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Authors
Mangrich, M E
Martinez-Font, R T
Saltveit, Mikal E

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Radicle Length and Osmotic Stress Affect the Chilling Sensitivity of Cucumber Radicles

Mary E. Mangrich, Rafael T. Martinez-Font, and Mikal E. Saltveit*

ABSTRACT

Cold soil and air temperatures reduce germination, seedling growth, and stand establishment of many important agronomic and horticultural crops. Chilling tolerance of many seeds is lost as they germinate and grow into young seedlings. Cucumber (*Cucumis sativus* cv. Poinsett 76) seedling radicles lose chilling tolerance as they emerge and elongate. Chilling at 2.5°C for 72 h reduced subsequent elongation at 25°C by 12, 41, and 77% for 1-, 10-, and 20-mm-long radicles, respectively. Radicle elongation followed an exponential decline with increasing mannitol concentration ($r^2 > 0.9$) for radicles initially 1 or 10 mm in length. Chilling 1-mm radicles treated for 24 h in 0.3 or 0.6 *M* mannitol inhibited elongation 90 or 40%, respectively. During mannitol treatment, 0.3 and 0.6 *M* treated radicles increased 12.5 and 0.7 mm in length. Chilling 10-mm radicles treated for 24 h in 0.3 or 0.6 *M* mannitol inhibited elongation 99 or 33%, respectively. During mannitol treatment, 0.3 and 0.6 *M* treated radicles increased 23.8 and 1.1 mm in length. The percentage inhibition of chilling-induced radicle elongation was related to the initial radicle length when chilled by a curve of the form $C_L = C_o \times (1 - e^{-KL})$ that describes many biological reactions where the rate of change is constant and proportional to the amount of reactants present. The increase in chilling-induced inhibition of radicle elongation with increasing radicle length was consistent with the progressive loss of protective compounds, possibly through dilution as the tissue expanded in volume during radicle elongation.

Low temperatures during germination and seedling emergence impose significant environmental limitations on crop production and the spatial distribution of species. Some of the most important agricultural crops are chilling sensitive {e.g., banana (*Musa* × *paradisiaca* L.), bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], and tomato (*Lycopersicon esculentum* Mill.)}, and suffer physiological injury when exposed to nonfreezing temperatures below ≈12°C (Saltveit, 2000). The extent of chilling injury is dependent on the tissue's susceptibility (which varies among tissues and during development), the duration of exposure, and the temperature. Prolonged exposure of meristematic tissue to lower temperatures usually produces the most severe injury.

Symptoms of chilling injury include reduced growth, tissue discoloration, and increased water loss, respiration, ethylene production, and disease susceptibility. Recovery of tissue from moderate levels of chilling is often accompanied by transient increases in respiration and ethylene production as the plant resumes normal rates of growth (Saltveit and Morris, 1990). The development of chilling injury symptoms can be ameliorated by treatments applied before (e.g., conditioning, heat shock), during (e.g., controlled atmospheres, intermittent warming), or after (e.g., high humidity, antioxidants) chilling (Cabrera and Saltveit, 1990; Kang and Saltveit, 2001; Saltveit, 1991, 2000).

Chilling sensitivity of cucumber (Poinsett 76) radicles decreases with distance from the apical tip, with the apical 3-mm tip being the most chilling-sensitive tissue (Rab and Saltveit, 1996). Under a constant level of chilling stress, the percentage inhibition of radicle elongation is also dependent on radicle length when chilled. Imbibed seeds with radicles 1 mm in length are significantly more chilling tolerant than seeds with radicles 10 mm in length. For example, chilling 1-mm-long cucumber seedling radicles at 2.5°C for 72 h inhibited subsequent elongation at 25°C by 2%, while chilling radicles 10 mm in length inhibited subsequent elongation by 85%. Species for which 1-mm radicles were more chilling tolerant than 10-mm radicles (e.g., cucumber, maize, tomato) were also those species in which a heat shock induced significant chilling tolerance (Mangrich and Saltveit, 2000b). We have recently identified dehydrin-like proteins and heat-shock proteins whose appearance and disappearance during cucumber seedling radicle elongation and heat-shock treatments were highly correlated with changes in chilling tolerance (Kang et al., 2005). The ability of other abiotic stresses to induce chilling tolerance may be a common physiological response of chilling-sensitive plant tissues (Saltveit, 2000).

While the literature on the appearance of protective proteins (e.g., late-embryogenesis abundant proteins and heat-shock proteins) during the development and maturation of seeds is extensive (Haslbeck, 2002; Ingram and Bartels, 1996), there is a paucity of information on the changes in these compounds during seedling germination and radicle elongation. Soluble sugars and specific proteins (e.g., dehydrins, which are a family of late-embryogenesis abundant proteins) accumulate during seed development and participate in protecting the maturing embryo during seed desiccation (Blackman et al., 1995; Chen and Burris, 1990; Close, 1996). In the series of experiments reported in this paper, radicle elongation was inhibited after germination by holding the seedlings in various concentrations of mannitol. The purpose of this research was to determine whether continued metabolic activity or the increase in tissue volume accompanying radicle elongation caused loss of chilling tolerance. We show that radicle elongation, not radicle age or increased respiration, was correlated with the loss of chilling tolerance.

MATERIALS AND METHODS

Plant Material

Poinsett 76 seeds were obtained from a local vendor. After imbibition in aerated water overnight at 25°C, the seeds were

Mann Laboratory, Dep. of Plant Sciences, Univ. of California, Davis CA 95616-8631. *Corresponding author (mesaltveit@ucdavis.edu).

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transferred to moist paper toweling overlying capillary cloth that was sandwiched between two 15- by 30-cm Plexiglas plates, held together with rubber bands. The seeds were oriented with the radicle down and the units were held in a vertical position at 25°C in a humid, ethylene-free atmosphere until the radicles reached the appropriate length (i.e., 1, 10, 20, and 30 mm).

Osmotic Treatments

Seedlings with radicles 1 or 10 mm in length were removed from the Plexiglas sandwich and placed in beakers containing 100 mL of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, or 1.0 *M* aqueous solutions of mannitol. The seedlings were held for 0.2 or 24 h at 25°C in the respective mannitol treatment solution. We had previously established that at 25°C it took approximately 24 h for 1-mm-long cucumber radicles to elongate to 10 mm in length. After the mannitol treatment, the seeds were washed three times in deionized water and six seedlings were gently transferred to moist paper toweling overlying capillary cloth and sandwiched between 7- by 13-cm Plexiglas plates as before.

Chilling Treatments and Measurements

The small Plexiglas sandwiches were put into 20- by 15- by 10-cm plastic tubs lined with wet paper towels, the top of the tubs were loosely covered with aluminum foil, and the tubs placed at 2.5 or 25°C. The extent of chilling injury was measured as the subsequent linear elongation of the radicle after chilling (Rab and Saltveit, 1996). After the 0.2- or 24-h mannitol treatment, the rinsed seeds were either held at 25°C for 72 h or chilled at 2.5°C for 72 h before holding them at 25°C for 72 h. Radicle length was measured with a clear, transparent plastic ruler to the nearest mm before and after the osmotic treatment, after chilling, and after the 72-h elongation period at 25°C.

Measures of Carbon Dioxide Production

Germinated seeds with radicles 1 or 10 mm in length were placed in Plexiglas sandwiches that were irrigated with 0.0, 0.3, 0.5, or 0.6 *M* mannitol solutions. The plates were periodically irrigated with fresh solutions to maintain the correct mannitol concentration. After 0, 24, and 48 h, the plates were enclosed in glass jars and the carbon dioxide concentration measured after an appropriate period by analyzing 1-mL gas samples with an infrared carbon dioxide gas analyzer as previously described (Saltveit and Strike, 1989). Production of carbon dioxide was calculated per seedling fresh weight.

Statistics

All treatments were replicated three times within each experiment, and each experiment was done twice. Each Plexiglas sandwich with six seedlings was considered one replicate. The treatments were applied as a completely randomized design. Means and standard errors were calculated for each treatment within an experiment and compared across experiments. An

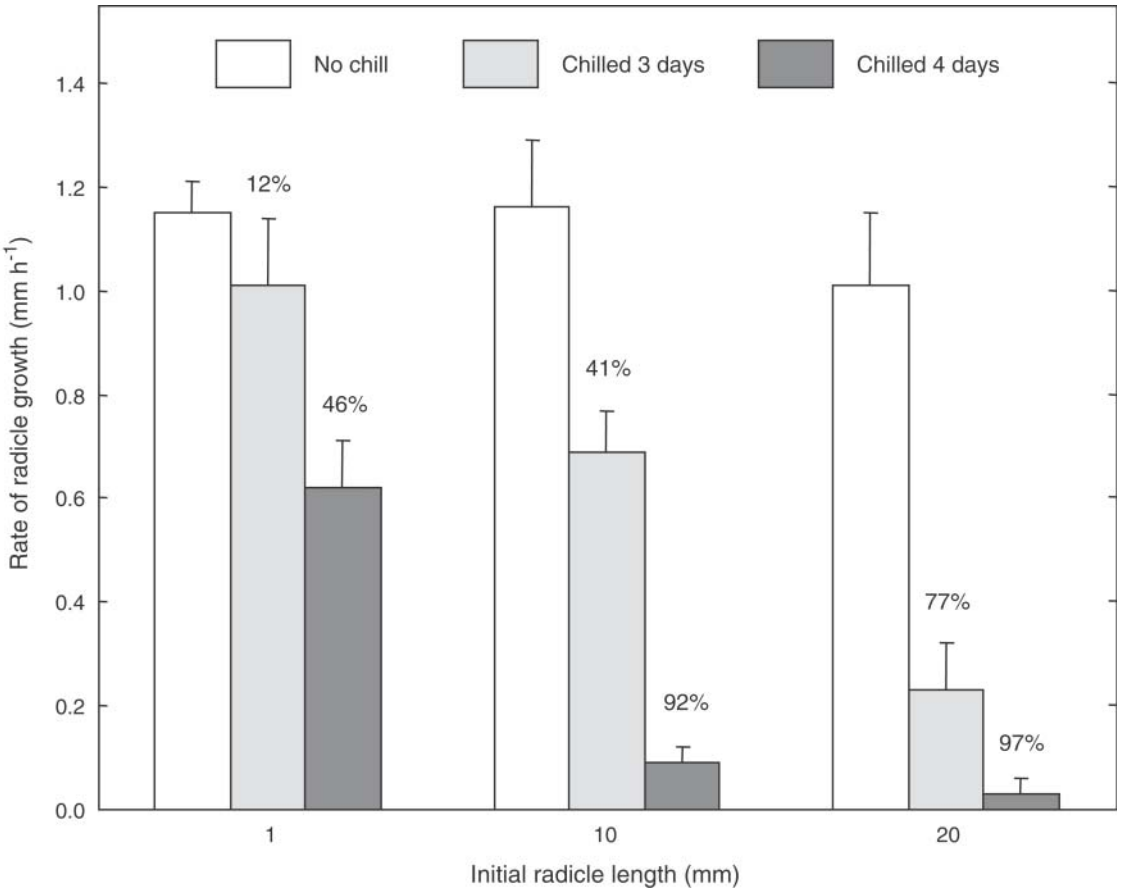

Fig. 1. Effect of radicle length at the time of chilling on the percentage inhibition of subsequent radicle elongation compared with nonchilled controls. The percentage above each shaded bar is the percentage reduction from the nonchilled control for that radicle length. The vertical line atop each bar represents ± SE (n = 6).

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ANOVA was done on the combined replicates for an experimental procedure and, when appropriate, LSD 0.05 values were calculated from the mean square error term.

RESULTS AND DISCUSSION

Radicle Length and Chilling Sensitivity

Radicles became more chilling sensitive as they elongated, and chilling at 2.5°C progressively inhibited subsequent radicle elongation at 25°C (Fig. 1). After chilling for 72 h, the 1-, 10-, and 20-mm-long radicles resumed linear rates of elongation ($r^2 > 0.95$) when returned to 25°C. These rates were significantly less (12, 41, and 77%, respectively) than the respective nonchilled controls during the 72-h postchilling period. For seedlings chilled for 96 h, the inhibition of the 1-, 10-, and 20-mm radicles were all significantly different from the controls (46, 92, and 97%, respectively). These data support previous studies that showed a progressive increase in chilling sensitivity as seeds completed germination and radicles elongated (Mangrich and Saltveit, 2000a; Rab and Saltveit, 1996). A chilling period of 72 h was used in all subsequent experiments.

Radicle Elongation in Mannitol Solutions

Radicles with an initial length of 1 mm were able to elongate in aqueous solutions of 0.0 to 1.0 *M* mannitol (Fig. 2A), while elongation of radicles with an initial length of 10 mm was effectively stopped by 0.6 *M* and higher mannitol concentrations (Fig. 2B). After an initial lag, 1-mm radicles elongated at a fairly stable rate for the remainder of the 96-h experiment (Fig. 2A). The lag was

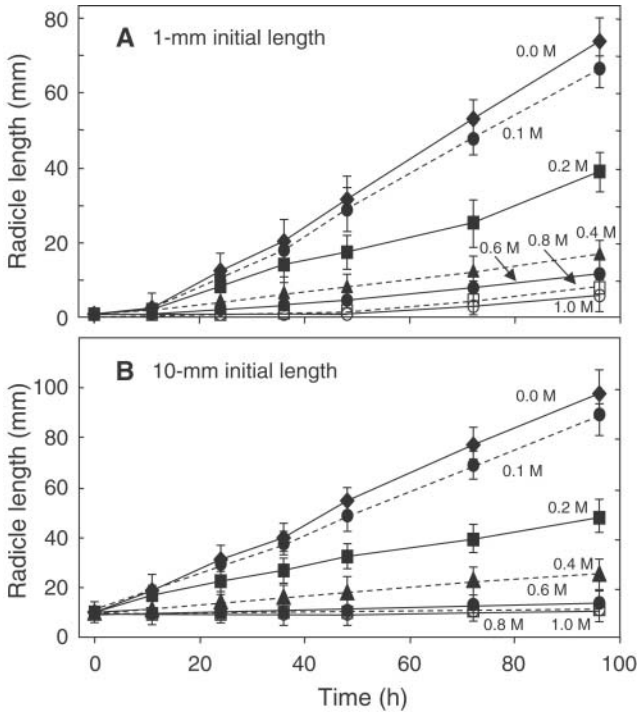

Fig. 2. Elongation of (A) 1-mm-long and (B) 10-mm-long cucumber radicles during continuous exposure to various concentrations of aqueous mannitol solutions at 25°C. The vertical line associated with each mean represents ± SE (n = 12).

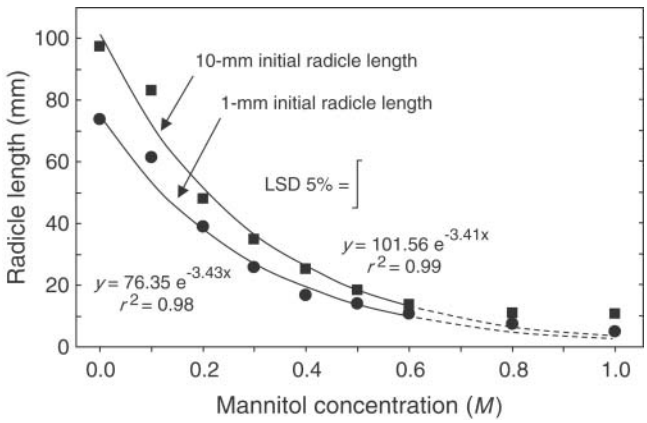

Fig. 3. Length of cucumber radicles initially at the 1- or 10-mm length after 96 h in various concentrations of aqueous mannitol solutions at 25°C. The vertical bar represents the 5% LSD value. An exponential curve was fitted to the data from 0.0 to 0.6 *M* mannitol. The dashed line is the extension of the exponential curve to 1.0 *M* mannitol.

12 h for 1-mm radicles in 0.0 to 0.4 *M* mannitol solutions, 24 h for 0.6 *M*, and 48 h for seedlings in the 0.8 and 1.0 *M* solutions. In contrast, radicles with an initial length of 10 mm showed no lag, but continued to elongate at a fairly constant rate in 0.0 to 0.4 *M* mannitol solutions for the 96 h of the experiment; albeit at a progressively slower rate as the mannitol concentration increased (Fig. 2B). The trauma incurred in moving the seedlings during treatment with the osmotic solutions and repositioning the seedlings after treatment may have contributed to the observed lag in resuming elongation.

The inhibition of elongation was surprisingly consistent across all mannitol concentration for radicles with initial lengths of 1 and 10 mm (Fig. 3). After 96 h, the 1- and 10-mm radicles had elongated to 73 ± 14 and 97 ± 26 mm, respectively. Although the final length of the 10-mm radicles was 33% greater than the 1-mm radicles, the 0.3, 0.4, 0.5 and 0.6 *M* mannitol solutions significantly reduced elongation of both by 66, 74, 82, and 85%, respectively, for radicles with initial lengths of either 1 or 10 mm.

The decrease in the rate of radicle elongation with increasing mannitol concentration from 0.0 to 0.6 *M* was exponential ($r^2 = 0.98$ for 1-mm, and $r^2 = 0.99$ for 10-mm radicles) (Fig. 3). The exponential constant showed that increasing mannitol concentrations produced a similar level of inhibition on the elongation of radicles initially at 10 mm in length (–3.41) and at 1 mm in length (–3.43). Radicle elongation in 0.1, 0.8, and 1.0 *M* mannitol deviated slightly from this general exponential decline. Mannitol concentrations of 0.0, 0.3, 0.5, and 0.6 were selected to further study the effect of reduced radicle elongation on chilling tolerance.

Effect of Mannitol Concentration on Chilling Sensitivity

Because chilling-tolerant 1-mm cucumber radicles became chilling sensitive as they elongated to 10 mm (Fig. 1) after 24 h at 25°C (Fig. 2A), radicles were held for 24 h in various mannitol concentrations to separate the

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effects of elongation from metabolism. A 0.2-h exposure to aqueous 0.0, 0.3, 0.5, or 0.6 *M* mannitol solutions did not significantly reduce subsequent elongation of nonchilled or chilled radicles with initial lengths of either 1 or 10 mm (data not shown). Since there was no statistically significant difference among the 0.0 and 0.3 *M* treatments, and 0.5 and 0.6 *M* treatments for radicles initially at 1 or 10 mm, for radicles treated for 0.2 or 24 h, or for chilled or nonchilled radicles, only data for 0.3 and 0.6 *M* treatments are shown to improve clarity of presentation (Fig. 4).

Chilling 1-mm radicles that had been held for 0.2 h in 0.3 or 0.6 *M* mannitol reduced subsequent elongation by 11 and 22%, respectively (Fig. 4A, 4B). Extending the mannitol treatments to 24 h increased elongation in nonchilled radicles by a small but significant 6% for the 0.3 *M* treatment, while decreasing elongation by 85% for the 0.6 *M* treatment (Fig. 4A, 4C). When chilled, elongation of the 0.3 *M* treated radicles was inhibited 90%, while it was only inhibited 40% in the 0.6 *M* treated radicles (Fig. 4C, 4D). During the 24 h mannitol treatment the 0.3 *M* treated radicles had increased an average of 12.5 mm in length, while the 0.6 *M* treated radicles had only increased 0.7 mm in length (Fig. 4C, 4D).

Chilling 10-mm radicles that had been held for 0.2 h in 0.3 or 0.6 *M* mannitol reduced subsequent elongation by 58 and 60%, respectively (Fig. 4E, 4F). Extending the mannitol treatments to 24 h decreased elongation in nonchilled radicles by 10 and 84% for the 0.3 and 0.6 *M* treatments, respectively (Fig. 4E, 4G). When chilled, elongation of the 0.3 *M* treated radicles was inhibited 99%, while it was only inhibited 33% in the 0.6 *M* treated radicles (Fig. 4G, 4H). During the 24-h mannitol treatment, the 0.3 *M* treated radicles had increased an average of 23.8 mm in length, while the 0.6 *M* treated radicles had only increased 1.1 mm in length (Fig. 4G, 4H).

When the percentage inhibition of chilling-induced radicle elongation data was plotted against the initial radicle length when chilled, most of the data points fell on a smooth curve which extended from a low of 14%

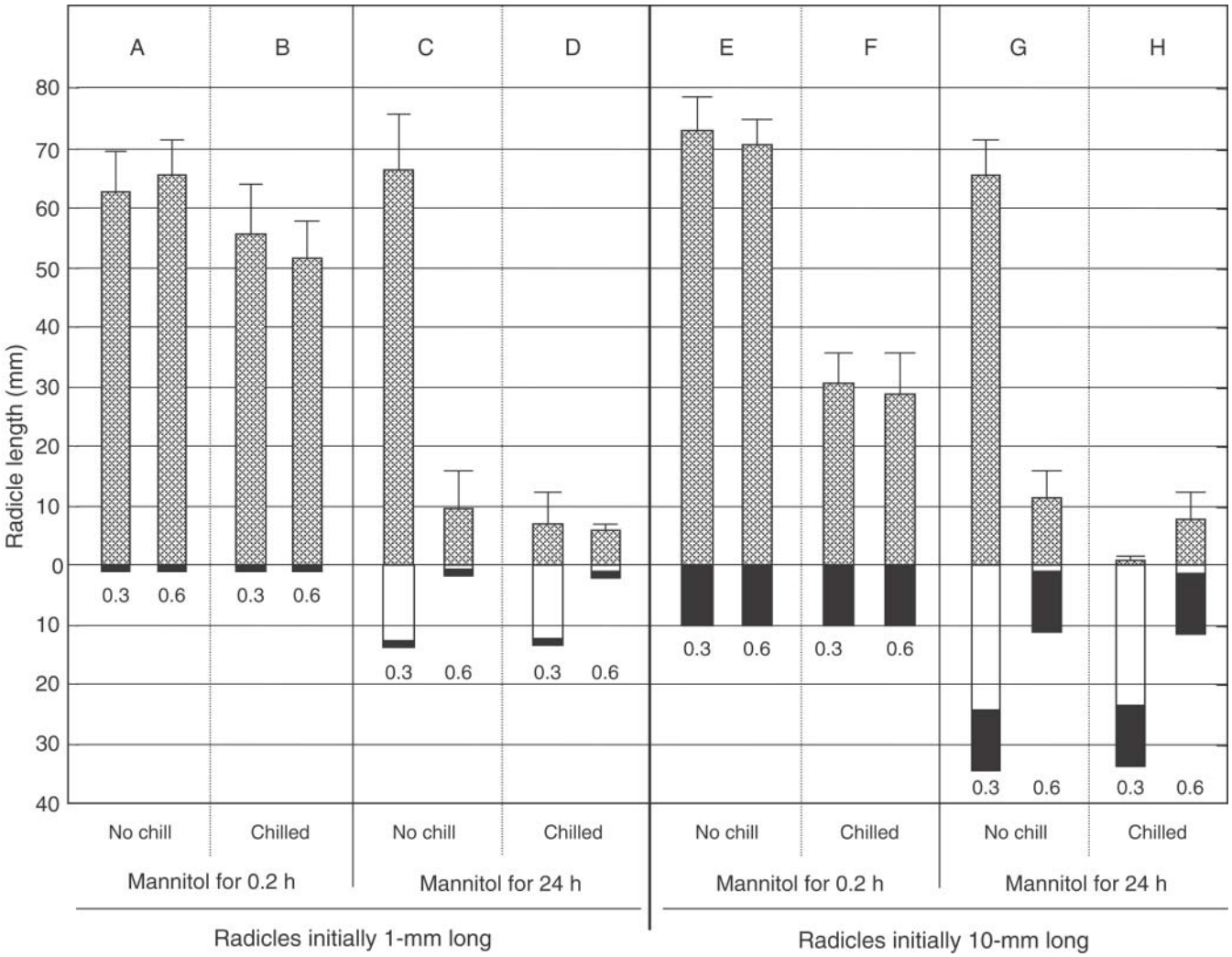

Fig. 4. Elongation of 1- and 10-mm-long cucumber radicles after they were either held in 0.3 or 0.6 *M* mannitol for 0.2 or 24 h at 25°C before being grown at 25°C for 72 h or chilled at 2.5°C for 72 h and then held at 25°C for 72 h. Lengths were adjusted so that 0 represents the length of the radicle when they were moved into 25°C for 72 h of elongation. The portion of the bar above 0 is the elongation that occurred during the final 72 h at 25°C. The solid portion of the bar below 0 is the initial radicle length, while the open bar is the elongation that occurred during the mannitol treatment. The vertical line atop each bar represents ± SE (n = 12).

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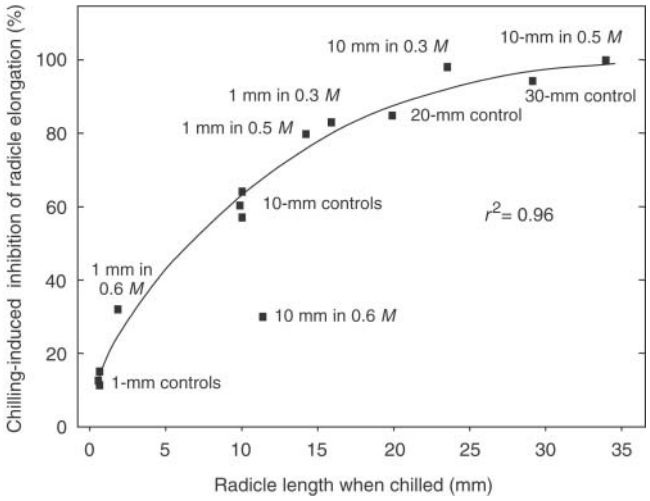

Fig. 5. Effect of radicle length when chilled on the chilling-induced inhibition of subsequent radicle elongation. Points are identified by the radicle length at the beginning of the experiment (1, 10, 20, or 30 mm in length) and the mannitol concentration to which they were exposed before chilling (0.3, 0.5, 0.6 *M*; control is 0 *M*). Points were calculated from data presented in Fig. 4, and other data. Curve of the form $C_L = C_o (1 - e^{-KL})$ fitted to data points (C_o = 100, K = 0.12, L = radicle length when chilled) had an *r* of 0.96.

inhibition (1-mm-long control radicles) to a high of 99% inhibition (10-mm-long radicles that had elongated to 34.1 mm in 0.5 *M* before chilling) (Fig. 5). The curve is of the form $C_L = C_o \times (1 - e^{-KL})$ where K is a constant (0.12), L is the length of the radicle in mm, C_o is 100% inhibition, and C_L is the inhibition of a radicle with length L. The r^2 value for this curve is 0.96. Equations of this form describe many biological reactions where the rate of change is constant and proportional to the amount of reactants present (Nobel, 2005). A good example is the dilution of a substance in a volume expanding at a constant rate, or the metabolism of a compound when its rate of loss is dependent on its concentration.

Effect of Mannitol Concentration on Respiration

Respiration (i.e., production of CO_2) was significantly lower from seedlings held in higher mannitol concentrations (Fig. 6). Holding seedlings with 1-mm radicles for 24 h in mannitol solutions reduced respiration in control radicles from 0.25 ± 0.05 μL CO_2 g^{-1} h^{-1} (i.e., 100%) to 0.10 ± 0.02 (40% of control), 0.07 ± 0.03 (28% of control), and 0.05 ± 0.02 μL CO_2 g^{-1} h^{-1} (20% of control), for 0.3, 0.5, or 0.6 *M*, respectively. A similar significant decline was seen when seedlings with 10-mm radicles were held for 24 h in mannitol solutions. Respiration was reduced from 0.46 ± 0.04 μL CO_2 g^{-1} h^{-1} (100% of 0.0 *M* mannitol control) to 0.21 ± 0.03 (46% of control), 0.15 ± 0.03 (33% of control), and 0.14 ± 0.03 μL CO_2 g^{-1} h^{-1} (30% of control), for 0.3, 0.5, or 0.6 *M*, respectively. Holding seedlings with either 1- or 10-mm radicles in 0.5 or 0.6 *M* mannitol for 24 h did not significantly alter their rate of respiration (28 vs. 20%, and 33 vs. 30%), however, there was a significant change in the chilling sensitivity brought on by these two treatments for both radicle lengths.

Effect of Respiration on Chilling Sensitivity

In 1- and 10-mm radicles, 0.3 and 0.5 *M* mannitol significantly suppressed respiration 34.8 ± 7.1%, and 39.6 ± 7.6%, respectively, while the chilling-induced inhibition of elongation was significantly suppressed 65.0 ± 5.6% and 67.9 ± 3.2%, respectively. In contrast, the 0.6 *M* treatment significantly suppressed respiration 31.0 ± 3.6%, and 21.2 ± 1.6% in the 1- and 10-mm radicles, respectively, while the chilling-induced inhibition of elongation was significantly suppressed 22.2 ± 8.2% and 32.4 ± 5.7%, respectively. The response of the 1- and 10-mm radicles overlapped such that the 0.6 *M* treatment reduced respiration 30% from 37.2 ± 7.3% in all other mannitol concentrations to 26.1 ± 6.1%, while it reduced chilling-induced inhibition by 60% from 66.4 ± 4.5% to 27.3 ± 8.2%. If the rate of metabolism is proportional to the rate of respiration, then the 30% reduction in metabolism cannot account for the observed 60% increase in chilling tolerance.

Possible Link between Radicle Length and Chilling Tolerance

It appears that radicles lose their chilling tolerance at a steadily decreasing rate as they elongate from 1 to 35 mm in length. About half of the chilling tolerance was lost for each 6.5 mm increase in radicle length (Fig. 5). The 24 h treatments in mannitol did not significantly alter this pattern, since the data from most of the treated radicles lay on the regression line according to their length when chilled, and almost completely irrespective of their initial length, or the mannitol treatment to which they were exposed. This loss was independent of the rate of respiration, and except for the 0.6 *M* treatment on 10-mm radicles, independent of prior mannitol treatment. The only treatment that did not fit on the curve was the 10-mm radicles in 0.6 *M* that grew very little (10.0 to 11.3 mm), but exhibited an inhibition of subsequent radicle elongation of only 33% compared with the 67% that would be expected from their length when chilled. The chilling sensitivity of 10-mm-long radicles

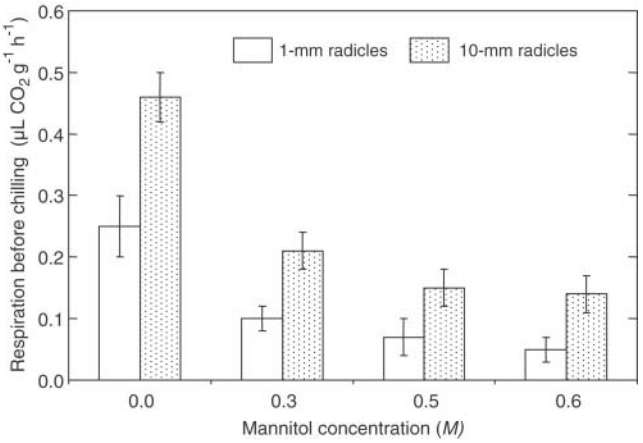

Fig. 6. Relation between the rate of respiration and prior mannitol treatment. Respiration was measured after 1- or 10-mm-long radicles were exposed to 0.0, 0.3, 0.5, or 0.6 *M* mannitol for 24 h at 25°C. The vertical line atop each represents ± SE (*n* = 6).

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held for 24 h in 0.6 *M* mannitol was similar to that of 1.0-mm radicles. Ismail et al. (1997) found that under chilling conditions, the presence of a 35-kDa dehydrin protein was associated with greater maximal percent emergence of chilling-sensitive cowpea [*Vigna unguiculata* (L.) Walp.] seedlings than in a genetically similar line in which the dehydrin protein was absent. If the chilling tolerance of 1-mm-long radicles was the result of dehydrins, then inducing the accumulation of dehydrin-like proteins could account for the increased chilling tolerance.

A number of abiotic stresses, including osmotic, temperature, and oxidative stresses, increase chilling tolerance (Toivonen, 2003). The induced increase in chilling tolerance could be through the induced synthesis and accumulation of stress-induced protective proteins (e.g., dehydrins, heat-shock proteins, and late-embryogenesis abundant proteins) (Collins et al., 1995; Egerton-Warburton et al., 1997; Sabehat et al., 1996; Wehmeyer and Vierling, 2000). Osmotic stresses that reduced radicle elongation are similar to those that induce the synthesis and accumulation of protective stress proteins (Almoguera and Jordano, 1992; Close, 1997).

There appears to be two components to the chilling-induced inhibition of radicle elongation. Mangrich and Saltveit (2000a) showed that the first component caused a linear decrease in subsequent radicle elongation as the duration of chilling increased, and was unaffected by heat shock treatments that increased chilling tolerance. Their analysis showed that a second component appeared after 72 h of chilling and caused an exponential decline in elongation that was completely reversible by the heat-shock treatment. For example, 120 h of chilling reduced subsequent radicle elongation by >95%, but seedlings heat-shocked before chilling had subsequent elongation that would be consistent with the linear decline projected from the response of seedlings chilled for 0 to 72 h. The first, linear component may arise through reactions that exhibit first order kinetics (e.g., oxidative damage to membranes), while the subsequent exponential decline may be enzymatically driven reactions with second-order kinetics (e.g., metabolism of protective compounds in the germinating seed).

We have shown that the kinetics by which chilling tolerance is lost in elongating radicles is consistent with the metabolism or dilution of some protective compound (e.g., dehydrins). Differences in respiration (and presumably metabolism) among the mannitol treatments did not account for differences in chilling tolerance. Without continued synthesis of protective compounds, they would be diluted as the radicles grew (i.e., expanded in volume). The increase in chilling-induced inhibition of radicle elongation (Fig. 5) is consistent with the loss of a protective compound through successive dilution in elongating radicles. Subsequent research has characterized the level of dehydrin-like compounds in cucumber radicles as they elongate, and after treatments that induce chilling tolerance (Kang et al., 2005).

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