes and thus implicates the thylakoid membrane as the major source of trapped radicals.

It is generally accepted that the highly reactive free radicals produced during environmental stress are rapidly consumed in reactions either with antioxidants or with the target molecules of damage. Therefore, it is not likely that these radicals can be trapped after the cessation of the inducing stress. Still, our UWLE and EPR data show that this delayed, 'post-stress' free radical production exists in the chilling-sensitive plant. This phenomenon is different from, although probably not unrelated to, production of reactive oxygen species during chilling in the light (reviewed by Wise 1995). A possible explanation for our results is that the free radicals produced during chilling, including the superoxide radicals produced in the enhanced Mehler reaction (Hodgson and Raison 1991a) or any other reactive oxygen promoters of chilling-enhanced photooxidation (Wise and Naylor 1987a,b), initiate a radical chain reaction. This process may also involve less reactive species such as superoxide, hydrogen peroxide and/or lipid peroxy radicals, which are able to move in the membrane and maintain a detectable concentration of free radicals. Because the natural antioxidant systems are disabled by the chilling stress in sensitive plants (Wise 1995 and references therein), they are unable to cope with the increased radical production. Also, during chilling stress, the free radicals may not be generated at the same sites where radicals are generally produced (at very low concentrations) in the absence of the stress. Consequently the spin trap competes successfully for the free radicals, resulting in an EPR signal.

Although the observed EPR spectra are characteristic for hydroxyl radicals and thus invite conclusions as to the chemical nature of the radicals produced in the leaf, it should be kept in mind that the complexity of the sample (a reaction system containing free radicals, the natural targets, antioxidants and the spin trap) and the time delay in the observation do not allow such deductions. Because of these limitations, it is very likely that the radical species trapped are not the primary products.

Also, the radical adduct of the spin trap may undergo chemical modifications during the time between trapping and the EPR measurement (Finkelstein et al. 1979). Further studies with a different approach are required to identify the primary radical species formed during chilling stress.

In summary, the observation of increased UWLE and the direct detection of free radicals in chilling-sensitive plants demonstrate that, contrary to tolerant plants, the former undergo more severe oxidative damage during chilling. The comparison of UWLE from plant leaves before and after chilling provides a technique for the evaluation of chilling sensitivity. This method may also prove useful in studying sensitivity and tolerance towards other free radical-mediated stress conditions.

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