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REDUCTION OF LINOLENATE CONTENT IN SOYBEAN COTYLEDONS BY A SUBSTITUTED PYRIDAZINONE

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Key Word Index—*Glycine max*; leguminosae; fatty acids; linolenic acid; tissue culture; mutant; lipid biosynthesis.

Abstract—The effect of a substituted pyridazinone, 4-chloro-5-(dimethylamino)-2-phenyl-3(2*H*)-pyridazinone (San 9785), on the reduction of linolenate content was examined in the cotyledons of the soybean cv. Century and a low linolenate mutant of soybeans, C1640, cultured *in vitro*. No apparent changes in dry matter and total fatty acid accumulation were observed in the cotyledons developing in the presence of San 9785. However, a significant reduction of linolenate content with a corresponding increase in linoleate resulted from growth of the cotyledons in culture medium containing San 9785. San 9785 had a greater effect on decreasing the linolenate content in cotyledons excised in early developmental phases than later stages from both the wild type and mutant soybeans. This result supports other observations that the biosynthesis of linolenate relative to the other major fatty acids of soybean cotyledons declines in later developmental stages. The compound becomes progressively less effective in reducing the content of linolenate during seed development in the mutant than in the wild type. Both San 9785 and the mutation result in changes in phosphatidylcholine molecular species which indicates the presence of a number of linolenate desaturation systems in developing soybean cotyledons. The possible biochemical nature of the mutation is discussed.

INTRODUCTION

The formation of linolenate has been proposed to occur through consecutive desaturations of oleic and linoleic acids in plants [1]. However the biochemical and genetic information about the synthesis of this fatty acid is currently limited. During soybean seed development, the composition of fatty acids of storage triglycerides shows considerable changes from high levels of the tri-unsaturated linolenic acid to higher levels of the more saturated linoleic and oleic acids [2–4]. Phosphatidylcholine (PC) appears to be the fatty acid donor in triglyceride biosynthesis in developing seeds [5]. By maturation, storage triglycerides (oil) of soybean seeds of most cultivars contain 7–9% linolenate. This relatively high content of linolenic acid has been considered to be a major cause decreasing the flavour and autooxidative qualities of soybean oil [6]. Consequently, the manipulation of the fatty acid content of soybeans has received considerable attention [7].

A substituted pyridazinone, 4-chloro-5-dimethylamino-2-phenyl-3(2*H*)-pyridazinone (San 9785) has been reported to reduce the linolenic acid content in a number of plant tissues [8–11]. It has been suggested that the compound specifically affects the desaturation of linoleic acid [8]. A decrease in the linolenic acid content due to San 9785 was observed in soybean cotyledons cultured *in vitro* [12]. Another approach to manipulate the linolenic acid content would be to isolate mutants deficient in the synthesis of this fatty acid in soybean seeds.

Table 1. Biomass accumulation and total fatty acid content in cultured soybean cotyledons

Cotyledon length (mm)	Treatment (100 μM)	Fr. wt (mg/piece)	Dry wt (mg/piece)	Total F.A. in neutral lipid* (nmole/mg)
5	Control	74.80	18.77	72.50
	San 9785	48.30	13.61	67.65
7	Control	93.50	34.62	64.25
	San 9785	100.78	31.09	74.0
9	Control	237.72	57.35	146.75
	San 9785	235.60	53.18	131.0
11	Control	337.60	89.94	161.5
	San 9785	313.82	83.32	192.5

*External 17:0 was added during extraction of neutral lipid and the amount of 17:0 used as internal standard to calculate the total extracted F.A.

A number of such mutants low in linolenate content have been isolated [13, 14]. The seed linolenate content is reduced by *ca* 50% in one of these, C1640 [14]. Inheritance studies have shown that the low linolenic acid content of C1640 is controlled by a nuclear factor [15], but the physiological and biochemical mechanism of the low linolenate content is not understood.

The present investigation of the effect of San 9785 on the reduction of the linolenic acid content of Century and C1640 cotyledons was undertaken with the following objectives: (1) the examination of the effect of San 9785 on the decrease in linolenic acid content at different stages of development to determine the nature of the change in the synthesis of linolenic acid during soybean cotyledon development; (2) the comparison of the fatty acid composition of Century vs the mutant and the effect of San 9785 on the two genotypes to gain further information concerning linolenate biosynthesis in soybeans.

RESULTS

Accumulation of dry matter and total fatty acids of cotyledons developed in vitro

Soybean embryos were excised at different stages of development based on the length of their cotyledons. The results (Table 1) are the average of five and four replications for dry matter and total fatty acid content, respectively. The content of total fatty acids increased as the cotyledons developed. San 9785 had no significant effect on the total fatty acid content nor on the dry matter accumulation except in the very early stages (5 mm). The total protein synthesis (incorporation of [^{35}S]methionine into TCA precipitable material) was also examined (data not shown) and no effect was observed.

Fatty-acid composition of neutral lipids and PC in soybean cotyledons of the control and low linolenate mutant

The profile of fatty acids of neutral lipids of soybean cotyledons (Table 2) shows that as development proceeds, linolenate and palmitate decline while linoleate and oleate increase. The sum of polyunsaturated fatty acids (18:2 + 18:3) remains similar across the different developing stages examined. The fatty acid composition of PC and neutral lipids differs with neutral lipids containing substantially higher levels of linoleate and lower levels of linolenate than PC (Tables 2 and 3).

The low linolenate mutant, C1640, showed a lower level of linolenate in neutral lipids and PC in cotyledons at various developmental stages of Century, with the difference being largest toward maturity (Tables 2 and 3). No alteration in fatty acid composition of vegetative tissue was observed. There was no significant change in the other major fatty acids except linoleate. A corresponding increase in linoleate was seen with the reduction of linolenate in C1640 (Fig. 1).

C1640 also showed large differences in certain phosphatidylcholine molecular species relative to Century (Table 4). The 16:0/18:3 and 18:2/18:2 are the predominant lipid molecular species in C1640; whereas, only 16:0/18:3 is in Century. The 16:0/18:3 PC molecular species is higher in Century; however, the 18:2/18:2 PC molecular species is greater in C1640.

Reduction of linolenic content by San 9785 *in Century and* C1640 *cotyledons cultured* in vitro

Although the general growth and total fatty acid accumulation were not altered by San 9785 in soybean cotyledons cultured *in vitro*, a significant reduction of linolenate synthesis occurred in the presence of 100 μM San 9785 (Tables 3 and 5). This compound also resulted in a relative increase in linoleate, but had little effect on the other fatty acids. These results suggest a specific effect on the rate of linoleate desaturation.

A greater decrease of linolenate formation by San 9785 occured in the cotyledons excised at early developmental stages in both genotypes (Tables 3, 5 and 6, Fig. 2). In addition, a similar decrease of linolenate content in Century and C1640 was observed at the 5 and 7 mm length stages of cotyledon development. However, at the later stages, 9 and 11 mm, San 9785 was much less effective in decreasing the linolenate content of the mutant.

The content of linolenate in PC was also reduced by San 9785 (Table 6). Treatment with this compound resulted in

Table 2. Profile of fatty acids of neutral lipids in Century and C1640

Cotyledon length (mm)	Genotype	Fatty acid (%) 16:0	18:0	18:1	18:2*	18:3†	18:2 + 18:3
5	Century	21.58	2.77	10.78	37.82	26.20	64.04
	C1640	18.35	3.10	7.66	44.94	22.78	67.72
7	Century	18.61	1.31	11.32	47.58	20.99	68.57
	C1640	18.80	1.99	10.86	53.53	15.28	68.81
9	Century	15.77	1.09	16.30	50.39	16.42	66.81
	C1640	13.23	2.64	16.97	58.64	9.31	67.95
11	Century	13.86	2.08	21.61	51.5	12.37	63.87
	C1640	16.59	2.50	19.93	56.26	7.77	64.03
Seed	Century	13.12	3.48	13.32	57.78	10.85	68.6
	C1640	13.80	3.25	13.45	63.02	5.0	68.02
leaf	Century	7.49	1.19	2.67	11.66	58.19	69.85
	C1640	8.20	1.14	2.31	11.00	56.93	67.93

*Linoleate (18:2) LSD (0.05) = 3.68 (see Experimental).
†Linolenate (18:3) LSD (0.05) = 3.39.

Table 3. Fatty acid composition of phosphatidylcholine at two stages of seed development in century and C1640

Variety	Developmental stages (mm)	Fatty acid (% by wt)					
		14:1	14:2	16:0	18:1	18:2	18:3
Century	9	1.44	3.48	26.92	13.51	15.23	38.92
	11	1.76	2.40	30.51	18.36	20.89	26.08
C1640	9	1.78	3.00	24.51	14.51	32.01	24.18
	11	2.66	4.12	21.78	18.36	31.00	22.16
Century	9	1.33	3.01	21.70	19.81	30.87	23.28
+ SAN 9785 (100 μm)	11	1.99	2.30	28.01	19.15	26.21	22.34
C1640	9	1.33	2.28	26.11	13.88	36.04	20.36
+ SAN 9785 (100 μm)	11	1.47	2.55	26.78	12.78	36.44	19.98

Table 4. Phosphatidylcholine molecular species at two stages of seed development in century and C1640.

Phosphatidylcholine molecular species	Century		C1640	
	9 mm (Wt%*)	11 mm (Wt%)	9 mm (Wt%)	11 mm (Wt%)
16:0/14:1†	3.79	3.62	3.21	3.01
16:0/14:2	4.14	4.60	5.21	6.55
16:0/18:2	5.98	21.48	7.64	7.86
16:0/18:3	39.72	30.00	30.80	25.40
18:1/18:1	6.37	10.11	7.23	11.68
18:1/18:2	6.82	10.34	6.49	5.86
18:1/18:3	3.80	3.68	3.99	4.55
18:2/18:2	6.84	3.55	20.28	19.20
18:2/18:3	4.66	5.12	5.12	6.86
18:3/18:3	17.88	7.00	10.03	9.03

*Values reported are a percentage by weight of the total phosphatidylcholine molecular species.

†Pairs of numbers representing fatty acids and separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. The positional distribution of the fatty acids was determined by hydrolysing the eluted molecular species with lipase from *R. arrhizus*.

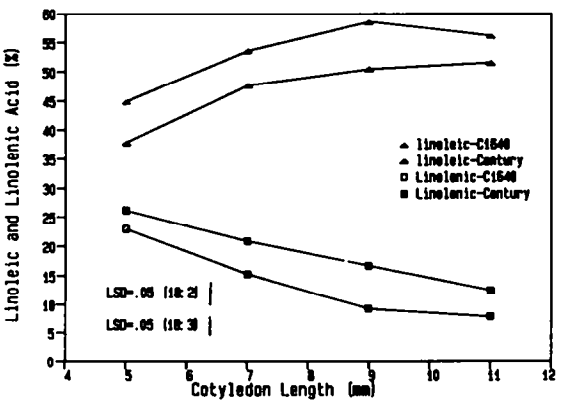

Fig. 1. Composition (%) of linoleic and linolenic acids of *in vivo* developing cotyledons of the soybean genotypes Century and C1640 [14].

an increase in most 18:2 containing PC molecular species (16:0/18:2, 18:1/18:2 and 18:2/18:2) and a decrease in most 18:3 bearing species (16:0/18:3, 18:1/18:3 and 18:3/18:3).

DISCUSSION

More than one site for linoleic desaturation has been proposed leading to the formation of linolenate in plants [16]. One (eukaryotic) occurs in the cytosol phase and involves microsomal phosphatidylcholine (PC) as the substrate and the other (prokaryotic) is located in chloroplast and uses glycolipid intermediates as precursors [17, 18].

The studies on the decrease of linolenate synthesis by San 9785 in plant leaves [10, 11] have revealed that this compound only inhibits the desaturation of diacylgalactosylglycerol, a proposed substrate for linoleic desaturation in chloroplasts. This suggests that San 9785

Table 5. Effect of San 9785 on the linolenate content in Century and C1640

Cotyledon length (mm)	Genotype	Linolenic content (%) Control (A)	+ San 9785 (B)	B/A × %
5	Century*	25.94	15.44	59.52
	C1640†	20.43	12.52	61.28
7	Century	24.73	14.52	58.71
	C1640	16.69	9.50	56.90
9	Century	18.07	12.56	69.51
	C1640	9.54	7.53	78.90
11	Century	13.17	9.87	74.94
	C1640	6.19	5.39	87.0

*Century LSD (0.05) = 2.54.
†C1640 LSD (0.05) = 1.12.

Table 6. Effects of SAN 9785 (100 μm) in Century and C1640 and the mutation on phosphatidylcholine molecular species at two stages of seed development

Phosphatidylcholine molecular species	San 9785/Century* 9 mm	11 mm	San 9785/C1640 9 mm	11 mm	C1640/Century 9 mm	11 mm
16:0/14:1†	79‡	104	93	88	85	83
16:0/14:2	104	108	92	78	126	142
16:0/18:2	279	106	131	126	128	37
16:0/18:3	56	66	87	102	78	85
18:1/18:1	150	94	103	64	114	116
18:1/18:2	215	138	122	142	95	57
18:1/18:3	91	82	75	88	105	124
18:2/18:2	178	186	110	117	297	541
18:2/18:3	86	189	125	87	110	134
18:3/18:3	35	75	81	88	56	129

*San 9785/Century and San 9785/C1640 are the ratios of values of cultured Century and C1640 cotyledons in the presence of 100 μm San 9785 (see Experimental) vs Century and C1640 cotyledons in control culture media. C1640/Century is the ratio of the values of C1640 vs Century.

†Pairs of numbers representing fatty acids and separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. The positional distribution of the fatty acids was determined by hydrolysing the eluted molecular species with lipase from *R. arrhizus*.

‡Values reported are expressed as a percentage of control values.

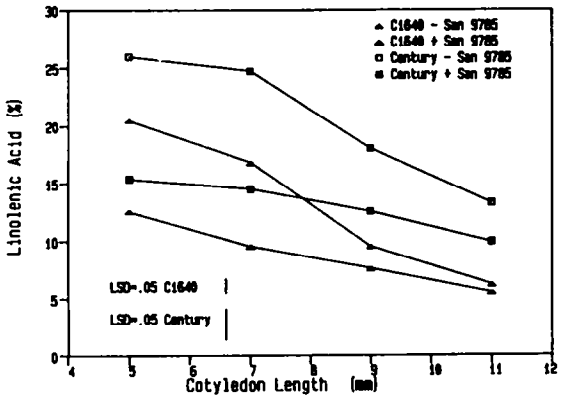

Fig. 2. Linolenic acid content (%) of Century and C1640 cultured *in vitro* in the presence and absence of San 9785 [12].

specifically inhibits linolenate synthesis in chloroplasts. However, in the present study, this compound resulted in a reduction of the linolenate content of PC in developing soybean cotyledons. PC is thought to be an intermediate in the synthesis of polyunsaturated fatty acids destined to be stored in seed triglycerides [5]. It is possible that in soybean seeds San 9785 does not have a stringent selective effect on the subcellular compartmentation of linolenate formation as is thought to occur in leaves and /or that PC has a metabolic role during the synthesis of linolenate in both the cytosolic and plastid desaturation systems in developing cotyledons.

The changes in fatty acid composition during soybean seed development has been thoroughly investigated [2–4]. In the early stages of development, the relative content of linolenate decreases while those of oleate and linoleate increase. These observations led to a suggestion that the rate synthesis of linolenate declines as seed development

proceeds [4]. Cell-free assays of desaturase activity in soybean cotyledons also showed a high rate of fatty acid desaturation at early stages of development and an irregular and rapid decline in desaturation at later stages [19]. In the present study, San 9785 effected a decrease in the linolenate content differentially in cotyledons excised at different stages of development, supporting previous suggestions that the synthesis of linolenate is more active at early stages [4, 9]. The differential reduction of linolenate might partially result from a differential expression of two different desaturation systems, suggesting that the chloroplast fatty acid desaturation system which is inhibited by San 9785 is more effective in early stages of development of soybean cotyledons. The differential effect of San 9785 on PC molecular species is consistent with multiple desaturation pathways as PC serves as a major substrate for linoleate desaturation as well as triglyceride biosynthesis [5].

The low linolenate mutant C1640 has about a 50% lower concentration of linolenate in mature seeds. However, no difference in fatty acid profiles was seen in leaf tissues. This inconsistent expression of low linolenate content in vegetative and reproductive tissues have also been observed in other low linolenate mutants [20]. These results suggest a differential regulation of desaturation systems in vegetative and reproductive tissues. The differences in PC molecular species in Century and C1640 also suggests the existence of several desaturation pathways. Examination of the fatty acid composition across different stages of development based on cotyledon length in control and mutant cotyledons showed an increasing difference in the relative amount of linolenate as development proceeds. When C1640 cotyledons were treated with San 9785, similar reductions of the relative amount of linolenate occurred on those excised at early stages in both genotypes while San 9785 was much less effective in reducing the linolenate content of the mutant at later stages of development. This mutation results in a buildup of 18:2/18:2 and a decrease of 16:0/18:3 PC molecular species which resembles the changes seen in the Century cotyledons treated with San 9785 (Tables 4 and 6). These results suggest that C1640 either has greatly reduced overall linolenate synthesis activity in later stages or shifts more to the microsomal desaturation system on which San 9785 has little effect. The reduction of linolenate with a corresponding increase in linoleate (Fig. 1) suggests a specific blockage in the desaturation of linoleate to linolenate.

EXPERIMENTAL

Culture of developing cotyledons. The soybean genotypes Century and the low linolenate mutant C1640 were grown in a greenhouse. Pods at different stages of development based on cotyledon length were excised and surface-sterilized in 95% EtOH for 2 min and 40% chlorox for 15 min. Seed coats were removed aspetically. The cotyledons were separated and the embryonic axis removed. One cotyledon was placed in the medium containing 100 μM San 9785 and the other cultured in control medium without San 9785. The culture medium was previously described by Hsu and Obendorf [21]. The solutions were sterilized by filtration. The cotyledons were incubated on a continuously illuminated shaker at 22°. The cultured cotyledons were collected after 2 weeks in culture and then lyophilized. For analysis of the *in vivo* developing cotyledons, embryos at the same cotyledon length of Century and C1640 were harvested and lyophilized for subsequent fatty acid analysis.

Lipid extraction and fatty acid analysis. Neutral lipids of lyophilized cotyledon tissues (*ca* 10 mg) were extracted with petrol (2 ml) containing 0.01% BHT (butylated hydroxytoluene). During the extraction 10 nmole of exogenous heptadecanoic acid (17:0) was added to each sample as an internal standard to calculate the content of total fatty acids extracted. MeOH containing 1% H_2SO_4 (1 ml) was added to the petrol extracts and the petrol was evaporated in an oven at 45°. Fatty acid methyl esters (FAME) were prepared by heating the lipid extracts in 1% H_2SO_4 in MeOH at 90° until 0.5 ml of solvent in each tube was left. The FAME were then reextracted into petrol for subsequent analysis.

The fatty acid methyl esters were analysed by GC using a modification of the procedure of Chaven *et al.* [22]. A Varian 3700 gas chromatograph was used with a 183 × 3.2 mm stainless steel column with a 20 cm pre-column. The column packing was GP 3%, SP-2310/2% SP2300 on 100/120 chromosorb WAW (Supelco, Inc.). The injector temperature was set at 170° and the column temperature was initially 165° and held at 165° for 2 min., then raised 5°/min to a final temperature of 210°. The detector temperature was 250°. The gas flows were 300 cm^3/min air, 30 cm^3/min H_2 and the carrier gas, N_2 was 24 cm^3/min. The sensitivity was usually set at 4×10^{-10}. The data were subject to statistical analysis by computing the restricted least significant differences (LSD) of the ranked treatment means [23].

Analysis of PC molecular species. Total lipids were extracted from lyophilized cotyledons using the procedure of Bligh and Dyer [24]. The extract was loaded onto a silica Sep-Pak cartridge (Waters Assoc., Milford, MA) and neutral lipids and galactolipids removed by successively washing with $CHCl_3$ and $CHCl_3$–MeOH (17:3), respectively, phospholipids were then eluted with MeOH, and PC was resolved by TLC on silica gel H using $CHCl_3$–HOAc–MeOH–H_2O (75:25:5:2.2). PC was detected by exposure of the plate to iodine vapour and eluted from the silica gel using $CHCl_3$–MeOH–H_2O (3:5:1). The eluates were adjusted to give proportions of $CHCl_3$–MeOH–H_2O (4:7:8), and the lower phase then collected and dried in N_2.

PC (0.2–0.4 μmol) was separated into constituent molecular species using a Waters Assoc. (Milford, MA) HPLC system (Model 6000A) equipped with a Model U6K universal injector, and a 25 cm × 4.6 mm Rainin Microsorb (5 μm) reverse-phase column. A Tractor 945 Flame Ionization LC Detector (Tracor Instruments, Austin, TX) operated at a block temperature of 160° was used for detection. The HPLC solvent was MeCN–MeOH–HOAc–H_2O–PrNHEt (89.8:6.8:1.5:1.0:0.9) delivered at a flow rate of 1.1 ml/min for routine quantitative analyses. The flow rate was increased to 1.9 ml/min in some cases so that 45% of the column eluated could be split away for recovery of individual lipid molecular species [25]. Each eluted lipid collected was dried in N_2 and fatty acid methyl esters were prepared with BF_3/MeOH/methanol (Sigma) [26]. The methyl esters were identified with a Hewlett Packard (Model 5880A) gas chromatograph using the procedures of Lynch and Thompson [27]. The positional distribution of fatty acids was determined by hydrolysing the eluted lipids with phospholipase A_2 from *Crotalus adamanteus* venom (Boehringer, Mannheim) [28]. Lipid phosphorus was determined using the method of Bartlett [29] as modified by Marinetti [30].

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