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Reduced chilling tolerance in elongating cucumber seedling radicles is related to their reduced antioxidant enzyme and DPPH-radical scavenging activity

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Cucumber seedling radicles become more chilling sensitive as they elongate. Chilling seedlings with radicles 20 mm long for 48 h at 2.5°C inhibited subsequent growth by 36%, while it reduced the growth of 70 mm-long radicles by 63%. Although the growth rate of non-chilled cucumber radicles at 25°C is constant from 20 to 80 mm, tissue viability [i.e. reduction of TTC (2,3,5-triphenyltetrazolium chloride) to formazan] and DPPH (*α,α*-diphenyl-*β*-picrylhydrazyl) radical scavenging activity of apical tissue declines as radicles elongate from 20 to 80 mm in length. TTC reduction, DPPH-radical scavenging activity and protein content of apical tissue were higher in 20 than in 70 mm radicles immediately after chilling and after an additional 48 h of growth at 25°C. Catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11) activity was higher in the apical tissue of 20 than in 70 mm radicles before chilling. Immediately after chilling and after an additional 48 h at 25°C, superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.6.4.2), and guaiacol peroxidase (GPX; EC 1.11.1.7) activity increased more rapidly in 70 mm radicles than in 20 mm radicles (SOD, GR, and GPX activity in 70 mm radicles was 1.5-, 1.9- and 8.6-fold higher, respectively, than in 20 mm radicles). However, APX and CAT activity in 20 mm radicles were always higher than in 70 mm radicles. Growth after chilling enhanced the activity of all antioxidant enzymes compared to that found in non-chilled tissue; however, CAT activity in 70 mm radicles did not recover to levels found in non-chilled tissue. Higher levels of CAT, APX and DPPH-radical scavenging activity are correlated with higher chilling tolerance of 20 mm-long cucumber radicles compared to 70 mm-long radicles.

Introduction

Exposure to non-freezing temperatures below approximately 12°C induces certain physiological disorders in chilling sensitive plants (Saltveit and Morris 1990). Chilling symptoms include growth suppression, abnormal ripening, reduced photosynthetic capacity, necrosis and discoloration, and increased disease susceptibility. Radicle growth after chilling is a sensitive indicator of chilling stress; more sensitive than the frequently used measure of ion leakage (Rab and Saltveit 1996a,1996b). Chilling induced reduction in subsequent radicle growth has been used to compare the chilling tolerance of corn, cucumber, rice and tomato seedlings (Rab and Saltveit 1996b, Mangrich and Saltveit 2000a,2000b, Saltveit 2000).

Chilling increases the level of active oxygen species (AOS) in chilling sensitive plants. AOS are highly reactive and can damage membrane lipids, proteins, and nucleic acids; thus disrupting cellular homeostasis (Scandalios 1993). Plants have both enzymatic and non-enzymatic antioxidant systems to prevent or alleviate the damage from AOS. Several enzymes can efficiently detoxify AOS, whereas superoxide radicals are disproportionately detoxified by superoxide dismutase (SOD). Hydrogen peroxide is destroyed by catalase (CAT) and different kinds of peroxidases such as guaiacol peroxidase (GPX). A major hydrogen peroxide-detoxifying system in plants is the ascorbate-glutathione cycle that includes ascorbate peroxidase (APX) and glutathione reductase (GR) (Asada 1994). Several small antioxidant molecules, such as ascorbic acid, glutathione, *α*-tocopherol, carot-

Abbreviations – APX, ascorbate peroxidase; CAT, Catalase; DPPH, *α,α*-diphenyl-*β*-picrylhydrazyl; GPX, guaiacol peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; TTC, 2,3,5-triphenyltetrazolium chloride.

enoids, and flavonoids, can quench many kinds of AOS (Halliwell and Gutteridge 1989).

A relationship exists between antioxidant enzyme activity and chilling tolerance. The chilling tolerance of rice cultivars is closely linked to the cold stability of CAT and APX (Saruyama and Tanida 1995). Activity of CAT, APX, and MDHAR (mono-dehydroascorbate reductase) may contribute to chilling tolerance at early stages of development in maize (Hodges et al. 1997). Increased activity of antioxidant enzymes, such as SOD, CAT, and APX, in heat shocked tissue enhanced the chilling tolerance of cucumber seedling radicles (Kang and Saltveit 2001). Increased CAT, APX, GR and DPPH-radical scavenging activity after heat shock, and the protection of CAT activity during chilling and subsequent growth in heat shocked rice seedling radicles was correlated with chilling tolerance induced by heat shock (Kang and Saltveit 2002).

Many horticultural crops show differences in chilling sensitivity according to their maturity and stage of growth or development. The stage of fruit ripeness and age of leaf tissue at the time of low-temperature storage significantly influences the plant's sensitivity to developing symptoms of chilling injury (Bramlage 1982, Couey 1982, King and Ludford 1983, Saltveit and Morris 1990). The pre-climacteric stage is generally more sensitive than the post-climacteric stage for avocado, papaya, honeydew melon, tomato, and mango (Robert 1990). The chilling sensitivity of seedling radicles of some chilling sensitive plants increases as they elongate (Rab and Saltveit 1996a,1996b, Mangrich and Saltveit 2000a, 2000b).

The objects of this study were to examine the relationships among antioxidant activity and chilling tolerance in cucumber seedling radicles as they elongate. We show that a correlation exists between increased chilling sensitivity with elongation and the loss of antioxidant capacity.

Materials and methods

Plant material

Cucumber (*Cucumis sativus* L., cv. Poinsett 76-S) seeds were obtained from a local vendor. Two g of seeds was imbibed in 1-l aerated water overnight at 20°C. Imbibed seeds were transferred to moist paper towels overlying capillary cloth and sandwiched between two 15 × 30 cm Plexiglas plates (6-mm thick) that were held together with rubber bands. The plates were held in a vertical position at 25°C in a humid, dark, ethylene-free atmosphere for about 24 h, or until the radicles were about 10 mm long. Germinated seeds with 10 ± 1 mm-long radicles were removed from the large Plexiglas sandwich and gently transferred to moist paper towels overlying capillary cloth and sandwiched between 7 × 30 cm Plexiglas plates (3-mm thick) as before. Each smaller plate held 6 seedlings and was treated as a unit of replication. The plates were positioned vertically in a 20 × 26 × 14 cm tall white translucent plastic tub and covered with aluminium foil. The three temperature treatments were non-chilled (i.e. grown at 25°C in the dark), chilled for 48 h at 2.5°C, and grown at 25°C for 48 h after chilling.

Measurement of chilling injury

The extent of chilling injury of the cucumber seedlings was measured as the subsequent linear growth of the radicles after chilling (Rab and Saltveit 1996a) by a method modified from that previously described (Jennings and Saltveit 1994). Radicle length was measured with a clear ruler to the nearest mm before and after treatment, after chilling, and periodically during growth at 25°C. In some experiments the small plates were disassembled and the radicles gently straightened before measurement. The growth measurements for each seedling were regressed over time and the slope and correlation coefficient calculated. Only data from radicles with linear rates of growth ($r^2 > 0.99$) were used in the analysis.

Preparation of enzyme fraction

Radicle tips (10 ± 3 mm in length) were excised from the seedlings with 20 or 70 mm-long radicles. About 150 tips (0.3 g FW) were homogenized at 4°C in 2.7 ml of extraction buffer (50 m*M* Tris-HCl buffer, pH 7.5, 3 m*M* $MgCl_2$, and 1 m*M* EDTA) with mortar and pestle. The homogenate was then centrifuged at 25 000 *g* for 20 min and the supernatant was used as the crude extract for the five antioxidant enzymes activity assay described below.

Enzyme assays

The activity of APX, CAT, GPX, GR, SOD, and tissue protein content was assayed as previously described (Kang and Saltveit 2001).

Measurement of the DPPH (α,α-diphenyl-β-picrylhydrazyl)-radical scavenging activity

The apical 10 mm of approximately 100 radicles (0.2 g FW) were excised and homogenized at 4°C in 2.0 ml of absolute ethanol with mortar and pestle (i.e. ethanol solution). A 0.5-ml aliquot was mixed with a 0.5-m*M* DPPH ethanol solution (0.25 ml) and 100 m*M* acetate buffer (pH 5.5; 0.5 ml). After standing for 30 min, the absorbance of the mixture was measured at 517 nm (Abe et al. 1998).

Measurement of TTC (2,3,5-triphenyltetrazolium chloride) reduction

The apical 5 mm of approximately 40 radicles (approximately 50 mg FW) was incubated for 24 h in the dark with 2 ml of 0.6% TTC solution that was dissolved in

50 m*M* phosphate buffer (pH 7.4). The radicle tissue was then recovered by filtration through No.1 Whatman filter paper and the water-insoluble red formazan was extracted from the tissue by heating to 80°C for 15 min in 5 ml of 95% (V/V) ethanol. The absorbance of the extract was read at 485 nm using a Shimadzu UV 160 U spectrophotometer (Shimadzu Scientific Instrument, Columbia, MD, USA) and used to calculate the TTC viability index of the radicle tissue (i.e. absorbance at 485 nm/50 mg FW) (Lutts et al. 1996).

Results

The growth rate of 20–80 mm-long radicles at 25°C was not influenced by their initial length (Fig. 1A). However, their growth rate after chilling at 2.5°C for 48 h (Fig. 1B), radicle tissue viability (Fig. 1C), and DPPH-radical scavenging activity (Fig. 1D) were significantly higher in 20 mm-long radicles than in longer radicles. The reductions in these measures (Fig. 1B-D) were greatest between 20 and 60 mm, while the difference between 60 and 80 mm radicles was not significant. We therefore selected radicle lengths of 20 and 70 mm for further experimentation.

The growth kinetics of 20 and 70 mm-long radicles was not different at 25°C (Fig. 2). However, subsequent radicle growth at 25°C was inhibited 36% in radicles chilled for 48 h at 2.5°C when they were 20 mm in length, while the subsequent growth of radicles chilled when they were 70 mm-long was inhibited by 63%.

The TTC viability index remained relatively constant at 0.88 ± 0.04 in the apical 10 mm of 20 mm radicles before chilling, immediately after chilling and after an additional 48 h of growth at 25°C following chilling (Fig. 3A). In contrast, the TTC viability index of 70 mm radicles showed a progressive decline from 0.61 to 0.43 with chilling and to 0.34 with subsequent growth. Because of this, differences in the TTC viability index between 20 and 70 mm-long radicles increased after chilling and during subsequent growth at 25°C. In non-chilled radicles, the TTC viability index of the apical 5 mm of 70 mm-long radicles was 33% lower than for apical tissue from 20 mm-long radicles (Fig. 3A). This difference increased to 51% after chilling and increased further to 61% after 48 h of growth at 25°C.

In non-chilled radicles, DPPH activity in the apical 10 mm of 70 mm-long radicles was 50% of that in 20 mm-long radicles (Fig. 3B). The level of activity in 70 mm radicles decreased to approximately $36 \pm 2\%$ of that for apical tissue from 20 mm radicles assayed either immediately after chilling or after 48 h at 25°C. Unlike the TTC viability index, DPPH activity of chilled 20 mm radicles was 20% less than non-chilled tissue and that reduced level of activity remained relatively constant at $81 \pm 2\%$ of the 20 mm non-chilled control for both treatments involving chilling. The 70 mm radicles behave in a similar fashion, with a 46% drop upon chilling and a relatively constant $29 \pm 2\%$ of the 20 mm non-chilled control level of DPPH activity for the two chilling treatments.

The protein content of the 10 mm radicle tips remained relatively constant before and immediately after chilling at 7.3 ± 0.2 and 6.6 ± 0.2 mg g^{-1} FW for 20 and 70 mm-long radicles, respectively (Fig. 4A). However, during subsequent growth at 25°C, the protein content decreased 11% and 16% from that of non-chilled 20 and 70 mm-long radicles, respectively.

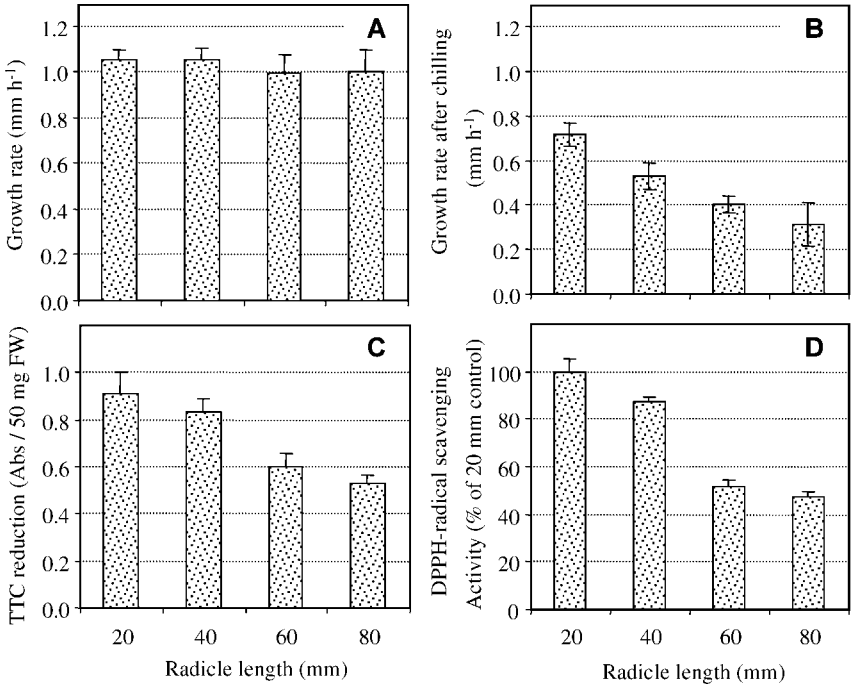

Fig. 1. Effect of radicle length on growth, chilling sensitivity, and measures of tissue viability. Growth rate at 25°C (A), Growth rate after chilling at 2.5°C for 2 days (B), TTC reduction (C), and DPPH-radical scavenging activity (D) of cucumber seedlings radicles according to length at 25°C. Vertical bars represent mean ± SD, n = 6.

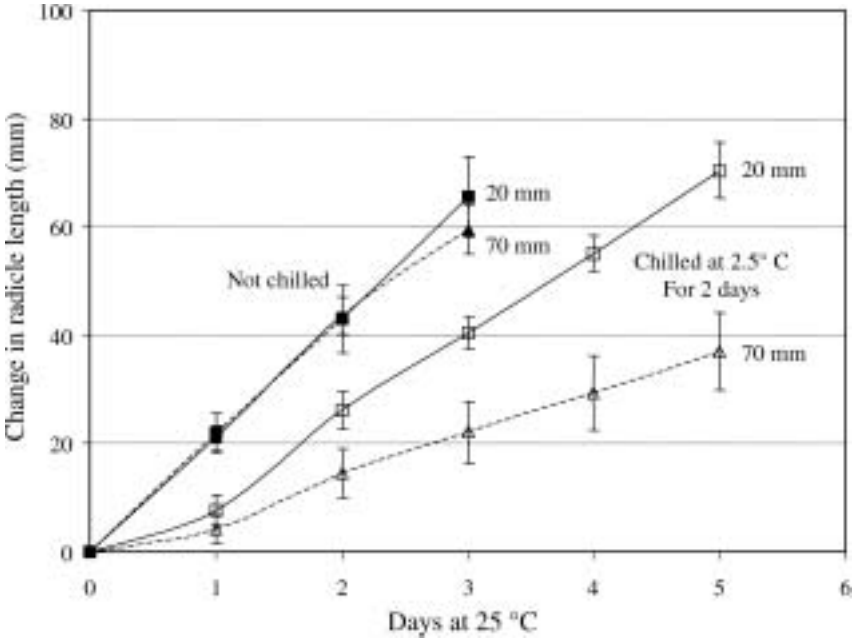

Fig. 2. Change in radicle length of whole cucumber seedlings transferred to 25°C following 48 h of chilling at 2.5°C or not chilled. Radicles were either 20 or 70 mm in length at the start of the experimental treatments. Vertical bars represent mean ± SD, n = 12.

The activity of SOD, CAT and APX were lower in the apical 10 mm of non-chilled 70 mm-long radicles than in 20 mm-long radicles: SOD (80 ± 6%), CAT (75 ± 4%), and APX (68 ± 9%) (Fig. 4B,C,D). This difference between radicle lengths was roughly maintained in the apical tissue immediately after chilling. In contrast, the activity of GR and GPX were similar in tissue from the two radicle lengths before and immediately after chilling (Fig. 4E,F). Only CAT activity was reduced by chilling in both 20 mm (61% of non-chilled) and 70 mm (70% of non-chilled) long radicles. The activity of the other enzymes remained at the same level as the non-chilled radicles.

Growth after chilling had a remarkable effect on the activity of SOD, GR and GPX in the apical 10 mm of 70 mm-long radicles compared to that in non-chilled tissue: SOD increased 206 ± 15%, GR increased 239 ± 4%, and GPX increased 1008 ± 28%. The activity of SOD, CAT and APX was higher in 20 mm than in 70 mm radicles immediately after chilling, but by 48 h after chilling the activities of SOD, GR and GPX were 1.5, 1.9 and 8.6 times higher in 70 mm than in 20 mm-long radicles (Fig. 4B,E,F). Although CAT activity increased in both radicle lengths as they grew at 25°C after chilling, their level of activity did not recover to that of non-chilled controls. Recovery of 70 mm-long radicles (73% of non-chilled) was less than 20 mm radicles (99% of non-chilled) (Fig. 4C). However, APX activity in both radicle lengths showed similar values and patterns in non-chilled and chilled radicles (Fig. 4D). Compared to the 20 mm-long non-chilled radicles, only the activities of SOD and GR increased during the 48 h of growth after chilling at 25°C; (SOD increased 1.4 times, while GR increased 1.2 times) (Fig. 4B,E).

Discussion

The viability of cucumber seedling radicles was measured by two techniques: subsequent growth at 25°C, and

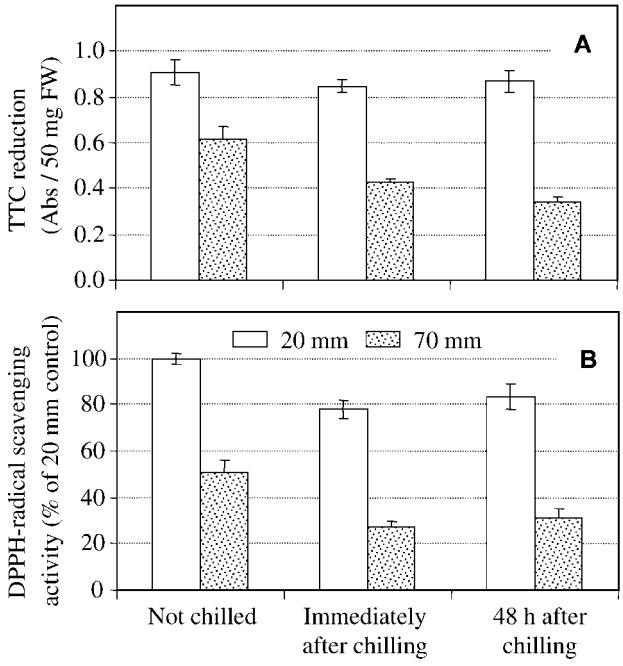

Fig. 3. Effect of radicle length on (A) TTC reduction and (B) DPPH-radical scavenging activity (% of control). Radicles were 20 or 70 mm in length at the start of the experimental treatments; not chilled, chilled at 2.5°C for 2 days, and chilled and then grown at 25°C for 48 h. Vertical bars represent mean ± SD, n = 4.

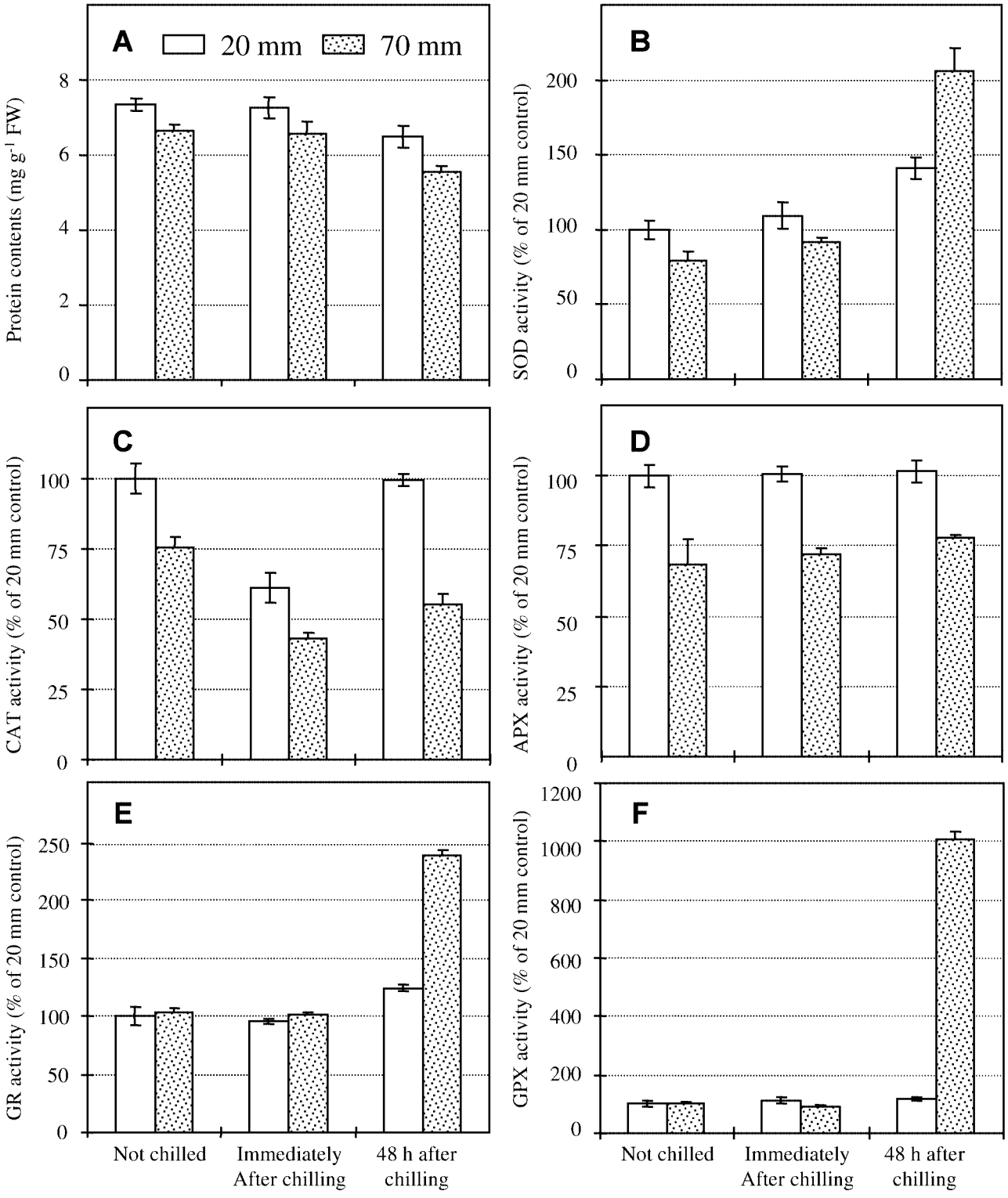

Fig. 4. Effect of radicle length on protein content, and antioxidant enzyme activity of the apical 10 mm of radicles that were 20 or 70 mm-long at the beginning of the treatments. Protein content and activity (A), superoxide dismutase (SOD) (B), catalase (CAT) (C), ascorbate peroxidase (APX) (D), glutathione reductase (GR) (E), and guaiacol peroxidase (GPX) (F). Seedlings were not chilled, chilled at 2.5°C for 2 days, or chilled and grown at 25°C for 48 h. Values are mean ± SD, n = 6.

the TTC viability index. While the growth rate did not change as the radicles elongated from 20 to 70 mm in length (Fig. 1A), the TTC viability index did show a significant reduction in the apical tissue as the radicles elongated (Fig. 1C). TTC reduction has been used to determine tissue viability in freezing tests (Stattin and Lindström 1999), and cold injury tests of horticultural crops (Steponkus and Lanphear 1967). TTC activity is associated with NAD dehydrogenase activity during aerobic respiration of roots (Shimada 1969). However, the growth of *Cattleya* orchid seedlings did not correspond to TTC reduction during experiments with light quality (Islam et al. 1999).

DPPH-radical scavenging activity is a measure of non-enzymatic antioxidant activity. Higher levels of DPPH activity have been correlated with increased chilling tolerance in heat-shocked rice seedling radicles (Kang and Saltveit 2002). There also appears to be a high correlation between DPPH activity in cucumber radicles and the loss of chilling tolerance during elongation (Fig. 3B).

CAT and APX activity was higher in 20 mm-long radicles before chilling than in 70 mm-long radicles (Fig. 4C,D). Saruyama and Tanida (1995) reported that the tolerance of rice cultivars to chilling injury is closely linked to the cold stability of CAT and APX. A chilling tolerant cucumber cultivar showed higher APX activity in leaves than the sensitive cultivar, but CAT activity was similar between the two cultivars (Shen et al. 1999). APX has been reported to be one of the most important antioxidant enzymes in defense against low temperature injury (Saruyama and Tanida 1995, Shen et al. 1999, Kang and Saltveit 2001).

The activity of SOD, GR, and GPX in the more chilling sensitive 70 mm-long radicles rapidly increased during the 48 h of growth at 25°C following chilling (Fig. 4B,E,F). The activity of these three enzymes has been reported to increase after oxidative stress. An increase in SOD mRNA levels was observed after low temperature stress in *Nicotiana plumbaginifolia* (Tsang et al. 1991). Kaminaka et al. (1998) suggested that expression of the rice cytosolic GR gene could be regulated via an ABA-mediated signal transduction pathway during environmental stresses, such as chilling, drought, and salinity. GR activity also increased during chilling and recovery of chilling sensitive maize seedlings (Kocsy et al. 1997). However, exposure to chilling temperatures also increased the activities of GR in chilling tolerant *Arabidopsis thaliana* (Kubo et al. 1999). Transcript levels of GOR2 (a GR cDNA) in chilling tolerant *Pisum sativum*, increased in the recovery (post-stress) phases after both drought and chilling by about 10- and 3-fold, respectively (Stevens et al. 1997). Compared to non-chilled plants, guaiacol peroxidase activity increased 55% in chilled *Coffea arabica* roots (Queiroz et al. 1998).

SOD, GR and GPX also rapidly increased in 70 mm-long radicles during 48 h of growth at 25°C following chilling. Shen et al. (1999) suggested that higher antioxidant enzyme activity in chilling tolerant cultivars, than in sensitive cultivars, during a post-chilling period of growth could be attributed to post chilling oxidative stress responses. Hariyadi and Parkin (1991) also found that peroxidation of thylakoid lipids was induced during the period of growth following chilling, and not during the period of chilling itself. An abrupt increase in H_2O_2 levels occurs after a cold treatment in wheat seedlings (Okuda et al. 1991). Warming chilled tissue to 25°C might stimulate the production of O_2^-, and that could trigger SOD synthesis. During the 48 h of growth at 25°C following chilling, SOD activity increased in both 20 and 70 mm radicles, but the increase was greatest in 70 mm radicles (Fig. 4B). This rise in SOD activity in 70 mm radicles might be more reflective of the damage done to the radicles than an indication of their chilling tolerance since chilling inhibited subsequent growth by 63% in 70 mm radicles, but only 36% in 20 mm radicles. A similar rise occurred in GR and GPX following chilling. The activity of these three antioxidant enzymes appears to be negatively correlated with chilling tolerance in elongating cucumber radicles.

The activities of APX, CAT, and DPPH appear to be positively correlated with chilling tolerance. The DPPH-radical scavenging activity of 70 mm radicles was around half of that in 20 mm radicles for the duration of the experiments. It appears that higher APX, CAT and DPPH-radical scavenging activities, and sustained APX activity during chilled and during subsequent growth at 25°C following chilling in 20 mm-long radicles corresponds with higher chilling tolerance. In contrast, elevated levels of SOD, GR, GPX appear to be correlated with chilling injury since they only showed substantial increases in activity in the more chilling sensitive 70 mm radicles after chilling. Increasing the constitutive or inducible level of APX, CAT, and/or DPPH activity by selection, treatments or genetic engineering could produce plants with superior and persistent chilling tolerance.

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