Electronic Journal of Plant Breeding, 1(6): 1396- 1404 (Dec 2010)
ISSN 0975-928X

Research Article

Rapid, cost-effective screening of flax genotypes to identify desirable fatty acid compositions

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(Received:25 Sep 2010; Accepted: 5 Nov 2010)

Abstract:
Seeds of oil flax (*Linum usitatissimum*) are rich in polyunsaturated fatty acids and their composition is one of the major criteria for flax selection. Conventional breeding schemes take many years and need a lot of space in greenhouses and/or in fields to achieve genotypes with the desired traits. We have improved the half-seed method that allows flax line selection from their oil seed composition. Cotyledon tips were analysed by GC to determine their FAME profiles while the remaining seed was sown to regenerate flax plant. An optimisation of the GC enabled a sample to be analysed every 4 minutes. Such a technique could be applied without loss of germination rate for excised seeds. Further experiments showed that both seed parts had the same fatty acid profile as cotyledon tips. This non-destructive procedure based on the half-seed method and ultrafast GC analysis provides an efficient screening of flax genotypes according to their seed fatty acid composition over only one generation.

Keywords
Gas chromatography, oil flax, half-seed, screening, fatty acid profiles, linoleic acid

Abbreviations: ALA, alpha-linolenic acid; LA, linoleic acid; GC, gas chromatography; PUFA, polyunsaturated fatty acids; FAME, fatty acid methyl ester

Introduction

The two primary uses of flax (*Linum usitatissimum*) are as an oil seed crop and as a fibre crop. The seed of flax contains between 35% and 50% oil known as linseed oil (Oomah and Mazza, 1998). Regarding the fatty acid composition of linseed, a high content of α-linolenic acid (18:3n-3, ALA), generally from 45% to 65%, is desirable for oleochemical purposes (Bickert et al., 1994). The high polyunsaturated fatty acid (PUFA) content makes linseed oil susceptible to polymerisation reactions upon exposure to oxygen in air and/or under the influence of heat and ultraviolet radiation. The result of this polymerisation is the rigidification of the material, which gives the appearance of "drying." This high drying quality makes it useful for industrial purposes. Most applications of linseed oil exploit its drying property, such as substitutes for petroleum-based solvents in a wide range of paints, stains and other coatings, and in the manufacture of particleboard and of the floor covering linoleum. Conversely, this high sensitivity to oxidative processes is detrimental and problematic for direct food use (rancidity). Indeed, under the thermal effects of industrial processes, compounds undesirable or toxic to humans can be engendered (Trautwein, 2001; Overeem et al., 1999). Flax cultivars with a low ALA-content, less than 2%, were obtained by mutagenesis (Green and Marshall, 1984; Green, 1986; Rowland, 1991). These "Solin" genotypes, which took the "Linola" generic name (Frith, 1994), are less sensitive to oxidation and were firstly bred for industrial uses but may be considered for food use.

Nowadays, linseed oil composition is a major criterion for flax genotype selection for industrial as well as food uses. Indeed, oil composition is of major importance to guide the future uses of new cultivars and their potential markets. To maintain and develop the competitiveness of the linseed oil sector,

genotypes should be selected on their oil composition from the early stages of the breeding scheme. Mutation induction and interspecific hybridisation have been used to expose variations in fatty acid composition in flax (Bergmann and Friedt, 1997). The use of analytical techniques such as gas chromatography (GC) may be helpful to accelerate breeding programmes by improving the efficiency of selection. To achieve this goal, we have renewed and improved the well-known half-seed method. This is based on an analytical approach that enables the selection of interesting plants through their seeds while preserving their regenerative capacity. Cotyledon tips were cut and used for gas chromatography (GC) analysis while parts that contain embryos were sown in a greenhouse and seedlings were selected according to their fatty acid profile. This method has already been used for fatty acid determination in many crops such as mustard (Kaushik and Agnihotri, 1997), flax (Bhatty and Rowland, 1990), sunflower (Conte et al. 1989) and rapeseed (Daun et al. 1983). With the recent developments in GC instrumentation, the determination of fatty acid methyl ester (FAME) could be achieved in a few minutes and a hundred samples could be analysed each day.

The main objective of this study was to highlight the major benefits of this fast method in a linseed breeding scheme. First, the reliability of the half-seed method was shown by studying a control high ALA-content genotype "Baïkal". Then, the half-seed method was applied to select low ALA-content genotypes from cross-breeding of flax genotypes contrasted for their seed ALA-contents.

Material and Methods

Plant material:

The oil flax cv. Baïkal with a high ALA-content was used as a control for preliminary studies in order to validate our method. Then, four oil flax genotypes were chosen for parental cross-breeding: a Solin line and three classical cultivars. The Solin flax cultivar L100.42 has a low ALA-content and was produced by Laboulet. Among the three classical cultivars with a high ALA-content, two varieties, Lutéa® and Lirina, are marketed while the third one, named LS-L 119, was produced by Laboulet but not marketed. The Solin flax L100.42, obtained from Laboulet Semence, was pollinated with the three classical cultivars. Flax is an autogamous plant so flowers were manually castrated in order to facilitate intermating by using the male sterility (Brim and Stuber, 1973; Mutschler and Bliss, 1980). Anthers from female lines were cut in order to avoid self-pollination. Cross-breeding was carried out using the male parental line anthers, according to the studies of Sun and Fu (1981). The first generation (F1) seeds obtained were all sown to give the second generation (F2). Seeds of the F2, around one hundred plants per breeding-cross, were harvested plant by plant and analysed by the half-seed method. Genotypes with a low ALA-content, less than 10 g of C18:3 per 100 g of FAME, were kept and sown for the next generation. This represented 10 genotypes for the breeding-cross L100.42 x Lutéa, 5 genotypes for the breeding-cross L100.42 x Lirina and 7 genotypes for the breeding-cross L100.42 x LS-L 119. Then, from F3 to F5, a classical single seed descent programme was carried out. Genotypes were selected according to agronomic traits such as yield, abiotic stress resistance and biotic stress tolerance.

Half-seed method:

Seeds were harvested at physiological maturity. The whole seed was cut into two unequal parts using a razor blade (Fig. 1). The first seed part containing the cotyledon tips (around one-third to one-quarter of the seed) was used for GC analyses while the second seed part, containing most of the cotyledons and the embryo, was sown in a greenhouse. GC analyses were carried out on seeds produced on one hundred F2 plants. The F3 seedlings were selected according to their fatty acid profiles determined from the GC analyses.

Gas chromatography

Immediately after dissection of the cotyledon tips, each sample was ground into 1 mL of hexane using a glass tube as a mortar and a homemade glass pestle and left to rest for 5 min. Triacylglycerols in the hexanic phase were directly trans-esterified by addition of 50 µL of tetra methyl ammonium hydroxide from Sigma-Aldrich (Steinheim, Germany). After 2 min, samples were centrifuged for 5 min at 2000 rpm and the supernatant was directly injected into the gas chromatograph for analysis. Samples were analysed with a Trace^{TM} GC Ultra chromatograph (Thermo Fischer Scientific, Villebon sur Yvette, France) equipped with the TriPlus autosampler and a hydrogen flame ionisation detector (FID) using a modified method according to Gontier et al. (2000). Gas chromatography was performed on a 30 m capillary column Trace TR-WAX-MS of 0.25 mm inner diameter and 0.25 µm film thickness (Thermo Fischer Scientific, Villebon sur Yvette, France). The injection was operated in PTV splitless mode. The injection temperature was 230°C and the detector temperature was 250°C. Helium was used as carrier gas with a constant flow of 2.5 mL.min^{-1}. A gradient of oven temperature from 230°C to 290°C was used (230°C to 245°C at 15°C.min^{-1}; 245°C to 290°C at 90°C.min^{-1} and 290°C for 2 min). The system was then temperature equilibrated for 30

seconds at 230°C prior to injection of the next sample. With this ultrafast method, a sample was analysed every 4 minutes. Quantification was performed using standard FAMEs (Sigma-Aldrich, France). Retention times for C16:0, C18:0, C18:1, C18:2 and C18:3 were 1.49, 1.76, 1.81, 1.89 and 2 min respectively. Linolenic acid content was expressed relative to the FAME composition.

Propagation of seedlings

After seeds had been cut in two, the seed part containing the majority of the cotyledons and the embryo was immediately sown at a depth of 2-3 cm in loam in a greenhouse. Flax plants were cultivated in a greenhouse under semi-controlled conditions: natural light day/night (between 12 h/12 h and 16 h/8 h), a mean temperature of 18-20°C, moisture content over 60% and a 16 h-photoperiod provided by sodium lamps placed every metre. Plants were regularly watered with a similar volume of water to maintain the soil water close to pot capacity.

Half-seed method validation

An initial study was completed with cv. Baïkal in order to validate the half-seed method protocol. To validate the GC step, the fatty acid profile i.e. the relative proportions of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), LA (C18:2) and ALA (C18:3), were compared between the two seed parts: (i) cotyledon tips and (ii) the majority of the cotyledons and the embryo. Then, to validate the half-seed seedlings, the germination rate between cut and uncut seeds was determined at 0, 3, 5 and 10 days after sowing.

Results and Discussion

Half-seed method validation

Before using our method in a demonstration of high-throughput screening of cross-breeding, we validated our half-seed method on seeds of cv. Baïkal. The distal end of seeds from the cv. Baïkal were cut and the two parts, cotyledon tips and the majority of the cotyledons and the embryo, were analysed for FAME determination (Fig. 1, Fig. 2). Our results showed that no significant differences were observed between the two parts of seeds in terms of oil FAME profile. Removing cotyledon tips from seeds had no effect on the fatty acid composition and the fatty acid profile was uniform in the whole flax seed. This result is in accordance with a previous study carried out on linseed by Green and Marshall (1984). Thus, FAME distal seed part analysis can be considered as representative of the whole seed fatty acid profile. In rapeseed, several studies using GC analysis of seed lipid content obtained similar results (Downey and Harvey, 1963; Thies, 1971). Nehlin et al. (1996) showed that the fatty acid composition of rapeseed embryos was similar to that of regenerated cotyledons. After showing that fatty acid composition was similar whichever seed part was analysed, we tested whether removing cotyledon tips affected the seed germination ability (Fig. 3). Our results showed no significant difference between the relative germination rate of uncut and cut seeds from which the distal part had been excised. Five days after sowing, around 9% of cut and uncut seeds had germinated. A study performed on lettuce half-seeds containing the embryonic axis and a portion of the cotyledons showed that the germinative vigour was the same in both intact and cut seeds (Scheibe and Lang, 1965). Our results have validated the two steps of the half-seed method and showed that it can be applied to screen flax plants according to their oil seed composition.

Characterisation of the four parental genotypes

The classical flax parental genotypes (Lutéa, Lirina and LS-L 119) displayed similar oil fatty acid profiles with a high content of ALA, around 65 $g.100g^{-1}$ FAME, whereas the Solin genotype L100.42 contained less than 4 g of ALA per 100 g of FAME (Fig. 4). These results showed that the proportions of palmitic, stearic and oleic acids were very similar in both classical and Solin genotypes but the LA and ALA-contents were different. The Solin genotype contained around eighteen times less ALA and five times more LA than classical genotypes. This is consistent with previous works showing that the Solin mutants had very low levels of ALA (less than 2%) and high levels of LA (more than 46%; Green, 1986). Our results confirmed that the Solin genotype had a very low ALA content and the classical flax genotypes had a high ALA content.

Selection of low ALA-content genotypes with the half-seed method

The Solin genotype (L100.42) was hybridised with the three classical genotypes (Lutéa, Lirina and LS-L119). The self-pollination of the homogenous F1 plants gave the second generation F2. The mean fatty acid profile of F2 seeds involving high ALA-content parents was similar (Fig. 5). In F2 seeds, the mean ALA content of all the genotypes was intermediate between both parental genotype contents and was around 39.9 ± 2.7 $g.100g^{-1}$ FAME. Selection of genotypes between F2 and F3 was carried out by applying the half-seed method. Distribution of ALA-content was obtained from GC analyses of F2 seeds (Fig. 6) and F2 genotypes with an ALA-content less than 10 g of ALA per 100 g FAME were sown to regenerate F3. The next generations, F3 to F5, were classically bred according to interesting agronomic

traits with a single seed descent programme. Two genotypes were kept until the F5 for the L100.42 x Lutéa breeding-cross, 5 genotypes for the L100.42 x Lirina breeding-cross and 4 genotypes for the L100.42 x LS-L 119 breeding-cross. The selected genotypes in F5 had kept a very low ALA-content, less than 5 g per 100g FAME (Fig. 5). The use of the half-seed method in a flax breeding scheme presents major benefits. Firstly, it reduces the number of plants by a generation. The half-seed method hugely reduces the number of plants moved forward as it enables an early identification of the desirable genotypes by analytical methods. Here, for three breeding-crosses, only 22 genotypes were kept in the F3 generation to obtain ultimately 11 genotypes in F5. Secondly, one or more generations are saved in the breeding process by using the half-seed method to screen flax genotypes from their seed fatty acid composition, as has already been shown in other crops (Conte et al., 1989; Jönsson, 1977). We showed that low ALA-content genotypes were selected early in the breeding scheme in one generation and time was also saved by regenerating plants from the same seeds as those used for GC analysis. Finally, with the optimisation of GC analysis, a hundred samples could be analysed every day. Analysis time could be further reduced by using a shorter column, as has been done for the analysis of complex matrices such as subcutaneous pork fat (Ficarra et al., 2010).

The half-seed method, which combines screening seeds for their oil composition determined by GC with simultaneous sowing of the remaining seeds, is clearly a very efficient and fast method of selecting flax genotypes according to their fatty acid profiles. Chunwongse et al. (1993) previously highlighted the great usefulness of the half-seed method for a variety of applications in plant breeding and genetic studies. In our study, we focused on the benefits of this method for the selection of low ALA-content genotypes, but it can also be applied to the selection of genotypes with other seed fatty acid compositions or to the selection of other species.

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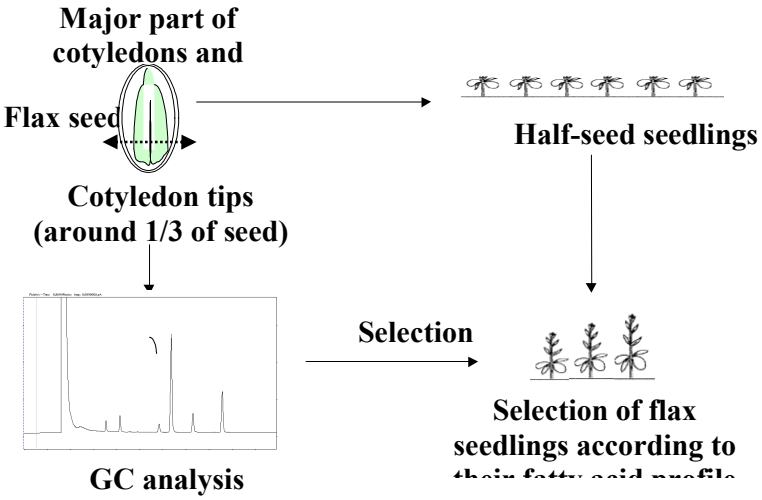

Figure 1. Diagram of the half-seed method. Flax seed was cut into two parts: the first part contains the major part of the cotyledons and the embryo and the second part contains the cotyledon tips. The part containing the embryo was sown and cotyledon tips were used for gas chromatography (GC) analysis. Flax seedlings were selected depending on their agronomic traits and their fatty acid profile.

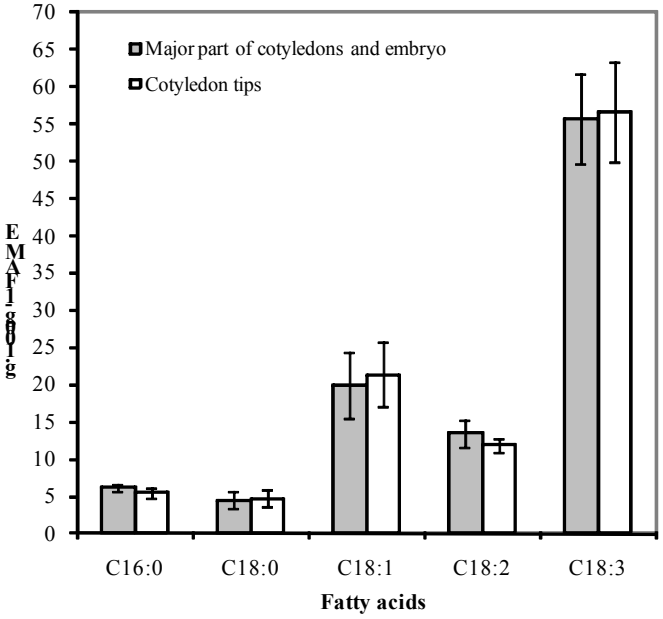

Figure 2. Comparison of the fatty acid profile of the main part of the Baïkal seed, containing the majority of the cotyledons and the embryo, and the cotyledon tips. The abundance of each fatty acid is expressed relative to FAME. The values are means of 10 seeds ± SE taken from a batch of certified seeds. A Student Newman Keuls test ($P<0.01$) showed that means did not differ between both seed parts.

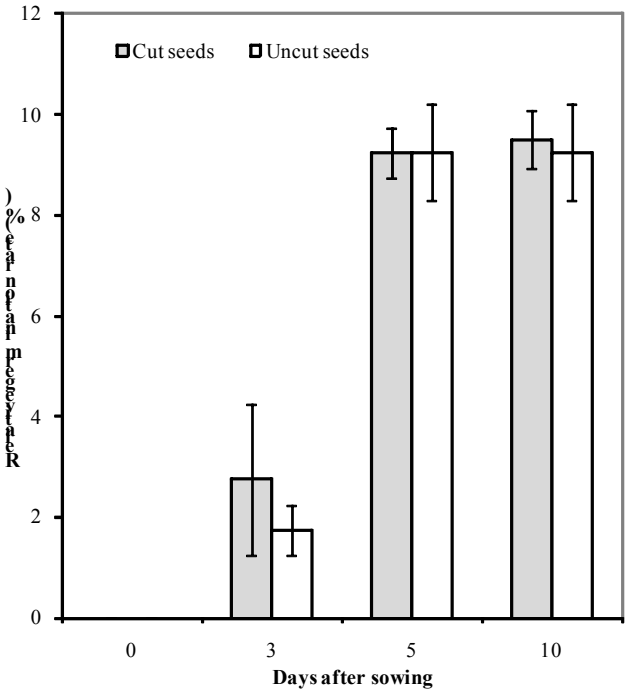

Figure 3. Comparison of the relative germination rate between cut and uncut Baïkal seeds. The values are means of 100 seeds ± SE. A Student Newman Keuls test ($P<0.01$) showed that means did not differ between cut and uncut seeds.

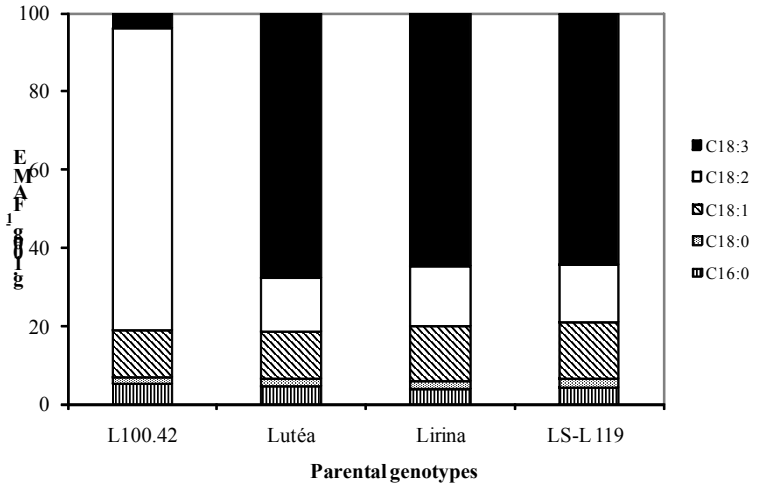

Figure 4. Proportions of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (LA; C18:2) and linolenic (ALA; C18:3) acids in linseed oil of the four parental genotypes expressed as a proportion of fatty acid methyl ester.

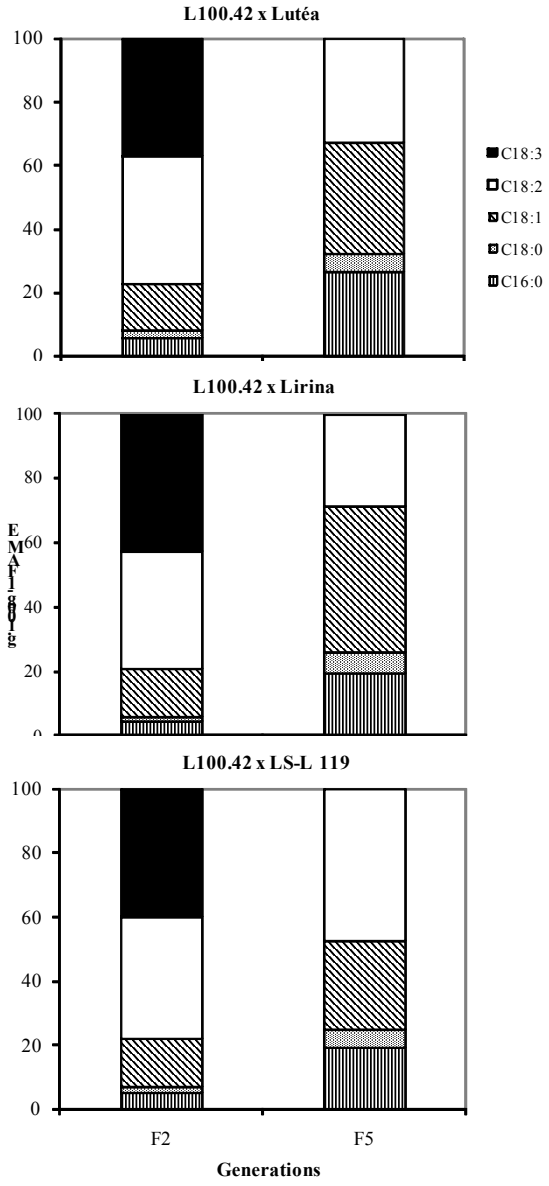

Figure 5. Relative mean fatty acid profiles (C16:0, C18:0, C18:1, C18:2, C18:3) in F2 and F5 seeds of the crosses L100.42 x Lutéa, L100.42 x Lirina and L100.42 x LS-L 119.

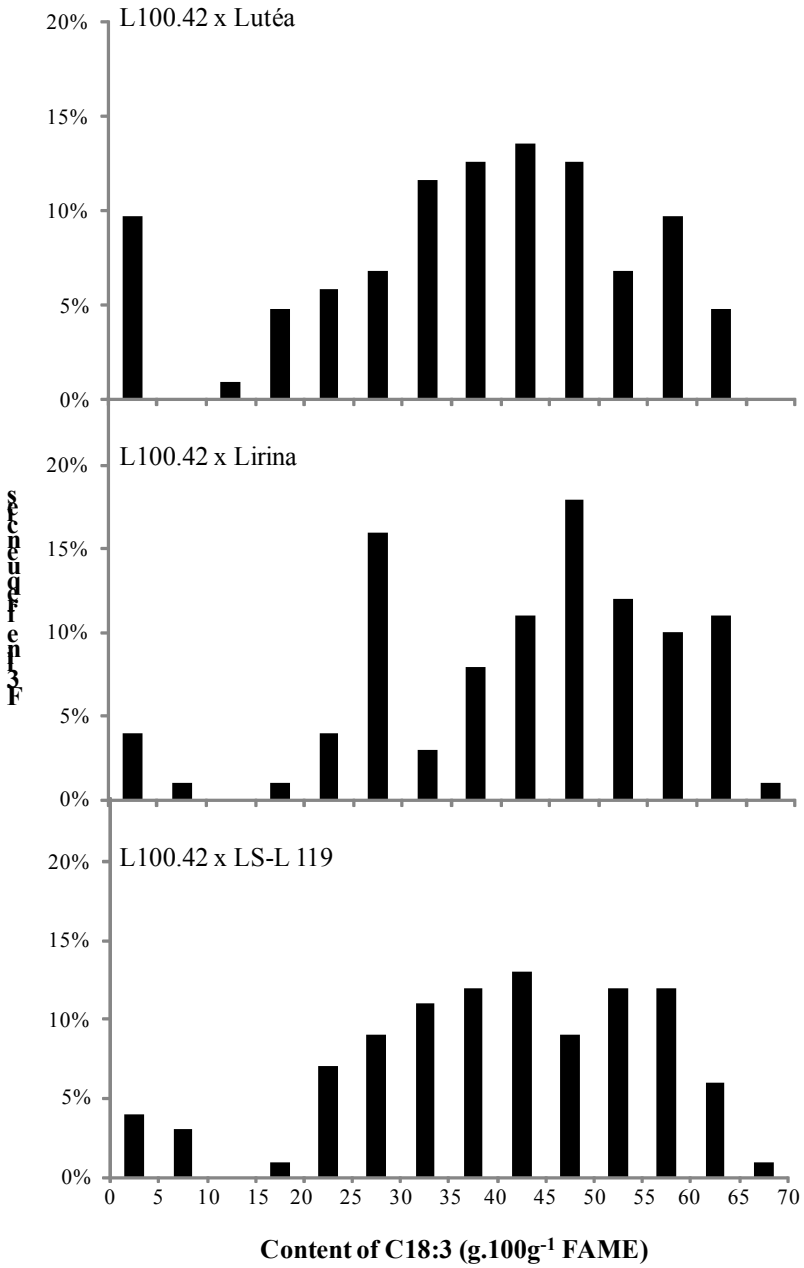

Figure 6. Linolenic acid content distribution of seeds F3 from F2 plants of the three breeding-crosses L100.42 x Lutéa, L100.42 x Lirina and L100.42 x LS-L 119. The first cross contained 103 genotypes and the following two contained 100 plants each.