In Vivo and In Vitro Lipid Accumulation in *Borago officinalis* L.

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Seeds of 13 accessions of borage (*Borago officinalis*) varied in total fatty acid content from 28.6 to 35.1% seed weight, with linoleic, γ-linolenic, oleic and palmitic as the predominant fatty acids, averaging 38.1%, 22.8%, 16.3%, and 11.3% of total fatty acids, respectively. There was an inverse relation between γ-linolenic acid (25.0 to 17.6%) and oleic acid (14.5 to 21.3%). Fatty acid content of leaf tissues was 9.1% dry weight, with α-linolenic acid 55.2% and γ-linolenic acid 4.4% of total fatty acids. Cotyledons were the major source of fatty acids in seeds. Seed fatty acid content increased from <1 mg at six days postanthesis to about seven mg at maturity (22 to 24 days). Individual fatty acid content of seed was relatively constant after day 8. When immature embryos from 6 to 16 days postanthesis were cultured in a liquid or semisolid basal medium, fatty acid composition was similar to that of in vivo-grown seeds. Growth of cultured embryos decreased as sucrose concentration was increased from 3 to 20% in the basal medium, and most embryos did not survive 30% sucrose; fatty acid as a percentage of dry weight was maximal at 6% sucrose.

Borage (*Borago officinalis* L., Boraginaceae) is an herbaceous annual native to Europe, Asia Minor and North Africa that has been used for centuries as a culinary herb of purported medicinal value (1). Recent interest in borage is due to the high concentration of γ-linolenic acid, 18:3 Δ6, 9, 12 found in the seed (2, 3). Evening primrose oil contains γ-18:3, a prostaglandin precursor (4, 5), and is marketed as a nutritional supplement for medicinal purposes (6, 7). While various plant species contain γ-18:3, common commercial sources are from seeds of *Oenothera* spp. (8), and *Ribes nigrum* L. (9). Although borage is one of the richest plant sources of γ-18:3, field production is limited by seed shattering and indeterminate growth habit. The demonstration of somatic embryogenesis in borage led Janick et al. (10) to suggest the possibility of in vitro production of γ-18:3. The objective of the present study was to compare in vitro and in vivo embryo lipid accumulation with emphasis on γ-18:3.

EXPERIMENTAL PROCEDURES

Plant material. Borage seed from 13 sources was evaluated for lipid and γ-18:3 content in the present study. All field-grown plants originally were derived from seed obtained from PGE Technology, Danvers, Massachusetts.

Fatty acid extraction. Lipids were extracted from tissues using a procedure modified from Folch et al. (11). Tissue samples were homogenized in five ml chloroform/methanol (2:1, v/v), and solvent was added to bring the total volume to 20 ml. Pentadecanoic acid (15:0) was added (0.5 mg) as an internal standard for gas chromatographic (GC) analysis. Samples were sonicated for 30 min, five ml of aqueous 0.58% sodium chloride was added, and samples were resonicated for 10 min. After centrifugation of the sample for five min at 1000 rpm, the chloroform phase was removed with a pipette and dried using an analytical evaporator.

Triglycerides were saponified and soaps were methylated using the procedure of Metcalfe and Schmitz (12). Samples were boiled for five min in 0.5 ml toluene and two ml 0.5 N sodium hydroxide in methanol. Boron trifluoride (2.5 ml) was added and samples placed in a hot water bath for three min. When cool, 15 ml petroleum ether and 20 ml distilled water were added. Following sonication of the samples for 10 min, the petroleum ether phase was filtered through Whatman No. 1 filter paper with one g sodium sulfate to remove water. Samples were dried with the evaporator and resuspended in one ml petroleum ether.

Fatty acid composition was analyzed with a Varian 3700 gas chromatograph using a 30-m × 0.32-mm fused silica capillary column with SP-2330 as the stationary phase. Initial column temperature was 150 C for two min and was increased by 4 C per min to 220 C. Peak areas of fatty acids were determined with a Hewlett Packard 3390A integrator. The order of peak elution was: 15:0 (internal standard), 16:0, 18:0, 18:1, 18:2, γ-18:3, 20:0, α-18:3, 20:1, 22:1 and 24:1. Peaks were identified by comparing retention times with pure standards. The identity of each fatty acid constituent was confirmed by gas chromatography (Finnigan 9610) and mass spectroscopy (Finnigan 4000) using electron impact analysis.

Tissue culture. Borage plants were grown in the greenhouse, lathhouse or field. Individual flowers were tagged at anthesis and ovaries were harvested from 6 to 16 days after anthesis at two-day intervals. Ovaries were disinfested with 0.5% (v/v) sodium hypochlorite for 30 min, rinsed three to five times in sterile distilled water, and the embryos were excised.

Explants were cultured in plastic petri dishes (60 × 15 mm) or in glass culture tubes (22 mm i.d. × 150 mm high) using plastic closures. Each culture vessel contained 10 ml of basal medium consisting of Murashige and Skoog salts (13) supplemented with 0.3 μM (0.1 mg/l) thiamine-HCl, 2.4 μM (0.5 mg/l) pyridoxine-HCl, 0.9 mM (100 mg/l) i-inositol, 4 μM (0.5 mg/l) nicotinic acid, 26.6 μM (2.0 mg/l) glycine, 1 g/l casein hydrolysate, sucrose at 88, 175, 292, 584 or 876 mM (30, 60, 100, 200 or 300 g/l) and 0 or 6 g/l agar (Sigma Chemical Co., St. Louis, Missouri), with pH adjusted to 5.7. Embryos were grown at 26 C under low-intensity illumination (45 μmol $s^{-1}m^{-2}$ from cool-white fluorescent lamps) with a 16-hr photo- period for 14 days or until 24 days postanthesis (18 days for six-day-old embryos and six days for 18-day- old embryos).

RESULTS

Lipid content of accessions. The 13 accessions of borage varied in average seed weight from 16.1 to 24.5 mg, but total fatty acids varied only from 28.6 to 35.1% of mature seed weight (Table 1). The major fatty acids

TABLE 1

Lipid Content of Borage Accessions[a]

Seed source	Seed wt (mg)	Crude lipid (mg)	Tot. FA (mg)	Tot. FA (%)	Fatty acid content (% of total fatty acids) 16:0	18:0	18:1	18:2	α18:3	γ18:3	20:0	20:1	22:1	24:1
									Seed					
Twilley	17.9	6.3	6.00	33.5	10.8	3.7	14.5	37.5	0.2	25.0	0.2	4.0	2.7	1.5
Stokes	18.5	6.8	6.49	35.1	10.6	3.4	15.0	38.2	0.2	24.4	0.2	4.0	2.8	1.4
The Netherlands (Borntraeger & Schlemmer)	20.8	6.9	6.55	31.5	11.2	3.7	15.3	38.3	0.2	24.0	0.2	3.7	2.3	1.4
Richters	17.2	5.7	5.59	32.5	11.8	3.8	15.6	37.9	0.2	23.3	0.2	3.9	2.5	1.4
The Netherlands (Agri Borretoch)	20.8	6.8	6.52	31.3	11.7	3.8	15.6	37.6	0.2	23.2	0.3	3.8	2.3	1.2
Denmark (Mouster Zonder Waarde Ohlsens)	19.2	6.2	5.81	30.3	11.5	2.8	17.6	40.1	0.2	23.1	0.2	4.0	2.2	1.2
Burpee	18.5	6.9	5.91	31.9	10.8	3.7	16.1	38.6	0.2	23.0	0.2	3.9	2.2	1.4
Harris Moran	18.5	7.0	5.93	32.1	11.8	3.7	16.0	38.1	0.2	22.9	0.2	3.7	2.2	1.2
The Netherlands (Agri Saaten)	19.2	7.3	5.69	29.6	11.4	3.5	16.3	38.0	0.2	22.7	0.2	3.9	2.5	1.4
PGE Technology	24.5	7.3	7.02	28.8	11.8	3.8	15.8	38.0	0.2	22.7	0.2	3.6	2.3	1.5
Abbott and Cobb	18.5	6.6	6.23	33.7	10.9	2.9	16.9	38.4	0.2	22.5	0.3	4.0	2.6	1.4
Agway	20.0	6.4	6.06	30.3	11.9	4.0	16.0	38.3	0.2	22.3	0.2	3.6	2.3	1.3
The Netherlands (Schobbers)	16.1	4.8	4.61	28.6	11.3	5.0	21.3	36.4	0.2	17.6	0.2	4.1	2.4	1.5
Mean	19.2	6.5	6.03	31.5	11.3	3.7	16.3	38.1	0.2	22.8	0.2	3.9	2.4	1.4
	(Sample wt)								Leaf					
PGE Technology	103.1	10.3	9.43	9.1	15.9	3.4	5.7	11.7	55.2	4.4	1.1	0.4	1.1	1.0

[a]Seed sample weight was 0.5 g.

TABLE 2

Lipid Content of Borage Seed Parts One Day After Germination[a]

Seed part	Dry wt (mg)	Crude lipid (mg)	Total fatty acids (mg)	Total fatty acids (%)	Fatty acid content (% of total fatty acids) 16:0	18:0	18:1	18:2	α18:3	γ18:3	20:0	20:1	22:1	24:1
Exocarp	6.1± 0.2[b]	0.3± 0.1	0.06± 0.01	1.0± 0.2	20.7± 1.5	6.4± 0.3	14.8± 1.3	33.8± 0.8	1.3± 0.4	14.5± 1.1	1.2± 0.4	3.2± 0.3	2.5± 0.2	1.6± 0.3
Cotyledon	10.8± 0.2	6.9± 0.2	6.0± 0.2	55.6± 1.3	10.6± 0.4	4.2± 0.3	16.0± 0.02	38.4± 0.4	0.2± 0.01	23.0± 0.03	0.2± 0.1	3.7± 0.03	2.3± 0.1	1.3± 0.03
Hypocotyl	0.8 0.1	0.6± 0.03	0.3± 0.01	32.7± 2.2	13.1± 0.2	3.9± 0.4	10.9± 0.5	35.4± 0.4	1.1± 0.2	25.3± 0.3	0.3± 0.1	3.8± 0.1	3.3± 0.1	2.8± 0.1

[a]Lot 1, PGE seed.
[b]± SE.

were 18:2, 18:1 and γ-18:3. Distribution of fatty acids among accessions was very similar with the exception of γ-18:3 and 18:1. There was an inverse relation between γ-18:3 [25.0 to 17.6% total fatty acids (TFA)] and 18:1 (14.5 to 21.3%); γ-18:3 plus 18:1 varied only from 38.8 to 40.7%. In contrast, TFA of leaf tissue were 9.1% dry weight with a different distribution: 55.2% α-18:3, 15.9% 16:0, 11.7% 18:2, and 4.4% γ-18:3.

Seed lipid distribution. One-day-old germinated seeds were divided into three parts, exocarp, cotyledons and hypocotyl, and each part was analyzed for lipids (Table 2). The cotyledons were the richest source of lipid (55.6% dry weight), with 32.7% in the hypocotyl and 1.0% in the exocarp. Although fatty acid distribution was similar in cotyledon and hypocotyl, the exocarp was lower in γ-18:3 and higher in 16:0.

TABLE 3

Lipid Content of Cotyledons of Germinating Borage Seed[a]

Days from germination	Dry wt (mg)	Total fatty acids (mg)	Total fatty acids (%)	Fatty acid composition (% total fatty acids) 16:0	18:0	18:1	18:2	α18:3	γ18:3	20:0	20:1	22:1	24:1
0	10.0	6.3	63.0	10.6	4.1	15.4	38.7	0.2	23.7	0.2	3.5	2.2	1.3
2	10.7± 0.4[b]	6.2± 0.2	58.3± 1.4	10.8± 0.2	4.2± 0.2	16.6± 0.8	37.8± 0.2	0.3± 0.03	22.7± 0.8	0.2± 0.01	3.8± 0.05	2.3± 0.04	1.2± 0.02
4	10.2± 0.5	5.5± 0.5	54.0± 2.6	10.4± 0.3	4.1± 0.1	16.0± 0.2	37.0± 0.4	0.5± 0.03	23.5± 0.4	0.2± 0.01	4.1± 0.2	2.5± 0.1	1.5± 0.1
6	7.6± 0.5	3.1± 0.3	40.3± 1.4	10.5± 0.2	4.9± 0.2	16.2± 0.4	35.5± 0.4	1.0± 0.05	23.7± 0.5	0.3± 0.02	4.2± 0.05	2.4± 0.1	1.4± 0.02
8	6.6± 0.2	2.3± 0.1	34.4± 1.2	10.8± 0.2	4.2± 0.2	15.2± 0.7	35.1± 0.4	1.7± 0.1	24.3± 0.8	0.3± 0.02	4.3± 0.1	2.7± 0.1	1.5± 0.03

[a]Initial data are based on a single sample of 10 seeds. Days 2 to 8 are based on 5 samples of 5 seeds each (Lot 1, PGE seed).
[b]± SE.

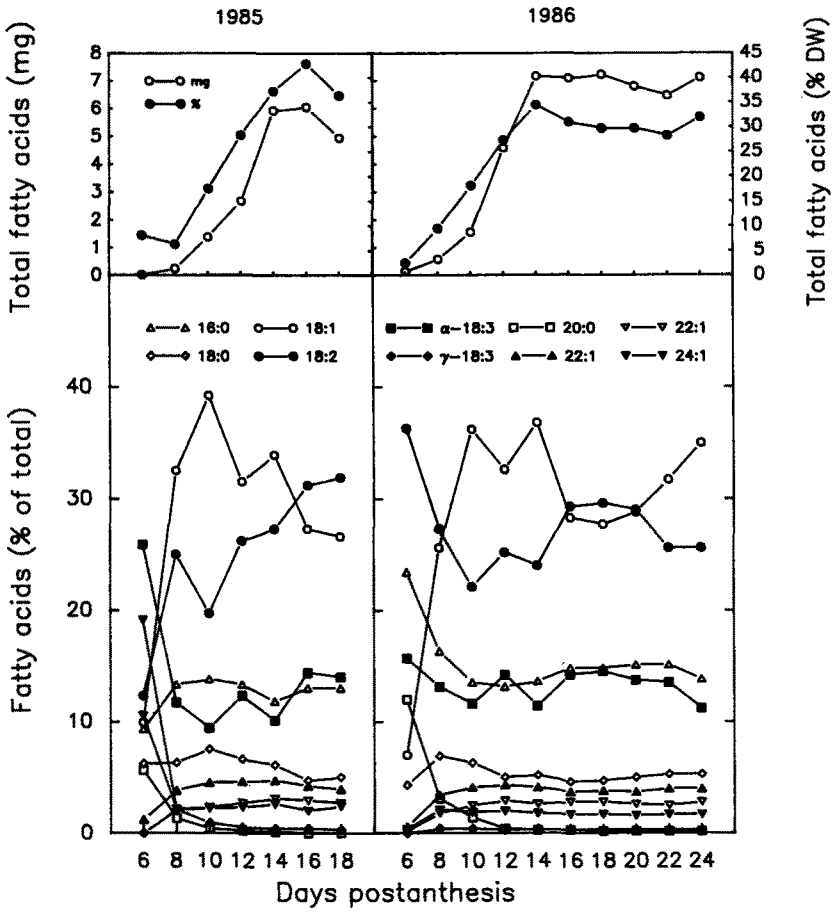

FIG. 1. Accumulation of total and percent fatty acids (top) and changes in fatty acid composition (bottom) of developing zygotic embryos of *Borago officinalis* collected 6 to 24 days postanthesis in 1985 and 1986.

Lipid content in cotyledons of germinating seed. Seeds were germinated for eight days, and the fatty acid content of cotyledons was analyzed at two-day intervals. Although TFA concentration decreased from 63.0 to 34.4% dry weight over the eight-day period, there was little change in fatty acid composition except for an increase in α-18:3 from 0.2 to 1.7% (Table 3).

In vivo lipid composition of developing embryos. Lipid composition of developing embryos from plants grown from PGE Technology seed was measured in 1985 and 1986. In 1985, embryos were extracted from ovules 6 to 18 days postanthesis; in 1986, immature seeds were evaluated from 6 to 24 days postanthesis. Results were similar for both years. Total fatty acids reached a peak of 42.8% dry weight at 16 days in 1985 and 34.4% at 14 days in 1986 (Fig. 1). The distribution pattern of individual fatty acids in vivo (Fig. 1) indicates that 18:0, α-18:3, 20:0, 20:1, 22:1 and 24:1 remained relatively constant after eight days, but that an inverse relation existed between 18:1 vs 18:2 and γ-18:3. In 1985, γ-18:3 reached 14.0% TFA and 18:1 reached 26.6% at 18 days. In 1986, γ-18:3 and 18:1 attained 14.5% and 27.7% TFA, respectively, at 18 days and 11.2% and 35.0% at 24 days.

In vitro lipid accumulation by embryos. Embryos were excised from 6- to 16-day-old seeds and cultured in liquid or semisolid basal medium supplemented with 3, 6, 10, 20 or 30% sucrose for either 14 days or until 24 days postanthesis. Lipid accumulation by 10-day-old embryos cultured for 14 days was compared with that by embryos grown in vivo for 10 or 24 days (Table 4). Embryos cultured with 30% sucrose died; therefore, this treatment is excluded.

Dry weight of 10-day-old embryos cultured for 14 days increased but was less than 24-day-old in vivo embryos. Dry weight decreased as sucrose increased from 3 to 20%. Dry weight of embryos cultured in liquid was greater than that of embryos on semisolid medium at 3% sucrose, but the difference decreased as sucrose increased.

TABLE 4

Comparison of Fatty Acid Composition in vivo and in vitro in *Borago officinalis*

			10-day embryos + 14 days in vitro							
	In vivo[a]		Liquid				Semisolid			
			Sucrose				Sucrose			
	10 days	24 days	3%	6%	10%	20%	3%	6%	10%	20%
Embryo dry weight (mg)	8.1± 0.8[b]	22.2± 1.3	16.4± 2.6	12.3± 1.2	12.6± 0.8	8.3	12.3± 0.8	10.7± 1.4	11.4± 0.7	8.6± 1.6
Total fatty acids (mg)	1.6± 0.2	7.1± 0.5	3.9± 0.7	5.0± 0.6	4.8± 0.4	3.1	4.2± 0.4	3.5± 0.6	4.3± 0.4	2.8± 1.0
(%)	18.1± 1.9	31.9± 0.8	24.4± 3.4	40.8± 1.4	38.2± 2.4	37.5	33.9± 3.0	33.5± 3.6	36.8± 2.0	30.9± 5.8
Fatty acid composition (%)										
16:0	13.5± 0.6	13.8± 0.3	14.5± 0.4	12.6± 0.2	11.7± 0.4	10.9	16.2± 0.5	14.7± 0.3	13.8± 0.4	13.0± 1.4
18:0	6.3± 0.1	5.3± 0.2	4.3± 0.3	4.1	4.1± 0.2	4.7	3.1± 0.2	3.4± 0.2	3.6± 0.2	4.5± 0.1
18:1	36.2± 1.2	35.0± 1.3	33.2± 2.2	40.5± 2.6	52.1± 2.4	53.2	21.3± 1.9	25.6± 2.3	33.5± 2.5	43.5± 2.3
18:2	22.1± 0.7	25.6± 1.0	26.1± 1.4	23.8± 1.5	17.4± 1.6	15.2	31.8± 0.7	31.1± 1.5	28.2± 1.6	24.2± 1.4
α18:3	1.4± 0.2	0.2± 0.0	0.4± 0.1	0.3± 0.0	0.3± 0.0	0.3	0.6± 0.1	0.4± 0.0	0.3± 0.0	0.4± 0.1
γ18:3	11.2± 0.2	11.2± 0.4	13.3± 0.8	10.7± 0.9	7.9± 0.7	9.0	17.8± 1.0	15.7± 0.8	13.0± 0.8	8.4± 0.0
20:0	0.4± 0.0	0.3± 0.0	0.2± 0.0	0.3± 0.0	0.2± 0.0	0.3	0.2± 0.0	0.3± 0.0	0.2± 0.0	0.4± 0.2
20:1	4.1± 0.2	4.0± 0.1	3.9± 0.1	4.0± 0.1	3.6± 0.1	3.5	3.8± 0.1	0.4± 0.1	3.6± 0.1	2.9± 0.3
22:1	2.5± 0.2	2.8± 0.2	1.8± 0.1	1.7± 0.1	1.1± 0.1	1.5	2.3± 0.1	2.2± 0.1	1.6± 0.1	1.2± 0.1
24:1	1.9 0.0	1.7± 0.0	2.2± 0.1	2.0± 0.2	1.6± 0.1	1.5	2.9± 0.1	2.6± 0.1	2.1± 0.1	1.4± 0.1

[a]Fig. 1, 1986 data.
[b]± SE.

In vivo, TFA content of 10-day-old embryos was 18.1% dry weight and TFA of 24-day-old embryos was 31.9%. TFA content of 10-day-old embryos cultured in vitro for 14 days reached a peak of 40.8% dry weight in liquid medium at 6% sucrose. The concentration of γ-18:3 in vivo was 11.2% of TFA at 10 and 24 days postanthesis, but γ-18:3 concentration varied from 7.9 to 17.8% when 10-day-old embryos were cultured for 14 days depending on media state and sucrose concentration. The highest concentration of γ-18:3 was produced on semisolid medium at 3% sucrose.

Content of γ-18:3 in mg/embryo from all ages for each sucrose concentration is averaged over media state and culture time in Figure 2. The γ-18:3 was produced in vitro in all treatments except in 14- and 16-day-old embryos at 3% sucrose, but embryos did germinate in this regime. The greatest increase in γ-18:3 production was achieved by 6- to 12-day-old embryos on basal medium supplemented with 3% sucrose, but γ-18:3 accumulation decreased with increasing sucrose concentration.

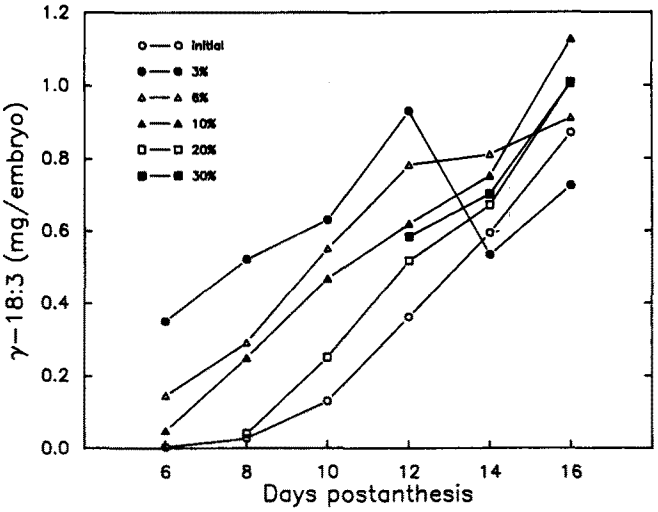

FIG. 2. Accumulation of γ-18:3 by zygotic embryos of *Borago officinalis* cultured in basal media supplemented with various sucrose concentrations from 6 to 16 days postanthesis. Data from liquid and semisolid media and from 14 days in culture and 24 days postanthesis are combined.

DISCUSSION

Lipid accumulation in borage seed increased markedly during development from day 6 to 14 and then leveled off (Fig. 1). This increase in lipid accumulation is accompanied by dramatic changes in lipid composition from 6-10 days, with percentage declines in 16:0, 18:2, α-18:3 and γ-18:3 and a corresponding percentage increase in 18:1. In this respect, borage resembles cocoa (14), where fatty acid composition changes during development, in contrast to jojoba, where fatty esters remain relatively constant during development (15). Throughout development an inverse relation appears to exist between 18:1 vs 18:2 and γ-18:3. This corroborates results obtained by Cherry et al. (16) for 18:1 vs 18:2 in soybean. The inverse relation between 18:1 and γ-18:3 suggests that high and low γ-18:3 lines are due to desaturation of 18:1 and 18:2.

Although dry weight accumulation was lower in vitro than in vivo, percent TFA was generally higher in vitro (Table 4). In vitro and in vivo patterns of fatty acid

TABLE 5

Lipid Content of Borage Seed after Four Weeks of Storage at Various Temperatures

Temp (°C)	Seed wt (mg)	Crude lipid (mg)	Total fatty acids (mg)	Total fatty acids (%)	Fatty acid content (% total fatty acids) 16:0	18:0	18:1	18:2	α18:3	γ18:3	20:0	20:1	22:1	24:1
Initial														
+27	21.0± 0.4[a]	8.7± 0.3	6.3± 0.2	30.1± 0.8	13.8± 0.2	5.0± 0.1	27.2± 1.0	30.2± 0.6	0.3± 0.0	14.3± 0.5	0.3± 0.0	4.1± 0.1	2.9± 0.1	1.9± 0.0
4 weeks														
-20	20.0± 0.5	8.7± 0.3	6.0± 0.1	30.1± 0.6	13.8± 0.2	5.2± 0.1	27.1± 0.9	30.7± 0.4	0.3± 0.0	13.9± 0.6	0.3± 0.0	3.9± 0.0	2.8± 0.0	2.0± 0.0
+ 2	20.5± 0.8	8.2± 0.5	5.9± 0.3	28.8± 0.7	13.8± 0.2	5.3± 0.2	28.4± 0.7	29.5± 0.7	0.4± 0.0	13.6± 0.4	0.3± 0.0	4.0± 0.1	2.7± 0.1	1.9± 0.1
+20	21.5± 0.6	8.9± 0.3	6.4± 0.2	29.6± 0.5	14.0± 0.2	5.2± 0.1	28.4± 0.9	29.7± 0.5	0.3± 0.0	13.6± 0.4	0.3± 0.0	3.9± 0.0	2.7± 0.1	1.9± 0.0
+27	20.4± 0.5	8.2± 0.5	6.1± 0.3	29.6± 1.2	13.4± 0.3	5.2± 0.1	28.0± 0.8	29.8± 0.4	0.3± 0.0	14.1± 0.6	0.3± 0.0	4.0± 0.1	2.8± 0.1	2.1± 0.0
+60	20.0± 0.4	7.9± 0.3	5.9± 0.3	29.5± 0.9	13.4± 0.2	5.1± 0.1	30.2± 1.2	29.1± 0.7	0.2± 0.0	13.0± 0.5	0.3± 0.0	4.0± 0.1	2.6± 0.0	1.9± 0.0

[a]± SE.

accumulation were similar, suggesting that individual fatty acids such as γ-18:3 could be produced by culturing somatic embryos.

In the in vivo fatty acid accumulation study, γ-18:3 reached only 11.2% of TFA at 24 days (Fig. 1), half the concentration observed in the original PGE seed source (Table 1), while 18:1 reached 35.0%, more than twice as high. Results were consistent in 1985 and 1986. There are several explanations why these seeds failed to produce the γ-18:3 levels of the original PGE seed source, but overproduced 18:1. Climatic conditions have been shown to account for differences in fatty acid distribution (17). However, in seeds harvested in 1986 from adjacent plots, γ-18:3 varied from 18.1 to 20.4% TFA and 18:1 varied from 17.6 to 18.3% (18). Although postharvest changes also can cause shifts in the composition of seed oils (19), the seed low in γ-18:3 demonstrated stability of fatty acid composition after storage for six mo at 4 C and for 30 days at temperatures from -20 to 60 C (Table 5). Resampling of seed lots with high and low γ-18:3 in 1987 gave consistent results. We suggest that a genetic difference is responsible for variation in γ-18:3 and 18:1.

Our results indicate that cotyledonary fatty acids including γ-18:3 are produced similarly in vivo and in vitro. If somatic embryos behave as zygotic embryos of borage in culture, then in vitro γ-18:3 production should be feasible.

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