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Short Communication

Physiological and Metabolic Responses of Winter Wheat to Prolonged Freezing Stress[1]

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ABSTRACT

Survival and cold hardiness declined gradually when cold-hardened Fredrick winter wheat (*Triticum aestivum* L.) was maintained at −6°C for several weeks. Moisture content of crown and root tissue did not change significantly during this period. Uptake of O_2 and accumulation of ^{86}Rb by root tissue declined abruptly upon exposure to −6°C, whereas a concomitant negative effect of freezing on these metabolic processes was not observed in crown tissue. Electron spin resonance spectroscopic analysis of microsomal membrane preparations from crown tissue revealed no evidence of gross changes in the physical properties of the bulk lipids even when seedlings were killed. The results provide biochemical evidence that seedling damage due to prolonged exposure to a mild freezing stress is due to disruption of key metabolic process in the root while cells within the crown remain viable.

Plants which overwinter in temperate climate zones may be exposed to several distinctly different forms of environmental stresses, including freezing, desiccation, ice encasement, and low temperature flooding (1, 12, 17, 25). Controlled environment studies have demonstrated an overall negative effect of interactions among these stresses on survival, cold hardiness, and a number of physiological and biochemical parameters in winter cereals. Tolerance to ice encasement is increased by previous hardening to cold, but both survival and the cold hardiness of surviving plants are reduced by extended exposure to ice encasement at −1°C (1). Brief exposure of winter wheat to low temperature flooding reduces cold hardiness but increases subsequent tolerance to icing stress (2, 3). Freezing to low temperatures (−8 to −10°C) for 1 week decreases subsequent cold hardiness and ice tolerance; however, when the freezing is preceded by low temperature flooding, there is a further decrease in cold hardiness, but an increase in ice tolerance (8). These observations demonstrate the complexity of interactions among different low temperature stresses in winter cereals, and illustrate the inherent difficulties in attempts to define precisely the nature of low temperature stress injury in plants. However, these studies do show that detectable metabolic changes occur well before the applied stresses are sufficiently severe to reduce survival, and that further changes in metabolic activities occur concomitantly with reduced viability.

This paper extends our previous studies of low temperature stress injury in winter cereals by examining the effect of prolonged exposure of seedlings to a freezing temperature (−6°C) to which brief exposure (up to several days) does not elicit visible symptoms of injury. The differential effect of freezing at −6°C on metabolic processes of root and crown tissues also is described.

MATERIALS AND METHODS

Plant Material and Freezing Treatments. Seeds of Fredrick winter wheat (*Triticum aestivum* L.) were sown in potting soil in 10- × 20-cm fiber flats and placed in a growth room at 20°C day (16 h/525 μE m^{-2} s^{-1})/15°C night for 5 d. The flats were then transferred to a cold-acclimating chamber at 2°C day (145 μE m^{-2} s^{-1})/0°C night for 6 weeks.

Plants were washed free of soil under cold tap water, trimmed to approximately 10 cm leaf and 5 cm root, divided into groups of 10, the roots wrapped in moist filter paper, and placed in unsealed plastic bags prior to being transferred to the freezer. The temperature was decreased 1°C/h to −6°C and maintained in the dark up to 5 weeks. Groups of plants were removed from the freezer at various intervals and thawed overnight at 2 to 4°C. Freezing resistance of seedlings was determined as the temperature required to kill 50% of the plants (LT_{50}, °C) following a programmed 1°C/h decrease in temperature (4). Viability of seedlings following freezing treatments was determined by transplanting the seedlings into vermiculite and recording survival after 2 weeks at 20°C day/15°C night. Where survival was reduced by the freezing treatments, cold hardiness values were calculated based on the number of plants shown to have survived the treatment. Moisture content of crown (1-cm nodal shoot segments directly above the epicotyl) and root tissues was obtained by blotting dry tissue segments, determining a fresh weight, drying the tissue for 24 h at 90°C, and reweighing to obtain a dry weight. Moisture content of tissues from seedlings frozen at −6°C for 3 and 5 weeks in sealed bags also was determined, and was not significantly different from that obtained from seedlings in unsealed bags.

Respiration and ESR[2] Spectroscopy. Respiratory activity was determined on crown and root tissues, and mitochondria isolated from crown tissue of seedlings from all stress treatments. Crown and root tissues were cut into 1- to 2-mm segments, weighed, infiltrated with water under reduced pressure for 5 min, and O_2

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[2] Abbreviations: ESR, electron spin resonance; 16NS and 5NS, *N*-oxyl-4, 4-dimethyloxazolidine derivatives of 16- and 5-ketosteric acids, respectively; R.C., respiratory control.

consumption determined at 24°C on 0.1-g lots using a conventional Clark O_2 electrode (18). Mitochondria and a crude microsomal membrane fraction were isolated from all stress treatments. Approximately 3 g fresh weight of tissue was homogenized with a mortar and pestle in a medium containing 0.4 M sucrose, 1mM EDTA, 67 mM KH_2PO_4, 0.75 mg/ml BSA, and 10 mM DTT at pH 7.5. Mitochondria were isolated and O_2 uptake measured polarographically at 24°C with the O_2 electrode (18). Protein was determined by the method of Lowry *et al.* (13).

The 20,000*g* supernatant from which the mitochondria were pelleted was centrifuged at 80,000*g* for 30 min. The sedimented membranes were resuspended in 10 ml of isolation medium, divided into two equal portions, spun at 80,000*g* for 30 min, and resuspended in approximately 200 μl of a medium containing 50% ethylene glycol, 0.1 M Tris-acetate, and 5 mM EDTA at pH 7.2. The membranes were labeled for ESR spectroscopy by incubating for at least 15 min with either 3 μl of 16NS or 6 μl of 5NS (10 mg/ml ethanol) and 2 μl of 50 mM potassium ferricyanide (to prevent reduction of the label) per 100 μl of membrane suspension. Approximately 10 μl of the labeled membrane preparation was drawn into a 50-μl micropipet by capillary action and the end sealed with a lipid-free inert material. ESR spectra were recorded at 0°C with a Bruker ER 200_{++} Spectrometer fitted with a Bruker B-VT-1000 temperature control assembly, using a mid-field set of 3335 G scan range of 100 G, microwave power of 16 mw, and modulation amplitude of 1.6 G. The molecular motion parameter (correlation time) for 16NS was calculated from the spectral data according to the method of Kivelson (11, 14). Motion of the 5NS spin label was determined as the separation of the inner and outer extrema ($2A_{11}$) on the ESR spectra (24).

Uptake of ^{86}Rb. Six groups of five plants from each freezing treatment and from plants killed with Dry Ice were trimmed to 5-cm shoot and 2-cm root and placed in beakers with the roots and lower crowns immersed in 5 ml water containing 2 μCi ^{86}RbCl, and transferred to a growth cabinet at 2°C for 24 h. Preliminary experiments had shown that uptake at 2°C was approximately linear for 48 h, and then the rate of uptake sharply declined. Following the 24 h uptake period, plants were thoroughly rinsed with water, and divided into roots, 1-cm crown segments, and leaves. The tissues were then placed on a shaker in 20 ml water at 2°C and washed for 16 h with two changes of the wash water. Prolonged washing was necessary since preliminary experiment revealed that passive diffusion of ^{86}Rb from both uninjured and dead tissue continued for several hours. The roots and leaves were then cut into 1-cm segments, and tissues were placed in scintillation vials in 10 ml water, and counts determined for 5 min in a Beckman LS 8000 scintillation counter.

RESULTS

Survival and cold hardiness declined gradually when cold-hardened Fredrick winter wheat plants were maintained at −6°C for 5 weeks (Table I). After 3 weeks, survival was reduced to 56% and cold hardiness had declined about 3°C, while after 5 weeks survival was reduced to only 13% and the LT_{50} of surviving seedlings increased to approximately −8°C. Moisture content of crown and root tissue did not change significantly during the 5-week period of exposure to −6°C (Table I). Uptake of O_2 by crown tissue segments declined slightly after only 3 d exposure to −6°C and then remained relatively constant up to 21 d (Table II). After 5 weeks, O_2 uptake dropped sharply, but still remained at about 50% of control level. In contrast, O_2 uptake by root tissue maintained at −6°C decreased markedly after only 3 d and continued to decline with increased duration of exposure to −6°C. After 5 weeks, O_2 consumption had declined to less than 10% of the control level. Uptake of O_2 by mitochondria isolated from crown tissue increased slightly after 3 d exposure to −6°C, concomitant with a loss in respiratory control (Table II). State III respiration then declined sharply afte 7 d and continued to decline for the remainder of the treatment period.

Table I. *Survival, Cold Hardiness (LT_{50}), and Moisture Content of Cold-Hardened Winter Wheat Seedlings following Exposure to −6°C*

Each value represents an average ± SE of at least three replications from three or four experiments.

Exposure to −6°C	Survival	Cold Hardiness	Moisture Content	
			Crown	Root
d	%	°C	%	
0	100	−14.5 ± 0.3	79.9 ± 0.5	83.2 ± 0.8
3	100	−14.2 ± 0.4	80.1 ± 0.6	81.0 ± 0.8
7	90 ± 5	−12.9 ± 0.7	81.4 ± 1.0	82.9 ± 0.9
14	84 ± 3	−11.8 ± 0.4	80.5 ± 0.3	82.6 ± 0.6
21	56 ± 4	−11.4 ± 0.4	81.5 ± 0.3	83.1 ± 0.6
35	13 ± 2	−7.9 ± 0.6	81.2 ± 0.3	81.8 ± 0.8

The effect of freezing stress on the physical properties of cellular membranes was determined by examining motion parameters of nitroxide spin labels intercalated into microsomal membrane preparation isolated from the shoot portions of stressed and unstressed seedlings. ESR spectra obtained from membranes from unstressed seedlings and from those exposed to −6°C for 5 weeks using two different spin labels were virtually identical (spectra not shown). Furthermore, similar spectra were obtained from membrane preparations which had been exposed to liquid N_2 or heated to 100°C. Rotational correlation times of spin label 16NS did not change during the 5-week period of freezing stress, indicating that no major change in the physical properties of the membranes was immediately induced, even by the severely damaging 5-week exposure to −6°C (Table II). Similarly, no significant differences were observed among treatments in hyperfine splitting ($2A_{11}$), a molecular order parameter, using the spin label 5NS (Table II). Rotational correlation times and hyperfine splitting values obtained from membrane preparations exposed to liquid N_2 and 100°C were similar to those obtained from the −6°C treatments.

Uptake and accumulation of ^{86}Rb by the roots of seedlings during 24 h at 2°C declined sharply following brief exposure to −6°C (Fig. 1). After only 3 d at −6°C, uptake was reduced by 50% and continued to decline until leveling off at 75 to 80% inhibition after 1 to 2 weeks. In contrast, accumulation of ^{86}Rb in crowns remained relatively constant up to 2 weeks exposure to −6°C and then increased by 30 to 40% during the next 3 weeks. Translocation of label into the leaves was unexpectedly low even in unstressed plants, and did not change appreciably following exposure to increasing duration of freezing stress (data not shown).

DISCUSSION

The survival of cold-hardened winter cereals under natural and controlled environment conditions is greatly affected by duration and severity of various types of low temperature stresses (2, 8, 10, 16, 19). The results obtained in the present study show that exposure of Fredrick winter wheat to −6°C does not initially reduce survival or cold hardiness of the plant, but that viability and cold hardiness decrease with increasing duration of exposure. An earlier investigation (22) had shown that survival of the hardier winter wheat cultivar Kharkov was unaffected by exposure to −6°C for 5 d, but that survival was reduced by the more severe freezing stresses of −12 and −16°C. Gusta and Fowler (9) reported that plants collected in the fall and stored at −2.5°C maintained the same level of hardiness for 17 weeks. Clearly then, winter cereals can withstand prolonged periods of mild

Table II. *Effect of Duration of Freezing at −6°C on Tissue and Mitochondrial Respiration and Physical Properties of Isolated Microsomal Membranes of Winter Wheat*

The values for state 3 and tissue respiration are averages ± SE of two to four determinations, respectively, from each of two separate experiments. The values for rotational correlation time and hyperfine splitting are averages ± SE of three spectra each from two separate membrane preparations from a single experiment. Similar data were obtained in a second experiment.

Duration	O_2 Uptake				Physical Properties	
	Crown					
	Mitochondria		Tissue	Root tissue	Rotational correlation time	Hyperfine splitting
	State 3	R.C.				
d	*nmol/min·mg protein*		*nmol/min·100 mg fresh wt*		$s \times 10^{-10}$	*g*
0	27.1 ± 2.0	1.8	40.2 ± 0.9	19.2 ± 1.1	11.1 ± 0.2	60.8 ± 0.1
3	31.7 ± 1.6	1.0	34.6 ± 0.4	13.1 ± 0.4	11.0 ± 0.1	61.4 ± 0.2
7	18.6 ± 1.5	1.0	32.7 ± 1.5	3.8 ± 0.4	11.0 ± 0.2	60.9 ± 0.2
14	15.4 ± 1.0	1.0	33.6 ± 1.4	3.0 ± 0.4	11.2 ± 0.1	61.5 ± 0.1
21	9.2 ± 0.5	1.0	31.2 ± 1.2	2.1 ± 0.2	11.3 ± 0.2	61.1 ± 0.1
35	4.5 ± 0.2	1.0	22.0 ± 1.0	1.3 ± 0.3	11.1 ± 0.2	60.9 ± 0.1

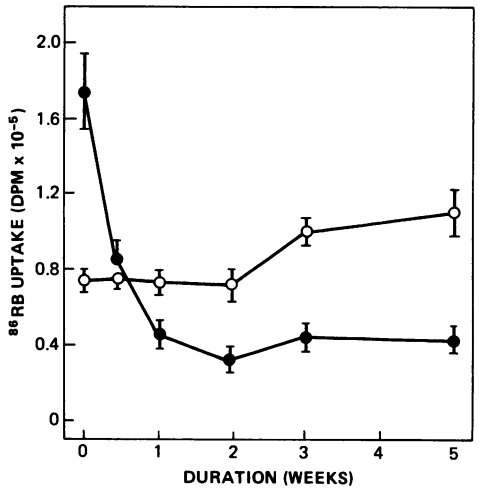

FIG. 1. Uptake and accumulation of ^{86}RbCl by root (●) and crown (○) tissue (0.1 g fresh cut) of cold-hardened winter wheat seedlings following exposure to −6°C. Bars, SE. Uptake and accumulation by root and crown tissue was reduced by 95 and 71%, respectively, following exposure to Dry Ice.

subfreezing temperatures, but damage occurs as the severity and duration of subzero exposure is increased. This could account for wide variations in year to year winter field damage where laboratory estimated LT_{50} temperatures are not reached at crown level, but the duration and magnitude of sublethal freezing temperatures may vary widely.

The decline in survival and cold hardiness induced by prolonged freezing stress was not accompanied by a significant change in moisture content of seedling crowns or roots, and hence it does not appear that observed seedling damage was the result of desiccation stress. Other studies (2, 7, 15) have demonstrated a close correlation between crown moisture levels and freezing resistance, but it has been suggested (6, 25) that changes in cold hardiness are more complex than simply the regulation of tissue water content. Our results support this view, and it is now evident that stress-induced reduction in cold hardiness is frequently accompanied by an increase in water content, but that is not a necessary prerequisite for dehardening.

The limited capacity of roots of winter cereals to develop cold hardiness has been suggested as a major cause of freezing damage to winter cereals (5, 17). This observation has been extended recently by a microscopic study by Tanino and McKersie (26) which examined viability of cells in specific regions of the crown of winter wheat in relation to freezing and icing stress. They observed that the majority of cells in cold-acclimated crowns were still viable after a lethal freezing stress. However, a relatively small number of cells in the vascular transition zone in the basal portion of the crown, critical for root regeneration, were killed. The results obtained in the present study provide biochemical evidence in support of the above observations. The rapid decline in root tissue respiration and uptake of ^{86}Rb during the first few days of exposure to −6°C indicates severe damage to key metabolic processes of root cells. In contrast, the results obtained from crown tissue and mitochondrial respiration, and from ion uptake experiments suggest that major disruption of these metabolic processes does not occur in the crown during the early stages of freezing at −6°C. The absence of any decline in ion uptake by crown tissue even when seedling viability was drastically reduced indicates that a sizable proportion of the crown cells remains viable throughout the freezing treatment, consistent with the observations of Tanino and McKersie (26). The observed increase in ion uptake after 3 and 5 weeks exposure to −6°C is not understood, but may be related to an adaptation mechanism in the uninjured cells. Crown tissue respiration decreased only slightly until 5 weeks exposure to −6°C, thus confirming the viability of at least a major proportion of crown cells. The more rapid decline in O_2 uptake by isolated mitochondria than by tissue segments may be related to deleterious effects of exposure to −6°C that are not manifested until the additional stress of subcellular fractionation is imposed on the mitochrondria.

The data obtained from ESR analysis of microsomal membranes from crown tissue indicated no major change in physical properties of the lipids in these membranes during exposure to −6°C. The spectra of 5NS spin label indicate no differences in molecular ordering in the polar region of the membranes, while 16NS spectra indicate no significant change in order in the hydrocarbon region of the membrane due to stress treatments. Furthermore, the similarity among spectra obtained from membranes isolated from seedlings exposed to various freezing treatments and those obtained from membrane preparations subjected to liquid N_2 or 100°C suggest that no irreversible change in properties of the bulk lipids occurs even when the membranes are severely disrupted. However, since membrane proteins are

not directly involved in these ESR measurements, denaturation of membrane proteins would not necessarily result in altered spectra. An earlier study on wheat (21) revealed that neither membrane lipid fluidity nor transition temperature changed significantly during exposure of seedlings to cold acclimating temperatures, even though structural and functional properties of the membranes are altered during cold hardening. Also, Yoshida (27) recently reported that phase transitions of plant plasma membranes during freezing appeared to depend on membrane proteins, not on the lipids.

The results obtained in this study have shown that prolonged exposure of winter wheat seedlings to a subfreezing temperature (−6°C) to which relatively brief exposure is not visibly damaging, gradually reduces cold hardiness and viability. The observed damage is primarily due to injury to the roots, as evidenced by early inhibition of key metabolic processes, but this damage does not appear to be related to desiccation stress. Previous studies (20, 23) have demonstrated extreme sensitivity of ion uptake by isolated cells to low temperature stresses, and hence the absence of a similar effect on ion accumulation by crown tissue in this study indicates little damage to at least a sizable proportion of these cells during exposure to the stress.

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