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Ice Encasement Injury to Microsomal Membranes from Winter Wheat Crowns[1]

I. COMPARISON OF MEMBRANE PROPERTIES AFTER LETHAL ICE ENCASEMENT AND DURING A POST-THAW PERIOD

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ABSTRACT

The functional and physical properties of cellular membranes isolated from *Triticum aestivum*, cvs Norstar and Fredrick, were altered coincident with changes in composition after a lethal ice-encasement stress and further during a 6 hour post-thaw period. Crowns encased in ice for a duration which inhibited regrowth, exhibited enhanced rates of electrolyte leakage. Furthermore, the recovery of total microsomal protein and phospholipid declined, suggesting that some membrane degradation had been induced during the anoxic stress. The microviscosity of microsomes and liposomes prepared from such membranes increased during stress, and this was correlated with a 2- to 4-fold increase in the free fatty acid levels in the microsomal fraction. There was, however, only a relatively minor change in fatty acid unsaturation during the ice-encasement stress. The process continued during a 6 hour aerobic post-thaw treatment, but the pattern was somewhat different. During this phase, the leakage of electrolytes was further increased and the recovery of microsomal protein and phospholipid continued to decline, indicating general degradation; but, in contrast to the anoxic phase, the degree of fatty acid unsaturation declined markedly, indicating lipid peroxidation.

Overwintering cereals grown in temperate regions are frequently exposed to various kinds of environmental stress, including freezing, low temperature flooding, and ice-encasement. Exposure of winter wheat seedlings to ice-encasement reduces the cold hardiness of surviving plants and prolonged exposure causes plant death (1). Tolerance of ice-encasement may be enhanced by environmental growth conditions. For example, acclimation at low growth temperatures which increases the seedling's tolerance of freezing stress, also promotes increased tolerance of ice-encasement. In addition, a nonlethal low temperature flooding treatment has also been reported to enhance tolerance of ice-encasement (2, 4).

One of the first injury symptoms observed during the early stages of ice-encasement, before the loss of viability, is a decline in the uptake of ^{86}Rb, suggesting that potassium ion uptake, presumably mediated by a plasma membrane ATPase, is adversely affected by the stress (23). Moreover, the availability of ATP does not appear to be limiting ATPase enzyme activity, indicating that ice-encasement promotes a direct inactivation of the ion transport system itself (24). As the duration of ice-encasement increases, a number of other modifications to membrane organization and function occur. At the ultrastructural level, there is a proliferation of existing cytoplasmic membranes and the formation of membrane whorls believed to be of endoplasmic reticulum (22). Eventually, the selective permeability of the plasma membrane declines and the leakage of cytoplasmic solutes including total electrolytes and amino acids is greatly enhanced (23). This injury to the plasma membrane resulting in leakage of cytoplasmic solutes can be simulated by the application of CO_2 and ethanol—two metabolites which accumulate during ice-encasement (3). Many of these changes are also observed in the plasma membrane following a lethal freezing stress (29), suggesting that there may be some degree of similarity in the nature of the injury to the cellular membranes following these two distinctly different stresses.

In this paper, the effect of a lethal ice-encasement stress and a time interval after thawing are examined in relation to the physical properties and chemical composition of microsomal membranes isolated from the crown region of two winter wheat cultivars.

MATERIALS AND METHODS

Plant Growth Conditions. Seeds of winter wheat (*Triticum aestivum* L.), cv Norstar and cv Frederick, were planted in small plastic trays as described previously (31) and grown at 20°C/15°C (day/night) temperature with an 18 h photoperiod and a photon flux density of 200 μmol m^{-2} s^{-1}. For the nonacclimated treatment, plants were grown under this environment for 10 d, while for the acclimated treatment, plants were grown in the 20°C/15°C environment for the first 7 d, then transferred to an acclimation chamber at 2°C (day and night) with a 12 h photoperiod of 350 ± 20 μmol m^{-2} s^{-1} for 10 d.

Stress Treatments. Acclimated and nonacclimated plants were assessed for their survival of varying durations of total ice-encasement using the method of Tanino and McKersie (31). The mean percentage of plants surviving a particular ice-encasement treatment was calculated from the survival of three replicates of 25 plants per pot from three separate experiments. The regrowth of surviving plants was estimated by measuring shoot length after a 3 week recovery period.

The damage to acclimated crowns by ice-encasement was also estimated by electrolyte leakage. Plants were carefully removed from the growing medium, and the leaves and roots trimmed to give 2 cm crown sections. Crowns were washed twice with distilled water and 5 crowns per test tube were immersed in 15 ml of ice-cold distilled water. Tubes were then placed in a freezer

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at −1°C. For the ice-encasement treatments, ice nucleation was induced by adding a small ice chip to each tube. For control treatments, the addition of ice was omitted which allowed the crowns to supercool to the same temperature but without ice formation. Tubes were then covered with aluminum foil caps. After various times, tubes were removed from the freezer and warmed at 2°C. When the bathing solution was totally thawed (where applicable) and at 2°C, its conductivity was measured at 2°C using a conductance meter (model 32, Yellow Springs Instrument Co.) either immediately or after an additional post-thaw period of 6 h at 2°C in darkness. The bathing solution was then returned to the crowns in the tube. Tubes were sealed with aluminum foil and autoclaved for 20 min at 1.2 kg/cm^2, and the conductivity of the bathing solution again measured at 2°C. The conductivity of the first reading (after ice-encasement or supercooling) was expressed as a percentage of the conductivity of the second reading (after autoclaving) and is referred to as '% leakage.'

For treatments used in membrane isolation, about 15 to 20 g fresh weight of crown tissue were prepared as for the electrolyte leakage measurements. For controls, the prepared crowns were used immediately for membrane isolation. For ice-encased treatments, crowns were weighed, placed in beakers with ice chips, and ice-cold distilled water was added giving total ice-encasement within 2 h. After various durations of ice-encasement at −1°C, crowns were thawed under cold running tap water in less than 2 min and membranes were isolated either immediately after thawing, or after a 6 h post-thaw period at 2°C in darkness.

Membrane Isolation. Crown tissue was homogenized using a chilled pestle and mortar in 50 ml of the isolation buffer described by Yoshida *et al.* (33) but without BSA. The brei was squeezed through two layers of cheesecloth and centrifuged at 10,000*g* for 20 min. Microsomal membranes were pelleted from the supernatant by centrifugation at 165,000*g* for 2 h. All operations were performed at 0 to 4°C. Subsamples of the microsomal membrane pellet (15–25 mg) were resuspended in 2 ml of 10 mM Hepes buffer (pH 7.5). Protein content was measured using the method of Bradford (7) with BSA (Sigma) as a standard.

Lipid Extraction and Analysis. Lipids were extracted from the membrane pellets by the method of Nichols (18), using hot isopropanol, followed by chloroform-methanol. Phospholipid content of the lipid extract was determined colorimetrically as inorganic phosphate after perchloric acid digestion (8). Fatty acid methyl esters were prepared from the total lipid extract using 14% BF_3 in methanol (Chromatographic Specialties) and quantified by gas chromatography as described previously (25). Neutral lipids were separated by loading aliquots of the total lipid extracts on to silica cartridges (Sep-Pak, Waters Assoc., Mississauga, Ontario, Canada) and separated from the polar lipids by eluting with a mixture of petroleum ether:diethyl ether:acetic acid (70:30:2 by volume). Fatty acids were methylated by heating with 14% BF_3 in methanol at 90°C for 10 min under N_2 and quantified as described above. Fatty acids were identified by comparison with authentic standards and quantified using heptadecanoic acid (17:0) as an internal standard.

Fluorescence Polarization Measurements. Microsomal membranes and liposomes prepared from total microsomal membrane lipid extracts were labeled with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma). DPH was prepared in tetrahydrofuran at 2 mM and diluted to 2 μM with 10 mM Hepes (pH 7.5) prior to labeling. Labeled membranes were prepared by resuspending pellets in 10 mM Hepes buffer (pH 7.5), with the DPH at a final concentration of 1 μM. Liposomes were prepared by vortexing the total lipid extract with 4 ml of the DPH-buffer suspension at 36°C.

Membrane suspensions and liposomes were incubated at 36°C for 60 min, the liposomes being vortexed every 20 min. Fluorescence polarization measurements were conducted using an SLM spectrofluorometer (model 4800) at 21°C as described previously (5, 6, 30). Fluorescence intensity was measured at 428 nm with excitation at 350 nm. From the polarization values obtained, microviscosity values were calculated (27).

RESULTS

Acclimation for 10 d at 2°C enhanced the tolerance of both winter wheat cultivars to ice-encasement (Fig. 1). Nonacclimated plants showed almost no survival after 3 d of ice-encasement, whereas after the same stress, acclimated seedlings of Norstar and Fredrick showed 95 and 60% survival, respectively. In addition to reducing survival, increasing the duration of ice-encasement caused a decrease in the vigor of the surviving plants, as shown by measurements of shoot length at 3 weeks of regrowth (data not shown). Longer periods of acclimation provide an even greater degree of stress tolerance (data not shown), but 10 d acclimation was chosen as a compromise. At this time, acclimated seedlings were similar in size to nonacclimated ones, yet exhibited significantly greater icing tolerance, and cultivar differences were also apparent.

Damage to crowns prepared from acclimated plants after ice-encasement was also assessed by measuring electrolyte leakage (Table I). Ice-encasement, for the time period which caused

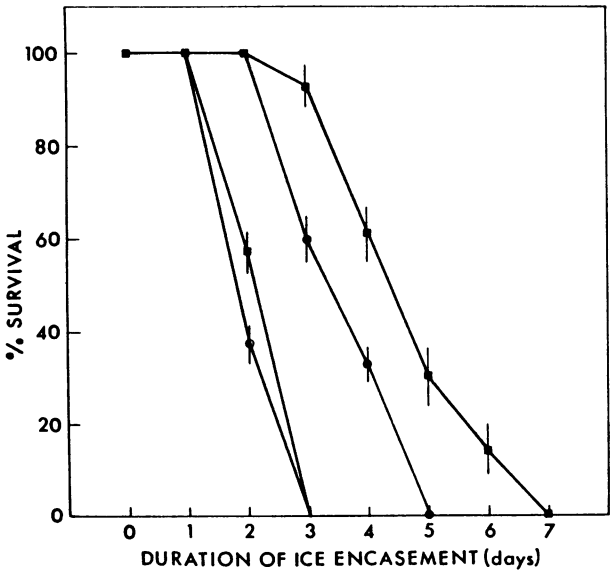

FIG. 1. Survival of two winter wheat cultivars after total ice-encasement at −1°C. Norstar nonacclimated (□) and acclimated (■). Fredrick nonacclimated (○) and acclimated (●). Mean values ± SE.

Table I. *Leakage of Electrolytes from Acclimated Crowns of Two Winter Wheat (T. aestivum L.) Cultivars*

Crowns were encased in ice for 7 d for Norstar and 5 d for Fredrick. Control crowns were supercooled at −1°C for the same period. Crowns were removed from ice-encasement by thawing at 2°C (ice-encased) or by thawing at 2°C with an additional 6 h post-thaw period at 2°C (ice-encased/post-thaw). Values are expressed as % of total electrolytes and are means of three experiments ± SE.

Treatment	Leakage	
	Norstar	Fredrick
	%	
Control	5.9 ± 2.5	2.9 ± 2.6
Ice-encased	52.4 ± 2.9	50.7 ± 2.2
Ice-encased/post-thaw	70.3 ± 3.5	56.5 ± 0.3

complete loss of plant regrowth in both cultivars (Fig. 1), resulted in approximately a 50% loss of total electrolytes, when measured immediately after thawing. Enhanced amounts of electrolyte leakage were apparent, particularly with Norstar, after a subsequent 6 h post-thaw period. Control treatments of crown sections supercooled at −1°C for the same time period had substantially lower levels of leakage, *i.e.* 5.9 and 2.9% for Norstar and Fredrick, respectively, indicating that injury to the cellular membranes was not due to exposure to low temperature *per se*.

When both cultivars were exposed to a duration of ice-encasement which prevented further regrowth, *i.e.* 5 d for Fredrick and 7 d for Norstar (Fig. 1), the amount of membrane material recovered in the microsomal fraction was substantially reduced, indicating a net loss of membrane components during ice-encasement (Table II). Further loss of membrane components occurred during the post-thaw period, with both cultivars showing a loss of protein, phospholipid and fatty acids from the microsomal fraction.

Microviscosity of microsomal membranes, and liposomes prepared from the total lipid extracted from the microsomal fraction, was determined using fluorescence polarization of the probe DPH (Table III). Ice-encasement induced a substantial increase in microviscosity of membranes isolated immediately following treatment. When liposomes were prepared from the total lipid extracted from the microsomal fraction, the microviscosity of these liposomes was substantially lower than that of the intact membrane, but the same trends of increased microviscosity were found in response to the icing treatment. The increase in microviscosity observed in the microsomes following ice-encasement, was correlated with higher ratios of protein:phospholipid, and lower protein:total fatty acid and phospholipid:total fatty acid ratios as a result of the differential loss of phospholipid, protein, and fatty acid from the fraction (Table IV). After a 6 h post-thaw period, further changes were noted. The protein:total fatty acid ratio was increased in both cultivars, and the phospholipid: total fatty acid ratio increased in Norstar, but decreased in Fredrick, whereas the protein:phospholipid ratio followed a reciprocal pattern.

Immediately after thawing, there were no major changes in the fatty acid composition of total lipid extracts from the microsomal fraction, relative to controls for either cultivar (Table V). However, after an additional 6 h post-thaw period there was a 9 to 12% decline in linolenic acid (18:3), accompanied by proportional increases in palmitic acid (16:0) for Norstar, or palmitic and oleic acid (18:1) for Fredrick. When compared to controls, membranes isolated either directly after lethal icing or after a 6 h post-thaw period contained elevated levels of nonesterified, free fatty acids, whether expressed relative to total fatty acids, phospholipids or protein (Table IV).

DISCUSSION

The microsomal membrane fraction was chosen for studies of the changes in the physical properties of cellular membranes which were associated with the loss of membrane semipermeability following a lethal ice-encasement for a variety of reasons. The microsomal fraction contains a heterogeneous mixture of small, vesicular membranes originating from various organelles including the plasma membrane, tonoplast, and endoplasmic reticulum, and to varying degrees from mitochondria and plastids. Isolation of purified fractions, such as those enriched in the plasma membrane, employ separations based on buoyant density, or alternatively surface charge. It was expected that both properties might be altered during ice-encasement and therefore, the origin of membranes at a specific buoyant density, for example, would be expected to differ when the specific purified fraction was isolated following different degrees of stress. Alternatively, membranes exhibiting symptoms of deterioration might well be excluded from a purified fraction if, for example, ice-encasement induced changes in the protein:phospholipid ratio. By sampling the entire microsomal fraction, an average estimate can be made of the physical properties and composition of cellular membranes in the crown tissue, which itself is quite heterogeneous in regard to cell type and degree of differentiation. Admittedly, by this procedure it is not possible to ascribe measurable changes to any specific organelle, and changes which might occur in only one organelle, such as the plasma membrane, may be masked by the inclusion of other membranes in the fraction.

Increasing the duration of ice-encasement for the two winter wheat cultivars studied caused a progressive decline in survival, and a reduction in the vigor of any surviving plants. After a lethal ice-encasement, there was a marked increase in the leakage of electrolytes from crowns, indicative of membrane damage. Moreover, this damage was associated with changes in both the physical properties and composition of microsomal membranes isolated from crown tissue, including a relative increase in the quantity of free fatty acids, and a reduction in levels of phospholipids and proteins. These changes were accompanied by a reduction in the amount of membrane, phospholipid, and protein that could be recovered from stressed tissues, and it therefore

Table II. *Effect of Ice-Encasement on the Amounts of Microsomal Membrane, and Microsomal Protein, Phospholipid and Total Fatty Acid Recovered per Gram Fresh Weight from Acclimated Crown Tissue of Two Winter Wheat (T. aestivum L.) Cultivars*

Crowns were encased in ice for 7 d for Norstar and 5 d for Fredrick, and membranes isolated after rapid thawing (ice-encased) or after rapid thawing and an additional 6 h post-thaw period at 2°C in darkness (ice-encased/post-thaw). Data are expressed per g fresh weight of crown prior to ice encasement. The data are mean values ± SE ($n = 4$).

Treatment	Total Microsomes[a]	Protein	Phospholipid	Total Fatty Acids
	mg/g		*μmol/g*	
Norstar				
Control	10.1 ± 1.1	0.96 ± 0.06	0.088 ± 0.004	0.54 ± 0.03
Ice-encased	6.5 ± 0.8	0.58 ± 0.05	0.040 ± 0.003	0.64 ± 0.04
Ice-encased/post-thaw	4.9 ± 0.4	0.43 ± 0.03	0.035 ± 0.003	0.33 ± 0.03
Fredrick				
Control	12.1 ± 0.7	1.08 ± 0.04	0.159 ± 0.017	0.72 ± 0.08
Ice-encased	8.4 ± 0.8	0.57 ± 0.06	0.063 ± 0.010	0.46 ± 0.07
Ice-encased/post-thaw	6.3 ± 1.1	0.18 ± 0.03	0.008 ± 0.002	0.10 ± 0.03

[a] Weight of microsomal packed pellet.

Table III. *Effect of Ice-Encasement on Microviscosity of Microsomes Isolated from Acclimated Crown Tissue of Triticum aestivum L. and Liposomes Prepared from Total Lipid Extracts of the Microsomal Membranes*

Crowns were encased in ice for 7 d for Norstar and 5 d for Fredrick and then removed from ice-encasement by rapid thawing. Values (poise) were calculated from fluorescence polarization values obtained at 21°C for DPH labeled membranes. The data are mean values ± SE ($n = 3$).

Treatment	Membrane	Liposome
Norstar		
Control	1.98 ± 0.06	1.46 ± 0.003
Ice-encased	2.43 ± 0.02	1.97 ± 0.008
Fredrick		
Control	1.95 ± 0.01	1.45 ± 0.005
Ice-encased	2.72 ± 0.08	2.15 ± 0.03

seems likely that there is a deesterification of membrane phospholipids leading to a loss of lipid phosphate from these membranes, with a concomitant accumulation of free fatty acids.

Decreasing levels of membrane lipid phosphate together with the accumulation of free fatty acids suggest that phospholipid deesterification may be responsible for the increase in membrane microviscosity after lethal ice-encasement. Possible agents which could bring about phospholipid deesterification include the combined action of certain phospholipases, lipolytic acyl hydrolases (10), or attack by the superoxide free radical (19, 26). It is not known whether the loss of protein from stressed membranes is associated with the lipolytic activity previously described, or is due to some independent proteolytic activity.

This altered composition apparently caused a significant increase in membrane microviscosity as measured by fluorescence polarization with DPH, a hydrophobic probe which partitions in the acyl region of the membrane of both gel and liquid-crystalline lipid domains (27) with minimal perturbation of membrane structure (13). The protein:lipid ratio, the degree of fatty acid unsaturation and components in the neutral lipid fraction including free fatty acids and sterols are factors known to alter membrane microviscosity (9, 16, 28). Evidence that the changes in membrane microviscosity with icing stress originate from changes in the lipid component is shown by the observation that increases in microviscosity in the intact membranes were also seen in liposomes prepared from the lipid extracts of those membranes. As no major changes in the degree of fatty acid unsaturation were found immediately after the icing stress, this can be eliminated as a possible factor in altering membrane fluidity. However, the substantial increase in free fatty acid levels of stressed membranes may be an important factor contributing to the increase in microviscosity. Neutral lipids which accumulate in membranes of senescing tissues induce the formation of gel phase lipid domains (16). Also, the addition of saturated free fatty acids to liposomes composed of a total lipid extract from the microsomal membranes of soybean axes caused an increase in the lipid phase transition temperature and an increase in microviscosity—indicative of a rigidification of the membrane bilayer (26). Essentially similar effects have been elicited by the incorporation of saturated fatty acids into less complex lipid mixtures (15).

Immediately following lethal ice-encasement the crowns of winter wheat are still able to reduce tetrazolium, but lose that ability during the post-thaw period (31). It is apparent that further and quite different deteriorative changes occur in cellular membranes during this post-thaw period. Microsomal membranes isolated after a 6 h post-thaw period had greater protein:total fatty acids ratios and a lower degree of unsaturation in the total fatty acid fraction than membranes isolated immediately after ice-encasement. One important factor contributing to a reduction in the degree of fatty acid unsaturation is a decline in linolenic acid perhaps as a result of lipid peroxidation. Lipoxygenase catalyses the oxidation of free fatty acids containing the 1,4-cis-pentadiene system including linoleic and linolenic acid (11). The products of this degradation include fatty acid peroxides and hydroperoxides which themselves alter the physical properties of membranes (21). In addition, they are suitable initiators of free-radical chain reactions in biological systems (32) which also have damaging effects upon cellular membranes (14).

It is noteworthy that an increase in levels of free fatty acids as found after lethal ice-encasement has been associated with the formation of gel phase lipid, increased membrane microviscosity, and loss of viability after both desiccation (25) and freezing (12) stresses, and during senescence (16). There are other similarities. Freezing and ice-encasement have both been reported to modify potassium transport across the plasma membrane (20, 23) and promote increased leakage of cytoplasmic solutes. During acclimation of winter wheat seedlings, tolerance of freezing and tolerance of ice-encasement increase concurrently. In a comparison of over 30 moderately to very winterhardy wheat cultivars grown in a field environment, a highly significant correlation between freezing and ice-encasement tolerances was observed (17). Thus, there may be some elements of similarity in the molecular mechanism of injury to the cellular membranes and perhaps in the molecular mechanism of tolerance, between freezing and ice-encasement stress.

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Table IV. *Effect of Ice-Encasement on the Proportions of Protein, Total Fatty Acids (TFA), Free Fatty Acids (FFA), and Phospholipid (PL) in Microsomal Membranes Isolated from Acclimated Crown Tissue of Two Winter Wheat (T. aestivum L.) Cultivars*

Crowns were encased in ice for 7 d for Norstar and 5 d for Fredrick and then removed from ice-encasement by rapid thawing (ice-encased) or after rapid thawing and an additional 6 h post-thaw period at 2°C in darkness (ice-encased/post-thaw). The data are mean values ± SE (n = 4).

Treatment	Protein:TFA	PL:TFA	Protein:PL	FFA:PL
	mg:μmol	*mol:mol*	*mg:μmol*	*mol:mol*
Norstar				
Control	1.79 ± 0.03	0.166 ± 0.002	10.8 ± 0.3	0.94 ± 0.02
Ice-encased	0.90 ± 0.03	0.062 ± 0.002	14.5 ± 0.5	3.95 ± 0.10
Ice-encased/post-thaw	1.32 ± 0.05	0.107 ± 0.001	12.3 ± 0.5	3.66 ± 0.03
Fredrick				
Control	1.49 ± 0.06	0.220 ± 0.005	6.8 ± 0.3	0.88 ± 0.03
Ice-encased	1.24 ± 0.13	0.137 ± 0.002	9.1 ± 0.6	2.08 ± 0.08
Ice-encased/post-thaw	1.82 ± 0.19	0.083 ± 0.005	21.9 ± 2.3	3.85 ± 0.05

Table V. *Effects of Ice-Encasement on the Total Fatty Acid Composition of Microsomal Membranes Isolated from Acclimated Crown Tissue of Two Winter Wheat Cultivars*

Crowns were encased in ice for 7 d for Norstar and 5 d for Fredrick and then removed from ice-encasement by rapid thawing (ice-encased) or after rapid thawing and an additional 6 h post-thaw period at 2°C in darkness (ice-encased/post-thaw). Mean values ($n = 4$).

Treatment	Fatty Acid Composition					Unsat/Sat[a]
	16:0	18:0	18:1	18:2	18:3	
	mol %					ratio
Norstar						
Control	25.8	1.2	13.0	20.1	39.9	2.7
Ice-encased	21.6	1.8	13.9	22.9	39.8	3.1
Ice-encased/post-thaw	31.4	1.6	10.9	25.4	30.7	2.0
Fredrick						
Control	27.1	0.9	10.0	21.9	40.1	2.6
Ice-encased	27.8	1.1	8.3	22.6	40.2	2.5
Ice-encased/post-thaw	32.2	1.1	12.2	25.6	28.9	2.0
LSD ($P<0.05$)[b]	3.0	0.6	5.1	6.0	8.6	0.4

[a] Unsaturated:saturated fatty acid ratio, *i.e.* 18:1 + 18:2 + 18:3/18:0 + 16:0. [b] Least significant difference for comparison of treatment means at 5% probability level.

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