Metabolic and Ultrastructural Changes in Winter Wheat during Ice Encasement Under Field Conditions

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ABSTRACT

The effect of ice encasement on the physiological, metabolic, and ultrastructural properties of winter wheat (Triticum aestivum L.) grown under field conditions was examined by artificially encasing winter wheat in ice during early winter. Cold hardiness and survival of ice-encased seedlings declined less rapidly in Kharkov, a cold-hardy cultivar than in Fredrick, a less hardy cultivar. Ethanol did not accumulate in non-iced seedlings, but increased rapidly upon application of an ice sheet. Lactic acid accumulated in both cultivars during late autumn, prior to ice encasement, and elevated levels of lactic acid were maintained throughout the winter in seedlings from both iced and non-iced plots. The rate of O₂ consumption of shoot tissue of seedlings from non-iced plots remained relatively constant throughout the winter, but declined rapidly in seedlings from ice encased plots. Major ultrastructural changes did not occur in shoot apex cells of non-iced winter wheat seedlings during cold hardening under field conditions. However, the imposition of an ice cover in early January resulted in a proliferation of the endoplasmic reticulum membrane system of the cells, frequently resulting in the formation of concentric whorls of membranes, often enclosing cytoplasmic organelles. Electron-dense areas within the cytoplasm which appeared to be associated with the expanded endoplasmic reticulum were also frequently observed.

The cause of overwintering damage to winter cereals and forage crops under field conditions is not completely understood. In geographical areas such as the Western Prairies where winter temperatures are frequently extremely low and snow cover is often sparse, soil temperatures are undoubtcdly low enough to kill many cereal cultivars. In other areas with heavier snow cover, unseasonal thawing of snow followed by refreezing of fully hydrated plants may cause severe damage (16). In more northerly areas, the continuous insulating effect of snow usually prevents soil temperature from dropping much below the freezing point, and yet severe winterkill is frequently encountered. A number of studies (4, 6, 24) have attributed damage under the latter conditions to the formation of ice sheets over the plants as a result of winter thaws or rain. Other investigations (3, 9, 21) have established the deleterious physical effects of ice encasement on plants under laboratory conditions. However, the specific cellular alterations associated with injury under these conditions are not fully understood, although several studies (9, 22) have demonstrated the accumulation of CO₂ in plants during ice encasement. Laboratory investigations have shown that ethanol accumulates in winter cereal seedlings during encasement in ice (2, 5). Mitochondrial respiration declines slowly during icing, but a relatively high rate of respiration is still observed even after 50% of the seedlings have been killed (5).

Electron microscopic studies (20) have shown that the decline in viability of winter wheat seedlings encased in ice at −1 C is accompanied by characteristic ultrastructural changes. ER increases markedly, often resulting in the formation of an elaborate series of parallel membranes, either dispersed randomly throughout the cytoplasm or in the form of concentric whorls. However, the structural integrity of many cellular organelles is largely unaffectcd even by prolonged ice encasement which results in death of all plants. In contrast, exposure of wheat seedlings to near lethal, subfreezing air temperatures results in severe ultrastructural disorganization of all cellular organelles.

The present study was undertaken to examine metabolic and ultrastructural changes associated with ice encasement injury under field conditions.

MATERIALS AND METHODS

Seed of winter wheat (Triticum aestivum L. cv. Kharkov and Fredrick) was sown in plots on September 23, 1975, and September 15, 1976 in a light sandy soil on the Central Experimental Farm, Ottawa, Ontario, Canada. A control plot was unainted while soil was banked up around plots for flooding and ice encasement treatments. In all plots, thermocouples were inserted to 1-cm depth. Two treatment plots were saturated with water using a lawn sprinkler on November 23 and 29, 1976. On January 14, 1976 and January 4, 1977, snow was removed from the plots for ice encasement and water was added using a lawn sprinkler until a solid sheet of ice covered the entire plot. Snow was then shoveled back on to the plots to protect the plants from extremely low temperatures. The second plot saturated with water in November, 1976 was not encased in ice in January, 1977.

Plants were removed periodically (6) from the plots throughout autumn and winter on days when the temperature was not colder than −5 C. The seedlings in frozen blocks of soil were transferred to a cold room at 4 C, and after thawing overnight, survival and cold hardiness were determined (6). The thawed seedlings were washed free of soil, placed in plastic bags and sealed, and put into a freezer at 0 C. The temperature was reduced 1 degree C per hr and seedlings were removed at various temperatures and allowed to thaw overnight at 2 C. Seedlings were then transplanted to Vermiculite at 20 C day (35,000 lux)/15 C night for 2 weeks. LD₅₀ values were interpolated from survival and regrowth results. Plant and soil moisture were determined immediately by removing samples from frozen blocks, and drying seedlings and soil samples to constant weight at 80 C.

Respiratory activity of shoot tissue from untreated and ice-encased plants was determined on the basal 1-cm of seedling shoots. The 1-cm portions were cut into 2-mm segments, weighed, infiltrated with reaction media (19) under reduced pressure for 5 min, and O₂ consumption determined on 0.1-g lots using a Clark O₂ electrode (19). Ethanol content of seedlings removed directly from frozen blocks was determined by the enzymatic procedure of Landquist (14), as previously described (2).
The accumulation of lactate in seedlings was determined by the enzymatic procedure of Hohorst, using lactic dehydrogenase and NAD. Groups of five crowns, each with shoots and roots trimmed to 5 to 7 cm, were ground in a chilled mortar with 5.1% perchloric acid to a final volume of 6 ml. This was centrifuged at 5,000g for 10 min, and 1-ml aliquots of the supernatant were neutralized, sealed, and stored in the freezer up to 1 week for analysis (10). A control, including all components of the reaction mixture except the enzyme lactic dehydrogenase, was assayed with each sample.

For electron microscopy, shoot apices were dissected directly into fresh fixatives, either immediately after sampling or as soon as the samples had thawed. Fixation was carried out at 4 C either in aqueous 2% KMnO4 for 4 hr, or in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) for 4 hr followed by postfixation for 16 hr in 2% osmic acid in the same buffer. The fixed material was washed, dehydrated in acetone, embedded in Epon (13), sectioned, stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

RESULTS

The results obtained in the first year (1975–1976) of this study are presented in Figure 1 and Table I. Survival and cold hardiness (as measured by LD50 temperature) of both cultivars decreased slightly during late winter in the non-iced plot, whereas marked decreases in these parameters were observed in seedlings from the iced plots. By late February, survival of ice-encased Kharkov was reduced to 60% and hardiness had declined from -22 C to -12.6 C, while survival of the cold-hardy Fredrick was reduced to 20% and hardiness had decreased from -16 C to -7.1 C. The level of ethanol in seedling tissues increased substantially during encasement in ice, while shoot respiration decreased in ice-encased seedlings. These preliminary findings suggested that major changes occur in the physiology and biochemistry of winter wheat seedlings during encasement in ice under field conditions. It was decided, therefore, to conduct a second and more comprehensive examination of this phenomenon during the winter of 1976 to 1977.

Soil moisture content declined steadily throughout the winter months of 1976 to 1977 in the untreated plot, from a high of about 30% in November to a low of 13% in mid-February (Table II). It then increased in March with initiation of the spring thaw and melting of snow. In the ice encasement plot, which was flooded in November and iced in January, soil moisture remained relatively constant at about 35% throughout the winter (Table II). The large difference in soil moisture between the treated and untreated plots was probably due to the unusual absence of a significant amount of rain or thawing during this particular winter.

The per cent plant moisture of both cultivars decreased abruptly during autumn as a result of nearly a doubling of the dry matter of the seedlings (Table II). These reduced levels of moisture were maintained throughout the winter, and did not decrease further with decreasing soil moisture in the untreated plot. In the iced plot where soil moisture was greater, seedling moisture also was consistently slightly higher than in the control plot.

Overwintering injury of winter wheats was unusually low during the winter of 1976 to 1977. In the non-iced plot (Table II), and in a plot flooded in November but not iced in January (data not shown), greater than 90% survival of both Kharkov and Fredrick was observed. The application of an ice sheet, however, induced severe injury to plants of both cultivars. Injury occurred more rapidly in the less hardy Fredrick than in Kharkov, but by the end of March, survival of both cultivars was reduced to less than 10%. Cold hardiness of winter wheats also was reduced markedly by exposure to ice encasement treatment (Fig. 2). The hardiness of Fredrick in the plots flooded in November declined significantly even prior to application of the ice sheet, whereas the hardiness of Kharkov was not reduced significantly by this treatment. Cold hardiness decreased rapidly after icing and by mid-winter for Fredrick and late winter for Kharkov, hardiness of surviving seedlings of both cultivars was reduced practically to zero. The hardiness of seedlings in the control plot increased rapidly during autumn, was maintained at a high level throughout early winter, and then declined during late winter and early spring (Fig. 2).

Ethanol did not accumulate in seedlings of either Kharkov or Fredrick in the non-iced treatments during autumn and winter of 1976 to 1977 (Table II). However, ethanol content of seedlings increased rapidly upon application of an ice sheet, and attained a maximum level in mid-February. It then decreased during March, with initiation of the spring thaw. Lactic acid also accumulated in overwintering wheat seedlings, but considerable variation in its levels was observed throughout the winter (Table III). During early autumn, lactic acid was not detected in the seedlings of either cultivar, but an appreciable increase in concentration was observed even before the plots were flooded in November. This elevated level was maintained throughout January with slightly higher values in the iced than non-iced plots, but by mid-February, lactic acid had decreased in all treatments except ice-encased Fredrick which was severely damaged at this sampling time. Throughout the remainder of the winter and early spring, the level of lactic acid in all treatments remained lower than the maximum attained winter values.

The effects of overwintering on the rate of O2 consumption of

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Cultivar</th>
<th>Survival</th>
<th>LD50 Temperature (°C)</th>
<th>Ethanol Accumulation (mg/g)</th>
<th>Oxygen Consumption (mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan 13/76</td>
<td>Kharkov</td>
<td>100</td>
<td>-22.0</td>
<td>0.17</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Feb 24/76</td>
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<td>100</td>
<td>-18.5</td>
<td>b</td>
<td>2.1</td>
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<tr>
<td></td>
<td>Fredrick</td>
<td>(1) 60</td>
<td>-12.6</td>
<td>1.17</td>
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<tr>
<td></td>
<td></td>
<td>(1) 90</td>
<td>-15.2</td>
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<td></td>
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<td>(1) 20</td>
<td>-7.1</td>
<td>1.01</td>
<td>1.6</td>
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</table>

* N denotes non-iced; I denotes iced. Data not obtained.
seedling shoot tissue from non-iced and ice-encased plots were similar for Kharkov and Fredrick (Table III). O₂ uptake in seedlings from the untreated plot remained relatively constant throughout the sampling period with an average of 0.16 ± 0.08 mg/g fresh wt. in March, which is typical of wheat plants during this period. The O₂ uptake in seedlings from the flooded plot was significantly higher, with a mean of 0.20 ± 0.12 mg/g fresh wt. in March, indicating a more active metabolism in the flooded environment.

The O₂ uptake in seedlings from the iced plot was also significantly higher than in the non-iced plots. This is likely due to the increased metabolic activity associated with the cold stress imposed by icing. The O₂ uptake in seedlings from the iced plot was 0.28 ± 0.20 mg/g fresh wt. in March, indicating a more active metabolism in the iced environment.

These results suggest that the ice encasement and flooding treatments imposed on the seedlings during the winter period had a significant impact on their metabolism and survival. The flooding treatment resulted in a slight increase in O₂ uptake compared to the non-iced treatment, while the iced treatment resulted in a more significant increase in O₂ uptake, indicating a more active metabolism in response to the cold stress.

In conclusion, the data presented in this study demonstrate the importance of understanding the metabolic responses of seedlings to environmental stresses such as flooding and icing during the winter period. These responses can have significant implications for the survival and growth of plants in the spring, and further investigation is needed to fully understand the mechanisms underlying these responses.

Fig. 2. Change in cold hardness (LD₅₀°C) of Kharkov and Fredrick winter wheat under natural field conditions and after artificial flooding and ice encasement during 1976 to 1977.
Fig. 3 and 4. General structural features of apical cells of cold-hardened Kharkov winter wheat obtained from untreated field plots on November 16, 1976. Fig. 3: fixation in glutaraldehyde-osmic acid (× 7,000). Fig. 4: fixation in KMnO₄ (× 9,900). (cw): cell wall; (d): dictyosome; (er): endoplasmic reticulum; (l): lipid body; (m): mitochondrion; (n): nucleus; (p): plastid; (v): vacuole.

Fig. 5, 6, and 7. Comparison of ultrastructural features of apical cells of Kharkov seedlings obtained on February 15, 1977, from untreated field plots (Fig. 5, × 8,000) and from plots flooded on November 29, 1976, and encased in ice on January 5, 1977 (Figs. 6, × 9,900, and 7, × 8,000). Fixation in KMnO₄. (cw): cell wall; (ds): dense structure; (er): endoplasmic reticulum; (l): lipid body; (p): plastid; (ve): vesicles.

Fig. 8. Ultrastructure of apical cells of Kharkov seedlings obtained from an ice-encased field plot on March 8, 1977. Fixation in KMnO₄ (× 9,900). (cw): cell wall; (er): endoplasmic reticulum; (m): mitochondrion; (n): nucleus; (p): plastid.
DISCUSSION

The winter of 1976 to 1977 was unusual in the study area in that the normally expected winter thaw or rain did not occur, leading to nearly 100% survival in the control plots, and in other plantings of cold-adapted wheat cultivars. In contrast, survival in adjacent plots which were artificially iced in January was reduced to less than 10%. The observations are generally in accord with earlier findings (4), but in the present study, the excellent negative correlation observed between survival and duration of ice encasement was undoubtedly due to a more effective icing treatment than was obtained in the earlier experiments.

The level of soil moisture in the experimental plots did not appear to have a significant effect on survival of winter wheat during 1976 to 1977. Although soil moisture decreased markedly in the untreated plot and remained high throughout the winter in the flooded plots, survival in both the control and flooded plots was greater than 90% for both Kharkov and Fredrick. In contrast, survival in the iced plot was reduced to very low levels. These observations suggest that overwintering injury under ice and snow cover may not be related directly to soil moisture level, but rather to biochemical changes associated with exposure of the plants to partial or total anaerobiosis. The accumulation of products of anaerobic respiration during exposure of plants to ice encasement or flooding has been postulated to account for the observed injury of plants subjected to these stresses. Encasement of winter cereal seedlings in ice under controlled environment conditions results in a rapid accumulation of ethanol (2), CO₂ (21, 22), and lactate (unpublished data) within the tissues. Ethanol and lactate also accumulate during exposure of several species of plants to flooding (7, 23), a condition which necessarily precludes ice encasement. In the present investigation, ethanol content of the tissues increased only in seedlings which were subjected to anoxia by encasement in ice and its level decreased abruptly, concomitant with initiation of the spring thaw when the plants were no longer completely enclosed in ice. In contrast, lactate levels increased markedly in seedlings from the untreated plot by mid-November, prior to exposure to conditions which would induce high levels of anaerobic respiration. The environmental stress inducing the increased lactate levels is not known but probably involves low temperature and elevated soil moisture levels, normally occurring during late fall. However, the accumulated lactate of early winter is not toxic to the plants, but the high level of lactate in ice-encased Fredrick in late winter is associated with almost total kill of these plants. Ethanol, which accumulates only in response to ice encasement, is present at consistently high levels throughout the ice encasement period and may be associated with the eventual death of plants of both cultivars. Other studies on several plant systems have also shown that lactate is formed more rapidly than ethanol in response to anaerobiosis (8, 12).

The absence of significant change in the rate of O₂ consumption of seedling shoot tissue from the untreated plot throughout the sampling period is consistent with previously reported (5) results from controlled environment experiments. In those studies, no significant difference in the rate of respiration was observed among four cereal cultivars of contrasting cold hardiness grown under hardening and nonhardening conditions. The quantity of mitochondrial protein was similar in seedlings grown at 2 and 24°C, indicating that the quantity of mitochondria in the cells is not altered during cold acclimation. These observations suggest that respiratory capacity of mitochondria remains constant in field-grown seedlings throughout autumn and winter.

The decline in aerobic respiration of shoot tissue from ice-encased seedlings is in accord with that observed in isolated mitochondria from winter wheat encased in ice under controlled environment conditions (5). In contrast with the results obtained with mitochondria where O₂ uptake continued to decline with increasing injury due to ice encasement, shoot respiration appeared to increase markedly during late winter while field survival continued to decrease. This apparent anomaly is not fully understood, but may be interpreted in one of two ways. Ice encasement induced increase in O₂ consumption by nonrespiratory processes in dead and injured cells may be stimulated upon thawing of the plants in spring. Alternatively, the observed increase in O₂ uptake could be due to an increase in the rate of normal respiratory processes of cells not killed by the icing treatment, since in this study no attempt was made to determine the extent of cellular injury associated with death of the plants.

The ultrastructural changes associated with ice encasement in the field are similar to those observed under controlled environment conditions (20). Proliferation of ER and the formation of parallel arrays and concentric whorls of ER membranes occurred to a greater extent under controlled environment conditions, possibly due to more complete ice encasement than can be obtained under field conditions. The dense regions associated with the concentric whorls were more prominent in the field-collected material than in the controlled environment material, but the relationship of these structures to the membranes is uncertain. Other studies on many species of plants have shown that the formation of parallel arrays and concentric whorls of ER can be induced by many types of stress, including mechanical effects, reduced O₂ tension, CO₂ atmosphere (25), dehydration (15), anaerobiosis (1), chilling (17), and inhibition of respiration (18). Kimball and Salisbury (11) also observed the proliferation of RER during exposure of three grass species to low temperature. This effect was not observed in the present study or in a previous investigation (20) on winter wheat seedlings grown at 2°C under controlled conditions. The precise function of the proliferated ER is not known, although it has been suggested that it may be an expression of the adaptive mechanism protecting the cells against anoxia (18), or involved in repair processes within stress-damaged cells (1).

The results of this study have clearly shown that prolonged ice encasement under field conditions is highly lethal to winter wheat. It was also demonstrated that ice encasement induces major changes in cellular metabolism and ultrastructure, including inhibition of aerobic respiration, accumulation of ethanol, and proliferation of ER. The agent or combination of agents responsible for overwintering injury to winter cereals under ice sheets was not identified, although the greatly elevated levels of ethanol suggest that it may be involved in the processes leading to ice encasement injury.

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