Plant Physiology, March 2000, Vol. 122, pp. 861–865, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Thermal Effect of CO_2 on Apoplastic Ice in Rye and Oat during Freezing

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Meristematic tissues from rye (*Secale cereale*) and oat (*Avena sativa*) were studied in an isothermal calorimeter at −3°C. When the frozen tissue was placed in the calorimeter, the pressure increased within 4 d to 25 and 9 kPa above ambient pressure in the sample vessels containing crowns of rye and oat, respectively. Concurrently, the thermal output went down to −194 μW in rye over the 4-d period; this negative thermal activity could be accounted for by ice melting in the plants. When the pressure was released, the output from the calorimeter went from −194 to 229 μW within 1 h, suggesting that water had frozen in the plants. We propose that CO_2 from respiration had dissolved in the water in the plants and caused melting of ice (heat absorption) due to the colligative properties of solutions. When the pressure was released, the CO_2 came out of solution and the water froze (heat evolution). These thermal observations were duplicated in a simplified, nonbiological system using a glycol/water mixture that was partially frozen at −3°C.

Mechanisms used by plants to counter stresses during freezing are interactive and therefore are very difficult to characterize. Biochemical and biophysical adaptation of plants that occur at below-freezing temperatures but before injury occurs (second phase of hardening, 2PH) have been reported (Trunova, 1965; Steponkus, 1978; Olien, 1984; Livingston, 1996; Livingston and Henson, 1998). These adaptations conferred hardiness to 2PH plants by allowing them to survive stresses that plants hardened only at above-freezing temperatures (first phase of hardening, 1PH) could not withstand (Olien, 1984).

Ice formation in plants begins in the apoplast and must remain there if injury to the plant is to be avoided. Griffith et al. (1997) reported an increase in proteins with antifreeze activity in the apoplastic fluid from rye (*Secale cereale*) leaves that had been cold hardened at above-freezing temperatures. They suggested that these proteins provided protection down to freezing temperatures, and that other protective mechanisms allowed the plant to survive lower temperatures.

Olien (1973, 1974, 1977, 1984) described a form of freezing stress called adhesion that occurs at around −10°C. Adhesion is the result of a slow rate of freezing such that very small displacements from equilibrium occur. Under these freezing conditions, the advancing ice lattice, upon reaching the vicinity of the cell wall, competes with it for the intervening liquid water; this competition causes adhesion between ice and the cell wall or between the cell wall and the plasmalemma. As the protoplast shrinks during freezing, adhesions to it can cause damage that results in the death of the plant (Olien, 1977).

Adhesive stress may be relieved through the release of solutes, presumably from the hydrolysis of fructan (Olien, 1984). This would virtually convert adhesive stress to osmotic stress, as melting increases the amount of interfacial liquid. Solute release into the apoplast during 2PH was reported in rye, barley, and oat (*Avena sativa*), and was correlated with an increase in freezing tolerance (Olien, 1984; Livingston and Henson, 1998). Olien (1992) calculated that the rate of heat absorption (melting of ice) by frozen rye crowns corresponded to the rate of fructan hydrolysis in terms of the melting (from the sugars produced by fructan hydrolysis) required to maintain a solution with a freezing point of −3°C.

The calorimetric measurements previously reported (Olien, 1992) were conducted in closed sample vessels. Depending on respiratory rates at −3°C, CO_2 production could affect the partial pressure in sample vessels. This would increase CO_2 solubility in water, which could reduce plant survival since high CO_2 has been shown to be toxic to some plants (Andrews and Pomeroy, 1991). In this study we wanted to see how pressure changes in sample vessels would affect thermal output and plant survival during 2PH in rye compared with the less-hardy winter cereal oat.

MATERIALS AND METHODS

Plant Culture

Seeds of rye (*Secale cereale* cv Rosen) and oat (*Avena sativa* cv Wintok) were grown as described previously (Livingston and Henson, 1998). Plants were grown for 5 weeks at 13°C day/10°C night temperatures with a 12-h photoperiod. Five weeks after planting, plants were transferred to a chamber at 3°C with a 12-h photoperiod. Plants remained under these conditions for 3 weeks; this constituted 1PH. After 1PH, plants were removed from tubes and washed free of planting medium in ice water. Roots were trimmed

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to 2 cm, shoots to 10 cm, and both were subjected to the experiments described below.

Thermal Analysis

Plants

The rye and oat crowns were studied in a Calvet isothermal calorimeter (Setaram MS 80, Lyon, France)[1] with four sample vessels, each with a volume of 12 cm. The signal from the calorimeter was recorded on a strip-chart recorder, and areas under curves were measured using a hand-held planimeter (to calculate joules). The average of five measurements (less than 3% variation between measurements) was used in all calculations. When the calorimeter was set to its full sensitivity (Seebeck circuit), 1 μV of output from the calorimeter equaled a 17.6 μW displacement from baseline and, 1 J corresponded to 41 cm^2 measured on the strip-chart recorder.

The lids of the sample vessels were fitted with a silicone/Teflon septum that permitted insertion of a 1-m-long needle (distance from access port to sample vessel) to sample pressure without disturbing the thermal output of the calorimeter by more than 10 μW.

Four grams (fresh weight) of crown tissue was placed in each sample vessel. Vessels were kept open and plant material was frozen in a freezer at −3°C. Within 3 h, vessels were sealed and placed into the calorimeter that was also at −3°C.

After 2 d, samples had attained a steady thermal output. At d 4, a pressure gauge (DPI 701, Druck, New Fairfield, CT) was used to sample the pressure of each vessel. The pressure gauge was attached to the end of the needle and the needle was pushed through the septum on top of the sample vessel. The total volume of the needle and pressure gauge was 0.15 cm^3. The volume of the vessels (taking plant material into account) was 8 cm^3. Therefore, the percentage volume change upon inserting the needle was an increase of 1.9%. This means that the pressures in Figure 1 and Figure 2 as well as those in the text are 1.9% higher than reported.

Once the pressure had been recorded, the pressure was reduced in each sample vessel at a rate of 0.25 kPa per min. In preliminary experiments this rate had a minimal effect on the thermal output of empty vessels.

Plants under 1 atm reached a new equilibrium state within a day. After 4 d at 1 atm (8 d total at −3°C), plants were removed and transplanted in soil to evaluate regrowth.

Water/Glycol

Four grams of 5% (v/v) ethylene glycol/water and 1 g of #3 filter paper (Whatman, Clifton, NJ) were placed in an empty sample vessel and frozen at −3°C. The vessel was then placed into the calorimeter that was also at −3°C. After the sample had equilibrated, pure CO_2 was passed through the vessel and the vessel was pressurized to 5 kPa (0.05 atm) above ambient pressure. This pressure was held until the thermal output stabilized. After recording the output, the pressure was released and the thermal output was again recorded. Identical experiments were repeated using pure N_2, pure CO_2 in an empty vessel, and pure CO_2 over pure ethylene glycol/filter paper.

[1] Mention of a proprietary product does not constitute an endorsement nor recommendation for its use by the U.S. Department of Agriculture.

RESULTS AND DISCUSSION

Thermal Stabilization

Samples reached thermal equilibrium in 2 d after inserting into the calorimeter (Fig. 1). The change in thermal activity in the first 2 d was from three sources: (a) dissipation of mechanical heat (from inserting the vessel into the calorimeter); (b) heat flow into and out of plants and the vessel as their temperature equilibrated with the calorimeter; and (c) biochemical changes occurring in the plants within the first 2 d (Livingston and Henson, 1998). Empty sample vessels usually equilibrated in about 6 h, while the most rapid biochemical changes occurred in the first day or two when plants were transferred from 3°C to −3°C (D.P. Livingston, C.R. Olien, and R. Premakumar, unpublished data). Therefore, most of the large decrease in net thermal activity between d 1 and 2 was probably due to biochemical and biophysical adaptation by the plant.

Once equilibrated, the net thermal output of the oat samples remained at about 0 J d^{-1}, while the rye samples remained at about −12.6 J d^{-1}. This is very close to the −11.5 J d^{-1} reported by Olien (1992) in rye plants that had been cold hardened at 2°C for 3 weeks under high light.

The most prominent sources of thermal activity in frozen plants are respiratory heat (Hansen et al., 1998), water freezing (exothermic), and ice melting (endothermic). Ice can be melted at an ice/liquid interface in plants at −3°C when solutes dissolve in the liquid and lower its freezing point. When winter cereals were exposed to −3°C for 3 d,

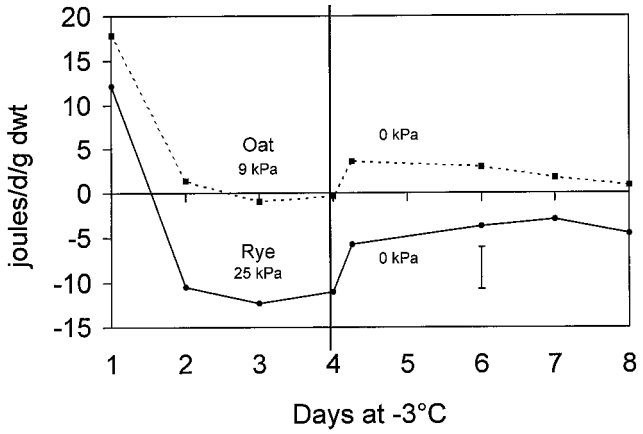

Figure 1. Thermal output in joules per day from rye and oats at −3°C. Each point is the mean of five replications. The vertical line at d 4 is the point at which the pressure in sample vessels was released. The above-ambient pressure produced by oat and rye after 4 d is given in kPa under the labels. The error bar represents the LSD at $P = 0.05$.

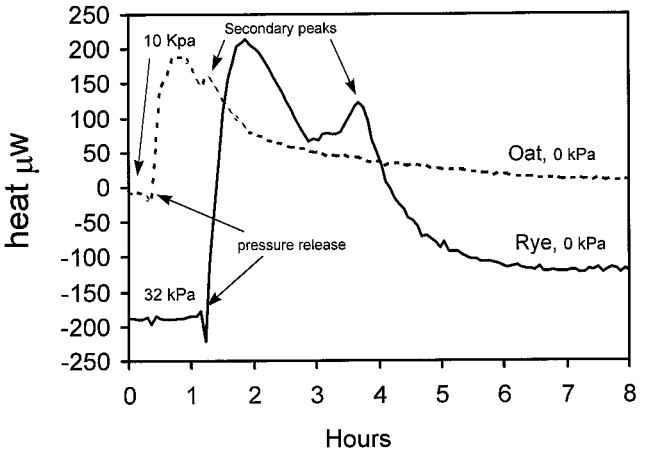

Figure 2. Detailed view of the thermal effect from the released pressure at d 4 in Figure 1 from one experiment. The area under the curve for rye corresponds to 3.4 J, and the area under the curve for oat to 0.54 J. Secondary peaks to the right of the major peaks are probably a result of additional melting from the release of trapped CO_2.

rye had a more rapid increase in simple sugars than barley or oat and was about the same as wheat (Livingston, 1996). If the amount of sugar increase at −3°C is related to the amount of ice melted, it would at least partially explain the more negative thermal output of rye than that of oat (Fig. 1).

CO_2 Effects on Thermal Activity

When air movement is restricted, such as ice encasement in the field during winter, CO_2 levels were reportedly high enough to be toxic to some plants (Andrews and Pomeroy, 1991). Rakitina (1970) reported that CO_2 levels in wheat plants under ice were as high as 44% of the total gas measured.

The solubility of CO_2 in water increases under pressure, but it is not known how much of the elevated CO_2 concentration in plants under ice encasement is due to effects of pressure. To measure pressure changes in the atmosphere surrounding plants that had been frozen, the vessels containing plant samples were sealed with a rubber septum; this allowed us to measure the pressure during 2PH with a relatively minor disruption to thermal activity. Vessels were subsequently returned to ambient pressure at a rate that had very little effect (<10 μW) on thermal activity in empty vessels.

After 4 d at −3°C, the average (of five replicates) pressure in the vessel containing oats and rye was 9 and 25 kPa, respectively (Fig. 1). The increase in pressure suggests the possibility of at least some level of anaerobic respiration, since a respiratory quotient (CO_2 produced: O_2 used) of 1, as in aerobic respiration, would presumably result in no increase of pressure.

When the pressure was released, a distinct exothermic event occurred in both oat and rye (Fig. 2). This exothermic effect could be explained if water in the plants had frozen when the pressure was released. We suggest that CO_2 from respiration had dissolved in the liquid water in the plants and caused ice at the ice/liquid interface to melt due to the colligative properties of solutions; this effect was enhanced by preventing CO_2 diffusion from the system and allowing pressure to increase over a 4-d period. Ice melting would induce an endothermic event, and if the heat absorption from ice melting was greater than the heat evolution from respiration, it would result in the net negative output at d 2, 3, and 4, as was seen in rye (Fig. 1). Since very little heat is produced in anaerobic respiration compared with aerobic respiration, it is doubtful that this exothermic source of heat would override the endothermic effect of ice melting.

We also suggest that when the pressure was released, some of the dissolved CO_2 came out of solution, raising the freezing point of the liquid. This resulted in freezing, which released the heat, shown in Figure 2. This phenomenon would be analogous to placing an unopened bottle of a carbonated beverage in a freezer. One observes that the beverage fails to freeze between −3°C and −5°C until the top of the bottle is opened and dissolved CO_2 is out-gassed. At this point the beverage rapidly freezes.

The areas under the curves shown in Figure 2 corresponded to 3.4 J (±0.1 J) for rye and 1.2 J (±0.1 J) for oat. If this heat was generated solely by water freezing, then, based on the heat of fusion of water (335 J d^{-1} H_2O), 10.2 mg of water froze in rye and 3.6 mg in oat when the pressure in the sample vessel was released. The solubility of CO_2 in water at 0°C and 1 atm is 0.076 mol in 1 L of water (Budavari, 1989). This amount of CO_2 would melt about 47 g of ice at −3°C. We measured only a fraction of this amount of melting. Therefore, CO_2 did not reach its full capacity to melt ice under our experimental conditions. It is possible that the pH of the liquid in the plants was not favorable for maximum formation of carbonates and subsequent solubility of CO_2.

Considering that the total water content of oat was 4,930 mg and rye 3,913 mg (from fresh/dry weight measurements), the amount of water apparently melted by CO_2 represents less than 1% of the total water in either oat or rye. The biological significance of a change in less than 1% of the liquid water could be argued, but we have calculated that the thickness of the water layer surrounding the crown cells that would have melted and subsequently frozen when the pressure was released was an average of 1,768 water layers. The following assumptions were made in the calculations: (a) the 20 oat crowns used in the analysis were cylindrical and of uniform size (height = 20 mm, diameter = 6 mm); (b) all the cells in the crown were spheres with a diameter of 0.01 mm; (c) the 3.6 mg of water freezing in the 20 oat crowns had a volume of 3,600 mm^3; and (d) a liquid water mono-layer is 3 Å thick. (Morrison and Dzieciuch, 1959).

Olien (1974) reported that a liquid water layer 6 monomers thick would result in strong adhesions, and that no adhesions were present with a liquid water layer 15 monomers thick. Therefore, if adhesions were present in plant crowns, then a change in the liquid water layer of 1,768 monomers would be more than adequate to relieve this form of stress. However, since this is an average, it is certainly possible that no change occurred in some regions of the crown, while in other regions the change was higher than 1,768 monomers.

Non-Biological Simulation

To simulate the effect of pressure and its release on thermal output from plants, 5% (v/v) ethylene glycol/water and filter paper were frozen at −3°C and inserted into the calorimeter. The atmosphere above the frozen water was flushed with pure CO_2, and the vessel was pressurized to 5 kPa (Fig. 3). The thermal output went below zero (Fig. 3), indicating heat absorption from ice melting. When all the ice that could had melted, the thermal output slowly returned to zero. When the pressure was subsequently released, the thermal output went above zero, which is characteristic of heat evolution from water freezing, similar to that observed in oat and rye. When the experiment was repeated using N_2 (Fig. 3) or pure CO_2 in an empty vessel (not shown) or pure CO_2 with pure ethylene glycol/filter paper (not shown), no thermal activity was observed. The difference in thermal activity between CO_2 and N_2 is presumably due to a difference in water solubility. CO_2 is approximately 80 times more soluble in water than is N_2 (Lide, 1995). Since this was a non-biological system containing only CO_2 (or N_2), water/ice/ethylene glycol/filter paper, the only explanation for the observed thermal activity is the melting of ice due to dissolution of CO_2 in water when the vessel was pressurized (heat absorption), and the freezing of liquid water (heat evolution) when the pressure was released and CO_2 was out-gassed.

The heat of solution of CO_2 in water (−20 kJ/mole) was not considered to be a significant error in the system for two reasons: (a) the heat resulting from pressurizing an empty, CO_2-filled vessel was nearly identical to the heat resulting from pressurizing a CO_2-filled vessel with liquid water supercooled at −3°C (data not shown); the difference between the two measurements could be considered an approximation of the heat of solution and (b) the area under the melting curve in Figure 3 corresponded to 0.54 J, which could result from the melting of 1.6 mg of water. Assuming that CO_2 acted purely colligatively and the heat of solution is negligible (see "a" above) this would mean that 2.6 μmol of CO_2 had dissolved in the water to cause the melting observed in Figure 1. The heat of solution from this amount of CO_2 would have an insignificant effect on the heat of fusion from water melting.

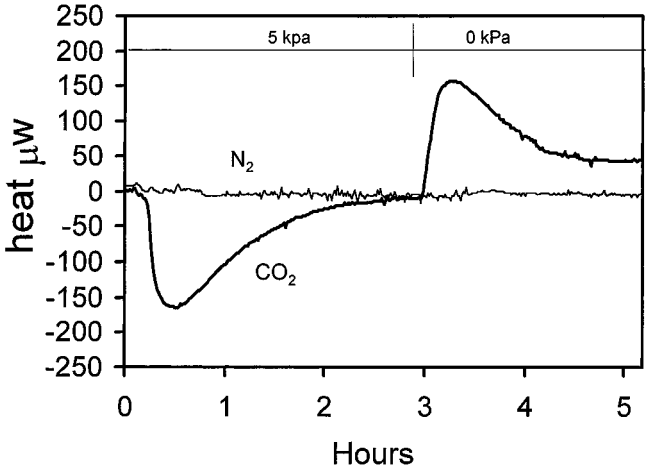

Figure 3. The thermal effect on 4.0 g of 5% (v/v) ethylene glycol/water/filter paper under pure CO_2 and pure N_2 at 2.5 kPa and ambient pressure at −3°C.

Post Pressure Release

After the pressure was released, plants re-equilibrated at a higher thermal level (Fig. 1). The difference between the pressurized and non-pressurized condition was 7.1 J d^{-1} g^{-1} dry weight in rye and about 2.9 J d^{-1} g^{-1} dry weight in oat. This corresponds to an average of 21 mg of ice melting/freezing per day in rye and 9 mg d^{-1} in oat. It is possible that CO_2 gradually induced more ice to melt as the pressure increased, resulting in the 7.1 J d^{-1} absorption of heat in rye. The continued negative thermal output in rye after the pressure was released may have been due to sugar-induced ice melting, since sugars continued to increase in rye plants up to 7 d at −3°C (D.P. Livingston, C.R. Olien, and R. Premakumar, unpublished data). Therefore, the net endothermic activity observed before the release of pressure may be accounted for by ice melting, but the melting may have occurred from CO_2 as well as from sugar dissolving in the water. We are attempting to determine the separate contributions of CO_2 and sugar to the apparent melting.

Another explanation for the continued endothermic activity of plants after the pressure was released is that not all the dissolved CO_2 was out-gassed when the pressure was released (Fig. 3). In the non-biological system, the thermal output did not return to zero after the pressure was released unless vacuum was applied (not shown).

The extent to which this phenomenon may be a protective mechanism in plants that are tolerant of high CO_2 levels is not known. Since ice can restrict gas diffusion (Andrews, 1996) from plants and the solubility of CO_2 increases as the temperature decreases, it is possible that CO_2 levels could be high enough, even under ambient pressures, to cause significant dissolution in water. This would reduce the freezing point of the liquid water at an ice/liquid interface and could induce ice melting similar to that proposed for sugars (Olien, 1992).

CO_2 Toxicity

It is likely, however, that toxic effects caused by high CO_2 counteract any protective effect from ice melting, particularly in oat. When rye and oat plants were frozen at −3°C under normal air for 8 d, both had 100% survival (Table I). When kept in closed vessels (pressure was allowed to increase) for 3 d at −3°C, rye survived as well as it did under normal air but oat was completely killed. In fact, oat could not survive 3 d at −3°C under pure CO_2 at ambient pressure (Table I). It did, however, survive −3°C for 3 d under pure N_2 at ambient pressure, albeit in a more damaged condition than under normal air (Table I). This suggests that toxic effects from CO_2 were more important than damage associated with the lack of O_2, which agrees with results cited by Andrews and Pomeroy (1991) for isolated wheat cells.

Table I. *Visual assessment of re-growth of oat and rye following indicated treatments*

Plants were exposed to the treatments for 4 d. Scale = 0, Dead; 5, undamaged. Each value is the mean of 10 plants.

Treatment	Temperature	Oat	Rye
Normal air	−3°C	5.0	5.0
Closed cells	−3°C	0.0	5.0
Pure CO_2	−3°C	0.0	5.0
Pure N_2	−3°C	1.8	5.0
Pure CO_2	1°C	1.9	nd[a]
Pure N_2	1°C	4.0	nd

[a] nd, Not determined.

When exposed to 1°C under pure CO_2 for 3 d, oat plants were more damaged than normal but did survive (Table I). The presence of ice at −3°C may have restricted CO_2 diffusion enough to increase tissue CO_2 to toxic levels and/or the higher solubility of CO_2 at −3°C may have been enough to induce toxic tissue CO_2 levels. It is also possible that while high CO_2 is obviously toxic to cv Wintok oat, it may have been a combination of damage from CO_2 and damage from ice that caused death at −3°C under pure CO_2.

Differences between oat and rye that make rye more tolerant of high CO_2 are not known. Uemura and Steponkus (1994) reported a difference in the plasma membrane lipid composition between oat and rye leaves. While this may not be related to the difference in CO_2 tolerance, it indicates at least some level of difference in membrane composition between the two species. Andrews and Pomeroy (1991) suggested that membrane ATPase inhibition by carbonate and bicarbonate ions (resulting from CO_2 dissolution under ice encasement) caused membrane dysfunction during freezing. If true, then a difference in the tolerance of membrane ATPases to carbonate and bicarbonate ions may partially explain the difference between rye and oat in their tolerance to CO_2.

CONCLUSION

The increased pressure during exposure to −3°C in a sealed system appeared to induce ice melting in plants and in a non-biological system; the subsequent release of that pressure seemingly induced freezing. While the thermal effect was much lower in oat than in rye, the treatment was lethal to oat but had no effect on the regrowth of rye. It is possible that the low level of freezing tolerance in oat is at least partly due to CO_2 toxicity from reduced gas diffusion and/or increased solubility in plants that are frozen. It is also possible that in addition to the effects of CO_2 toxicity, the increased liquid water content from melting by CO_2 dissolution caused severe damage in oats when it froze rapidly after the pressure was released. Altering liquid water content while plants are frozen by changing air pressure may help in understanding the complex interaction of water/ice with plant tissues and how plants withstand stresses induced at below-freezing temperatures.

Received July 26, 1999; accepted November 1, 1999.

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