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Mitochondrial Activity and Ethanol Accumulation in Ice-encased Winter Cereal Seedlings[1]

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ABSTRACT

Cold-hardened dark-grown seedlings of winter wheat (*Triticum aestivum* L.) and winter rye (*Secale cereale* L.) are killed during total encasement in ice at −1 C at a rate related to the initial cold hardiness of the cultivars. Few plants remain alive after 7 days of encasement. Nonhardened seedlings are rapidly killed in ice. The respiratory properties of mitochondria isolated from plants after increasing periods of ice encasement decline slowly, and activity is little impaired when intact plants are about 50% killed. Electron microscopy indicates that mitochondrial structure is not disrupted until 3 weeks of ice encasement. Ethanol accumulates in hardened and nonhardened plants in ice, but at levels which are not toxic to the plants.

Plants encased in ice are subject to a decrease in O_2 tension (6, 17), and an increase in CO_2 (6, 17, 21). Cold hardiness and survival decrease rapidly during ice encasement (2), and considerable accumulation of ethanol occurs in light-grown plants under ice. The concentrations of ethanol attained do not appear to be sufficiently high to be solely responsible for death of plants in ice (1). The accumulation of ethanol has been implicated previously in the damage to plants by flooding (5, 10), a stress with characteristics similar to ice encasement. Low concentrations of ethanol inhibit growth of bacteria (7) but a rapid adaptation restores growth to control levels.

The functional properties of mitochondria in aerobically grown cold-hardened wheat seedlings are well documented (11, 14, 15). During ice encasement, mitochondria could be expected to cease functioning efficiently and to be damaged by factors of the anaerobic environment. Analysis of respiratory activity in encasing ice is difficult as ice is impermeable to respiratory gases (16), and isolation of mitochondria can effectively be made only after thawing of the ice. Indications that mitochondria are alive in tissues killed by freezing (20) do not necessarily extend to the ice encasement system because during short low temperature exposures, little metabolic accumulation occurs which might disorganize mitochondrial structure.

This paper examines the relationship between mitochondrial efficiency, ethanol accumulation, and plant death under conditions of anaerobiosis induced by ice encasement.

MATERIALS AND METHODS

Experiments were carried out with dark-grown seedlings of Cappelle Desprez, Frederick and Kharkov 22 MC winter wheats (*Triticum aestivum* L.) and Puma winter rye (*Secale cereale* L.). The cultivars represent a wide range of cold hardiness. Sterilized seeds were grown on moist filter paper for 24 hr at 20 C and then at 2 C for 4 weeks, or continuously at 20 C for 3 days, when seedling shoots were approximately 1 cm long. Seedlings were placed in plastic saucers, 150/treatment, immersed in ice-cold water, and frozen at −1 C. After various periods, ice blocks were thawed, seedling shoots harvested for mitochondrial preparations, and whole seedlings sampled for ethanol, survival, and cold hardiness determinations.

Mitochondria were isolated from seedling shoots by grinding and differential centrifugation (14), and respiratory parameters determined by a conventional Clarke electrode using α-ketoglutarate as substrate. Aliquots of the mitochondrial preparations of Kharkov wheat were fixed for electron microscopy. Glutaraldehyde in 0.05 M K-phosphate buffer was added to mitochondrial suspensions to a final concentration of 4%. After 15 min at 4 C, mitochondria were pelleted at 12,000*g* for 10 min, and fixation of the pellets continued for 2 hr at 4 C. Pellets were post fixed for 2 hr in 2% osmic acid in 0.05 M K-phosphate buffer (pH 7.4). The fixed material was washed, dehydrated in acetone, embedded in Epon (8), and sectioned. Sections were stained in uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

Ethanol was determined by the method of Lundquist (9) using alcohol dehydrogenase (EC 1.1.1.1.) (Sigma Chemical Co.) and NAD (United States Biochemical Corp.). Fresh weighed samples of 10 plants were ground in a total of 6 ml 5.1% perchloric acid and the neutralized supernatant stored frozen until analysis. Aliquots of the thawed ice containing ethanol leached from plants during thawing were stored directly.

Survival of plants during ice encasement was determined by transplanting seedlings, after thawing, to flats of moist vermiculite and recording live and dead plants after 2 weeks at 20 C day/15 C night (2). Cold hardiness of plants before and after ice encasement was measured by their survival after exposure to decreasing freezing temperature at a rate of 1 C/hr (3).

The toxic effect of exogenous ethanol on dark-grown seedlings was observed by immersing groups of 10 seedlings in 0, 2.5, 5, and 10 (v/v) aqueous ethanol for 1.5 day (20 C-grown material) or 7 day (2 C-grown material). Plants were removed from ethanol, rinsed, transplanted to moist vermiculite to measure survival, and their ethanol content determined as above. The plant ethanol content at the interpolated 50% kill point due to exogenous ethanol was then compared with the plant ethanol content at the 50% kill point due to ice encasement, to estimate the toxicity of endogenously generated ethanol in iced plants as reported previously for light-grown plants (1).

RESULTS

Cold-hardened dark-grown cereal seedlings were damaged by encasement in ice at −1 C to the extent that about half of the seedlings in each cultivar were killed by a 3.5-day exposure (Table I). The damage was directly related to the initial cold

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hardiness of the cultivars, with the exception of the relatively low survival of Puma rye after 3.5 days. Warm-grown seedlings of all cultivars were rapidly damaged by ice encasement, and few plants survived 2 days of exposure (Table II). Cold hardiness of 2 C-grown seedlings (Table I) after ice encasement decreased rapidly with increasing ice exposure and was not retained at a higher level in Puma despite its higher initial hardiness. All cultivars showed negligible cold hardiness after 7 days.

The respiratory characteristics of mitochondria isolated from seedlings of all four cultivars exposed to increasing periods of ice encasement were similar (Table III). A general decline in respiratory control and ADP/O ratios was observed but at 3.5 days in ice, equivalent to approximately 50% survival, there was only a slight reduction in respiratory control due predominantly to a decrease in state 3 respiration. Respiratory efficiency, as measured by ADP/O ratios, was only slightly reduced after 3.5 days. After 14 days icing when all except a few plants of Puma were dead, mitochondrial preparations still showed respiratory control values about 40% of control levels, and with significantly higher values from the two hardier cultivars, Puma and Kharkov. The ADP/O ratios were also considerably higher in these two cultivars than in the less hardy Frederick and Cappelle. Three weeks icing was lethal to all plants, but there remained some respiratory activity which was totally uncoupled in the wheat mitochondria.

Electron microscopic examination of mitochondria isolated from Kharkov seedlings encased in ice up to 14 days (Fig. 1-4) revealed no major structural changes despite the fact that plant survival at this stage was negligible. The micrograph of mitochondria from seedlings iced for 14 days (Fig. 4) was not fortuitously taken from the living 2% of the sample, but rather from a sample of mitochondria from hundreds of pooled seedlings, most of which were dead. By 21 days, internal mitochondrial structure was almost totally lost, but a few mitochondria retained identifiable membranes without evidence of cristae.

Table I. The survival and cold hardiness of 2 C dark grown wheat and rye seedlings during ice encasement at -1 C

Cultivar	Ice Encasement	Survival	Cold Hardiness
	days	% ± SE	LD_{50} °C
Puma	0	100	-18.3 ± .66
	3.5	48 ± 4	- 7.0 ± .57
	7	28 ± 5	< -1
	14	14 ± 3	-
	21	1	
Kharkov	0	100	-13.3 ± .30
	3.5	62 ± 5	- 8.4 ± .60
	7	22 ± 5	< -1
	14	2	-
	21	0	-
Frederick	0	100	-11.4 ± .28
	3.5	61 ± 6	- 6.8 ± .20
	7	9 ± 3	< -1
	14	0	-
	21	0	-
Cappelle	0	100	- 6.5 ± .45
	3.5	39 ± 5	- 5.8 ± .69
	7	3	-
	14	0	-
	21	0	-

Table II. The survival of 20 C dark-grown seedlings of wheat and rye during ice encasement at -1 C

Ice encasement	Puma	Kharkov	Frederick	Cappelle
days		survival %		
0	100	100	100	100
5	100	100	100	100
1	17 ± 2	42 ± 7	0	53 ± 12
2	6 ± 2	0	0	0

Table III. The respiratory properties of mitochondria isolated from 2 C grown wheat and rye seedlings following ice encasement at -1 C

Cultivar	Ice Encasement	Oxygen Uptake State 3	Oxygen Uptake State 4	Respiratory Control	ADP/O
	days	n moles/min/mg protein		ratio	
Puma	0	53.7 ± 3.1	14.3 ± 1.1	3.8 ± 0.1	2.8 ± 0.1
	3.5	34.8 ± 3.0	15.1 ± 1.2	2.3 ± 0.1	2.3 ± 0.2
	7	33.3 ± 2.1	14.9 ± 1.0	2.2 ± 0.1	2.3 ± 0.1
	14	13.7 ± 1.1	9.0 ± 1.0	1.5 ± 0.1	1.4 ± 0.1
	21	9.0 ± 1.3	6.4 ± 1.1	1.4 ± 0.1	1.0 ± 0.2
Kharkov	0	53.5 ± 4.3	15.3 ± 1.4	3.5 ± 0.3	2.8 ± 0.1
	3.5	32.3 ± 1.5	15.1 ± 0.4	2.2 ± 0.2	2.5 ± 0.0
	7	31.8 ± 4.2	11.5 ± 2.0	2.8 ± 0.6	2.5 ± 0.2
	14	9.7 ± 1.0	6.7 ± 0.5	1.5 ± 0.1	1.3 ± 0.1
	21	2.8 ± 1.1	2.8 ± 1.1	1.0 ± 0.0	0.0 ± 0.0
Frederick	0	53.6 ± 3.4	14.7 ± 1.8	3.7 ± 0.5	3.1 ± 0.2
	3.5	34.6 ± 2.2	14.8 ± 0.3	2.3 ± 0.1	2.3 ± 0.1
	7	22.0 ± 2.7	11.8 ± 1.1	1.9 ± 0.1	2.0 ± 0.1
	14	6.0 ± 0.2	5.1 ± 0.3	1.2 ± 0.1	0.4 ± 0.1
	21	4.5 ± 1.5	4.5 ± 1.5	1.0 ± 0.0	0.0 ± 0.0
Cappelle	0	53.5 ± 4.2	17.6 ± 1.0	3.0 ± 0.2	2.8 ± 0.2
	3.5	32.0 ± 8.4	12.5 ± 1.3	2.6 ± 0.4	2.6 ± 0.1
	7	19.4 ± 3.4	9.9 ± 1.1	2.0 ± 0.2	2.1 ± 0.2
	14	6.0 ± 0.7	4.7 ± 0.5	1.3 ± 0.1	0.7 ± 0.2
	21	3.2 ± 0.3	3.2 ± 0.3	1.0 ± 0.0	0.0 ± 0.0

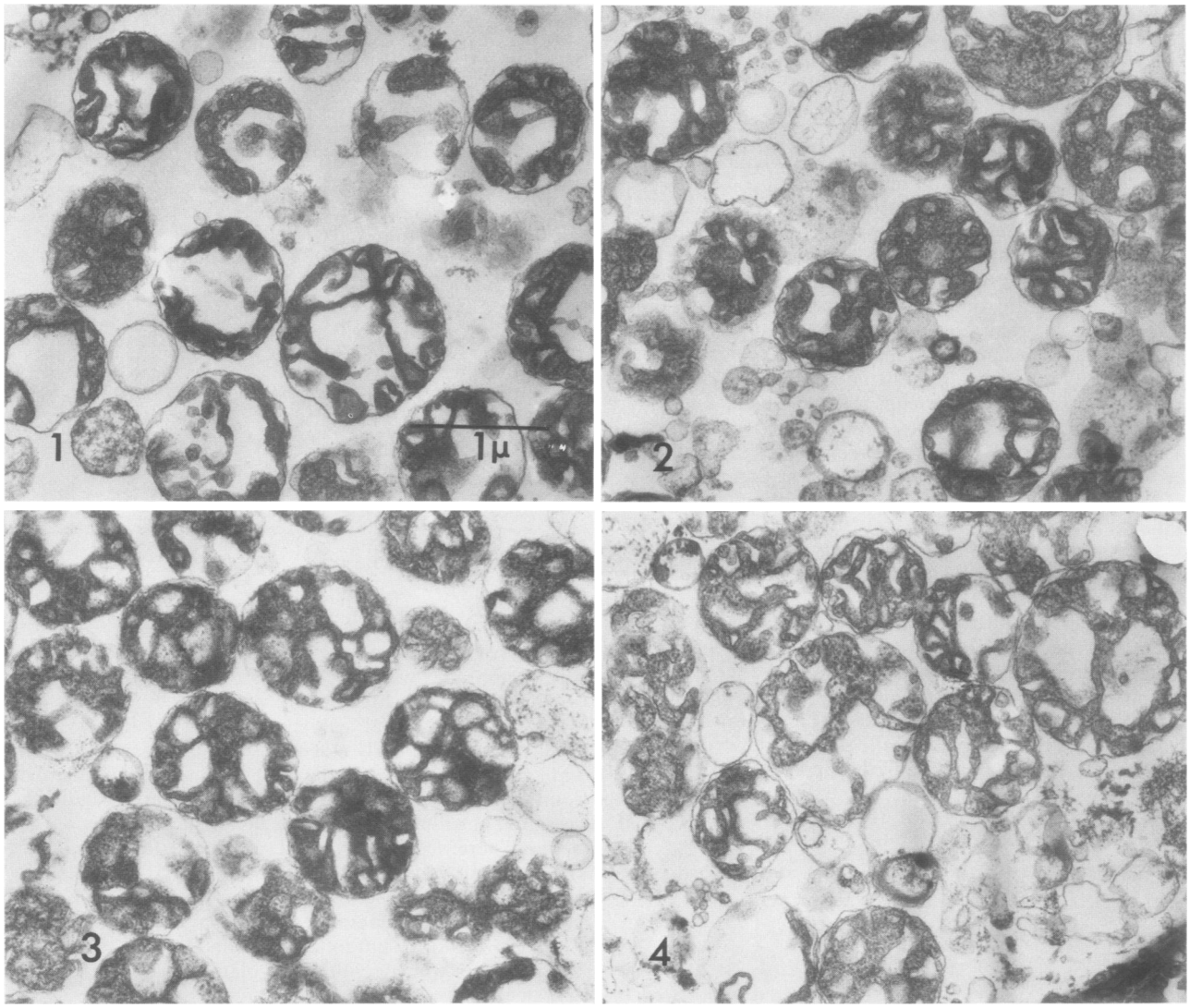
During exposure to ice, there was an accumulation of ethanol within the tissues of all cultivars (Table IV). Accumulation was most rapid in the early stage of ice encasement and continued until after the plants were dead. This observation, together with continued mitochondrial activity and integrity, indicates that cessation of cellular metabolism and plant death are not simultaneous. There was little difference in ethanol level accumulated in the four cultivars, and this was associated with the relatively small differences in survival between cultivars in ice. Seedlings grown at 20 C accumulated ethanol in ice at a similar rate to those grown at 2 C, but total accumulation was low during the 2-day period in which death of the plants occurred (Tables II and V).

The level of ethanol in cold-hardened and warm-grown seedlings soaked in ethanol concentrations which induced 50% kill, was considerably higher than the ethanol at 50% kill due to ice (Table VI). This indicated that ethanol, although partially toxic to wheat seedlings, was not solely responsible for death of plants during ice encasement.

DISCUSSION

The preservation of ultrastructure and at least partial function of mitochondria despite death of the plants during ice encasement was unexpected, but is in agreement with the maintenance of mitochondria in anaerobically grown rice coleoptiles (12), in differentiating phloem elements (13), and the transient survival of mitochondria in freeze-killed wheat shoots (20). These observations all indicate the relative resistance of mitochondria to stress. There was no evidence in the present study of swollen and atypical mitochondria reported from yeast (24) and rice (23) in anaerobic conditions.

Mitochondria of Kharkov winter wheat showed no structural deterioration after 2 weeks, during which time their respiratory control and ADP/O ratios decreased about 50%, but nearly all plants were killed. During this period, intact apical cells showed no deterioration but rather a synthesis of lamellar membranes (unpublished data). These observations suggest that plants are not killed during ice encasement by the formation of ice within the cells, because this process is destructive both to cell structure



FIGS. 1-4. Electron micrographs of mitochondria isolated from 2 C dark-grown seedlings of Kharkov winter wheat encased in ice at −1 C. 1: Control, before ice; 2: 3.5 days in ice; 3: 7 days in ice; 4: 14 days in ice.

Table IV. Ethanol content of 2 C grown wheat and rye seedlings during ice encasement at -1 C

	Ice encasement	Ethanol	
	days	mg/g fwt.	mg/10 plants
Puma	0	.07 ± .002	.05 ± .002
	3.5	1.14 ± .03	.81 ± .02
	7	1.71 ± .05	1.29 ± .04
	14	3.69 ± .04	2.69 ± .05
	21	4.12 ± .16	2.86 ± .07
Kharkov	0	.05 ± .002	.04 ± .002
	3.5	1.07 ± .02	.80 ± .02
	7	1.76 ± .03	1.33 ± .02
	14	3.34 ± .07	2.45 ± .04
	21	4.00 ± .08	2.86 ± .03
Frederick	0	.05 ± .002	.05 ± .002
	3.5	1.30 ± .02	1.28 ± .02
	7	2.25 ± .05	2.15 ± .03
	14	3.45 ± .08	3.21 ± .05
	21	3.93 ± .15	3.44 ± .06
Cappelle	0	.05 ± .003	.06 ± .003
	3.5	.83 ± .01	.85 ± .01
	7	1.36 ± .04	1.40 ± .04
	14	2.44 ± .05	2.45 ± .03
	21	2.55 ± .03	2.56 ± .05

(19) and to the function of mitochondria in contact with ice (18). Death of plants in the presence of potentially active aerobic respiration, and active anaerobic respiration, as measured by the production of ethanol, supports the concept that disruption of energy supply is not the cause of plant death in ice.

There was little difference in respiratory parameters between cultivars of contrasting hardiness levels during early stages of ice encasement. Higher respiratory control and ADP/O ratios of the hardier cultivars after more than 1 week in ice, when survival values approach zero, are probably a reflection of the activity of a few living cells in the hardier cultivars, rather than a difference in activity of the whole population of each cultivar. The final decline in respiratory control and ADP/O values lags the total death of plants by an approximately similar time period in each cultivar. This similarity of function of mitochondria from cultivars of differing hardiness levels has been shown previously to exist at ambient (11) and low (15) temperatures and here is demonstrated after exposure of cultivars to a lethal stress. It further indicates that disruption of energy supply is not the causative agent of damage during ice encasement.

A relationship between the decline in aerobic respiration due to deficiency of O_2 and the increase in anaerobic respiration was not established from this study because the activity of mitochon-

Table V. Ethanol content of 20 C grown wheat and rye seedlings during ice encasement at -1 C

	Ice encasement	Ethanol	
	days	mg/g fwt.	mg/10 plants
Puma	0	.09 ± .003	.08 ± .002
	.5	.23 ± .001	.14 ± .001
	1	.39 ± .020	.29 ± .005
	2	.64 ± .019	.55 ± .007
Kharkov	0	.08 ± .003	.06 ± .004
	.5	.31 ± .008	.25 ± .004
	1	.45 ± .014	.33 ± .006
	2	.74 ± .012	.54 ± .012
Frederick	0	.07 ± .001	.07 ± .002
	.5	.31 ± .014	.31 ± .008
	1	.52 ± .004	.47 ± .007
	2	.81 ± .012	.72 ± .002
Cappelle	0	.07 ± .002	.08 ± .003
	.5	.19 ± .005	.22 ± .004
	1	.34 ± .006	.37 ± .004
	2	.50 ± .001	.52 ± .007

Table VI. Comparison of the ethanol content of cereal seedlings grown at 2 C and 20 C at the LD_{50} of external ethanol solution, and at the LD_{50} of ice encasement duration.

	Growth temp.	Ethanol exposure LD_{50} ethanol	ethanol	Ice Exposure LD_{50} ice	ethanol
	°C	%	mg/g. fwt.	days	mg/g. fwt.
Puma	2	4.9†	5.5	3.36	1.04
	20	>10.0†	13.87	0.81	0.34
Kharkov	2	8.4	11.65	4.61	1.29
	20	8.3	11.36	0.93	0.43
Frederick	2	7.0	11.65	4.48	1.56
	20	7.6	10.49	0.75	0.42
Cappelle	2	3.6	7.34	3.02	0.67
	20	8.6	9.55	1.04	0.35

† ethanol exposure of 2 C grown seedlings for 7 days; of 20 C grown seedlings for 1.5 days.

dria in plants within the ice remains unclear. However, it is apparent in ice encasement that pyruvate utilization shifts from aerobic oxidation via the TCA cycle and electron transport, to its anaerobic reduction to ethanol and CO_2. There is also likely to be a stimulation of the glycolytic rate, as occurs in flooded plants (5), by the decline of aerobically generated ATP leading to the derepression of P-fructokinase (4). A stimulated glycolysis increases energy supply, but further contributes to the formation of ethanol and CO_2.

The toxicity of ethanol to dark-grown cereal plants (Table VI) was similar to its toxicity to hardy light-grown plants (1) but its accumulation does not solely account for the damage during ice encasement to plants in either condition. It is possible that highly localized accumulations occur, damaging organelles in the crown meristem which is vital to the overwintering of cereals. However, ethanol concentration of isolated ice-encased cereal crowns (1) was no higher than that from whole seedlings, suggesting that such local accumulations do not occur. Damage caused by ethanol within cells may be an important factor contributing with other aspects of anaerobiosis to the death of plants in ice. Low concentrations of ethanol cause a decrease in bacterial membrane fluidity and an inhibition of growth (7). Such decreases also occur during anaerobiosis (22, 24). At high incubation temperatures, rapid adaptations in the fatty acid composition of the membrane increase its fluidity and permeability and allow a resumption of growth (7). Cereal seedlings in ice at subfreezing temperatures accumulate ethanol, but it is unlikely that any adaptive process operates at low temperature in anoxia to prevent damage to membranes and the eventual loss of cellular integrity.

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