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Inhibition of Linolenic Acid Synthesis and Modification of Chilling Resistance in Cotton Seedlings

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ABSTRACT

The temperature at which cotton seeds (*Gossypium hirsutum* L.) germinated influenced the fatty acid composition of the polar lipids of developing root tips. Seeds were germinated at 15, 20, 25, and 30 C. As the temperature decreased the linolenic acid content of the polar lipid fraction increased. Sandoz 9785[4-chloro-5-(dimethylamino)-2-phenyl--3(2*H*)-pyridazinone] reduced the low temperature-induced increase in linolenic acid content of the polar lipids and reduced seedling ability to withstand 8 C chilling. The results are consistent with the conclusion that chilling resistance in cotton seedlings is related to the level of linolenic acid in the polar lipids in the developing root tips.

Chilling resistance is a biological process marked by changes in many chemical constituents. Chilling resistance has been correlated with changes in sugars, amino acids, nucleic acids, and proteins. These correlations only support the fact that submarginal temperatures alter the entire metabolism of living organisms. At present the unsaturated fatty acids in the membrane polar lipids are believed to play a major role in the mechanism of chilling resistance in plants. Gerloff *et al.* (5) have demonstrated dramatic chilling-induced changes in the fatty acid composition of the polar lipid fraction. Lyons *et al.* (11) have correlated chilling resistance with fatty acid composition of the mitochondrial lipid in chill-hardy plant species. They found that the linolenic acid content of the primary mitochondrial membrane lipid (phosphatidylethanolamine) increased in chilled tissue. This increase in linolenic acid was associated with increased membrane fluidity. They suggested that survival of plants at low temperatures is associated with increases in linolenic acid accompanied by increased membrane fluidity.

Alteration of the linolenic acid content of membrane polar lipid in plants offers a means of elucidating the role of unsaturated polar lipids in resistance to chilling and in the hardening process. Hilton *et al.* (7) reported that Sandoz 6706 [4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone] prevented maturation of chloroplasts of germinating mustard and barley by inhibiting production of linolenic acid, thereby preventing galactolipid synthesis necessary for chloroplast lamellae. The present work is an extension of this research to determine: (*a*) if another pyridazinone analog can also inhibit linolenic acid synthesis in nonphotosynthetic tissue, especially altering the increased synthesis during chill hardening; and (*b*) if the inhibition of linolenic acid synthesis alters chilling resistance and the hardening process, thereby firmly implicating a role for fatty acid unsaturation in these processes.

MATERIALS AND METHODS

Germination Procedure for Lipid Analysis Samples. Seeds of *Gossypium hirsutum* L., genetic selection M8, were placed between germination papers rolled in a waxed paper outer covering. Seed germination rolls were moistened with distilled H_2O in control treatments and with Sandoz 9785 [4-chloro-5-(dimethylamino-2-phenyl-3(2*H*)-pyridazinone] at 10 μM concentration in the chemical treatments. Seeds were germinated at 15, 20, 25, or 30 C. Time sequences were used to produce seedlings of equal size at all temperatures, *i.e.*, germination time was 24 hr at 30 C, 40 hr at 25 C, 70 hr at 20 C, and 168 hr at 15 C.

Tissue Preparation. One-cm root tips and 2-cm hypocotyl sections were cut and frozen immediately on dry ice, freeze dried, and stored at −15 C until analyzed for lipid content.

Extraction and Quantitative Analysis of Polar Lipid. Lipids were extracted and recovered from 1 g of freeze-dried root tips by the procedures of Folch *et al.* (4). Polar lipids were separated from neutral lipids by the rubber membrane dialysis method of Böttcher *et al.* (2) and saponified with alcoholic KOH as outlined by Burchfield and Storrs (3). Methyl esters of the resulting fatty acids were prepared by the boron trifluoride in methyl alcohol method of Metcalfe and Schmitz (13). The esterified acids were analyzed by gas chromatography as previously described (7) with the exception that a 1.83-m glass column packed with 10% SP-216-PS on 100/120 mesh Supelcoport[1] (Supelco, Inc., Bellefonte, Pa.) was used in the present studies. Heptadecanoic acid was included in all samples as an internal standard. As nearly as was possible, the lipids were maintained under a nitrogen atmosphere throughout the procedure to minimize oxidation of the unsaturated lipids. Preliminary data accumulation and reduction was achieved with an Autolab System IV computing integrator (Spectra-Physics, Parsippany, N. J.).

Growth and Hardening Experiments. All growth and hardening studies were conducted in growth chambers in which the environment was closely controlled. Forty seeds were planted at 2-cm depth in plastic trays (30 × 20 × 10 cm) containing a commercial potting mixture. The plantings were moistened with water or water containing sufficient pyridazinone compound to produce a 20 μM concentration with the potting mixture. This concentration approximated 5 μg/g (w/w) with the potting mixture.

Control and Sandoz 9785 plantings were germinated at 30 C for 48 hr. Then a control and a Sandoz 9785 planting were chill-hardened by growing the seedlings for 2 days at 30 C, 2 days at

[1] Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

25 C, 2 days at 20 C, and 2 days at 15 C. Another control and another Sandoz 9785-treated planting were not chill-hardened before exposure to chilling temperatures. These plantings were grown to 30 C for 8 days.

Growth conditions were 16-hr (200 ft-c) day, 8-hr night, with a 70% relative humidity. After the 8-day growth period, all treatments were chilled for 4 days at 8 C with a 12-hr day at 2000 ft-c, 70% relative humidity. The seedlings were returned to 30 C after chilling to observe postchilling injury symptoms and recovery. Photographs and seedling counts were taken immediately after chilling and after 7 days of recovery at 30 C.

RESULTS

Polar Lipid Fatty Acid Studies. As the temperature of growth decreased from 30 to 15 C, the linolenic acid (C18:3) content of the polar lipid fraction of untreated cotton seedling root tips increased (Table I). Treatment with Sandoz 9785 reduced the low temperature-induced increases in linolenic acid. In comparison to fatty acid levels in untreated root tips, Sandoz 9785 treatment lowered the levels of linolenic acid at all temperatures and raised the levels of linoleic acid (C18:2) at all temperatures. This suggests a blockage of C18:3 acid formation (Table II). In contrast to the results of fatty acid analysis of root tips, analysis of hypocotyl tissue of seedlings germinated at 30 and 15 C showed no effect of temperature or of the Sandoz 9785 on fatty acid unsaturation (Table III), perhaps indicating little turnover of the lipid components of embryonic tissue during germination except at the site of active cell division in the root tips.

Temperature—Growth Studies. Cotton seedling response to chilling (8 C) was related to linolenic acid levels measured in root tips. Nonhardened control and Sandoz 9785-treated seedlings (germinated 8 days at 30 C) wilted within 24 hr after start of chilling. The nonhardened control seedlings rehydrated to a limited degree after 4 days of chilling, but the nonhardened Sandoz 9785-treated seedlings wilted completely (Fig 1). The hardened control and hardened Sandoz 9785-treated seedlings showed no response to chilling for 2 days. On the 3rd day, many Sandoz 9785-treated seedlings lost turgor. The hardened controls showed no evidence of chilling injury throughout the 4-day treatment.

Table I. *Fatty Acid Composition of Polar Lipids of 1-cm Root Tips of Cotton Seedling Radicles*

The data are the average of three experiments.

Treatment	Growth Temperature	Fatty Acid				
		C 16	C 18	C 18:1	C 18:2	C 18:3
	degree C	*% by weight*				
Control (water)	30	32.6	3.7	7.5	36.3	13.6
	25	35.3	2.8	5.7	33.4	22.9
	20	32.9	3.3	5.4	31.7	26.8
	15	31.6	4.8	4.2	31.9	27.5
Sandoz 9785 (10 μM)	30	34.7	2.8	7.6	44.0	10.9
	25	34.4	2.7	5.7	43.1	14.0
	20	34.5	3.7	6.4	41.7	13.7
	15	30.9	4.1	4.6	43.7	17.1

Table II. *C 18:2/C 18:3 Fatty Acid Ratios of Cotton Seedling Root Tip Polar Lipids*

Growth Temperature	Treatment	
	Control	Sandoz 9785 (10 μM)
degree C		
30	2.67	4.04
25	1.46	3.08
20	1.18	3.07
15	1.16	2.56

Table III. *Fatty Acid Composition of Polar Lipids of Cotton Seedling Hypocotyls*

The data are the average of three experiments.

Treatment	Growth Temperature	Fatty Acid				
		C 16	C 18	C 18:1	C 18:2	C 18:3
	degree C	*% by weight*				
Control (water)	30	25	6	7	37	11
	15	26	4	6	35	19
Sandoz 9785 (10 μM)	30	27	5	6	39	11
	15	29	3	5	36	17

FIG. 1. Sandoz 9785 effect on chilling sensitivity in hardened and nonhardened cotton seedlings. Cotton germinated 8 days at 30 C, control-9785 germinated 8 days at 30 C with 2 μM Sandoz 9785 in media. Hardened control germinated 2 days each at 30 C, 25 C, 20 C, and 15 C. Hardened 9785 germinated in like manner with 20 μM Sandoz 9785 in media. Pictures taken following 4 days chilling at 8 C.

After 4 days at 8 C and 7 days at 30 C, all nonhardened Sandoz 9785-treated seedlings lost cotyledons and only a few were normal (Table IV). After chilling, the nonhardened control seedlings showed marginal necrosis of cotyledons but most survived and resumed growth when returned to 30 C. The hardened control and hardened Sandoz 9785-treated seedlings exhibited fewer visible latent symptoms of chilling injury, although a greater number of abnormal seedlings occurred in the latter. The wilted Sandoz 9785-treated seedlings regained turgor within 24 hr after return to 30 C. Perhaps if the chilling treatment had been continued for a longer period greater seedling injury would have occurred.

DISCUSSION

The relationship between chill hardiness and fatty acid levels in plants is a subject of controversy. The present research may help clarify the situation. Wilson and Crawford (15) believe that unsaturated fatty acid levels are not well correlated with chill hardiness or with changes in plant cell membranes. A number of researchers (6, 8, 14) report increases in unsaturated fatty acid levels, and increases in total fatty acid or total phospholipid content concurrent with hardening. Some of the most relevant

Table IV. *Cotton Seedling Survival 7 Days after 96 Hr of Chilling at 8 C*

Growth Conditions	Seedling Classification		
	Normal	Abnormal[1]	Dead
		%	
Control 30 C, 8 days	62	35	3
Sandoz 9785 (10 μM) 30 C, 8 days	0	60	40
Control, hardened[2]	92	9	0
Sandoz 9785 (10 μM), hardened	80	20	0

[1] Seedlings with 50% of cotyledon necrotic were classed abnormal.

[2] Grown 2 days at 30 C, 2 days at 25 C, 2 days at 20 C, and 2 days at 15 C.

work has been reported by Lyons and fellow workers (9–11). They associated differences in chilling resistance among plant species with degree of fatty acid unsaturation of membrane lipids. The present research clearly relates the level of linolenic acid in polar lipids to chilling resistance in cotton seedlings. Sandoz 9785 blocks synthesis of linolenic acid and greatly reduces resistance to chilling. This clearly indicates that polar lipid linolenic acid is correlated with chilling resistance in plants.

Failure to alter fatty acid quality or quantity in hypocotyl tissue in the present research points out that selection of tissue for analysis can materially alter results and conclusions. Wilson and Crawford (15) and Guinn (6) used mature leaves for lipid assay and recorded no increase in phospholipid. Gerloff *et al.* (5) used 10-cm root sections cut from below the crown of alfalfa and showed increases in linolenic acid associated with low temperature. Kuiper (8), using mature tissue, reported little change in alfalfa leaf fatty acids induced by chill hardening. A part of the diversity of results lies in choice of material. It appears that low temperatures alter only fatty acid unsaturation in newly developing tissue. These findings are an expected consequence of the repeated demonstration (12) that little or no net synthesis of membrane lipids occurs in mature tissues.

One important factor in chilling injury of leaf tissue may be water status and plant ability to extract water from the soil and transport it to the leaves. The first symptom noted in the present seedling chilling study was loss of leaf turgor. Reduction in root tip water uptake at low temperature (such as Sandoz 9785-alteration of membrane lipid fatty acids) could alter leaf water status leading to initial marginal necrosis of the leaves and possibly to death of the leaves. The desiccating effect of chilling noted by Guinn (6) and the inability of cotton seedling roots to absorb water at temperatures below 10 to 12 C noted by Arndt (1) were well demonstrated in the present study. Also demonstrated was the chill-hardening ability of cotton as previously noted by Guinn (6). Although he could demonstrate chilling resistance in cotton, he could not relate chill hardiness to phosphate fixation in the lipids of cotton. The difference is resoluble on either the choice of tissue or perhaps because changes are not in total phospholipid but in the fatty acid complement of some specific phospholipid.

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