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Increase in Linolenic Acid Is Not a Prerequisite for Development of Freezing Tolerance in Wheat[1]

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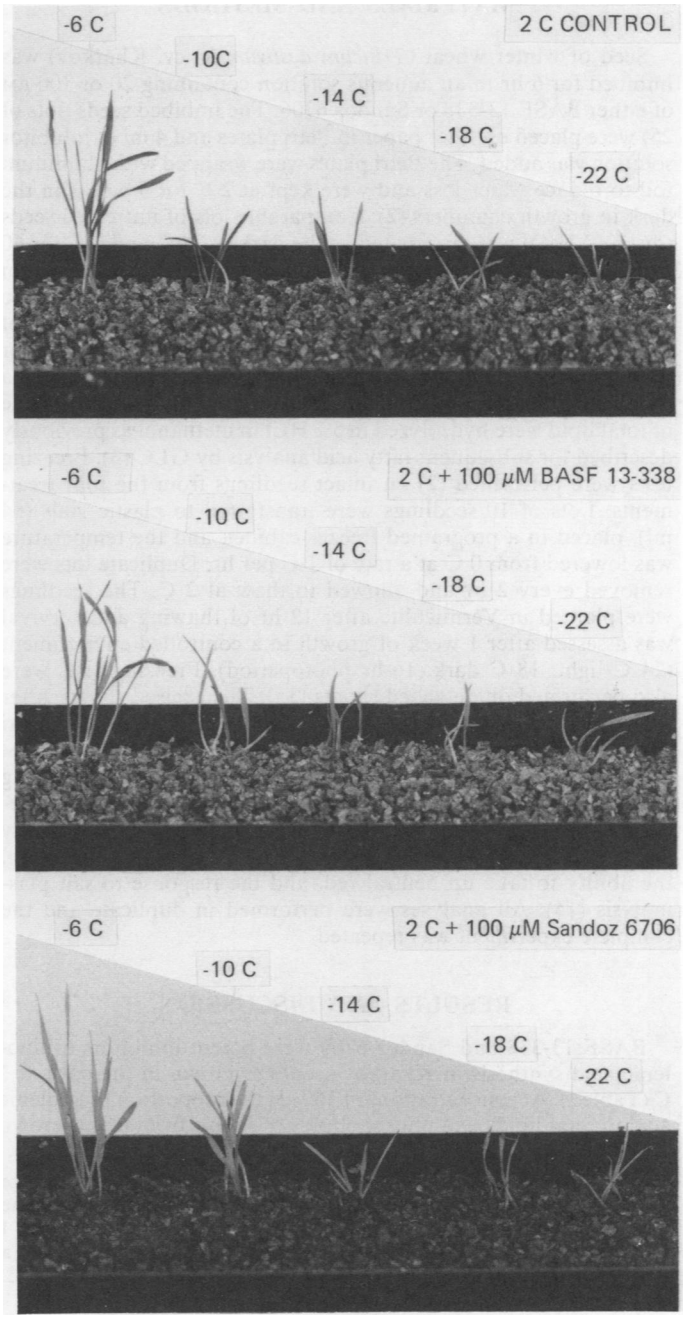
A. IAN DE LA ROCHE
Ottawa Research Station, Research Branch, Agriculture Canada, Ottawa, Ontario, Canada K1A OC6

ABSTRACT

Seedlings of winter wheat (*Triticum aestivum* L. cv. Kharkov) were acclimated at 2 C in the dark in the presence of two inhibitors of linolenic acid synthesis, 4-chloro-5(dimethylamino)-2-phenyl-3(2H)pyridazinone-(BASF 13-338) and 4-chloro-5(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)pyridazinone (Sandoz 6706). Although the increase in the proportion of linolenic acid generally observed at low temperature was completely inhibited, the development of freezing tolerance was unaffected. These results demonstrated that an enrichment in linolenic acid is not a prerequisite for low temperature acclimation.

During low temperature acclimation of herbaceous plants there is a preferential synthesis of unsaturated fatty acids (2, 4, 10). In young shoots of wheat and rye an increase in linolenic acid is observed, while in alfalfa roots the proportion of linoleic acid increases. Physical, chemical, and functional analyses of mitochondrial membranes from freezing-resistant and freezing-susceptible tissues of wheat have suggested a possible relationship between freezing tolerance and the unsaturation and fluidity of cellular membranes (9). A later study (3) demonstrated that the proportions of linolenic acid among cold-hardened seedlings of several cultivars of wheat were not correlated with their levels of freezing tolerance. These results were later confirmed by Willemot *et al.* (17) with wheat plants at a more advanced stage of development.

Willemot (16) observed that when wheat plants were pretreated with an inhibitor of linolenic acid synthesis (BASF 13-338)[2] prior to cold acclimation, both the accumulation of linolenic acid and the development of freezing tolerance were completely inhibited. He concluded that low temperature stimulation of linolenic acid synthesis was a necessary prerequisite to the development of freezing resistance even though it was not the factor responsible for the difference in tolerance observed among the various strains of wheat studied previously (3, 17). Willemot (16) also found that BASF 13-338 inhibited the increases in both dry weight and phospholipid content generally associated with low temperature hardening (13). This observation, coupled with the considerable evidence (6, 11) demonstrating that pyridazinone herbicides inhibit chloroplast function, may be an indication that a reduced level of photosynthetic carbon, and not the inhibition of linolenic acid synthesis, is the causal factor inhibiting cold hardening. The



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[2] Abbreviations: BASF13-338: 4-chloro-5(dimethylamino)-2-phenyl-3-(2H)pyridiazinone; Sandoz 6706: 4-chloro-5(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)pyridazinone.

requirement of photosynthesis for cold hardening has been well documented in several systems (7). A notable exception is the dark-germinated wheat seedling system utilized by de la Roche *et al.* (2) which derives its source of fixed carbon for cold hardening from the endospermal reserve. Germinating seedlings provide a system for studying the effect of pyridazinone inhibitors on linolenic acid accumulation and cold hardening without the necessity of a functional photosynthetic system.

In this communication I report the effects of two pyradazinone derivatives, BASF 13-338 and Sandoz 6706, on the synthesis of linolenic acid and the development of freezing tolerance during cold hardening of seedlings of Kharkov wheat. The development of freezing tolerance proceeded normally in every instance even though the low temperature stimulation of linolenic acid synthesis was completely inhibited.

MATERIALS AND METHODS

Seed of winter wheat (*Triticum aestivum* L. cv. Kharkov) was imbibed for 6 hr in an aqueous solution containing 20 or 100 μM of either BASF 13-338 or Sandoz 6706. The imbibed seeds (lots of 25) were placed on filter paper in Petri plates and 4 ml of inhibitor solution was added. The Petri plates were wrapped with aluminum foil to reduce water loss and were kept at 2 C for 5 weeks in the dark in growth chambers (2). Comparable lots of untreated seeds (distilled H_2O) were incubated in the dark at 25 C and 2 C for 60 hr and 5 weeks, respectively. Primary shoots approximately 2 cm long were excised from the young seedlings grown at the two temperatures and their lipids were extracted by a modification of the Bligh and Dyer method as previously described (3). The major lipid classes were separated by TLC on Silica Gel H using a double development system (3). The isolated lipids and a sample of total lipid were hydrolyzed in 5% HCl in methanol as previously described for subsequent fatty acid analysis by GLC (3). Freezing tests were performed (2) on intact seedlings from the four treatments. Lots of 10 seedlings were transferred to plastic vials (64 ml), placed in a programed freezer cabinet, and the temperature was lowered from 0 C at a rate of 1 C per hr. Duplicate lots were removed every 2 hr and allowed to thaw at 2 C. The seedlings were planted in Vermiculite after 12 hr of thawing and survival was assessed after 1 week of growth in a controlled environment (24 C light, 18 C dark, 16-hr photoperiod). Freezing tests were also performed on detached shoots (15). The excised shoots, after thawing, were incubated under continuous light at 22 C in Petri plates containing 10 ml of dilute salt solution (containing 45 mM NaCl and 5 mM $CaCl_2$). After 48 hr the extent of shoot greening was recorded. Shoots were also examined microscopically immediately after thawing and after 48 hr of incubation. Cell vitality was assessed by the presence or absence of protoplasmic streaming, the ability to take up neutral red, and the response to salt plasmolysis (15). All analyses were performed in duplicate and the complete experiment was repeated.

RESULTS AND DISCUSSION

BASF 13-338 and Sandoz 6706 were potent inhibitors of linolenic acid synthesis in Kharkov seedlings grown in the dark at 2 C (Table I). At concentrations of 100 μM the proportion of linolenic acid in total lipids and phospholipids of shoots from plants grown at 2 C was even less than the level observed in the untreated control grown at 24 C. These data were similar to those obtained by Willemot (16) for roots and leaves of the same cultivar grown to a more advanced stage of development.

The fatty acid compositions of the other major lipid classes (mono- and digalactosyldiacylglycerol, triacylglycerol, and free fatty acids) were also examined and were found to exhibit the same reduction in the proportion of linolenic acid as found in the phospholipids. The inverse relationship between linoleic and linolenic acids in the six treatments (Table I) suggests that the two pyridazinone inhibitors are specifically inhibiting the activity of linoleic acid desaturase which apparently is stimulated by incubation at low temperature.

Measurements of plants survival 1 week after freezing showed that there was no significant difference in freezing resistance between the 2 C-grown seedlings treated with inhibitors and the untreated 2 C control, all being tolerant to −22 C but killed by freezing to −24 C (Table I). The unhardened control grown at 24 C was killed by freezing to −4 C. Closer examination of the plants revealed that the tips of the leaves of the seedlings grown at 2 C and frozen to −22 C were necrotic (Fig. 1). The sublethal damage at higher freezing temperatures, −10, −14, and −18 C, shown in reduced regrowth (Fig. 1), is similar in all three treatments. Freeze tests on detached shoots further demonstrated that the development of freezing tolerance was unaffected by inhibitor treatments. Approximately 10% of the cells in the detached leaves were killed by freezing to −21 C while all cells survived freezing to −17 C (Fig. 2). Similar results were obtained with Capelle-Desprez, a less hardy cultivar of wheat, and *Secale cereale* L. cv. Puma, a hardy winter rye.

Sandoz 6706, but not BASF 13-338, prevented regreening of etiolated seedlings when exposed to light during the regrowth test (Fig. 1). In the case of Sandoz 6706 treatment, regrowth was able to proceed for 1 week because of the presence of an endospermal reserve. After 7 to 8 days this reserve was exhausted and the seedling died shortly thereafter. The ability of detached leaves floating in dilute salt solution to regreen after being frozen to −17 C was due probably to Sandoz 6706 leaching out of the tissue. The absence of regreening in Sandoz-treated tissues frozen to −21 C may reflect an interaction with the partial damage which occurs at this temperature. With this exception, no other morphological differences were apparent between seedlings treated with inhibitors and the untreated control.

The data from the freeze tests are in direct contrast to the results reported by Willemot (16) which showed that plants treated with BASF 13-338 were incapable of developing a tolerance to freezing when exposed to low temperature. The difference in the results is probably due to the different physiological stages of the plants used; dark-grown seedlings relying on endospermal reserves for growth and development, and 14-day-old plants depending on photosynthesis for their source of fixed carbon. Consequently the more advanced light-grown plants may not be able to develop freezing tolerance in the presence of pyradazinone inhibitor because of an impairment of normal photosynthesis by this compound (6, 11).

It may be argued that the chloroplasts are the primary target of freezing injury in light-grown plants (5) and that an increase in the proportion of linolenic acid in the membrane lipids of chloroplasts during acclimation is a prerequisite to development of resistance. There are several lines of evidence to indicate that this is not the case. Senser and Beck (12) showed that freezing isolated chloroplasts of cold-hardened and nonhardened spruce needles to temperatures that were lethal to the intact cells did not markedly alter their photochemical activity, a measure of membrane integrity. Heber and Santarius (5) found no difference in the freezing response of chloroplasts isolated from spinach grown at acclimat-

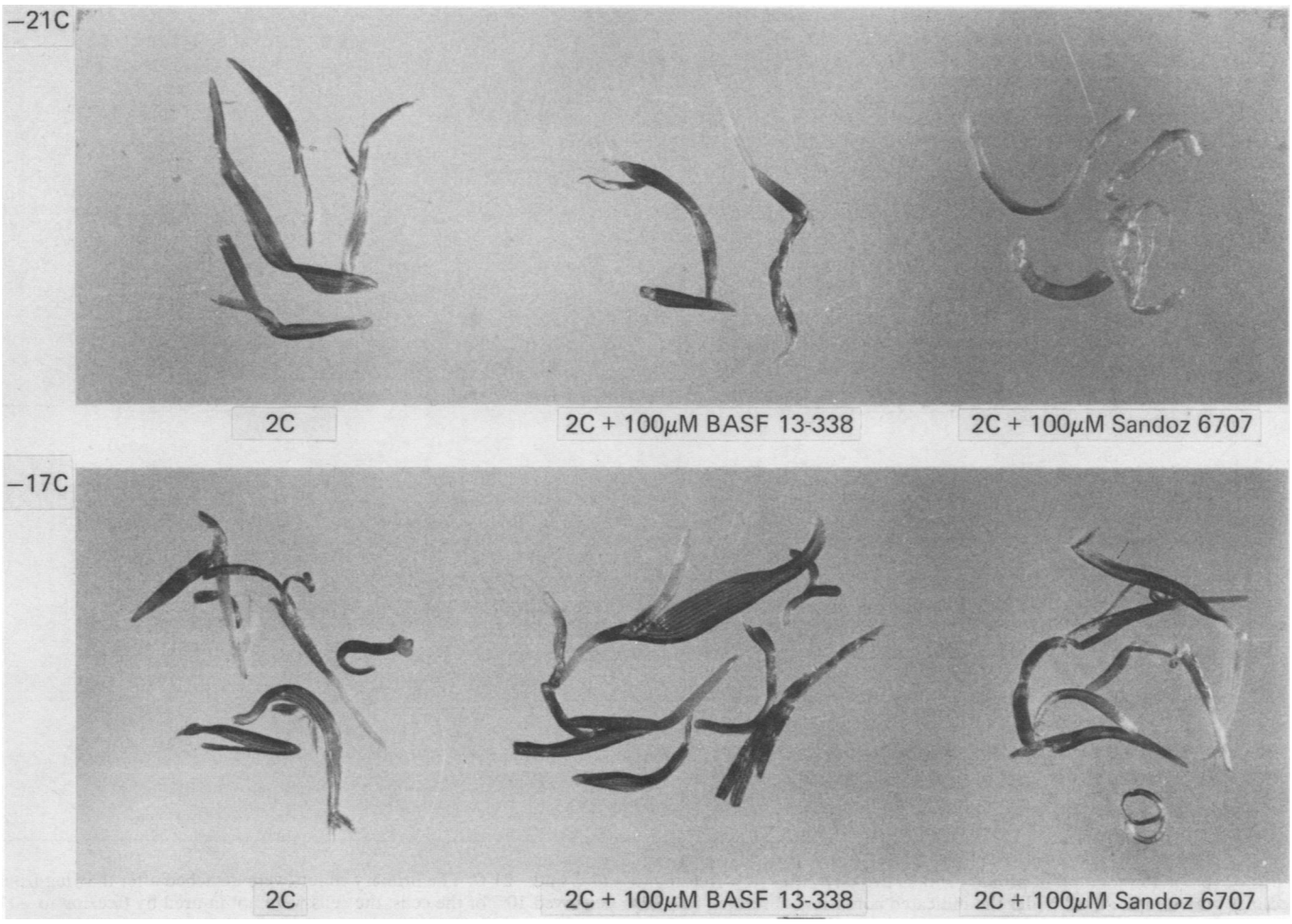
FIG. 1 (p. 5). Survival of Kharkov seedlings after exposure to a range of freezing temperatures. The seedlings were grown in the dark under the indicated conditions for 5 weeks before freezing. Photographed after 1 week regrowth in a controlled environment (24 C light, 18 C dark with a 16-hr photoperiod). All seedlings were killed by freezing to −24 C. Seedlings grown at 24 C were killed by freezing to −4 C.

Table I. The effect of BASF 13-338 and Sandoz 6706 on the fatty acid composition of total lipids and phospholipids and on the freezing tolerance of Kharkov wheat seedlings.

Treatment	Concentration of Inhibitor	LD_{50}[1]	Lipid Component	Fatty Acid Composition[2]				
				16:0	18:0	18:1	18:2	18:3
	μM	C		% by weight				
24 C, Control	0	-4	Total lipids	17.6	0.5	8.3	23.7	49.9
			Phospholipids	23.1	0.7	9.6	31.1	35.5
2 C, Control	0	-23	Total lipids	15.7	0.7	5.9	14.1	63.6
			Phospholipids	21.9	0.5	7.9	17.6	52.1
2 C, BASF 13-338	20	—	Total lipids	16.0	0.4	5.5	25.3	52.7
			Phospholipids	21.0	0.7	7.5	23.6	47.2
2 C, BASF 13-338	100	-23	Total lipids	15.2	0.7	8.9	51.8	23.3
			Phospholipids	19.3	0.2	11.1	47.1	22.3
2 C, Sandoz 6706	20	—	Total lipids	14.8	0.6	6.6	21.8	56.2
			Phospholipids	21.6	0.4	8.2	23.1	46.6
2 C, Sandoz 6706	100	-23	Total lipids	14.7	0.4	10.4	38.4	36.1
			Phospholipids	18.5	0.6	12.6	35.3	33.0
Average SD				0.5	0.1	0.2	0.1	0.7

[1]Temperature at which 50% of the tested population is killed

[2]Number of carbon atoms: no. of double bonds



ing and nonacclimating temperatures although the chloroplast lipids of the acclimated plants were presumably more unsaturated. Lyons and Asmundson (8) demonstrated that variations in the mol % of linoleic and linolenic acids have similar effects on the freezing points of mixtures of palmitate and linoleate, or palmitate and linolenate, the predominant fatty acids in plants. Differential scanning calorimetry revealed that the large differences in linolenic acid content of organelles from cold-hardened and nonhardened rye had no significant effect on the membrane lipid phase transition temperature (14), the membrane property generally considered critical in the mechanism of cold resistance.

Desiccation studies with wheat seedlings offer further evidence in support of the conclusion that an increase in unsaturation is not a prerequisite to the development of freezing tolerance. When Kharkov seedlings were germinated under reduced water potential for 12 days at 24 C, then rehydrated and subjected to freeze tests, they were capable of withstanding slow freezing to −13 C. The unhardened control grown in an atmosphere of 100% RH at 24 C was killed at −5 C. Lipid analysis of shoots showed that the proportion of linolenic acid in the tissues hardened by desiccation did not increase but actually decreased to less than that found in the unhardened control (1).

The present results with seedlings grown in the dark at 2 C demonstrate clearly that an increase in fatty acid unsaturation is not a prerequisite for the hardening process at lower temperatures. Kharkov wheat seedlings when grown in the presence of the inhibitors, although exhibiting less lipid unsaturation than the unhardened control, were able to cold harden to the same extent as the more highly unsaturated hardened control.

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FIG. 2 (p. 7). Photograph of primary shoots two days after slow freezing to −17 C and −21 C. The primary shoots were detached after thawing from seedlings grown in the dark under the indicated conditions. Freezing to −21 C destroyed 10% of the cells; the cells were not injured by freezing to −17 C.