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Refreshed Delayed Light Emission and Fluorescence for Detecting Pretreatment Effects on Chilling Injury in Coleus

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Abstract. **Intact plants of a green-leafed strain of *Coleus blumei* Benth. (PI 354190) were exposed to 5°C for 48 or 72 hr after pretreatment for 48 hr at two levels of photosynthetic photon flux (PPF) (8 or 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$) at two temperatures (13° or 20°). Plants were sprayed with two abscisic acid (ABA) levels (0 or 200 $g \cdot m^{-3}$) either 0 or 48 hr before chilling. Postchilling condition of the plants was assessed by comparing the time courses of refreshed (cyclically excited and measured) delayed light emission (RDLE) and fluorescence (FLU) from dark-equilibrated leaves. Greater suppression of RDLE and FLU indicates greater injury. Plants pretreated at 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF showed less suppression of RDLE and FLU, contained more chlorophyll, and showed less injury than did plants pretreated at 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF. Increasing the duration of chilling from 48 to 72 hr reduced the maximum RDLE and FLU slightly. Pretreatment temperatures and ABA concentration had negligible effects on RDLE and FLU levels. The maximum RDLE, the RDLE level at 7.5 sec, the maximum FLU, the FLU at 1.5 sec, and variable FLU were the measurement variables most responsive to individual and combined treatment effects. Maximum RDLE from upper leaf surfaces was the measurement most responsive to the combined effects of all treatments. Chemical name used: [*S*-(*Z*,*E*)]-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid [abscisic acid (ABA)].**

Coleus and many other tropical and subtropical crops are susceptible to chilling injury at temperatures above freezing. In most chilling-susceptible species, the extent of injury caused by exposure to chilling depends on many factors, including temperature and duration of exposure, genotype (16), light intensity (14, 18), and prior hardening (7). Abscisic acid has been reported to ameliorate chilling injury in some species (18).

Chloroplasts progressively lose their photoreductive capacity (17) in chilling-susceptible plant tissues exposed to chilling stress and eventually chlorophyll content decreases; therefore, measurements of photosynthetic activity and chlorphyll content can provide information about chilling stress response. Measurements of chlorophyll fluorescence (FLU) or delayed light emission (DLE) can be used to estimate nondestructively the photosynthetic activity and chlorophyll content of leaves and other tissues (6, 9, 15, 19–21).

Melcarek and Brown (15) reported that temperature at the time of measurement affected the times of peak emission and the steady-state levels of both refreshed (cyclically excited) delayed light emission (RDLE) and FLU from intact leaves of chilling-sensitive species. Smillie and Nott (20) demonstrated that FLU maxima were sensitive to measurement temperature in chilling-susceptible species. Havaux and Lannoye (8) reported a maximum in the steady-state levels of RDLE near the temperature at which thylakoid membranes undergo a phase transition in chilling-sensitive species. Abbott and co-workers (1–3) exposed vegetables, fruits, leaves, and cotyledons to different temperatures, equilibrated them to ≈23°C in the dark, and then measured RDLE and FLU. They found that, in chilling-susceptible tissues, chilling exposure had caused a quantitative decrease in peak RDLE and, in some species, also caused a qualitative increase in the initial rise of RDLE.

The present study evaluated whether PPF levels, ABA, and temperature treatments before chilling exposure could condition plants against chilling stress and to determine the effects of these pretreatments on postchilling levels of RDLE and fluorescence from leaves. Coleus was chosen as the subject of this study because of its extreme sensitivity to chilling stress (16). RDLE and fluorescence data are presented here; morphological and physiological data have been reported previously (13).

Materials and Methods

Plant material and treatments. A strain of *Coleus blumei* (PI 354190) containing minimal anthocyanin was selected to eliminate potential interference by red pigments with detection of RDLE and FLU. [Chlorophyll emits at wavelengths between 660 and 800 nm and anthocyanins absorb in the range from 450 to 750 nm, depending on the anthocyanin and the pH, so some of the energy from DLE or fluorescence could be absorbed by some anthocyanins (K.H. Norris, personal communication).]

Plant materials and treatments summarized here are described in detail by Krizek et al. (13). Individual plants (experimental

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units) grown in a greenhouse were pretreated for 48 hr at either 13° or 20°C (TEMP) under continuous 8 or 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF provided by cool-white fluorescent lamps. Plants were sprayed to dripping with 0 or 200 $g \cdot m^{-3}$ racemic abscisic acid (ABA_{conc}) at either the beginning or the end of the pretreatment period (ABA_{time}). Analysis of the effect of application time was confounded because the two application times were tested in successive weeks rather than simultaneously. Following pretreatment, all plants were chilled at 5° under 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF for a duration of 48 or 72 hr (DUR). The relative humidity was maintained at 75% ± 5% during pretreatments and during chilling. No measurements were made before chilling because our interest was comparison of pretreatment effects on post-chilling condition.

Chlorophyll content and visual symptoms. Upon removal of the plants from chilling treatments, the fourth and fifth leaf pairs with lengths ≥1.0 cm were harvested for analyses and the plants were returned to the greenhouse. Chlorophyll was extracted from the fourth leaf pair and measured spectrophotometrically (13); not all treatments were tested in the first week of the experiment (ABA_{time} = 48 hr). The fifth leaf pair was used for RDLE and fluorescence measurements.

Visual observations of chilling symptoms (chlorosis and necrosis) were made 3 days after returning the plants to the greenhouse (13).

Delayed light emission and fluorescence. A prototype RDLE instrument described by Abbott and Massie (3) was modified to permit measurement of FLU. Each measurement consisted of 1000 successive readings made over 15 sec, each reading being one datapoint in the computer record.

Leaves for RDLE and FLU measurement were folded in moist paper towels as they were excised. They were held in the dark at about 23°C for 1 hr before RDLE was measured on the upper (abaxial) surface of each leaf to one side of the midrib. They were replaced in the damp towels, held in the dark for 1 hr, and then RDLE was measured on the lower (adaxial) surface of the leaf on the other side of the midrib. They were replaced in the damp towels, again kept dark for 1 hr, then FLU was measured on the upper leaf surface only. Measurements were made on an area 25.4 mm in diameter.

Measurements of RDLE from upper and lower leaf surfaces are designated RDLE-U and RDLE-L, respectively; no designation is given for FLU because it was measured only on upper surfaces. When a specific datapoint is discussed, the datapoint number is hyphenated to the measurement designation.

Statistical analyses. We analyzed RDLE-U, RDLE-L, and FLU data at selected datapoints, chlorophyll concentrations, and injury scores within ABA_{time} (weeks) using a factorial analysis of variance (ANOVA) with four main effects (PPF, DUR, TEMP, and ABA_{conc}) and all possible interactions. The following datapoints were selected for all three measurement modes by preliminary analyses: 2, 5, 10, 20, 100, 400, 500, 1000, the maximum value (MAX) and its location (LOC, expressed as datapoint number). We also analyzed variable fluorescence (FLU-VAR) (4), calculated as

$$(\text{FLU-MAX} - \text{FLU-2})/\text{FLU-2},$$

where FLU-2 represents initial fluorescence.

From the ANOVA for each measurement, we calculated the relative sums of squares (%SS), i.e., the percentage of the total sum of squares accounted for by each significant treatment or interaction effect and by the combination of all treatments, as %SS = 100× (treatment sum of squares/corrected total sum of squares). These relative sums of squares indicate the relative importance of each effect on the measured response and are similar to coefficients of determination (expressed as $r^2 \times 100$); however, the relative sums of squares should not be interpreted as necessarily showing linear regressions, because each factor was tested at only two levels. Within each mode of measurement, the datapoints that best showed the responses to treatments were divided by chlorophyll concentration to obtain the RDLE or FLU per unit chlorophyll, and these chlorophyll-adjusted data were analyzed by ANOVA. The relative sums of squares for chlorophyll-adjusted data were compared by inspection with those for the raw data to assess whether the suppression of RDLE and FLU by chilling was due to decreased chlorophyll concentration or inhibition of photosynthesis.

Simple linear correlations were calculated between RDLE or FLU at selected datapoints and chlorophyll concentration.

Results

Overall responses to treatments. RDLE and FLU responded similarly to the combined effects of pretreatments and duration of chilling (Table 1). In the second week of the experiment (ABA_{time} = 0 hr), within which all treatments and all measurements can be compared, the proportions of variance for which the combined treatments accounted were similar for maximum RDLE, maximum FLU, and chlorophyll content. The RDLE from upper leaf surfaces was a slightly better indicator of the combined treatment effects than that from lower leaf surfaces at both ABA_{time}s (Table 1).

Before presenting results for specific treatments, we must consider which measurements best demonstrate the treatment effects. The percentage of total variance attributable to combined treatment effects differed relatively little among data-

Table 1. Amount of variation attributable to treatment effects[z] for refreshed delayed light emission[y], fluorescence[y], and chlorophyll concentrations from leaves of chilled *Coleus blumei* (PI 354190) in two successive weeks (ABA_{time}s[z]).

	Relative sums of squares[x]	
	ABA_{time}s	
Measurement	48 hr	0 hr
RDLE-U-500	84.7	94.3
RDLE-U-MAX	84.7	94.3
RDLE-L-500	76.8	89.5
RDLE-L-MAX	77.1	89.4
FLU-100	86.7	90.3
FLU-MAX	86.5	90.1
FLU-VAR	79.3	91.8
Chlorophyll concn	---	87.5

[z]Treatments were PPF = 8 or 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux, DUR = 48 or 72 hr duration of chilling at 5°C, TEMP = 13° or 20° pretreatment temperature, ABA_{time} = 0 or 48 hr pretreatment time with abscisic acid (separate weeks), and ABA_{conc} = 0 or 200 $g \cdot m^{-3}$ abscisic acid. All leaves were equilibrated to about 23° before measurement.

[y]RDLE-U- and RDLE-L- represent refreshed delayed light emission from upper and lower leaf surfaces, respectively. FLU represents fluorescence from upper leaf surfaces. MAX is amplitude at maximum or peak emission; numbers indicate specific datapoints or measurement cycles. FLU-VAR is variable fluorescence = (FLU-MAX − FLU-2)/FLU-2.

[x]Treatment responses significant at $P \leq 0.05$. Values shown are relative sums of squares for combined treatments expressed as percentage of corrected total sum of squares.

points in the range between datapoint numbers 100 and 1000 for all three modes of measurement (data not shown). The specific datapoints that gave the highest relative sums of squares due to combined treatments were RDLE at datapoint 500 and FLU at datapoint 100. However, because the relative sums of squares for RDLE-500 and FLU-100 were nearly identical to those obtained using RDLE-MAX and FLU-MAX (Table 1), and because maxima are more commonly reported, the maxima will be used to evaluate the treatment effects. RDLE-500 and FLU-100 will be referred to again in our concluding recommendations. Treatments accounted for a smaller proportion of the variance in amplitudes of RDLE and of FLU at datapoints before 100 than at the maxima. The time at which peak emission occurred was relatively insensitive to treatment effects, giving relative sums of squares of 63% to 67% (data not shown).

Measurement of variable fluorescence presents a practical problem in that the value for "initial" fluorescence, which begins within picoseconds upon illumination, is dependent on the time of measurement. Our FLU-2 reading does not begin until the specimen has been illuminated for 7.5 ms, but that is much earlier than the average time of maximum emission at datapoint 218, or about 3.3 sec. Because variable fluorescence is a common way of expressing fluorescence responses, some FLU-VAR responses also are shown (Tables 1 and 2).

Photosynthetic photon flux pretreatment effects. The PPF pretreatments exerted the greatest effect on all measurement variables (Table 2). Postchilling levels of RDLE and FLU were suppressed more by the 48-hr pretreatment at 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF than by pretreatment at 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF (Table 3), indicating that 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF resulted in either lower chlorophyll concentrations or lower photosystem II activity than did the lower PPF level. The chlorophyll concentrations were indeed reduced after the 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF pretreatment (Table 3), but not enough to account for the entire suppression in emissions (Table 2, chlorophyll-adjusted values vs. raw values). Over all treatments for which chlorophyll concentrations were measured, the relationship between chlorophyll concentration and maximum RDLE or fluorescence was significant but rather low, the highest being $r^2 = 0.44$ for upper leaf surfaces (n = 72). Apparently, both chlorophyll concentration and chloroplast activity after chilling were affected by the PPF pretreatment. For example, PPF levels accounted for 72% of the variance in maximum RDLE values of lower leaf surfaces (RDLE-L-MAX) and for 48% after adjusting for differences in chlorophyll concentrations (Table 2); the differences in chlorophyll-adjusted RDLE presumably are attributable to differences in chloroplast activity.

Kaniuga and Michalski (12) showed that low or moderate PPF levels during chilling could prevent inhibition of the water-oxidizing side of photosystem II, the source of energy for RDLE (9, 10). Krizek et al. (13) proposed two explanations for the lesser chilling injury in coleus after 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF pretreatment compared to 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF: the dynamic balance between synthesis and destruction of chlorophyll shifts at 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF to favor synthesis or, at 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF, there may be an increase in free radicals that could shift the chlorophyll balance in favor of destruction. These proposals address the greater postchilling RDLE, FLU, and chlorophyll content that we measured, but do not address directly other symptoms usually associated with chilling, such as wilting and membrane leakage.

Duration of chilling effect. Increasing the duration of chilling from 48 to 72 hr consistently decreased RDLE and FLU values (Figs. 1 and 2) and chlorophyll concentrations (13), including trends in those instances where the change was not statistically significant (Table 2). Diminished RDLE and FLU would be expected with increasing time under very low PPF levels, such as our 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF. The difference between weeks may be due to some effect of spraying the plants with water and/or ABA, or may be due to differences in plants and envi-

Table 2. Relative sums of squares from analyses of variance of treatment effects[z] on maximum refreshed delayed light emission (RDLE-MAX), maximum fluorescence (FLU-MAX), and chlorophyll concentrations from leaves of chilled coleus in two successive weeks (ABA_{time}s), based on measured values (raw) or on values adjusted for chlorophyll concentration (chl adj). Chlorophyll was not measured on all treatments in ABA_{time} = 48 hr, thus chlorophyll adjustments could not be calculated.

Treatment effects (Sources of variation)	Relative sums of squares[y]												
	Week 1 ABA_{time} = 48 hr				Week 2 ABA_{time} = 0 hr								
	RDLE-U-MAX (raw)	RDLE-L-MAX (raw)	FLU-MAX (raw)	FLU-VAR (raw)	RDLE-U-MAX (raw)	RDLE-U-MAX (chl adj)	RDLE-L-MAX (raw)	RDLE-L-MAX (chl adj)	FLU-MAX (raw)	FLU-MAX (chl adj)	FLU-VAR (raw)	FLU-VAR (chl adj)	CHLOROPHYLL (concn)
PPF	60.7	66.0	61.2	66.5	69.0	54.3	72.1	47.9	61.8	34.4	81.9	74.5	77.9
DUR	3.1	NS	NS	NS	15.3	22.3	6.2	7.7	16.5	26.9	3.4	2.0	2.6
TEMP	8.3	3.4	10.2	3.1	NS	NS	5.3	13.3	1.9	4.8	1.6	4.0	NS
PPF × DUR	NS	NS	NS	NS	3.8	NS	NS	NS	2.5	NS	2.5	NS	NS
PPF × TEMP	3.2	NS	6.9	NS	NS	NS	NS	NS	NS	4.2	NS	4.3	2.0
DUR × ABA_{conc}	NS	NS	1.9	NS	0.9	NS	NS	NS	NS	NS	NS	NS	NS
PPF × DUR × ABA_{conc}	NS	NS	2.1	NS	1.7	2.4	2.2	3.0	1.4	NS	NS	NS	NS
DUR × TEMP × ABA_{conc}	NS	NS	NS	2.0	1.0	NS	NS	NS	NS	NS	NS	NS	NS
PPF × DUR × TEMP × ABA_{conc}	NS	NS	NS	2.3	0.9	NS	NS	NS	NS	NS	NS	NS	NS
Combined treatments (total model)	84.7	77.0	86.5	79.3	94.3	86.1	89.4	78.6	90.1	78.6	91.8	87.6	87.5

[z]See Table 1 for explanation of treatments and abbreviations.
[y]Within a column, the data shown are sums of squares for each significant effect ($P \leq 0.05$), expressed as a percentage of corrected total sum of squares.

Table 3. Effects of photosynthetic photon flux (PPF) and temperature pretreatments[z] on maximum refreshed delayed light emission (RDLE-MAX) and fluorescence (FLU-MAX) from chilled leaves of coleus.

Measurement	Relative sums of squares[y]			Mean values			
				PPF = 8 μmol·s^{-1}·m^{-2}		PPF = 320 μmol·s^{-1}·m^{-2}	
	PPF	TEMP	PPF × TEMP	13°C	20°C	13°C	20°C
			Week 1 (ABA_{time} = 48 hr)				
RDLE-U-MAX	60.7	8.3	3.2	2848	4479	753	1130
RDLE-L-MAX	66.0	3.4	NS	3225	4138	1208	1385
FLU-MAX	61.2	10.2	6.9	15136	25285	6073	7046
			Week 2 (ABA_{time} = 0 hr)				
RDLE-U-MAX	69.0	NS	NS	3420	3384	996	967
RDLE-L-MAX	72.1	5.3	NS	4123	3195	1454	1079
FLU-MAX	61.8	1.9	NS	2522	2102	915	830
Chlorophyll (μg·g^{-1})	77.9	NS	2.0	3.5	3.8	2.2	2.1

[z]See Table 1 for explanation of treatments and abbreviations.
[y]Sum of squares for treatment or interaction effect expressed as a percentage of corrected total sum of squares.
[NS]Not significant at $P > 0.05$. All other responses, significant at $P \leq 0.05$.

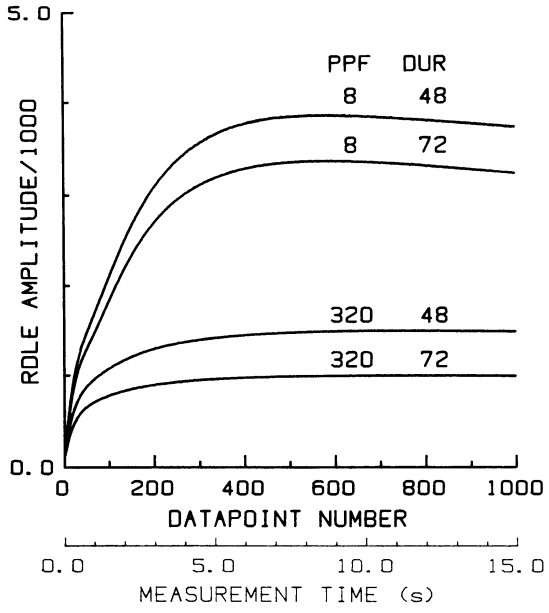

Fig. 1. Effects of two levels of photosynthetic photon flux pretreatment (PPF = 8 or 320 μmol·s^{-1}·m^{-2}) and two chilling durations (48 or 72 hr) at 5°C on postchilling refreshed delayed light emission from lower leaf surfaces (RDLE-L) of *Coleus blumei* averaged over both weeks of the test (ABA_{time}s).

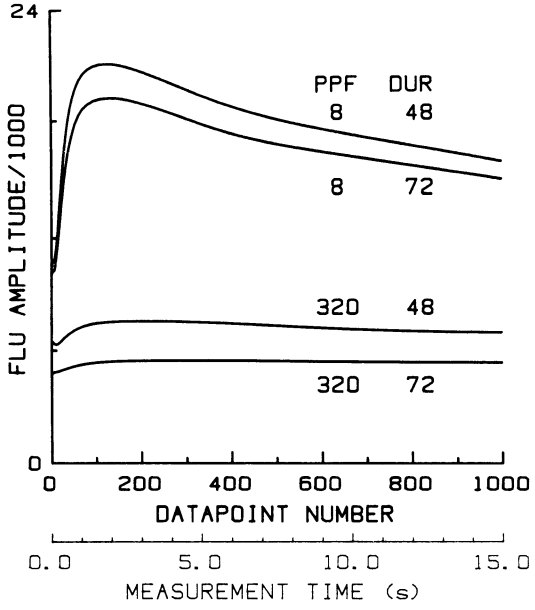

Fig. 2. Effects of two levels of photosynthetic photon flux pretreatment (PPF = 8 or 320 μmol·s^{-1}·m^{-2}) and two chilling durations (48 or 72 hr) at 5°C on postchilling fluorescence (FLU) from *Coleus blumei* leaves (ABA_{time} = 48 hr).

ronment between successive weeks. Seasonal differences in chlorophyll content normally occur in this coleus strain, perhaps reflecting natural variation in the extent of photoinhibition.

Temperature pretreatment effect. The effect of temperature pretreatments alone on RDLE differed between the two weeks (Table 3), being significant for RDLE-U in the first week but not in the second. During the first week, RDLE-U, RDLE-L, and FLU were higher after the 20°C pretreatment than after 13°; the second week, values were lower after 20° pretreatment than after 13°. The reversal of trends between weeks may have been caused by environmental effects, perhaps ambient PPF levels before initiation of pretreatments, or may be random variation, since the temperature effect was small (Figs. 3 and 4).

Abscisic acid effect. There was no significant main effect of ABA applications (ABA_{conc}) on RDLE or FLU levels (Table 2). ABA did not ameliorate loss of chlorophyll or other chilling symptoms (21). ABA contributed to some minor interaction effects, but these effects were negligible. Krizek et al. (13) suggest that the unusually high level of endogenous ABA found in the green coleus strain used in this study may account for its failure to respond to exogenous ABA.

PPF pretreatment interactions. Some of the interactions involving PPF were significant for RDLE and FLU measurements, but the magnitude of differences among treatments within a PPF level was negligible in comparison to the main effect of PPF levels. PPF × DUR and PPF × TEMP interactions each accounted for <7% of the total variance (Table 2). These differences are too small and too erratic to appear meaningful within our experiment, but might become important if all plants were to be pretreated at a single PPF level.

Leaf surfaces. On the basis of the number of significant re-

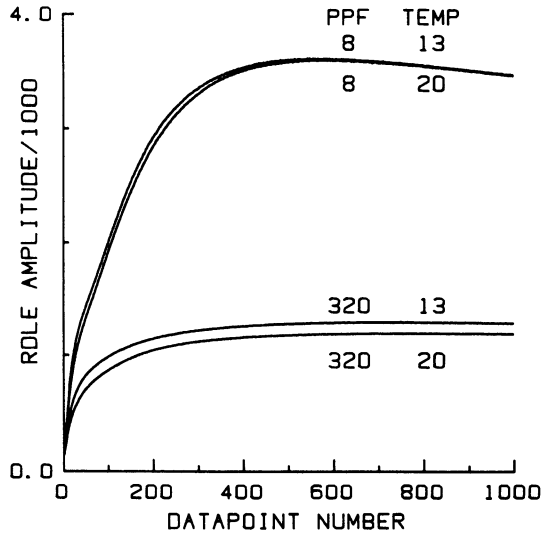

Fig. 3. Effects of pretreatment interactions at two levels of photosynthetic photon flux (PPF = 8 or 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$) and two temperatures (13° or 20°C) on postchilling refreshed delayed light emission from lower leaf surfaces (RDLE-L) of *Coleus blumei* averaged over both weeks of the test ($ABA_{time}s$).

sponses for refreshed delayed light emission for all datapoints analyzed (not all shown), upper-leaf-surface responses were more complex than those of the lower surfaces; however, most of the effects that were significant for the upper surface but not for the lower, account for very small portions of the total variance. Fork et al. (5) found that cells from the upper surface of bean leaves showed significant photoinhibition at high light intensities, while cells from the shaded lower surface did not. The slight differences in response between leaf surfaces that we observed in coleus may have been caused by small differences in chlorophyll concentrations of upper and lower surfaces, differences in chilling susceptibility or photoinhibition between cells of the upper and lower surfaces, or later measurement of the lower surfaces, causing either better dark equilibration of the lower surfaces or longer incubation of the injury (the lower surfaces were dark-equilibrated an additional hour while RDLE-U was measured). Refreshed delayed light emission was slightly greater from lower leaf surfaces than from upper, averaging 2476 and 2247 units, respectively. Jacob et al. (11) and Abbott (unpublished data) have shown that delayed light emission increases as the thickness of the chlorophyll-containing portion of the sample increases to a depth of 2 to 5 mm, so the measurements from each surface of these coleus leaves contain some proportion of delayed light emission originating in cells on the opposite surface of the leaf. Surfaces differed more in RDLE at early datapoints, such as at RDLE-20, than at later datapoints.

Discussion

Plants pretreated at 8 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF for 48 hr before chilling at 5°C showed less suppression of RDLE and FLU, less loss of chlorophyll, and less injury than plants pretreated at 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF. PPF pretreatments affected RDLE and FLU levels in part by affecting chlorophyll concentrations. However, PPF levels also apparently affected the accumulation or equilibrium of photophosphorylation products, thereby causing differences in RDLE and FLU levels.

Pretreating plants at 13° or 20°C or applying ABA had negligible effect on RDLE, FLU, chlorophyll concentration, or chilling injury.

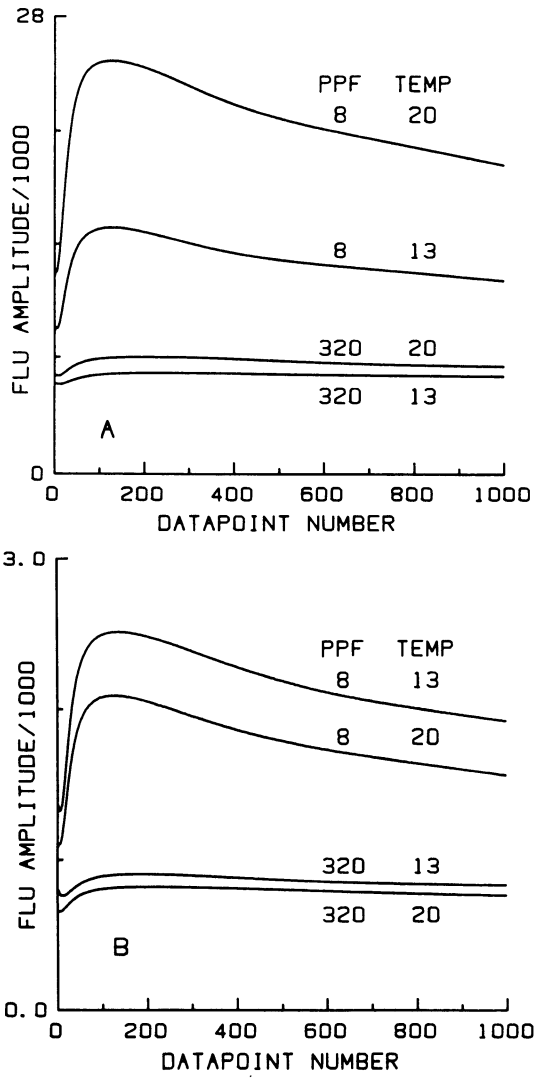

Fig. 4. Effects of pretreatment interactions at two levels of photosynthetic photon flux (PPF = 8 or 320 $\mu mol \cdot s^{-1} \cdot m^{-2}$) and two temperatures (13° and 20°C) on postchilling fluorescence (FLU) from *Coleus blumei* leaves. (**A**) Abscisic acid (ABA) pretreatments applied 48 hr before chilling (week 1 of experiment). (**B**) ABA pretreatments applied immediately before chilling (week 2).

Based on our results, both maximum RDLE and maximum FLU are sensitive, nondestructive measurements for detecting and quantifying chilling stress in coleus. The maximum FLU occurred earlier than maximum RDLE, but time of occurrence of the peak was relatively insensitive to the pretreatments that we tested. It is possible to measure RDLE or FLU at a specific time rather than tracking emission over a time long enough to exceed the time of maximum emission; in that case, it is critical to control the time of measurement and to select the optimum time point. If RDLE or FLU is to be measured at a single specified time, we recommend that RDLE be sampled at 7.50 sec and that FLU be sampled at 1.50 sec. These points correspond to our datapoints RDLE-500 and FLU-100, where we obtained the greatest sensitivity to our pretreatments. There was no clear superiority between RDLE and FLU for detecting the effects of the treatments which we tested.

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