Temperature Effects on Tocopherol Composition in Soybeans with Genetically Improved Oil Quality

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ABSTRACT: Tocopherol, a natural antioxidant, typically accounts for a small percentage of soybean (*Glycine max* L. Merr.) oil. Alleles that govern the expression of polyunsaturated fatty acids in soybean germplasm are influenced by temperature. However, little is known about the environmental influences on tocopherol expression. The objective of this study was to assess the influence of temperature on tocopherol composition in soybean germplasm that exhibit homozygous recessive and dominant alleles that govern the predominant ω-6 and ω-3 desaturases. The control cv. Dare and three low-18:3 genotypes (N78-2245, PI-123440, N85-2176) were grown under controlled-temperature environments during reproductive growth. Analysis of crude oil composition at various stages of seed development revealed a strong negative correlation between total tocopherol content and growth temperature. The relative strength of this correlation was greater in the germplasm that exhibited homozygous alleles governing the ω-6 desaturase than those governing the ω-3 desaturase. The decline in total tocopherol with reduced temperature was attributed predominantly to loss of γ-tocopherol. However, γ-tocopherol concentration also was directly related to 18:3 concentration in all genotypes. Thus, low-18:3 oils contained both a lower content and a lower concentration of γ-tocopherol. Although the biochemical basis for this observation is unknown, the antioxidant capacity of γ-tocopherol appeared to be directly associated with changes in oil quality that were mediated more by genetic than by environmental influences on 18:3 concentration. Another aspect of this work showed that low-18:3 soybean varieties should be expected to contain more α-tocopherol, especially when grown under normal commercial production environments. This condition should be regarded as another beneficial aspect of plant breeding approaches to the improvement of soybean oil quality.
JAOCS 75, 591–596 (1998).

KEY WORDS: Environmental variation, genetics, *Glycine max* L. Merr., linoleic acid, oil quality, α-tocopherol, γ-tocopherol, tocopherol composition.

Tocopherols are considered antioxidants because they limit the availability of oxidants that decompose polyunsaturated fatty acids. In membranes of plant cells, it is presumed that, as in animal cells, tocopherols interrupt the chain reaction of lipid peroxidation by scavenging peroxyl radicals and thus enhance membrane stability (1–3). Tocopherols also inhibit triacylglycerol peroxidation at initiation by accepting free radicals (4,5). Soybean oil typically contains three primary types of tocopherol: δ [2,8-dimethyl-2-(4,8,12-trimethyltridecyl)]-, γ [2,7,8-dimethyl-2-(4,8,12-trimethyltridecyl)]-, and α [2,5,7,8-dimethyl-2-(4,8,12-trimethyltridecyl)]-tocopherol (6). In decreasing order, the relative effectiveness of these compounds as antioxidants is: δ-, γ-, and α-tocopherol (5).

Although a considerable amount of tocopherol may be removed during refining, especially in the deodorization process, the oxidative stability of soybean oil may be enhanced by hydrogenation, which lowers the level of 18:2 and 18:3 (7,8). In addition, emerging biotechnologies have led to the development of soybean oils that are naturally low in 18:3 (9,10). These genetically modified oils exhibit improved stability under high-temperature frying conditions, resulting in superior flavor quality compared to foods fried in hydrogenated soybean oil (11). However, hydrogenation is a controlled process, whereas biological reduction of 18:3 may be subject to the environmental conditions under which the plants are grown. In that regard, polyunsaturated fatty acid composition in vegetable oils tends to be negatively correlated with temperature (12,13). In other words, 18:3 concentrations may be higher when seeds develop under cooler temperatures. Mazliak (14) suggested that this phenomenon may be due to different temperature optima for the desaturase enzymes that catalyze 18:2 and 18:3 synthesis. Subsequently, Martin *et al.* (15) showed that the conversion of 18:1 to 18:2 was more sensitive to temperature than the conversion of 18:2 to 18:3 in soybean germplasm (N78-2245) that carries homozygous recessive alleles that encode the ω-6 desaturase. Mating N78-2245 with PI-123440, a germplasm with recessive alleles that encode the ω-3 desaturase, led to selection of N85-2176 that carries homozygous recessive alleles for both desaturases (16). Combination of these genes (where *A* or *a*, and *B* or *b* denoted dominant or recessive alleles for the ω-6 and ω-3 desaturases, respectively) resulted in a threefold reduction in 18:3 concentration and reduced the influence of temperature effects on oil quality.

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Given the development of soybean germplasm that is naturally low in 18:3, one may wonder whether tocopherol content also is decreased. Mounts *et al.* (17) recently reported the first estimates of tocopherol content in soybean oils with genetically modified 18:3 composition. However, the genotypes analyzed were from different geographic regions in the United States with markedly different growth environments. Because there is no available literature on the impact that environmental conditions may have on tocopherol composition in soybean, this investigation was conducted to determine the effect of growth temperature on the deposition of individual tocopherols in soybeans with altered 18:3 concentration.

MATERIALS AND METHODS

Soybeans (*Glycine max* L. Merr.) of cultivars N78-2245, PI-123440, N85-2176, and Dare were grown in the Southeastern Plant Environment Laboratory at North Carolina State University (Raleigh, NC). Seedlings were grown under 26°C/22°C day/night temperatures and a 15-h photoperiod until flowering was induced *ca.* 30 d after germination. After pod initiation, plants of each genotype were exposed to 30/26, 26/22, 22/18, or 18°C/14°C day/night treatments under a 9-h photoperiod throughout reproductive growth. The daily mean temperature (weighted for day and night photoperiod) for each treatment was 27.5, 23.5, 19.5, and 15.5°C, respectively. Developing seed was harvested at 30, 45, and 60 (maturity) d after flowering (DAF) and lyophilized at 60°C for 72 h.

Tocopherols were extracted by a modification of the procedure described by Priestly *et al*. (18). One gram of finely ground lyophilized material was extracted in 50 mL chloroform/methanol (2:1, vol/vol). After filtering, the extract was partitioned against 1% NaCl; the organic phase was passed through a column containing Na_2SO_4 and taken to dryness under N_2. Oil was saponified by refluxing with 5 mL 20% KOH in 60% methanol that contained 0.2% pyrogallol for 1.5 h under N_2. The mixture was washed twice with 10 mL ethyl ether to remove tocopherols.

Tocopherols were separated by thin-layer chromatography on plates coated (250 μm) with silica gel and developed with petroleum ether/ethyl ether/acetic acid (50:50:1, vol/vol/vol). Tocopherols were visualized with 0.2% 2,7-dichlorofluorescein in 95% ethanol under ultraviolet (UV) radiation and identified by cochromatography with authentic standards. Total tocopherols were eluted from the gel with absolute ethanol. After drying under N_2, tocopherols were converted to trimethylsilylether derivatives (TMSE) with bis(trimethylsilyl)-trifluoroacetamide and pyridine at 65°C for 30 min. After addition of petroleum ether, samples were dried under N_2 and redissolved in isooctane. Individual tocopherols were separated by flame-ionization gas chromatography (GC) in a fused-silica SPB-1 capillary column (30 m × 0.32 mm × 0.25 μm; Supelco Inc., Bellefonte, PA). Operating temperatures were 230°C (column), 280°C (injector), and 300°C (detector). Carrier flow (helium) was maintained at 76 cm/s (19).

Fatty acid composition of total lipid extracts was determined after transesterification with sodium methoxide in chloroform/methanol (2:1, vol/vol) by GC analysis as previously described (13). All data were reported as the mean of three replicate treatments. Least significant difference and regression analysis, based on means, were used to analyze data.

RESULTS AND DISCUSSION

Oil deposition in soybean seed typically follows a sigmoidal pattern during reproductive growth. Although environmental factors, such as temperature, are often associated in a positive relation with oil concentration, the effect of growth temperature on oil content usually is minimal (Burton, J.W., personal communication). Apparently, changes in oil concentration that are induced by environmental factors may be attributed to effects on other seed constituents that determine seed mass. Greater seed mass also may be expected at lower growth temperatures owing to the reduced number of seeds produced per plant. These trends were observed in the present study, where the genotypes Dare and three germplasm lines (N78-2245, PI-123440, and N85-2176) with various combinations of homozygous recessive or dominant alleles to govern ω-6 and ω-3 desaturase activity were grown under different temperatures during reproductive growth (Fig. 1). At all temperatures (reported as the daily mean temperature, which is weighted for photoperiod), the most active period of lipid accumula-

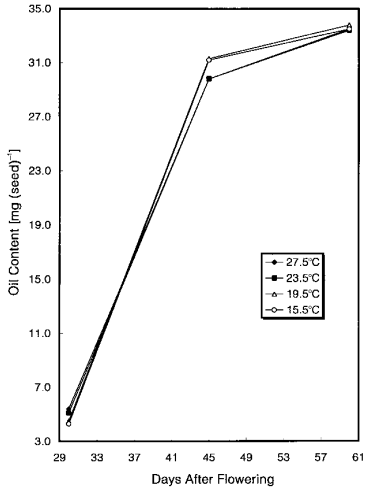

FIG. 1. Effect of growth temperature on oil accumulation during soybean seed development. Temperature treatments were expressed as daily mean temperature (weighted for photoperiod) throughout reproductive development. Data shown for cv. Dare were representative of other genotypes.

tion was between 30 and 45 DAF. The apparent rate of oil synthesis (as judged by accumulation) during that period was relatively insensitive to differences in growth temperature among these genotypes.

Growth temperature did influence unsaturated fatty acid composition among these genotypes. At seed maturity, 18:3 concentration, which is a crude indicator of oil quality, increased in an inverse relationship with growth temperature (Table 1). This trend was evident in both normal and low-18:3 genotypes in this study. Indeed, in the lowest-temperature treatment, the 18:3 concentration of N78-2245 and PI-123440 approached that of the control in the 23.5 and 27.5°C treatments. These treatments (23.5 and 27.5°C) bracket the average temperatures that are normally experienced under field conditions in most U.S. soybean production areas. Barring advent of unusual weather, soybeans in commercial production rarely would be exposed to continuous daily mean temperatures of 19.5°C or less throughout reproductive growth. The temperatures used in this study, however, were selected to determine the response of recessive alleles that govern 18:1- and 18:2-desaturation under extreme conditions. In that regard, 18:3 concentration in crude oil N85-2176, which carries homozygous recessive alleles for both desaturases, was remarkably stable. No statistically significant differences were found in expression of the low-18:3 trait in N85-2176 among the 27.5, 23.5, and 19.5°C treatments. By using the ratio %[18:2 + 18:3)/(18:1 + 18:2 + 18:3)] as a practical means of distinguishing genotypes with dominant (*A*) or recessive (*a*) alleles that govern expression of the predominant ω-6 desaturase and the ratio %[18:3/(18:2 +18:3)] to distinguish dominant (*B*) or recessive (*b*) alleles that govern the ω-3 desaturase in soybean seed, these data indicate that expression of homozygous recessive alleles for each desaturase was sensitive to growth temperature when paired with the homozygous dominant allele for the alternate desaturase. However, fatty acid composition in germplasm with double homozygous recessive or homozygous dominant alleles was less sensitive to wide ranges in growth temperature. Although this phenomenon cannot yet be explained, it was apparent that both homozygous recessive alleles are needed to ensure the lowest 18:3 concentration, regardless of temperature.

Another factor that influences oil quality is tocopherol composition, and soybean is the predominant source of vitamin E (α-tocopherol). The biological activity of tocopherols generally is believed to be manifested in protection of polyunsaturated triacylglycerol molecular species by terminating peroxidation at initiation (5). Few investigations have reported the nature of tocopherol deposition during soybean development, the effect of growth temperature on tocopherol levels, or, perhaps more importantly, the effect of genetically altered soybeans with reduced levels of 18:3 on tocopherol composition.

Total tocopherol accumulation tended to parallel oil accumulation during development of soybean seeds (Fig. 2). The most active rate of tocopherol synthesis occurred between 30 and 45 DAF in these genotypes. However, unlike the minimal response of oil deposition to growth temperature, both the rate of accumulation and total amount of tocopherol were significantly diminished by lower temperatures. At seed maturity (60 DAF), tocopherol content exhibited a strong positive correlation with growth temperature in all genotypes (Fig. 3). Each genotype showed *ca.* 1.5-fold change in total tocopherol over the temperature range, and the slope of these trends was also similar among genotypes. A striking feature

TABLE 1
Temperature Effects on Expression of Alleles That Govern Linolenic Acid Concentration of Oil from Mature Soybean Seed

Genotype[a]		Temp[b] (°C)	(% of total lipid) 16:0	18:0	18:1	18:2	18:3	Total (mg seed^{-1})	(%) 18:1D[c]	18:2-D
Dare	*AABB*	27.5	11.4	3.8	24.6	51.9	8.2	33.42	71.0	13.6
		23.5	10.7	3.3	21.9	55.6	8.6	33.35	74.6	13.4
		19.5	10.2	3.1	16.9	60.6	9.2	33.74	80.5	13.2
		15.5	10.1	3.1	16.1	61.3	9.4	33.45	81.5	13.3
N78-2245	*aaBB*	27.5	11.4	3.6	54.4	26.2	4.4	39.52	36.0	14.4
		23.5	11.5	3.3	47.4	32.4	5.4	43.42	44.4	14.3
		19.5	11.6	3.6	36.4	41.1	7.3	46.20	57.1	15.1
		15.5	10.9	3.5	30.0	47.6	8.0	48.62	65.0	14.4
PI-123440	*AAbb*	27.5	11.6	3.1	26.2	54.9	4.2	30.09	69.3	7.1
		23.5	11.2	3.1	25.0	55.6	5.1	31.38	70.8	8.4
		19.5	11.0	3.4	22.1	56.7	6.8	32.14	74.2	10.7
		15.5	10.7	3.3	19.2	58.2	8.5	32.94	77.6	12.7
N85-2176	*abba*	27.5	11.5	3.1	43.5	38.8	3.1	32.41	49.1	7.4
		23.5	11.7	3.2	39.2	42.3	3.6	34.20	53.9	7.8
		19.5	12.9	3.4	34.5	44.8	4.4	36.47	58.8	8.9
		15.5	12.9	3.4	31.7	46.2	5.7	38.62	62.1	11.0
$LSD_{0.05}$			0.5	0.1	7.0	6.4	1.3	3.4	8.3	1.7

[a]*AA/aa,* homozygous dominant or recessive alleles that govern 18:1-desaturation; *BB/bb,* homozygous dominant or recessive alleles that govern 18:2-desaturation in selected soybean germplasm.
[b]Temp, daily mean growth temperature during reproductive development, weighted for photoperiod.
[c]18:1-D, relative estimate of 18:1-desaturation: %[(18:2 + 18:3)/(18:1 + 18:2 + 18:3)]·100; 18:2-D, relative estimate of 18:2-desaturation: %[(18:3)/(18:2 + 18:3)]·100. Abbreviation: $LSD_{0.05}$, least significant difference ($P < 0.05$).

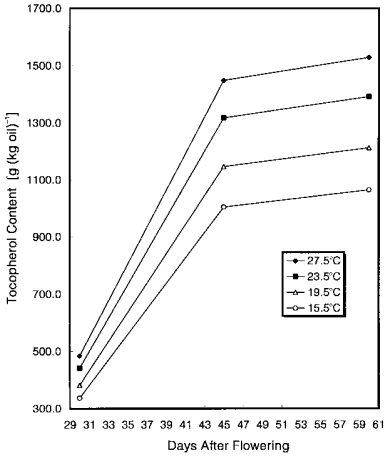

FIG. 2. Effect of growth temperature on total tocopherol accumulation during soybean seed development. Data shown for cv. Dare were representative of other genotypes.

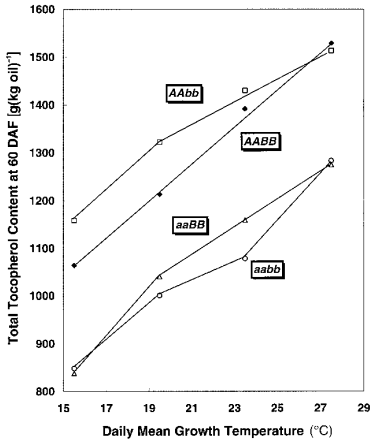

FIG. 3. Effects on total tocopherol content in mature seed of soybean genotypes with genetically modified linolenic acid composition. The gene descriptors *AA* or *aa* represent homozygous dominant or recessive alleles that encode the predominant ω-6 desaturase; *BB* or *bb* represent homozygous dominant or recessive alleles that encode the ω-3 desaturase in soybean seed. DAF, days after flowering.

of these data was the apparent effect of alleles that govern expression of the ω-6 desaturase on the response of tocopherol content to growth temperature. Regardless of the alleles (*BB* or *bb*) that govern ω-3 desaturase activity, tocopherol content at each temperature was significantly greater in genotypes that carried the homozygous dominant alleles (*AA*) for ω-6 desaturase than those with the homozygous recessive alleles (*aa*). Indeed, the mean total tocopherol levels for cv. Dare and PI-123440 were *ca.* 1.3-fold greater than those for N78-2245 and N85-2176 within temperature treatments. Thus, it appears that genetic effects exerted by, or associated with, alleles that determine ω-6 desaturase activity may impose a considerable influence on tocopherol levels in addition to the effects of widely divergent growth temperatures.

As shown in Table 2, the concentration of individual tocopherol isomers remained constant within genotypes during seed development at a given temperature. In view of inherent genotypic differences in tocopherol composition, each genotype responded to temperature treatments in a similar manner. γ-Tocopherol concentration tended to be positively correlated to growth temperature, apparently at the expense of δ- and α-tocopherol. When expressed on an absolute basis, the change in amount of γ-tocopherol accounted for 73.4 ± 2.7% of the variation in total tocopherol within all genotypes over the temperature treatments. Among and within all temperature/genotype combinations, δ-tocopherol averaged 280.4 ± 11.0 g/kg oil and α-tocopherol 139.0 + 5.1 g/kg oil. Given the relatively minor response of α- and δ-tocopherol content to altered growth temperature, the change in total tocopherol content appeared to be essentially attributed to temperature effects on the amount of γ-tocopherol.

Given the negative relation between 18:3 concentration and growth temperature, it was somewhat surprising to observe a positive response in γ-tocopherol concentration to temperature. However, regressing γ-tocopherol concentration against the 18:3 concentration among genotypes in a given temperature treatment showed the expected response (Fig. 4). A strong positive correlation was found between γ-tocopherol and 18:3 among genotypes in each temperature treatment with little variation in slope (1.59 ± 0.09) whereas the intercept ranged from 57.8% γ-tocopherol at the highest growth temperature (R^2, 0.95) to 46.1% γ-tocopherol in the lowest temperature (R^2, 0.89). Therefore, lower γ-tocopherol concentrations may be expected in soybean varieties with genetically reduced 18:3 concentration. Expression of this trend is further influenced by environmental factors. Although the biochemical basis for this observation is unknown, it appears that γ-tocopherol may be associated with the changes in 18:3 concentration by both genetic and environmental influences.

Based on these data, low-18:3 soybean oils, especially N852176, appear to have inherently elevated levels of α-tocopherol or vitamin E. The apparent enrichment in α-tocoph-

TABLE 2
Effect of Growth Temperature on Tocopherol Composition in Developing Seed of Soybean Genotypes with Genetically Altered Linolenic Acid Composition

Genotype	Temp. (°C)	DAF[a]	(% of total tocopherol) δ	γ	α	Total (g/kg oil)	Genotype	Temp. (°C)	DAF	(% of total tocopherol) δ	γ	α	Total (g/kg oil)
Dare	27.5	30	19.3	71.3	9.5	484.75	N78-2245	27.5	30	22.7	65.3	12.0	403.21
		45	19.4	71.2	9.5	1448.79			45	22.8	65.2	12.0	1205.67
AABB		60	19.4	71.2	9.4	1529.16	*aaBB*		60	22.6	65.4	11.9	1274.53
	23.5	30	20.0	70.7	9.3	441.67		23.5	30	23.6	64.4	12.0	367.88
		45	20.1	70.6	9.4	1318.14			45	23.6	64.5	11.9	1104.30
		60	20.0	70.7	9.3	1391.98			60	23.5	64.5	12.0	1158.94
	19.5	30	20.9	68.8	10.4	382.06		19.5	30	24.4	63.4	12.2	330.39
		45	21.0	68.7	10.3	1146.88			45	24.6	63.2	12.3	983.79
		60	20.8	68.9	10.3	1213.15			60	24.4	63.4	12.2	1040.37
	15.5	30	21.7	68.0	10.3	337.44		15.5	30	25.1	62.6	12.3	265.70
		45	21.8	67.8	10.4	1005.07			45	25.2	62.3	12.4	793.33
		60	21.7	68.0	10.4	1064.18			60	25.1	62.5	12.4	838.16
PI-123440	27.5	30	23.9	65.8	10.4	480.42	N85-2176	27.5	30	23.7	61.9	14.4	406.76
		45	23.8	65.8	10.4	1442.04			45	23.8	61.7	14.5	1214.14
AAbb		60	23.9	65.8	10.4	1513.52	*aabb*		60	23.7	61.9	14.4	1283.13
	23.5	30	24.7	64.3	11.0	450.83		23.5	30	24.3	61.4	14.3	342.29
		45	24.7	64.3	11.0	1354.89			45	24.5	61.2	14.3	1019.34
		60	24.6	64.4	11.0	1429.94			60	24.3	61.4	14.3	1078.30
	19.5	30	25.6	63.2	11.2	419.51		19.5	30	25.6	60.4	14.0	317.45
		45	25.6	63.2	11.2	1259.45			45	25.8	60.3	14.0	946.55
		60	25.7	63.1	11.2	1322.18			60	25.6	60.4	13.9	1000.63
	15.5	30	25.9	62.4	11.6	368.83		15.5	30	26.8	59.3	13.9	269.10
		45	25.9	62.4	11.6	1104.43			45	27.0	59.2	13.8	802.20
		60	26.0	62.4	11.7	1158.25			60	26.8	59.4	13.8	848.33

[a]DAF, days after flowering. See Table 1 for other abbreviations.

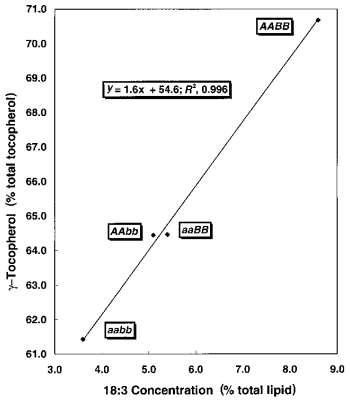

FIG. 4. Relation between γ-tocopherol concentration and 18:3 concentration in oils of mature soybean seed grown at different temperatures. 18:3 Concentration was expressed as a percentage of total unsaturated fatty acids in crude oil. The gene descriptors *AA* or *aa* represent homozygous dominant or recessive alleles that encode the predominant ω-6 desaturase: *BB* or *bb* represent homozygous dominant or recessive alleles that encode the ω-3 desaturase in soybean seed.

erol was a function of loss of γ-tocopherol. The percentage difference in the actual amount of α-tocopherol between Dare and N85-2176 increased in a linear relation, where percentage difference = 1.225 (daily mean temperature) + 12.504 [R^2 = 0.993] from the 15.5°C treatment (2.8% difference) to the 27.5°C treatment (12.5% difference). Therefore, low-18:3 soybean varieties should contain more α-tocopherol, especially when grown under normal commercial production environments. This condition should enrich the amount of extractable vitamin E in deodorization distillates compared to normal or high-18:3 soybean oils, and should be regarded as another beneficial aspect of plant breeding approaches to improve soybean oil quality.

ACKNOWLEDGMENTS

This investigation was a cooperative research effort between the North Carolina Agricultural Research Service and the USDA Agricultural Research Service. Funding for this work was received in part from the United Soybean Board, project number 5045.

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[Received August 16, 1996; accepted September 29, 1997]