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FREE STEROL AND PHOSPHOLIPID EVOLUTION IN *TRITICUM AESTIVUM* SEEDLINGS AT OPTIMUM AND LOW TEMPERATURES

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Abstract—The free sterol and phospholipid of the leaves and roots of *Triticum aestivum* var MEC seedlings, grown at different temperatures, were determined During growth, free sterols increased in the leaves and roots at optimum temperature (21°) whereas in the cold treatment (1°) they remained significantly unchanged despite an increase of cholesterol of the leaves indicating a higher degree of regulation of membrane structure under cold conditions Phospholipid from both groups of plants increased in the leaves and in the roots during all the experimental period, although at a lower degree in the cold treated plants The molar ratio of free sterol/phospholipids suggested a less ordered membrane structure in the cold treated leaves and roots

INTRODUCTION

It has been inferred by many authors that winter wheat exposed to low temperatures undergoes metabolic changes which lead to an increase in resistance This increase during cold acclimatization was at first attributed to increased total phospholipid and higher levels of unsaturated fatty acid [1] in the membranes synthesized by seedlings growing and adapting to low temperature [2] These changes were assumed to have increased membrane fluidity

Later De La Roche *et al* [3] found, at least in wheat seedlings, that increased unsaturation of membrane fatty acids is not essential for the adaptation to low temperatures Furthermore Pomeroy and Raison [4] observed small changes in the fluidity of membrane polar lipids in wheat seedlings during acclimatization, but these changes were not correlated with freezing tolerance Other lipid factors, such as sterols, also might vary and hence alter the properties of membranes Indeed, it has been suggested that interaction of free sterols with phospholipids stabilizes membranes and permeability control [5]

Very little is known about the effect of temperature on sterol metabolism in wheat even though its importance on membrane structure is obvious Davis and Finkner [6] showed that shoots of wheat plants grown at 10° had higher total sterol content than shoots from plants grown at 1°, whereas roots from the same plants had the reverse pattern Willemot [7] found that low temperature caused an increase in total sterol content but had little effect on sterol composition and total sterol to phospholipid ratio

Knowledge of the changes in amounts of individual sterols of the free sterol fraction in wheat seedlings grown at optimum-temperature and under cold-acclimatization conditions is required in order to clarify further the biochemical mechanism underlying cold-acclimatization

For this reason we were interested in whether the free sterol content and composition, and the free sterol to phospholipid molar ratios were affected, when comparing *Triticum aestivum* seedlings grown at optimum temperatures (21°) and at below optimum temperatures (1°) for cold acclimatization

RESULTS

Growth of wheat seedlings was more pronounced under the optimum temperature (control, 21/15° day/night) than under the low temperature treatment (1° day/night) (Fig 1 and Table 1) The height of seedlings increased in the control whereas in the treated plants it remained unchanged over all the experimental period The shoot dry weight of the first group of plants increased five times whereas the cold-treated plants showed about a three-fold increase during the same period

In wheat roots the dry matter was significantly lower than in shoots and its increase followed the same pattern as in shoots for the 21/15° day/night treatment On the contrary, in the cold-treated plants only the shoots showed a dry matter increase

Measurements of solute leakage showed that there were no significant differences in the relative amount of leakage between the treatments (Fig 2) The relative amount of leakage at 1° was indeed similar to that observed at 21° at every sampling date throughout the experimental period Furthermore the injury evaluation by Sakumaran and Weiser [8] showed no damage in cold treated seedlings

The free sterol content of the shoot and root tissues, measured on a μg/plant basis, was higher in the control than in the treated plants (Table 2) Under optimum temperature conditions, sterol content increased up to the third harvest date, afterwards it remained unchanged, whereas the cold treatment left the sterol level significantly unmodified

The major sterols of winter wheat from both groups of plants were sitosterol and campesterol, followed by

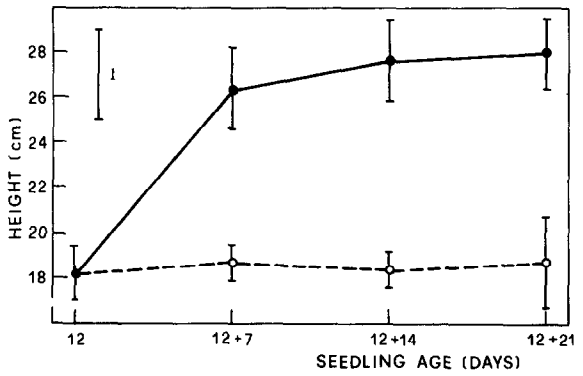

Fig 1 Effect of two temperatures on growth of *T aestivum* seedlings over the experimental period (●—●, 21°, O--O, 1°)

stigmasterol, cholesterol was present in small quantities (Tables 3 and 4) During plant growth, in the leaves of the control sitosterol and campesterol had a behaviour very similar to that of free sterols, stigmasterol increased up to the same time as free sterols afterwards it decreased, and cholesterol increased significantly over all the experimental period In the low-temperature plants sitosterol, campesterol and stigmasterol levels remained the same but the cholesterol level increased up to the second harvest date after which it remained unchanged

In the roots of the control, cholesterol, campesterol and sitosterol contents showed an increase while stigmasterol had a similar pattern to that observed in the leaves The changes in response to cold temperature were similar to those for free sterols

Phospholipid content was always higher in shoots than in roots, and these showed lower values under low temperature than the control (Table 5) Besides in these two parts from both groups of plants, phospholipid increased during all the experimental period, although to a lower degree in the treated plants The low temperature induced a decrease of free sterol to phospholipid molar ratio (Table 6) and so this ratio appeared very low in the leaves In roots, although this ratio was higher than in shoots, we noticed a similar behaviour

DISCUSSION

Leakage at 1° was similar to that of the 21° treatment throughout the experimental period, indicating that the winter wheat plant viability was the same at both temperatures (Fig 1)

From the data in Tables 2–6 we can determine if changes in free sterol content, sterol composition, phospholipid and free sterol to phospholipid ratio occur after transfer of the plants from 21° to 1° In this experiment on winter wheat the amounts of free sterols were always higher in shoots than in roots and they increased in both plant parts during the plant growth in the optimum temperature treatment.

Free sterol comparative values for the two parts of plant have not been reported, but Davis and Finkner [6] found a very similar pattern for total sterols When we consider what happens at 1°, the free sterol content of shoots and roots appears significantly unchanged over all the experimental period even though the dry matter increases

With regard to the absolute amounts of each sterol we noticed that in both shoots and roots low temperature influences their evolution Whereas at 21° every sterol increased during plant growth, at 1° significant differences did not appear either during successive growth stages or for the first stage, with the exception that cholesterol increased in the shoots about two fold after a week of treatment, thereafter remaining at the same level

The relative amounts of the sterols (calculated from Tables 2–4) did not vary greatly among the plant parts or between treatments, as was observed by Davis and Finkner [6] Davis and Finkner [6] and De La Roche [9] observed a shift towards sitosterol in low temperature treated winter wheat but our data did not show any increase in this sterol Because the only observed variation was in the cholesterol content of the shoots two explanations can be considered Low temperature could influence either (a) a common intermediate for sterols other than cholesterol or (b) the movement of mevalonic acid from its site of synthesis to the site of synthesis of the other three sterols as suggested by Davis and Finkner [6]

It has been suggested that free sterols play an important role in the structure of biological membranes and the increase of permeability and fragility of erythrocytes is well known [10] In higher plants, among free sterols it was found that cholesterol, at low concentration, was

Table 1 Effect of temperature on dry matter levels (mg/plant) of *T aestivum* shoots and roots over the experimental period

	Dry matter					
Seedling age (days)	Shoots			Roots		
	21°C	1°C	Means	21°C	1°C	Means
12	24 8 Aa	—	24 8 Aa	13 2 Aa	—	13 2 Aa
12+7	46 6 Bc	32 6 Aab	39 6 Bb	29 2 Ba	15 8 Aa	22 5 Ba
12+14	74 3 Cd	42 8 Bbc	58 5 Cc	61 7 Cb	18 8 ABa	40 2 Cb
12+21	117 4 De	67 9 Cd	92 6 Dd	75 0 Db	24 7 ABa	49 8 Db
Means	65 8 Bb	42 0 Aa		44 8 Bb	18 1 Aa	

For comparisons among means the analysis of variance test was used Means with capital letters in common are not significantly different at the $P = 0.05$ Means with small letters in common are not significantly different at the $P = 0.01$

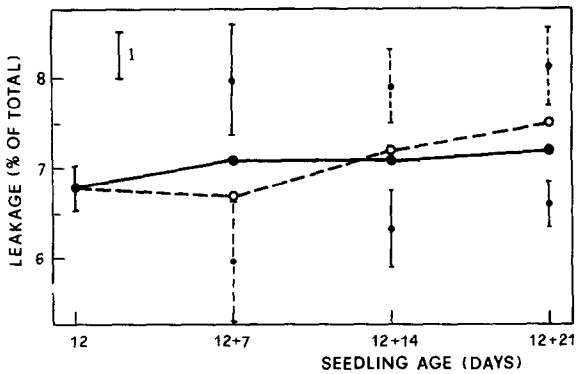

Fig 2 Cellular leakage as a percentage of total conductivity for *T aestivum* seedlings at two different temperatures over the experimental period (●—●, 21°, ○—○, 1°)

more effective than calcium chloride, the classic membrane stabilizer, in preventing the methanol-induced leakage of betacyanin from red beet disk and the effectiveness decreased in the order cholesterol, campesterol, stigmasterol, sitosterol [11, 12] This hypothesis has been confirmed [13] in micelles with different proportions of phospholipids and phytosterols A critical point for higher plants is that cholesterol is generally present in small amounts, while the major sterols are sitosterol and stigmasterol Nevertheless for cholesterol Wood and Paleg [13] found that the concentration needed to act is very low, because only slight changes in its content could change the permeability and function of the membrane

When we examine the data from the shoots, we find support for this suggestion This behaviour might be related to the greater effectiveness of cholesterol than other sterols in stabilizing membrane structure, and this fact may be a contribution in overcoming the negative effect of low temperature This hypothesis did not find corroboration in the root sterol content which did not show any significant change in free sterols during the growth at 1° The phospholipid of shoots and roots increased during cold acclimatization as already pointed out by Willemot [7], although his data, expressed in μg/g fr wt, are not really comparable with ours Also Horváth [14] showed a strong relationship between phospholipid content and level of hardiness

As the amount of phospholipids reflects the amount of membranes the increase in the phospholipid content could be interpreted as cold acclimatization dependent augmentation of membraneous materials in leaves and roots of wheat, even if it appears unlikely without a parallel increase in free sterols that are structural components of membranes The molar ratio of free sterol to phospholipid in the shoots of plants grown at 21° shown in Table 6 closely resembled that obtained by De La Roche [9] in a total membrane fraction of winter wheat, suggesting that most free sterols are localized in the cell membranes The ratio was at its lowest during acclimatization in the roots and in the shoots and this suggested a less ordered membrane structure

EXPERIMENTAL

Plant material Winter wheat (*Triticum aestivum* L cv MEC) was germinated in pots with basal perforation, filled to a uniform level with a steam sterilized perlite Uniformly sized seeds were planted in each pot at a depth of 1 cm and maintained in the dark at 24° for 2 days Then they were grown at 21° and 15° day/night, a 16 hr photoperiod, an 85% relative humidity and a light intensity of 18 000 lux

Half strength Hoagland's 2 soln was used during seedling growth Twelve day-old seedlings were divided in two groups one group continued to grow under unchanged conditions as the control, and the second group was transferred to a cold chamber 1° day/night with the other conditions unchanged Plant material (three replicates of 150 seedlings each) was collected after the twelve day period and on the 1st, 2nd and 3rd week of further growth from both temperature regimes

At each harvest date, the aerial parts were separated from roots, the wet wt, seedling height and number were recorded, and all samples were lyophilized and stored under N_2 at −20° until analysed

Lipid extraction and sterol analysis Extraction, purification and precipitation of the sterols were as previously described [15] Individual sterols were identified by GLC, cholestane was the internal standard, and for their quantitation, corrections were made for differences in detector response The column was glass (3 m × 4 mm) and packed with GP 3% SP-2250 AW-DMCS 100/120 mesh The operating conditions were column, 235°, flash heater, 280°, detector, 300°, N_2 was the carrier gas at 40 ml/min Phospholipid was determined by the method of Allen [16] after sample extraction with $CHCl_3$–MeOH (2 1) [17]

Table 2 Free sterol evolution (μg/plant) of *T aestivum* shoots and roots at two different temperatures over the experimental period

	Free sterols					
Seedling age (days)	Shoots			Roots		
	21°C	1°C	Means	21°C	1°C	Means
12	26 0 Aa	—	26 0 Aa	16 6 Aa	—	16 6 Aa
12+7	55 2 Bb	27 6 Aa	41 4 Bb	40 2 Bb	12 0 Aa	26 1 Bab
12+14	101 2 Cc	30 8 Aa	66 0 Cc	62.3 Cc	7 9 Aa	35 1 Cbc
12+21	107 9 Cc	33 1 Aa	70 5 Cc	69 9 Cc	14 5 Aa	42 2 Cc
Means	72 6 Bb	29 4 Aa		47 2 Bb	12 8 Aa	

For comparisons among means the analysis of variance test was used The significance of the letters is the same as in Table 1

Table 3 Free sterol composition (μg/plant) of *T aestivum* shoots at two different temperatures over the experimental period

Seedling age (days)	Cholesterol			Campesterol			Stigmasterol			Sitosterol		
	21°C	1°C	Means	21°C	1°C	Means	21°C	1°C	Means	21°C	1°C	Means
12	0 37 Aa	—	0 37 Aa	7 03 Aa	—	7 03 Aa	1 30 Aa	—	1 30 Aa	17 17 Aa	—	17 17 Aa
12+7	0 63 Bb	0 73 Bb	0 68 Bb	16 33 Bb	8 10 Aa	12 22 Bb	3 20 Bb	1 10 Aa	2 15 Ba	38 30 Bb	17 67 Aa	27 98 Bb
12+14	0 73 Bb	0 80 Bb	0 77 Bb	28 20 Cc	8 73 Aa	18 47 Cc	5 87 Dc	1 00 Aa	3 43 Cb	66 40 Cc	20 20 Aa	43 30 Cc
12+21	1 27 Cc	0 67 Bb	0 97 Cc	29 63 Cc	8 73 Aa	19 18 Cc	4 43 Cb	1 67 Aa	3 05 Cb	72 60 Cc	22 07 Aa	47 33 Dc
Means	0 75 Bb	0 64 Aa		20 30 Bb	8 15 Aa		3 70 Bb	1 26 Aa		48 62 Bb	19 27 Aa	

For comparisons among means the analysis of variance test was used The significance of the letters is the same as in Table 1

Table 4 Free sterol composition of *T aestivum* roots (μg/plant) at two different temperatures over the experimental period

Seedling age (days)	Cholesterol			Campesterol			Stigmasterol			Sitosterol		
	21°C	1°C	Means	21°C	1°C	Means	21°C	1°C	Means	21°C	1°C	Means
12	0 14 Aab	—	0 14 Aa	5 20 Aa	—	5 20 Aa	0 81 ABa	—	0 81 Aa	10 44 Aa	—	10 44 Aa
12+7	0 36 Bcd	0 09 Aa	0 23 ABab	12 33 Bb	3 55 Aa	7 94 ABa	2 22 Ba	0 39 Aa	1 30 Aa	25 29 Bb	8 01 Aa	16 65 Bab
12+14	0 35 Bbcd	0 10 Aa	0 22 ABab	14 97 Bb	2 39 Aa	8 68 Ba	6 10 Cb	0 18 Aa	3 14 Bb	31 58 Bb	5 29 Aa	18 44 Bb
12+21	0 49 Bd	0 16 Aabc	0 33 Bb	22 76 Cc	4 04 Aa	13 40 Cb	1 74 Aba	0 96 ABa	1 35 Ab	44 45 Cc	8 86 Aa	26 65 Cc
Means	0 34 Bb	0 12 Aa		13 82 Bb	3 79 Aa		2 72 Bb	0 59 Aa		27 94 Bb	8 15 Aa	

For comparisons among means the analysis of variance test was used The significance of the letters is the same as in Table 1

Table 5 Phospholipid content (μg/plant) of *T aestivum* shoots and roots at two different temperatures over the experimental period

Seedling age (days)	Shoots			Roots		
	21°	1°C	Means	21°C	1°C	Means
12	22 67 Aa	—	22 67 Aa	6 34 Aa	—	6 34 Aa
12+7	36 29 BCab	29 21 ABab	32 75 Bb	9 53 BCab	6 79 ABab	8 16 Aa
12+14	39 80 Cc	35 46 BCab	37 63 Bb	18 24 Dc	7 22 ABab	12 73 Bb
12+21	75 47 Dc	44 43 Cd	59 95 Cc	18 43 Dc	10 93 Cb	14 68 Bb
Means	43 56 Bb	32 94 Aa		13 13 Bb	7 82 Aa	

For comparisons among means the analysis of variance test was used The significance of the letters is the same as in Table 1

Table 6 Free sterol/phospholipid molar ratio of *T aestivum* shoots and roots at two different temperatures over the experimental period

Seedling age (days)	Free sterols/phospholipid molar ratio					
	Shoots			Roots		
	21°C	1°C	Means	21°C	1°C	Means
12	0 08 Bb	—	0 08 Aa	0 20 Cbc	—	0 20 ABab
12+7	0 12 Cc	0 06 Aa	0 09 Aa	0 32 Ed	0 14 Bab	0 23 Bb
12+14	0 19 Dd	0 06 Aa	0 12 Bb	0 26 Dcd	0 08 Aa	0 17 Aa
12+21	0 11 Cc	0 06 Aa	0 08 Aa	0 29 DEd	0 10 ABa	0 19 ABab
Means	0 12 Bb	0 06 Aa		0 27 Bb	0 13 Aa	

For comparisons among means the analysis of variance test was used The significance of the letters is the same as in Table 1

Leakage A solute leakage technique was used to assess membrane integrity during cold acclimatization of winter wheat [8] The relative amount of leakage was expressed as percentage of the maximum conductivity, measured after killing the samples by immersion in liquid nitrogen All observations are the means of three replicates Statistical analysis was performed using the two way analysis of variance

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