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Toxicity of Anaerobic Metabolites Accumulating in Winter Wheat Seedlings during Ice Encasement[1]

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ABSTRACT

Ice encasement damages cold-hardened winter wheat without major disruption of cellular organelles. CO_2 accumulates during total ice encasement to higher levels in Kharkov than in less hardy Fredrick wheat. Partial ice encasement and exposure to a nitrogen atmosphere at −1 C allows greater CO_2 accumulation but neither treatment is as damaging as total ice encasement. Lactic acid accumulates to low levels only during the 1st day of encasement and thereafter remains constant. Exposure of plants to a combination of 50% CO_2 and 5% ethanol reduces survival, with a cultivar difference similar to that found in ice-encased plants. Plants in CO_2 and ethanol show a proliferation of membranes and nuclear condensation similar to that in cells of ice-encased plants. Permeability increases markedly in the presence of CO_2 and ethanol, to levels similar to or greater than those of iced plants. Ethanol alone does not increase permeability but in combination with CO_2 raises permeability of the less hardy Fredrick, although not of Kharkov, but reduces survival of both cultivars. A comparison of the endogenous levels of ethanol, CO_2, and lactic acid at the 50% kill point of plants due to ice encasement or due to externally supplied metabolite indicates that only CO_2 accumulates to independently toxic levels. Permeability and ultrastructural evidence suggest that CO_2 and ethanol in combination are the agents reducing plant viability during ice encasement.

Damage to winter cereal plants during ice encasement at mild subfreezing temperatures is associated with a decline in cold hardiness (2) and a gradual decrease in mitochondrial aerobic respiration (3). Mitochondrial activity and structure are retained after plants are dead, indicating that mitochondrial membranes are not the primary site of damage. As aerobic respiratory capacity decreases, products of anaerobiosis accumulate. Ethanol levels increase but not to levels that are independently toxic (1), while CO_2 has been shown to accumulate but without estimation of its toxicity (20). Both metabolites change cellular permeability, but the molecular mechanisms are not known (7, 10, 23). Lactic acid is a major metabolite of animal systems, but also accumulates to low levels in a wide range of plant species under anaerobic stress (21).

Ice encasement damage is not accompanied by major disruptions of cellular ultrastructure (18). The maintenance of structure of at least the major cellular components demonstrates that cells are not destroyed during encasement by intracellular ice, or by strong dehydrative stresses that may develop in the presence of extracellular ice at lower freezing temperatures. Ultrastructural changes which do occur within cells of ice-encased plants involve the proliferation of cytoplasmic membranes and the formation of membrane whorls which are believed to be of ER origin (18). These membranes persist after whole plants have lost the ability to regrow after treatment but whether or not they are involved in cellular damage is not clear.

We here report the accumulation of CO_2 and lactic acid in ice-encased tissues, and relate the effects of these metabolites, and ethanol, to the survival and ultrastructure of winter wheat plants.

MATERIALS AND METHODS

Plant Material. Cold-hardened, light-grown seedlings of winter wheat (*Triticum aestivum* L.), cvs. Kharkov and Fredrick were used in this investigation. Two hundred seeds, sown in potting soil in fiber flats (10 × 20 cm), were grown for 5 days at 20 C day/15 C night with a 16-h day of 35,000 lux. Flats were then transferred to 2 C day 0 C night with a 16-h day of 25,000 lux. After 7 weeks the LD_{50}[2] in a programed 1 C/h temperature decrease was −22 C for Kharkov and −17.5 C for Fredrick. In preparation for further testing, plants were washed free of soil, endosperms removed, and roots trimmed to 1 cm and shoots to 2 cm.

CO_2 Analysis. Groups of 10 plants were placed in 35-ml septum bottles with 1 or 20 ml of glass-distilled H_2O. Those in 20 ml of water were either left with their cut tips just emerged from the water (partially encased) or were totally submerged. Bottles were placed at −1 C, and after freezing of the water, filled with N_2 and immediately capped with septum stoppers. After various periods at −1 C, 0.5-ml samples were withdrawn by syringe from the nonencased and partly encased units and injected into a CO_2 analyzer. Totally encased units were allowed to thaw for 3 to 4 h with intermittent agitation to allow CO_2 to diffuse from the plants, acidified with 0.5 ml 1 N H_2SO_4 to release CO_2 from solution, and the gas phase sampled a further 30 min with agitation. Samples were injected into a N_2 stream entering a Beckman 864 infrared CO_2 analyzer at a flow rate of 500 ml/min and deflections related to standard CO_2 samples. Results were adjusted to account for residual CO_2 dissolved in water. This proportion was obtained by determining in a similar experimental arrangement the disappearance of CO_2 from a known amount of CO_2 injected into the gas phase over acidified water. Under these conditions, CO_2 dissolved in water increased linearly at a ratio of 0.97:1 with increasing concentration in the gas phase up to 0.4 mg/ml. Fresh weights of plants were recorded after CO_2 analysis and thawing, and groups of 10 plants transplanted to moist Vermiculite to determine survival. Numbers and heights of live plants were recorded after 2 weeks at 20 C day/15 C night with a 16-h day of 35,000 lux, and with daily supply of Hoagland nutrient solution.

Lactic Acid Analysis. Lactic acid was determined enzymically using lactate dehydrogenase and NAD (9). Groups of five plants were totally immersed in 3 ml of water in disposable culture tubes, placed at −1 C, seeded with an ice chip, and capped. After various periods of ice encasement, tubes were thawed, plants were weighed, ground in 6 ml of 5.1% perchloric acid, centrifuged, neutralized, and immediately analyzed. Thaw water also was

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[2] Abbreviation: LD_{50}: median lethal dose.

analyzed. A control with all components of the reaction mixture except the enzyme was run with each sample. Other groups of five plants were similarly ice encased and transplanted to determine survival.

Toxicity of Metabolites. Groups of 10 plants were placed in 20-ml vials with 4 ml of 0.5 M phosphate buffer (pH 7.0) containing 0, 5, or 10% ethanol with 0, 0.25, or 0.5% lactic acid. Vials were placed in desiccators, evacuated, and refilled with air alone, 50% CO_2 + 50% air, or 100% CO_2, to give various combinations or environment. Ice-encased plants were included as controls. Desiccators were chilled to −1 C, and the gas phase adjusted periodically during chilling with the relevant gas mixture to maintain atmospheric pressure. After 1 week desiccators were warmed, ice was thawed, and washed plants transferred to moist Vermiculite to determine survival.

In a further series of experiments, plants were exposed to concentrations of lactic acid from 0 to 1.0%, and their survival and endogenous lactate determined after 1 week at −1 C. Thus an LD_{50} of exogenous lactate concentration was determined and the endogenous lactate concentration at the LD_{50} was interpolated. This could then be compared with the lactate concentration at the LD_{50} due to ice encasement, following the procedure previously outlined in detail for ethanol (1). A similar comparison was made between the endogenous CO_2 derived from external application, and that derived from metabolic pathways during ice encasement. To determine endogenous CO_2, plants exposed to various concentrations of the gas were rapidly submerged in 20 ml water in septum bottles with a N_2 atmosphere, capped, maintained in the cold for 3 to 4 h with agitation, acidified, and after 30 min, the gas phase sampled by syringe and injected into the Beckman analyzer. Adjustments for solubility of CO_2 in water were made as described above.

Permeability Measurements. Plants were exposed to concentrations of CO_2 and water with or without 5% ethanol for 1 week at −1 C, together with iced controls. After thawing of the iced treatment and killing of a sample in liquid nitrogen, plants were transferred to 20 ml water in Erlenmeyer flasks and shaken for 24 h at 2 C. As a measure of relative cell permeability, material leached out of the plants was analyzed for ninhydrin-positive substances (22), and for total electrolytes, as conductivity measured on a wheatstone bridge.

Ultrastructure. Plants were taken from environments of CO_2, ethanol, and lactic acid described above, the apices dissected into aqueous 2% $KMnO_4$ and fixed for 4 h at 4 C. The fixed material was washed, dehydrated in acetone and embedded in Epon (14). Sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

RESULTS

CO_2 Accumulation. Plants of both hardy Kharkov and the less hardy Fredrick winter wheats accumulated CO_2 when totally encased in ice at −1 C, but at different rates (Fig. 1). Fredrick consistently yielded less CO_2 than Kharkov, but even at the earliest sampling time fewer live plants contributed to CO_2 evolution. Survival declined more rapidly in Fredrick than in Kharkov. Plants partially emerged from the ice were killed less rapidly and produced more CO_2 (Table I) than totally encased plants. An ice-less anaerobic system (N_2 atmosphere) showed a slower killing rate, higher CO_2 levels, and a production of CO_2 that was similar in the two cultivars for 3 weeks of treatment (Table I). The lower levels of CO_2 obtained from the totally encased plants in comparison with those partially encased were associated with the more rapid death during total ice encasement.

Lactic Acid Accumulation. An alternate end product of respiration of plants under anaerobic conditions is lactic acid. This metabolite accumulated to a low level during the 1st day of encasement, and thereafter remained relatively stable as viability

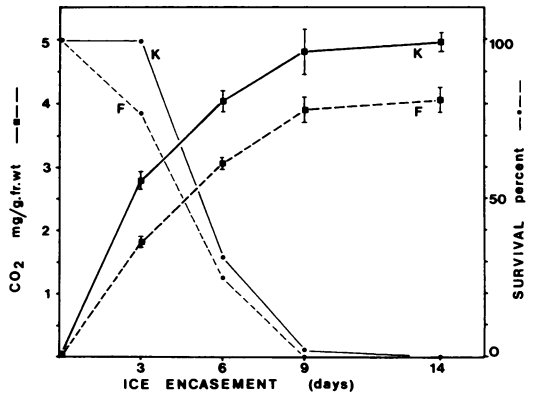


FIG. 1. Accumulation of CO_2 and survival of seedlings of Fredrick (F) and Kharkov (K) winter wheats during total ice encasement at −1 C. Plants cold-hardened for 6.5 weeks.

Table I. Accumulation of CO_2 and survival of seedlings of Frederick and Kharkov winter wheat during partial ice encasement and during exposure to a nitrogen atmosphere at -1 C. Plants were cold-hardened for 8 weeks

Cultivar	Time of exposure	Partial ice encasement Survival	Partial ice encasement CO_2	Nitrogen atmosphere Survival	Nitrogen atmosphere CO_2
	days	%	mg/g fr wt	%	mg/g fr wt
Fredrick	3	64	*1.1 ± .16	100	†2.6 ± .05
	7	20	2.7 ± .18	100	4.9 ± .18
	10	16	4.8 ± .46	92	7.3 ± .24
	14	14	6.8 ± .14	80	9.8 ± .33
	21	10	8.4 ± .52	63	13.1 ± .76
Kharkov	3	94	1.0 ± .14	100	2.7 ± .09
	7	44	3.2 ± .16	100	4.8 ± .18
	10	44	5.2 ± .20	97	6.7 ± .74
	14	26	8.2 ± .18	97	9.9 ± .18
	21	22	10.1 ± .34	95	13.3 ± .25

*Values are a mean of 5 replicates ± standard error.
†Values are a mean of 4 replicates.

declined (Table II). There was no consistent difference in lactate concentrations produced by the two cultivars, although Fredrick seedlings declined in viability faster than those of Kharkov.

Toxicity of Metabolites. Cold-hardened plants exposed to combinations of ethanol, CO_2, and lactic acid showed varying degrees of survival (Table III). Each component alone reduced survival more at high than at low concentration, and generally, survival was reduced more when two components were applied together at low concentration. Only when all three components were present at low concentrations did damage occur at a level similar to that in ice, but in this condition the differential survival between the two cultivars was not demonstrated. This differential survival was simulated in several combinations of CO_2 without lactate, suggesting that CO_2 may be a major toxicant to plants at −1 C. Exposure of plants to a N_2 atmosphere for 1 week at −1 C caused no survival reduction in Fredrick or Kharkov.

CO_2 increased cell permeability of cold-hardened wheat at concentrations lower than those which caused decreased survival (Table IV). The survival of Kharkov plants in ice and in 100% CO_2 remained high in this experiment because of the young age and relatively high cold hardiness of the plants. Ethanol alone did not reduce survival or change permeability but ethanol in combination with CO_2 reduced survival considerably in both cultivars and contributed to greater cell leakage in Fredrick though not in Kharkov. In Fredrick, permeability and survival of CO_2 + ethanol-treated plants was similar to that of the iced treatment. In contrast, permeability and damage of Kharkov plants were higher from metabolite-treated tissues than iced tissues. The higher survival of seedlings treated with CO_2 alone was accompanied by high permeability values and may indicate that hardy cells tolerate CO_2-enhanced membrane permeability, while permeability induced by CO_2 and ethanol is more damaging, leading to increased

Table II. Accumulation of lactic acid and survival of cold-hardened seedlings of Fredrick and Kharkov winter wheats during total ice encasement at -1 C

Cultivar	days of ice encasement						
	0	1	2	4	7	10	14
	lactic acid mg/g fr wt						
Fredrick	*0.11 ± .03	0.66 ± .06	0.73 ± .05	0.61 ± .02	0.69 ± .05	0.80 ± .08	0.66 ± .03
Kharkov	0.11 ± .01	0.64 ± .06	0.68 ± .05	0.58 ± .06	0.64 ± .07	0.75 ± .05	0.74 ± .05
	survival %						
Fredrick	100	73	57	43	23	0	0
Kharkov	100	90	80	73	30	7	7

*Values are means of two experiments with plants cold-hardened for 6.5 and 9 weeks, each with 4 replications.

Table III. Survival of cold-hardened seedlings of Fredrick and Kharkov winter wheat exposed to CO_2 ethanol and lactic acid at -1 C for 1 week

	Components %#			Survival %	
	CO_2	ethanol	lactic acid	Fredrick	Kharkov
1	100	0	0	15* ± 8.2	46 ± 11.1
2	50	0	0	79 ± 6.4	92 ± 2.3
3	0	10	0	48 ± 8.4	64 ± 9.3
4	0	5	0	87 ± 6.0	93 ± 2.2
5	0	0	0.5	67 ± 12.1	51 ± 7.9
6	0	0	0.25	96 ± 2.4	92 ± 1.6
7	50	5	0.25	6 ± 3.9	8 ± 4.6
8	50	5	0	27 ± 7.9	45 ± 6.4
9	50	0	0.25	64 ± 6.6	71 ± 7.3
10	0	5	0.25	84 ± 4.5	80 ± 7.0
11	0	0	0	100	96 ± 1.5
12	ice encased			7 ± 3.3	23 ± 5.2

*Values are means of three experiments with plants cold hardened for 7.5, 8, and 10 weeks, each with 4 replications.

#Percentages in solution (v/v) of 4 ml 0.05 M phosphate buffer (pH 7.0) for ethanol and lactic acid, and as percentage CO_2 in atmosphere, balance - air.

killing. The concentration of leachate from iced plants as a proportion of the leachate from liquid nitrogen-killed plants was far greater in Fredrick than in Kharkov, corresponding to the difference in survival in ice between the two cultivars.

Relative Toxicity of Metabolite Accumulations. Internal concentrations of the three metabolites at the LD_{50} due to ice encasement, as compared with the internal concentrations due to external application of the metabolite are shown in Table V. Both ethanol and lactic acid were considerably higher at the 50% kill point induced by metabolite exposure than by ice, whereas the level of CO_2 accumulating and damaging plants in ice surpassed that entering and causing damage to plants in an external CO_2 atmosphere. This different pattern for CO_2-related plant kill indicates a more significant role of CO_2 in ice encasement damage than of the other two metabolites that have been shown to accumulate.

Ultrastructure. Shoot apex cells of aerobically treated cold-hardened Kharkov seedlings had large nuclei and were without vacuoles (Fig. 2). Cells were tightly packed with small intercellular spaces and frequent plasmodesmata. Small amounts of ER were distributed throughout the cytoplasm in discontinuous irregular sheets. Nuclei showed small discrete dark and light staining areas (heterochromatin, euchromatin) in approximately equal proportions. After ice encasement for 1 week, there was a proliferation of ER forming concentric whorls and sheets in those parts of the cell devoid of major organelles (Fig. 3). Examination of many sections confirmed that these structures occurred in nearly all apical cells. The nucleus of ice-encased cells showed dark and light staining areas as in the controls, but areas of each staining reaction were more continuous giving a characteristic condensed, or clumped appearance. A similar pattern occurred in material fixed in glutaraldehyde-osmic acid (unpublished results).

Plants exposed to the three metabolites described above (Table III, treatment 7) showed completely and partially disorganized cells in juxtaposition (Fig. 4), and partially disorganized cells with strongly condensed nuclei (Fig. 5). There was no evidence of increased membrane in the cytoplasm. Structure of cells exposed to CO_2 and ethanol together (treatment 8) however, did show a

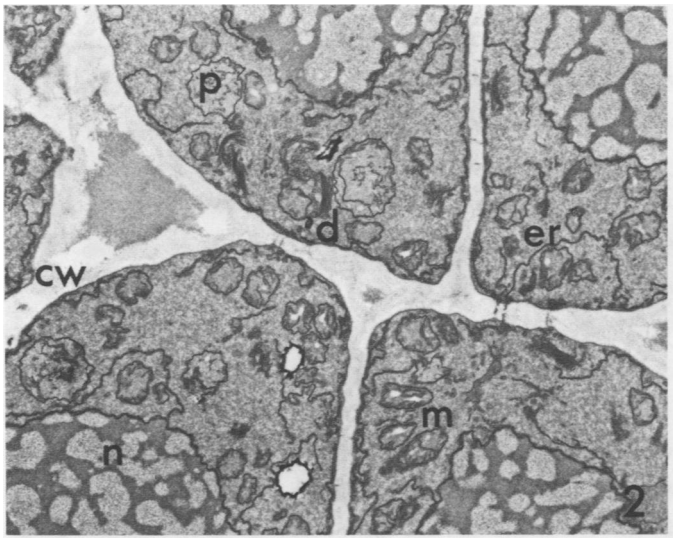
Table V. Comparison of the endogenous metabolite concentration at the 50% kill point of cold-hardened seedlings of Fredrick and Kharkov winter wheats induced by ice encasement or the exogenous metabolite. Metabolite concentrations at the LD_{50} ice exposure was interpolated from the concentrations occurring at decreasing survival levels during ice encasement: ethanol values reported previously (1), lacate values derived from Table II and CO_2 values from Fig. 1. Metabolite concentrations at the LD_{50} metabolite exposure were interpolated from the concentrations occurring at decreasing survival levels due to increasing level of externally applied metabolite. Ethanol values reported previously (1).

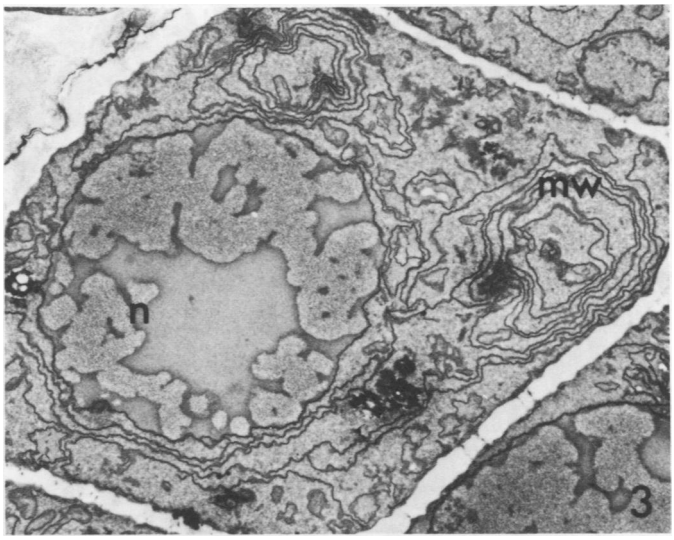
Cultivar	Metabolite	LD_{50} ice exposure	LD_{50} metabolite exposure
		mg/g fr wt	
Fredrick	Ethanol	2.94	14.01
	Lactate	0.67	2.17
	CO_2	2.45	1.24
Kharkov	Ethanol	4.15	13.87
	Lactate	0.61	2.46
	CO_2	3.70	1.14

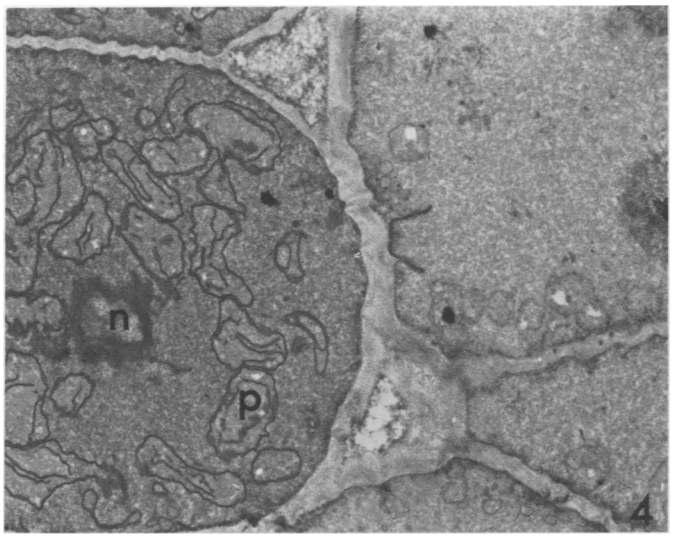
Table IV. The effects of CO_2 with and without 5% ethanol on survival and cell permeability of seedlings of Fredrick and Kharkov winter wheats, as measured by ninhydrin-positive substances (glycine equivalents) and electrolytes (conductivity) in a 24-h leachate. Plants were cold-hardened for 6 weeks.

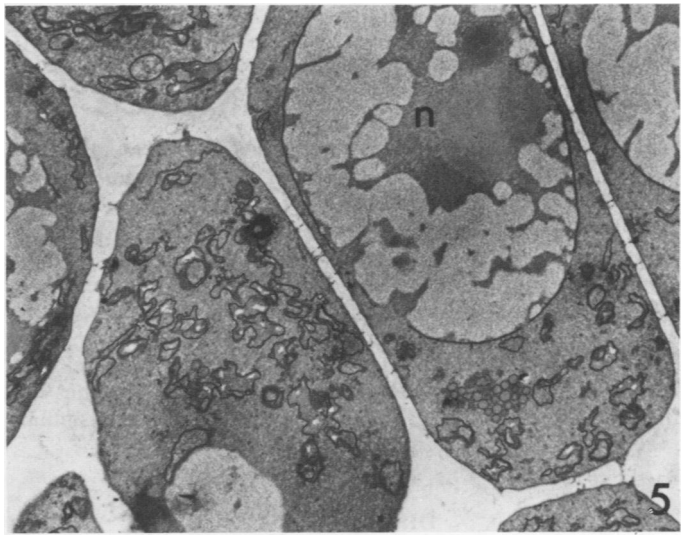
	CO_2 %	-Ethanol			+Ethanol		
		survival %	leachate		survival %	leachate	
			glycine equivs. µmole/g fr wt	conductivity mhos/g fr wt		glycine equivs. µmole/g fr wt	conductivity mhos/g fr wt
Fredrick	0	100	0.1*	1.5 ± 0.4	98	0	1.2 ± 0.1
	50	100	3.8 ± 0.7	6.7 ± 1.1	45	6.2 ± 1.2	8.8 ± 0.5
	100	65	7.1 ± 0.4	11.5 ± 0.6	5	11.4 ± 1.2	13.1 ± 0.5
	Iced	4	13.8 ± 1.6	11.9 ± 1.1			
	Total kill	0	25.2 ± 1.6	27.1 ± 2.4			
Kharkov	0	100	0.2 ± 0.1	0.9 ± 0.03	100	0	1.1 ± 0
	50	100	9.2 ± 0.7	6.9 ± 0.5	43	9.9 ± 1.6	6.3 ± 0.7
	100	95	17.8 ± 2.1	13.1 ± 1.1	33	15.4 ± 1.3	12.5 ± 0.3
	Iced	85	10.0 ± 0.4	10.6 ± 0.5			
	Total kill	0	32.7 ± 2.9	32.7 ± 2.5			

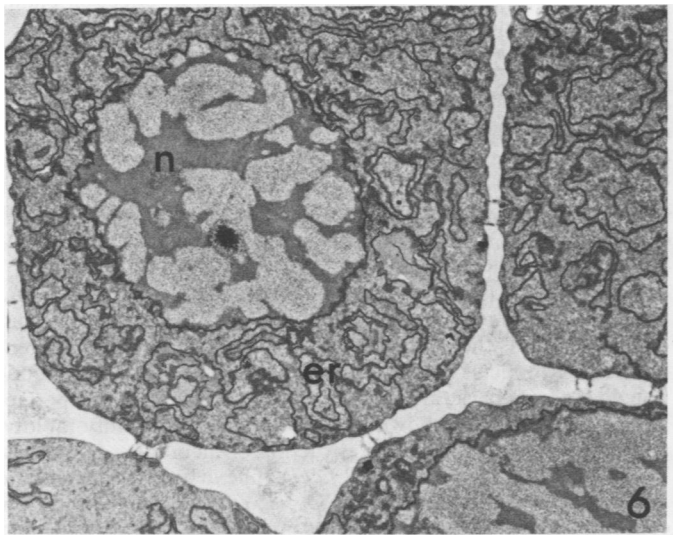
*Values are a mean of 4 replicates.

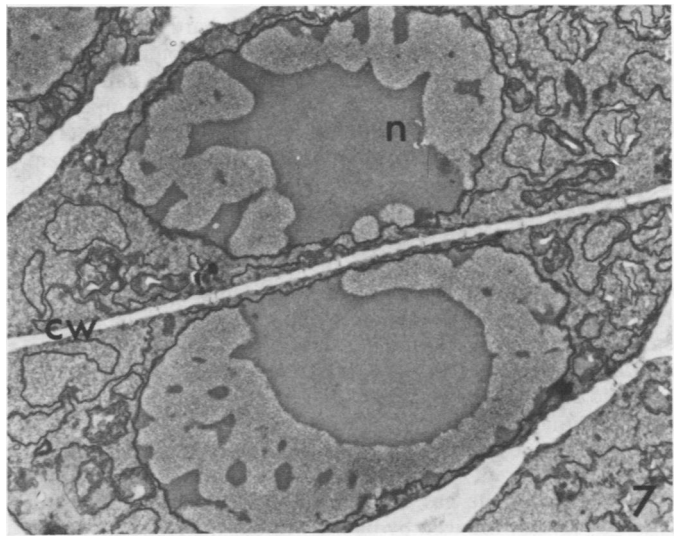












FIGS. 2 to 7. Ultrastructural features of apical cells of cold-hardened Kharkov winter wheat seedlings fixed in $KMnO_4$. (cw) Cell wall; (d) dictyosome; (er) endoplasmic reticulum; (mw) membrane whorls; (m) mitochondrion; (n) nucleus; (p) plastid.

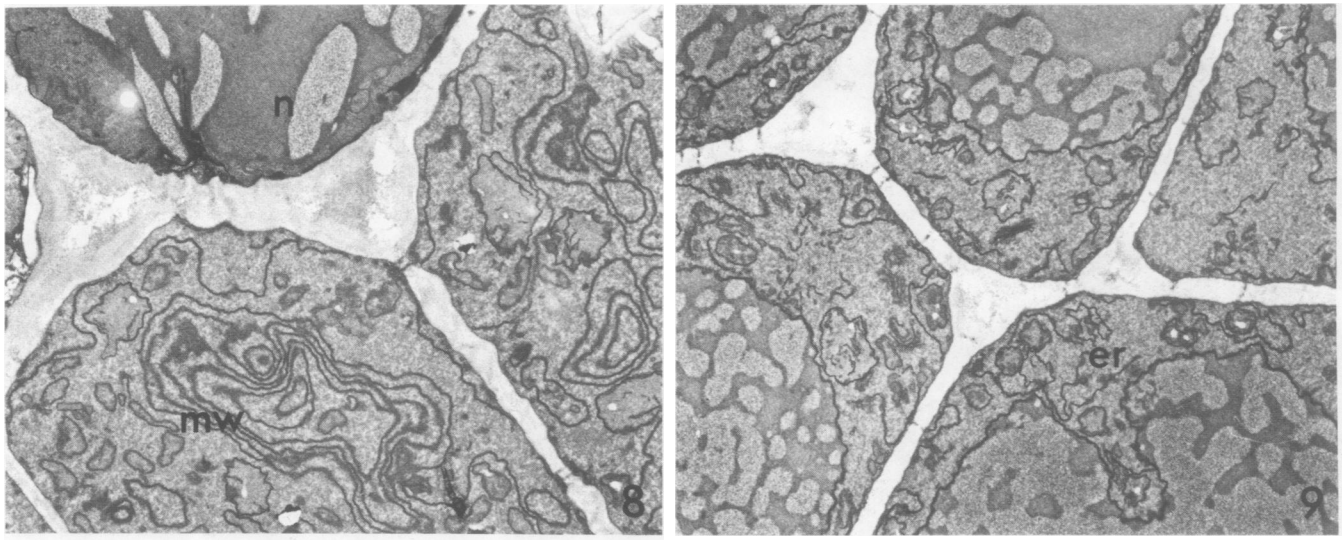
FIG. 2. Noniced control (× 6690).

FIG. 3. Ice-encased for 1 week at 1 C (× 5440).

FIGS. 4 and 5. Treated with 50% CO_2, 5% ethanol, 0.25% lactate for 1 week at −1 C (Fig. 4, × 6690; Fig. 5, × 4422).

FIG. 6. Treated with 50% CO_2 and 5% ethanol for 1 week at −1 C (× 6690).

FIG. 7. Treated with 100% CO_2 alone (× 5440).



FIGS. 8 and 9. Ultrastructural features of apical cells of cold-hardened Kharkov winter wheat seedlings fixed in $KMnO_4$. (er) Endoplasmic reticulum; (mw) membrane whorls; (n) nucleus.

FIG. 8. Treated with 10% ethanol alone (× 6690).

FIG. 9. Treated with 0.5% lactate alone (× 6690).

markedly increased incidence of membrane (Fig. 6) though it was not organized into whorls or stacks as in the iced treatment. The nuclear material was condensed, but not as severely as in response to the combination of CO_2 ethanol and lactate.

High concentration of CO_2 alone (Fig. 7) did not induce proliferation of membrane, but was associated with the condensed form of nucleus. Ten per cent ethanol alone (Fig. 8) caused the proliferation of membrane whorls in cells, together with nuclear rearrangement. Lactate, at 0.5%, induced little change in cellular structure (Fig. 9). Lower concentrations of these metabolites resulted in either no change of structure, or weaker responses similar to those of the high concentration.

DISCUSSION

During the relatively short period in which hardy wheat plants are killed by ice encasement, at least three potentially toxic metabolites—ethanol, lactic acid, and CO_2—accumulate within the plants as a result of anaerobic respiration and the impermeability of the surrounding ice (19). Ethanol has been shown (1) to accumulate, but not to be the sole toxic agent in ice encasement. Both ethanol and CO_2 increase within the plants for several weeks of ice encasement, but the rate of increase slows as the metabolic function of an increasing porportion of the cells ceases. Lactic acid accumulates during the 1st day of encasement, but thereafter remains relatively stable. This cessation of lactate production has been shown in root tissues (5) to be due to the lowering of pH by the lactic acid to a level which is suboptimal for the activity of lactic dehydrogenase. This pH is, however, optimal for the activity of pyruvic decarboxylase which reduces pyruvate to acetaldehyde and hence ethanol.

The different concentrations of CO_2 observed in ice-encased plants under different experimental conditions make it difficult to establish levels of the gas that are physiologically active in the plant. CO_2 levels produced by plants without the restraints of ice, and with partial and total encasement (Table I and Fig. 1) are progressively lower, but are also associated with an increased rate of kill of the plants. With increasing degree of encasement, endogenously produced CO_2 is more confined to the tissues in which it is produced, thus markedly increasing its relative concentration. From the present study, it is not known in which tissues the CO_2 accumulates, or the ionic form of the gas. CO_2 is soluble in water to give in solution both CO_2 molecules and carbonate ions. Furthermore, bicarbonate ions are also produced, and this reaction has been shown to be catalyzed by carbonic anhydrase (4). CO_2 as the bicarbonate ion accelerates senescence in cotton plants (4), and as CO_2 in solution increases cell permeability to water and ultimately leads to destruction of cell membranes (6).

The activity of CO_2 and bicarbonate and the endogenous proportions of the two forms were not investigated in this work, so that the levels of CO_2 recorded after diffusion out of the tissues may not reflect total CO_2-related accumulations, but the values are valid for comparisons within the experimental system. Thus, the toxicity of endogenous CO_2 is greater than that of applied CO_2 (Table V) indicating that it accumulates in sufficient quantities to be independently toxic. Survival data (Table III) and comparisons of ultrastructure (Figs. 3 and 6) also indicate that CO_2 or the combination of CO_2 and ethanol, is similar in its effect on plants to that of ice encasement. High concentrations of CO_2 supplied independently, however, do not induce large amounts of cytoplasmic membranes as is found in ice-encased plants. Such proliferation of membrane is found in treatments of CO_2 and ethanol, and high concentrations of ethanol alone induce formation of considerable numbers of membrane whorls. These results, together with the increased permeability caused by CO_2 and ethanol at concentrations lower than those reducing survival (Table IV) demonstrate the toxicity of the combined accumulations of CO_2 and ethanol in the encased plants.

The nature of this toxicity is not known. The differing survival with similar permeability levels induced by CO_2 with and without ethanol supports the view that the two components have different but additive effects on cells, since ethanol alone at this concentration does not increase, although it may decrease membrane permeability. CO_2 has been shown (6) to decrease permeability rapidly, and then increase it prior to destruction of membrane properties. These responses also occur at 2 C (7). The activity of membrane ATPase is impaired by both CO_2 (15) and ethanol (8). Low concentrations of ethanol decrease membrane fluidity (10) and decrease cell permeability (11). It has been proposed (10) that the effect is due to ethanol molecules penetrating the hydrophobic layer and inserting between the acyl chains to decrease their mobility. Higher concentrations are toxic (7) probably by membrane and enzyme protein denaturation (8, 23). Responses such as these presumably occur in ice-encased wheat cells but their details are not yet known.

The appearance of the nucleus in electron micrographs of cells of the iced treatment and in those of most of the metabolite combinations is unusual and its origins uncertain. Material throughout this study was fixed in $KMnO_4$ which stabilizes membranes to the eventual detriment of nuclear and cytoplasmic structure (13), but the nuclear appearance was not an artifact of $KMnO_4$ fixation, because similar condensation was seen in glutaraldehyde-fixed material (unpublished results). The condensation appears to occur in response to the anaerobic environment but has not been commonly reported. Similarly condensed nuclei were shown in frozen apical cells of *Festuca* (16), but were suggested to be a secondary response of damaged cells. Frozen apical cells of wheat (18) showed no such contraction. In iced cells, it may be linked with the damage to the nuclear membrane reported previously (18), consisting of the formation of enlarged nuclear pores and separation of the membrane layers. No experimental connection has been established between the two conditions. Condensation of chromatin is known (12) to be markedly affected by a number of cellular and fixation factors, but the interrelationships of these factors are not sufficiently well established to explain the characteristic appearance of the nuclei.

The conclusion that ice encasement damage is caused by the combined accumulations of both CO_2 and ethanol explains at least in part the lack of major ultrastructural disruption during ice encasement (17, 18). Damage by these metabolites occurs at the membrane level, changing permeability and destroying membrane function *in situ*. The effects of such changes become manifest only after thawing, as an inability of cells to metabolize normally leading to cell death. The role of the membrane whorls which are organized during ethanol and CO_2 accumulation remains unclear.

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