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Diurnal Changes in the Chilling Sensitivity of Seedlings

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ABSTRACT

Seedlings of tomato (*Lycopersicon esculentum*, Mill.) varied diurnally in their sensitivity to chilling temperatures. If chilled near the end of the dark period when they were most sensitive, the time taken to kill half of the seedlings was approximately 3 days, whereas in samples taken 4 hours after the onset of dark, a period of 6 days of chilling was required. Sensitivity dropped rapidly after the onset of the light period. This rhythm was exogenously controlled by the diurnal changes in light, rather than in the temperature. The susceptibility of predawn seedlings could be reduced by exposure to light, by water stress, or by abscisic acid applied to the leaves. However, the subsequent changes in sensitivity to chilling did not correlate with stomatal aperture. Six other chilling-sensitive species showed similar diurnal changes in their chilling sensitivity.

Many investigators studying the chilling sensitivity of plants have used seedling tissues or whole seedlings (5) because the response of seedlings to temperatures below their chilling threshold is easily monitored, and fairly rapid. For example, Creencia and Bramlage (2) showed that although corn seedlings held for 36 h at 0.3°C were undamaged when returned to ambient temperatures, those held for 48 h or more were irreversibly damaged.

Patterson *et al.* (7) showed that there were diurnal changes in the sensitivity of tomato seedlings to low temperature. They found that seedlings chilled from the end of the dark period were injured several days earlier than those chilled starting later in the day or during the first hours of the dark period. In this paper we report a study of the diurnal nature of chilling sensitivity in seedlings of a number of species, and the environmental factors that influence this diurnal response.

MATERIALS AND METHODS

Plant Material. The following chilling-sensitive plants were grown from seed and used in the seedling stage: *Capsicum annuum*, L. cv. 'Bell Boy'; *Cosmos bipinnatus*, Cav. cv. 'Sensation'; *Lycopersicon esculentum*, Mill. cv. 'Ace'; *L. esculentum*, Mill. cv. 'Beefsteak'; *L. esculentum*, Mill. cv. 'Grosse Lisse'; *L. esculentum*, Mill. cv. 'Rutgers'; *Phaseolus aureus*, Roxb.; *P. vulgaris*, L. cv. 'Blue Lake'; *P. vulgaris*, L. var. humilis, Alef. cv. 'Stringless Green Pod'; *Solanum Melongena*, L. var. esculentum, Nees. cv. 'Black Beauty'; and *Zea mays*, L. cv. 'Early Sunglow Hybrid'. Chilling-resistant seedlings used were: *Brassica oleracea* var. *botrytis*, L. cv. 'Green Duke'; *Digitalis purpurea*, L.; *Lathyrus odoratus*, L. cv. 'Royal Mixed Colors'; and *Raphanus sativus*, L. cv. 'French Breakfast'.

Seeds were germinated under mist and were planted in one-third sand, one-third peat, and one-third redwood sawdust with five seedlings per 4-inch pot. Seedlings were grown in a growth chamber, 21°C day/15.6°C night and 70% to 80% RH, until the time of treatment. In specified experiments, seedlings were grown at 21°C continuously. Half-strength Hoagland solution (3) was given at every watering. Most seedlings were 2 to 4 weeks old at the time of treatment.

Lighting conditions in the growth chamber consisted of a 9-h d (0800–1700 h) and 280 w m^{-2} of combined incandescent (100 w Sylvania) and cool-white fluorescent (1500 w F96T12-CW-1500, General Electric).

Chilling Conditions. Plants were chilled by placing them in a covered styrofoam box, the bottom lined with crushed ice, which was held in a cool room. The temperature in the box was 2.0 ± 0.3°C, RH was 97% to 100%. All chilling treatments were in the dark.

Postchilling Conditions. On removal from chilling, plants were placed under continuous light (100 w m^{-2}, cool-white fluorescent tubes) at 23 ± 1°C, and approximately 50% RH. Surviving seedlings were counted 7 to 10 d after removal from chilling. Leaf necrosis on these seedlings was scored as absent, moderate (necrosis on approximately 25–50% of the leaf area), or severe (necrosis on greater than 50% of the leaf area).

Diurnal Changes in Chilling Sensitivity. To establish a survival curve of tomato seedlings exposed to chilling conditions at different times of the day/night cycle, tomato seedlings (cv. Beefsteak) were taken from the growth chamber and put into chilling conditions at 2-h intervals throughout a 24-h period. Twenty seedlings were used per treatment. Each group of plants was removed from chilling exactly 96 h after the time of initial chill. Nonchilled control plants were kept in the dark for 96 h.

This experiment was also conducted using groups of seedlings chilled at the same chronological age. Seeds were planted, transferred to the growth chamber, and placed in chilling conditions at 2-h intervals.

Time Course of Chilling. To evaluate the interaction between chilling duration and the time at which chilling commenced, seeds of *L. esculentum* cv. 'Grosse Lisse' were sown at 0700 and 2200 h and grown in a growth chamber (12 h dark, 1800–0600 h 18°C, 22°C during the light period). Exactly 9 d after sowing (*i.e.* at 0700 or 2200 h), batches of seedlings were placed in the chilling conditions. Plants were withdrawn at intervals and placed in the postchilling conditions.

Electrolyte Leakage following Chilling. Seedlings were chilled for 48 or 72 h, beginning at 0700 and 1900 h, then 0.5 g fresh weight leaf samples were taken from each treatment, sliced into 2-mm (after 48 h) or 3-mm (after 72 h) wide strips, and slowly shaken in 10-ml deionized H_2O for 1 h. Electrical conductivity of the medium was then measured. Leaf samples were boiled for 5 min, then frozen and thawed to determine total conductivity. Samples from nonchilled control plants were taken at the two time intervals from plants that had been kept in a 21°C dark growth chamber for 48 or 72 h.

Measurement of Stomatal Aperture. The possibility that

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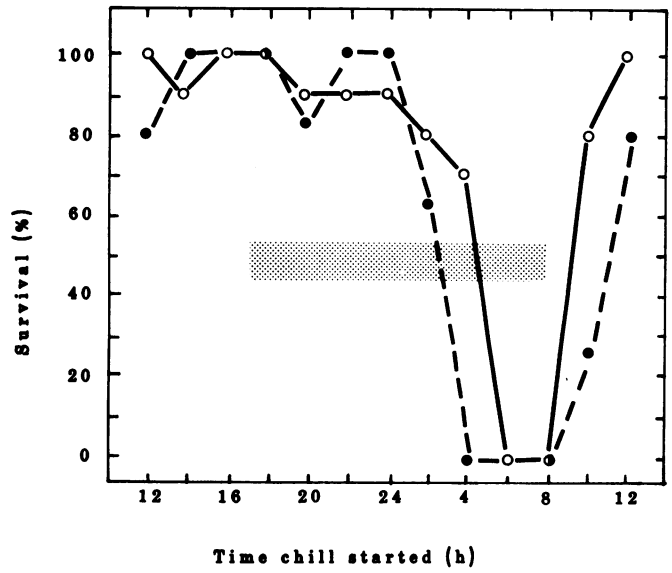

FIG. 1. Survival of tomato seedlings following chilling. Chilling was started at 2-h intervals for a period of 96 h, then placed at room temperature for 10 d. Plants 2 weeks old at beginning of diurnal cycle (○); plants 2 weeks old when put into chilling (●). Shaded bar shows dark period.

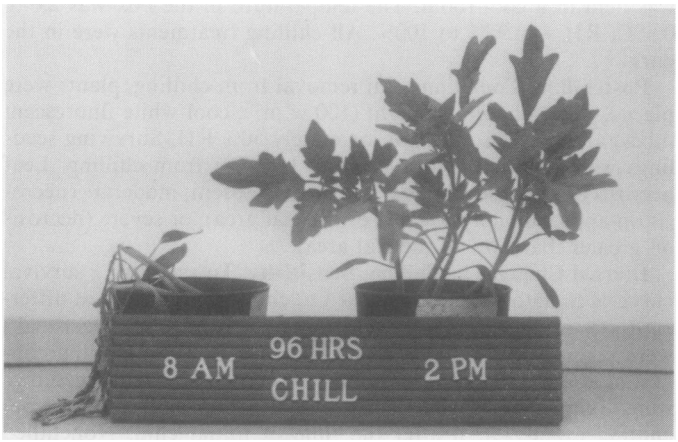

FIG. 2. Effect of chilling for 96 h on tomato seedlings placed in chilling conditions at different times of day. Photograph taken 7 d after placing chilled plants at room temperature.

Table I. *Effect of Chilling Tomato Leaves from Different Times of the Day on Subsequent Leakage of Electrolytes*

Leaves were chilled for 48 or 72 h. The mean values of three measurements are given. Values with no common subscript letter are significantly different ($p = 0.05$).

	Chilling Started at:	
	0700 h	1900 h
	% leakage	
Nonchilled, 48 h	18.0_b	14.4_{bc}
Chilled, 48 h	14.3_{bc}	18.4_b
Nonchilled, 72 h	10.8_{bc}	10.1_c
Chilled, 72 h	38.7_a	13.2_{bc}

changed stomatal aperture was involved in changed resistance to chilling was examined by measuring stomatal aperture at different times of the day. Diffusive resistance of bean leaves to water vapor was determined with a LI-COR model LI-65 Resistance Meter (LI-COR, Inc, Lincoln, NE) Measurements were made just prior to chilling, and 48 h after the start of chilling.

Table II. *Survival of Various Species following Chilling and the Effect of Time of Start of Chilling*

Plant	Starting Time of Chill	Length of Chill	No. Plants Chilled	Survival
	h			*%*
Mung bean	0600	48	60	2
	0900	48	60	60
	1200	48	60	93
	1800	48	60	82
	2400	48	60	83
Bell pepper	0600	97	20	20
	2200	97	16	100
Cosmos	0700	60	10	0
	1600	60	10	100
Corn	0600	99	46	6
	1900	99	37	57
Green bean	0600	99	39	56
	1900	99	41	83
Eggplant	0600	96	51	0
	2200	96	35	66

Table III. *Effect of Light on Survival of Tomato Seedlings following a 96-h Chill*

Plants were given the light treatment during the 'night' prior to chilling, *e.g.* between 1700 and 0800 h.

Starting Time of Chill	Night Treatment before Chill	No. Plants Chilled	Survival
h			*%*
0700	Dark	35	0
0700	Light	35	74

Table IV. *Diurnal Differences in Stomatal Aperture in Chilled and Nonchilled Bean Seedlings*

Mean Values of Five readings per treatment are given.

Starting Time of Chill	Resistance at Time of Chilling	Resistance after 48-h Chill
h	*sec cm*$^{-1}$	
0700	7.24 Dark	3.45 Dark
1200	1.45 Light	2.27 Dark
1800	9.40 Dark	8.10 Dark
2300	30.09 Dark	5.68 Dark

Table V. *Effect of Water Stress on the Survival of Tomato Seedlings Subsequently Chilled from 0800 or 1400 h*

Starting Time of Chill	Treatment	No. Plants Chilled	Survival
h			*%*
0800	Control	15	0
0800	Stressed	20	100
1400	Control	15	80
1400	Stressed	20	100

Effect of Water stress and ABA Treatments. Water was with-

Table VI. *Effects of Pretreatment with ABA on Survival of Tomato Seedlings after a 96-h Chill*

Starting Time of Chill	Treatment	No. Plants Chilled	Survival
h			%
0600	−ABA	23	0
0600	+ABA	30	87
2200	−ABA	18	100
2200	+ABA	29	100

Table VII. *Electrolyte Leakage from ABA-Treated Tomato Seedlings following a 72-h Chill*

Plants were chilled starting at the indicated times. Mean values of three measurements are given. Values with no common subscript letter are significantly different ($p = 0.05$).

Treatment	Time Started	Leakage	
		−ABA	+ABA
	h	%	
Nonchilled	1900	10.1_b	12.6_b
Nonchilled	0700	10.8_b	15.3_b
Chilled	1900	13.2_b	10.9_b
Chilled	0700	38.7_a	11.8_b

held from tomato seedlings until the seedlings first showed loss of turgor (4–5 d). Thirty minutes before the start of chilling, the seedlings were watered to allow turgor reestablishment. Chilling was started at 0800 and 1400 h, and continued for 98 h.

Plants were sprayed twice with an ABA solution (2×10^{-4} M) containing 2 drops/L Tween-20 at 12 h and again at 30 min before the start of chilling.

RESULTS

Diurnal Changes in Tomato Chilling Sensitivity. When plants were placed in chilling conditions at mid-day, more than 90% of the plants survived the 96-h chilling treatment (Fig. 1). Survival was high for plants chilled at subsequent stages of the diurnal cycle up to 2400 h. Thereafter, survival declined, falling to 0% after 0400 h. None of the plants whose chilling started at 0600 and 0800 h survived the 96-h chill. Within a few hours of removal from chilling, these plants were completely wilted and collapsed (Fig. 2). In contrast, if chilling was started 4 h after the start of the light cycle, at 1200 h, more than 70% of the plants survived. Similar results were obtained when this experiment was repeated using groups of seedlings at the same chronological age at the start of the chilling period (Fig. 1). Seedlings placed in a dark growth chamber for 96 h beginning at 0600 and 1000 h resumed normal growth after removal from the dark.

Time Course of Chilling. If plants were chilled from 0700 h, only 3 d of chilling were required to kill half the plants (Fig. 3). In contrast, plants chilled from 2200 h required 6 d of chilling to kill half the plants.

Electrolyte Leakage following Chilling. No significant differences in leakage of solutes from leaf slices were found after 48 h between plants placed in chilling conditions at different times during the diurnal cycle (Table I). After 72 h of chilling, a marked increase in leakage was evident only in slices of leaves from plants chilled starting at 0700 h.

Comparison of Species. Seedlings of cool-temperate crops (broccoli, radish, foxglove, and sweet pea) were not injured by the chilling treatment, regardless of the time at which it was started (data not shown). In the 10 chilling-sensitive species and varieties tested, exposure to chilling temperatures at the end of the dark period caused severe injury. Plants chilled from 1200 to 2400 h were much less damaged and some species, for example Bell Pepper and Cosmos, were unaffected by this chilling treatment (Table II).

Effect of Constant Temperature. When seedlings grown at constant temperature were chilled at different times of the light/dark cycle, a diurnal rhythm was still apparent in the response to chilling. Plants chilled starting at 1600 and 2400 h developed no visible injury, whereas those chilled beginning at 0600 h collapsed and died (data not shown).

Effect of Changing Light Regimes. Exposure to light during the night prior to chilling reduced the injury on tomato seedlings chilled beginning at 0700 h (Table III). The majority of the plants given light for one 'night' prior to being chilled showed only minor leaf damage, whereas those given the standard dark treatment were killed.

Relationship to Stomatal Aperture. At 0700 h, 1 h before 'dawn,' when bean seedlings were most sensitive to low temperature

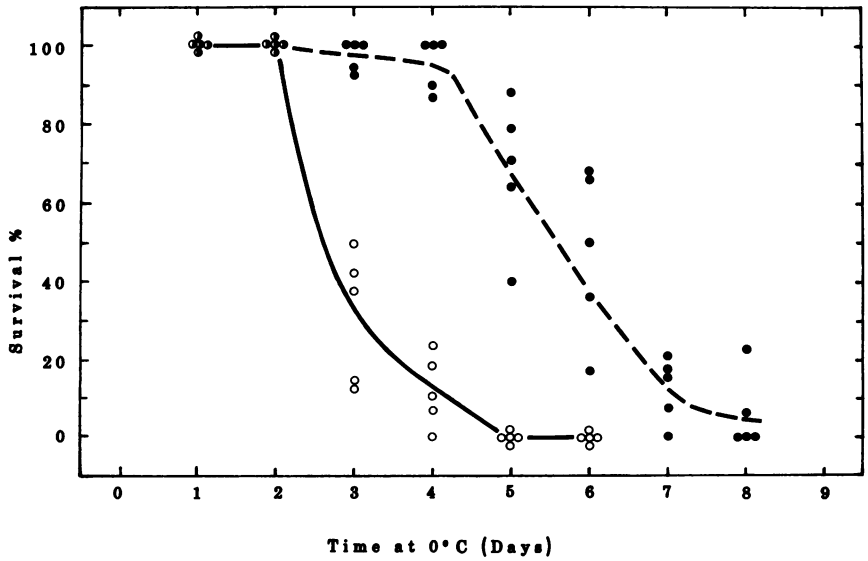

FIG. 3. Time-course of chilling of tomato seedlings. Seedlings were chilled from 0700 h (○) or 2200 h (●) for times indicated, and survival assessed after 1 week under postchilling conditions.

(Table II), their stomata were partially open (Table IV). At 1200 h, a time of low sensitivity, their stomata were maximally open and stomatal resistance was low. After 2 d at chilling temperatures, the stomata of all plants were partially open.

Humidity after Chilling. After chilling for 96 h, plants were removed under 50% RH and under 100% RH. Although the time at which chilling was started influenced survival, as in the previous experiments, the differences in RH did not (data not shown).

Water Stress. Previously water-stressed plants, which had been watered so as to regain turgor, whether placed in chilling conditions at 0800 or 1400 h, were not damaged by the chilling stress (Table V). In the nonstressed plants, damage was slight in those chilled from 1400 h, and severe in those chilled from 0800 h.

ABA Treatments. Although control plants chilled from 0600 h were killed, ABA-treated plants chilled at this time were only slightly damaged (Table VI). Plants chilled from 2200 h, with or without ABA, suffered no visible damage after chilling.

Application of ABA to nonchilled plants did not significantly alter electrolyte leakage (Table VII). In the plants chilled starting at 1900 h, leakage did not rise above the levels of the nonchilled plants, nor did ABA affect leakage. An increase in leakage was evident, however, from the control plants chilled from 0700 h. However, if ABA was applied before the chilling period beginning at 0700 h, leakage was reduced to the level shown by unchilled plants.

DISCUSSION

Our results show that diurnal fluctuations in response to chilling temperatures occur in a wide variety of chilling-sensitive plants, although the fluctuations are not endogenous, but are determined by the light/dark regime. In the species tested, sensitivity to chilling temperatures was highest at the end of the dark period. Plants chilled from this time showed increased leakage of electrolytes and necrosis. Time courses of chilling started at different times of the day did not indicate that chilling sensitivity was entirely absent at some times. Rather, they indicated that the duration of chilling required to elicit symptoms of injury varied considerably through the day/night cycle, although all of the chilling-sensitive species eventually died after extended exposure to low temperature.

Sensitivity to chilling still varied diurnally when plants were grown at uniform temperatures under a light/dark cycle, but when a dark period was missed the variation was removed. Clearly, light is an important factor in the diurnal changes of response to chilling. Recently, Rikin *et al.* (10) reported that light protects cotton seedlings against chilling injury. Our results also show that light reduces the effect of chilling, and that the response is general for a number of unrelated chilling-sensitive species.

Light might be influencing the plant in several ways—by changing stomatal aperture and internal water balance, by changing metabolism, or by increasing the carbohydrate status of the plant. If water loss from plants were primarily responsible for chilling injury, it would be expected that most injury would occur when stomata were maximally open. However, no correlation between stomatal aperture and chilling sensitivity was found.

Water stressing plants prior to chilling has been reported to reduce to the severity of injury (9, 13). Wilson (13) proposed that water stressing increases endogenous ABA levels, thus conditioning stomata to close when chilled. ABA levels are known to increase in water-stressed plants (6, 9). Rikin and Richmond (11) showed that spray applications of ABA to chilling-sensitive cucumber seedlings resulted in reduced injury following chilling. How ABA reduces chilling injury is unclear (1, 9, 11), but the recent finding that ABA significantly reduced cellular leakage from *Nicotiana tabacum* pith explants (1) suggests that ABA is not acting solely on stomatal regulation.

The irreversible damage that accompanies prolonged chilling may result from the accumulation of toxic metabolites (4, 5, 8). Carbon flux through the pathways that produce toxic metabolites could vary during the light/dark cycle, and in this way such diurnal changes within the plant could alter its sensitivity to chilling temperatures. No matter what time of the day chilling is started, all seedlings will be injured if chilled long enough. Therefore, rather than some change in the primary lesion, it seems likely that the diurnal change in sensitivity is related to diurnal changes in metabolic rates, particularly those responsive to light fluctuations.

After seedlings have been in the dark for an extended period of time, a metabolite may be depleted or accumulated to the point of reducing plant tolerance to chilling temperatures. With the onset of light, the compound might be synthesized or metabolized rapidly, thus decreasing chilling sensitivity. Alternatively, it could be that metabolic rates at chilling temperatures are a reflection of those at growth temperatures. If the metabolic rate during the diurnal light cycle shows substantial variations, then this carry over would result in diurnal variations in the rate of accumulation of toxic metabolites during chilling.

The mitigation of chilling injury in seedlings that were water stressed or sprayed with ABA might also be a reflection of involvement of metabolism in the expression of symptoms. Inhibition of metabolism by ABA (12) could reduce the rate of accumulation of toxic metabolites in the same way as diurnal variations in metabolic rates. Since ABA levels in nonstressed cotton plants have been reported to vary diurnally, with lowest levels measured during the predawn hours (6), it is possible that the diurnal variations reported here may be a response to diurnal changes in ABA, mediated perhaps through its effect on metabolic rates.

It could be argued that the effects reported here relate to the cyclic depletion of endogenous carbohydrate during the dark phase. Certainly the seedlings were grown in relatively short days. However, the rapidity with which the sensitivity to chilling disappeared after the onset of light would refute this explanation. In addition, the closed stomata of waterstressed seedlings would be expected to result in low carbohydrate status, yet these plants were highly resistant to the chilling treatment. Elucidation of the cause of the diurnal response requires further investigation of diurnal metabolic changes in seedling tissues.

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