INHERITANCE AND CHARACTERIZATION OF EMS-INDUCED FATTY ACID MUTATIONS IN McGREGOR FLAX

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ABSTRACT

Genetic studies were carried out under controlled phytotron conditions, on McGregor (a Canadian flax cultivar) and four EMS-induced fatty acid mutant lines of McGregor. The objective of this study was to determine the inheritance of the mutated genes in the mutants (which, prior to this study, were described as: E67 - high in palmitic acid; E1747 - low in linolenic acid; E1929 - elevated oleic acid; and E1536 - high in both oleic and linoleic acids). Crosses among these five flax lines were made in all possible combinations.

Fatty acid analysis of the seed oil from F_1, F_2 and backcross populations indicated the control of the characteristic fatty acids in the mutant lines to be under simple Mendelian inheritance. The high palmitic acid character in E67 and the moderately low linolenic-high oleic acid character of E1929 were each controlled by a single partially dominant gene, whereas the very low linolenic-high linoleic character of E1747 was controlled by two partially dominant genes at independent loci. The E1929 gene apparently is allelic to one of the two genes in E1747. A partial maternal effect was observed for the control of the palmitic acid trait in E67. E1536 is a true mutant. However, its mutated character could not be detected, due to its "chameleon-like" behaviour. A moderately low linolenic-elevated oleic and linoleic acid phenotype was vaguely inferred. The flax mutants showed varying degrees of interaction when they were crossed with each other; this interaction(s) being inversely proportional to the closeness of the characteristic fatty acid phenotypes of the

mutants (involved in a cross), in the fatty acid synthesis pathway. Since the inheritance of these mutant characters are controlled by one or two genes, breeding for these characteristics should be relatively simple.

Two ^{14}C radio-labelled investigations were conducted to determine enzymes in the fatty acid biosynthesis pathway that may have been affected to produce these mutant phenotypes: i) ^{14}C acetate (*in vivo*) assays and ii) *in vitro* target enzyme assays using ^{14}C-labelled precursors. Gas chromatography was used for the analysis of fatty acid composition, whereas a scintillation counter and high performance liquid chromatography were used for assaying radioactive fatty acids. ^{1}H-NMR analyses were also conducted on intact seeds to determine oil contents of the flax lines. The ^{14}C-sodium acetate experiments could not help in the identification of the fatty acid synthesis enzymes which were affected in producing these mutant lines. Based on the *in vitro* enzyme assays, none of the following enzymes appeared to be responsible for the high palmitic acid character in E67: 16:0-ACP thioesterase, 18:1-ACP thioesterase and

β-ketoacyl-ACP synthetase II.

^{1}H-NMR data on oil content showed that whereas the mutations in E67 and E1929 had no effect on oil content (relative to McGregor), the mutation in E1536 resulted in a drastic 47% reduction in oil content. A relative 30% increment in oil content was shown by E1747. This higher oil content character, coupled with the very low linolenic-high linoleic acid trait of E1747, should boost the edible oil candidacy of this mutant flax line.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

1. INTRODUCTION

Flax (*Linum usitatissimum* L.) is noted for the high quality "drying" oil produced in its seeds. Upon exposure to air, linseed[1] oil is easily oxidized to form a durable film. It is, therefore, used industrially for manufacturing paints, stains, inks, lacquers, varnishes, linoleum, etc. The industrial importance of linseed oil results from its high content of α-linolenic acid (about 50%). Conversely, flavour reversion problems (offensive odour, rapid oxidation and rancidity) imparted by the same high linolenic acid content, excludes linseed oil from the large and continually expanding market of edible vegetable oils.

Scientists in Australia and Canada are pioneering research to develop edible linseed oil by reducing the linolenic acid content to less than 3%. Having flax as a second vegetable oilcrop in western Canada (in addition to canola) will not only diversify oilcrop production, but also help to arrest some of the pest problems and build-up of diseases in canola fields. Flax fits well into a cereal/canola cropping rotation for two reasons: 1) few additional or specialized production techniques are required, and 2) though a poor competitor with weeds, flax is one of the few crops that tolerate herbicides used for the control of both grassy and broadleaf weeds, and

[1] Linseed - cultivars of flax grown for their seed oil (as opposed to fibre flax - cultivars of flax grown for their fibre).

thus can serve as a "clean-up crop" in rotations (Braidek, 1975).

Green (1986) produced two mutants (with slightly lowered linolenic acid contents) by ethyl methanesulphonate (EMS) mutagenesis in the Australian cultivar Glenelg, crossed them and obtained some F_2 genotypes whose seed oil contained very low levels of linolenic acid (2%). Rowland and Bhatty (1990) also used EMS to induce mutations in seeds of the Canadian flax cultivar McGregor, and identified three mutant genotypes with varying fatty acid profiles; low linolenic, high palmitic and elevated oleic. The differences in fatty acid profiles among these mutant genotypes suggest the possibility of developing many types of edible oil fatty acid profiles to meet different market demands (e.g. low linolenic-high linoleic, low linolenic-high palmitic, low linolenic-high oleic). The knowledge needed to achieve such goals necessitated the initiation of this research. The objectives were to:

- Determine the inheritance patterns of fatty acid profiles in these McGregor EMS-induced mutant lines.
- Determine possible linkages and/or allelic relationships among the mutated genes.
- Characterize the mutations by determining the biochemical site(s) affected by these mutations.
- Determine the relative proportions of oil content in these flax lines.

2. LITERATURE REVIEW

2.1. Flax

2.1.1. History and Adaptation

Flax is a member of the Linaceae family. It is the only species in this family with nondehiscent or semidehiscent capsules suitable for commercial farming (Lay and Dybing, 1989). Flax is an annual crop grown for either its fibre (fibre flax) or seed oil (linseed) and has a chromosome number 2n=30. Fibre flax cultivars are taller and less branched than oilseed types, and are adapted to cool-temperate regions. The largest producer of fibre flax is the USSR. Linseed cultivars on the other hand, are shorter, more branched and larger-seeded. Canada is the leading exporter of flaxseed; others are Argentina, India, USA and the USSR. Flax is well adapted to the prairies of western Canada (Manitoba, Saskatchewan and Alberta) and usually 0.4 to 1.2 million hectares of flax are cultivated annually in the region (Runciman and Olson, 1975). Canadian flaxseed is preferred on the world market due to its high oil content and quality (Kenaschuk, 1975). The cool temperatures of western Canada are conducive to the production of high oil content and unsaturated fatty acid-rich flaxseed.

Due to the introduction of synthetic fibres which are cheaper and easier to care for, world production of fibre flax has declined over the years (McHughen, 1990) with

a virtual cessation in North America. In the United States, cultivation of fibre flax became unprofitable and ceased in the mid-1950s (Hartmann *et al.*, 1981). Since all of the North American flax is produced for seed, the remainder of this review will deal only with linseed flax.

The centre of origin of cultivated flax is not certain (Lay and Dybing, 1989). However, it is believed to be around Mesopotamia, Assyria, Egypt and the districts included between the Persian Gulf, the Caspian Sea, and the Black Sea (De Candolle, 1964).

2.1.2. Mode of Reproduction

Flax is a highly self-pollinated species with perfect, complete and pedicellate flowers. Its "sticky" pollen is rarely transferred by insects (Lay and Dybing, 1989). However, as much as 2% of natural outcrossing, resulting from insect activity may occur under field conditions (Dillman, 1938).

2.1.3. Botany and Phenology

The flax flower parts, petals, sepals and stamens, all occur in units of 5; and the ovary also has 5 carpels each of which is capable of developing 2 seeds. The flower colour (colour of petals) can range from dark blue to light blue or may be white or pale pink (Poehlman, 1959).

Under field conditions, flowers open shortly after sunrise on clear warm days and the petals are shed in the early afternoon (Beard and Comstock, 1980). Under

phytotron conditions germination, flowering, pollen shedding and seed setting occur under a wide range of environments and photoperiods; day lengths exceeding 12h and temperatures between 12 and 24°C are considered optimum.

With complete seed set, 10 seeds can be obtained in a boll[2] (or capsule). Variable ripening of bolls occurs since individual plants bloom over a 2- to 3-week period (Kenaschuk, 1975). The seeds are 'flat', oval, and pointed at one end (embryo axis end). The seed colour of different cultivars ranges from light to dark reddish brown or yellow; others may be mottled[3].

In general, the average life cycle of the flax plant is about 100 days. The vegetative period occupies the first half of the cycle and the other half is shared between flowering and maturation periods (Anon., 1984). Oil content is about 40%, under western Canadian growing conditions (Carr, 1989).

2.2. Fatty Acid Composition and Utilization of flaxseed

2.2.1. Fatty acid composition of linseed oil

Most vegetable oils are composed largely of fatty acids with 16- and 18-carbon chains (C_{16},C_{18}) (Table 2.1). The 5 major fatty acid components of linseed and most other vegetable oils are: palmitic (16:0)[4] , stearic (18:0), oleic (18:1), linoleic (18:2)

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2 matured flax ovary.

3 a combination of yellow and brown colour in the same seed (a physiological condition).

4 Number of carbon atoms : Number of double bonds.

Table 2.1 Fatty acid composition (%) of linseed and other major vegetable oils (Anon., 1984)

Oilcrop	Fatty acid				
	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3
Sunflower	7.2	4.1	16.2	72.5	0.0
Corn	12.1	2.3	28.7	56.2	0.7
Soybean	11.5	3.9	24.6	52.0	8.0
Canola	3.5	1.5	60.0	20.0	10.0
Linseed	7.0	4.0	20.0	17.0	52.0

and α-linolenic[5] (18:3) acids.

Linseed oil is composed of about 90% unsaturated fatty acids (18:1, 18:2 and 18:3) and 10% saturated fatty acids (16:0 and 18:0). The sum of 18:1 and 18:2 is 76.6% for soybean, 88.7% for sunflower, 84.9% for corn and 80.0% for canola, but less than 40% for linseed oil (Table 2.1). However, 18:3 content in linseed oil is over 50% as compared to 10% or less in each of the other mentioned vegetable oils. The percentage of the unsaturated fatty acids, particularly 18:3 determines the quality of linseed oil. Cultivar and temperature are two important factors which determine the proportion of these fatty acids; for example, the 18:3 content in linseed may vary from

[5] α-linolenic:- cis -9,12,15 octadecatrienoic acid (as opposed to γ-linolenic:- cis -6,9,12 octadecatrienoic acid).

45% under warm to 60% under cool temperature regimes (Anon., 1984).

2.2.2. Utilization of Linseed

The suitability of a vegetable oil for either industrial or edible use is determined by its fatty acid composition. High content of a specific fatty acid imparts unique qualities to certain vegetable oils (Table 2.2).

The industrial importance of linseed oil is attributed to the fact that it contains more than 50% α-linolenic acid, the 18-carbon fatty acid with 3 double bonds at the Δ9, Δ12 and Δ15 positions. Upon exposure to air, the double bonds in this fatty acid react rapidly with oxygen to polymerize into a relatively soft, flexible and durable film. This unique property of linseed oil accounts for its extensive use in the manufacture of protective coatings such as paints, stains, lacquers, and varnishes. The oil is also used in soaps, linoleum, oilcloth, printer's ink, putty, patent and imitation leather, automobile brake linings, as a core oil in making sand castings, and also as an anti-spalling and curing agent (salt-resistant coating) for concrete surfaces on highways and bridges (Kenaschuk, 1975; McHughen, 1990).

Linseed meal or oil cake (the residue after oil extraction) contains about 36% crude protein, and is, therefore, used as a protein supplement in livestock feed. The meal is deficient in lysine (an essential amino acid), and high in crude fibre (Bell, 1989).

Mature flax straw (prussic acid-free) serves as a roughage diet when it is fed to livestock. Paper companies also utilize mature flax straw for manufacturing

Table 2.2 Major fatty acids of selected industrial oilseeds

Oilcrop	Fatty acid	Chemical nature	Uses (Reference)
Castor	Ricinoleic	Hydroxy	Paints, varnishes (Atsmon, 1989)
Jojoba	Eicosenoic	Liquid wax esters	Cosmetics (Benzioni and Forti, 1989)
Rapeseed	Erucic	Long chain	Plastics (Princen and Rothfus, 1984)
Linseed	α-linolenic	Drying oil	Paints, varnishes (Poehlman, 1959)

cigarette and currency papers (Lay and Dybing, 1989).

The use of linseed in products for human consumption is limited. Because of its high mucilage and fibre content (which aids digestion) small quantities of flaxseed are sometimes used in human food products (e.g. bread, cereals). Consumption of large quantities of linseed may be hazardous to animals because of the presence of two natural toxicants; linamarin (a cyanogenic glucoside) and linatine (an antagonist of vitamin B_6) (Bell, 1989).

2.3 Modifications of fatty acid composition of plant oils

2.3.1 Edible oil

An inverse relationship exists between the percentage of linolenic acid in an edible oil and its flavour stability. High quality edible oils contain low levels of linolenic acid; for example sunflower oil (0.0%) and corn oil (0.7%), as opposed to 52% in linseed (Table 2.1). The breeding for low linolenic acid content in an oilcrop that contains linolenic acid in its seed oil may be attained through i) screening existing cultivars, ii) making interspecific crosses and iii) inducing mutations. Any research leading to a significant decrease of linolenic acid content in an oilcrop is likely to improve the competitive position of that crop, due to the elimination of flavour reversion problems and/or processing costs to impart flavour stability (Smith, 1984).

2.3.2. Induction of fatty acid mutations

Genetic variability in fatty acid contents is essential for the development of novel and specific plant genotypes. The existence of a wide range of variation in erucic acid content (0.2 to ~45%) in natural populations of summer rape (*Brassica napus*), permitted the development of erucic acid-free genotypes in this species, through selection of the desired genotypes (Stefansson *et al.*, 1961).

The modification of fatty acid profiles has been a major objective in oilseed breeding programs since an increase or decrease of a particular fatty acid determines the end use of the oilseed crop (Table 2.3) (Rattray, 1991). The primary emphasis has been the reduction in the percentage of linolenic acid, which is associated with the unacceptable flavours in oilseeds (Dutton *et al.*, 1951; Evans *et al.*, 1971;

Table 2.3 Breeding objectives in the modification of fatty acid composition of plant oils (Rattray, 1991)

Plant oil	Fatty acid	Objective	End use
Soybean	18:0	Increase	Margarine industry
	18:3	Reduce	Flavour stability/ edible oil
Rapeseed	8:0	Increase	Oleochemical industry
	10:0	Increase	Oleochemical industry
	16:0	Increase	Margarine industry
	18:3	Reduce	Edible oil
	22:1	Increase	Oleochemical industry
	22:1	Reduce	Edible oil
Sunflower	18:1	Increase	Olive oil substitute
Safflower	18:1	Increase	Olive oil substitute
Linseed	18:3	Reduce	Edible oil

Downey and McGregor, 1975).

The industrial process of hydrogenation (which converts unsaturated fatty acids to more stable fatty acids) is one method of improving flavour stability in oils with intermediate levels of 18:3 (e.g., soybean) (Table 2.1) (Cowan *et al.*, 1970). This industrial process, however, is not only costly, but can result in the formation of nutritionally inferior *trans* isomers (Wilcox *et al.*, 1984).

The use of explants, cell suspensions or tissue culture (which could permit

selection and intermating of selections in the same season) has not been successful in screening for a desired seed fatty acid composition. Seed oil composition is developmentally controlled by mechanisms which are not consistently expressed in vegetative tissues (Martin and Rinne, 1985). Thus *in vitro* systems, though effective in selecting for cell lines that are resistant to or tolerant of some herbicides, environmental stresses or diseases, are not suitable for such traits as oil quality and quantity (McHughen, 1990).

Low levels of linolenic acid in natural populations of soybean were utilized by White *et al.* (1961) to produce an F_2 plant with 3.4% linolenic acid content. However, the low linolenic acid level proved unstable in succeeding generations. In flax the presence of strong interspecific crossing barriers has prevented the hybridization of *L. usitatissimum* (the cultivated species) to low linolenic (wild) species like *L. campanulatum, L. catharticum, L. flavum* and *L. mucronatum* (Green, 1985; Nichterlein *et al.*, 1989). The latter species contains only 2% linolenic acid in its seed oil (although 15% ricinoleic acid is also present) (Kleiman and Spencer, 1971).

A common and more satisfactory method for modifying fatty acid profiles of oilseeds is by chemical mutagenesis (Table 2.4). A particular kind of mutagen seems to produce similar effect(s) in seeds of different oilcrops (Table 2.4), e.g., EMS produces low 18:3 seed types in soybean, rapeseed and flax, though the mechanisms involved are not understood.

Wilcox *et al.* (1984) demonstrated the use of chemical mutagenesis (with soybean seeds) as a tool for producing, not only novel oilseed types but also,

Table 2.4 Seed oil fatty acid modification as a consequence of chemical mutagenesis and breeding.

Crop	Mutagen*	Seed type	Reference
Soybean	EMS	Low 18:3	Hammond & Fehr, 1983a
		High 16:0	Fehr *et al.*, 1991b
	NaN_3	High 18:0	Hammond & Fehr, 1983b
	NMU	High 16:0	Fehr et al., 1991b
Rapeseed	EMS	Low 18:3	Rakow, 1973
Sunflower	DMS	High 18:1	Fick, 1989
Linseed	EMS	Low 18:3	Green, 1985; Rowland & Bhatty, 1990
		High 16:0	Rowland & Bhatty, 1990
		High 18:1	Rowland & Bhatty, 1990

* DMS - dimethyl sulphate.

EMS - ethylmethane sulphonate.

NaN_3 - sodium azide.

NMU - N-nitroso-N-methylurea.

variability (range) in fatty acid composition for further exploitation. They obtained a 2-fold increase in variability of stearic and linolenic acid and a 3- to 4-fold increase in the variability of palmitic, oleic and linoleic acid content. It would appear that treatment of oilseeds with EMS in most cases results in simple blocks in the

biochemical pathway of fatty acid biosynthesis (Fig. 2.1), causing alteration of only a few genes. Wilcox *et al.* (1984) found M_4 soybean fatty acid mutants to be uniform and visually indistinguishable from the parent plant. E1747 and E1929 mutants used in the present study were visually indistinguishable from the parent McGregor flax plant. EMS-treated oilseeds seem to have similar maturity (with some exceptions, e.g. rapeseed, McGregor, personal communication) as the parents (Constantin *et al.*, 1976; Wilcox *et al.*, 1984). Most of the novel mutants generated by chemical mutagenesis have been usually identified and isolated from M_3 or M_4 populations (Wilcox *et al.*, 1984; Green, 1985; Rowland, 1991). This may be explained by the fact that most mutants (about 99%) are due to recessive mutations and, hence, start to become discernible in homozygous forms after the M_2 generation (Gottschalk and Wolff, 1983; Knowles, 1989). EMS-treated seeds on the other hand, may produce weak seedlings, albinism and other phenotypic abnormalities, especially in M_1 and M_2 generations (Wilcox *et al.*, 1984; Green, 1985).

2.3.3. Inheritance of fatty acid mutations

Fatty acid composition of several oilseed crops is simply inherited and controlled by the genotype of the embryo rather than the genotype of the maternal parent (Wilcox and Cavins, 1985). Work by Yermanos and Knowles (1962) established that in linseed the fatty acid composition of the seed is controlled by the genotype of the embryo and not by the maternal sporophyte, though this embryonic control is not absolute. Similar reports by Downey and Harvey (1963), and Knowles and Hill (1964) on rapeseed and safflower respectively, indicated that the control of

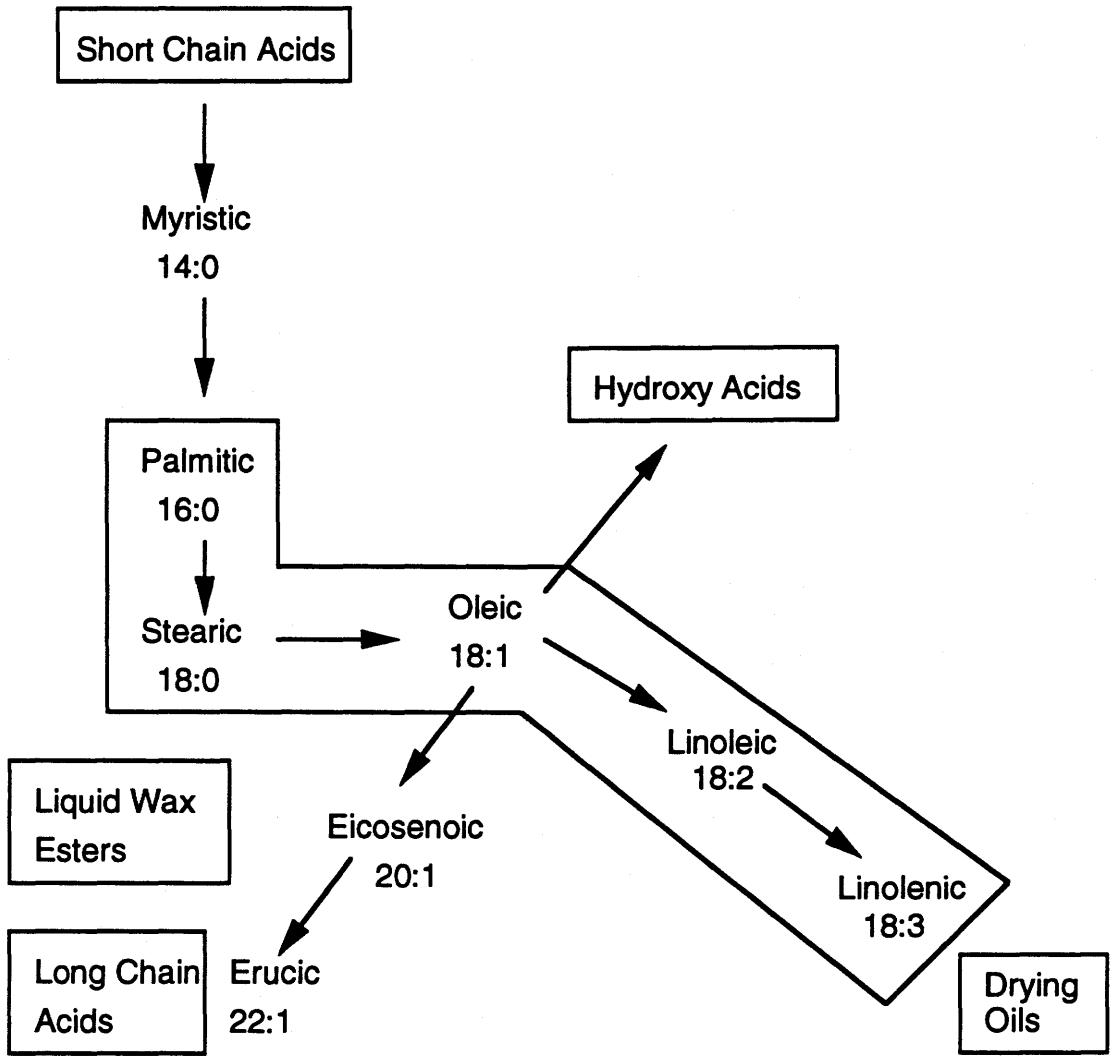

Fig. 2.1 Steps in the biosynthesis of fatty acids (Knowles, 1989)

fatty acid composition is by the genotype of the embryo. This embryonic control of fatty acid composition of oilseed crops implies that 1) the seed of an F_1 plant represents an F_2 population, 2) selection among individual seeds from a heterozygous plant can be successfully employed (Graef *et al.*, 1985), and 3) genetic analysis can be obtained an apparent generation earlier by analyzing oil from each individual seed produced on an F_1 plant (Downey and Harvey, 1963).

Brim *et al.* (1968) found that 18:1 and 18:2 composition of soybean oil was under maternal control. However, three subsequent studies on several soybean mutants found no significant maternal effects on the inheritance of saturated fatty acids (16:0 and 18:0) (Bubeck *et al.*, 1989; Graef *et al.*, 1985; Fehr *et al.*, 1991 a,b). Both Erickson *et al.* (1988) and Fehr *et al.* (1991 a,b), working with different mutant soybean lines with altered (both reduced and elevated) levels of 16:0, observed that two independent loci segregated for palmitic acid content and the alleles at each locus exhibited additive gene action.

Using a chemically induced low 18:3 soybean mutant (C1640), Wilcox and Cavins (1985) observed that the inheritance of linolenic acid content was under embryonic control. This result differed from that previously reported by Brim *et al.* (1968). Wilcox and Cavins (1985) attributed these differences to the germplasms used for these studies [naturally occurring genotypes (Brim *et al.* (1968) as opposed to chemically induced mutants (Wilcox and Cavins, 1985)]. The low 18:3 content of C1640 was controlled by a single gene. In contrast, Graef *et al.* (1988) using a soybean mutant (A5) with low 18:3 content, observed a partial maternal effect for low

18:3 inheritance. Such differences suggest that inheritance studies of specific mutants are essential and paramount for their use in breeding programs.

Green (1985) found that the desaturation of oleic to linoleic acid in flax was controlled completely by the maternal genotype, whereas further desaturation to linolenic acid was under the control of the embryo, and only slightly affected by the maternal parent. Several other crosses produced varying results: Somaco X Avantgarde (non-mutants) showed complete embryo control of linoleic acid content, whereas a partial maternal effect was observed in M1722 (mutant) X Avantgarde. The two low 18:3 linseed mutants developed by Green (1985) through EMS application possessed two independent genes (one in each mutant) which upon crossing, acted additively and equally to reduce 18:3 content to <2% in the progeny (Zero). A direct EMS treatment of McGregor (a Canadian flax) produced the low 18:3 linseed mutant (E1747) used in this study (Rowland and Bhatty, 1990). The linolenic acid data from the F_2 of E1747 x McGregor gave a good fit to a 15:1 ratio (Rowland, 1991), indicating that two independently inherited mutations were responsible for the low 18:3 content of E1747.

2.4 Biosynthesis of fatty acids in oilseeds

2.4.1. General concepts of plant lipid biosynthesis

Sucrose from photosynthesis is converted to and stored as starch in low lipid-containing seeds (e.g. peas and beans). In high lipid-containing seeds (e.g. canola and linseed) however, lipid (chiefly triacylglycerols) is the principal storage product.

In oilseeds the synthesis of fatty acids apparently occurs in two compartments: the synthesizing compartment (proplastid) and the modifying compartment (cytosol; probably on the endoplasmic reticulum (ER)) (Fig. 2.2). Each compartment contains all enzymes necessary for its operation. The principal product of plastidic fatty acid synthesis is free oleic acid (18:1), which then undergoes modification in the cytosol. The type of modification that 18:1 is subjected to is oilcrop-specific; e.g., hydroxylation for castor bean seed, elongation for rapeseed, elongation/reduction/condensation for jojoba seed and desaturation for linseed (Stumpf, 1989).

Acyl carrier protein (ACP) in the proplastid, acts as the thioester acyl-carrier moiety during the synthesis of the C_{16} and C_{18} fatty acids, in their transfer to suitable acceptors and in the introduction of the first double bond ($\Delta 9$ position)[6] in the hydrocarbon chain, whereas acyl-CoA derivatives are involved in the reactions in the cytosol (Stumpf, 1989).

2.4.2. Biosynthesis of Palmitic, Stearic and Oleic Acids

β-ketoacyl-ACP synthatase I and β-ketoacyl-ACP synthatase II (also called condensing enzymes) are unique among the fatty acid synthetase (FAS) enzymes. Each of the condensing enzymes operates in concert with the other FAS enzymes. β-ketoacyl-ACP synthetase I has a broad specificity for acyl-ACPs having chain lengths from C_2 to C_{14}, leading to the formation of palmitoyl-ACP (Stumpf, 1989).

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[6] Between numbers 9 and 10 carbon atoms (counting from the carboxyl group end).

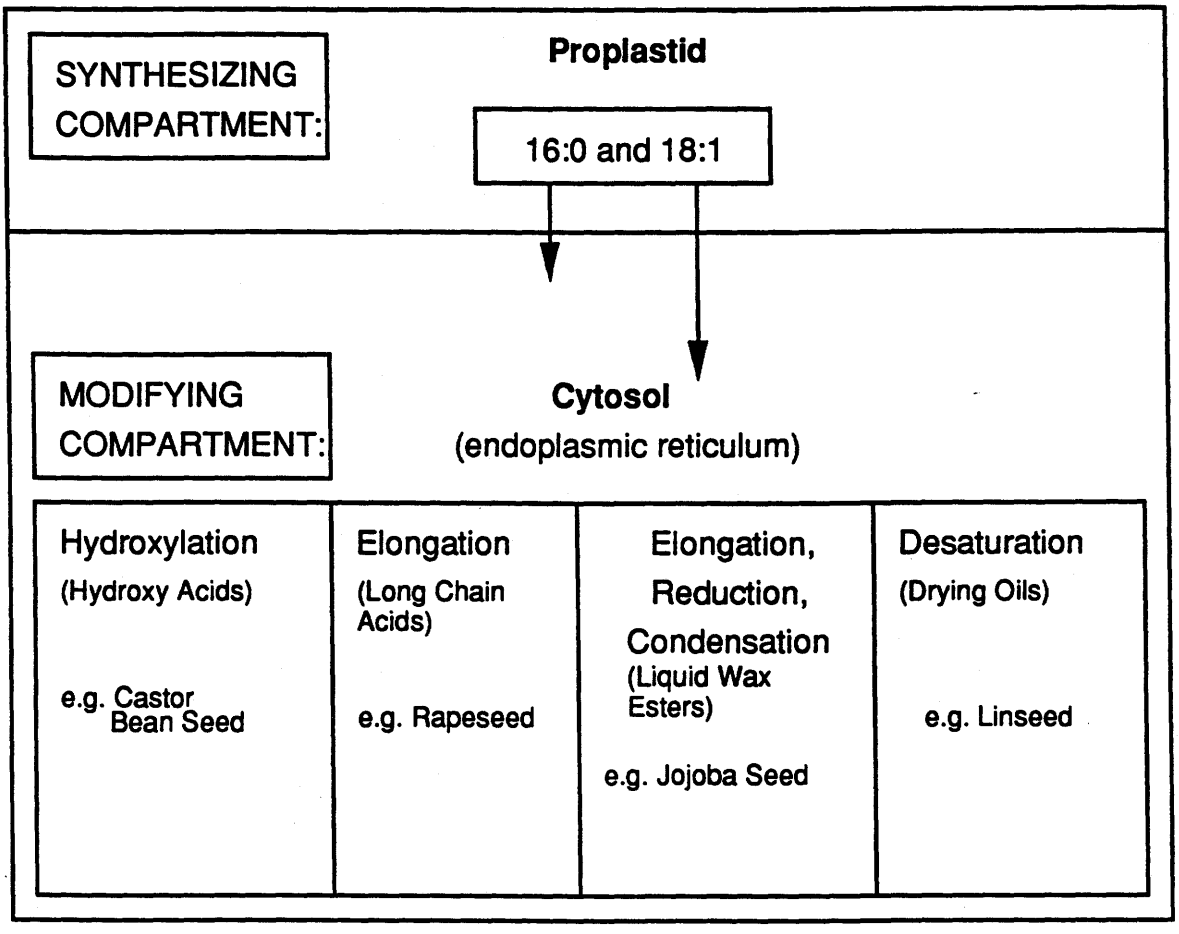

Fig. 2.2. A general scheme of fatty acid synthesis in oilseeds (Stumpf, 1989)

β-ketoacyl-ACP synthetase II on the other hand is highly specific for the conversion (or elongation) of palmitoyl-ACP to stearoyl-ACP. Stearoyl-ACP is then used as a substrate by a very active and efficient stearoyl-ACP desaturase for conversion to oleoyl-ACP (introduction of a double bond at Δ9 position). The final step of the plastidic reactions involves the hydrolysis of oleoyl-ACP by an oleoyl-ACP hydrolase (thioesterase) to yield ACP, which is recycled into the synthesizing system, and free oleic acid, which is readily transported to the cytosol for modification. The high affinity of stearoyl-ACP desaturase for stearoyl-ACP, and favourable kinetics, combined with the conserved nature of its gene sequence (Somerville and Browse, 1991), may be responsible for the consistently low stearic acid content in vegetable oil crops (Table 2.1). Palmitate and stearate can also be released from the plastid by thioesterase hydrolysis of their corresponding ACP-derivatives, which are then converted to acyl-CoAs for synthesis of triacylglycerols in the cytosol.

2.4.3. Biosynthesis of Linoleic and Linolenic Acids

Linoleic and linolenic acids are formed by consecutive desaturations of oleic acid (Green, 1985). The synthesis of these two polyunsaturated fatty acids occurs in the modifying compartment (cytosol). Oleoyl desaturase is responsible for the introduction of the second double bond at the Δ12 position in the conversion of oleic acid to linoleic acid. Linoleoyl desaturase further converts linoleic to linolenic acid, by the insertion of a third double bond at the Δ15 position.

Lack of a consistent pattern has been observed in the formation of these two

polyunsaturated fatty acids. Several workers have indicated that the expressions of activity of oleoyl desaturase and linoleoyl desaturase are related to the type and age of tissue, the temperature at which the tissue was grown, as well as the light regime(s) to which the tissue was exposed (Stumpf, 1989; Green, 1985). Some of the frustrations faced by scientists in the breeding of low linolenic acid genotypes of oilseed crops are often blamed on these environmental influences on fatty acid composition (Downey and McGregor, 1975).

2.4.4 Bioassembly of Triacylglycerols (TAGs)

After synthesis, the various fatty acids are stored in the seed as TAGs, which are naturally important as reserves of energy [TAGs contain twice the energy of carbohydrates or proteins (Downey and McGregor, 1975)] to fuel germination of the seed (Gurr, 1980). These TAGs, which make up about 95% of crude seed oil, are of enormous economic importance (Carr, 1989).

TAGs are esters of glycerol in which each of the three hydroxyl groups is esterified with a fatty acid. In higher plants, C_{16} and C_{18} fatty acyl moieties are incorporated into TAGs via the glycerol-3-phosphate (G-3-P) pathway (sometimes called the Kennedy pathway) (Gurr, 1980; Stymne and Stobart, 1987). It has been recently demonstrated that in *Brassica napus* (Taylor *et al.*, 1992), *Sinapis alba* and *Lunaria annua* (Fehling *et al.*, 1990), TAGs containing very long chain fatty acids (VLCFA) such as eicosenoic and erucic acids, are biosynthesized via the same Kennedy pathway, following their (VLCFA) biosynthesis from 18:1-CoA.

In oilseeds, the acylation of position *sn*-1[7] of glycerol-3-phosphate by acyl transferase I (AT I) (EC 2.3.1.15)(Fig. 2.3) occurs with a predominant selectivity for the saturated fatty acids (16:0 and 18:0). Conversely, the subsequent acylation of *sn*-2 of the lysophosphatidic acid shows strong preference for 18:1, 18:2 and 18:3. The specificity of acyl transferase II (AT II) (EC 2.3.1.51) is so strong that saturated fatty acids are almost completely excluded from the *sn*-2 position (Stymne and Stobart, 1987). The *sn*-3 position is prepared for acylation by hydrolysis of phosphatidic acid which is catalyzed by phosphatidate phosphohydrolase (Fig. 2.3). A diacylglycerol acyltransferase (AT III) (EC 2.3.1.20) then catalyses the acylation of the *sn*-3 position. AT III utilizes the common cytoplasmic pool of acyl-CoAs and exhibits a broad acyl specificity. Collaborative work between the plant breeder and the genetic engineer, utilizing such knowledge, could result in the production of triacylglycerols of definite structure to meet specific market demands; for example, the production of 1-palmitoyl,2-oleoyl,3-stearoyl glycerol in flax or any other oilcrop would yield fats which could serve as a cocoa butter substitute in the confectionery industry (Rattray, 1991; Somerville and Browse, 1991).

2.4.5. Polyunsaturated-rich triacylglycerols

Many seed oils are rich in polyunsaturated C_{18} fatty acids (18:2 and 18:3); e.g., linseed contains about 70% and sunflower contains 72% (Table 2.1). Evidence from

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[7] Stereo-specific numbering system:- Carbon # 1, 2, and 3 on the G-3-P backbone.

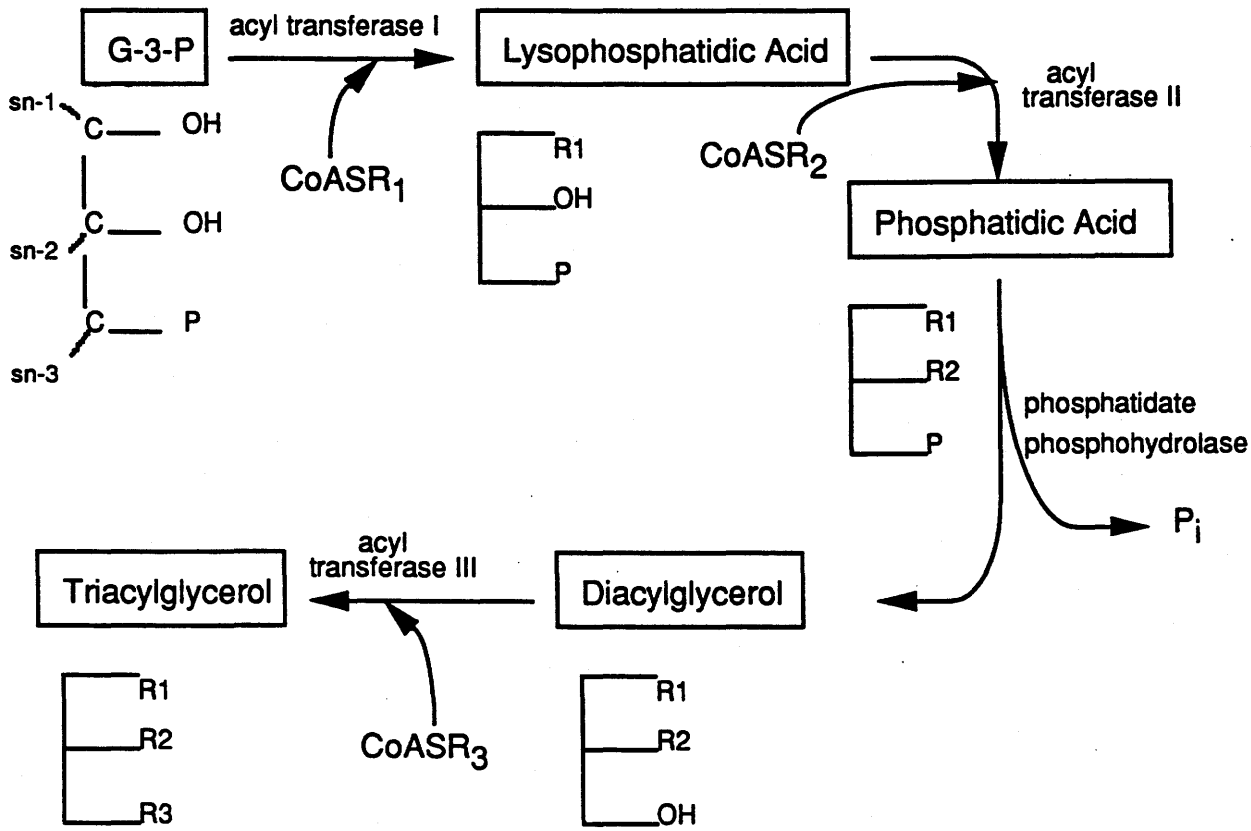


Fig. 2.3 Schematic representation of triacylglycerol biosynthesis

radio-labelled biochemical studies suggest that such oilseeds possess a mechanism which forms part of the triacylglycerol bioassembly pathway, and results in the production of polyunsaturated-rich acyl-CoA pools (Stymne and Stobart, 1985; Slack *et al.*, 1978; Griffiths *et al.*, 1985; Stobart and Stymne, 1985a,b). This mechanism centres around phosphatidylcholine (PC) which is involved in two equilibria reactions (A and B) (Fig. 2.4).

Radio-labelled studies of microsomal preparations of developing linseed (Dybing and Craig, 1970; Stymne and Stobart, 1985; Stymne *et al.*, 1992), safflower (Stobart and Stymne, 1985b; Griffiths *et al.*, 1985) and sunflower (Griffiths *et al.*, 1988) have led to the establishment of the following concepts:

a) Sequential desaturations of oleate (18:1) to linoleate (18:2) and linolenate (18:3) occur primarily at the *sn*-2 position of phosphatidylcholine.

b) PC is involved in two equilibria reactions:- acyl exchange with the acyl-CoA pool, and diacylglycerol:phosphatidylcholine interconversion (reactions A and B, respectively) (Fig. 2.4).

c) During the movement of G-3-P through the Kennedy pathway (Fig. 2.3) diacylglycerol can equilibrate with phosphatidylcholine. This reversible reaction [(B) (Fig. 2.4)] offers a channel through which any oleate present in the diacylglycerol can be returned to the PC pool for further desaturation. The oleate already in position 1 is also desaturated *in situ* (Fig. 2.5).

d) An exchange of acyl groups occurs between the acyl-CoA pool and phosphatidylcholine [reaction A (Fig. 2.4)]. This exchange reaction is responsible for

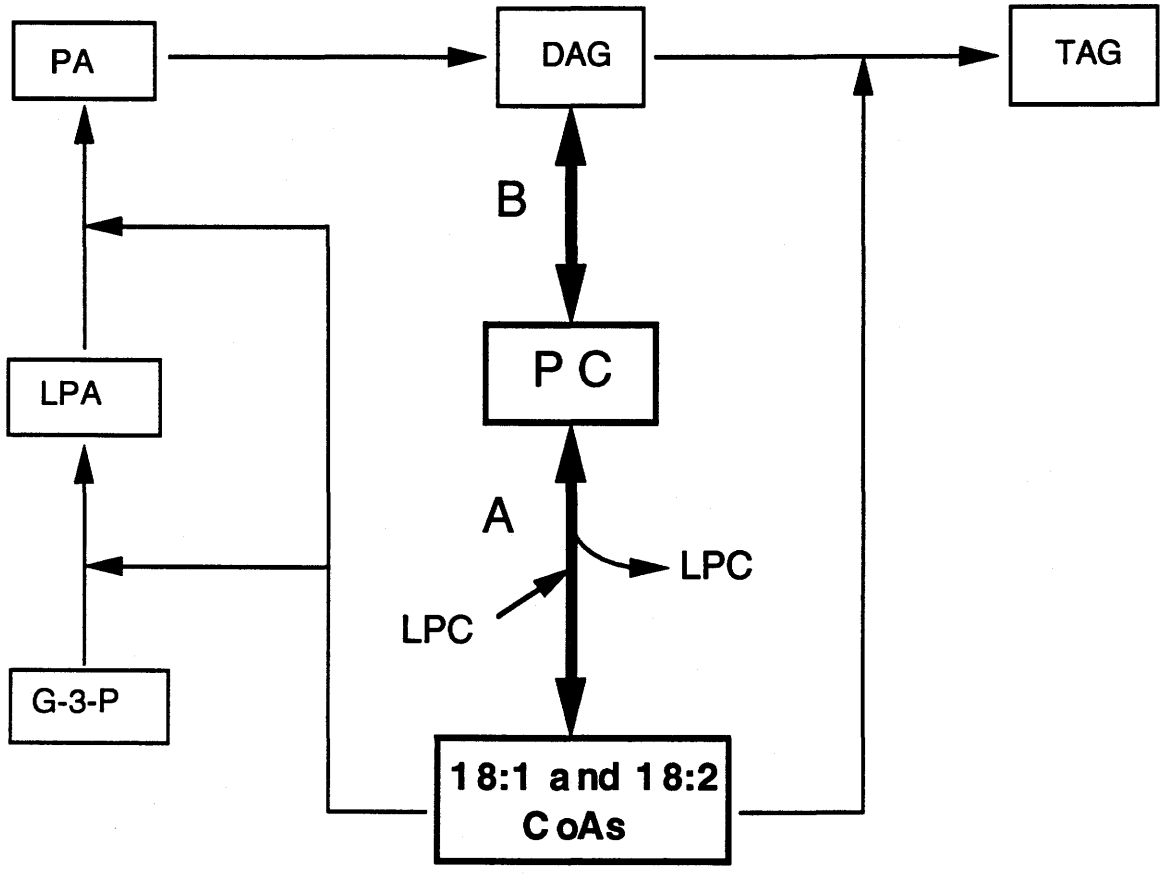
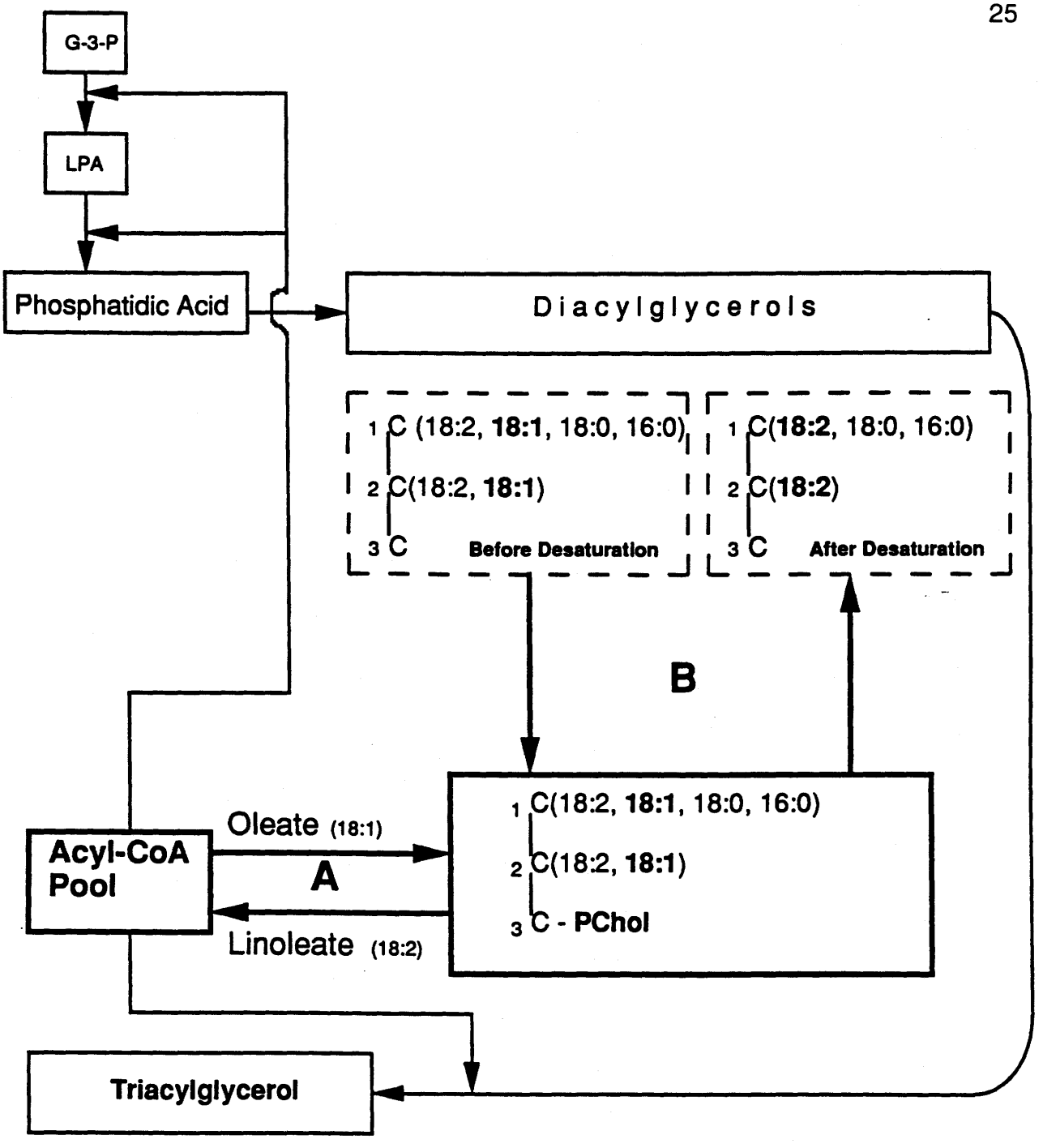


Fig. 2.4 Schematic representation of Phosphatidylcholine involvement in the synthesis of polyunsaturated-rich triacylglycerols (Stymne and Stobart, 1984)



NB **A further desaturation of 18:2 to 18:3 via this mechanism operates in oilseeds rich in 18:3 (e.g. flax).**

Fig. 2.5 Acyl quality regulation of C18-polyunsaturated fatty acid-rich triacylglycerols (Stobart and Stymne, 1985b).

the release of desaturated products from the phospholipid, resulting in the return of linoleate and linolenate to the acyl-CoA pools, enriching the latter with polyunsaturated fatty acids which can then be incorporated by acyltransferases. Stymne and Stobart (1985) demonstrated that the acyl exchange onto PC is relatively more selective for oleoyl-CoA than for other acyl-CoA entities.

e) The enzymes responsible for these two equilibria reactions are:

i) cytidine 5'-diphosphate (CDPcholine):1,2-diacylglycerol cholinephosphotransferase (E.C. 2.7.8.2) for the catalysis of DAG-PC interconversion [reaction B (Fig. 2.5)] and ii) acyl-CoA:lysophosphatidylcholine acyltransferase (E.C.2.3.1.23), catalyzing the acyl exchange reactions [reaction A (Fig. 2.5)].

These two equilibria reactions working in concert are responsible for the continuous enrichment of the glycerol backbone with polyunsaturated fatty acids. Thus, the triacylglycerols which accumulate are enriched in 18:2 and 18:3.

2.4.6. Developing embryos of oilseeds; materials for radio-labelled experiments

Lehberg *et al.* (1939) found that about 80-90% of the total oil was present by the 15th -18th day after flowering in flaxseeds grown at Saskatoon and Brandon (western Canada), while at other locations (also in western Canada) the same process took 30 days. They also found that the desaturation process occurred later in development. This observation agrees with later findings that showed that linoleic and linolenic were the last two unsaturated fatty acids in the fatty acid biosynthesis pathway of linseed (Knowles, 1989). For radio-labelled experiments, developing

oilseed embryos must be sampled at the 'right age' to ensure that the enzymes responsible for fatty acid synthesis are active within the selected maturation period.

Several studies in this area clearly depict that the 'appropriate' sampling age or developmental stage varies from crop to crop, and that it is also affected by the temperature and photoperiod regimes to which the plant is exposed (Table 2.5). In linseed the 'appropriate' sampling age of developing embryos for radio-labelled studies ranges from 17 to 28 days post anthesis (Table 2.5). Stymne *et al.* (1992) sampled both mutant and non-mutant (high- and low-linolenate) linseed lines at the same age for any particular temperature/photoperiod regime (Table 2.5).

It seems that for linseed any sampling age falling between the 17 to 28 days post anthesis range is suitable. However, it may be desirable to determine the 'appropriate' harvesting age for mutants whose characteristic fatty acids are not at the end of the fatty acid biosynthesis pathway (Fig. 2.1).

Table 2.5 Influence of growing conditions (temperature and photoperiod) on days to sampling of developing embryos of various oilseeds for radio-labelled experiments.

Oilseed	Growing conditions*	Days to sampling (d.p.a.)**	Reference
Linseed:	Heated, naturally-lit glasshouse	18-25	Slack *et al.* (1978)
	18/13°C;natural photoperiod, glasshouse	21	Stymne *et al.* (1992)
	24/16°C; "	17	
	28/22°C;16/8hr	21	Stymne & Stobart (1985)
	18/18°C;naturally-lit greenhouse	20-28	Dybing & Craig (1970)
Safflower:	28/22°C;16/8hr	14-18	Stobart & Stymne (1985a,b)
	Heated, naturally-lit glasshouse	18-25	Slack *et al.* (1978)
	30/20°C;16/8hr	14-18	Griffiths *et al.* (1988)
	25/15°C;16/8hr	14-18	Stymne & Stobart (1984)
Soybean:	Heated, naturally-lit glasshouse	25-30	Slack *et al.* (1978)
Sunflower:	30/20°C;16/8hr	14-18	Griffiths *et al.* (1988)
	In the field	18-20	Stymne & Stobart (1984)

* Day/night temperatures; day/night photoperiod.

** Days post anthesis.

3. MATERIALS AND METHODS

3.1. Characteristics of plant materials

The Canadian flax cultivar McGregor (McG), and the following flax mutants developed by Rowland and Bhatty (1990) were used for this project: 1) E67 (high palmitic acid content) 2) E1747 (very low linolenic acid content) 3) E1929 (elevated oleic content) and 4) E1536 (elevated oleic and linoleic levels) (Rowland, personal communication).

3.2 Growing conditions

All seeds used for this project were obtained from plants grown under controlled phytotron conditions. Seeds were sown into Terra-lite Redi-earth[8] (W.R. Grace & Co. of Canada Ltd.) in pots (16 cm diameter) and grown in growth chambers controlled at a day temperature of 20°C for 16h and at a night temperature of 15°C for 8h.

Seeds were first sterilized in 75% ethanol for 2 min. followed by 20 min. in 25% bleach and finally rinsed with distilled water. They were then cut transversely with a razor blade into two parts. The part containing the embryonic axis (approximately two-thirds of the seed) was put in a petri dish on an agar-water medium and kept in

[8] Plant growing media (W.R. Grace & Co. of Canada Ltd.).

a Hotpack Programmed Incubator (Philadelphia, PA., USA) at 18°C for germination. After 6 to 10 days the seedlings were transplanted into the pots of Redi-earth. The other "half seed" was analyzed for fatty acid composition to establish the phenotype of the corresponding "half seed" to be planted. This half-seed technique, first proposed by Downey and Harvey (1963), offers some advantages: 1) the possibility of planting the 'wrong' seed is greatly reduced and 2) inadequate sampling is avoided because the true genetic characteristics of the plants are pre-determined. Although the relative fatty acid concentrations of different parts of the seed can differ, the half-seed analysis technique has been found suitable and representative of the oil quality of the whole seed (Dybing, 1968; Dorrell, 1970; Conte *et al.*, 1989).

Fertilizer was applied 2 weeks after transplanting of the seedlings, at the rate of 250 ml per pot of a solution of 0.06 g $CuSO_4$ and 15 g Rx-15[9] (Garden Research Laboratories, Ontario, Canada) in 6 litres of water. Each pot contained 1 to 5 plants (depending on the number of seedlings for each cross and the total number of available pots). The same fertilizer dosage was repeated just before flowering. Identity seeds and plants was maintained throughout this study.

3.3 Procedure of Crosses (Inheritance studies)

Crosses involving E67, E1747, E1929, E1536 and McGregor were made in all possible combinations to produce F_1 seeds which were then grown to produce segregating F_2 populations, and in some cases backcross populations as well. For

[9] All purpose fertilizer (15(N)-30(P)-15(K), with B, Mo, and chelated Cu, Fe, Mn and Zn).

all crosses, flowers with about half the petals protruding, but not yet opened, were selected and tagged for use as female parents. Immediately after emasculation the stigmas were hand-pollinated, using a flower from a male-designated plant. These crosses were usually done in the morning before pollen shedding occurred. The instruments for emasculation (a pair of scissors and tweezers) were sterilized in 75% ethanol after each cross. Identification (male and female parents and date) of each cross was recorded. At maturity each F_1 boll was harvested separately while the selfed seeds for a particular plant were bulked. To prevent spoilage during storage the harvested bolls were oven-dried at 35°C for 14-16 h, and threshed. Some of the F_1 seeds were used for the production of F_2 seeds. Due to the shortage of some F_1 seeds, backcrosses (in both directions) were done for only E67 x (E67xMcG), E1747 x (E1747xMcG), and E1536 x (E1536xE1747).

3.4 Statistical analysis

Frequency distribution patterns of fatty acids that segregated into more than one phenotypic class in F_2 (420 seeds per family) and backcross (19-70 seeds per family) populations were studied. This offered an objective procedure of classification into various fatty acid phenotypic classes for further analysis. In cases where class limits were not clearly separated, depressions in the histograms were use for their estimation.

Using a one- or two-gene segregation model, chi-squared analyses were computed to test goodness-of-fit of F_2 and backcross data to hypothesized genetic

ratios. Where two phenotypic classes were involved (degrees of freedom = 1), Yates correction for continuity was applied in the chi-squared calculation. The *t*-test for unpaired observations was used to determine significant differences between means of reciprocal F_1 (as well as F_2) populations.

According to Steel and Torrie (1980), by analyzing more than 150 F_2 seeds the probability of making a wrong chi-squared test decision (P_w) for a two-gene model for example, is quite small; e.g., P_w for a 15:1 ratio (among 9:7 and 13:3) is 0.006.

3.5 Biochemical studies

3.5.1. Developmental investigations

Due to lack of seeds of some of the flax mutant lines at the time when this part of the project was conducted, only McGregor and E1929 were involved in the developmental studies. The aim was to determine the suitable 'embryo sampling age' for the radio-labelled studies. Flowers were tagged on the morning of the day on which the petals were fully opened. The date and identity of the plants were carefully recorded. About 20-30 bolls of the same age were sampled (at different times) for each of the following ages: 17, 19, 21, 23, 25, 27 and 29 days post anthesis (d.p.a). The bolls were dissected, the developing seeds removed, kept in vials and stored in a fridge until approximately 100 mg fresh weight was obtained for each age. Fatty acid composition analyses were then performed, as described in section 3.6.3.

3.5.2. Radio-labelled studies

All five lines were used for this study. Flowers were tagged on the first day of anthesis as a means of recording age. Based on results from the developmental studies, developing seeds were harvested in bulk for each line at 21-25 d.p.a., put in a container and placed on ice. The developing seeds were removed from the bolls and used within 30-45 min. after sampling, for the ^{14}C-sodium acetate feeding experiments.

3.6. Analytical procedures

3.6.1. Lipid extraction methods

Several basic methods are used for lipid extraction. Bligh and Dyer (1959) introduced the chloroform-methanol method while the hexane-isopropanol method is credited to Hara and Radin (1978). A more rapid one-step acidic-methanol extraction-methylation method was proposed by Browse *et al.* (1986). Investigations by Pomeroy *et al.* (1991) on the above three methods did not establish any significant differences in the distribution of the major fatty acids obtained by these different methods.

In this study, a modification of the method proposed by Browse *et al.* (1986) was used to analyze the fatty acid composition of parental, F_1, F_2 and backcross matured seeds. In the case of the developmental and radio-labelled studies a modified version of the Bligh and Dyer method described by Holbrook *et al.* (1992) was used.

3.6.2. Fatty acid composition analyses for parental, F_1, F_2 and backcross seeds

Parental and F_1 seeds were subjected to "half-seed" analysis, while analyses on F_2 and backcross seeds were by individual whole seed. In both cases the same protocol was used:

a) The seed (half or whole) was crushed with a pair of tweezers and placed in a 3.5 ml vial.

b) 1 ml 2% sulphuric acid in methanol was added to each vial and covered with a teflon-lined cap.

c) The oil within each vial was methylated by being kept in an oven at 100°C for an hour.

d) After cooling, 1 ml petroleum ether was added to each vial and mixed by vigorous shaking.

e) The petroleum ether layer (top layer) was removed and placed into new correspondingly labelled 2-ml vials for injection into a gas chromatograph (GC) to analyze the proportions of fatty acids in the oil.

The GC instrument used was a Hewlett Packard model 7671A Automatic Sampler/5710A Gas Chromatograph with a 3385A Automatic system integrator. The gas chromatograph was fitted with a 1.8 m column, packed with GP 3%, SP-2310/2%, and SP-2300 on 100/120 Chromosorb W AW (Supelco Inc., Oakville, Ontario, Canada). GC conditions were as follows: oven temperature, 190°C isothermal; flame ionization detector temperature, 250°C; injector port temperature, 250°C. The carrier gas was nitrogen (oxygen free).

3.6.3. Fatty acid composition analyses for developing embryos

About 60 mg of immature seeds (section 3.5.1) was weighed into a test tube for each of the different ages of each flax line. For oil extraction and analysis the following protocol was used:

a) 2 ml of IPA/BHT[10] was added, capped and heated in a boiling water bath for 5 min. (to terminate enzyme activity).

b) After cooling on ice the tissues were homogenized with a Polytron for 1-2 min. A second 2 ml aliquot of IPA/BHT was used to rinse the probe of the Polytron.

c) To extract the lipids, and destroy phospholipases the test tube was capped and heated in boiling water for 10 min.

d) After cooling, 2 ml DCM was added, the solution was then vortexed and centrifuged at 2000 RPM for 5 min (all centrifugation were done at this speed and time) to precipitate the residue. The supernatant, as one phase, was decanted into labelled test tubes for phase separation.

e) Phase separation:- 2 ml DCM was added and gently mixed, then 2 ml 1M KCl in 0.2M H_3PO_4 was also added and mixed. After centrifugation the lower organic (lipid) phase was pipetted into a labelled test tube. The remaining aqueous phase was backwashed twice with 2-ml portions of DCM, vortexed, centrifuged and the lower phase pipetted and combined with the previous lipid phase. 1-2 ml C:B:M

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[10] **Abbreviations:-** IPA/BHT -Isopropyl alcohol/butylated hydroxy toluene (IPA containing 2.25 g/l BHT); RPM -revolutions per minute; DCM -Dichloromethane; KCl -Potassium chloride; H_3PO_4 -Phosphoric acid; C:B:M - Chloroform:Benzene:Methanol; N_2 -Nitrogen; 17:0 FFA -Heptadecanoic (margaric) free fatty acid; MeOH/HCl -Methanolic hydrochloric acid; NaCl -Sodium chloride; Na_2CO_3 -Sodium carbonate; DPM -disintegrations per minute; TLE -Total lipid extract; FAME -Fatty acid methyl ester.

(1:1:1) was added before this combined organic extract was dried down under N_2 on reactitherm at 35-40°C. A portion (85%) of the TLE was methylated to determine FAME composition.

f) Methylation:- To permit quantitative analysis 17:0 FFA (2 µg) was added as an internal standard to each sample. Next, 2 ml of 3N MeOH/HCl followed by 1 ml benzene were added. Samples were capped and reacted in an oven at 80°C for 1 hour. After cooling on ice, 2 ml 0.9% NaCl followed by 2 ml 5% Na_2CO_3 were added. Three 3-ml hexane extractions were performed, each time removing the upper layer. The combined hexane fractions were dried down under N_2 on reactitherm without heat.

g) Samples were then reconstituted in 200 µl DCM for GC analysis on a Hewlett Packard model 5880 gas chromatograph fitted with a DB-23 column (30 m x 0.25 mm, film thickness 0.25 µm; J & W Scientific, Folsom, CA). GC conditions were as follows: injector temperature, 250°C; flame ionization detector temperature, 250°C; running temperature program, 180°C for 1 min, then increasing at 4°C/min to 240°C and held at this temperature for 10 min.

3.6.4 Radio-labelled experiments

About 100-120 mg fresh weight (exact weight was recorded) of immature seeds was used for each of the time course incubation studies. Steps in the experimentation were:

a) Weighed seeds were sliced lengthwise (once) with a razor blade, placed in a test tube (marked A).

b) 1 ml stock solution of 1-^{14}C sodium acetate [NEN-DuPont (Boston), 59 μCi/μmol; 4.86 μCi/ml=10,692,000 DPM; final concentration=72.2 μM] was added and placed in a constantly shaking water bath. All incubations were done at 30°C, 100 RPM and 10,000 lux[11] illumination, leaving the test tubes uncapped. Incubation periods were 30 min, 1h, 2h, 4h and 18h for each line.

c) After each incubation period the labelled acetate solution was quickly removed to a new tube (B). Three 3-ml portions of distilled water were quickly used to rinse off any excess isotope. Vortexing was done each time, before pipetting off the washes into tube B. The combined solution in tube B (approx. 10 ml) was saved for further analysis (See section 3.6.4.1).

d) To the ^{14}C-labelled seeds remaining in tube A, 2 ml IPA/BHT was added. The tube was capped and placed in a boiling water bath for 5 min.

e) After a brief cooling on ice, samples were homogenized with a Polytron. 1 ml distilled water was added and 2 x 1 ml IPA/BHT was used to rinse the probe of the Polytron and combined with the homogenate in tube A. The probe was carefully cleaned after each grinding (using IPA).

f) 2 ml DCM was added to tube A which was then capped and vortexed.

g) Phase separation protocol as previously described was then followed (section 3.6.3.e). The organic phase was pipetted into a new tube (C).

h) The total incorporation/recovery of ^{14}C was estimated at this point, as described further in section 3.6.4.1.

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[11] 1 lux = 0.0195 μmole m^{-2} s^{-1} = 0.093 foot candles.

i) The ^{14}C total lipid extract (TLE) in tube C was then dried under N_2 at 35°C.

j) A portion of the the ^{14}C TLE (95%) was methylated (TMe) (section 3.6.3.f).

k) After methylation the ^{14}C TMe sample was reconstituted in 1 ml hexane and 50 µl used for analysis on a GC which was vented to the atmosphere.

l) 1% of the remainder (about 10 µl) was assayed for radioactivity on a scintillation counter (see section 3.6.4.1). This was necessary to ascertain the volume of acetonitrile (AcCN) solvent needed for reconstitution of the ^{14}C TMe for high performance liquid chromatography (HPLC) analysis.

m) The remaining ^{14}C TMe was dried down under N_2 without heat to remove any residual hexane. The residue was dissolved in AcCN such that the volume contained 500,000 DPM/ml, based on the ^{14}C TMe count above. The exact volume of AcCN used for each sample was recorded.

n) 100 µl aliquot was then injected onto the HPLC. The HPLC system consisted of an SP8700 liquid chromatograph (Spectra Physics, San Jose, CA) furnished with an ACS model 750/14 Evaporative Analyzer (Applied Chromatography Systems Ltd., Macclesfield, Cheshire, UK) and a Flo-One/Beta radioactive flow detector and data processing program (Radiomatic Instruments and Chemical Co., Tampa, FL). The HPLC columns consisted of a Whatman RP guard cartridge followed by two Whatman PartiSphere C_{18} 5 µm reverse-phase cartridges (4.6 mm id. x 12.5 cm) (Whatman Inc., Clifton, NJ) in series. The HPLC flow rate was 1 ml/min, and the elution profile/solvents are depicted in Table 3.1.

Table 3.1 Elution profile and solvents used for HPLC analysis.

Time (min)	Acetone (%)	Acetonitrile (%)	Flow rate (ml/min.)
0.0	0	100	1
5.0	0	100	1
17.5	25	75	1
20.0	50	50	1
25.0	100	0	1
30.0	100	0	1
35.0	0	100	1
45.0	0	100	1

3.6.4.1. Estimation of total ^{14}C incorporation/recovery

A 1% portion each of the volumes of the aqueous phase (tube A), recovery (tube B) and TLE (tube C) were assayed for radioactivity. A 4-ml portion of Aquasol-2 scintillation cocktail (NEN-DuPont) was added to the 1% aliquot (aliquot of tube C was first dried down under N_2 at 35°C), vortexed and counted on program A1 on an LKB Liquid Scintillation Counter. Blanks consisting of dried down DCM (same quantity as the 1%) and 4 ml of Aquasol 1-2 scintillation cocktail were used to correct

for any background (quenching/chemiluminescence) effects.

3.7. Target enzyme assays on a high palmitic acid line

Phenotypic inconsistencies from the findings of the 1-^{14}C-sodium acetate experiments prompted these target enzyme pilot assays. Due to lack of growth-chamber plant materials, and the availability of ample field-grown seeds, field-grown McGregor flax (wild type) and a field-grown line which was phenotypically high in palmitic acid and low in linolenic acid (E67/E1747 - F_7 homozygous line) were used for these studies. Immature flax bolls were harvested at 18-25 d.p.a. from Kernen Farm, University of Saskatchewan, Saskatoon in August 1992, then frozen in liquid nitrogen and stored at -80°C (four months in advance), until they were needed for this experiment. The fatty acid profile phenotypes of the two flax lines were determined from gas chromatography analysis of fatty acid methyl esters (FAME) by direct transmethylation of embryo samples (approximately 1 g fresh weight).

The roles of thioesterase enzymes in cleaving the -ACP and -CoA components from carrier moieties to yield free fatty acids, as well as the activity of β-ketoacyl-ACP synthetase II, which uses palmitoyl-ACP as its substrate, have been previously discussed (section 2.4.2). Since differences in activity of these enzymes could affect the palmitic acid content in a flax line, these enzymes were targeted for this experiment. The -ACP and -CoA substrate were used to test plastidic and extraplastidic thioesterase activities, respectively. The ^{14}C 16:0-ACP and ^{14}C 18:1-ACP substrates were synthesized and supplied by Dr. J. Ohlrogge (Michigan State

University), and were prepared using the method of Ohlrogge *et al.* (1978), whereas the ^{14}C 16:0-CoA and ^{14}C 18:1-CoA were synthesized from their corresponding ^{14}C FFA, using the method of Taylor *et al.* (1990). Time did not permit similar target assays of other mutant lines; for example linoleoyl-PC desaturation (Δ15 desaturase) assays of McGregor versus E1747 (the low linolenic acid mutant), as reported by Stymne *et al* (1992) on the Australian flax cultivar Glenelg and two linolenic acid-deficient mutants derived from Glenelg.

The two assays conducted were:

i) Thioesterase assays:- The substrates used for this test were ^{14}C 16:0-ACP, ^{14}C 16:0-CoA, ^{14}C 18:1-ACP, and ^{14}C 18:1-CoA.

ii) β-ketoacyl-ACP Synthetase II (β-KAS II):- The substrate used for this test was ^{14}C 16:0-ACP in the presence of malonyl-CoA and reductants [the method was modified from Jaworski *et al.* (1974)].

3.7.1 Preparation of homogenate

a) The translucent waxy envelope surrounding the flax embryos was removed (with a razor blade) to prevent gummy extracts. Homogenates were prepared by grinding embryos (1.0 g embryo fresh weight) in a mortar and pestle in the presence of grinding medium [100 mM Tricine-NaOH, pH 8.1, containing 1 mM thiothreitol (DTT)] (5 ml/g fresh weight) and a little acid-washed silica. Homogenates were filtered through a Miracloth and the final supernatant volume recorded. All operations were carried out on ice.

3.7.2 Protein determination

To justify subsequent comparison between the two flax lines, the amount of protein in homogenate fractions was estimated by the Bio-Rad protein assay (Bradford, 1976), using 5 µl aliquot of the supernatant, 95 µl of grinding medium and 5 ml dye reagent/sample. 100 µl grinding medium was used as the control sample. The optical density at 595 nm was determined on a spectrophotometer. The protein content for each sample was calculated from a standard curve for bovine serum albumin, using the following equation: Protein (µg) per 5 µl = $O.D._{595}/0.00746$. Protein concentrations were adjusted so that equal quantities of total protein were used in each assay.

3.7.3 Thioesterase assays

To 50 µl of McGregor or E67/E1747 protein fraction (or 50 µl grinding medium as control), the following radiolabelled substrates were added to start the reactions: 5 µl 16:0-ACP (56 nCi/nmol; final concentration=1.5 µM) or 5 µl 16:0-CoA (9.8 nCi/nmol; final concentration=18 µM) or 5 µl 18:1-ACP (56 nCi/nmol; final concentration=1.5 µM) or 5 µl 18:1-CoA (12.1 nCi/nmol; final concentration=18 µM), at 1 min intervals. Each was incubated at 30°C and 100 RPM in a shaking water bath for 15 min. Reactions were terminated by adding 1 ml 0.2 M H_3PO_4. 5 ml heptane:IPA (3:2) was added, vortexed and centrifuged to cause phase separation. The upper layer (the organic phase - about 3 ml) which contained the hydrolysed FFA was pipetted into a scintillation vial and dried under nitrogen at 35°C for 1 h to remove organic solvents. 4 ml of scintillation cocktail (Aquasol-2, NEN-DuPont) was

then added, the solution was mixed and counted on an LKB Liquid Scintillation Counter using a quench correction program for ^{14}C. The assays were performed in duplicate.

3.7.4 β-KAS II assay

20 µl 16:0-ACP (56 nCi/nmol=123,200 DPM/nmol; final concentration=325 nM) was added to 250 µl McGregor or E67/E1747 protein fraction (or 250 µl grinding medium as control), in the presence of malonyl-CoA (final concentration=100 µM) and NADH + NADPH[12] (reductants) (final concentration=500 µM) in tricine buffer (pH=8.1; final concentration=170 mM), at 1 min intervals. Conditions for incubation were: 30°C, 100 RPM for 30 min. Reactions were stopped by the addition of 3 ml 10% KOH in MeOH and 1 ml IPA/BHT, and then heated at 80°C for 1.5 h to saponify the lipids. After cooling the reaction mixtures on ice for 10 min, 2 ml 6N HCl was added, followed by 3 x 3 ml hexane extractions. The combined extracts, which contained the FFA, were dried down under N_2 at 35°C, transmethylated (as per protocol in section 3.6.3f, without the addition of an internal standard). Samples were dissolved in 60 µl acetonitrile to separate radiolabelled methyl esters of ^{14}C-16:0 (precursor) from ^{14}C-18:0 (product) on radio-HPLC (as per section 3.6.4.n). The assays were done in triplicate.

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[12] NADH, NADPH - reduced forms of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, respectively. These are enzyme co-factors.

3.8. Quantitative determination of oil content (by ^{1}H-NMR)

To determine the oil content of each line, quantitative (^{1}H-NMR)[13] analyses were carried out on intact seeds using a Bruker AM wide-bore spectrometer operating at 360 MHz. To reduce anisotropic line broadening, the seeds were rotated at 1KHz in a Zirconium rotor oriented at 54.7° to the magnetic field (magnetic angle spinning, MAS). This NMR technique is a non-destructive method which provides a good distinction between the liquid and solid parts of the seeds, such that intensities of ^{1}H signals quickly determine the oil content (Alexander *et al.*, 1967).

[13] Proton Nuclear Magnetic Resonance.

4. RESULTS

4.1 Fatty acid composition of parent materials

Table 4.1 describes the fatty acid composition of seed lipids in parent materials (phytotron-grown). Linolenic acid constituted approximately 50% of the total fatty acids in McGregor seed oil. E67 not only had a high palmitic acid content (about 3 times that of McGregor), but also palmitoleic acid which was found in only trace amounts in McGregor and the other mutants. E67 also had a relatively high linolenic acid content (42%), and also reduced stearic and linoleic acid contents. E1747 contained only 2% linolenic acid and 65% of the immediate precursor, linoleic acid. E1929 had a high oleic acid content which was accompanied by a lowered content of linolenic acid (16%) (relative to McGregor), while the linoleic acid content was similar to that of McGregor. E1536 on the other hand, showed elevated levels of both oleic (41%, as opposed to 18% in McGregor) and linoleic (25%, as opposed to 15% in McGregor) acids as well as a lowered linolenic acid content (10%) (Table 4.1).

4.2 Fatty acid profiles of F_1 seeds

In some cases significant differences were observed in the fatty acid profiles

of the F_1 seeds produced from respective reciprocal crosses (Table 4.2), whereas in others the difference was either non-significant or minor when the ranges of fatty acid levels were considered (Appendix A).

Table 4.1 Fatty acid composition of phytotron-grown McGregor flax and EMS-derived McGregor mutant lines (E67, E1747, E1929 and E1536) used in the present study.

Flax line	Fatty acid (%)					
	16:0‡	16:1	18:0	18:1	18:2	18:3
McGregor	9.4† ± 0.3	-	5.1 ± 0.1	18.4 ± 0.7	14.6 ± 0.4	**49.5**Φ ± 1.1
E67	**27.8** ± 0.7	**4.8** ±0.3	**1.8** ± 0.1	17.5 ± 0.7	**6.0** ± 0.2	42.0 ± 1.3
E1747	9.5 ± 0.4	-	4.6 ± 0.1	15.6 ± 0.8	**65.3** ± 0.9	**2.1** ± 0.1
E1929	9.5 ± 0.3	-	3.4 ± 0.1	**51.7** ± 1.2	16.3 ± 0.6	**16.2** ± 0.5
E1536	13.2 ± 0.6	-	6.2 ± 0.3	**41.3** ± 2.0	**25.1** ± 1.2	**9.8** ±0.9

† Mean and standard error of 15 half-seed analyses.

‡ Fatty acid symbols: 16:0=palmitic, 16:1=palmitoleic (typically associated with high palmitic acid lines; occurs only in trace amounts in the other lines), 18:0=stearic, 18:1=oleic, 18:2=linoleic, 18:3=linolenic.

Φ Bold figures are the characteristic fatty acid(s) of the particular flax line (relative to McGregor, the wild type).

Table 4.2 Fatty acid composition of phytotron-grown F_1 reciprocal crosses between McGregor flax and EMS-derived McGregor mutant lines (E67, E1747, E1929 and E1536).

Cross (No. of seeds)	Fatty acid (%)				
	16:0‡	18:0	18:1	18:2	18:3
McGxE67(8)	14.2[a**]	2.6[a]	14.0[a]	12.2[a]	55.8[a]
E67xMcG(13)	15.3[b]	3.5[b]	13.6[a]	10.6[b]	55.3[a]
McGxE1747(10)	10.0[c]	5.5[c]	15.3[c]	27.3[c]	38.4[c]
E1747xMcG(12)	11.3[c]	5.0[c]	17.0[c]	30.9[d]	34.3[c]
McGxE1929(4)	13.0[e]	6.4[e]	18.1[e]	19.5[e]	40.6[e]
E1929xMcG(4)	7.4[e]	4.8[f]	21.9[e]	20.6[e]	45.4[e]
McGxE1536(7)	10.2[g]	5.4[g]	16.9[g]	14.6[g]	50.3[g]
E1536xMcG(8)	9.4[g]	5.0[g]	21.1[h]	15.1[g]	47.3[h]
E67xE1747(7)	14.7[i]	3.0[i]	12.4[i]	21.2[i]	47.3[i]
E1747xE67(7)	15.3[i]	2.9[i]	11.5[j]	21.1[i]	47.5[i]
E67xE1929(1*)	16.7	4.0	14.4	14.6	48.4
E1929xE67(2)	13.4	4.1	14.7	18.0	48.5
E67xE1536(8)	15.1[k]	2.8[k]	12.5[k]	10.0[k]	57.8[k]
E1536xE67(6)	19.1[l]	4.1[l]	21.2[l]	22.8[l]	31.2[l]
E1747xE1929(6)	8.6[m]	4.7[m]	14.6[m]	47.7[m]	22.2[m]
E1929xE1747(8)	7.4[n]	3.0[n]	25.1[n]	44.9[m]	18.2[n]
E1747xE1536(10)	9.8[p]	4.4[p]	16.6[p]	31.8[p]	34.9[p]
E1536xE1747(7)	8.5[p]	3.5[q]	20.7[q]	29.6[q]	36.2[p]
E1929xE1536(6)	9.1	3.5	27.8	20.4	37.3
E1536xE1929(1*)	12.6	3.5	23.1	44.3	16.3

* Not enough data to allow for computation of *t*-test.

** Mean values of reciprocal crosses in the same column having a common superscript (*t*-test) are not significantly different at the 5% level.

‡ See footnote of Table 4.1.

4.3 F_2 populations of crosses involving McGregor and each of the mutant lines

Since all flax mutants used in this study were obtained from chemical mutagenesis of McGregor, F_2 seeds of crosses involving McGregor and each of the mutants should reveal the major difference(s) between McGregor and each of the mutants. The fatty acid segregation pattern(s) of each cross should, therefore, give some indication as to which gene(s) mutated.

4.3.1 E67

4.3.1.1 Palmitic acid

Trimodal distribution patterns were obtained when the palmitic acid data of the reciprocal F_2 populations (involving McGregor and E67) were individually plotted (due to non-homogeneity of chi-squared values for the two populations) in frequency histograms (Fig. 4.1 a,b). Since the observed numbers for the three classes suggested a 1:2:1 [(low:intermediate:high), respectively] monohybrid model, the data were tested for goodness-of-fit to this genetic ratio. Both populations gave a satisfactory fit to this 1:2:1 ratio (Table 4.3).

4.3.1.2 Palmitoleic acid

A close association between levels of palmitic and palmitoleic acids was clearly depicted by scatter graphs (Fig. 4.2). The three observed phenotypic groups were: i) low in both palmitic and palmitoleic acids ii) intermediate in both fatty acids and iii) high in both fatty acids. The correlation between levels of palmitic and palmitoleic

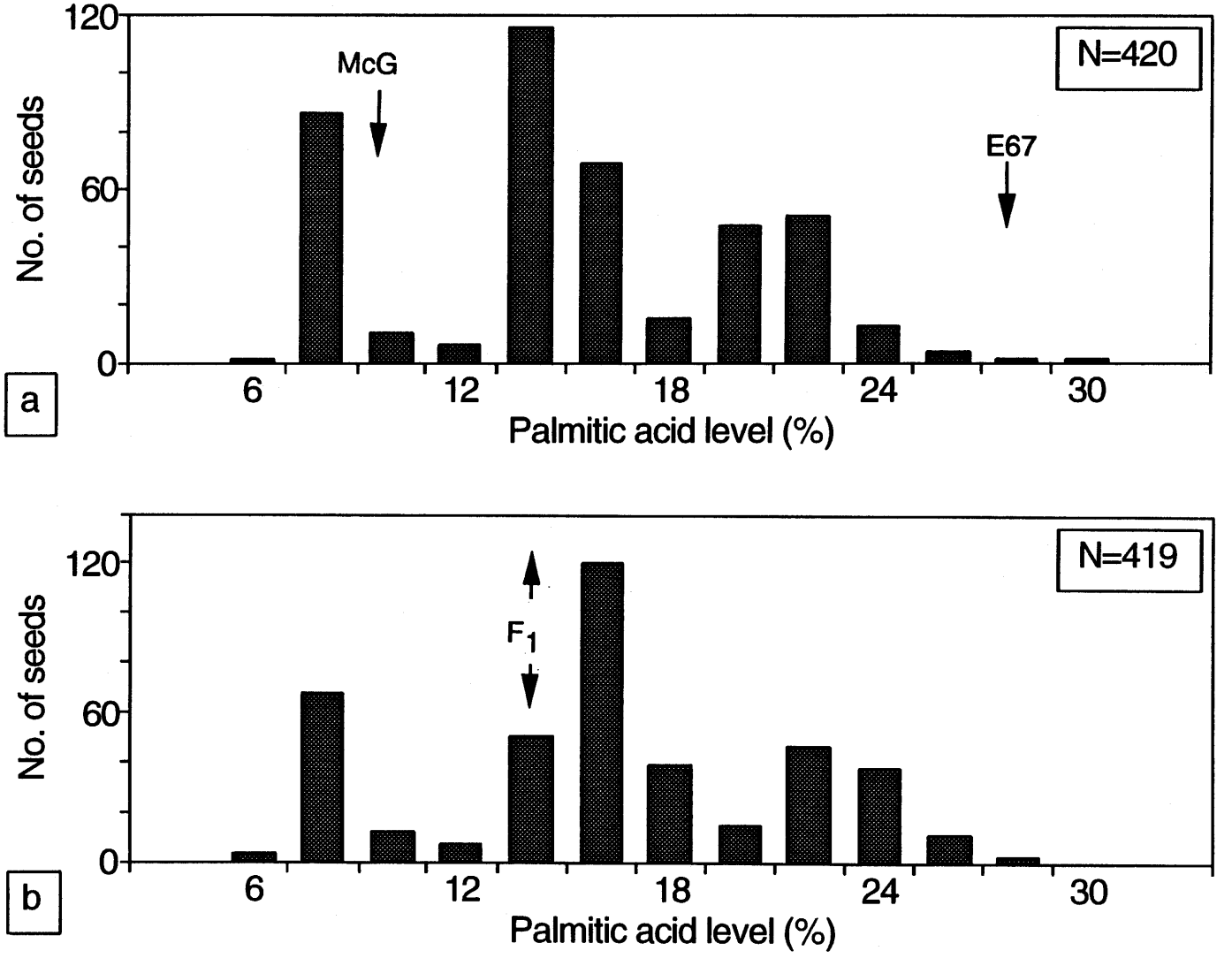

Fig. 4.1 Frequency distribution of palmitic acid in F_2 flaxseeds of a) McGregor x E67 and b) E67 x McGregor.

Table 4.3 Chi-squared (χ^2) analyses of palmitic acid levels of individual F_2 flaxseeds of crosses between McGregor and E67 (EMS-derived mutant line).

Cross (No. of seeds)	Palmitic acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
McG x E67	<11	97	105	**(1:2:1)**	
	11-19	206	210		
(420)	>19	117	105	2.06	**0.36**
E67 x McG	<11	83	104.75		
	11-19	217	209.5		
(419)	>19	119	104.75	4.78	**0.09**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

acids was r=0.94 for McGregor x E67 and r=0.95 for E67 x McG. Chi-squared values for a 1:2:1 ratio in the two populations were non-homogeneous, and therefore they were analyzed separately. Observed numbers in McGregor x E67 gave a good fit to a 1:2:1 ratio but not E67 x McGregor (Table 4.4). This appears to lend support to the implication of maternal influence in the fatty acid control of the mutated gene in E67.

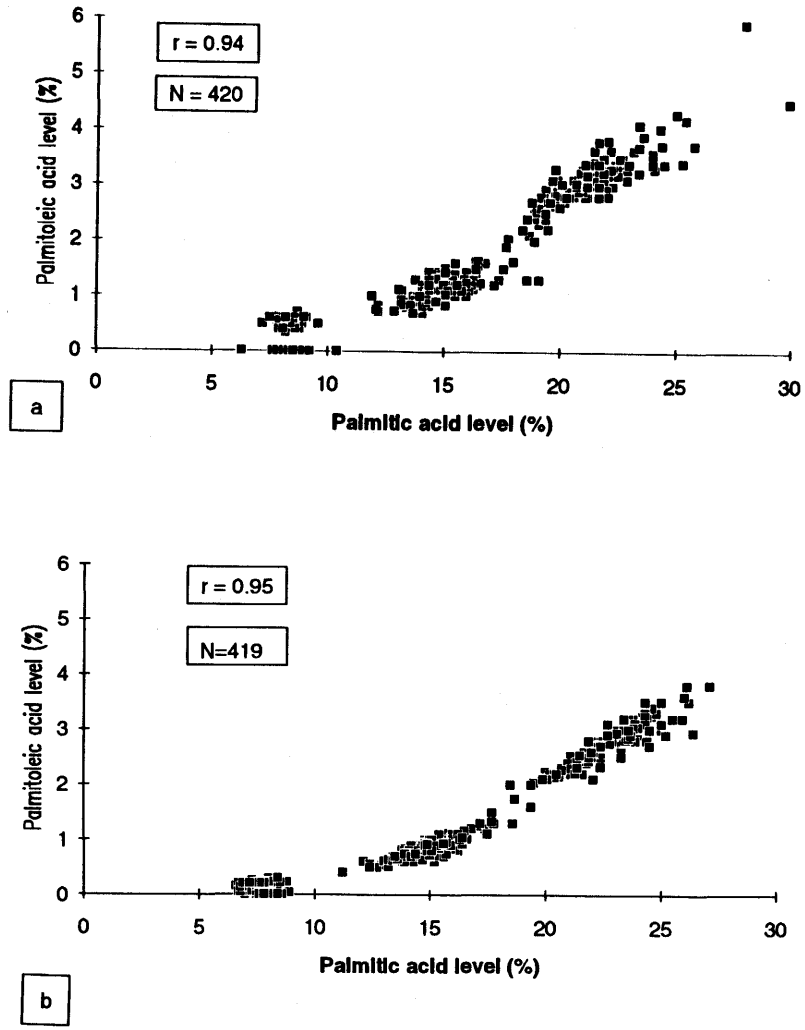

Fig. 4.2 Correlation between palmitic and palmitoleic acid levels in oil from F_2 flaxseeds of a) McGregor x E67 and b) E67 x McGregor.

Table 4.4 Chi-squared (χ^2) analyses of palmitic and palmitoleic acid levels of individual F_2 flaxseeds of crosses between McGregor and E67 (EMS-derived mutant line).

Cross (No. of seeds)	Fatty acid class (%)***	Observed no.	Expected no.	(T.R.)* χ^2	P**
McG x E67	<11 (<0.4)	97	105	**(1:2:1)**	
(420)	11.5-18 (0.4-1.7)	197	210		
	>18 (>1.7)	126	105	5.61	**0.06**
E67 x McG	<11 (<0.4)	82	104.75		
(419)	11.5-18 (0.4-1.7)	211	209.50		
	>18 (>1.7)	126	104.75	7.9	**0.02**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at P<0.05.

*** - Palmitic (palmitoleic) acid levels.

4.3.2 E1747

The chi-squared values for the F_2 reciprocal populations involving McGregor and E1747 were homogeneous for linolenic acid content [heterogeneity chi-squared ($H_{\chi 2}$)=0.49 and P=0.48] (Table 4.5), and therefore the two populations were combined for analysis. The combined linolenic acid data showed a bimodal frequency distribution pattern (Fig. 4.3a). Based on the observed numbers the data were tested for goodness-of-fit to a 1:15 ratio [low (<7.5%):high (>12.5%), respectively]. The chi-squared analysis indicated a good fit to the 1:15 ratio (Table 4.5). The combined linoleic acid data (Fig. 4.3 b) gave a "mirror image " segregation pattern and test ratio to that of linolenic acid (Fig. 4.3 a and Table 4.5). All other fatty acids produced unimodal segregation patterns (only one phenotypic class) (Appendix B1).

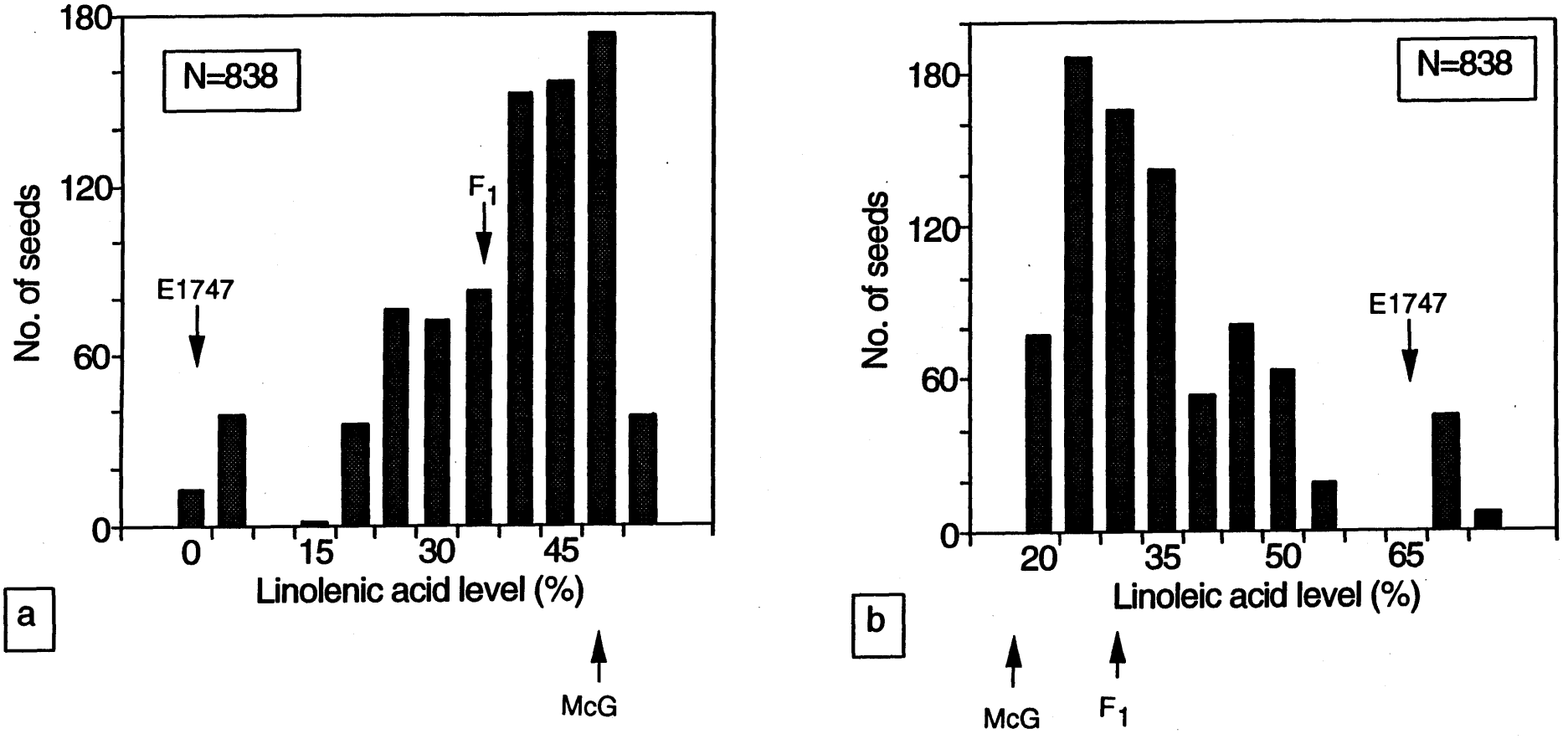

Fig. 4.3 Frequency distribution of a) linolenic and b) linoleic acids in oil of F_2 flaxseeds of combined reciprocal crosses involving McGregor and E1747.

Table 4.5 Chi-squared (χ^2) analyses of linolenic and linoleic acid levels of individual F_2 flaxseeds of crosses between McGregor and E1747 (EMS-derived mutant line).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Linolenic				
McG x E1747	< 7.5	29	26.1875	**(1:15)**	
(419)	>12.5	390	392.8125	0.20	**0.64**
E1747 x McG	< 7.5	23	26.1875		
(419)	>12.5	396	392.8125	0.29	**0.59**
Pooled data	< 7.5	52	52.375		
(838)	>12.5	786	785.625	0.0003	**0.99**
Heterogeneity				0.49	**0.48**
	Linoleic				
McG x E1747	<17.5	390	392.8125	**(15:1)**	
(419)	> 7.5	29	26.1875	0.20	**0.64**
E1747 x McG	<12.5	396	392.8125		
(419)	> 7.5	23	26.1875	0.29	**0.59**
Pooled data	<15	786	785.625		
(838)	> 5	52	52.375	0.0003	**0.99**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

4.3.3 E1929

In each of the separate reciprocal sets of data involving McGregor and E1929, only linoleic acid produced a segregation pattern that yielded more than one distinct phenotypic class. The two F_2 reciprocal chi-squared values were homogeneous for

linoleic acid content ($H_{\chi 2}$=3.31 and P=0.07) (Table 4.6) and therefore the reciprocal populations were combined. From the frequency distribution pattern of the combined data, three phenotypic groups were classified (Fig. 4.4a), and a chi-squared test to a 1:2:1 ratio indicated a good fit (Table 4.6). Linolenic acid produced a unimodal segregation pattern (Appendix B2a). The oleic acid desaturation ratio (ODR)[14] and the linoleic acid desaturation ratio (LDR) were also analyzed (separately for the two reciprocal populations, because they were non-homogeneous for LDR). Segregation for ODR was unimodal (Appendix B2 b,c), whereas that of LDR was trimodal (Fig. 4.4 b,c), with observed numbers that indiicated a good fit to a 1:2:1 ratio (Table 4.6).

4.3.4 E1536

All fatty acids in F_2 populations of crosses involving McGregor and E1536 produced unimodal segregation patterns (Fig. 4.5 a-e), with F_2 individuals (and F_1) close to McGregor, and the distribution tailing toward E1536. For palmitic, stearic oleic and linoleic acids, transgressive segregants possessing lower levels than McGregor were obtained, whereas for linolenic acid over 80% of the population possessed levels which were greater than the parent McGregor. The transgressive segregation patterns suggest that the parents differ in alleles, at least at one locus

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[14] $\text{ODR} = \frac{\text{linoleic acid (\%)} + \text{linolenic acid (\%)}}{\text{oleic acid (\%)}}$

$\text{LDR} = \frac{\text{linolenic acid (\%)}}{\text{linoleic acid (\%)}}$

These are alternative methods of determining the composition of unsaturated fatty acids (Green, 1985). The magnitudes of these ratios are directly proportional to the activities of Δ12 and Δ15 desaturase enzymes, respectively.

for fatty acid control.

Table 4.6 Chi-squared (χ^2) analyses of linoleic acid level and linolenic desaturation ratio (LDR) of individual F_2 flaxseeds of crosses between McGregor and E1929 (EMS-derived mutant line).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Linoleic				
McG x E1929	<21	97	105	**(1:2:1)**	
(420)	21-31	215	210		
	>31	108	105	0.81	**0.67**
E1929 x McG	<21	118	105		
	21-31	194	210		
(420)	>31	108	105	2.91	**0.23**
Pooled data	<21	203	210		
(840)	21-31	421	420		
	>31	216	210	0.41	**0.82**
Heterogeneity				3.31	**0.07**
	LDR				
McG x E1929	<1.3	108	105	**(1:2:1)**	
(420)	1.3-2.5	224	210		
	>2.5	88	105	3.77	**0.15**
E1929 x McG	<1.3	109	105		
	1.3-2.5	225	210		
(420)	>2.5	86	105	4.66	**0.10**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

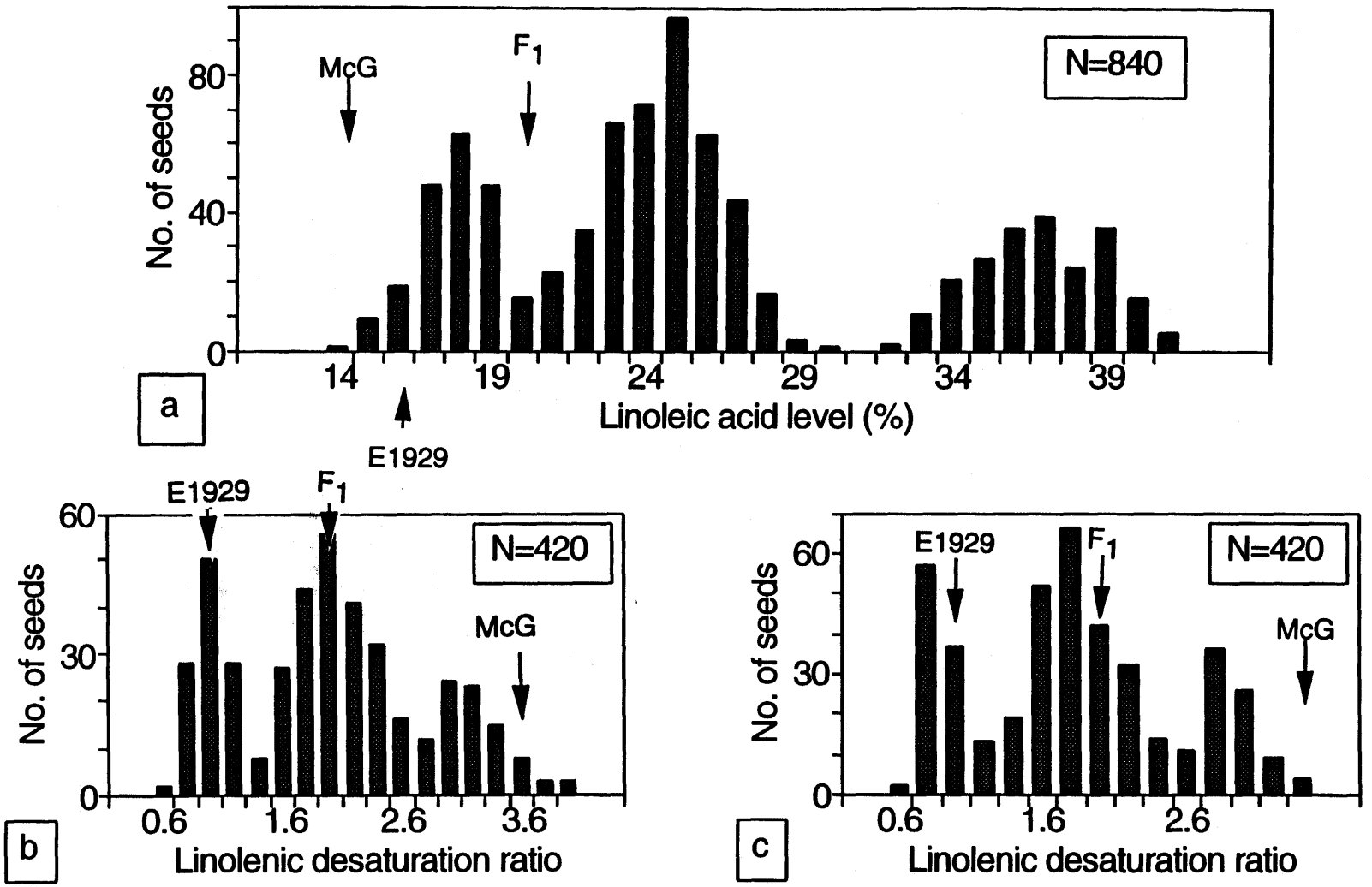
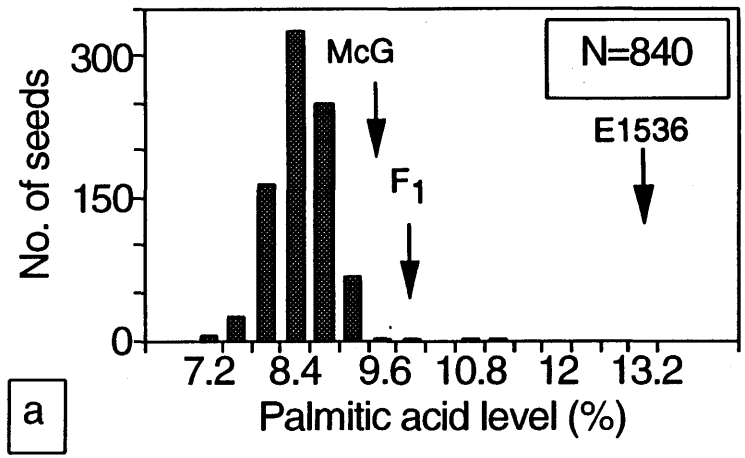
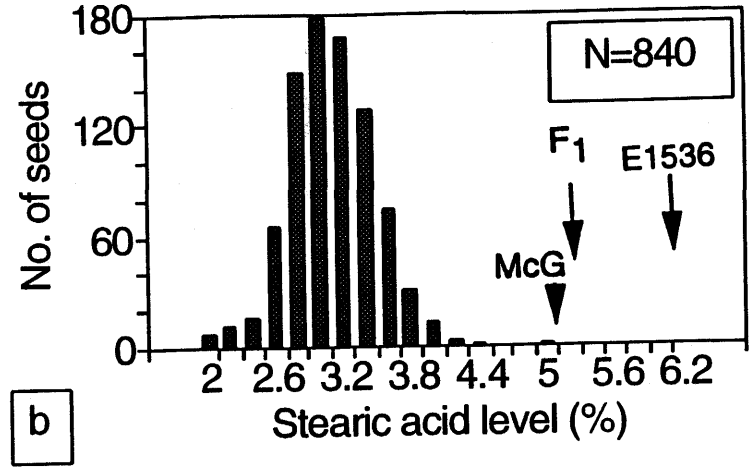
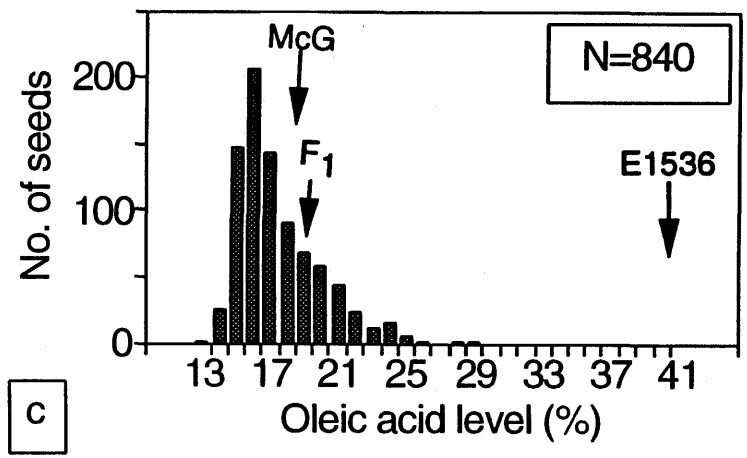
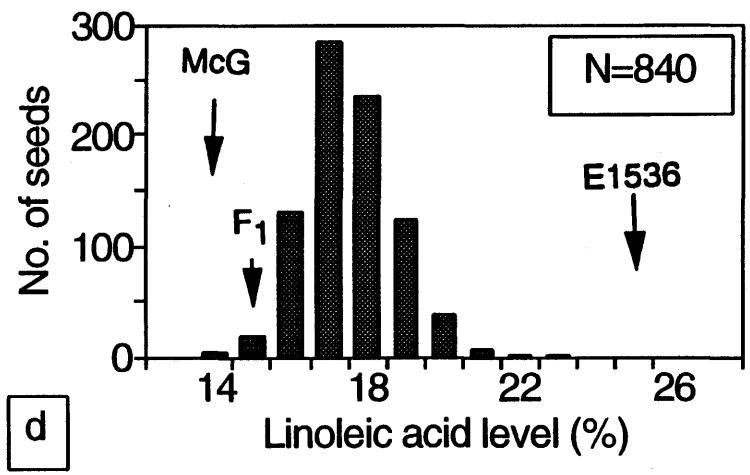


Fig. 4.4 Frequency distribution of
a) linoleic acid in combined reciprocal crosses involving McGregor and E1929,
b) linolenic desaturation ratio of McGregor x E1929 and
c) linolenic desaturation ratio of E1929 x McGregor
in oil of F_2 flaxseeds.









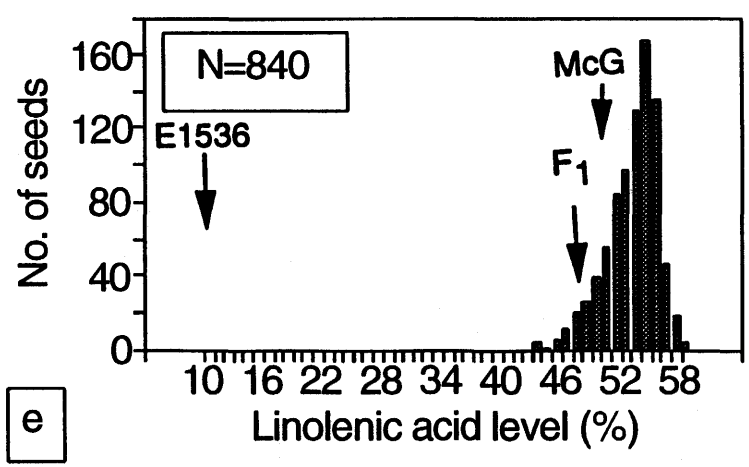


Fig. 4.5 Frequency distribution of a) palmitic, b) stearic, c) oleic, d) linoleic and e) linolenic acids in oil of F_2 flaxseeds of combined reciprocal crosses involving McGregor and E1536.

4.3.5 Mean fatty acid composition of reciprocal F_2 populations of crosses involving McGregor and a mutant line

Among the distinctly segregating fatty acids, no major significant reciprocal F_2 differences were shown by the high palmitic (E67) and the low linolenic (E1747) acid lines (Table 4.7). A small difference was observed for linoleic acid in the reciprocal crosses involving E1929 and McGregor. The F_1 reciprocal difference for palmitic acid for the crosses involving E67 and McGregor (Table 4.2), may therefore be attributable to true maternal effect (no abnormal fatty acid profile was observed in the F_1 seed lot).

Table 4.7 Characteristic fatty acid composition of oil in flaxseed (whole-seed analysis) of reciprocal F_2 populations involving McGregor and EMS-derived mutant lines (E67, E1929 and E1747).

Cross	No. of seeds	Fatty acid (%)
		Palmitic
McGxE67	420	15.3[a**]
E67xMcG	419	15.7[a]
		Linoleic
McGxE1929	420	25.3[c]
E1929xMcG	420	26.8[d]
		Linolenic
McGxE1747	419	37.8[e]
E1747xMcG	419	37.5[e]

** Mean values of reciprocal crosses in the same column (*t*-test) having a common superscript are not significantly different at the 5% level.

4.4 F_2 populations of crosses involving two mutant lines

4.4.1 E67 and E1747

4.4.1.1 Independent segregation of E67 and E1747 mutant genes

The reciprocal populations were combined since their chi-squared values were homogeneous for palmitic and linolenic acid contents [$H_{\chi 2}$=2.58, P=0.11 ; $H_{\chi 2}$=0.16, P=0.69, respectively, (Table 4.8)]. A trimodal frequency distribution pattern for palmitic acid segregation was produced by the combined data (Fig. 4.6 a), with observed numbers that gave a good fit to a 1:2:1 ratio (Table 4.8). Linolenic acid values of the same combined data also produced a segregation pattern which was consistent with results in section 4.3.2 (Fig. 4.6 b). The observed numbers for linolenic acid segregation gave a satisfactory fit to a 1:15 test ratio (Table 4.8).

4.4.1.2 Joint action of E67 and E1747 mutant genes

Results from section 4.4.1.1 suggest that three genes (one for the high palmitic acid trait and two for the low linolenic acid trait) are expected to segregate in a cross involving E67 and E1747. Since the high palmitic acid gene and the low linolenic acid genes in E1747 segregate to 1:2:1 and 1:15 phenotypic classes respectively, a 30:15:15:2:1:1 F_2 ratio or some modified form is expected . Five phenotypic groups instead of six, were observed (Fig. 4.7). The 'missing' seed group was that expected to possess both high palmitic (>20%) and low linolenic (<5%) acid characters. The observed numbers fitted a 30:16:15:2:1 ratio (a slightly modified form of the expected ratio) (Table 4.9).

Table 4.8 Chi-squared (χ^2) analyses of palmitic and linolenic acid levels of individual F_2 flaxseeds of crosses between E67 and E1747 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Palmitic				
E67 x E1747	<11.5	108	105	**(1:2:1)**	
	11.5-18.5	207	210		
(420)	>18.5	105	105	0.13	**0.94**
E1747 x E67	<11.5	93	105		
	11.5-18.5	220	210		
(420)	>18.5	107	105	1.88	**0.39**
Pooled data	<11.5	201	210		
	11.5-18.5	428	420		
(840)	>18.5	211	210	0.54	**0.76**
Heterogeneity				1.47	**0.48**
	Linolenic				
E67 x E1747	< 7	20	26.25	**(1:15)**	
(420)	>11	400	393.75	1.34	**0.25**
E1747 x E67	< 7	24	26.25		
(420)	>11	396	393.75	0.12	**0.72**
Pooled data	< 7	44	52.5		
(840)	>11	796	787.5	1.30	**0.25**
Heterogeneity				0.16	**0.69**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

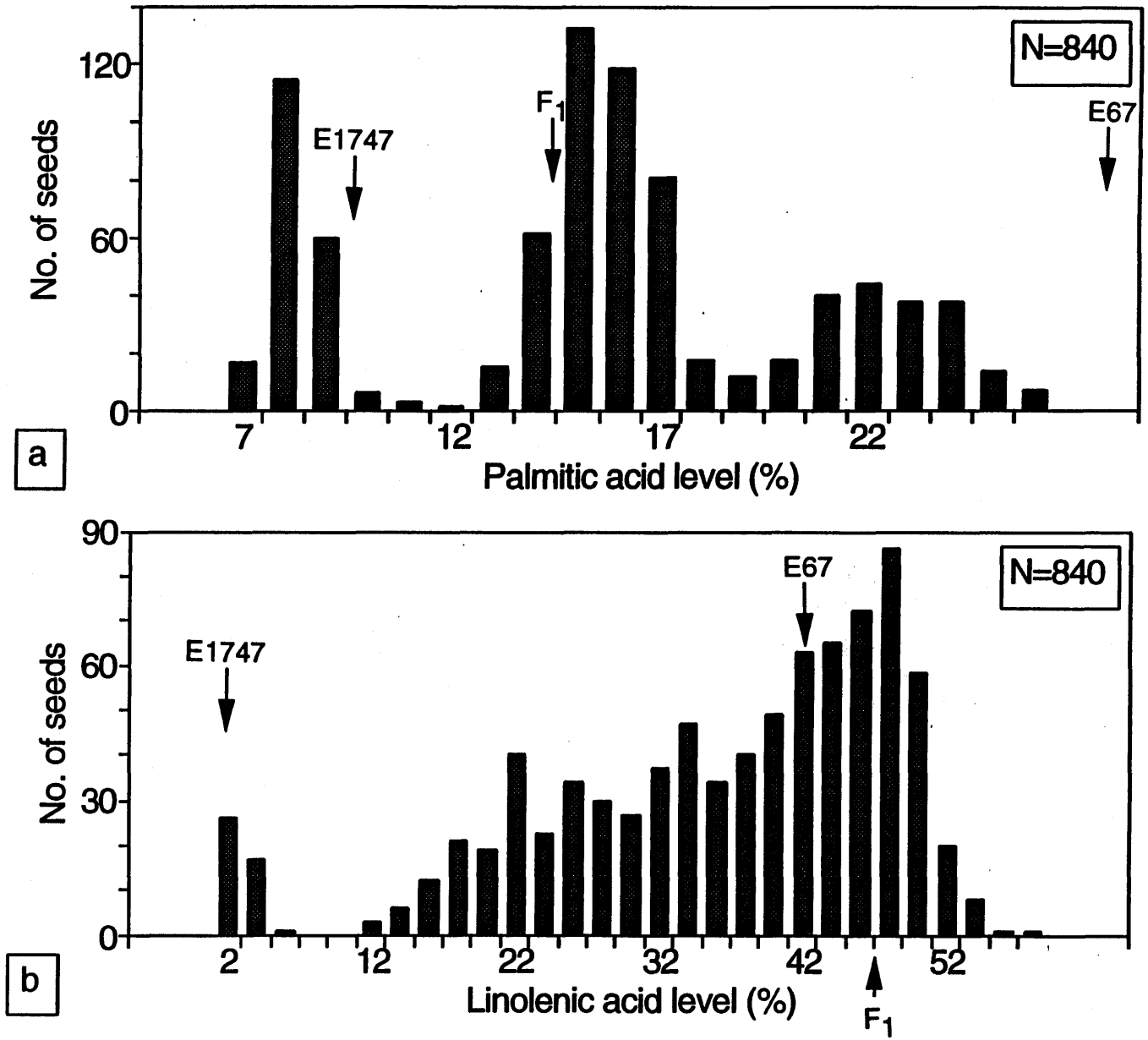


Fig. 4.6 Frequency distribution of a) palmitic and b) linolenic acids in oil of F_2 flaxseeds of combined data of reciprocal crosses involving E67 and E1747.

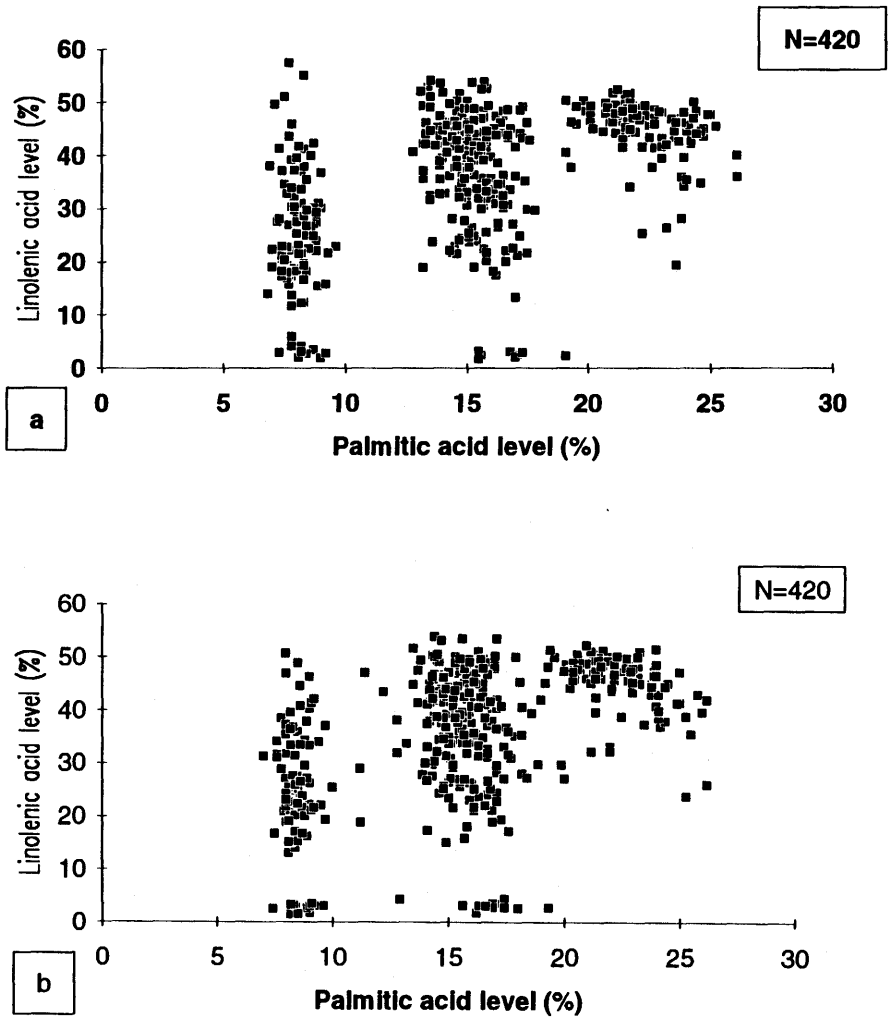


Fig. 4.7 Scatter graph of palmitic versus linolenic acid in oil from F_2 flaxseeds of a) E67 x E1747 and b) E1747 x E67.

Table 4.9 Chi-squared (χ^2) analyses of joint action of palmitic and linolenic acid genes in individual F_2 flaxseeds of crosses involving E67 and E1747 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid phenotype[ψ]	Observed no.	Expected no.	(T.R.)[*] χ^2	P[**]
E67 x E1747	IH	200	196.875	**(30:16:15:2:1)**	
(420)	HH	104	105.000		
	LH	96	98.4375		
	IL	8	13.1250		
	LL	12	6.5625	6.63	**0.16**
	HL	0	0.0		
E1747 x E67	IH	214	196.875		
(420)	HH	102	105.000		
	LH	80	98.4375		
	IL	12	13.1250		
	LL	12	6.5625	9.63	**0.05**
	HL	0	0.0		

ψ IH = Intermediate palmitic and high linolenic.
HH = High palmitic and high linolenic.
LH = Low palmitic and high linolenic.
IL = Intermediate palmitic and low linolenic.
LL = Low palmitic and low linolenic.
HL = High palmitic and low linolenic.

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

4.4.2 E67 and E1929

4.4.2.1 Independent segregation of E67 and E1929 mutant genes

Each population gave a trimodal segregation pattern for palmitic acid (Fig. 4.8 a,c), and a satisfactory fit to a 1:2:1 ratio was obtained in both populations (Table 4.10). The low and intermediate linoleic acid classes were indistinguishable

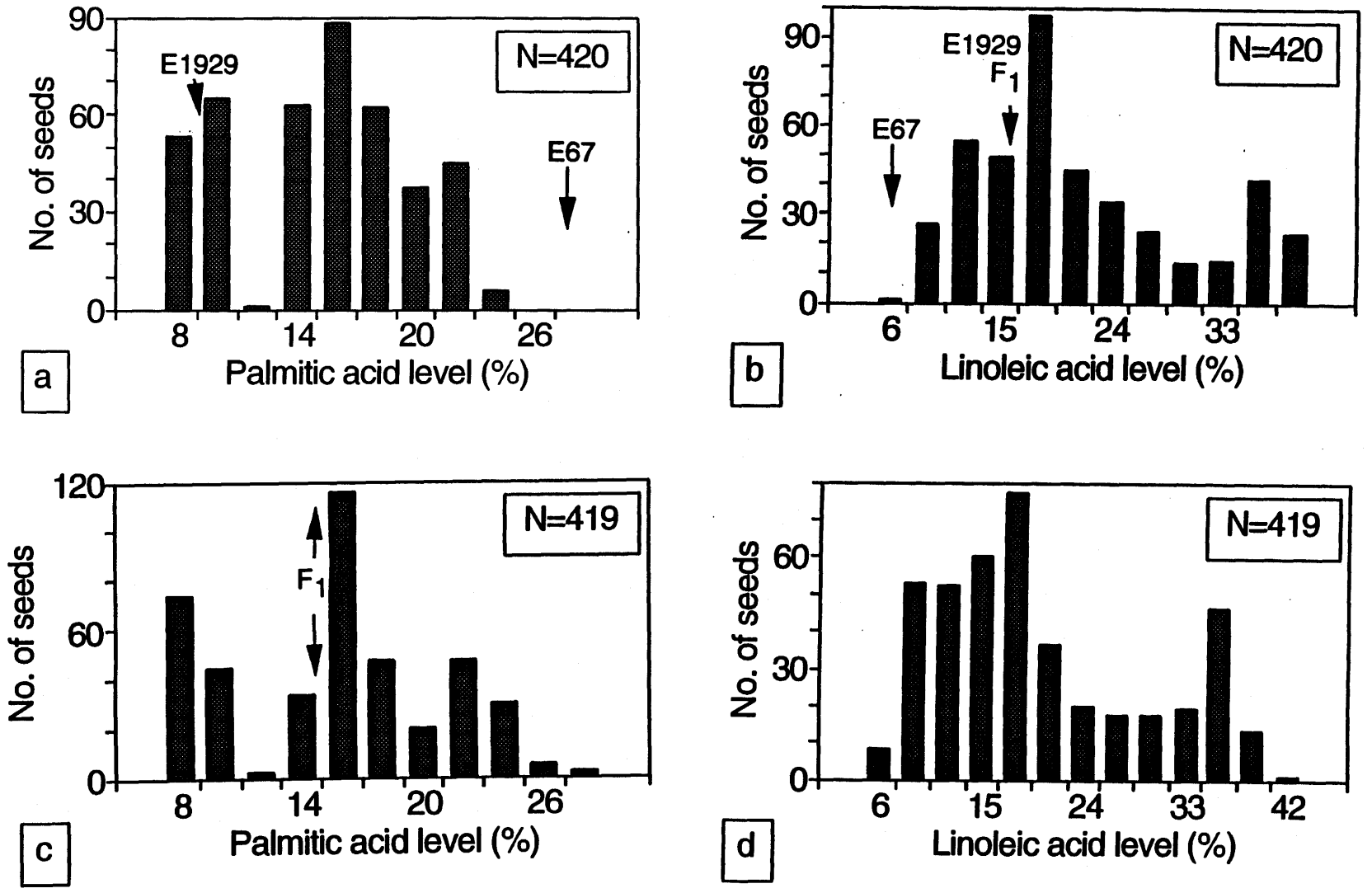


Fig. 4.8 Frequency distribution of
a) palmitic acid in E67 x E1929 b) linoleic acid in E67 x E1929
c) palmitic acid in E1929 x E67 and d) linoleic acid in E1929 x E67
F_2 flaxseeds.

Table 4.10 Chi-squared (χ^2) analyses of palmitic and linoleic acid levels of individual F_2 flaxseeds of crosses between E67 and E1929 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Palmitic			**(1:2:1)**	
E67 x E1929	<11	118	105		
	11-19	214	210		
(420)	>19	88	105	4.44	**0.11**
E1929 x E67	<11	117	104.75		
	11-19	198	209.5		
(419)	>19	104	104.75	2.07	**0.36**
	Linoleic			**(3:1)**	
E67 x E1929	<28.5	329	315		
(420)	>28.5	91	105	2.31	**0.13**
E1929 x E67	<28.5	323	314.25		
(419)	>28.5	96	104.75	0.87	**0.35**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

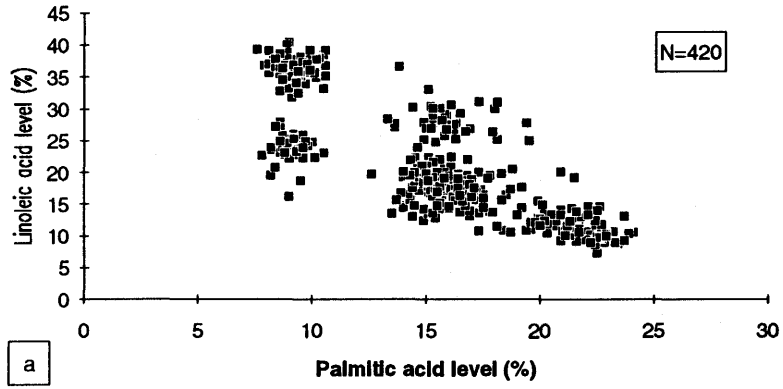
(Fig. 4.8 b,d). The linoleic acid data of each population were, therefore, tested for goodness-of-fit to a 3:1 ratio, and a satisfactory fit was obtained for each population (Table 4.10).

4.4.2.2 Joint action of E67 and E1929 mutant genes

An apparent five or six phenotypic groups were observed (Fig. 4.9 a,b). However, the high palmitic acid groups (in relation to 18:2 content) were difficult to classify. All low palmitic acid genotypes contained intermediate or high 18:2 levels (<11% 16:0 & >15% 18:2), whereas the intermediate and high palmitic acid genotypes contained low to intermediate 18:2 levels (>11% 16:0 & 5-35% 18:2). The observed numbers gave a satisfactory fit to an apparent 1:3 ratio (Table 4.11). It is worth noting that some high palmitic-low linoleic acid genotypes which possessed similar palmitic acid levels to the E67 parent (between 25%-30%, and linoleic acid content as low as 10%) were produced, when E1929 was used as the maternal parent (Fig. 4.9 a,b). This observation appears to implicate some degree of maternal influence in the combination (substrate-product inter-relationships) of the high palmitic acid content in E67 and the moderately low linolenic-high oleic acid level in E1929.

4.4.3 E67 and E1536

The reciprocal F_2 populations were combined since their chi-squared values were homogeneous for palmitic acid levels [$H_{\chi 2}$=0.41; P=0.52, (Table 4.12)]. The combined palmitic acid data gave a trimodal segregation pattern (Fig. 4.10), and a good fit to a 1:2:1 ratio (Table 4.12). Other major fatty acids gave unimodal segregation patterns (Appendix B3).



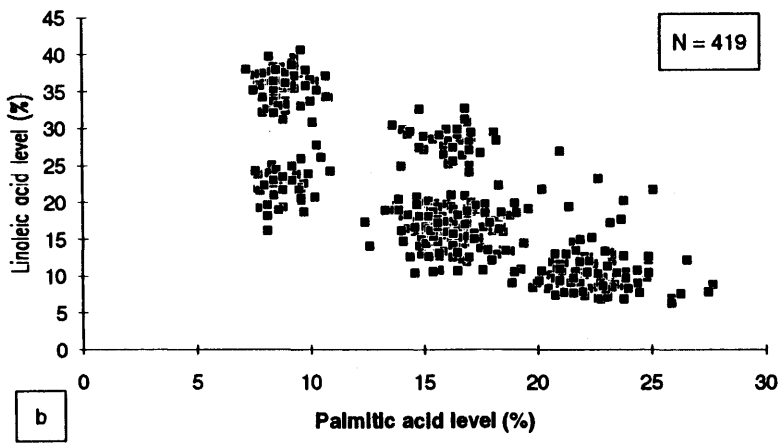


Fig. 4.9 Scatter graph of palmitic versus linoleic acid in oil from F_2 flaxseeds of a) E67 x E1929 and b) E1929 x E67.

Table 4.11 Chi-squared (χ^2) analyses of joint action of palmitic and linoleic acid levels in individual F_2 flaxseeds of crosses between E67 and E1929 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid phenotype[ψ]	Observed no.	Expected no.	(T.R.)[*] χ^2	P[**]
E67 x E1929	<11(& 15-45)	119	105	**(1:3)**	
(420)	>11(& 5-35)	301	315	2.31	**0.13**
E1929 x E67	<11(& 15-45)	119	105		
(420)	>11(& 5-35)	301	315	2.31	**0.13**

ψ - e.g. <12 (& 15-45) = less than 12(%) palmitic and between 15 and 45(%) linoleic acid levels.

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

4.4.4 E1747 and E1929

4.4.4.1 Independent segregation of E1747 and E1929 mutant genes

The reciprocal populations gave satisfactory fits to test ratios which were inconsistent with previous observations. In E1747 x E1929, linolenic and linoleic acid segregation patterns (Fig. 4.11 a,b) gave observed numbers that fitted 1:2 and 2:1 ratios, respectively (Table 4.13). In the reciprocal cross, segregation patterns (Fig. 4.11 c,d) produced numbers which gave a satisfactory fit to 1:3 and 3:1 ratios for linolenic and linoleic acids, respectively (Table 4.13). These mirror image ratios seem to suggest that linolenic acid may be the 'true' segregating fatty acid.

Table 4.12 Chi-squared (χ^2) analyses of palmitic acid levels of individual F_2 flaxseeds of crosses between E67 and E1536 (EMS-derived mutant lines).

Cross (No. of seeds)	Palmitic acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
E67 x E1536	<11.5	100	105	**(1:2:1)**	
	11.5-18.5	210	210		
(420)	>18.5	110	105	0.48	**0.79**
E1536 x E67	<11.5	104	105		
	11.5-18.5	204	210		
(420)	>18.5	112	105	0.65	**0.72**
Pooled	<11.5	214	210		
	11.5-18.5	408	420		
(840)	>18.5	218	210	0.72	**0.70**
Heterogeneity				0.41	**0.52**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

4.4.4.2 Joint action of E1747 and E1929 mutant genes

Three phenotypic groups were observed, but two clear phenotypic classes were were made (due to difficulty in classification of the last two groups); a low 18:3-high 18:2 group (<5% 18:3 and >60% 18:2) and high 18:3-intermediate 18:2 (>14% 18:3 and <55% 18:2) (Fig. 4.12 a,b). Identical numbers and chi-squared results to section 4.4.4.1 were obtained (Tables 4.13). The 1:3 ratio suggests a one gene segregation model, which seems to indicate that the mutated gene in E1929 is allelic to one of

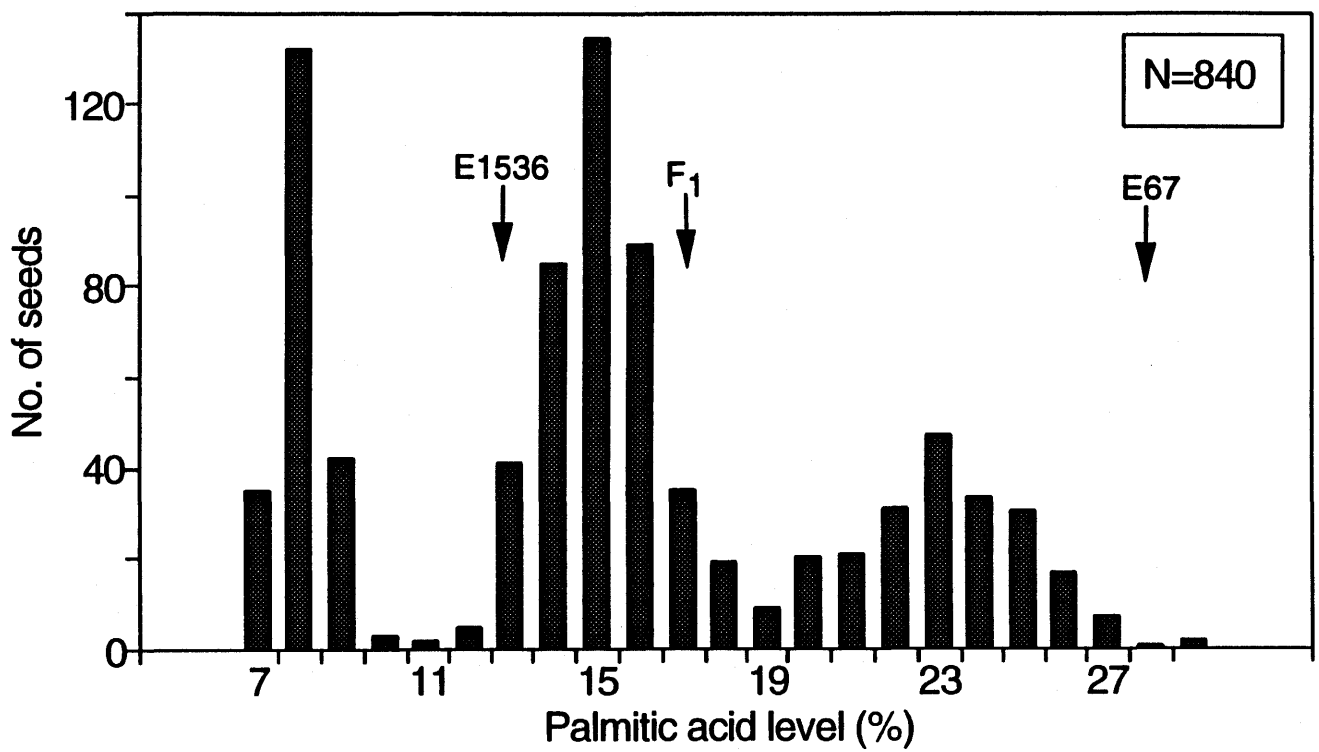


Fig. 4.10 Frequency distribution of palmitic acid in oil of F_2 flaxseeds of combined reciprocal crosses involving E67 and E1536.

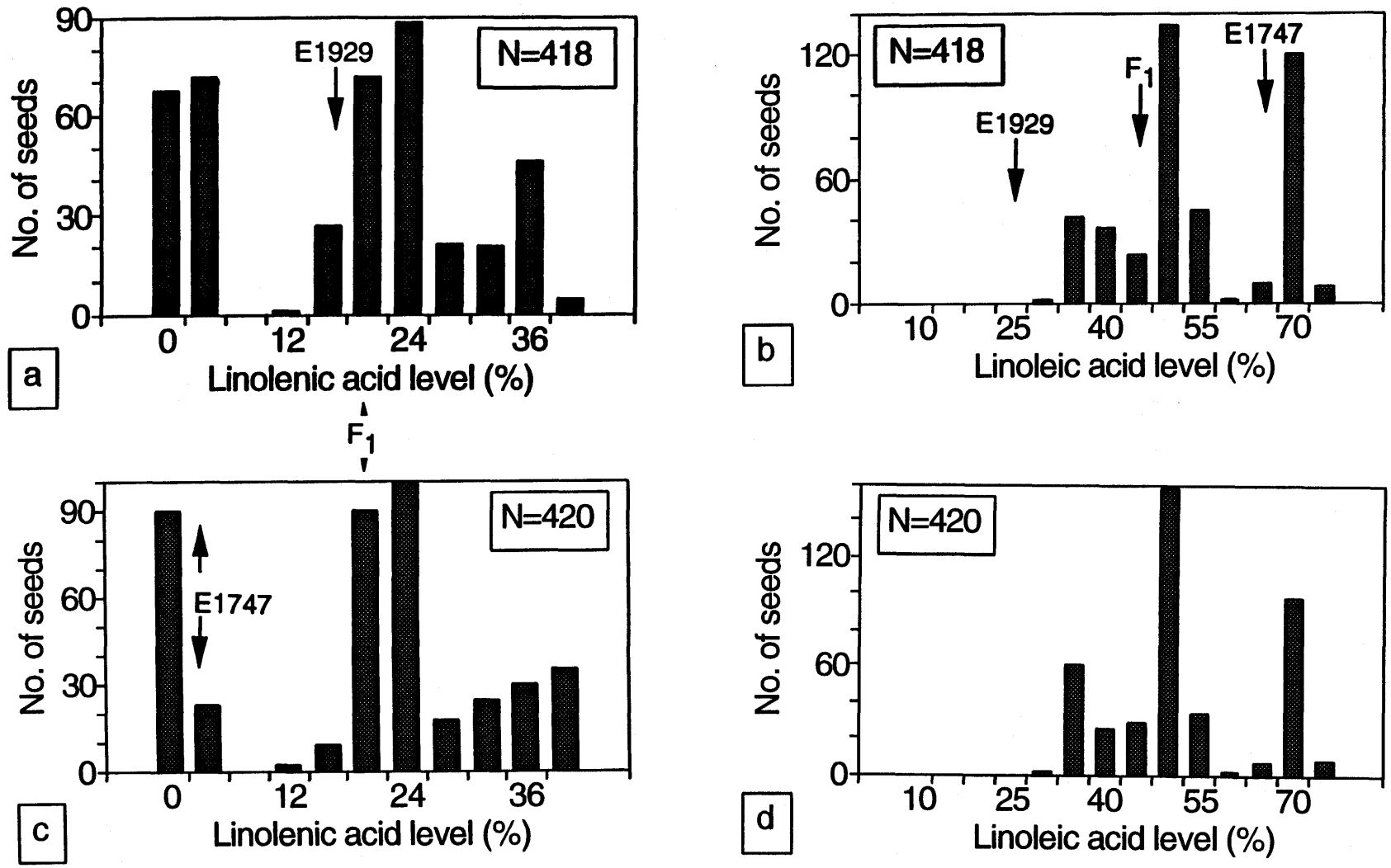


Fig. 4.11 Frequency distribution of
a) linolenic acid in E1747 x E1929 b) linoleic acid in E1747 x E1929
c) linolenic acid in E1929 x E1747 and d) linoleic acid in E1929 x E1747
F_2 flaxseeds.

Table 4.13 Chi-squared (χ^2) analyses of linolenic and linoleic acid levels of individual F_2 flaxseeds of crosses between E1747 and E1929 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Linolenic				
E1747 x E1929	< 6	139	139.33	**(1:2)**	
(418)	>14	279	278.67	0.0003	**0.99**
E1929 x E1747	< 6	113	105	**(1:3)**	
(420)	>14	307	315	0.71	**0.40**
	Linoleic				
E1747 x E1929	<57.5	280	278.67	**(2:1)**	
(418)	>57.5	138	139.33	0.007	**0.93**
E1929 x E1747	<57.5	307	315	**(3:1)**	
(420)	>57.5	113	105	0.71	**0.40**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

the linolenic acid genes in E1747. The 1:2 ratio however, suggests that one of the genotypes was non-expressed. This condition is unidirectional, since a 1:3 ratio is observed in E1929 xE1747 and not in the reciprocal cross.

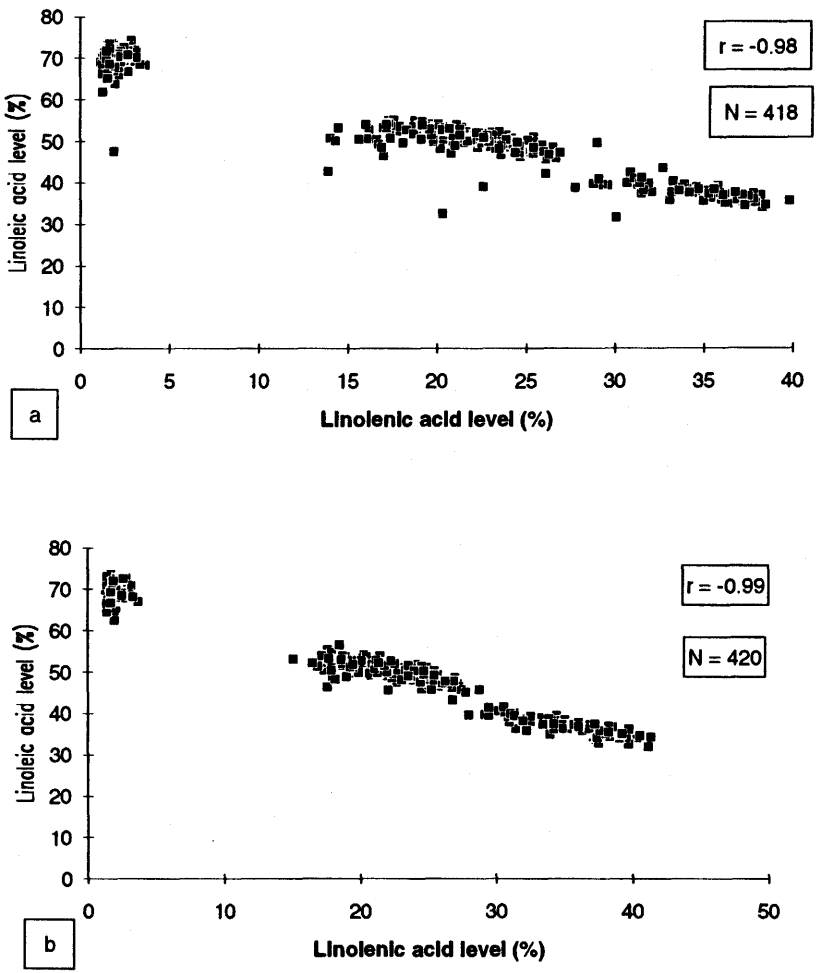


Fig. 4.12 Scatter graph of linolenic versus linoleic acids in oil from F_2 flaxseeds of a) E1747 x E1929 and b) E1929 x E1747.

4.4.5 E1747 and E1536

The pooling together of the two sets of data (reciprocals) was justified by the heterogeneity chi-squared and P values [$H_{\chi 2}$=0.8; P=0.37, (Table 4.14)]. The combined data showed a mirror-image segregation pattern for linolenic and linoleic acids (Fig. 4.13). The observed values for linolenic acid segregation gave a good fit to the presumed 1:15 ratio, and linoleic acid segregation gave a good fit to the expected 15:1 ratio (Table 4.14). The other fatty acids produced unimodal segregation patterns (Appendix B4).

4.4.6 E1929 and E1536

Chi-squared values for goodness of fit to a 3:1 ratio were homogeneous for linoleic acid levels [$H_{\chi 2}$=0.21; P=0.65, (Table 4.15)], and therefore were combined. The low and intermediate linoleic acid level classes overlapped (Fig. 4.14). The observed numbers suggested a 3:1 ratio, to which good fits were obtained (Table 4.15).

Table 4.14 Chi-squared (χ^2) analyses of linolenic and linoleic acid levels of individual F_2 flaxseeds of crosses between E1747 and E1536 (EMS-derived mutant lines).

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Linolenic			**(1:15)**	
E1747 x E1536	< 7.5	28	26.25		
(420)	>17.5	392	393.75	0.06	**0.80**
E1536 x E1747	< 7.5	21	26.25		
(420)	>17.5	399	393.75	0.92	**0.34**
Pooled data	< 7.5	49	52.5		
(840)	>17.5	791	787.5	0.18	**0.67**
Heterogeneity				0.80	**0.37**
	Linoleic			**(15:1)**	
E1747 x E1536	<57.5	392	393.75		
(420)	>57.5	28	26.25	0.06	**0.80**
E1536 x E1747	<57.5	399	393.75		
(420)	>57.5	21	26.25	0.92	**0.34**
Pooled data	<57.5	791	787.5		
(840)	>57.5	49	52.5	0.18	**0.67**
Heterogeneity				0.80	**0.37**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

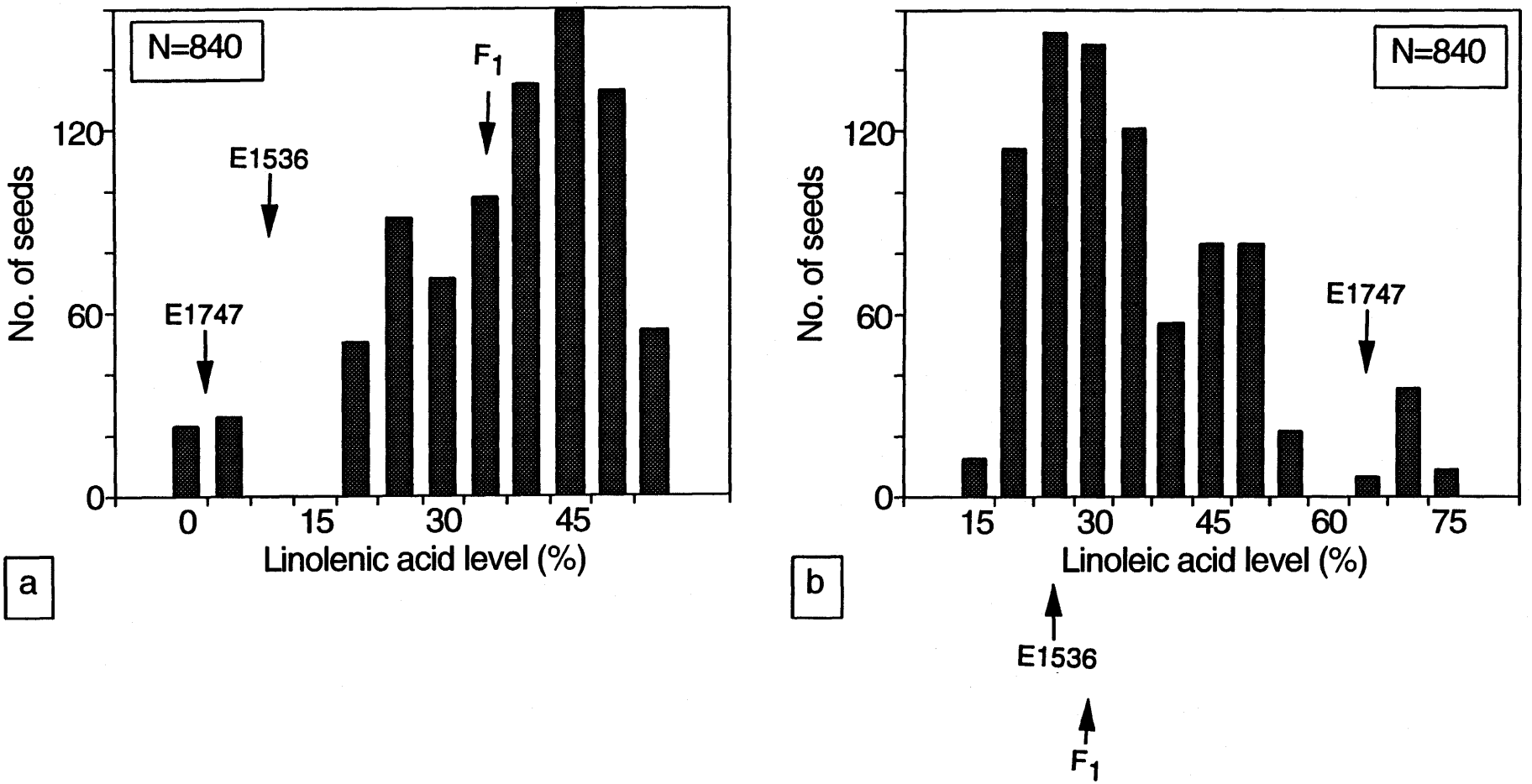


Fig. 4.13 Frequency distribution of a) linolenic and b) linoleic acids in oil of F_2 flaxseeds of combined reciprocal crosses involving E1747 and E1536.

Table 4.15 Chi-squared (χ^2) analyses of linoleic acid levels of individual F_2 flaxseeds of crosses between E1929 and E1536 (EMS-derived mutant lines).

Cross (No. of seeds)	Linoleic acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
E1929 x E1536	<29	319	315	**(3:1)**	
(420)	>29	101	105	0.16	**0.69**
E1536 x E1929	<29	311	314.25		
(419)	>29	108	104.75	0.10	**0.76**
Pooled	<29	626	629.25		
	>29	213	209.75	0.05	**0.83**
Heterogeneity				0.21	**0.65**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

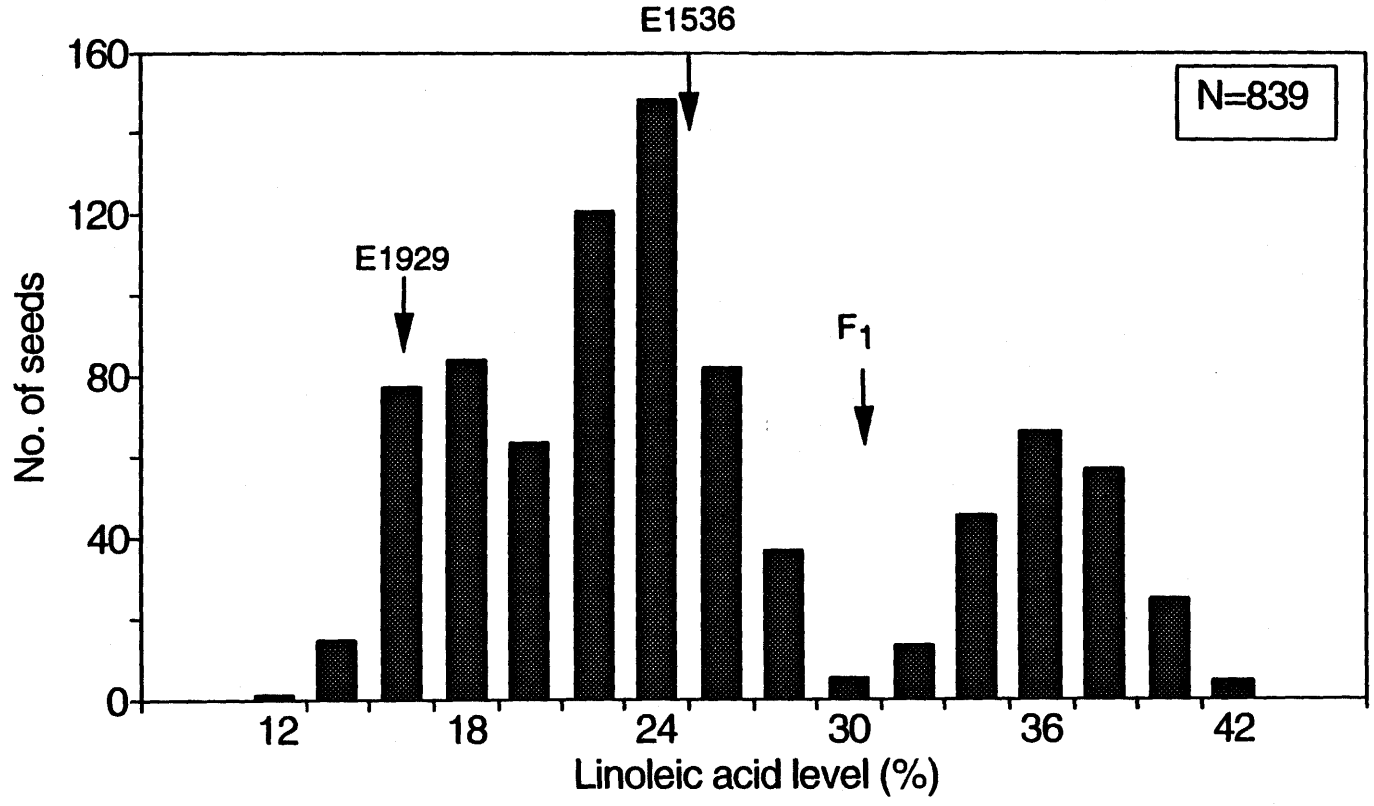


Fig. 4.14 Frequency distribution of linoleic acid in oil of F_2 flaxseeds of combined reciprocal crosses involving E1929 and E1536.

4.5 Backcross (test cross) data (BC_1F_1)

4.5.1 E67 and McGregor

Chi-squared values for goodness of fit to a 1:1 ratio were homogeneous for both palmitic and palmitoleic acid levels [$H_{\chi 2}$=0.98 and P=0.32 for each, (Table 4.16)]. Frequency distribution patterns of the combined data indicated segregation by both palmitic and palmitoleic acids into two clear phenotypic classes (Fig. 4.15 a,b). A good fit to a single gene test cross ratio (1:1) was obtained, with palmitic and palmitoleic acids showing identical segregation numbers, chi-squared and P values (Table 4.16).

4.5.2 E1747 and McGregor

The reciprocal populations were combined because the chi-squared values for goodness of fit to a 1:2:1 ratio were homogeneous for linolenic acid levels ($H_{\chi 2}$=1.59, P=0.21) (Table 4.17). A bimodal frequency distribution pattern for linolenic acid was produced by the combined data (Fig. 4.16). The observed numbers suggested a 1:3 ratio, to which a good fit was obtained (both reciprocal populations gave a good fit to a 1:2:1 ratio) (Table 4.17). The 1:3 (as well as 1:2:1) ratio is a modified form of the 1:1:1:1 test cross ratio for a two independent gene with no epistasis.

Table 4.16 Chi-squared (χ^2) analyses of palmitic and palmitoleic acid levels of backcross (BC_1F_1) flaxseeds of E67 x (E67xMcG) and reciprocal.

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Palmitic				
E67 x (E67xMcG)	<16.5	12	9.5	**(1:1)**	
(19)	>16.5	7	9.5	0.64	**0.42**
(E67xMcG) x E67	<16.5	17	17.5		
(35)	>16.5	18	17.5	0.0	**1.00**
Pooled	<16.5	29	27.0		
(54)	>16.5	25	27.0	0.30	**0.59**
Heterogeneity				0.98	**0.32**
	Palmitoleic				
E67 x (E67xMcG)	<1.75	12	9.5	**(1:1)**	
(19)	>1.75	7	9.5	0.64	**0.42**
(E67xMcG) x E67	<1.75	17	17.5		
(35)	>1.75	18	17.5	0.0	**1.00**
Pooled	<1.75	29	27.0		
(54)	>1.75	25	27.0	0.30	**0.59**
Heterogeneity				0.98	**0.32**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

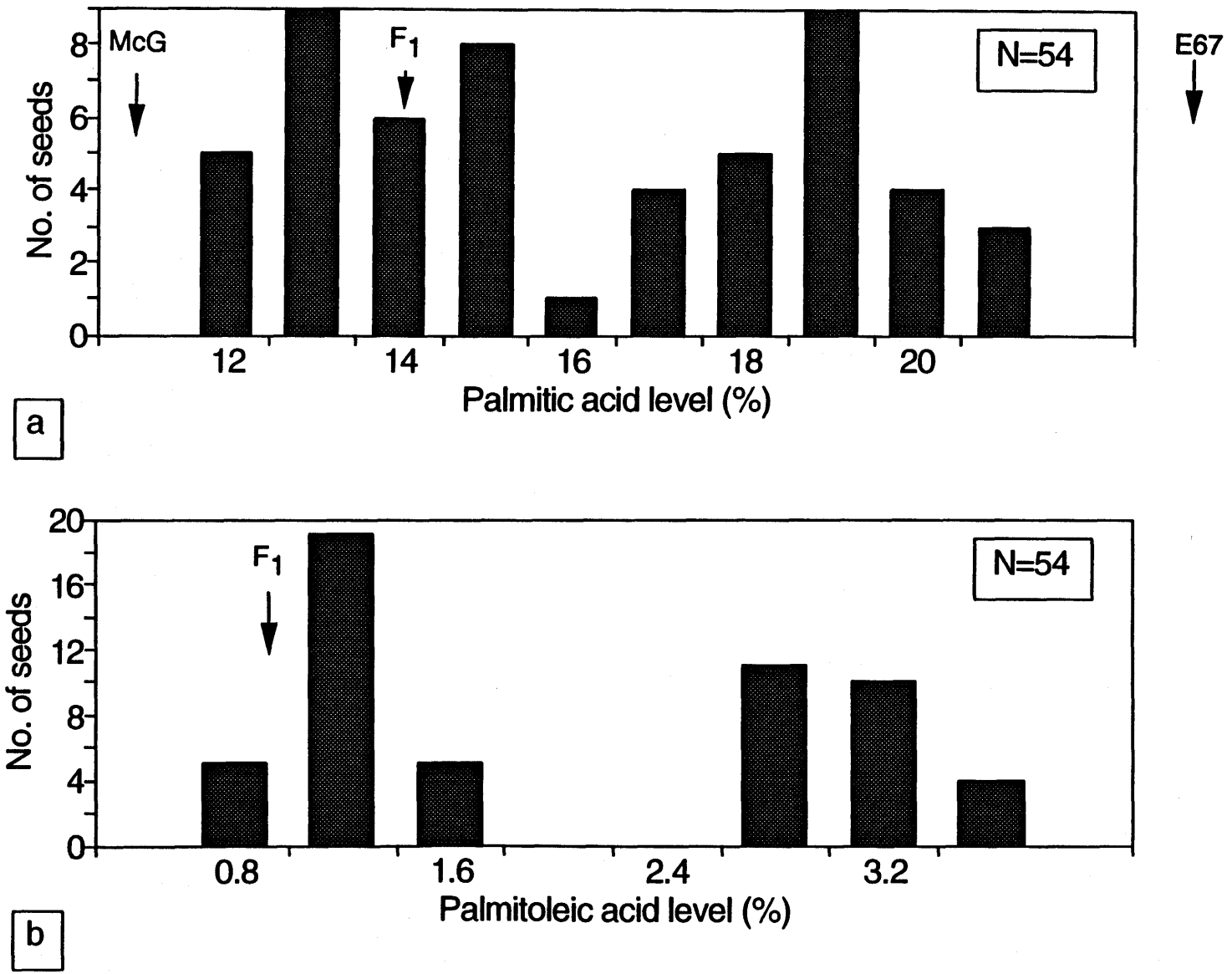


Fig. 4.15 Frequency distribution of
a) palmitic acid and b) palmitoleic acid
of BC_1F_1 flaxseeds of combined reciprocal crosses involving E67 and (E67xMcG).

Table 4.17 Chi-squared (χ^2) analyses of data on linolenic acid content of backcross (BC_1F_1) flaxseeds of E1747 x (E1747xMcG) and reciprocal.

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R.)* χ^2	P**
	Linolenic				
E1747x(E1747xMcG)	< 7.5	18	17	**(1:2:1)**	
(68)	12.5-37.5	37	34		
	>37.5	13	17	1.26	**0.53**
(E1747xMcG)xE1747	<7.5	20	17.25	**(1:2:1)**	
(69)	12.5-37.5	34	34.5		
	>37.5	15	17.25	0.74	**0.69**
Pooled	< 7.5	38	34.25	**(1:3)**	
(137)	> 7.5	99	102.75	0.41	**0.52**
Heterogeneity				1.59	**0.21**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

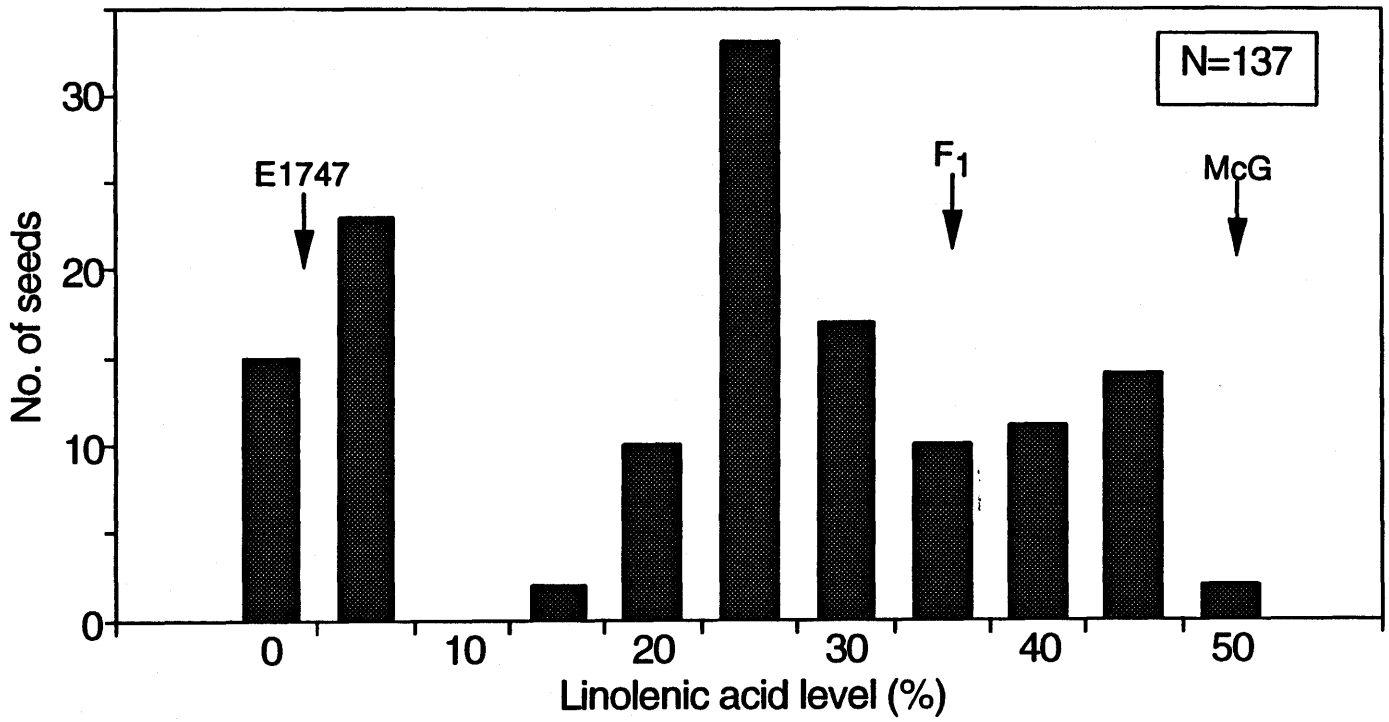


Fig. 4.16 Frequency distribution of linolenic acid in oil of BC_1F_1 flaxseeds of combined reciprocal crosses involving E1747 and (E1747xMcG).

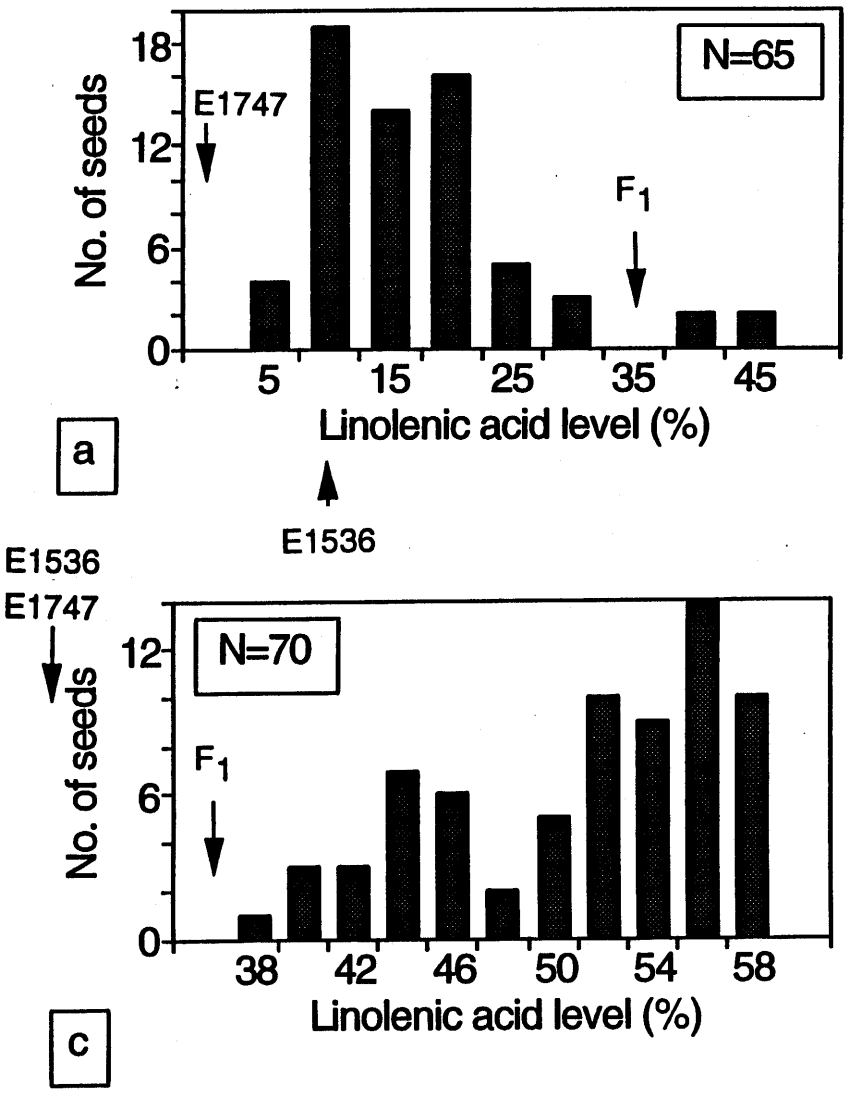
4.5.3 E1536 and E1747

Reciprocal differences were observed for linolenic and linoleic acid distribution frequencies of E1536 x (E1536xE1747) and (E1536xE1747) x E1536 (Fig. 4.17 a,b,c,d). The former cross gave a good fit to a 15:1 ratio (<33%:>37%, respectively) for linolenic acid segregation, whereas the latter gave a satisfactory fit to a 1:3 ratio (<47%:>47% respectively) (Table 4.18). This 15:1 ratio (<33%:>37% linolenic acid level, respectively) does not represent a test cross ratio, and is very different from the usual 1:15 ratio [<7.5%:>17.5% linolenic acid level, respectively(table 4.14)], in terms of phenotypic class range. The 1:3 and 3:1 segregation ratios (test cross) for linolenic and linoleic acids, respectively, suggest the segregation of two genes (a modified form of 1:1:1:1 test cross ratio).

4.6 Fatty acid composition of developing flaxseed at progressive stages of maturity

The fatty acid composition phenotype for each line was determined from day 16 to day 30 post-anthesis. McGregor already contained 50% linolenic acid by at 17 d.p.a. (Fig. 4.18 a). Similarly, at 17 d.p.a. E1929 contained 46% oleic acid peaked at 53% at 21 d.p.a. (Fig. 4.18 b). These values compare favourably with profiles obtained from mature seeds; McGregor had 50% linolenic acid and E1929 had 52% oleic acid (Table 4.1).

In both flax lines the saturated fatty acids (palmitic and stearic) remained virtually constant throughout the investigated maturity periods. The general trends of fatty acid accumulation with age were similar in both lines.



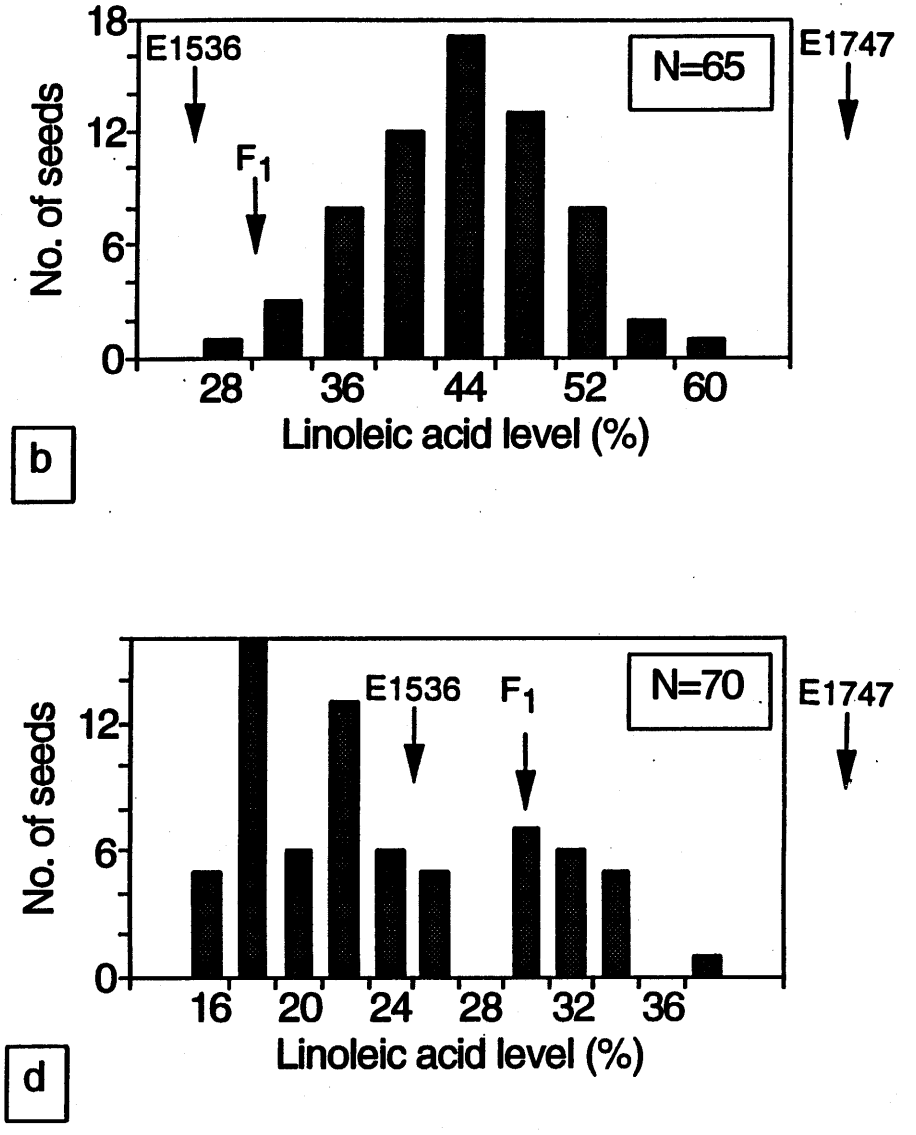


Fig. 4.17 Frequency distribution of
a) linolenic acid in E1536 x (E1536xE1747)
b) linoleic acid in E1536 x (E1536xE1747)
c) linolenic acid in (E1536xE1747) x E1536 and
d) linoleic acid in (E1536xE1747) x E1536
BC_1F_1 flaxseeds.

Table 4.18 Chi-squared (χ^2) analyses of linolenic and linoleic acid levels of backcross (BC_1F_1) flaxseeds of E1536 x (E1536xE1747) and reciprocal.

Cross (No. of seeds)	Fatty acid class (%)	Observed no.	Expected no.	(T.R)* χ^2	P**
	Linolenic				
E1536 x (E1536xE1747)	<33	61	60.9375	**(15:1)**	
(65)	>33	4	4.0625	0.05	**0.82**
	Linoleic				
	-	Unimodal segregation pattern (Appendix B5).			
	Linolenic				
(E1536xE1747) x E1536	<47	20	17.5	**(1:3)**	
(70)	>47	50	52.5	0.30	**0.58**
	Linoleic				
	<28	51	52.5	**(3:1)**	
	>28	19	17.5	0.08	**0.78**

* - Test ratio (T.R.) in bracket.

** - Probability (P) value at 95% confidence level. Differences between observed and expected frequencies are judged significant at $P<0.05$.

4.7 Radio-labelled studies

4.7.1 Gas chromatography analysis of ^{14}C flax embryos

Gas chromatography analysis of total lipids extracted from ^{14}C-labelled flax embryos was used to determine the endogenous fatty acid composition at the age chosen for the acetate feeding experiments (Table 4.19). McGregor and E67 were high in linolenic and palmitic acids, respectively. The low linolenic and high linoleic

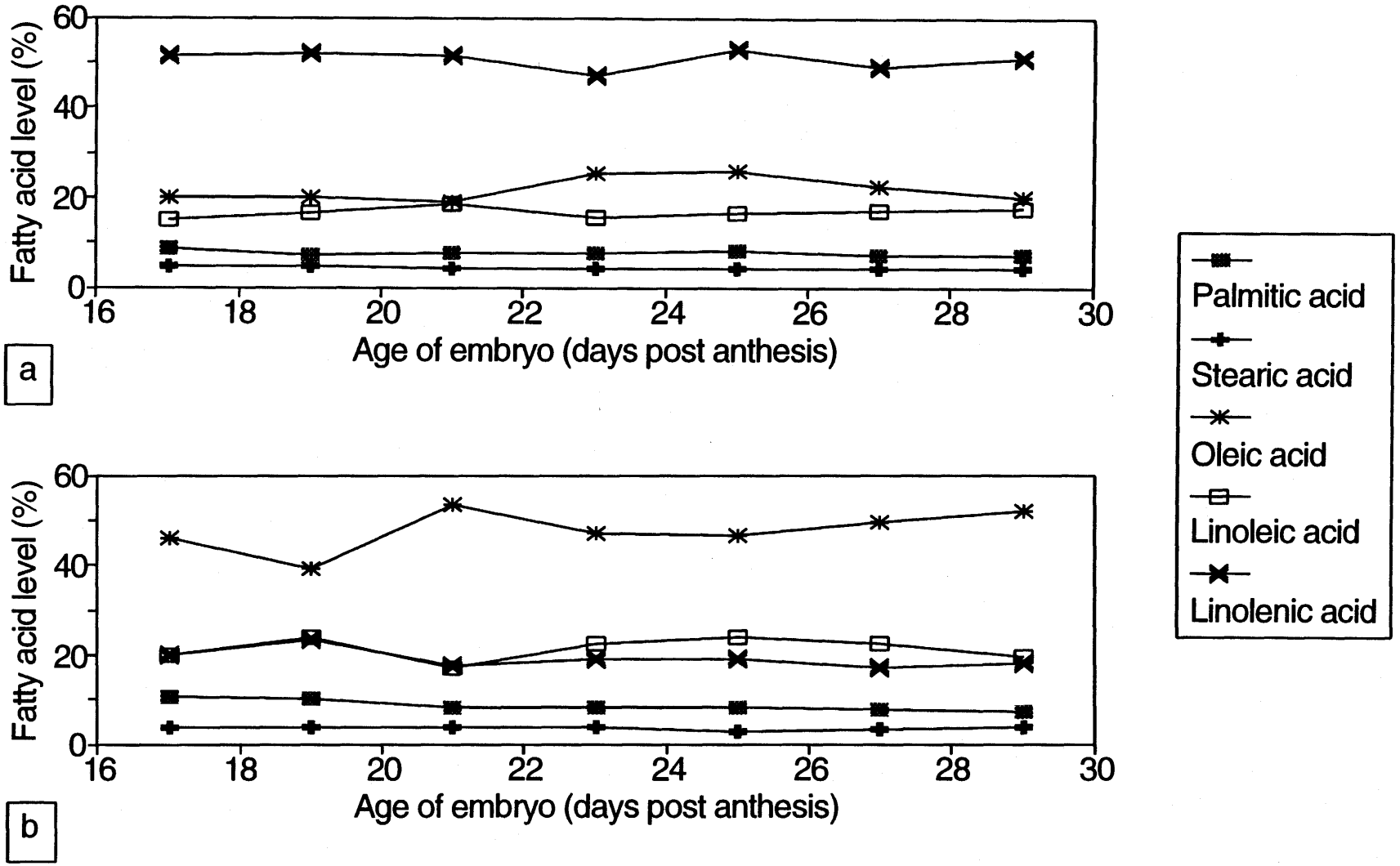


Fig. 4.18 Accumulation of fatty acids in developing flax embryo tissues of a) McGregor and b) E1929.

acid observation for E1747 was consistent with previous findings (Tables 4.1 and 4.19). However, profiles observed for E1929 and E1536 were different from the profiles of mature seeds; mature seeds of E1929 contained 52% oleic acid as opposed to 27% in immature embryos 20-25 d.p.a. embryos (Table 4.19). In mature seeds of E1536, the proportion of linoleic acid was lower than oleic acid, but vice versa in immature embryos 20-25 d.p.a. (Tables 4.1 and 4.19).

Table 4.19 Mean fatty acid composition and their standard errors for fatty acids present in ^{14}C-labelled embryos of McGregor flax and EMS-derived McGregor mutant lines (E67, E1747, E1929 and E1536).

Flax line	Fatty acid (%)					
	16:0‡	16:1	18:0	18:1	18:2	18:3
McGregor	6.7 ± 0.05	-	3.7 ± 0.02	15.4 ± 0.22	16.5 ± 0.13	**57.5** ± 0.26
E67	**22.9** ± 0.13	**2.5** ±0.09	2.1 ± 0.12	13.3 ± 0.30	5.6 ± 0.13	53.4 ± 0.27
E1747	7.4 ± 0.07	-	3.3 ± 0.05	13.1 ± 0.32	73.6 ± 0.26	**2.0** ± 0.05
E1929	7.5 ± 0.15	-	3.5 ± 0.04	**27.2** ± 1.5	31.2 ± 0.95	30.0 ± 0.62
E1536	8.9 ± 0.11	-	3.3 ± 0.04	**34.2** ± 0.68	**37.0** ± 0.35	15.9 ±0.28

‡ Fatty acid symbols: 16:0=palmitic, 16:1=palmitoleic (typically associated with high palmitic acid lines; occurs only in trace amounts in the other lines), 18:0=stearic, 18:1=oleic, 18:2=linoleic, 18:3=linolenic.

4.7.2 Net DPM ^{14}C FAMES

The LKB Liquid Scintillation Counter results showed a high efficiency (>90%) of ^{14}C recovery, and the percentage incorporation of ^{14}C increased with time (Appendix C). The patterns of total ^{14}C incorporation from acetate into fatty acids were similar in all five flax lines (Fig. 4.19 a-e). For any particular fatty acid, the ^{14}C content increased with incubation time. In all lines, oleic acid had the highest accumulation of ^{14}C at each incubation time while linolenic acid had the lowest (Fig. 4.19). 50-70% oleic acid, and less than 5% linolenic acid were observed in all lines (Fig. 4.20 a-e) when the relative proportions (% distribution) of labelled fatty acids (at any particular time point) were considered. The patterns of ^{14}C incorporation into palmitic and stearic acids (the saturated fatty acids) were either similar (as in McGregor, E67 and E1929; Fig. 4.19 a,b and d) or virtually identical (as in E1747 and E1536; Fig. 4.19 c and e).

No major differences were observed in the patterns of ^{14}C incorporation into specific fatty acids by the different flax lines (Fig. 4.21 a-e). However, it is surprising that a greater incorporation (expressed as DPM) of ^{14}C into linolenic acid was observed in E67 and E1536 than in McGregor, an observation that is inconsistent with McGregor's high linolenic acid phenotype. The lack of ^{14}C incorporation into linolenic acid by E1747 is however, consistent with the low linolenic acid phenotype of E1747 (Fig. 4.21 e).

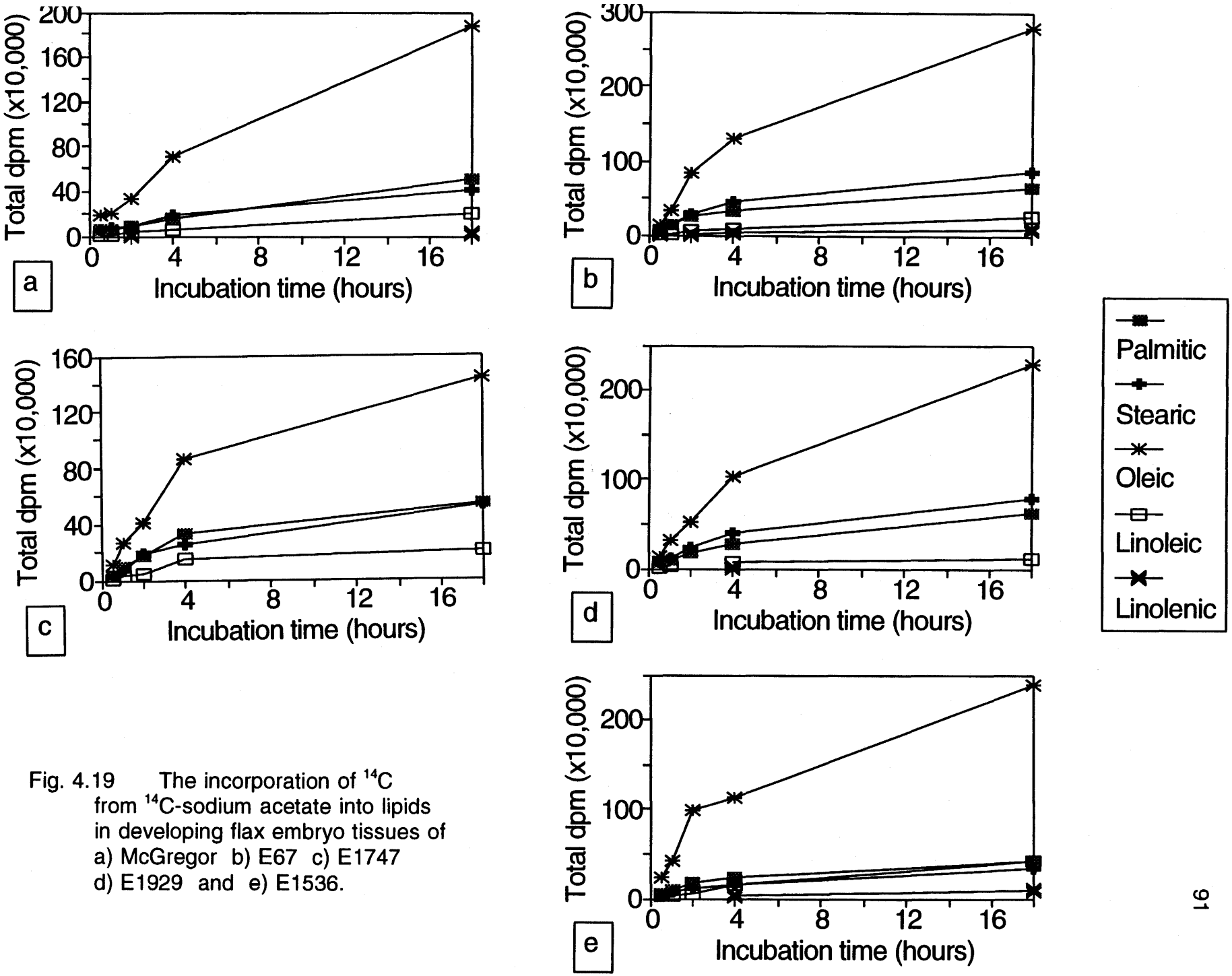


Fig. 4.19 The incorporation of ^{14}C from ^{14}C-sodium acetate into lipids in developing flax embryo tissues of a) McGregor b) E67 c) E1747 d) E1929 and e) E1536.

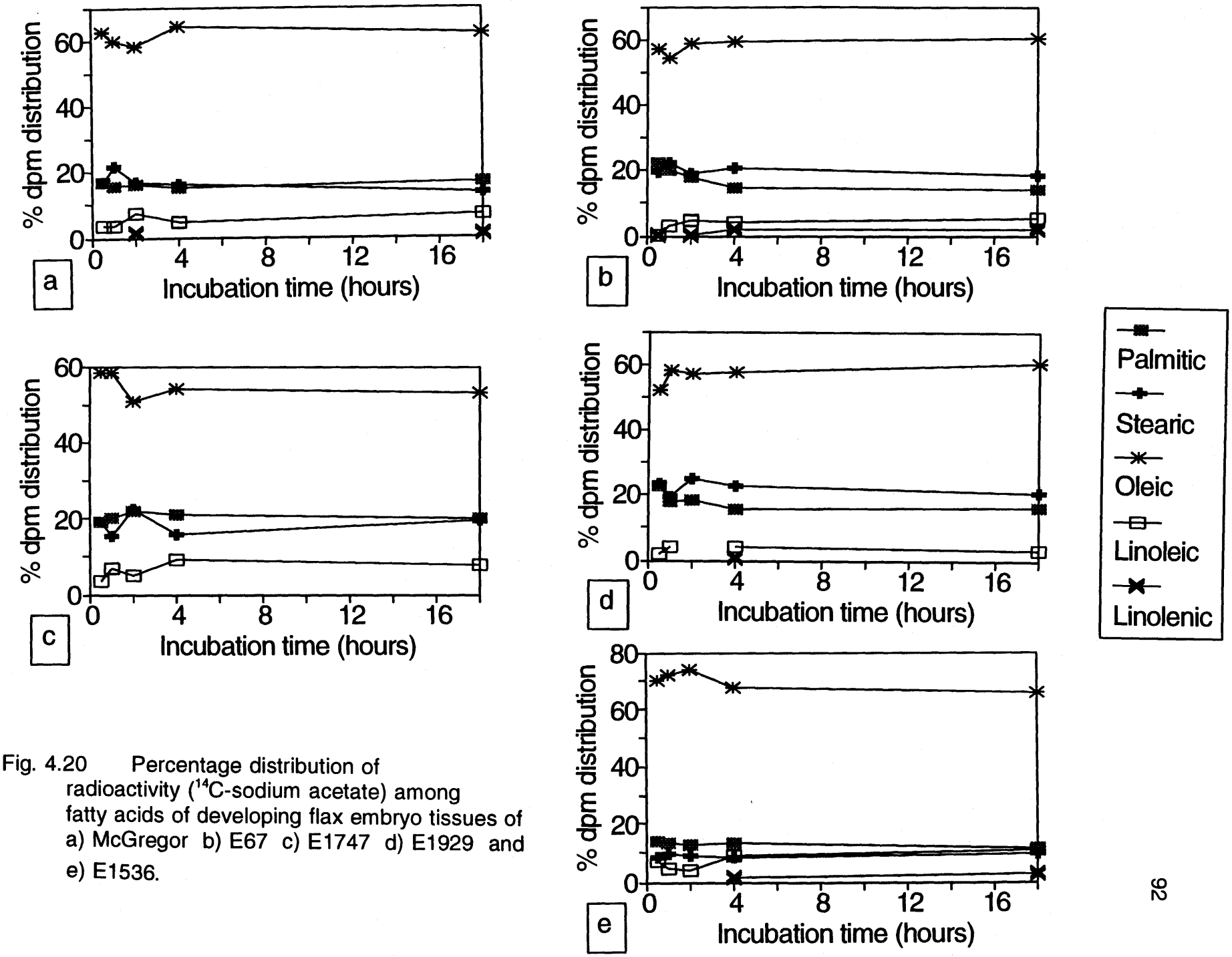


Fig. 4.20 Percentage distribution of radioactivity (^{14}C-sodium acetate) among fatty acids of developing flax embryo tissues of a) McGregor b) E67 c) E1747 d) E1929 and e) E1536.

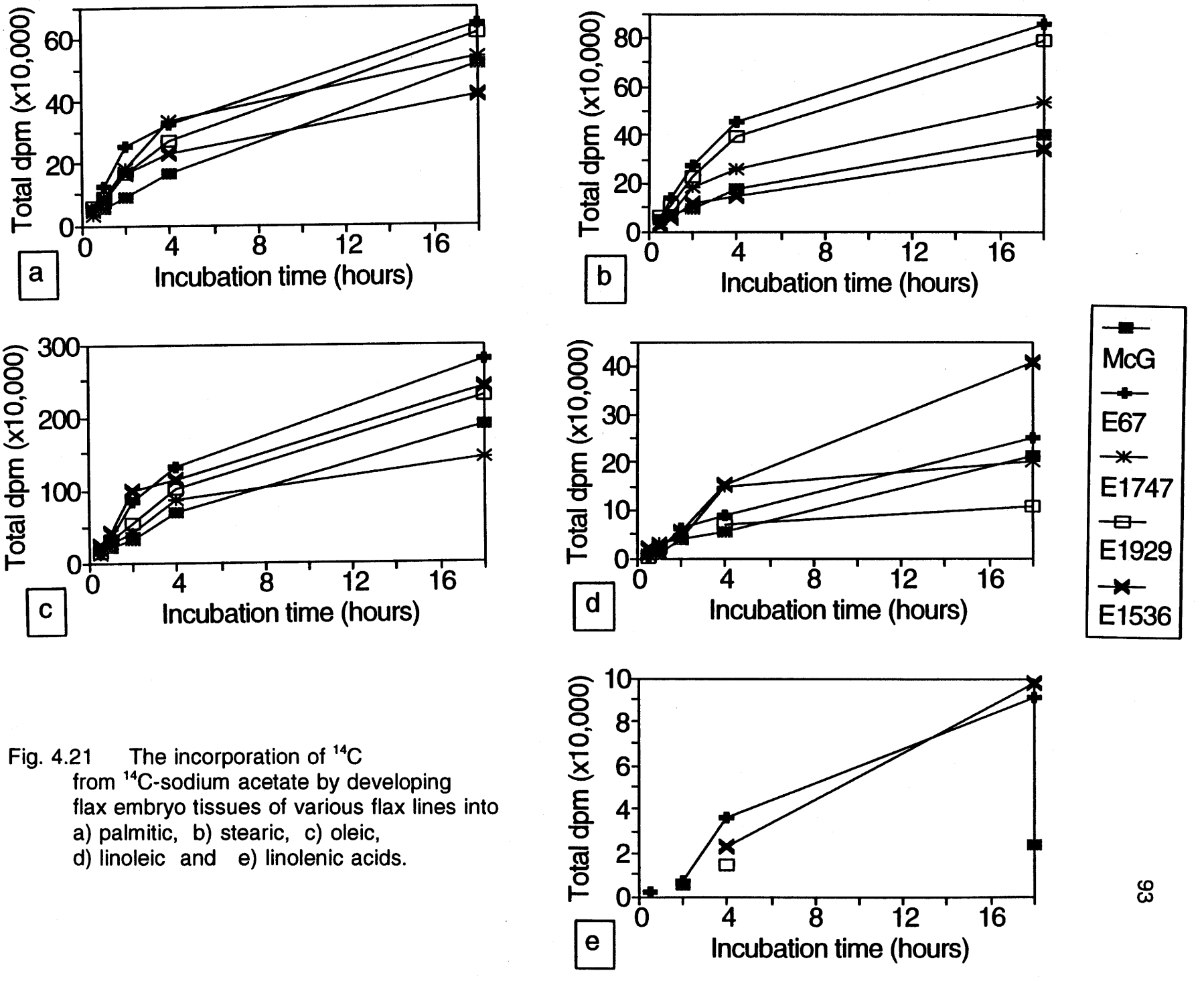


Fig. 4.21 The incorporation of ^{14}C from ^{14}C-sodium acetate by developing flax embryo tissues of various flax lines into a) palmitic, b) stearic, c) oleic, d) linoleic and e) linolenic acids.

4.8 Thioesterase and β-KAS II assays

4.8.1 Fatty acid phenotypes of field-grown embryos

The high linolenic acid level typical of McGregor was observed (Table 4.20). Palmitic acid level of the E67/E1747 line was high (about 3 times that of McGregor). These results confirm the suitability of E67/E1747 as a substitute for E67 for these assays. This was further supported by the independent segregation pattern of the high palmitic acid gene in the presence of the low linolenic acid genes, observed in the genetic studies (section 4.4.1.1).

Table 4.20 Fatty acid composition of immature field-grown flax embryos tested for thioesterase and β-ketoacyl-ACP Synthetase II specificity.

Flax line	Fatty acid (%)				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
McGregor	6.7	5.6	14.3	15.4	**56.8**
E67/E1747	**22.5**	3.1	12.3	51.9	8.4

4.8.2 Target enzyme assays

No significant differences were observed for thioesterase activities on ^{14}C 16:0-ACP, ^{14}C 18:1-ACP, ^{14}C 16:0-CoA and ^{14}C 18:1-CoA between McGregor and E67/E1747 (Table 4.21). However, a slight but perhaps noteworthy difference occurred in the rate of hydrolysis of 18:1-ACP to FFA between the two flax lines; thioesterase activity (on 18:1-ACP) in McGregor was about 4% higher than that of E67/E1747 when standard errors were taken into account. In general the activities of the -ACP thioesterases were lower than in the corresponding -CoA thioesterases. This observation may be a reflection of the lower concentration of the -ACP substrate (1.5 μM) in comparison to their -CoA analogs (18 μM). The β-KAS II activities in McGregor and E67/E1747 were not significantly different (Table 4.22).

These assays were initial surveys only, and time did not permit the optimization of these target enzyme assays in terms of reaction temperatures, assay time, and/or substrate and protein concentrations. However, since both McGregor (wild type) and E67/E1747 (mutant line) were extracted from immature seeds at about the same stage and tested at equal protein concentrations a direct comparison can be made.

Table 4.21 Mean thioesterase activities (Thioest. Act.) and their standard errors on ^{14}C 16:0-ACP, ^{14}C 18:1-ACP, ^{14}C 16:0-CoA and ^{14}C 18:1-CoA for McGregor (wild type) and a high palmitic acid mutant (E67/E1747) flax.**

Substrate/ line	Net avg DPM in *FFA†	pmol *FFA (released per 15 min)	Protein (mg)	Thioest. act. (pmol FFA/ min/mg pr)***
***16:0-ACP/ McGregor**	7,778±93 (±1.2%)	63.1±0.8 (±1.2%)	0.71±0.02 (±2.2%)	**5.92[a]±0.20** (±3.4%)
E67/E1747	7,943±533 (±6.7%)	64.5±4.3 (±6.7%)	0.80±0.05 (±6.4%)	**5.38[a]±0.70** (±13.1%)
***18:1-ACP/ McGregor**	10,010±23 (±0.2%)	81.3±0.2 (±0.2%)	0.71±0.02 (±2.2%)	**7.63[b]±0.18** (±2.4%)
E67/E1747	9,686±266 (±2.7%)	78.6±2.1 (±2.7%)	0.80±0.05 (±6.4%)	**6.55[c]±0.60** (±9.1%)
***16:0-CoA/ McGregor**	13,496±303 (±2.2%)	626±14 (±2.2%)	0.71±0.02 (±2.2%)	**58.8[d]±2.6** (±4.4%)
E67/E1747	13,294±828 (±6.2%)	617±38 (±6.2%)	0.80±0.05 (±6.4%)	**51.4[d]±6.5** (±12.6%)
***18:1-CoA/ McGregor**	5,628±150 (±2.7%)	211±6 (±2.7%)	0.71±0.02 (±2.2%)	**19.8[f]±1.0** (±4.9%)
E67/E1747	5,936±257 (±4.3%)	223±10 (±4.3%)	0.80±0.05 (±6.4%)	**18.6[f]±2.0** (±10.7%)

† - Corrected for control values.

* - ^{14}C-labelled acyl moiety on ACP or CoA thioester.

** - Means having a common superscript are not significantly different [i.e. their ranges (minimum to maximum values) overlap].

*** - Units of thioesterase activity: picomole free fatty acid (FFA) released per min per milligram protein (Appendix D).

Table 4.22 Mean[**] β-ketoacyl-ACP Synthetase II activities (β-KAS II Act.) and their standard errors on ^{14}C 16:0-ACP in the presence of malonyl-CoA, for McGregor (wild type) and a high palmitic acid mutant (E67/E1747) flax.

Line	Net avg DPM in 18:0 FFA[†]	pmol 18:0 FFA (released per 30 min)	Protein (mg)	β-KAS II Act. (pmol FFA/ min/mg pr)[***]
McGregor	35,077±3,673 (±10.5%)	284.7±29.9 (±10.5%)	3.54±0.08 (±2.2%)	**2.68[a]±0.34** (±12.7%)
E67/E1747	36,449±432 (±1.2%)	295.9±3.6 (±1.2%)	4.01±0.26 (±6.4%)	**2.46[a]±0.19** (±7.6%)

[†] - Corrected for control values.

[**] - Means having a common superscript are not significantly different [i.e. their ranges (minimum to maximum values) overlap].

[***] - Units of thioesterase activity: picomole FFA released per min per milligram protein (Appendix D).

4.9 Quantitative determination of oil content by ^{1}H-NMR

Results showed that E67 and E1929 have similar oil contents as McGregor. E1747 has a higher oil content than McGregor, whereas E1536 (compared to McGregor) has about 50% reduction in oil content (Table 4.23).

Table 4.23 Quantitative determination on oil content in seeds of flax lines by nuclear magnetic resonance (^{1}H-NMR).

Line	Mass/seed* (mg)	Oil/seed* (mg)	Oil/sample*	% of McGregor (Oil/sample)
McGregor	5.13	1.63	0.32	100
E67	6.10	1.93	0.32	100
E1747	5.58	2.58	0.42	130
E1929	7.70	2.52	0.33	100
E1536	3.85	0.66	0.17	53

* A sample of each line consisted of 12 seeds.

5. DISCUSSION

5.1 Genetic studies

5.1.1 E67

The consistent F_2 segregation patterns and goodness of fit of the palmitic acid trait in E67 to a 1:2:1 ratio suggest that the control of this character is by a single incompletely dominant gene with E67 having a mutant allele of the wild type. This one-gene inheritance of the high palmitic acid trait in E67 is supported by the good fit of its backcross (test cross) population to a 1:1 ratio. Since the palmitic acid content in the oil from F_1 seeds involving E67 and other lines was approximately intermediate between the parents, embryonic control of the synthesis of this fatty acid is inferred. However, a partial maternal influence on the control of high palmitic acid in E67 was also evident. Similar results indicating a single gene control have been reported by several workers who studied the inheritance of altered (both reduced and elevated) palmitic acid mutant lines in soybean (Erickson *et al.*, 1988; Bubeck *et al.*, 1989; Fehr *et al.*, 1991 a, b). However, no maternal effects were observed for palmitic acid control in any of the soybean mutants.

The conversion of palmitic to palmitoleic acid occurs in the plastid (Browse and Somerville, 1991). In *Arabidopsis thaliana*, this conversion is probably specific for the

sn-2 position of MGD[15], and is under the control of *fad* B desaturase enzyme. Fehr *et al.* (1991 b) in their inheritance studies on high palmitic acid mutants in soybean (23%-27%), did not report any appreciable palmitoleic acid level, in contrast to the present finding with flaxseed. Assuming that both flax and soybean seeds possess the plastidic desaturase enzyme responsible for the conversion of 16:0 to 16:1, then differences between flax and soybean mutants, in terms of the level of palmitoleic acid may be attributed to a) differences in the threshold concentrations of 16:0 required to induce the plastidic desaturation of 16:0 to 16:1 and/or b) differences in the relative thioesterase activities responsible for the effective removal of 16:0-ACP from the site of desaturation. These possibilities will require further comparative studies.

The appreciable content of palmitoleic acid associated with the high palmitic acid trait in E67 may imply that the desaturase enzyme responsible for the conversion of palmitic acid to palmitoleic acid in flaxseed (analogous to the *fad* B enzyme in *Arabidopsis thaliana*) is activated by a high level of its substrate (palmitic acid), and hence the high positive correlation (r=0.94) between proportions of palmitic and palmitoleic acids (Fig.4.2).

5.1.2 E1747

The 1:15 F_2 segregation ratio and the goodness of fit of the backcross data to a 1:3 test cross ratio (a modified form of 1:1:1:1) indicate that the low linolenic acid

[15] MGD - Monogalactosyldiacylglycerol.

character in E1747 is controlled by two independently segregating additive genes. These findings agree with earlier observations made by Rowland (1991) on this same flax mutant. Results on the control of the deficient linolenic acid character in a very low linolenic acid mutant developed from a cross of two mutants by Green (1986) are identical to the results obtained in this study.

The mirror-image segregation pattern observed between linoleic and linolenic acids and the strong negative correlation existing between these two fatty acids (r= -0.99) indicate that the mutation in E1747 resulted from simple blocking(s) in the desaturation of linoleic to linolenic acid (substrate-product relationship). This correlation value is close to the r= -0.95 and r= -0.97 reported by Rowland (1991) and Green (1986), respectively.

No maternal effects on linolenic acid control were indicated (Table 4.2). This contradicts the observation made by Rowland (1991), for the control of linolenic acid in E1747. Differences in the proportion of half-seeds cut and used, and the numbers of F_1 seeds used [10 and 12 F_1 reciprocal seeds were analyzed in this study as compared to 7 and 10 seeds analyzed in the earlier study by Rowland (1991)] for analysis could be possible reasons for such a disparity. Analysis of larger sample sizes may resolve this discrepancy.

5.1.3 E1929

When E1929 was crossed with McGregor (its non-mutagenized parent), linoleic acid was the only fatty acid that segregated into more than one distinct phenotypic

class. This observation to suggests that the mutation that occurred in E1929 resulted in a 'technically lowered' linoleic acid character with a consequent elevation of its immediate precursor, oleic acid. The fact that the linoleic acid content of E1929 (16%) is still similar to that of McGregor (15%) and also that a much lower content of linoleic acid (6%) is possessed by another mutant (E67) (Table 4.1) prompted the analysis of ODR and LDR. The segregation of LDR (and not ODR) to a 1:2:1 ratio suggests that the mutation in E1929 affected the Δ15 desaturase enzyme, and hence the low linolenic acid content (16%) in E1929 as compared to McGregor (50%). It is possible that the high inherent linolenic acid content of McGregor may have interfered and prevented the observation of linolenic acid segregation into distinct phenotypic groups, and that the observed linoleic acid segregation might be the usual 'mirror image' of a 'hidden' linolenic acid distinct segregation pattern. Presumably, the lack of segregation into clear phenotypic groups in the F_2 populations led Green (1985) to similarly use LDR of backcross populations in determining the fatty acid characteristics of M1589 and M1722, and their inheritance.

It could be speculated that the degree of demand for linoleic acid (as a substrate) by Δ15 desaturase enzyme, somehow determines the proportion of oleic acid left unsaturated by the Δ12 desaturase enzyme; i.e., the high Δ15 desaturase activity in McGregor resulted in about 18% oleic acid content, whereas the reduced Δ15 desaturase activity in E1929 resulted in about 52% oleic acid content. The 1:3 and 3:1 ratios of 18:3 and 18:2 segregation patterns, respectively, in E1929 x E1747 suggests that the two mutant lines differ in only one gene, i.e., the mutated gene in

E1929 appears allelic to one of the two mutated genes in E1747. These results are in concert with the 1:2:1 LDR segregation pattern observed for the cross involving McGregor and E1929. On the other hand the 1:2 and 2:1 ratios of 18:3 and 18:2 segregation patterns, respectively (Table 4.13), while rendering support to the inference that the two mutants differ in only one gene (substrate-product relationship), clearly show a unidirectional non-expression of one genotypic group (i.e., when E1747 was used as the maternal parent). The minor reciprocal difference observed for linoleic acid segregation in section 4.3.5 (Table 4.7), thus, appears important. The 1:3 ratio, compared to the 1:2 ratio, alternatively implicates unidirectional non-expression of a genotypic group. Comparing the linolenic fatty acid classes (<4%, >14% and <6%, >14%) and the observed numbers and their totals, for each respective class (Table 4.13), it is apparent that the maternal milieu of E1747 caused a modification, or lacked a factor (which might be present in E1929) that resulted in the 1:2 ratio.

Where desaturase enzymes are involved, perhaps the descriptive system adopted by Knowles and Hill (1964), which reflects the linearity of the fatty acid biosynthesis reactions, may be appropriate. E1929 could, therefore, be described as a moderately low linolenic-high oleic acid mutant (and likewise E1747 as a low linolenic-high linoleic acid mutant). Results of the cross involving E1929 and McGregor indicated that the low linolenic-high oleic acid character of E1929 is controlled by a single additive gene.

5.1.4 E1536

The fatty acid phenotypic characteristics of E1536 and its behaviour in crosses with other lines are difficult to explain. Compared to McGregor, E1536 has elevated oleic acid, elevated linoleic acid and low linolenic acid. Since E1536 is capable of perpetuating its fatty acid phenotype by selfing, it could be argued that a mutation indeed did occur in creating E1536. However, when E1536 was crossed with McGregor all fatty acids produced unimodal segregation patterns in the F_2 populations (Fig. 4.5) where maximum variability was expected. This suggests no gene difference between McGregor and E1536, an inference which is inconsistent with the capabilities of the two lines to perpetuate different fatty acid phenotypes through self pollination. However, the seemingly similar behaviour of these two lines in separate crosses with E1747 supports the above observation (Tables 4.5 and 4.14). In both crosses, the linolenic acid class levels were the same (<7.5% and >17.%%) and the observed numbers were quite similar: McGregor x E1747 ⇒ <7.5%=29, >17.5%=390; E1536 x E1747 ⇒ <7.5%=28, >17.5%=392, and E1747 x McGregor ⇒ <7.5%=23, >17.5%=396; E1747 x E1536 ⇒ <7.5%=21, >17>5%=399 (total number of seeds analyzed were virtually identical, 419 or 420), and in all cases a 1:15 good fit for linolenic acid segregation ratio was obtained. The lack of backcross data involving E1536 and McGregor in the present study is a limitation to the understanding of E1536.

Results of the backcross data involving E1536 and E1747 are difficult to explain. However, the drastic reciprocal differences on observed linolenic acid

segregation ratios (15:1 and 1:3) seem to implicate maternal influences. One common observation to both reciprocal backcrosses was the upper limit of the linolenic acid range of the first class; <33% and <47% (Table 4.18), both of which are high [as compared to the usual <4% or <7.5%, when E1747 is involved in a direct cross with other lines (Tables 4.5, 4.8, 4.13, 4.14 and 4.17)]. The 1:15 linolenic acid segregation ratio (which indicates segregation of two different genes) that resulted from F_2 seeds produced on a E1536 x E1747 F_1 plant (Table 4.14) suggests that the F_1 seed of E1536 x E1747 used for the backcross should be heterozygous for both low linolenic acid gene loci (AaBb)[16].

The 1:3 backcross (test cross) ratio could emerge from two possible ways: either a) E1536 was behaving like the wild type (AABB) or b) the mutated gene in E1536 is allelic to one of the two low linolenic acid mutated genes in E1747 (aaBB); [AaBb x AABB or AaBb x aaBB]. If the latter was the case then a one-gene model ratio (1:2:1 or 1:3) and not a two-gene (1:15) should have been observed in crosses involving E1747 and E1536 (Table 4.14). This makes the first scenario a probable option, with the following phenotypic ratio: 1(AaBb, <47%): 3(AABB + AABb + AaBB, >47%), while the mirror image segregation pattern of linoleic acid (3:1), renders support to the fact that Δ15 desaturase is the enzyme involved, and that two independent genes are involved [since 1:3 or 3:1 is a modified form of a two-gene model test cross ratio (1:1:1:1)].

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[16] Lower- and uppercase letters represent the mutant allele and the wild type allele, respectively, and not dominance or recessiveness (since genes under study are additive). Symbols were so chosen, merely for convenience and clarity.

Mistakingly picking an F_2 boll which might have matured from a selfed-flower on a E1536 x E1747 F_1 plant could produce a 1:15 ratio. The weakness in this argument is shown by the fact that the linolenic acid classes as well as the ratios in Tables 4.14 and 4.18 were entirely different.

Backcross data involving E1536 and McGregor, and E1536 and E1747, using all reciprocal F_1s may help introduce some vital pieces to this E1536 mutant puzzle. It is apparent that E1536 expresses the normal wild type phenotype in one genetic milieu and a different phenotype in another.

5.2 The joint action of mutant genes

Gottschalk and Wolff (1983) stated that "the combination of mutant genes does not always lead to the combination of the mutant characters controlled by the genes involved." Since the combination of specific genes occurs in a genetic milieu and not in isolation, the above statement becomes important and operative in an oilseed crop (e.g. flax), where sequential fatty acid biosynthesis occurs. Although it may be difficult to offer explanations in some cases, mutant gene(s) interactions (substrate-product relationships) may be responsible for some of the inconsistencies and observations made in this study.

Gottschalk and Wolff (1983) crossed two pea mutants possessing different characters (R20D - a high yielding mutant; R46C - an early maturing mutant) the R46C mutant failed to manifest its "earliness" gene in the presence of the R20D genome. Gottschalk and Kaul (1980) studied the performance of R46C and another

pea mutant, RM849 (which among other qualities, was homozygous for the "earliness" gene derived from recombinant R46C). In their observations the "earliness" gene in RM849 was not expressed in Northern India, even though positive results were obtained under the climatic conditions of Germany. Since all experiments in this present study were carried out under the same controlled (growth chamber) conditions, any unusual observations may not be ascribed to environmental effects.

5.2.1 Joint action of E67 and E1747 mutant genes

Results obtained from this study (Fig. 4.12) clearly show five phenotypic groups instead of the expected six groups, and a good fit to a 30:16:15:2:1 ratio, a slightly modified form of the expected 30:15:15:2:1:1 (Table 4.15). The 'absorption' of the high palmitic-low linolenic acid group into the high palmitic-high linolenic acid group is noteworthy. This may have resulted from modification of the linolenic acid level in such genotypes by the inherently high level of linolenic acid in the high palmitic acid line (E67) (Table 4.1). This suggests that screening for a genotype possessing palmitic acid as high as the E67 parent and linolenic acid as low as the E1747 parent from an F_2 population (by conventional crosses of E67 and E1747) may be difficult. However, Rowland (personal communication) has succeeded in isolating such genotypes from further generations (F_3, F_4, etc.).

When E1929 was used as the maternal parent in a cross with E67, genotypes possessing palmitic acid as high as the parent E67 (>25% and <30%) and also low

in linoleic acid were obtained in the F_2 population (Fig. 4.9). Such genotypes may also possess relatively low linolenic acid levels, due to the inherent low linolenic acid character of the E1929 parent. This observation suggests an alternative way by which the high palmitic acid trait of E67 and the low linolenic acid trait in E1747 may be combined (by using a high palmitic F_2 progeny of E1929(♀) x E67(♂) to cross E1747).

5.3 Developing flaxseeds at progressive stages of maturity

In both McGregor (wild type) and E1929 (mutant), seed fatty acid phenotypes characteristic of each line were established in the developing embryos by 17 to 21 d.p.a.; 50% linolenic and 53% oleic acids, respectively. These profiles were similar to those obtained in mature seeds. Similar trends of fatty acid accumulations with age were observed in both lines. These findings agree with observations made by Lehberg *et al.* (1939). In their study on the physical and chemical characteristics of flaxseed at progressive stages of maturity, the phenotypic fatty acid profile was established by 21 days after flowering. The results obtained in this present study indicate that even though the mutated gene in E1929 caused a difference in fatty acid phenotype between the mutant and the wild type, the general trends of fatty acid accumulation with age were similar.

5.4 Radio-labelled studies

5.4.1 ^{14}C-sodium acetate experiments (*in vivo* assays)

The non-invasive approach (*in vivo*) adopted for this work ensured that the embryo subcellular compartmentation was left undisturbed. The fatty acid profiles obtained from vented gas chromatography analysis of ^{14}C-acetate-labelled flax embryos provided a measure of the endogenous fatty acid pool sizes existing at the time when ^{14}C-sodium acetate substrate was fed to the developing embryos. Results obtained agreed with the findings of the embryo development studies (section 5.3), in that all flax lines had their characteristic fatty acid phenotypes well established between 14 and 21 days after anthesis. This suggests that the mass contribution by ^{14}C-labelled products to the endogenous fatty acid pool sizes was negligible.

Although E1747 failed to incorporate ^{14}C into linolenic acid, this could not be claimed as support for a reduced Δ15 desaturase enzyme activity in E1747 (a very low linolenic acid mutant), since McGregor which was expected to have an efficient Δ15 desaturase enzyme system also failed to show any appreciable ^{14}C incorporation into linolenic acid. Oleic acid had the highest ^{14}C incorporation at all treatment periods for all flax lines. The high accumulation of ^{14}C oleate in all lines coupled with the lack of appreciable conversion to ^{14}C-linoleate and linolenate seem to suggest that the products of the plastidic reactions failed to enter the extraplastidic desaturation reaction scheme during these *in vivo* studies. An efficient and rapid incorporation of newly-labelled ^{14}C-oleate and ^{14}C-palmitate into TAGs may occur by diacylglycerol acyltransferase (EC. 2.3.1.20) in the presence of a pre-existing DAG

pool, thus effectively causing a by-pass of the desaturase stream [the two equilibria reactions A and B, (Fig. 2.4)] involving PC. A stereo specific analysis is required to determine the type of labelled fatty acids and their *sn*- position specificity on TAGs. If the above speculation is probable, then most ^{14}C-18:1, ^{14}C-16:0 and/or ^{14}C-18:0 are expected in the *sn*-3 position. The high accumulation of ^{14}C labelled oleate agrees with an observation made by Dybing and Craig (1970). They observed fatty acid specific activities in flax embryos incubated in acetate, to be highest for oleic and lowest for stearic and linolenic acids. On the contrary, whereas the incorporation of oleate into linoleate and linolenate was generally low in the wild type (<9% 18:2 and <3% 18:3, at 2 hr incubation period), Dybing and Craig observed appreciable decline of oleate followed by its incorporation into linoleate and linolenate (31.6% 18:2 and 24.3% 18:3, at 1.5 hr). The reasons for such differences are not known, since the embryos used in both experiments were harvested at about the same age (20 to 25 d.p.a. for the present study as compared to 24 d.p.a. for the other). It is possible that some factor(s) (e.g., NADH or NADPH) which may be required for the effective desaturation of oleic acid to polyunsaturates may be missing in these excised embryos.

E1747, typical of its endogenous fatty acid phenotype (Table 4.20), did not show any ^{14}C incorporation into linolenic acid. The wild type, McGregor (endogenously high in 18:3) also did not produce any appreciable incorporation of ^{14}C into linolenic acid. In general, the acetate feeding experiments failed to aid in the identification of the mutated genes (biochemical lesions) which affected the fatty acid

biosynthesis in these flax mutants.

5.4.2 Target enzyme assays on E67 (*in vitro*)

A change in the relative specificities of the thioesterases responsible for hydrolysing acyl-ACPs could lead to an accumulation of palmitic acid in the TAGs of the E67 line. An increased 16:0-ACP thioesterase, a reduced 18:1-ACP thioesterase (which could cause a "back-up" and accumulation of 16:0-ACP), or a reduction in β-KAS II elongase activity, are all possibilities which could lead to an accumulation of palmitic acid in the E67 line. The lack of significant differences in the activities of these thioesterases and β-KAS II, between McGregor and the high palmitic acid line, indicate that none of these enzymes was responsible for the high palmitic acid character in E67. It is quite possible that in destroying compartmentation in the *in vitro* homogenate assays, important *in vivo* pools were disturbed. This could cause changes in enzyme kinetics as compared to the *in vivo* situation.

Similar findings were recently made by Ohlrogge (1993; personal communication to Taylor/Ntiamoah). In their studies (affiliated with USDA, Peoria, USA) on 18:0-ACP desaturase and thioesterase activities of a soybean mutant line high in 18:0, that produced 25% stearate in the seed oil, they did not observe any differences between the mutant and its parent, in a direct assay of these two target enzymes. Ohlrogge *et al.* (1993: personal communication to Taylor/Ntiamoah) stated "The data were a little messy, but we could see no difference in these enzymes between mutant and its parent. So, I guess this tells us that we have lots more to learn about control of

fatty acid composition." Increased efforts are required to examine the little-studied area of possible interactions between the fatty acid synthesis (FAS) and lipid bioassembly pathways, and, in particular, the changes to such interactions brought about by mutagenesis. Such changes, e.g., precursor (FAS)-product (TAG bioassembly) interactions [e.g., the "pull" by a highly specific acyltransferase on an acyl-pool] may have a bearing on the mutant phenotypes observed. Certainly, most of the mutants are not easily characterized by simple biochemical reaction assays, and require a more detailed study. A study of ^{14}C sodium acetate-labelled lipid species for general acyl composition and analysis of stereospecificity (i.e., TAG vs DAG, etc.) may reveal more about interactions between FAS and lipid bioassembly systems in these mutants.

5.5 Oil contents in flax lines

The mutations that occurred in E67 and E1929 had virtually no effect on their oil contents. Although the seed weights of E67 and E1929 were 19% and 50%, respectively greater than their parent McGregor, these did not translate into greater oil contents. The mutation in E1536 caused a 47% reduction (relative to McGregor) in oil content (E1536 has flat, non-glossy seeds, which weigh 25% less than McGregor). In Green's (1985) studies, both M1589 and M1722 had reduced oil contents when compared to their parent Glenelg. However, the percentage reductions were not as high as the one observed in E1536; the oil contents of M1589 and M1722 were 17% and 15%, respectively less than Glenelg.

The preference of Canadian flaxseed on the world market is not only due to its quality but also its high oil content. The higher oil content (30%) of E1747 than McGregor is encouraging. Should E1747 maintain such high oil content under field conditions, then these two important qualities (very low linolenic acid and high oil content) of E1747 should render it an extremely attractive candidate as an edible oil flax line.

6. CONCLUSIONS

The following conclusions could be deduced from the results reported in this study:

1. Five different mutations occurred to produce these four different mutant phenotypes (E67, E1747, E1929 and E1536).

2. The control of the palmitic acid trait is by a single incompletely dominant gene with E67 having a mutant allele of the wild type. Partial maternal influence on the control of this character was implicated.

3. High palmitoleic acid concentration is associated with a high palmitic acid concentration. The relative high level of palmitoleic acid in E67 apparently results from the elevation of its precursor, palmitic acid.

4. Two independent genes with incomplete dominance control the low linolenic-high linoleic acid trait in E1747. This character is under complete embryonic control.

5. E1929 is a low linolenic-high oleic acid mutant. This trait is controlled by a single incompletely dominant gene, which is allelic to one of the genes in E1747. When involved in a cross with E1747, maternal influence becomes important.

6. E1536 is a true mutant. However, its mutated character could not be detected, due to its "chameleon-like" behaviour. A moderately low linolenic-elevated

oleic and elevated linoleic acid phenotype was vaguely inferred in some instances, while a wild type behaviour was observed in other instances.

7. Combination of the mutant characters is not free of substrate-product interactions. Apparently, the degree of interaction depends on the closeness of the characteristic fatty acids of the mutants (involved in a cross) in the fatty acid biosynthesis pathway.

8. The ^{14}C-sodium acetate experiments failed to identify the enzymes (biochemical lesions) involved in the production of these flax mutant phenotypes.

9. Based on *in vitro* assays, 16:0-ACP thioesterase, 18:1-ACP thioesterase and β-KAS II (elongase) enzymes do not seem to be responsible for the high palmitic acid character in E67.

10. Oil contents in E67 and E1929 (compared to McGregor) were not affected by the mutations. The mutation in E1536 caused a drastic 47% reduction in oil content (relative to McGregor). If E1747 exhibits the high oil content character under field conditions, then its edible oil candidacy should be boosted.

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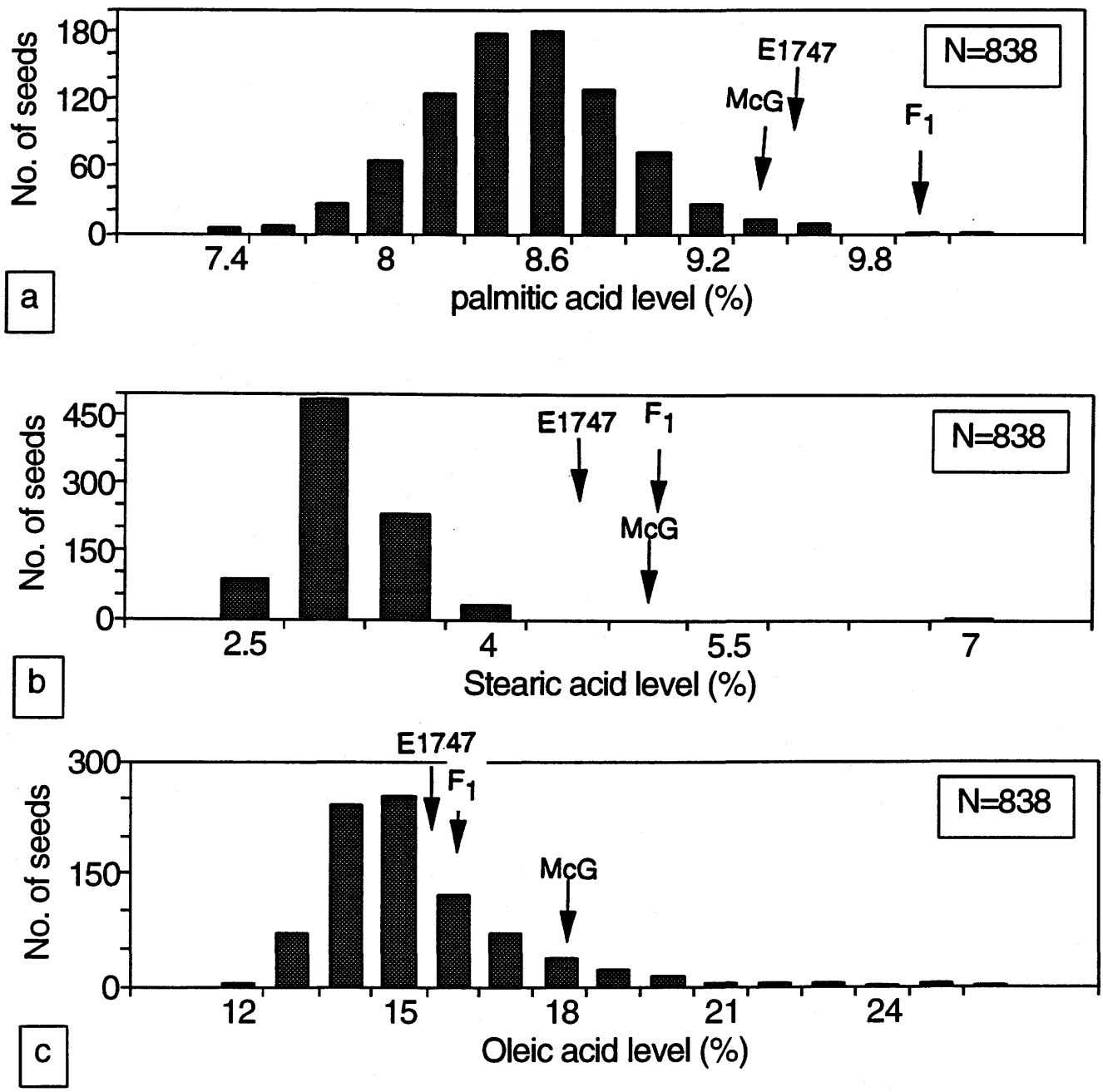
APPENDICES

Appendix A **Fatty acid range* in oil of F_1 flaxseeds (half-seed analysis) of reciprocal crosses.**

Cross (No. of seeds)	Fatty acid range (%)				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
McGxE67(8)	12.9-14.9	2.4-2.7		11.3-13.4	
E67xMcG(13)	13.5-17.0	3.0-4.5		8.6-12.6	
McGxE1747(10)				26.0-29.7	
E1747xMcG(12)				26.9-34.5	
McGxE1929(4)		5.8-7.1			
E1929xMcG(4)		4.2-5.1			
McGxE1536(7)			15.8-19.7		47.9-53.9
E1536xMcG(8)			17.7-25.0		43.1-51.9
E67xE1747(7)			12.1-12.9		
E1747xE67(7)			10.2-12.8		
E67xE1929(1)					
E1929xE67(2)					
E67xE1536(8)	13.3-16.9	2.5-3.3	11.1-13.4	8.4-11.4	55.4-61.2
E1536xE67(6)	17.6-20.0	2.9-5.2	19.5-23.6	20.1-26.9	27.8-35.4
E1747xE1929(6)	7.8-9.7	4.5-5.0	14.1-15.5		20.6-23.2
E1929xE1747(8)	7.0-8.4	2.7-3.6	15.5-32.3		14.6-26.3
E1747xE1536(10)		4.0-4.9	15.0-19.7	30.8-33.6	
E1536xE1747(7)		2.9-3.7	18.9-24.4	29.1-30.3	
E1929xE1536(6)					
E1536xE1929(1)					

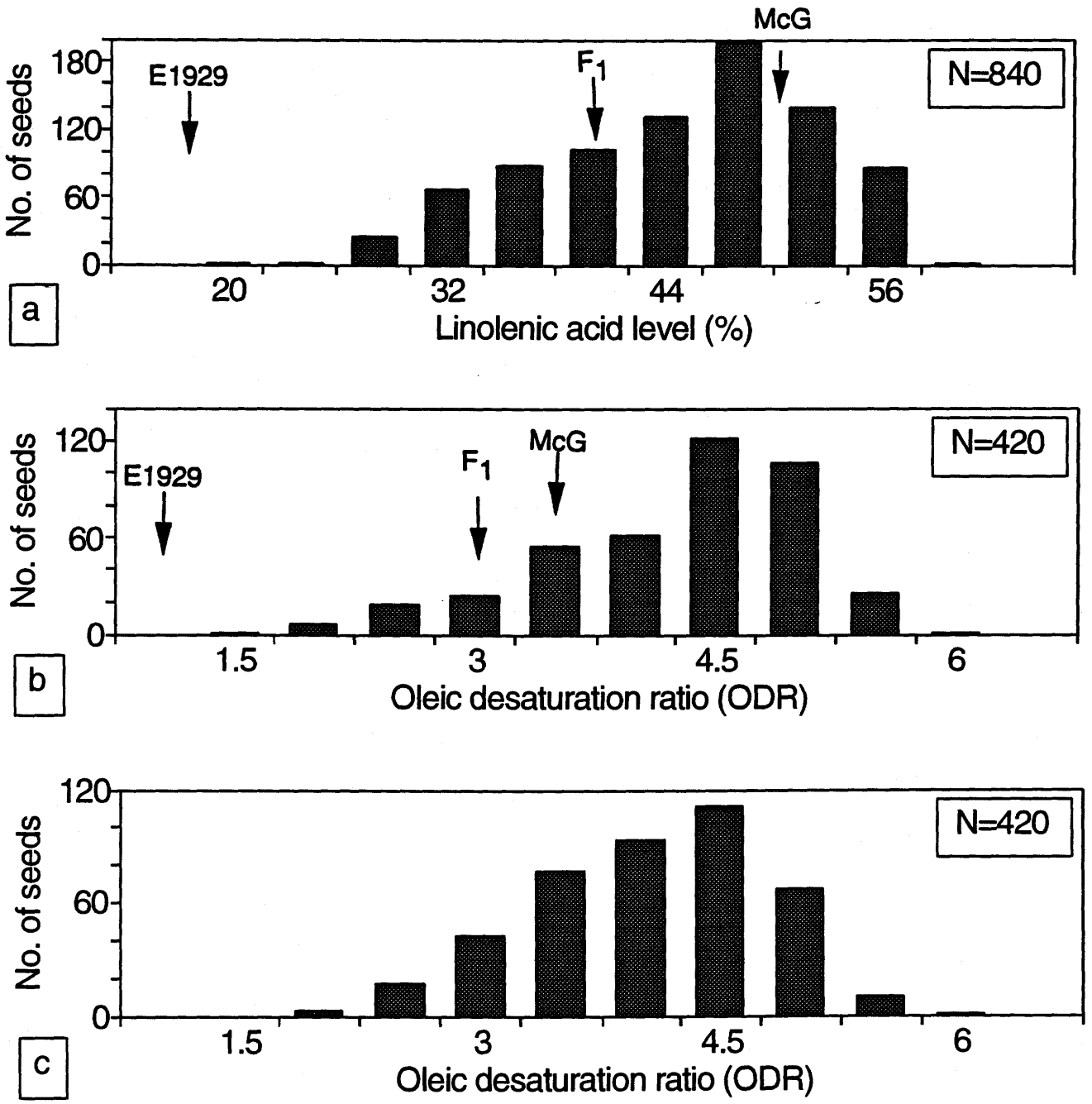
* Values were provided for fatty acids of reciprocal crosses for which significant difference was indicated in Table 4.2.

APPENDIX B



Appendix B1

Frequency distribution of
a) palmitic, b) stearic and c) oleic acids
in oil of F_2 flaxseeds of combined reciprocal crosses involving
McGregor and E1747.



Appendix B2

Frequency distribution of

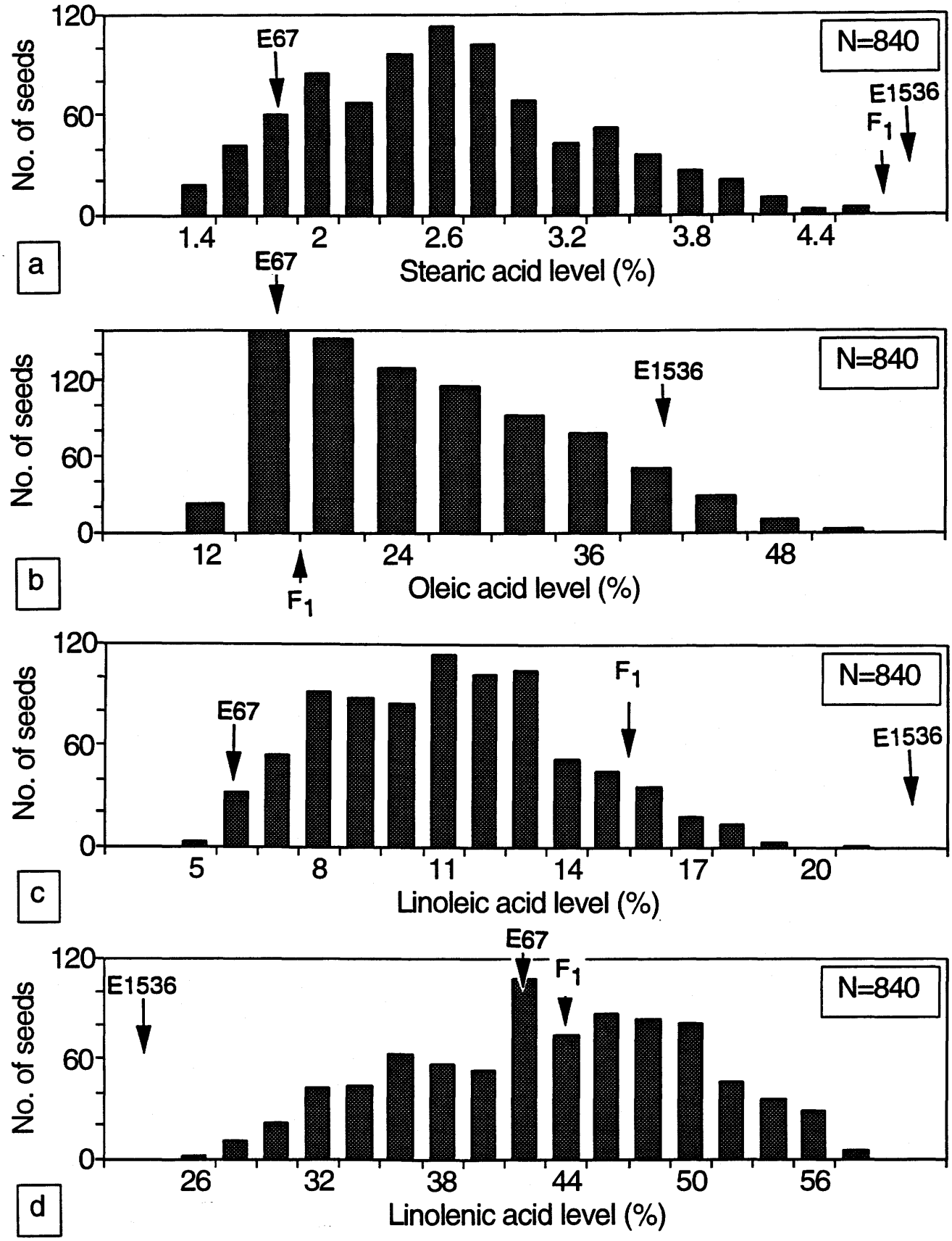
a) linolenic acid in oil of F_2 flaxseeds of combined reciprocal crosses involving McGregor and E1929,

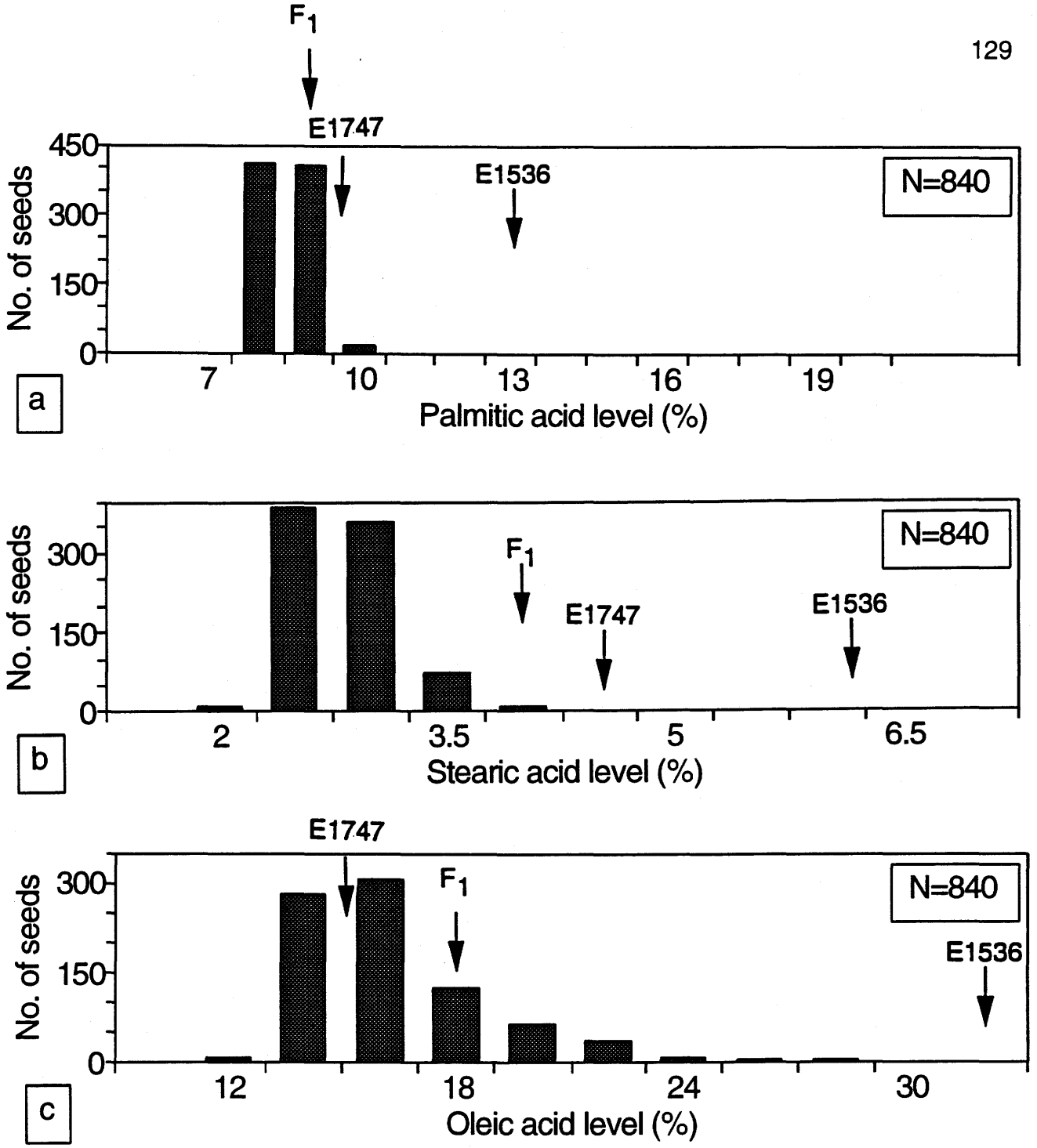
b) oleic desaturation ratio in oil of F_2 flaxseeds of McG x E1929 and

c) oleic desaturation ratio in oil of F_2 flaxseeds of E1929 x McG.

Appendix B3

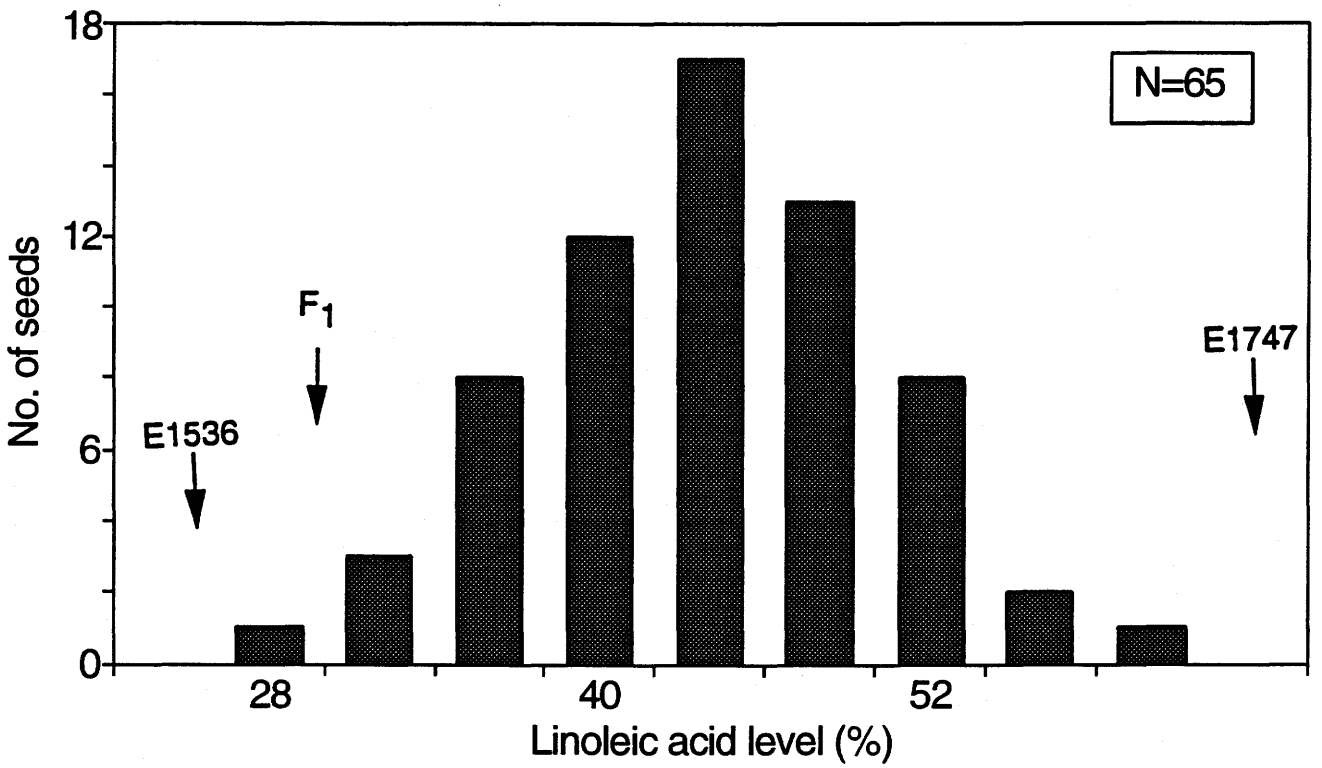
Frequency distribution of
a) stearic, b) oleic, c) linoleic and d) linolenic acids
in oil of F_2 flaxseeds of combined reciprocal crosses involving
E67 and E1536.





Appendix B4

Frequency distribution of
a) palmitic, b) stearic and c) oleic acids
in oil of F_2 flaxseeds of combined reciprocal crosses involving
E1747 and E1536.



Appendix B5

Frequency distribution of linoleic acid in oil of BC_1F_1 flaxseeds of combined reciprocal crosses involving E1536 x (E1536xE1747).

APPENDIX C

Sample data

1-^{14}C sodium acetate counts (DPM) from McGregor flax and EMS-induced McGregor mutant line (E1747).

Line	Incubation time	Total net DPM	% Recovery*	% Incorporation** [DPM in Tube C (TLE)]
McGregor				
	15 min	10,105,540	94.6	1.0 (110,840)
	30 min	9,660,640	90.4	0.7 (78,340)
	1 h	9,757,420	91.4	3.1 (328,820)
	2 h	9,672,570	90.6	4.6 (487,770)
	4 h	10,607,810	99.3	7.0 (744,110)
E1747				
	15 min	10,464,600	98.0	0.9 (98,700)
	30 min	10,230,970	95.8	1.3 (142,470)
	1 h	9,619,780	90.1	2.7 (293,380)
	2 h	9,329,220	87.3	4.8 (513,420)
	4 h	10,140,220	94.9	6.3 (671,620)

1 ml stock solution of ^{14}C sodium acetate ≡ 10,681,350 DPM

* - % Recovery of ^{14}C = $\frac{\text{DPMs in Tube A + Tube B + Tube C (see section 3.6.4.1)}}{10,681,350}$

** - % Incorporation of ^{14}C = $\frac{\text{DPM in Tube C (TLE)}}{10,681,350}$

Appendix D Sample calculation of enzyme specific activity.

Example: β-ketoacyl-ACP Synthetase II specific activity (Table 4.22).

McGregor: (^{14}C 16:0-ACP substrate)

a) Specific activity of substrate = 56 nCi/nmol ≡ 123,200 DPM[17]/nmol

b) Peak area of ^{14}C 18:0 methyl esters ≡ 35,077 DPM

Therefore:

$$\frac{35{,}077 \text{ DPM}}{123{,}200 \text{ DPM/nmol}} = 0.2847 \text{ nmol} = 284.7 \text{ pmol}$$

c) $\frac{284.7 \text{ pmol}}{30 \text{ min}} = 9.49$ pmol/min.

d) $\frac{9.49 \text{ pmol/min}}{3.45 \text{ mg pr}} =$ **2.68 pmol/min/mg pr**

[17] 1 nCi ≡ 2.2 x 10^3 DPM