UNIVERSITÀ DEGLI STUDI DI UDINE

Dottorato di Ricerca in Scienze e Biotecnologie Agrarie
Ciclo XXV
Coordinatore: prof. Mauro Spanghero

TESI DI DOTTORATO DI RICERCA

Inheritance of the high oleic trait and environmental effects on seed fatty acid composition in High Oleic Sunflower (*Helianthus annuus* L.)

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ANNO ACCADEMICO 2012/2013

Non passo notti disperate su quel che ho fatto o quel che ho avuto:

le cose andate sono andate ed ho per unico rimorso le occasioni che ho perduto...

Francesco Guccini

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Summary

Inheritance of the high oleic trait and environmental effects on seed fatty acid composition in High Oleic Sunflower (Helianthus annuus L.)

By

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High Oleic Sunflower oil has a wide range of applications, such as in the food industry and as raw material for non-food applications. The development of a high oleic sunflower mutant was first reported by the Russian researcher K. I. Soldatov (1976), who developed the first stable high oleic acid open-pollinated cultivar "Pervenets" through chemical mutagenesis with dimethyl sulfate (DMS) and further selections for high oleic acid content. High temperature enhances oleic acid content in the oil of normal or low oleic cultivars but conflicting results are reported about temperature effects on oleic acid content of high oleic acid cultivars: either no effect or an increase in oleic acid content with temperature. Researches on genetic control of the high oleic mutation led to several hypotheses on the number of genes (major genes, modifier genes and suppressors), on their dominance and maternal influence on the trait. Genetic variability among HO lines could be of importance to breeders working in the industry of high oleic types. A goal in HO breeding for industrial use is to select hybrids with an oleic acid content higher than 90% and stable across environments.

The approach to studying the inheritance of oleic acid content was based only on the cross between HO inbred lines. Thus, *Ol* (or Pervenets Allele) was homozygous. All variation in oleic acid content observed across generations was due to others genetic factors.

To investigate the effects of temperature in high oleic genotypes under natural field condition, a three-year field trial was carried out with two dates of sowing and three HO genotypes in Udine. Oleic and linoleic acids content was influenced by temperature in two genotypes (one inbred line and in the hybrid) while the other inbred line was insensitive to temperature. There was an increase

of about 3% in oleic acid content from 400 to 500 Growing Degree Days (accumulated from flowering to 25 days after flowering).

The HO inbred lines tested differed for some of the alleles that condition their high oleic content. The use of reciprocal hybrids and backcrosses led to the hypothesis that two genetic factors (one major gene and a combination of modifier genes), in addition to Pervenets allele, affected oleic acid content in HO genotypes. The cytoplasm masks the effects of this second major gene, designated as Ol_s. It could be a second FAD2 gene. The temperature may affect segregation ratio or, in other words, the phenotypical expression of some genes only in the male-fertile cytoplasm. Maternal effects could modify phenotypic expression of some genes and consequently the 90% threshold in oleic acid content.

In the F_2 plant generation some individual seeds showed a low oleic phenotype (oleic acid content <55%). In seeds collected from one plant a longitudinal gradient in oleic acid content was found from embryo to upper cotyledons. A maternal phenotypic effect on the HO trait was suggested: a third recessive gene, designated as Ol_l, acting on oleic acid content in the HO phenotypes. Temperature seems to modify the phenotypical expression of some genes.

It seems that three elements, two major genes and a combination of modifiers, in addition to the Pervenets allele, were involved in the genetic control of high oleic acid content.

Segregation patterns led to several hypotheses of gene interactions: epistasis, suppression or duplicate genes. Results suggest that epistasis and/or duplicate genes are the most probable type of gene interactions.

To obtain environment-insensitive hybrids, selection could be based on inbred lines that do not show any phenotypic variation in oleic acid content across years and locations. It was observed that the cytoplasmic effect might have an important role in the genetic control of these traits. Cytoplasmic effect could be used by breeders to obtain stable HO hybrids, insensitive to the environment. Selection for increased oleic acid composition on single seed basis and on half-seed technique in early self-pollinated generations should be avoided. Selection on single seed basis or on single plant basis should be made with increasing of inbreeding level. Recombinant inbred lines with a LO phenotype developed from the crosses between HO inbred lines could be used as tester lines to select against negative factors new High Oleic lines. To obtain hybrids with a content in oleic acid higher than 93-95% it is necessary to select inbred lines with a low content in saturated fatty acids.

Further studies are needed to elucidate the nature of suppression for Pervenets mutation effect, the number of these suppressors, the number of modifier genes and their interaction with the other genetic elements.

Key Words: High oleic acid content, Genetic Control, Maternal Effects, Environment

Aims of the work

The topic of research presented in this thesis is the evaluation of environmental and maternal effects on oleic acid content and its inheritability in High Oleic Sunflower. The environmental effects have been studied by using two sowing dates in different years. The effects of temperature are considered. Maternal effects have been studied by using reciprocal crosses and backcrosses between High Oleic inbred lines. Finally, inheritance of high oleic acid content has been studied by analyzing parents, their F_1, F_2 and partially F_3.

The main goal of this thesis is to determine that factors affect oleic acid content in HO genotypes and, consequently, to give some indications for HO sunflower breeding procedures.

1 Introduction and literature review

Sunflower (*Helianthus annuus* L.) is an annual species belonging to subtribe Helianthinae, subfamily Asteroideae and family Compositae (Seiler and Rieseberg, 1997). Sunflower originated in northern Mexico and south-western USA and domestication occurred about 3000 B.C. by the Native American Indians.

It was introduced into Spain from North America for floral decoration in the 16th century. Massal selection for oil content was first carried out in Russia and, through continuous breeding efforts, inbred lines with a 50% oil content were obtained.

There are three types of sunflower. These include oilseed type, non-oilseed type and ornamental sunflower. However, the production of sunflower is mainly devoted to oil extraction (Dorrell and Vick, 1997). With the discovery in France of a cytoplasmic male sterility system based on PET1 (Leclercq, 1969), and fertility restoration from a wild sunflower (Kinman, 1970), hybrid sunflower varieties have become predominant onto the market to produce seed oil. This characteristic revolutionized the sunflower industry by producing high-quality hybrid sunflowers.

Sunflower oil is the fourth most important vegetable oil in world trade at present with an annual production of around 9 million tons and a cultivated acreage of over 23 million hectares, mainly concentrated in the Russian Federation, Ukraine, India, and Argentina, which totalize more than 50% of sunflower world acreage (Faostat, 2012). In Italy, sunflower is cultivated on acreage of 110 thousand hectares, mainly concentrated in three Italian regions in central Italy: Marche, Umbria and Tuscany (Istat, 2012). The new European Federation with 27 countries is become the first sunflower producer (Romania, Bulgaria, Hungary, Spain, and France).

Oilseed sunflower has many applications in both the non-food and food industry. However, the use in non food industry or for human consumption required oils with different fatty acid composition (Vannozzi, 2006). Different mutagens (physical and/or chemical mutagenic agents) have been used to develop these mutants with a high content in saturated fatty acids (palmitic and stearic acids) and/or with a modified ratio between unsaturated fatty acids (oleic/linoleic ratio).

1.1   MUTANTS FOR FATTY ACID PROFILE

The properties of a vegetable oil are determined by the fatty acid composition of its lipids. Seed oil from cultivated sunflower is comprised primarily of the saturated fatty acids palmitic (16:0) and stearic (18:0) acids, and the unsaturated fatty acids oleic (18:1), linoleic (18:2) and linolenic (18:3) acids (Dorrel and Vick, 1997). Typically up to 90% of the fatty acids in conventional sunflower oil are unsaturated, namely oleic (16%-19%) and linoleic (68%-72%) fatty acids. Sunflower oil is

premium oil because of its relatively high level of unsaturated fatty acid contents. However, oils with different fatty acid composition are required depending on their use in industry (Vannozzi, 2006) or for human consumption.

Advances in modern genetics, most importantly induced mutations, have altered the fatty acid composition of sunflower oil to a significant extent. Different mutagens (physical or chemical mutagenic agents or combined treatment) and methods have been used to develop these mutants with an increase in saturated fatty acid and/or with a modified oleic/linoleic ratio.

With regard to the oil with a modified oleic/linoleic ratio, the most important mutations have been obtained by treatment with dimethyl sulfate (DMS), which produced genotypes with more than 80% oleic acid (Soldatov, 1976). Mutants have also been obtained that have a high linoleic acid content (>80%) by treating seeds with X-rays and ethyl methanesulfonate (Skoric *et al.*, 2008).

A second landmark in the genetic improvement of sunflower oil quality was the discovery of mutants with increased levels of saturated fatty acids, either with high palmitic acid (>25%) or high stearic acid (>25%) content (Ivanov *et al.*, 1988; Osorio *et al.*, 1995; Fernández-Moya *et al.,* 2002; Velasco *et al.*, 2008). For instance, as far as the use of mutagenic agents is concerned, treating sunflower seeds with γ- and X-rays has produced mutants with 25%-30% palmitic acid. Sunflower seed treatment with X-rays has also resulted in mutants having 30% palmitoleic acid, while treatments with mutagenic sodium azide have produced seeds containing 35% stearic acid (Skoric *et al.*, 2008).

1.2   HIGH OLEIC SUNFLOWER

Sunflower genotypes are often classified according to the potential oleic acid percentage in their oil. Oleic acid percentage in oil is 10–50% in traditional or low oleic or normal types, 50–80% in mid oleic types and more than 80% in high oleic types.

The development of a high oleic sunflower mutant was first reported by the Russian researcher K.I. Soldatov (1976), who developed the first stable high oleic acid open-pollinated cultivar "Pervenets" through chemical mutagenesis with dimethyl sulfate and further selections for high oleic acid content. The Pervenets mutant came from only one pollen grain and it has been screened as a dominant mutation (Lacombe and Bervillé, 2000). It was used worldwide as a high oleic acid content source in breeding programs to produce High Oleic (HO) lines and commercial hybrids with an oleic acid content over 80%. In practice now, all HO lines derived from the mutant population Pervenets display seed oil with the oleic acid content HO over 80%. In other words, all

High Oleic genotypes (Hybrids and Inbred lines) have the same High Oleic Source, the Pervenets mutant. The HO genotype carries not only the Pervenets mutation but also different factors that affect oleic acid content.

The high oleic trait is seed-specific and the mutation reduced the expression of a Δ12-desaturase gene (Garcés and Mancha 1989, 1991; Kabbaj *et al.* 1996; Hongtrakul *et al.* 1998; Martínez-Rivas *et al.*, 2001). The seed specific microsomal oleate desaturase encoded by the FAD2-1 (Fatty Acid Desaturase) gene was completely active, however, the level of gene expression was drastically reduced (Martínez-Rivas *et al.*, 2001). The microsomal oleate-desaturase (MOD) mRNA accumulation is reduced during the grain filling period, compared to the LO genotypes, leading to a decrease of microsomal oleate-desaturase activity in the seeds during lipid reserve elaboration steps (Hongtrakul *et al.*, 1998; Lacombe and Ber;villé, 2000).

More recently, Lacombe *et al.* (2009) demonstrated that Pervenets allele is organized in two parts: the first section present in both HO and LO genotypes carries a normal microsomal oleate desaturase gene, the second section is specific to HO genotypes and carries a part of MOD duplications. The study of mRNA accumulation in LO and HO seeds revealed that the mutation is expressed in trans and induces an oleate desaturase mRNA down-regulation. Furthermore, oleate desaturase small interfering RNA (23bp), characteristic of gene silencing, accumulated specifically in HO seeds.

Silencing mechanism is dominant (Lacombe *et al.*, 2009) and acts in trans in F_1 hybrid plant heterozygous for the Pervenets mutation. The absence of MOD oleate desaturase transcript explains the absence of the MOD oleate desaturase activity and consequently the high oleic traits (Ber;villé *et al.*, 2006).

1.2.1 Genetics

1.2.1.1 Origin of Pervenets mutant

Soldatov reported the development of a sunflower with high oleic acid content in 1976. About 200 sunflower heads from the population VNIIMK 8931 were treated with a 0.5% solution of dimethyl sulfate, a chemical mutagen. Thirty M_1 seeds from each of the 200 sectors were sown on one lane, plants were self-pollinated and the operation was repeated until the M_3 generation. To detect eventual fatty acid mutants, Soldatov collected pollen on 10 different M_3 progenies to pollinate one normal plant. Thus, Soldatov repeated this operation 200 times. He sowed 30 seeds per pollinated head and after self-pollination of each plant he used 30 seeds per plant to extract oil. Thus, he

analysed 6,000 oil samples per iodine method or gas chromatography and only one displayed an Oleic Acid Content (OAC) 50.3% higher than those of other plants. He sowed the remaining seeds from the plants that had such an OAC (50.3%) and self-pollinated each of them. He used again 30 seeds per head to extract oil and mixed the seeds from all the progenies, which displayed an OAC higher than 40%. Thus, he constituted the Pervenets population. After two cycles, by intercrossing plants in Pervenets, he enhanced the average OAC of Pervenets to 65% (range 60 to 80%). It was found that the high oleic content was stable and well maintained in the next generations, as indicated by a close relationship of plants with high oleic content and their progeny (r = 0.67). By bulking the superior plants with high oleic content, the Pervenets variety was created and released to producers in Russia by the VNIIMK research centre. The Pervenets variety had an oleic acid content of approximately 70-75%, whereas the oleic acid content of the original VNIIMK 8931 was 30-35%.

1.2.1.2 Genetic Studies

The high oleic trait in sunflower is complex and no general agreement on how oleic acid is inherited has been reached. In general, genetic modifications that alter the fatty acid profile have been found to be qualitative rather than quantitative. This means that they are controlled by a low number of genes and are less affected by the environment than quantitative traits such as oil content. They are mostly determined by the genotype of the developing embryo with little or no maternal influence.

It has never been considered if there is only one mutation in Pervenets or several mutations, all leading to a HO phenotype (Lacombe and Bervillé, 2000). However, no evidence for several mutations occurring in Pervenets is reported in literature (see Table 1).

Various levels of dominance were reported in different publications (Table 1). The HO trait may behave as a dominant, recessive or semi-dominant trait. In the subsequent generations, depending on the progenies, the HO trait may disappear, segregate as a Mendelian factor, or submitted to strong distortion in the segregation pattern either as an excess of LO or an excess of HO individuals (Lacombe and Bervillé 2000; Bervillé *et al.*, 2006). The behavior of the mutation depends on the background of the classical sunflower lines crossed onto the Pervenets source (Bervillé *et al.*, 2006). Some genetic variation for oleic acid content appeared in interaction with Pervenets mutation (Varès *et al.*, 2002).

The first genetic analyses on the high oleic sunflower mutant concluded that the high oleic acid trait was controlled by a partially dominant or dominant (Fick, 1984; Urie, 1985) single gene designated *Ol*. Urie (1985) detected also the presence of modifiers as well as an unexplained reversal of the

dominance of the *Ol* gene and he concluded that some LO parent lines do and some do not carry major factors and/or modifiers causing deviation. Miller *et al.* (1987) reported the action of a second gene, designated *Ml,* that appeared to modify the oleic acid content. These Authors found in F_2 seed a trimodal distributions for oleic acid content. An intermediate class was clearly evident, ranging from 48% to 72% in oleic content. The high oleic class ranged from 82% to 92%, whereas the low oleic class was similar to HA 89 (LO parent) and ranged from 11% to 18%. The number of seeds in the intermediate class was too large to support a single, dominant gene theory. However, this study did confirm the presence of a major gene with partially dominant gene action, as reported by Fick (1984) and Urie (1985). This gene produced oleic composition levels of 60-75% in seed oil. Therefore, a second locus, designated *Ml,* appears to modify the oleic content, when the recessive allele, *mlml,* and combined with the gene *Ol,* oleic levels in seed were 82% or higher.

Fernández-Martínez *et al.* (1989) proposed a model based on the presence of three dominant, complementary genes, designated Ol_1, Ol_2, and Ol_3. These Authors found by the analysis of F_2 and backcrossed seeds three kind of segregations, in both F_2 and backcrossed populations, with different proportions of low, intermediate and high oleic types. They reported in F_2 the following segregation patterns: 1:3, 7:9 and 37:27 for high oleic and low-intermediate classes. Genetic analysis of these data supported the hypothesis that the high oleic trait is controlled by three dominant complementary genes. Additional data showing F_3 seeds with intermediate oleic content and segregations for high oleic in progenies of intermediate types suggest the presence of major factors modifying high oleic acid content.

Demurin and Škoric (1996) could not confirm any of the previous hypotheses and concluded that the *Ol* locus exhibited genetically unstable expression.

Fernandez *et al.* (1999) reported also a two gene model, but they proposed that the recessive allele *ol* increases oleic acid content and the dominant *Ml* allele controls the feedback mechanism maintaining oleic/linoleic content ratio at the physiological level, in relation to the temperature during the seed maturation. They advanced the hypothesis that *Ml* is a polygene or a gene complex which works by a series of single genes closely influenced by temperature.

Only Demurin *et al.* (2000) reported the existence of another allele of *Ol* gene. According to these Authors a recessive allele of *Ol* gene, designated ol[1], controlled the mid-oleic acid content.

The first genetic study on oleic acid inheritance conducted under controlled environment was carried out by Alonso (1988). The author found a great effect of temperature on oleic acid levels in all generations. In all cases the conclusion was that oleic acid content was controlled by a single

gene *Ol*, which depending on the temperature can act either as dominant or partially dominant. No additional genes or modifiers were detected. A second genetic study under controlled environment was carried out by Velasco *et al.* (2000). They reported the existence of five genes controlling oleic acid content, designated Ol_1, Ol_2, Ol_3, Ol_4 and Ol_5. The genotypes Ol_1Ol_1, $Ol_1ol_1Ol_2Ol_2$ and $Ol_1ol_1Ol_2ol_2$ were high in oleic acid, whereas the genotype ol_1ol_1 had a low oleic phenotype. Conversely, the genotype $Ol_1ol_1ol_2ol_2$ could exhibit low, medium, or high oleic acid content depending on the Ol_3, Ol_4 and Ol_5 genes. They speculated that the phenotypic expression of some of these genes is dependent on the temperature during seed maturation and therefore the genetic hypothesis might not be useful to understand segregations for oleic acid under different conditions. The works done by Lacombe *et al.* (2000; 2001; 2002a,b; 2004) with molecular technique indicated that the high oleic acid content was directed by two independent loci, a locus carrying the oleHOS allele (that is exclusively correlated to the high oleic acid content status of the genotype) and another locus that carries a suppressor allele (supHOAC) that also directs the high oleic acid trait. All the HO lines derived from the Pervenets mutant carry a specific RFLP (oleHOS) revealed by an oleate desaturase cDNA used as a probe. The Low Oleic genotypes do not carry oleHOS, but another allele: oleLOR. They studied HO heredity in two segregating populations. In an F_2 population, the HO trait co-segregated with oleHOS. In a Recombinant Inbred line (RI) F_6 population, all HO RI lines carried oleHOS. The RI lines carrying oleHOS were either LO or HO (1:1 − low to high). The absence of HO RI lines with oleLOR eliminated the occurrence of a recombination event between the locus carrying oleHOS and the locus carrying the Pervenets allele. Thus, the HO trait is due to 2 independent loci: the locus carrying oleHOS allele and another locus. One allele at this other locus may suppress the effect of the oleHOS allele on the HO trait. Therefore, depending on the supHOAC allele in the segregating population, the high oleic acid trait is controlled by one or two loci. The suppressor allele could disturb the conventional segregating pattern for high oleic to linoleic acid and therefore other observations that reject the dominance of the high oleic acid trait might be due to segregation at this locus (Lacombe *et al.*, 2001).

1.2.1.3 Modifier genes

The combined experience of public and private sunflower researchers has led them to suspect that several modifier genes are present in inbred lines of sunflower, each having an effect on the intermediate level of oleic acid in hybrids (Vick and Miller, 2002).

Fig. 1. F_2 segregation for gamma-tocopherol content in absence (A) or presence (B) of modifiers from a cross Low gamma-tocopherol x High gamma-tocopherol. F_2 segregation for oleic acid content in absence (C) or presence (D) of modifiers from a cross Low oleic acid x High oleic acid. (Source: Velasco *et al.*, 2012).
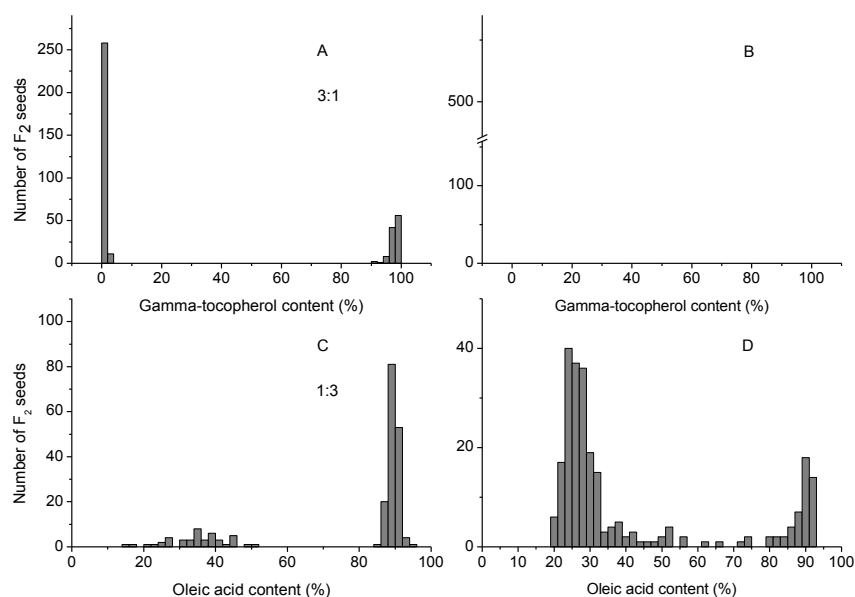
Modifying genes, also known as modifier genes or simply modifiers, are minor genes having no known effect except to intensify or diminish the expression of a major gene (Briggs and Knowles, 1967; Allard, 2001). The effect of modifiers is well known in some annual leguminous plants on seed mottling levels (Allard, 2001). Also in soybean, a role of modifying genes acting on oleic acid content was proposed (Alt *et al.*, 2005).

For sunflower oil quality traits, modifiers were first reported for the high oleic acid trait (Urie, 1985; Miller *et al.*, 1987). Breeders are making a beneficial use of modifiers to break complete dominance of high oleic acid in order to develop hybrids producing mid-oleic acid oil (Velasco *et al.*, 2012). Modifiers are not a phenomenon specific to high oleic acid, but they have been found to influence most of the oil quality traits developed (Velasco *et al.*, 2012). The presence of modifiers hinders breeding for oil quality traits.

As shown in Fig. 1, modifiers exert a dramatic effect upon the expression of alleles controlling modified oil quality traits. The genetic bases underlying modifiers action are poorly understood. Recently, García-Moreno *et al.* (2012) identified four modifiers of *tph2*, which underlies a gamma-tocopherol methyltransferase (gamma-TMT) enzyme, on four different linkage groups of sunflower genome and demonstrated that in most cases they corresponded to duplicated copies of gamma-TMT.

1.2.1.4 HO Suppressor or Suppressors

Suppression is a type of gene interaction. A suppressor is an allele that reverses the effect of a mutation of another gene, resulting in the normal (wild-type) phenotype. For example in sunflower, the action of a suppressor on Pervenets allele (HO mutant phenotype) could originated a LO phenotype (wild-type). Both recessive and dominant suppressors are found, and they can act on recessive or dominant mutations (Griffiths *et al.*, 2000). Suppression is sometimes confused with epistasis. However, the key difference is that a suppressor cancels the expression of a mutant allele and restores the corresponding wild-type phenotype while epistasis is inferred when an allele of one gene masks the expression of the alleles of another gene and expresses its own phenotype instead (Griffiths *et al.*, 2000).

The action of a suppressor on the Pervenets was first proposed by Lacombe *et al.* (2001). Suppressors may affect the expression of the silencing mechanism that may cause to save oleate desaturase transcript and thus to restore the LO trait (Bervillé *et al.*, 2006). Demurin (2003) and Demurin and Borisenko (2011b) verified the existence of suppression in normal and HO genotypes. Demurin and Borisenko (2011a) reported that the normal line RIL100 contained a high oleic mutation Ol in hypostatic condition (a suppressor masked HO phenotype). Inheritance of the high oleic mutation in the crosses of VK508 (HO) with suppressors (LO lines that carried a suppressor) in F_2 fitted a digenic model of epistatic action of Sup over Ol in the ratio of 13 normal (LO): 3 mutant (HO). L26 (HO) line showed resistance to suppressor with complete dominance of Ol mutation in the F_1 and monogenic inheritance (3:1 of HO to normal) in the F_2 when LG 26 was crossed with suppressor-carrying lines. Suppression seems to be common in several sunflower genotypes.

1.2.1.5 Other Desaturase genes

Another important issue is the following: how many FAD2 genes are there in sunflower? In sunflower three-different FAD2 (Fatty Acids Desaturase) were identified. One of these, FAD2-1, was expressed specifically in seeds (Martinez-Rivas *et al.*, 2001) and temperature may regulate this enzyme both by altering its expression and by regulating its activity. In HO sunflower the FAD2-1 gene was completely active, however the level of gene expression was drastically reduced (Martinez-Rivas *et al.*, 2001). Recently, García-Moreno *et al.* (2012) demonstrated that unstable expression of high gamma-tocopherol content in sunflower seeds was due to interaction between duplicated loci that revert the high gamma-tocopherol phenotype to intermediate-low gamma-tocopherol values. Duplicated FAD2 gene have been found in other oil crops (e.g. Schlueter *et al.*, 2007; Jung *et al.*, 2000). In soybean, FAD2 is a gene family (with duplicate genes) that consists of

at least five members (Schlueter *et al.*, 2007). It is known that duplicate genes provide alternative genetic determination of a specific phenotype (Griffiths *et al.*, 2000). At present, the literature contains no information on the presence of FAD2 duplicate genes in sunflower.

In conclusion, there is a general agreement on the presence of a major *Ol* gene controlling the high oleic acid content trait, but this trait is complex and involves several modifying genes and suppressors of HO phenotype whose number and function still need to be determined (Lacombe *et al.*, 2001; Pérez-Vich *et al.*, 2002; Demurin and Borisenko, 2011b). The level of dominance may depend on genetic background. Studies by Fernández-Martínez *et al.* (1989), Demurin and Skoric (1996), Velasco *et al.* (2000) and Pérez-Vich *et al.* (2002) confirmed the reversal of dominance that was first mentioned by Urie (1985). Furthermore, interpretative differences in these studies may be due to background genes in the parental lines used (suppressors), environments utilized to test the segregating generations and the number of modified genes present in the breeding material (Miller, 1992; Miller and Fick, 1997; Triboï-Blondel *et al.*, 2000). Finally, the molecular and genetic nature of the high oleic acid trait is still not well understood.

This lack of understanding causes difficulties in directing the conversion of traditional linoleic lines into high oleic lines during breeding programmes. Understanding the genetic control of HO trait will help to find solutions and more effective breeding procedures.

Table 1. Summary of the genetic studies dealing with the oleic acid content trait in sunflower (modified from Lacombe and Bervillé, 2000 and Varés *et al.*, 2002).

HO Line	LO Line	Dominant/recessive/ incomplete dominance	Maternal effect	Number of major (M) gene with or without modifiers	References
Pervenets selection		Incomplete dominance	Not checked	1M	Fick, 1984
Pervenets selection	P21	Dominant	No	1M + modiefer	Urie, 1985
Pervenets selection	HA89	Incomplete dominance	maternal influence	1M + modiefer	Miller *et al.,* 1987
Pervenets selection		Dominant	Not checked	1 M	Schmidt *et al.,* 1989
Pervenets	Cms HA89	Dominant	No	3M additive + modifiers	Fernández-Martínez *et al.*, 1989
AO-P-1	Cms HA89	Dominant	No	3M additive + modifiers	Fernández-Martínez *et al.,* 1990
6 different HO line	6 different LO line	Dominant but also sometimes recessive	Not checked	3 hypotheses with increasing gene numbers. gene *Ol* with incomplete penetrance determined by genotypic epistatic factors of reversion	Demurin and Skoric, 1996; Demurin, 2003
HAOL9	ROL71	Dominant but also sometimes recessive	Not checked	1 M + modifiers not clear	Dehmer and Friedt, 1999
R 978	HA89	Recessive	Not checked	2 interacting genes: Single recessive gene and recessive modifier (gene complex)	Fernandez *et al.,* 1999
7 different HO lines	3different LO lines	Dominant	Reciprocal effect	Not addressed	Varès *et al.,* 2000; 2002
HAOL-9	High stearic mutant CAS-3	1 Dominant	Not checked	Major QTL (85 % EV)	Pérez-Vich *et al.,* 2000
HO line from Monsanto	LO line from Monsanto	1 Dominant	Not checked	1 locus = Δ12 RFLP	Lacombe *et al.,* 2000
Different HO lines	Different LO lines	Dominant	No effect	Not addressed	Lacombe *et al.,* 2000
HAOL9	HA89	Complex some maybe dominant	No	Five genes + modifiers	Velasco *et al.,* 2000
LG-27	HA89A OL	Dominant and recessive but also sometimes recessive	No effect	1 Major locus with 3 alleles	Demurin *et al*., 2000
line BE78079 from Monsanto	line BD40713 from Monsanto	Dominant	Not checked	2 M (1 epistatic suppressor) + modifier (combination of)	Lacombe *et al.*, 2001; 2002a,b ; 2004
LG 26	LO suppressor-carrying lines	Dominant	Not checked	2M (1 epistatic suppressor)	Demurin and Borisenko, 2011
VK508	VIR721	Dominant	Not checked	Dominant epistatic action of the suppressor	Demurin and Borisenko, 2011

1.2.2 Maternal effects

Variation in an individual's phenotype may be determined not only by the genotype and environment of that individual but also by maternal effects. In this work, the terminology used to define the maternal effects is that proposed by Roach and Wulff (1987).

Roach and Wulff (1987) defined maternal effects as the contribution of the maternal parent to the phenotype of its offspring beyond the equal chromosomal contribution expected from each parent. Maternal effects in plants may also affect phenotypic expression of traits in subsequent generations and they can have a profound impact on selection, especially if selection is based on the phenotypic performance of seeds produced on a plant. Understanding how maternal effects influence selection can allow for the development of more efficient breeding strategies and an increase in genetic gain (Gilsinger *et al.* 2010).

Roach and Wulff (1987) described three types of maternal effects and classified them as cytoplasmic genetic, endosperm nuclear, and maternal phenotypic.

The cytoplasm is strictly maternally inherited (Rieseberg *et al.*, 1994) in sunflower. For instance, cytoplasmic male sterility (CMS) is a maternally inherited trait in sunflower (Rieseberg *et al.*, 1994; Miller and Fick, 1997). In the botanical context, cytoplasmic genetic maternal effects occur when the maternal parent passes, to her offspring, genes located in the cytoplasm that affect a certain trait. When these non-nuclear genes are passed from one generation to the next, the phenomenon is called cytoplasmic inheritance (Miko, 2008). Non-nuclear effects may play an important role in determining the phenotype of hybrids and thus breeding procedures. In self-pollinated species, these effects are heritable and normally are detected in each successive generation of inbreeding, unless cytoplasmic × nuclear genetic effects exist. In this case, the effects may dissipate or appear, depending on the interaction.

Endosperm nuclear maternal effects can occur as a result of 3N endosperm having two nuclei from the maternal parent. This can give rise to a higher dosage of maternal genes and is sometimes referred to as dosage maternal effects. In sunflower endosperm nuclear maternal effects were negligible (Seiler, 1997).

The third class of maternal effects are maternal phenotypic or maternal influence, resulting from the environment and/or genotype of the maternal parent itself. These influences may occur via structure or physiology and may be the most common type of maternal influence. In high stearic sunflower, Fernández-Moya *et al.* (2003) reported a mother plant control on high stearic trait in the high-stearic temperature-dependent mutant line CAS-14. These authors speculated that the lower expression of the stearate desaturase (high content in stearic acid at high temperature) was due to the existence of

a thermosensitive element in the fatty acid biosynthesis regulatory cascade originating from the maternal plant during capitulum and seed development. Gilsinger *et al.* (2010) found in soybean that offspring's phenotype was affected by maternal plant. Maternal effects between reciprocal crosses dissipated when soybean seeds were grown in vitro, while significant differences between the parents were maintained. This is evidence that factors translocated from the maternal plant may be causing the maternal effect.

It is also important to realize that observed maternal effects could be the result of any one or a combination of the maternal effects just described.

Cytoplasmic influences are normally detected via reciprocal crosses since, in sunflower, cytoplasmic genes are inherited maternally. If cytoplasmic effects are present, significant differences should be observed among reciprocal crosses in successive generations. If consistent effects are not observed (diminishing effects with inbreeding), likely causes are nuclear × cytoplasmic interaction effects, short-lived maternal effects, or Type I error.

The reciprocal hybrid (F_1) seeds would normally be phenotypically different from those obtained from self-pollination in female parents, because they have different genotypes. If F_1 seeds from reciprocal crosses have a phenotype equal to that of self-pollinated seeds of the maternal parents, than maternal effects occur. In the absence of maternal phenotypic and/or cytoplasmic effects, it is also expected that reciprocal F_1 have the same phenotype because they have the same genotype. If they are different, there could either be cytoplasmic inheritance, maternal phenotypic effects, or both**.** The absence of significant differences in the means of F_1 plants is not necessarily evidence that cytoplasmic effects are absent (Gilsinger *et al.*, 2010). Reciprocal effects due to the environment should produce significant differences between reciprocal crosses, but diminish in the subsequent generations or environments. However, environmental maternal effects on progeny phenotype often appears to be transitory.

Cytoplasmic and maternal effects may be distinguished by comparing F_2 seeds borne on reciprocal F_1 plants (Knowles and Mutwakil, 1963). Under the assumption that F_1 plants and seeds from reciprocal crosses have the same genotype on average, it is expected that they will have the same mean value for the trait under study whether maternal effects are present or not. If differences exist between reciprocal F_2 populations, they would be expected to be due solely to cytoplasmic effects (Mosjidis and Yermanos, 1984). The results obtained with these procedures may be influenced by environmental factors such as flower manipulation at the time crosses are made, and temperature during seed development.

If the parental lines used are very different in flowering time, or in the period from flowering to seed maturity, environmental effects on the oil composition may cause differences in reciprocal crosses. One way of getting around the problem is to use backcrosses that have the F_1 plants as maternal parents. It is expected that F_1 plants have similar or equal developmental timing because this is controlled by the nuclear genotype (Miller and Fick, 1997). It is normally expected that seeds borne on the F_1 A/B backcrossed to parent A will be different to those backcrossed to parent B, because the seeds have different genotypes. If they have the same value for the trait being studied, it indicates that the maternal genotype controls the trait. It is also expected that the backcross (A/B)A would be equal to (B/A)A. If this expectation is not fulfilled, it indicates that cytoplasmic inheritance is present, because the only factor that is different between these reciprocal crosses is the cytoplasm of the maternal plants (Mosjidis and Yermanos, 1984).

One limitation of both reciprocal crosses and reciprocal back-crosses is that the conclusions of the presence of cytoplasmic inheritance are obtained solely from F_2 populations. No information is obtained about the persistence of reciprocal differences in later generations (Mosjidis and Yermanos, 1984).

Early studies on HO trait have indicated maternal influence but not complete maternal inheritance (Miller *et al.*, 1987) and the complete influence of the embryo genotype on trait (Urie, 1985, Fernández-Martínez *et al.*, 1989). In other words, the absence of maternal effects. More recently, Varès *et al.* (2000; 2002), with a complete diallelic cross between seven lines, reported a reciprocal effect on oleic acid content.

1.2.3 Environment

Environmental factors (that may cause any type of stress) influence the proportions of fatty acids by altering the enzyme activity (Garcés *et al.*, 1992; Sarmiento *et al.*, 1998; Rondanini *et al.*, 2003; 2006) as well as transport between organelles (Steer and Seiler, 1990) and therefore a thorough understanding of the environmental factors influencing seed development and oil quality is necessary.

Sunflower oil content and composition is not only influenced by genetic factors, but also by environmental and agronomical factors including planting location, climate, temperature, water availability, planting date, nitrogen and irrigation applications (Steer and Seiler, 1990; Baldini *et al.*, 2002; Flagella *et al.*, 2002; Izquierdo *et al.*, 2002; Rondanini *et al.*, 2003; Roche *et al.*, 2004; Roche *et al.*, 2006; Zheljazkov *et al.*, 2008; Zheljazkov *et al.*, 2009; Anastasi *et al.*, 2010; Zheljazkov *et al.*, 2011).

In traditional hybrids, fatty acid composition varies with sowing date (Unger, 1980; Jones, 1984; Roche *et al.*, 2004; Zheljazkov *et al.*, 2011), year (Goyne *et al.*, 1979; Varès *et al.*, 2002; Roche *et al.*, 2004) and location (Benvenuti *et al.*, 1984; Lajara *et al.*, 1990; Zheljazkov *et al.*, 2008), though high oleic hybrids seem to be less affected by environmental conditions (Garcés *et al.*, 1989; Triboï -Blondel *et al.*, 2000; Baldini *et al.*, 2002; Flagella *et al.*, 2002; Izquierdo *et al.*, 2002; Roche *et al.*, 2004; Roche *et al.*, 2006; Anastasi *et al.*, 2010).

In normal type sunflower, oil composition (and especially oleic acid content) is highly influenced by environmental factors as the temperature (Lajara *et al.*, 1990; Izquierdo *et al.,* 2002; Rondanini *et al.*, 2003; Izquierdo *et al.*, 2006; Rondanini *et al.*, 2006; Izquierdo and Aguirrezábal, 2008), the intercepted solar radiation (Izquierdo *et al.*, 2009; Echarte *et al.*, 2010; Echarte *et al.*, 2012) and water availability (Baldini *et al.*, 2002).

1.2.3.1 Temperature

Temperature may have a major effect on sunflower oil characteristics during grain filling phase. Among sunflower seed components, the unsaturated fatty acid content (oleic and linoleic acids) is the most temperature sensitive. It has long been known that temperature is the main environmental factor affecting the fatty acid composition in the oil of traditional type sunflower (Canvin, 1965) mainly regulating the ratio of oleic and linoleic acid during sunflower seed development. There is an inverse relationship between temperature and linoleic acid content (Harris *et al.*, 1978; Izquierdo *et al.*, 2006).

Several studies have been conducted, under natural field conditions and in controlled environment, in order to clarify how temperature affects the fatty acid composition of sunflower seeds. Under field conditions, variation in oleic acid percentage was better explained by maximum (Seiler, 1983), minimum (Harris *et al.*, 1978) or daily mean temperature (Nagao and Yamazaki, 1983). In controlled environment studies, high temperatures during seed development (especially night temperature; Rochester and Silver, 1983) have been found to cause a decrease in the amount of linoleic acid and a corresponding increase in the amount of oleic acid in the oil (Izquierdo *et al.*, 2002). Seed maturation during periods of low temperature gave opposite results. The mechanism involved appeared to be the direct effect of temperature on the activity of the desaturase enzymes that are responsible for the conversion of oleic to linoleic acid (Canvin, 1965; Harris *et al.*, 1978; Silver *et al.*, 1984; Garcés and Mancha, 1991). Therefore, both temperature and genetic effects are mediated by changes in the activity of the microsomal ODS (Oleate Desaturase) or FAD2 (Fatty Acid Desaturase). According to Izquierdo *et al.* (2002), Izquierdo *et al.* (2006) and Echarte *et al.* (2010) variations in oil fatty acid composition were well related to night temperature. Izquierdo *et*

al. (2002) suggested that the effect of temperature during the dark period on fatty acid composition was an indication that light or a metabolite associated with the day/night cycle could affect the activity of the ODS enzymes. In a later work, Pleite *et al.* (2008) demonstrated that the rate of oleic acid desaturation increasing in the middle of the night indicates the influence of night temperature on the desaturation activity in sunflower seeds.

In sunflower seeds, temperature affects the activity of the ODS responsible for the synthesis of linoleic acid (Garcés *et al.*, 1992). The temperature may regulate this enzyme both by altering its expression and by regulating its activity since it is an enzyme that can be thermally inactivated (Garcés *et al.*, 1992; Sarmiento *et al.*, 1998).

The ODS enzyme is highly regulated by temperature in sunflower seed and according to García-Díaz *et al.* (2002), different mechanisms might be involved in the control of the microsomal ODS activity. These mechanisms include: 1) de novo enzyme synthesis or activation of ODS that is stimulated by low temperatures, 2) the rapid and reversible partial inhibition of the pre-existing enzyme at high temperatures and 3) the exchange of oleate and linoleate between TAGs and PC (Canvin, 1965; Garcés *et al.*, 1992; Sarmiento *et al.*, 1998). In addition, Martínez-Rivas *et al.* (2001) proposed two separate and independent mechanisms that could be involved in the temperature regulation of ODS activity in developing sunflower seeds: 1) The long-term direct effect of temperature, mostly related to the low thermal stability of the ODS enzyme and 2) the short-term indirect effect of temperature on the availability of oxygen. The internal oxygen level acts as a key regulator for the activity of the FAD2 enzyme (Rolletschek *et al.*, 2007). Higher solubility of oxygen in water at low temperatures may increase the total desaturase activity by increasing the availability of oxygen that acts as co-substrate for oleate desaturation. It is concluded that a major mechanism by which temperature modifies the unsaturation degree of the sunflower oil is through its effect on dissolved oxygen levels in the developing seed (Rolletschek *et al.*, 2007).

Both regulation mechanisms are of particular relevance as they act during field growth conditions of sunflower plants. However, temperature does not only regulate ODS activity, but also the amount of oleate (synthesised de novo and mobilised from preformed TAG) available as substrate for the enzyme (García-Díaz *et al.*, 2002).

Another topic of interest is quantifying the effects of temperature on oleic acid content at different phases in the grain-filling process in sunflower and thus selecting the critical period. Critical periods are those when the sensitivity to an environmental variable is highest. Fatty acid composition of sunflower oil has been related to temperature during various periods, such as mean

flowering to physiological maturity (Harris *et al.*, 1978), 10 days before harvest (Nagao and Yamazaki, 1984) and 40 days after the beginning of flowering to harvest (Seiler, 1986). Robertson *et al.* (1978) reported a significant correlation between oleic acid content and temperature between 21 and 70 days after flowering. However, from research on the enzymes involved in fatty acid synthesis it appears that temperature effects would be most important early during fruit filling (Garcés and Mancha, 1991; Garcés *et al.*, 1992; Kabbaj *et al.*, 1996). Rondanini *et al.* (2003) reported that the period of greatest sensitivity for oil quality was from 19 to 26 days after anthesis in normal line HA89. However, some difference among genotype seems to be present (Rondanini *et al.*, 2006). Izquierdo *et al.* (2002; 2006) and Izquierdo and Aguirrezábal (2008) found increments in oleic percentage with higher night temperatures applied during early stages of grain development (100-300 degree-days after flowering, base temperature 6 °C), with standard genotypes showing the greatest change and high oleic hybrids the least. Roche *et al.* (2006) hypothesized that changes in the level of oleic acid in seeds are modulated by the mean temperature during the flowering period and also by the temperature sums of all phases.

Linear relationships between oleic (or linoleic) acid concentration and temperature were established (Harris *et al.*, 1978; Goyne *et al.*, 1979; Silver *et al.*, 1984) for ranges of daily mean temperature between 15 and 27 °C. Trémolieères *et al.* (1982) reported a curvilinear relationship between oleic acid concentration and mean temperature, with a maximum value at approximately 27 °C. It appears then that there is an optimum temperature for maximum oleic acid concentration. Izquierdo *et al.* (2006) established that the response of oleic acid concentration to temperature was bilinear. Increases in night minimum temperature from 10.7 to 22.6 °C resulted in a strong increment of oleic acid concentration. Higher night temperatures did not increase the concentration of this fatty acid. According to Izquierdo and Aguirrezábal (2008) oleic acid percentage showed a sigmoid response to minimum night temperature between 100 and 300 °C days after flowering (base temperature 6 °C). These Authors demonstrated the existence of an interspecific genetic variability in the response of oleic acid percentage to temperature among sunflower hybrids.

1.2.3.2 Temperature effects in high oleic sunflower

Several researchers reported that oleic acid content showed a great stability in different environments in high oleic genotypes, even if genetic differences were present (Salera and Baldini, 1998; Roche *et al.*, 2006). Additionally, in high oleic mutants the oleic and linoleic acid contents were less influenced by temperature than standard genotypes (Flagella *et al.*, 2002; Roche *et al.*, 2004; Roche *et al.*, 2006). However, it is known that in high oleic hybrids Δ12-oleate desaturase is active only at the early days of the embryo development associated with synthesis activity of the

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lipids (Garcés and Mancha, 1991) and that its transcript is not accumulated during the grain filling period (Lagravére *et al.*, 2000).

However, Champolivier and Merrien (1996) suggested that temperature had an effect on oleic acid content in high oleic sunflower hybrids. In contrast, Lagravére *et al.* (2000; 2004) found that the high oleic hybrids they studied were insensitive to temperature conditions. The differences between these reports could be related to differences in hybrids studied as well as their genetic backgrounds. Oleic hybrids can be characterized as high or low oleic acid potential hybrids and the largest part of total variation in oleic acid percentage could be due to differences in potential acid percentages of the hybrids (Izquierdo *et al.*, 2002). Lagravére *et al.* (2000) suggested that hybrids with low oleic acid potentials could be more sensitive to environmental conditions such as temperature, while hybrids with a higher oleic acid content genetic potential were insensitive to temperature conditions.

1.2.3.3 Other Environmental factors

Environmental factors other than temperature have been shown to affect oil fatty acid composition. There is vast information about how temperature regulates oleic acid synthesis by both direct and indirect control of FAD2, the key enzyme involved in oleic acid biosynthesis (Garcés *et al.*, 1992; Kabbaj *et al.*, 1996; Rolletschek *et al.*, 2007). By contrast, light-mediated changes in sunflower oil oleic acid have been less explored. A positive correlation between oleic acid percentage and incident solar radiation has been reported (Seiler, 1986). Izquierdo *et al.* (2009) proposed that changes in oleic acid percentage in response to variations in Intercepted Solar Radiation (ISR) could be mediated by changes in the carbon supply to the grains, which would affect the level of saturation of key enzymes in lipid synthesis. At higher irradiances, FAD2, the main regulation point in this pathway, would be substrate saturated and thus increased carbon availability would lead to a relative accumulation of oleic acid. Echarte *et al.* (2012) confirmed that effects of ISR on fatty acid composition are a consequence of changes in assimilate availability for grain oil synthesis.

In standard type sunflower, differences in oleic acid percentages driven by ISR could be higher than 10 percentage points (Izquierdo *et al.*, 2009). No information is reported in literature on ISR effects in High Oleic Genotypes.

Studies based on different irrigation regimes reveal contrasting results (Flagella *et al.*, 2002; Roche *et al.*, 2006; Ananstasi *et al.*, 2010). However, in high oleic genotypes, when water stress was applied during the grain filling period, the oleic/linoleic acid ratio increased in sunflower grown in North-East Italy (Baldini *et al.*, 2002), with respect to a more favorable water regime. They concluded that water stress, causing accelerated and earlier embryo development and lipid

accumulation therefore determines a shorter duration of all enzymatic activities, including those of ODS and this could reflect on the final acid composition.

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2   Effects of accumulated Growing Degree Days on fatty acid composition in three High Oleic genotypes

ABSTRACT

High Oleic Sunflower oil has a wide range of applications, such as in the food industry and as raw material for non-food applications. High temperature enhances the oleic acid content in the oil of normal or low oleic cultivars but conflicting results are reported on temperature effects on oleic acid content of high oleic acid cultivars: either no effect or an increase in oleic acid content with temperature. To investigate the effects of temperature on high oleic genotypes under natural field conditions, a three-year field trial was carried out with two dates of sowing and three HO genotypes (2 inbred lines and 1 hybrid). To compare our results with previous works, Growing Degree Days were computed (tb = 6 °C). GDD accumulated during Flowering - 25 days after flowering period influenced fatty acid composition. Oleic and linoleic acids content was influenced by temperature in two genotypes (one inbred line and in the hybrid) while the other inbred line was insensible to temperature. There was an increase of about 3% in oleic acid content from 400 to 500 GDD. There was genotype by environment interaction that we suppose depends by modifier genes. This indicated the importance of breeding targeted to select hybrids with an oleic acid content higher than 90% and stable across environment and the role of other genetic factors that affected oleic acid content. Some difference between the two HO genotypes sensitive to temperature is detected. The inbred line 342mt and the hybrid seem to differ in the timing of sensitivity to temperature. Furthermore, oleic acid content is well related to mean daily temperature in 342mt and to minimum night temperature in the hybrid. This result suggests that temperature could act on different processes in HO genotypes. Also saturated fatty acids (palmitic and stearic) were influenced by temperature and there was genetic variability among genotypes. Generally, palmitic and stearic acid content increased with GDD accumulated with a variation of about 1%. These results suggested a genetic variability among HO genotype.

Key Words: High Oleic Genotypes, GDD, Date of Sowing, Year, Fatty Acid

2.1   INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in the world. Sunflower oil has a wide range of applications, such as in the food industry (e.g. margarine production) and as raw material for non-food application (biofuels, oleochemical). Sunflower oil quality is determined by content and ratio of fatty acids in the oil. Sunflower oil contains both saturated (palmitic and stearic acid) and unsaturated fatty acids (up to 90-95%), either monounsaturated (oleic acid) or polyunsaturated (mostly linoleic and traces of other minor fatty acids). Depending on unsaturated fatty acid composition, sunflower oil can be divided into traditional or normal or Low Oleic (LO) types, with an oil composition characterized by a majority of linoleic acid, Mid-Oleic (65-80% oleic acid) and High Oleic (HO) sunflower (80-94% oleic acid).

Previous research has demonstrated that the fatty acid composition of sunflower oil depends on genotypes (Low Oleic, Mid-Oleic and High Oleic) and environmental conditions during seed filling phase (Harris *et al.*, 1978; Champolivier and Merrien, 1996; Roche *et al.*, 2006; Izquierdo and Aguirrezábal, 2008).

HO sunflower was obtained by chemical mutagenesis with dimethyl sulfate (DMS) of LO sunflowers (Soldatov, 1976). HO genotypes cumulate Pervenets mutation effect and other independent factors acting on oleic acid content such as modifier genes (Miller *et al.*, 1987; Fernandez *et al.*, 1999; Velasco *et al.*, 2000; Lacombe *et al.*, 2004). Some genetic variation for oleic acid content among HO genotypes may depend on these independent factors (genetic background of parental lines).

Sunflower oil content and composition is not only influenced by genotypes, but also by environmental and agronomical factors (date of sowing, irrigation, fertilization, etc.). It has long been known that temperature is the main environmental factor affecting the fatty acid composition in the oil of LO sunflower (Canvin, 1965), mainly regulating the ratio of oleic and linoleic acid. Oleic acid content showed a great stability in different environments in high oleic genotypes compared to the LO genotypes. High temperature enhances the oleic acid content of normal cultivars but conflicting results are reported about temperature effects on oleic acid content of high oleic acid cultivars: either no effect (Lagravére *et al.*, 2000) or an increase in oleic acid content with temperature (Champolivier and Merrien, 1996; Triboï-Blondel *et al.*, 2000; Izquierdo and Aguirrezábal, 2008). The differences between these reports could be related to differences in hybrids studied as well as their genetic backgrounds. Lagravére *et al.* (2000) suggested that hybrids with low oleic acid potentials could be more sensitive to environmental conditions such as

temperature, while hybrids with a higher oleic acid content genetic potential were insensitive to temperature conditions.

Under natural field conditions, the effect of temperature on high oleic genotypes through the delay of sowing has not been extensively studied. Connor and Sadras (1992) reported that sowing date influences the fatty acid composition by modifying the ontogenesis. For standard hybrids, delay of sowing involves a reduction of oleic acid content and an increase in linoleic acid content (Jones, 1984; Unger, 1986). The same variation is observed for oleic hybrids (Flagella *et al.*, 2002; Roche *et al.*, 2004; 2006) but the variation was less intensive across date of sowing (a variation in oleic acid content of about 2%).

Another topic of interest is quantifying the effects of temperature on oleic acid content at different phases in the seed-filling phase in sunflower and thus selecting the critical period. Critical periods are those when the sensitivity to an environmental variable is highest. Rondanini *et al.* (2003) reported that the period of greatest sensitivity for oil quality was from 19 to 26 days after anthesis in normal line HA89. Izquierdo *et al.* (2002; 2006) and Izquierdo and Aguirrezábal (2008) found increments in oleic percentage with higher night temperatures applied during early stages of grain development (100-300 degree days after flowering, base temperature 6 °C), with standard genotypes showing the greatest change and high oleic hybrids the least. Roche *et al.* (2006) hypothesized that changes in the level of oleic acid in seeds are modulated by the mean temperature during the flowering period and also by the temperature sums of all phases. It is not known whether the period in which temperature has maximum effect on fatty acid composition differs among HO genotypes.

The saturated fatty acids, palmitic and stearic, are less influenced by environmental condition than unsaturated fatty acids with small variation across years and locations (Lajara *et al.*, 1990; Izquierdo *et al.*, 2002; 2006). However, to select HO hybrids with a high and stable oleic acid content, it is important to detect all factors that may reduce or increase the concentration of oleic acid.

This work aimed at assessing the grain composition of fatty acids of three high oleic sunflower genotypes in response to different growing degree days accumulation and to study genetic variability in seed fatty acids composition among HO genotypes as affected by temperature.

2.2   MATERIAL AND METHODS

2.2.1 Plant material

The sunflower (*Helianthus annuus* L.) seeds used in this work were from the inbred lines 342mt and R978, both high oleic inbred lines, and from their hybrid 342 x R978. Line 342mt is a selection, made at University of Udine, derived by Ha 342 USDA, and it is a male sterility maintainer with a single head. Line R978, selected by University of Udine, is a fully-branched type and it is a male fertility restorer.

2.2.2 Field trials

Inbred lines and hybrid were grown in 2009, 2010, 2011 at the experimental field of University of Udine (Fig. 2), Azienda Agraria Universitaria "A. Servadei" (46°04'N, 13°22'E, altitude 109 m), in North-East Italy. The experiment was designed as a complete block randomization scheme, with three replications, using two dates of sowing (Table 3). Plot size was 5 m x 2 m. The seeds were sown at a spacing of 0.75 m between rows. Plants were thinned after seedling emergence from 10 to 7.5 plants m^{-2}. Nitrogen was applied at 100 kg ha^{-1}. Weeds and diseases were controlled, and regular watering throughout the experiment ensured that plants were not subjected to water deficit during the entire growth period. All plants were covered with paper bags, at the R4 stage (Schneiter and Miller, 1981), to prevent cross-fertilization. Five plants per plot were studied.

Meteorological data (Table 2) for the experimental period were recorded at a station (Udine - S. Osvaldo Station; Osmer- FVG Region Meteorological Service) located 200 m away from the field site.

2.2.3 Sampling

Harvesting was done when all plants in a given treatment reached Physiological Maturity (PM), R9 phase (Schneiter and Miller, 1981). Seed from outer rings was separated for fatty acid and oil content determination and analyzed. Seed was dried in an oven at 60 °C for 48 h.

Fig. 2. Field trials at S.Osvaldo (Udine) Experimental Station – Azienda Agraria Universitaria "A. Servadei".

To find the right PM, and thus to investigate the relationship between fatty acids contents and temperature, plants were sampled two times a week over a period of 5 weeks, commencing 10 or 13 Days After Flowering (daf) for a total of 8 samplings. At each sampling ten seeds per plant from each of three plants were taken from the outer region of the capitulum (first six rings). Seed was dried in an oven at 60 °C for 48 h. Plants reached the real PM, when the seed weight becomes constant after three successive sampling.

2.2.4 Fatty acid analysis

100 seeds per plant from outer rings (1-6) were collected. Seed were dehulled. Kernels were ground to a fine powder using a coffee grinder. 200 mg of kernel powder were weighed to perform fatty acid analysis. Lipids were extracted in *n*-hexane. Fatty acids were converted in Fatty Acid Methyl Esters (FAMEs) by transesterification with a methanolic potassium hydroxide solution (2N). FAMEs composition was determined by gas chromatography and every fatty acid was expressed as a percentage of the total fatty acids detected in the oil. The gas chromatograph, equipped with Flame Ionization Detector (FID) and a split–splitless injector, was fitted with a 60 m HP-88 capillary column (Agilent Technologies, USA). Helium was used as carrier gas, and the injector, detector and oven temperatures were 280, 250 and 200 °C, respectively. 5 µL of sample were injected in split mode. Different FAMEs were identified by comparison with known standards.

2.2.5 Oil content analysis

Whole seeds (10 g) were analyzed for oil content by Nuclear Magnetic Resonance (NMR Oxford Instruments -4000).

2.2.6 Growing Degree Days accumulation

To study the environmental effects on fatty acids and to compare our results with previous works, data from years and date of sowing were divided according to Growing Degree Days accumulated. The accumulation of the Growing Degree Days (GDD), over a base temperature of 6 °C (Tb), was calculated using the following formula:

$$GDD = \Sigma[(Tmax + Tmin)/2] - Tb$$

where Tmax and Tmin were the daily maximum and minimum temperatures, respectively, in °C. GDD_1 is calculated from last flowering to Physiological Maturity and GDD_2 was calculated from last flowering to 25 daf. Two GDD_1 groups were created: A and B. In group A, there was inbred lines at 500 GGD and hybrid at 600 GGD. Group B was formed by inbred lines at 600 GGD and hybrid at 700 GGD. GGD_2 was also separated into two groups: the first one was constituted by plants that had accumulated 400 GGD and the second by plants that had accumulated 500 GGD.

2.2.7 Statistical analysis

Statistical analysis was performed using R version 2.15.0 (R Development Core Team, 2012). Shapiro-Wilk normality test was performed to test normality condition. A two-way ANalysis Of VAriance (ANOVA) was performed as fixed-effect model with GDD and genotypes. Significance of each source of variation was evaluated by F-test. When the F-ratio revealed significant differences, means were compared by the Least Significant Difference (LSD) at P≤0.05.

Table 2. Meteorological data for the field site during sunflower growth in 2009–2011.

	2009				2010				2011			
	Tmin (°C)	Tmean (°C)	Tmax (°C)	Rainfall (mm)	Tmin (°C)	Tmean (°C)	Tmax (°C)	Rainfall (mm)	Tmin (°C)	Tmean (°C)	Tmax (°C)	Rainfall (mm)
April	9.2	14.7	20.6	131.5	6.7	13.5	19.6	75.1	7.9	15.0	22.1	18.1
May	13.1	19.7	26.1	28	11.7	16.6	21.6	230.2	11.5	19.1	26.3	85.2
June	15.2	20.9	26.8	104.2	15.5	21.3	27.1	68.7	15.6	21.2	26.7	185.1
July	17.1	23.6	29.7	104.5	18.1	24.5	30.6	143.7	15.8	22.0	28.1	148.4
August	18.2	24.9	31.8	66.2	16.2	22.0	28.0	122.1	16.9	24.0	31.3	23.3
September	14.5	20.5	26.9	145.6	12.2	17.5	23.4	264.8	14.6	21.7	29.3	83.9
Mean	14.5	20.7	27.0		13.4	19.2	25.1		13.7	20.5	27.3	
Total				580.0				904.6				544.0

2.3 RESULTS AND DISCUSSION

Whole cycle duration (emergence – physiological maturity) and relative phases duration are reported in Table 2. Physiological maturity, expressed as daf when seed weight was constant, was reached some days before R9 phase (Schneiter and Miller, 1981). In inbred lines the difference between PM and R9 was less important than in the hybrid. In the hybrid true PM was reached about 10 days before R9 phase. Thus, the duration of end of flowering to PM phase was calculated on true PM expressed as daf when seed weight becomes constant.

The Growing Degree Days (GDD) from last-flowering to PM (GDD1) for the year 2010 (average 540 GDD; Standard Deviation 94) was different from that of the years 2009 and 2011 (average 618 ± 49 GDD and 630 ± 38 GDD respectively). Average GDD accumulated by inbred lines (average 567 °C days ± 67) was lower than GDD accumulated by hybrid (average 652 °C days ± 53). This compared with 699-836 (mid-flowering – PM) reported by Robertson and Green (1981), when these data are converted to a tb of 6 °C, and with degree days summation (R6-R9 phase) reported by Roche *et al.* (2006).

Oil content and stearic acid were influenced by interaction genotype by GDD1 accumulated from flowering to physiological maturity (Table 4). The effect of GDD1 on oil content was expected because seed oil concentration of sunflower is sensitive to environmental conditions during the grain-filling period (Connor and Hall, 1997). The effect of GDD1 on stearic acid content in the hybrid was unexpected.

The GDD accumulated from last-flowering to 25 daf (GDD2) was 439 ± 33, 426 ± 22, 405 ± 23 GDD2 for the year 2009, 2010, 2011 respectively. Average GDD2 accumulated by inbred line 342mt was 436 ± 34, 420 ± 28 by inbred line R978 and 414 ± 23 by hybrid.

Seed fatty acids (palmitic acid and unsaturated oleic and linoleic acids) composition was influenced by GDD2 accumulated from end of flowering to 25 daf (Table 5).

This results are in agreement with Rondanini *et al.* (2003) who reported that in sunflower the sensitive period for modifications in oil quality was 19-26 days after anthesis and with Izquierdo *et al.* (2002) who reported that the sensitive period for modification in fatty acid composition was 0-400 °C days after flowering.

Table 3. Date of sowing, cycle duration and Emergence to Flowering, and End of Flowering (F)-Physiological Maturity (PM) phase duration.

Genotype	Year	Code	Date of Sowing	Cycle duration (dd)	Emergence-Flowering (dd)	F-PM R9[1] (dd)	F-PM[2] (dd)
342mt	2009	I	6th May	106	68	31	31
		II	28th May	110	71	35	34
	2010	I	19th April	111	66	34	32
		II	1st June	91	52	33	32
	2011	I	18th April	110	62	41	35
		II	30th May	101	59	37	35
R978	2009	I	6th May	110	71	33	32
		II	28th May	115	74	34	34
	2010	I	19th April	119	73	35	32
		II	1st June	95	57	32	30
	2011	I	18th April	113	67	40	38
		II	30th May	101	60	37	33
Hybrid	2009	I	6th May	113	69	47	37
		II	28th May	113	72	46	36
	2010	I	19th April	124	64	53	42
		II	1st June	98	49	45	38
	2011	I	18th April	116	60	45	40
		II	30th May	106	56	40	38

[1] PM – R9 according to Schneiter and Miller (1981)

[2] PM as seed constant weight

Table 4. Analysis of Variance (Mean Square) for the main fatty acids and seed oil content. GDD_1 are the GDD accumulated from end of flowering to physiological maturity (daf when seed weight becomes constant).

Source of Variation	DF	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Oil
GDD_1	1	2.80*ns*	0.03*ns*	0.15*ns*	3.57*ns*	18.02*ns*
Genotype (G)	2	5.21**	1.87*ns*	146.44***	73.35***	204.19***
GDD_1 x G	2	1.17*ns*	3.53*	11.48*ns*	7.99*ns*	94.19**
Residuals	48	0.70	0.69	4.96	4.34	13.52

*, **, *** = Significant at the P<0.05, 0.01 and 0.001 levels, respectively. ns = not significant.

Table 5. Analysis of Variance (Mean Square) for the main fatty acids and seed oil content. GDD_2 are the GDD accumulated from end of flowering to 25 daf.

Source of Variation	DF	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Oil
GDD_2	1	12.60***	0.00*ns*	6.07*ns*	35.94***	0.39*ns*
Genotype (G)	2	2.86**	2.03*ns*	169.71***	98.87***	192.42***
GDD_2 x G	2	5.03***	1.60*ns*	19.34**	9.37*	50.21*ns*
Residuals	48	0.43	0.77	3.54	2.55	16.21

*, **, *** = Significant at the P<0.05, 0.01 and 0.001 levels, respectively ; ns = not significant.

2.3.1 Effect of GDD on saturated fatty acids content

Palmitic acid content was on average 4.20%. The oleic genotypes had lowest palmitic acid percentages compared with the standard sunflower types as reported in other works (Lajara *et al.*, 1990; Roche *et al.*, 2004; Anastasi *et al.*, 2010). The two high oleic inbred lines tested and their hybrid were different for palmitic acid content (Fig. 3). The highest value in palmitic acid was achieved by line 342mt with 500 GDD_2 (5.37%) and the lowest content was reached by R978 inbred line with 400 GDD_2 (3.28%). The hybrid showed a content in palmitic acid like the parental lines (4.00%; 400 GDD_2) or significantly lower (3.60%; 500 GDD_2). High oleic genotypes with high oleic acid potentials had the lowest palmitic acid contents. In literature, there is a small number of works on palmitic acid content inheritance and the trait appears to be complex (Vick *et al.*, 2004).

The hybrid did not show any significant difference among GDD_2 groups, while inbred lines showed an increase in palmitic acid content from 400 to 500 GDD_2. Several environmental factors like water availability (Roche *et al.*, 2006; Jalilian *et al.,* 2011), temperature (Rondanini *et al.*, 2003; Izquierdo and Aguirrezábal, 2008) and nitrogen (Zheljazkov *et al.*, 2009) could altered saturated fatty acid content in sunflower. In our experiment, the most variable environmental factor was temperature. The temperature modified the concentration of palmitic acid of the oil in normal sunflower type (Rondanini *et al.*, 2003) and in high oleic hybrids (Izquierdo and Aguirrezabal, 2008). We found a positive correlation between mean temperature of F-PM phase and palmitic acid content only in inbred line 342mt (Fig. 4) while we did not find any correlation between temperature and palmitic acid content in inbred line R978.

These data are in agreement with Izquierdo and Aguirrezábal (2008) who found a correlation between palmitic acid content and temperature only in some hybrids. Interestingly, in inbred line R978 oil seed percentage and palmitic acid were positively correlated (Table 6) and seed oil content increased from 500 to 600 GDD_1 (Fig. 10). We suppose that the rise in palmitic acid content was related to an increase in seed oil content and not to a rise in air temperature *per se*.

Fig. 3. Effects of Interaction genotype by GDD$_2$ on palmitic acid content. Means followed by the same letter are not significantly different (LSD at the 5% level).
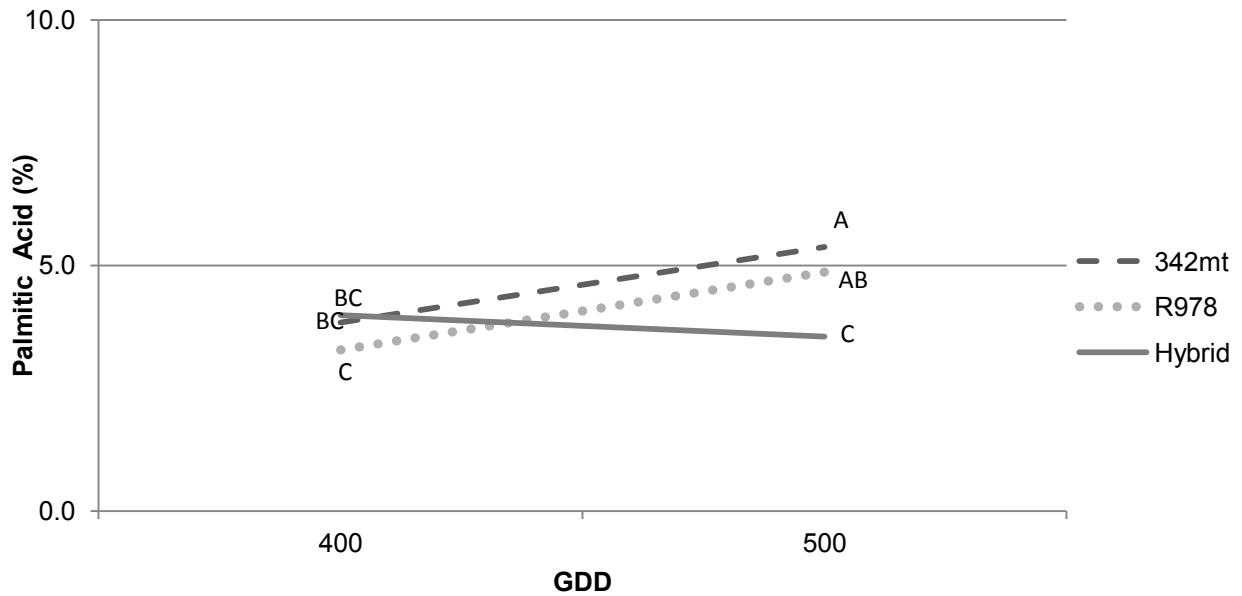
Fig. 4. Correlation between mean air temperature and palmitic acid in inbred line 342mt during F-PM phase (*p*-value 0.015).
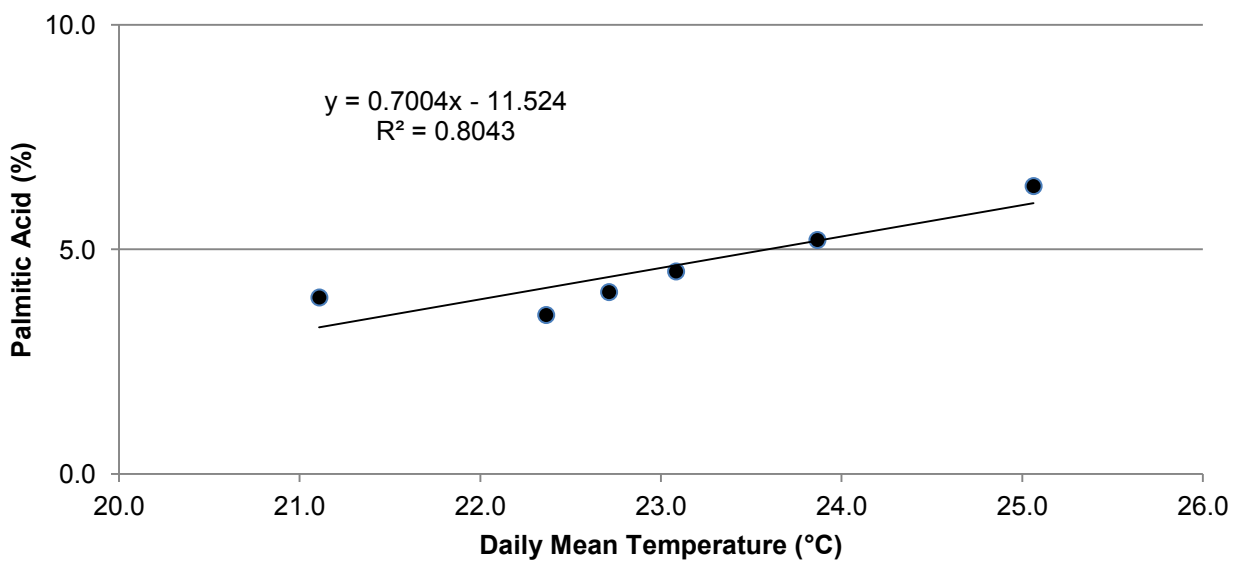
Fig. 5. Effects of Interaction genotype by GDD$_1$ on stearic acid content. Means followed by the same letter are not significantly different (LSD at the 5% level).
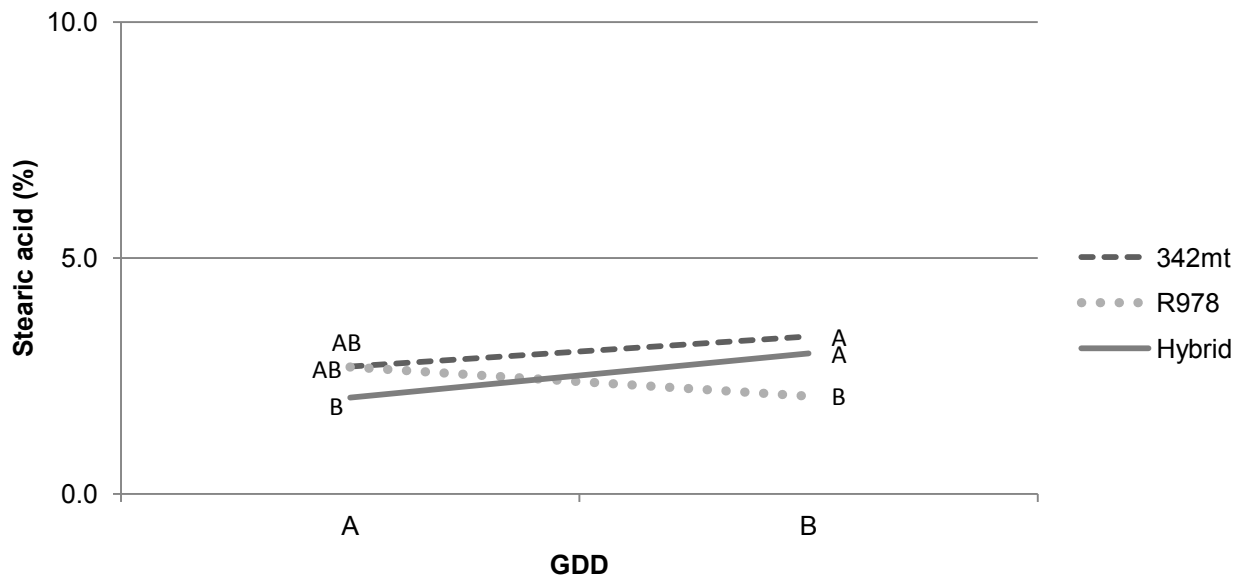
Stearic acid content was significantly affected by interaction genotype by GDD$_1$. The hybrid had a stearic acid content into the parental lines range and it showed the same interaction with GDD$_1$ as its female parental line. At A GDD$_1$ accumulated there was no significant difference among tested genotypes. At B GDD$_1$ accumulated inbred line R978 had the lowest stearic acid content (2.00%). There was a difference in interaction genotype by GDD$_1$. Inbred lines had the same stearic acid content with different GDD$_1$ levels, while the hybrid showed an increase of about 1% in stearic acid content from 600 GDD$_1$ to 700 GDD$_1$ (Fig. 5). Differences among genotypes could be related to isozymes of stearate desaturase (Fernandez-Moya *et al.*, 2003). Also thioesterase FatA and FatB could regulate stearic acid content as suggested by Byfield and Upchurch (2007) in soybean.

At A GDD$_1$, mean air temperature was lower than at B GDD$_1$ (21.9 °C and 23.6 °C respectively). Our results are in agreement with Fernandez-Moya *et al.* (2003) who suggested an inhibition of the stearate desaturase (SAD) enzyme responsible for the C18:0/C18:1 conversion at higher temperature in a high stearic sunflower mutant. In soybean, Byfield and Upchurch (2007) found that decreased SAD transcript accumulation at the warm temperature was positively associated with a significantly increased level of stearic acid but only in a high-stearic mutant line.

Fig. 6. Effects of Interaction genotype by GDD_2 on oleic acid content. Means followed by the same letter are not significantly different (LSD at the 5% level).
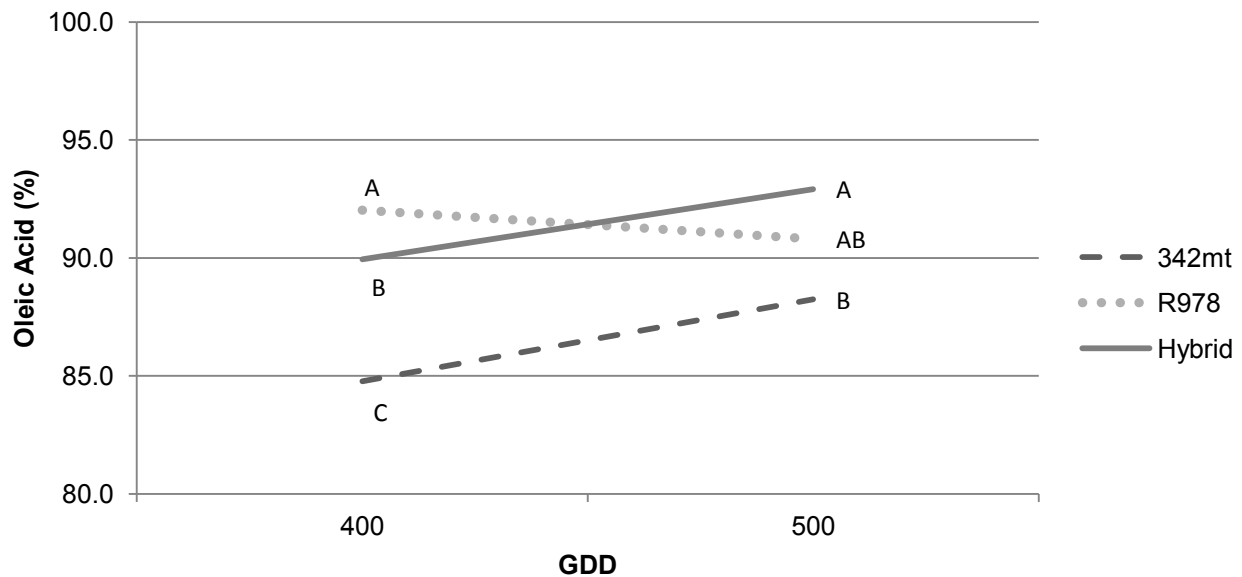
On the other hand, in a soybean genotype, the stearic acid percentage was negatively related to daily mean temperature during grain filling period (Zuil *et al.*, 2012). Thus, the effect of temperature on stearic acid content was unclear and this effect seems to be genotype-specific.

The response to GDD_1 and not to GDD_2 could be related to a different oil accumulation pattern among genotypes (inbred lines vs hybrid). Considering that variation in stearic acid amount was small, the response to GDD_1 could be related to a "long – term" effect of temperature on stearic acid.

2.3.2 Effect of GDD on C18 unsaturated fatty acids content

2.3.2.1 Oleic acid content

Oleic acid contents were relatively stable in the high oleic genotypes tested. These data are in agreement with Lagravére *et al.* (2004) and Roche *et al.* (2004). Small but significant differences in oleic acid content were manifest with different GDD_2 accumulated and with a difference among genotypes. The two high oleic inbred lines tested are different. Analysis of Variance showed that there was a relative prominence of genotype effects compared to GDD_2 (Table 5). Inbred line 342mt showed an average in oleic acid content lower than R978 inbred line and their hybrid (Fig. 6). Line R978 was insensible to GDD_2 tested. Line 342mt and hybrid showed the same interaction genotype by GDD_2 with an increase of 3% in oleic acid content from 400 GDD_2 to 500 GDD_2. This indicates the presence of genotype by environment interaction on fatty acid composition as reported by some authors (e.g. Izquierdo and Aguirrezábal, 2008). Flagella *et al.* (2002) reported a similar

variation in oleic acid content (about 2%) in other high oleic sunflower hybrids as affected by date of sowing.

High oleic inbred lines with different origins are not equivalent for high oleic trait. The hybrid showed an average in oleic acid content like its male parental line or intermediate to parental lines range. The basis for differences between high-oleic and normal sunflower genotypes is a differential activity of the enzyme Δ12-desaturase, which catalyzes the desaturation of oleic acid to linoleic acid (Garces and Mancha, 1991; Kabbaj et al., 1996).

High oleic mutants had substantially lower Δ12-oleate desaturase gene transcript accumulation than sunflower standard type (Kabbaj et al., 1996; Hongtrakul et al., 1998). High oleic trait was controlled by at least three loci: oleHL, a suppressor locus, and modifier loci (Lacombe et al., 2004). We suppose that inbred lines and hybrid tested were homozygous for the Pervernets allele. Thus, observed differences could be related only to modifier genes (Miller et al., 1987; Velasco et al., 2000) or to other genetic factors (Varès et al., 2002; Lacombe et al., 2004). We speculate that genotype x environment interaction was caused by modifier genes. The observed response to temperature in inbred line 342mt and in the hybrid (see 2.3.2.3; pag. 47) could be related to a residual activity of the Δ12-desaturase (mediated by modifier) or to some genes that have no phenotypic expression at higher temperatures (Velasco et al., 2000). From a plant breeder point of view, knowledge of G x E interaction, that it is not desirable, facilitates the efficient use of appropriate breeding and selection procedures. A hybrid is preferred to be stable in different growing conditions.

Fig. 7. Effects of Interaction genotype by GDD$_2$ on linoleic acid content. Means followed by the same letter are not significantly different (LSD at the 5% level).
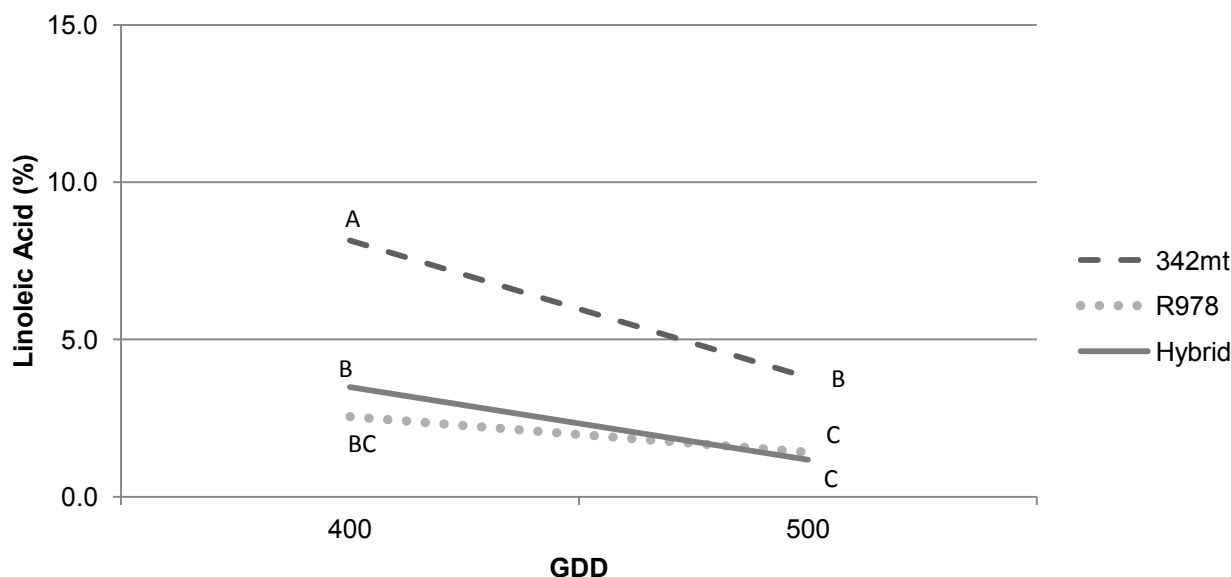
2.3.2.2 Linoleic acid content

Linoleic acid content was significantly affected by GDD2 only in two genotypes: 342mt and hybrid (Fig. 7). As for oleic acid content, inbred line R978 did not show any response to GDD2 tested. Line 342mt and hybrid showed a decrease in linoleic acid from 400 to 500 GDD2. As for oleic acid, the difference between genotypes could be related to modifier genes.

2.3.2.3 Relationship between temperature and unsaturated fatty acid content

The unsaturated fatty acid content (oleic and linoleic acids) is the most temperature sensitive seed component. There was no relationship between temperature and unsaturated fatty acids content in inbred line R978 (data not shown). On the other hand, oleic acid content was correlated positively with temperature in line 342mt and in the hybrid. High oleic sunflower may respond to temperature (Triboï-Blondel *et al.*, 2000; Izquierdo *et al.*, 2002; Izquierdo and Aguirrezábal, 2008), with a small variation respect to normal and mid-oleic types. There was a positive relationship between temperature and oleic and linoleic acids in the hybrid and in inbred line 342mt. In the hybrid, during 100-300°C degree-days (dd) after flowering period, there was a strong relation (*p*-value 0.012) between minimum night temperature and oleic acids content (Fig. 8).

This is in agreement with Izquierdo *et al.* (2006). At the same, linoleic acid content was linearly and negatively related with minimum night temperature (Fig. 8) during 100-300 dd after flowering (*p*-value 0.006).

Line 342mt was quite different (Fig. 9). Oleic acid content was linearly related with daily mean temperature during 200-300°C dd after flowering (*p*-value 0.053). Also linoleic acid content was linearly related with daily mean temperature during 200-300 dd after flowering (*p*-value 0.017).

Linear relationships between oleic acid concentration and temperature were recognized (Harris *et al.*, 1978; Goyne *et al.*, 1979; Silver *et al.*, 1984; Izquierdo *et al.*, 2006) for ranges of daily mean temperature between 15 and 27 °C. Linear responses of oleic acid percentage to daily mean temperature were also observed by Izquierdo *et al.* (2009) and Zuil *et al.* (2012) in soybean and maize.

The difference in the timing of sensitivity to temperature in 342mt inbred lines compared to the results of Izquierdo *et al.* (2006) and Izquierdo and Aguirrezábal (2008) could be related to a shorter cycle duration in inbred lines compared to the hybrid (Table 3). In fact, these authors tested in their studies oleic hybrids and not inbred lines. We cannot exclude that the difference in the timing of sensitivity, more simply, was related to a genetic variability among HO genotypes (Rondanini *et al.*, 2006).

Fig. 8. Oleic acid percentage (a) and linoleic acid (b) percentage in the hybrid as a function of minimum night temperature during 100-300 degree-days after flowering. 100-300 degree-days after flowering in the hybrid were accumulated in average from 8 to 21 days after flowering.

Tesi di dottorato di Claudio Ferfuia, discussa presso l'Università degli Studi di Udine
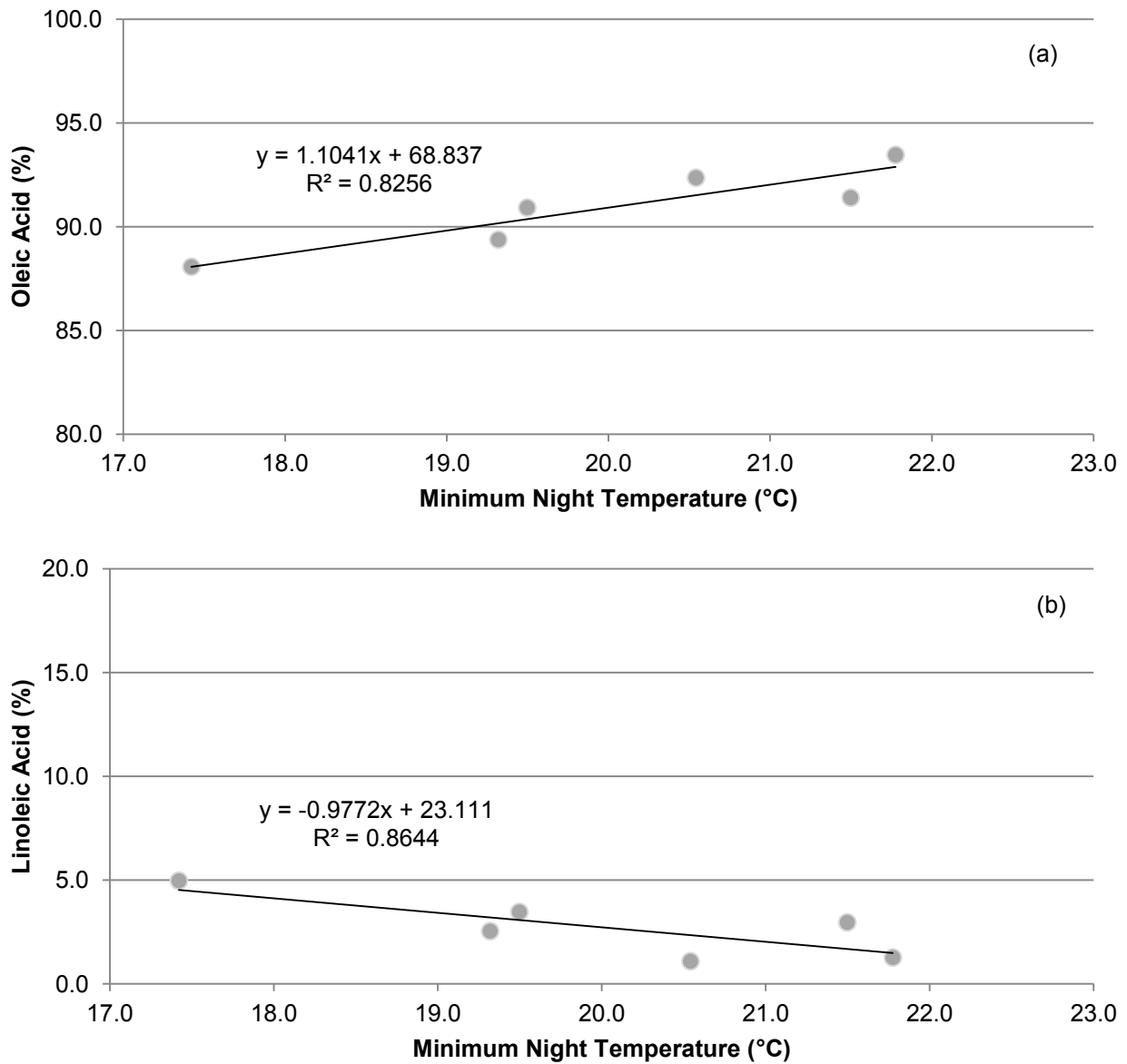
Fig. 9. Oleic acid percentage (a) and linoleic acid percentage (b) in the line 342mt as a function of daily mean temperature during 200-300 degree-days after flowering. In the line 342mt, 200-300 degree-days after flowering were accumulated in average from 15 to 20 days after flowering.
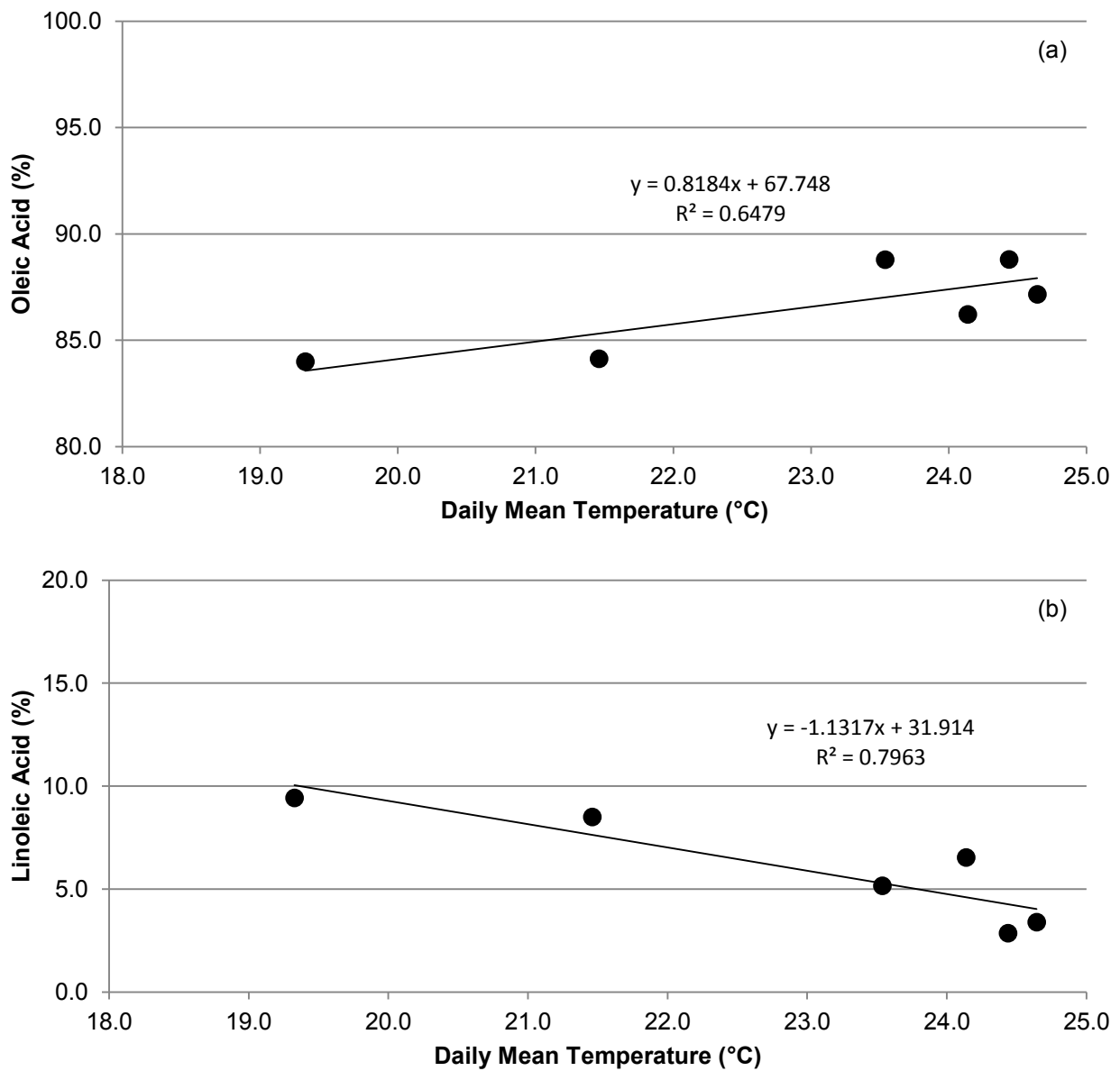
The differences in temperature response (mean daily temperature as best temperature predictor for fatty acid composition) in 342mt could be explained by physiological specific mechanisms. Regarding the specific physiological process, we hypothesized that temperature affected unsaturated fatty acids content in 342mt in two ways. First, temperature regulated phenotypic expression of some modifier genes, and these genes were not expressed at high temperature (Velasco *et al.*, 2000). We suppose that 342mt carries a recessive allele of "temperature instability gene", designated as Ols, and temperature affected its expression. Furthermore, temperature regulated Δ12-desaturase activity (Garcés and Mancha, 1991) and this was well related with night temperature (Izquierdo and Aguirrezábal, 2008). For these reasons we suppose that daily mean temperature was well related with oleic and linoleic acids contents in 342mt. The genetic variability between the female parental line and hybrid was associated to the fact that the hybrid was heterozygous at modifier locus while 342mt was homozygous. In addition, a maternal effect was found (see Chapter 3 and 4).

Izquierdo and Aguirrezábal (2008) reported a sigmoid relationships between the oleic acid percentage and minimum night temperature in several normal type sunflower and in one high oleic hybrid. These authors reported also a range of temperatures that altered fatty acid composition and this was specific for a given genotypes. Furthermore, they reported a high difference in upper and lower limits of range according to genotypes. In our trials the temperature range was 17-22 °C (night temperature) for hybrid and 19-25 °C (mean daily temperature) for 342mt. It is possible that the relationship between oleic acid percentage and temperature in our tested genotypes is also sigmoid but the temperatures, above and below which the concentration of oleic acid remains relatively constant, are lower and higher than those explored in this work for the tested genotypes.

Fig. 10. Effects of Interaction genotype by GDD$_1$ on seed oil content. Means followed by the same letter are not significantly different (LSD at the 5% level).
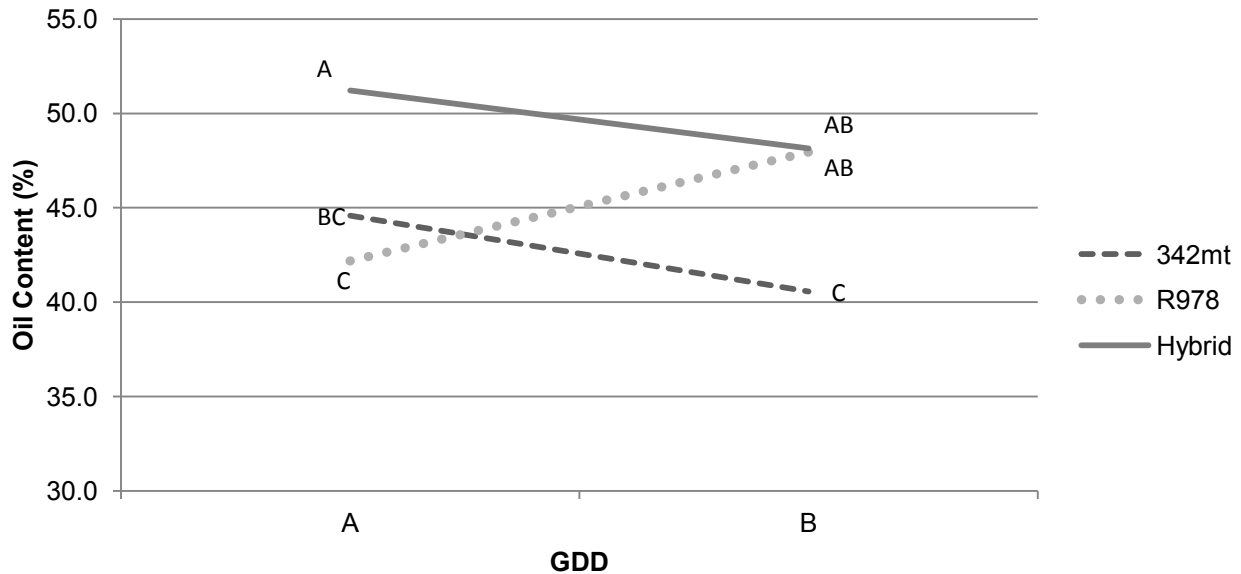
2.3.3 Seed oil content

Oil content varied from 40% to 51%. Seed oil concentration in this study was generally high and similar to previous reports (Roche *et al.*, 2004; Anastasi *et al.*, 2010).

Analysis of Variance showed that oil content was influenced by genotype effects and by interaction genotype by GDD$_1$ (Table 4). The hybrid showed a seed oil content higher than its parental lines. 342mt and hybrid did not show any significant variation with GDD$_1$ tested while R978 showed an increase in seed oil content from 500 to 600 GDD$_1$ (Fig. 10). Results suggest that sunflower seed oil concentration depends on genotype, but it may be expressed differentially under different environmental condition because seed oil concentration of sunflower is sensitive to environmental conditions during the grain-filling period (Connor and Hall, 1997). No relationship was found between seed oil content and temperature or rainfall. Thus, seed oil content may be affected by other variables not studied in this work. Differences, among genotypes, in response to GDD could be related to a different oil accumulation pattern (Mantese *et al.*, 2006) or due to the fact that R978 is a fully-branched type. Thus, it would be possible to suppose a different interaction between yield components (seed number, seed weight, number of heads per plant).

2.3.4 Seed oil content and fatty acid relationship

There is no significant relationship between seed oil content and oleic acid content in both lines and in the hybrid (Table 6). In inbred line 342mt, oil content was negatively correlated with stearic acid

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content. In high oleic hybrids a negative correlation between stearic acid and oil content was reported by Van der Merwe *et al.* (2012). In inbred lines R978, oil content was positively correlated with palmitic acid content. This result was not in agreement with Velasco *et al.* (2007) that reported a negative correlation between oil content and palmitic acid. In the hybrid, oil content was positively correlated with linoleic acid. Further studies are needed on oil content and its relationship with fatty acids in high oleic mutants.

With regard to the ratio of palmitic to oleic acid, a negative and significant correlation was observed in line R978 and in the hybrid and these results are in agreement with data by Champolivier and Merrien (1996), Roche *et al.* (2004) and Izquierdo *et al.* (2006), collected for oleic hybrids. Velasco *et al.* (2007) also reported a negative correlation between palmitic and oleic acids in high stearic mutants.

Flagella *et al.* (2002) showed that in sunflower an increase in palmitic acid is accompanied by a decrease in both oleic and stearic acids. Studies on soybean (Rebetzke *et al.*, 1996), peanut (Andersen and Gorbet, 2002), sesame (Were, 2006) and winter oilseed rape (Möllers and Schierholt, 2002) also revealed strong inverse relationships between palmitic and oleic acids. Line 342mt did not show any significantly correlation between palmitic and oleic acid, but we report an inverse significant correlation between palmitic and linoleic acids. Martinez *et al.* (2010) reported a negative correlation between palmitic and linoleic acid in oat.

Oleic and linoleic acids were negatively correlated. There was a difference between high oleic inbred lines (Fig. 11). Line 342mt showed a strong negative oleic linoleic relationship (p-value < 0.001) and indicating that increasing 1 point of oleic corresponded with the decrease of 1 point of linoleic acid. The correlation coefficient between oleic and linoleic acids was not significant in R978 (Table 6). The hybrid showed a strong negative oleic linoleic relationship and it had an average value of the regression parameter between parental lines (Fig. 11).

We suppose that the linoleic acid level reached by R978 was a physiological threshold (limit) for this genotype. We speculate that in R978 only the constitutive desaturase system (FAD2-2 and FAD2-3) present in the whole plant (Martìnez-Rivas *et al.*, 2001) could be active and responsible for low linoleic acid synthesis (physiological threshold) while seed-specific desaturase is fully inactive. In line 342mt, linoleic acid synthesis is probably due to a residual activity of the achene-specific desaturase system (Lagravére *et al.*, 2004). From a breeder point of view, increasing the oleic acid content over 93-95% is possible only with a reduction in saturated fatty acids content.

Table 6. Correlation coefficients between seed oil content and concentration of fatty acids in the three high oleic genotype tested (n = 18).

Genotypes		Palmitic	Stearic	Oleic	Linoleic	Oil
	Palmitic	1.00				
	Stearic	-0.27	1.00			
342mt	Oleic	0.31	-0.45	1.00		
	Linoleic	-0.55*	0.21	-0.92***	1.00	
	Oil	0.18	-0.81***	0.32	-0.12	1.00
	Palmitic	1.00				
	Stearic	0.15	1.00			
R978	Oleic	-0.65**	-0.53*	1.00		
	Linoleic	-0.10	-0.32	-0.39	1.00	
	Oil	0.57*	-0.41	0.00	-0.13	1.00
	Palmitic	1.00				
	Stearic	0.15	1.00			
Hybrid	Oleic	-0.49*	-0.55*	1.00		
	Linoleic	0.22	0.15	-0.87***	1.00	
	Oil	-0.39	-0.29	-0.19	0.54*	1.00

*, **, *** = Significant at the P<0.05, 0.01 and 0.001 levels, respectively.

Fig. 11. Relationship between oleic and linoleic acids in inbred lines and in the hybrid.
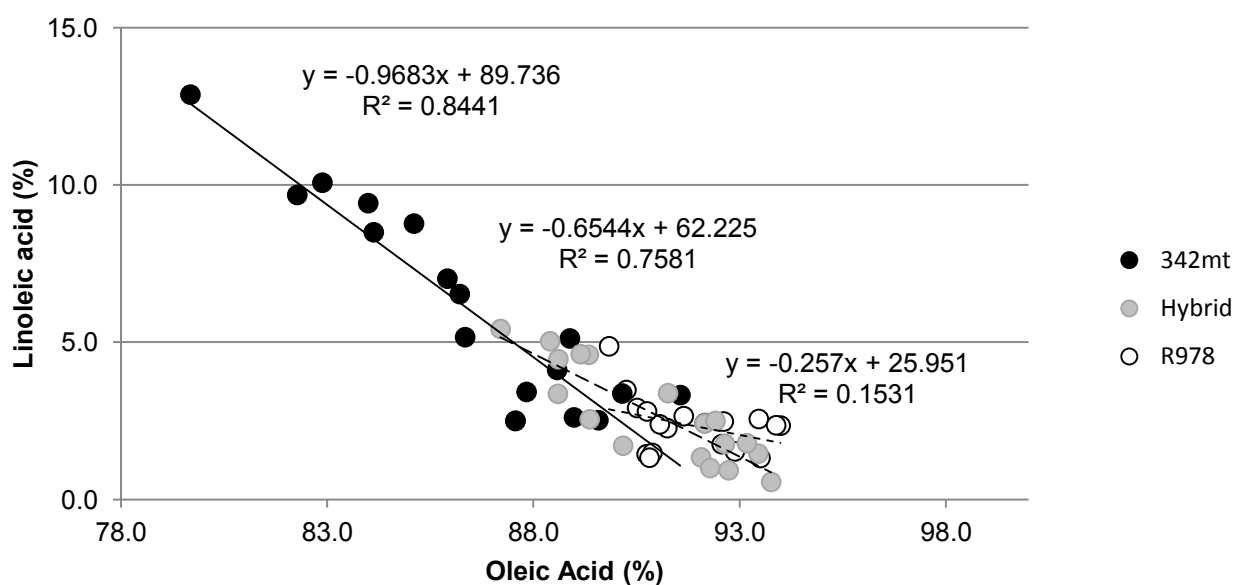
2.4   CONCLUSION

Fatty acid composition in high oleic sunflower depended mainly on the genotype. Environment could modify fatty acids profile in high oleic varieties, but the effects are smaller than in standard type sunflower. However, environmental effects are significant to achieve the 90% threshold of oleic acid content.

Seed fatty acid composition was mainly influenced by GDD_2 accumulated from end of flowering to 25 daf. Only stearic acid content was modified by GDD_1 accumulated from end of flowering to PM. Generally, at the highest GDD accumulated, palmitic, stearic and oleic acids increased with a genetic variability among high oleic genotypes. Linoleic acid decreased at the highest GDD_2 level.

Oleic acid contents was affected by temperature in two high oleic genotypes. To obtain environment-insensitive hybrids, selection could be based on inbred lines that not show any phenotypic variation in oleic acid content across years and locations. Genetic variability among high oleic genotypes tested in response to temperature could be related to modifier gene. Some differences are detected in the period in which temperature has the maximum effect on fatty acid composition. Furthermore, oleic acid content shows a good correlation with mean daily temperature in 342mt and to minimum night temperature in the hybrid. This result suggests that temperature could be acting on different physiological processes in HO genotypes. Further studies are needed.

Saturated fatty acid content was affected by GDD with a different response among genotypes. To obtain hybrids with a content in oleic acid higher than 93-95% it is necessary to select inbred lines with a low content in saturated fatty acids. Further studies on saturated fatty acid content in high oleic genotypes are needed.

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3   Maternal effects on oleic acid content in high oleic sunflower

ABSTRACT

High oleic sunflower is currently used in the food sector and as raw material for non food applications (biofuels, oleochemical). Non food applications, in particular, require a stable oleic acid content higher than 90%. Seed fatty acid composition depends on two factors, namely the genetic background of the embryo and the female plant condition (abiotic and biotic stresses). High oleic trait has been rarely studied in reciprocal crosses and in most of these cases maternal effects on the content of unsaturated fatty acids have not been identified (except for soya and rapeseed). The aims of this study are: (i) to check if any maternal effect on oleic acid content is present in high oleic mutants and (ii) if maternal effect and environment conditions could modify the 90% threshold for oleic acid content.

Two high oleic inbred lines with different origins were evaluated: 342mt, a selection derived by Ha 342 USDA (maintainer), and line R978 (male fertility restorer, branched), selected by University of Udine, and their reciprocal crosses (R978 x 342mt and 342mt x R978) obtained in 2010 by hand emasculation of female parent and controlled pollination. Field trials were conducted in 2011 at the experimental fields of the University of Udine using a complete randomization scheme with two dates of sowing.

Key results:

i) The two inbred lines respond differently to the same environmental conditions. R978 showed the same oleic acid content through sowing date, while 342mt showed variation in oleic acid content of 3% through sowing date;

ii) Reciprocal hybrids showed different accumulation patterns in the first sowing date and the same response as their female parent. Reciprocal hybrids were equal in the second sowing date. 342mt x R978 hybrid showed a variation in oleic acid content of 4% from first to second sowing date. In the first sowing date, 342mt x R978 hybrid showed an increase of 1.4% in linoleic acid content from 35 DAF to PM (Physiological Maturity);

iii) There is a maternal effect in the early stages of the seed filling phase. Low temperature increased linoleic acid and decreased oleic acid at 13 DAF only in 342mt inbred line and in 342mt x R978 hybrid. Low temperature treatment did not cause any variation on oleic acid content in line R978 and R978 x 342mt hybrid.

iv) Reciprocal hybrids and backcrosses showed a different content in oleic acid in the first sowing date. Difference in reciprocal generation was due to recombinant types with a Mid-oleic phenotype.

Temperature seems to modify segregation ratio or, in other words, the phenotypical expression of some genes. Reciprocal hybrids and backcrosses were equal in the second sowing date.

v) HO phenotype depends on three genetic factors: Pervenets allele, a second major gene (designated as Ols) and a combination of minor modifier. Their phenotypical effect seems to be influenced by cytoplasm and temperature.

High oleic inbred lines with different genetic backgrounds respond differently to the same environmental conditions. There is activity of a Δ12-desaturase in the early stages of accumulation of reserve lipids in one line and this is inherited from the mother plant. Results in reciprocal segregating and backcrosses populations suggest that oleic acid percentage was affected by cytoplasm or by cytoplasm x nucleus interaction.

The importance of female parental line choices in breeding to obtain hybrids insensitive to environmental conditions and with a stable oleic acid content over 90% was determined. Cytoplasmic effects could be used in breeding programs to select hybrids with an OAC insensitive to environment.

Key words: Environment, Fatty Acids, High Oleic Genotypes, Maternal Effect

3.1   INTRODUCTION

High oleic sunflower is currently used in the food sector and as raw material for non food applications (biofuels, oleochemical). Non food applications, in particular, require a stable oleic acid content higher than 90% (Vannozzi, 2006). In a breeding program targeted to select hybrids with an oleic acid content higher than 90% it is necessary to understand all phenomena that modify fatty acids accumulation and their ratio in the seed.

Seed fatty acid composition depends on two factors, namely the genetic background of the embryo and the female plant condition (abiotic and biotic stresses).

High Oleic mutants were obtained by chemical mutagenesis, with dimethyl sulfate, of normal sunflowers (Soldatov, 1976). High oleic trait is seed-specific and mutation reduced the expression of a Δ12-desaturase gene (Lacombe *et al.*, 2004). Its transcript is not accumulated during the grain filling period (Martínez-Rivas *et al.*, 2001). Some linoleic acid is still present in the achenes and it is synthesized by a constitutive desaturase system (Martínez-Rivas *et al.*, 2001; Lagravére *et al.*, 2004).

The high oleic trait was controlled by at least three loci: oleHL, supole, and modifier loci (Lacombe *et al.*, 2004). Genetic variability among HO genotypes is due to the alleles at these different loci. Many genetic approaches have been developed to study the HO mutation and in literature different conclusions are reported on the number of genes that control trait and on their dominance (Lacombe and Bervillé, 2000).

Oil composition in standard sunflower is clearly affected by the environment. It has long been known that temperature is the main environmental factor affecting the fatty acid composition in the oil of traditional type sunflower (Canvin, 1965), mainly regulating the ratio of oleic and linoleic acid during sunflower seed development. High temperature enhances the oleic acid content of normal cultivars (Low Oleic) but conflicting results are reported about temperature effects on oleic acid content of high oleic acid cultivars: no effect (Lagravére *et al.*, 2000) or an increase in oleic acid content with temperature (Triboï-Blondel *et al.*, 2000; Izquierdo and Aguirrezábal, 2008).

Variation in an individual's phenotype may be determined not only by the genotype and environment of that individual but also by maternal effects (Roach and Wulff, 1987). Maternal effects in plants can have a profound impact on selection, especially if selection is based on the phenotypic performance of seeds produced on a plant. Understanding how maternal effects influence selection can allow for the development of more efficient breeding strategies and an

increase in genetic gain. There are three types of maternal effects classified as cytoplasmic genetic, endosperm nuclear, and maternal phenotypic (Roach and Wulff, 1987). Cytoplasmic genetic maternal effects occur when the maternal parent passes, to her offspring, genes located in the cytoplasm that affect a certain trait. In self pollinated generations, these effects are heritable and normally are detected in each successive generation of inbreeding, unless cytoplasmic × nuclear genetic effects exist. In this case, the effects may dissipate or appear, depending on the interaction. Endosperm nuclear is not important in sunflower seed because endosperm is a tiny layer in sunflower seed (Seiler, 1997). The third class of maternal effects are maternal phenotypic, resulting from the environment and/or genotype of the maternal parent itself. These influences may occur via structure or physiology and may be the most common type of maternal influence. It is also important to realize that observed maternal effects could be the result of any one or a combination of the maternal effects just described.

A small number of studies investigated maternal effects on fatty acids in high oleic sunflower. Early studies on HO trait have indicated maternal influence but not complete maternal inheritance (Miller *et al.*, 1987) and the complete influence of the embryo genotype on trait (Urie, 1985; Fernández-Martínez *et al.*, 1989). Only one previous study has reported a maternal effect on high oleic trait (Lacombe and Bervillé, 2000). Reciprocal effects on oleic acid content have been reported by Varès *et al.* (2002). Interestingly, they conclude that some genetic variation for oleic acid content appeared in interaction with Pervenets mutation.

Maternal effect on fatty acids was also found in other oil crops such as canola and soybean (Thomas and Kondra, 1973; Erickson *et al.*, 1988; Gilsinger *et al.*, 2010).

Our approach to these problems was to use only high oleic genotypes with different genetic backgrounds. All tested genotypes were homozygous for the Pervenets allele and consequently every phenotypic variation in oleic acid content is due to different alleles at the other loci that acts on the high oleic trait.

In order to study the effect of the temperature on oleic and linoleic acid contents in HO genotypes with a different genetic backgrounds, portions of sunflower head were incubated in the air, in Petri dishes containing water, at 10 °C for 24 h. An index of the ODS (Oleate Desaturase) activity (Green, 1986) was calculated. The value of this index is directly proportional to the activity of the enzyme system believed to be responsible for the desaturation of oleic acid (Cherif *et al.*, 1975).

Cytoplasmic influences are normally detected via reciprocal crosses and backcrosses (Mosjidis and Yermanos, 1984), since, in sunflower, cytoplasmic genes are inherited maternally (Rieseberg *et al.*,

1994). Reciprocal backcrosses are used because they give more accurate results when differences in phenological development between parental lines may have an effect on the trait studied (Mosjidis and Yermanos, 1984). Furthermore, in our case, the use of F_1 plants as mother plants, permitted to overcome the problem of branching that may have an effect on the trait studied.

The aims of this study are: (i) to test for the presence of maternal effects on oleic acid content in high oleic mutants and (ii) if any occurred, how maternal effects and environment could modify the 90% threshold for oleic acid content.

3.2   MATERIALS AND METHODS

3.2.1 Experiment 1. Effects of low temperature

3.2.1.1 Plant materials

Two high oleic inbred lines and their reciprocal cross were used. Inbred lines used were: 342mt and R978. Line 342mt is a selection derived by Ha 342 USDA, and it is a male sterility maintainer with a single head. Line R978, selected by University of Udine, is a fully-branched type and it is a male fertility restorer. Reciprocal F_1 seeds were obtained by hand demasculation of female parent and controlled pollination in field conditions at Udine in 2010. All plants were covered with paper bags, during the R4 stage (Schneiter and Miller, 1981), to prevent cross-fertilization. The reciprocal F_1 seed was planted in field in 2011 and tested with parental inbred lines.

3.2.1.2 Field experiment

Inbred lines and their reciprocal hybrids (F_1 hybrids plants and F_2 seeds) were grown in 2011 at University of Udine, Azienda Agraria Universitaria "A. Servadei" (46°04'N, 13°22'E, altitude 109 m) in North-East Italy. The experiment was designed as a complete randomization scheme, with three replications, using two dates of sowing. The first date of sowing was 18th April (I) and the second was 31st May 2011 (II). Plants were thinned after seedling emergence from 10 to 7.5 plants m^{-2}. Nitrogen was applied at 100 kg ha^{-1}. Weeds and diseases were controlled, and regular watering throughout the experiment ensured that plants were not subjected to water deficit during the entire growth period. All plants were covered with paper bags, at the R4 stage, to prevent cross-fertilization.

3.2.1.3 Sampling

Two types of samples were taken: i) 5 seeds per plant and ii) pieces of sunflower head (see below) in several days during seed filling period. Seeds sampling were started at 13 DAF (Days After Flowering). Five seeds per plant (taken from the outer rings of the head) were taken every 7 days from 13 DAF to 35 DAF, in order to determine fatty acid accumulation. Seeds were dried in an oven at 60 °C for 24 h. The final harvest was done at physiological maturity (R9 phase; Schneiter and Miller, 1981).

3.2.1.4 Effect of Low Temperature

To simulate the physiological conditions of seed lipid synthesis in the plant, portions of sunflower head were collected at 13, 20, 27 and 35 DAF. Portions of head were incubated in the air, in Petri dishes containing water, at 10 °C for 24 h (García-Díaz et al., 2002). After cold treatment, pieces of

head were dried in an oven at 70 °C for 24 h. An index of the ODS (Oleate Desaturase) activity (Green, 1986) was calculated for each sampling using the formula:

$$ODS \text{ activity index} = \%18{:}2/(\%18{:}2+\%18{:}1)$$

where %18:2 and %18:1 are the percentage of linoleic and oleic fatty acids, respectively.

The value of this index is directly proportional to the activity of the enzyme system believed to be responsible for the desaturation of oleic acid (Cherif *et al.*, 1975).

3.2.2 Experiment 2. Reciprocal crosses and backcrosses

3.2.2.1 Plant materials

Maternal effects were evaluated by analyzing F_1 seeds resulting from the reciprocal cross between the high oleic inbred line 342mt and R978 and from the reciprocal F_2 segregating seeds and from the reciprocal backcrosses BC_1F_1 seeds.

3.2.2.2 Field experiment

F_1 seeds generation was tested on female parental plant in 2010 with three sowing dates: 19th April (I), 12th May (II) and 1st June 2010 (III; Table 9). Five F_1 plants from each reciprocal cross were self pollinated (covered with paper bags during R4 phase) to produce F_2 seeds and five F_1 plants were emasculated for each reciprocal backcross during 2011. F_2 generation and reciprocal backcross generation (BC_1F_1) were evaluated under natural field conditions in 2011 in two sowing dates. The first (I) was 26th April and the second (II) date of sowing was 10th June 2011 (Table 12).

3.2.2.3 Emasculation and Cross

All plant materials used were male-fertile. Female parental plants require emasculation to avoid self-pollination. Hand emasculation was done pinching off the anthers with tweezers. Five plants for each reciprocal backcross were emasculated. Emasculation began with first flowering (R5.1; Schneiter and Miller, 1981) and plants were emasculated for the next 4 days. Afterwards, the disk flowers in the center of the head were excised and controlled pollination was carried out. Half-head of male parent was cut from the stem and carried to female parent plants for pollination. The other half-head was self-pollinated to evaluate male parental line.

3.2.2.4 Sampling

Plants were harvested at physiological maturity (R9 phase; Schneiter and Miller, 1981). Seeds from head outer rings (1-6), with the same developmental timing, were separated. Two types of sampling

were taken. (1) 100 seeds were counted and dehulled. Kernels were ground to a fine powder using a coffee grinder. 200 mg of kernel powder were weighed to perform fatty acid analysis. (2) To verify the hypothesis of maternal effects, individual seeds were analyzed from the F_1, F_2, BCP_1, and BCP_2 reciprocal populations. To detect transgressive segregation in F_1 generation, 15 single-seeds were analyzed. To detect segregation in reciprocal F_2, BCP_1, and BCP_2 populations, 50 single seeds from outer ring (with the same development timing) of each plant were analyzed for fatty acids composition. 5 plants were studied from each population.

3.2.3 Fatty acids determination

Individual seeds were dehulled and grounded. Lipids were extracted in 1 mL of *n*-hexane. Fatty acids were converted in Fatty Acid Methyl Esters (FAMEs) by transesterification with a methanolic potassium hydroxide solution (2N). Fatty acid composition was determined by gas chromatography and every fatty acid was expressed as a percentage of the total fatty acids detected in the oil. The gas chromatograph was fitted with a 60 m HP-88 capillary column (Agilent Technologies, USA). Helium was used as carrier gas, and the injector, detector and oven temperatures were 230, 250 and 200 °C, respectively. 5 µL of sample were injected in split mode. A 5.0 mm ID precision inlet split liner with wool (Restek, USA) was used. Different FAMEs were identified by comparison with known standards.

3.2.4 Statistical Analysis

Statistical analysis was performed using R version 2.15.0 (R Development Core Team, 2012). Shapiro-Wilk normality test was performed to test normality condition. ANOVA (ANalysis Of VAriance) and LSD (Least Significant Distance) test with *p*-value adjusted using Bonferroni correction were applied to experimental results to determine the significance of differences among treatments. Segregation ratios were tested by a chi-square goodness of fit.

3.3   RESULTS

3.3.1 Experiment 1

Sowing date effects on duration of cycle and F-PM (Flowering to Physiological Maturity) phase in inbred lines and reciprocal F_1 plants are showed in Table 7. Later sowing date resulted in a shorter cycle, likely due to environmental differences during plant development. Within sowing date, reciprocal F_1 plants had the same developmental timing.

ANOVA results for the main and interaction effects of genotypes and sowing dates on seed fatty acids composition indicated a significant sowing date by genotype interaction effect. There are some differences between the inbred lines and reciprocal hybrids.

Sowing date did not alter the oleic acid concentration in line R978 and in its R978 x 342mt hybrid. On the other hand, sowing date altered the oleic and linoleic acids concentration in line 342mt and in 342mt x R978 hybrid (Table 8).

We also investigated the variation in saturated fatty acid concentration across sowing date. Palmitic and stearic fatty acids content did not alter oleic acid concentration (Table 8). Reciprocal hybrids were equal for saturated fatty acids content.

The concentration of oleic acid was affected by the concentration of linoleic acid. This was confirmed by ODS index due to the metabolism pathway (Lagravére *et al.*, 2004). In the first sowing date ODS activity was significantly higher than in the second sowing date only in 342mt and 342mt x R978. Reciprocal hybrids were not equal for unsaturated fatty acids content through sowing date.

The oleic acid accumulation showed some differences between inbred lines and hybrids. R978 and R978 x 342mt showed the same accumulation pattern for both sowing dates. 342mt and 342mt x R978 showed the same accumulation pattern of the other genotypes within the second sowing date. There were some differences within the first sowing date.

All the genotypes exhibited a synthesis of linoleic acid at early stages (13 DAF), but we reported a difference between the two high oleic inbred lines and among reciprocal hybrids.

Table 7. Genotypes, sowing date, cycle duration, duration of Flowering to Physiological Maturation (F-PM) and mean temperatures from F-PM for field experiments.

Genotype	Sowing Date	Cycle	F-PM	Tmin	Tmean	Tmax
		(dd)	(dd)	°C	°C	°C
342mt	I	110	41	16.2	22.4	28.7
R978	I	113	41	16.1	22.5	29.0
342mt x R978	I	123	53	16.2	23.2	30.5
R978x 342mt	I	123	53	16.2	23.2	30.5
342mt	II	101	37	16.7	23.9	31.5
R978	II	101	37	16.7	23.9	31.5
342mt x R978	II	108	42	16.7	23.8	31.4
R978x 342mt	II	109	43	16.7	23.8	31.4

Table 8. Fatty acids composition (%) of seed from self-pollinated inbred lines and hybrids. ODS is an adimensional index of oleato desaturase activity. Results are means ±SE.

Genotype	Sowing date	Fatty acid (%)				ODS
		Palmitic	Stearic	Oleic	Linoleic	
342mt	I	3.54±0.04c	3.05±0.22bc	83.99±0.64c	9.42±0.38a	0.11±0.006a
R978	I	3.12± 0.05c	2.76±0.36bc	91.92±0.44a	2.19±0.21cd	0.02±0.002c
342mt x R978	I	3.57± 0.12c	3.39±0.19ab	88.07±0.44b	4.97±0.28b	0.05±0.003b
R978 x 342mt	I	4.47± 0.13bc	3.31±0.13ab	90.62±0.25a	1.60±0.01cd	0.02±0.000c
342mt	II	5.21± 0.39a	4.25±0.09a	87.16±0.41b	3.39±0.88bc	0.04±0.011bc
R978	II	4.87± 0.04a	2.91±0.07bc	90.81±0.04a	1.42±0.04cd	0.02±0.003c
342mt x R978	II	3.86± 0.13bc	2.61±0.10bc	92.35±0.20a	1.09±0.12d	0.01±0.000c
R978 x 342mt	II	3.76± 0.15bc	2.19±0.06c	91.56±0.55a	1.40±0.23d	0.02±0.003c
	LSD at 5%	0.90	0.96	2.19	1.98	0.02

Values followed by the same letter are not significantly different (LSD at the 5% level, *p*-value adjustment Bonferroni).

Fig. 12. Oleic (a) and linoleic (b) fatty acids accumulation in lines and hybrid in the first sowing date.
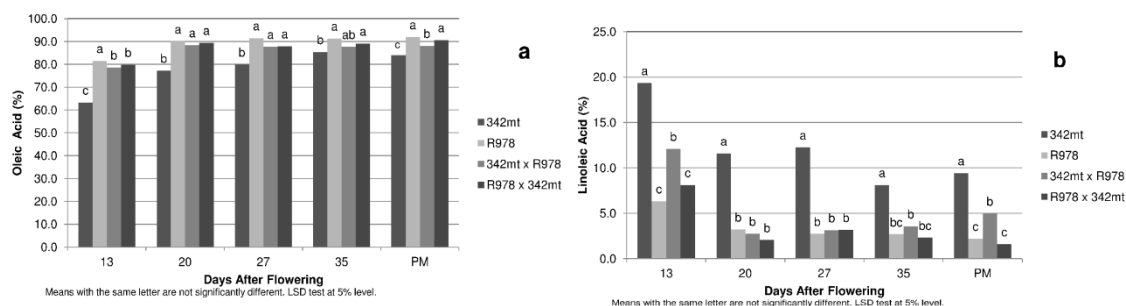
Line 342mt presented the highest initial linoleic acid content followed by a rapid decrease (Fig. 12). Line R978 presented a lower initial linoleic acid content than 342mt followed by a rapid decrease.

In 342mt and in 342mt x R978, the amount of linoleic acid synthesized is higher than in other genotypes. Reciprocal hybrids were not equal for oleic and linoleic fatty acids content at 35 DAF and at physiological maturity (Fig. 12). 342mt x R978 hybrid showed a continuous increase of linoleic acid accumulation (1.4% from 35 DAF to PM).

Cold treatment (10 °C for 24h) modified the fatty acid composition only at 13 DAF in line 342mt and 342mt x R978 hybrid (Fig. 13). A net increase in linoleic acid content occurred for both sowing dates. The lowest increase was observed in the seeds incubated at 10 °C in the second sowing date. The cold treatment on line R978 and hybrid R978 x 342mt did not modify oleic and linoleic acid content in the seeds incubated at 10 °C (Fig. 13).

Fig. 13. Low temperature (10 °C) effects on oleic acid content at 13 DAF in the first (a) and in the second sowing date (b).
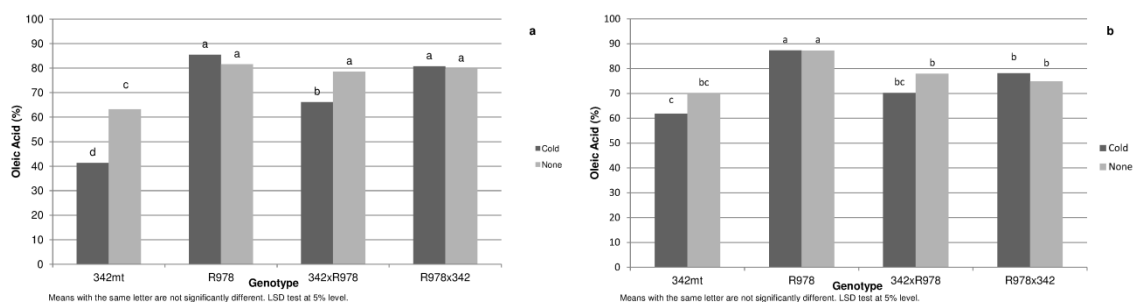
3.3.2 Experiment 2

Whole cycle duration (Emergence – Physiological Maturity), vegetative (Emergence – Flowering) and reproductive phases (End of Flowering – Physiological Maturity) duration are reported in Table 9. Physiological maturity was expressed as R9 phase (Schneiter and Miller, 1981).

No transgressive segregation for all fatty acid was detected in F_1 seeds generation (data not shown). Palmitic acid content depends only on genotypes and there was no significant difference between reciprocal hybrids seeds. Date of sowing influenced stearic, oleic and linoleic acids content as well as ODS activity with some differences among genotypes (Table 10). Mean oleic acid content of F_1 seeds resulting from the cross between 342mt x R978 and the reciprocal R978 x 342mt averaged 91.6% and 90.6% respectively, very similar to the content in this acid of the parent R978 (91%). In the first and in the second sowing date reciprocal hybrid seeds had the same oleic acid content (Table 11) while in the third sowing date a significant difference between reciprocal hybrids was detected. On the other hand, only in the inbred line 342mt, linoleic acid content and ODS index were modified by date of sowing. No significant difference was found in the reciprocal F_1 seed. Thus, variations in oleic acid content were related to decrease in stearic acid content. Hybrid 342mt x R978 showed a decrease in stearic acid content from first to third sowing date.

Significant differences between O/L means of reciprocal F_1 seeds were observed (Table 11). F_1 seeds from the cross 342mt x R978 had a higher O/L ratio than the reciprocal F_1. On the other hand, R978 x 342mt showed the same O/L ratio across sowing date and it was equal to O/L ratio of the female parent used in the cross. Significant differences between O/L means of reciprocal F_1 support a maternal effects hypothesis.

Hybrid plants and parental inbred lines cycle duration (Emergence – Physiological Maturity), vegetative (Emergence – Flowering) and reproductive phases (End of Flowering – Physiological Maturity) duration in 2011 are reported in Table 12.

Table 9: Sowing date, cycle duration, duration of Flowering to Physiological Maturation (F-PM) and GDD (Growing Degree Day, tb=6 °C) for inbred lines used as mother plants for F_1 hybrid seeds.

Date of Sowing	Female parental line	Male parental line	Cycle (dd)	E-F (dd)	F-PM (dd)	GDD F-25 daf	GDD F-PM
19/04/2010	342mt	R978	111	71	40	430	636
	R978	342mt	119	76	43	391	591
12/05/2010	342mt	R978	98	59	39	378	569
	R978	342mt	105	66	39	376	577
01/06/2010	342mt	R978	91	57	34	403	516
	R978	342mt	95	59	36	374	475

Table 10. Analysis of Variance (Mean Square) for the main fatty acids in F$_1$ generation.

SoV	DF	Palmitic	Stearic	Oleic	Linoleic	ODS	O/L ratio
Date of Sowing (D)	2	0.0746ns	0.5897*	7.76***	4.91***	0.000553***	92.6ns
Genotype (G)	3	0.8697***	1.4842***	119.55***	83.13***	0.010578***	2801.0***
D x G	6	0.2228ns	1.0128***	7.61***	6.35***	0.000808***	157.3ns
Residuals	24	0.1003	0.1116	0.44	0.39	0.000053	77.2

Table 11. Fatty acids composition (%) of seed from self-pollinated inbred lines and from reciprocal F$_1$ seeds. ODS (index of oleato desaturase activity) and O/L (Oleic/ Linoleic acids ratio) are adimensional. Results are means.

Genotype	Sowing date	Fatty acid (%)				ODS	O/L ratio
		Palmitic	Stearic	Oleic	Linoleic		
342mt	I	3.93	3.28 abcd	83.20 d	9.59 a	0.11 a	9
R978	I	3.42	2.54 cd	91.72 ab	2.72 c	0.03 c	40
342mt x R978	I	4.04	3.93 a	89.88 b	2.15 c	0.02 c	42
R978 x 342mt	I	4.04	2.41 cd	91.07 ab	2.48 c	0.03 c	37
342mt	II	4.20	4.05 a	81.50 d	10.25 a	0.11 a	8
R978	II	3.51	3.02 abcd	90.76 b	2.72 c	0.03 c	34
342mt x R978	II	3.29	3.22 abcd	91.85 ab	1.64 c	0.02 c	62
R978 x 342mt	II	3.91	3.43 ab	90.45 b	2.21 c	0.03 c	43
342mt	III	4.19	3.91ab	86.76 c	5.14 b	0.06 b	18
R978	III	3.57	2.86 bcd	90.50 b	3.06 c	0.03 c	30
342mt x R978	III	3.19	2.27 d	92.96 a	1.58 c	0.02 c	59
R978 x 342mt	III	3.91	3.17 abcd	90.44 b	2.48 c	0.03c	37
	LSD at 5%	ns	1.05	2.10	1.96	0.023	ns

Values followed by the same letter are not significantly different (LSD at the 5% level, *p*-value adjustment Bonferroni). Ns Not Significant

Sowing date affected all fatty acids content and the ODS and O/L index (Table 13). Palmitic acid content increased in all tested genotypes from the first to the second sowing date. The highest value was recorded by line 342mt (5.20% in the second sowing date) and lowest value was achieved by R978 (3.21% in the first sowing date). Reciprocal segregating generation did not show any significant difference within sowing date. Reciprocal backcrosses with 342mt as polliniser had the highest content of palmitic acid. No reciprocal differences were detected.

The highest stearic acid content (4.00% in the first sowing date) was achieved by [342mt x R978] x 342mt backcross and the lowest (2.03% in the second sowing date) was showed by [R978 x 342mt] x R978 backcross. Date of sowing did not alter stearic acid content in parental inbred lines and in reciprocal segregating generation. Inbred line 342mt had a higher stearic acid content than line R978. Reciprocal backcrosses with R978 as polliniser had a lower content of stearic acid. No maternal effect on stearic acid content was detected.

Oleic acid content increased from the first to the second planting date only in some genotypes. The highest value (93.02%) was recorded by backcross [342mt x R978] x R978 and the lowest by inbred line 342mt (84.42%). In 342mt inbred line, oleic acid content was affected by sowing date and it showed a lowest oleic acid content than inbred line R978. R978 did not show any variation through sowing date. A significant difference of about 6% in oleic acid content was detected among reciprocal segregating generations in the first sowing date. Also, a reciprocal effect was detected in the first sowing date. Backcross [342mt x R978] x 342mt showed a significantly lower content of oleic acid than the reciprocal backcross and equal to 342mt x R978 F_2 seeds.

Linoleic acid content varied across sowing date as oleic acid content. The concentration of oleic acid was affected by the concentration of linoleic acid. This was confirmed by ODS index. In the first sowing date ODS activity was significantly higher than in the second sowing date in 342mt, 342mt x R978 and in [342mt x R978] x 342mt.

O/L ratio was affected by sowing date in the inbred line 342mt, in the segregating population 342mt x R978 and in the backcross [342mt x R978] x 342mt. The highest value (95 in the second sowing date) was achieved by [R978 x 342mt] x R978 and the lowest (11) by 342mt and 342mt x R978 in the first sowing date. The O/L ratio in hybrid 342mt x R978 and in the backcross with 342mt was equal to female parental line in the first sowing date. The backcross with R978 showed a O/L ratio higher than female parent. On the other hand, reciprocal effects were detected. The backcross [342mt x R978] x 342mt was not equal to [R978 x 342mt] x 342mt for O/L ratio in the first sowing date.

Means and ranges of the main FAMEs, based on single seed analysis, in parental lines, reciprocal segregating generation and reciprocal backcrosses were reported in Table 14. Differences were found in oleic acid content in the reciprocal F_2 segregating populations and in the reciprocal backcrosses BC_1F_1. Recombinant individuals, that displayed an intermediate content of oleic acid, that were not found in parent lines and in the reciprocal hybrid generation, were detected in the F_2

seeds of the cross 342mt x R978 and in the BC_1F_1 seeds from the backcross [342mt x R978] x 342mt.

To study the segregation pattern in F_2 and in BC_1F_1 generations, ODS index was used to compare phenotypes to avoid an interference on unsaturated fatty acids by the other fatty acids content. The distributions for F_2 and BC_1F_1 seeds were divided into phenotypic classes on the basis of the ODS index of the parents grown in the same environment (Table 17). Any individual with an ODS index greater than 0.10 (the highest value recorded in inbred line 342mt) was classified as recombinant (individual that showed a phenotype not matched in inbred and reciprocal hybrid seeds). Recombinant individuals with an intermediate content of oleic acid were detected only in the first sowing date in the F_2 seed of 342mt x R978 and in backcross [342mt x R978] x 342mt with a frequency of 25%. In the second sowing date no segregation was detected in any of the tested genotypes and generations. Thus, variation in oleic acid content across sowing date was related to a different segregation pattern and not to an overall decrease or increase in oleic acid content (e.g. all seeds showed the same variation). All 5 plants had the same segregation pattern and fatty acids composition. No different pattern of segregation within genotypes was detected.

In the F_2 seeds of the cross 342mt x R978, we obtained a fit to a 3:1 ratio (Non recombinant – Recombinant). The F_2 seeds population supported the hypothesis of a gene, with two alleles, in addition to oleHL gene (in this case it is homozygous for the Pervenets allele) that controlled the HO trait. This gene, when it is homozygous for the recessive allele, affected oleic acid content in HO mutants. The backcross with female parent 342mt fitted the ratio 3:1 non recombinant – recombinant. The number of segregants in the recombinants ODS class was lower than expected if a single gene was controlling high oleic expression (1:1 expected segregation ratio in backcross). The backcross supported a two gene theory in addition to Ol gene. The backcross with male parent R978 showed only offspring with a parental phenotype. The backcrosses supported a hypothesis that the recessive allele of the first gene was in the 342mt inbred line. A first gene could affect the expression of a second gene. A different distribution in oleic classes of the F_2 and BC_1F_1 recombinant seeds was found (Table 15 and Table 16). The seeds with the lowest content of oleic acid (55-65%) had the same frequency in the F_2 and BC_1F_1 generations. The seeds in other intermediate oleic acid classes had a different frequency in the F_2 and BC_1F_1. The relationship oleic – linoleic acids content in the F_2 and in the BC_1F_1 with female parent 342mt (Fig. 14) showed some difference among generations. The F_2 generation could be divided into two main groups: recombinant and non recombinant. Recombinant class (58%-75%) showed a continuous variation in the oleic linoleic acids relationship. The BC_1F_1. generation could be divided into four discrete

groups: Non Recombinant and three type of recombinants (about 60%, 70% and 80% in oleic acid content). Considering the BC_1F_1 plants (replicates) separately we observed a slightly difference in the frequency distribution of the seeds in this three type of recombinants (data not shown). This suggest that the third genetic elements that controlled HO trait could be some minor genes.

Table 12. Sowing date, cycle duration, duration of Flowering to Physiological Maturation (F-PM) and GDD (Growing Degree Day, tb=6 °C) for parent lines and F_1 plants.

Date of Sowing	Genotype	Cycle (dd)	E-F (dd)	F-PM (dd)	GDD F-25 daf	GDD F-PM
26/04/2011	342mt	100	55	35	449	681
	R978	102	62	32	448	581
	342mt x R978	123	56	53	390	916
	R978 x 342mt	123	57	53	390	916
10/06/2011	342MT	101	59	37	456	674
	R978	101	60	37	416	674
	342mt x R978	111	59	47	458	760
	R978 x 342mt	111	58	47	458	760

Table 13. Analysis of Variance (Mean Square) for the main fatty acids in parent lines, reciprocal F_2 seed generation and Backcrosses.

SoV	DF	Palmitic	Stearic	Oleic	Linoleic	ODS	O/L ratio
Genotype	7	7.260***	14.672***	116.52***	93.48***	0.010811***	11496***
Date of Sowing	1	1.200***	1.002***	49.96***	27.04***	0.003321***	4249***
G x D	7	0.887***	1.626***	20.79***	16.74***	0.002077***	2045***
Residuals	64	0.076	0.143	1.39	1.20	0.000150	269

Table 14. Fatty acids composition (%) of seed from self-pollinated inbred lines and hybrids (F$_2$ seed) and from backcrosses. ODS and O/L are adimensional. Results are means.

Genotype	Generation	Sowing date	Plants number	Fatty acid (%)				ODS	O/L ratio
				Palmitic	Stearic	Oleic	Linoleic		
342mt	Inbred	I	5	4.53 bc	3.28 a	84.42 c	7.77 a	0.08 a	11 f
R978	Inbred	I	5	3.21 h	3.05 bcdef	91.30 a	2.44 c	0.03 d	38 cdef
342mt x R978	F$_2$	I	5	3.78 efgh	3.76 a	84.52 c	7.94 a	0.09 a	11 f
[342mt x R978] x 342mt	BC$_1$F$_1$	I	5	3.78 efgh	4.00 a	86.75 bc	5.47 ab	0.06 bc	21 ef
[342mt x R978] x R978	BC$_1$F$_1$	I	5	3.86 defg	2.91 bcdefg	91.77 a	1.47 c	0.01 d	65 abc
R978 x 342mt	F$_2$	I	5	3.58 gh	3.19 a	90.98 a	2.25 c	0.02 d	60 abcd
[R978 x 342mt] x 342mt	BC$_1$F$_1$	I	5	4.06 cdefg	3.59 a	90.46 a	1.88 c	0.02 d	51 bcde
[R978 x 342mt] x R978	BC$_1$F$_1$	I	5	3.68 fgh	2.87 cdefg	92.49 a	0.98 c	0.01 d	95 a
342mt	Inbred	II	5	5.20 a	3.78 a	87.52 b	3.50 bc	0.04 cd	25 def
R978	Inbred	II	5	4.90 ab	2.87 cdefg	90.70 a	1.53 c	0.02 d	60 ab
342mt x R978	F$_2$	II	5	4.44 bcde	2.18 fg	92.21 a	1.17 c	0.01 d	81 ab
[342mt x R978] x 342mt	BC$_1$F$_1$	II	5	4.47 bcd	2.09 g	92.32 a	1.12 c	0.01 d	86 ab
[342mt x R978] x R978	BC$_1$F$_1$	II	5	3.62 fgh	2.05 g	93.02 a	1.32 c	0.01 d	72 ab
R978 x 342mt	F$_2$	II	5	4.61 abc	2.42 defg	91.32 a	1.64 c	0.02 d	72 ab
[R978 x 342mt] x 342mt	BC$_1$F$_1$	II	5	4.23 cdef	2.03 g	92.22 a	1.51 c	0.02 d	63 abcd
[R978 x 342mt] x R978	BC$_1$F$_1$	II	5	3.84 defg	2.36 efg	92.68 a	1.12 c	0.01 d	84 ab
LSD at 5%				0.65	0.89	2.78	2.58	0.03	39

Means followed by the same letter are not significantly different (LSD at the 5% level, *p*-value adjustment Bonferroni).

Table 15. Mean and range for the main fatty acid contents of the reciprocal F_2 and backcrosses seeds. Data for any genotypes was from 5 plants and 50 seeds per plant from outer rings.

Genotype	Generation	Seeds	Fatty acid (%)			
			Palmitic	Stearic	Oleic	Linoleic
342mt x R978	F_2	250	3.78 [2.76-4.97]	3.65 [2.16-5.25]	84.98 [57.80-92.80]	7.58 [0.86-34.98]
[342mt x R978] x 342mt	BC_1F_1	250	3.85 [2.84-4.84]	3.78 [2.01-5.99]	86.26 [60.58-92.74]	6.11 [1.10-31.29]
[342mt x R978] x R978	BC_1F_1	250	3.76 [2.87-4.85]	2.87 [1.62-4.84]	91.77 [87.01-93.74]	1.60 [0.47-3.96]
R978 x 342mt	F_2	250	3.55 [2.25-4.49]	3.11 [1.80-4.75]	91.45 [85.35-93.50]	1.89 [0.64-7.78]
[R978 x 342mt] x 342mt	BC_1F_1	250	4.00 [3.15-5.51]	3.40 [1.61-5.77]	90.37 [81.22-93.07]	2.24 [0.49-11.00]
[R978 x 342mt] x R978	BC_1F_1	250	3.70 [3.03-4.26]	2.87 [1.74-3.88]	92.34 [90.77-93.42]	1.09 [0.60-1.97]

Table 16. Distribution of seeds in oleic class. From 81% to >90% individuals had an equal oleic acid content to parent lines and they were not recombinants. If oleic acid content was lower than 80%, the individual seed was recombinant.

Genotype	Generation	Seeds	Oleic Acid					
			55-65	66-75	76-80	81-85	86-90	>90
342mt x R978	F_2	250	22	37	2	0	16	173
[342mt x R978] x 342mt	BC_1F_1	250	20	18	6	14	59	133
[342mt x R978] x R978	BC_1F_1	250	0	0	0	0	18	232
R978 x 342mt	F_2	250	0	0	0	0	36	214
[R978 x 342mt] x 342mt	BC_1F_1	250	0	0	0	8	69	173
[R978 x 342mt] x R978	BC_1F_1	250	0	0	0	0	0	250

Table 17. Distribution of seeds in ODS Activity class. If ODS activity was higher than 0.10, the individual seed was recombinant.

Genotype	Generation	Seeds	ODS Activity			
			0.1	0.11-0.20	0.21-0.30	>0.30
342mt x R978	F_2	250	189	5	35	21
[342mt x R978] x 342mt	BC_1F_1	250	192	34	5	19
[342mt x R978] x R978	BC_1F_1	250	250	0	0	0
R978 x 342mt	F_2	250	250	0	0	0
[R978 x 342mt] x 342mt	BC_1F_1	250	250	0	0	0
[R978 x 342mt] x R978	BC_1F_1	250	250	0	0	0

Fig. 14. Oleic linoleic acids relationship in [R978 x 342mt] and in [342mt x R978] backcrosses with 342mt.
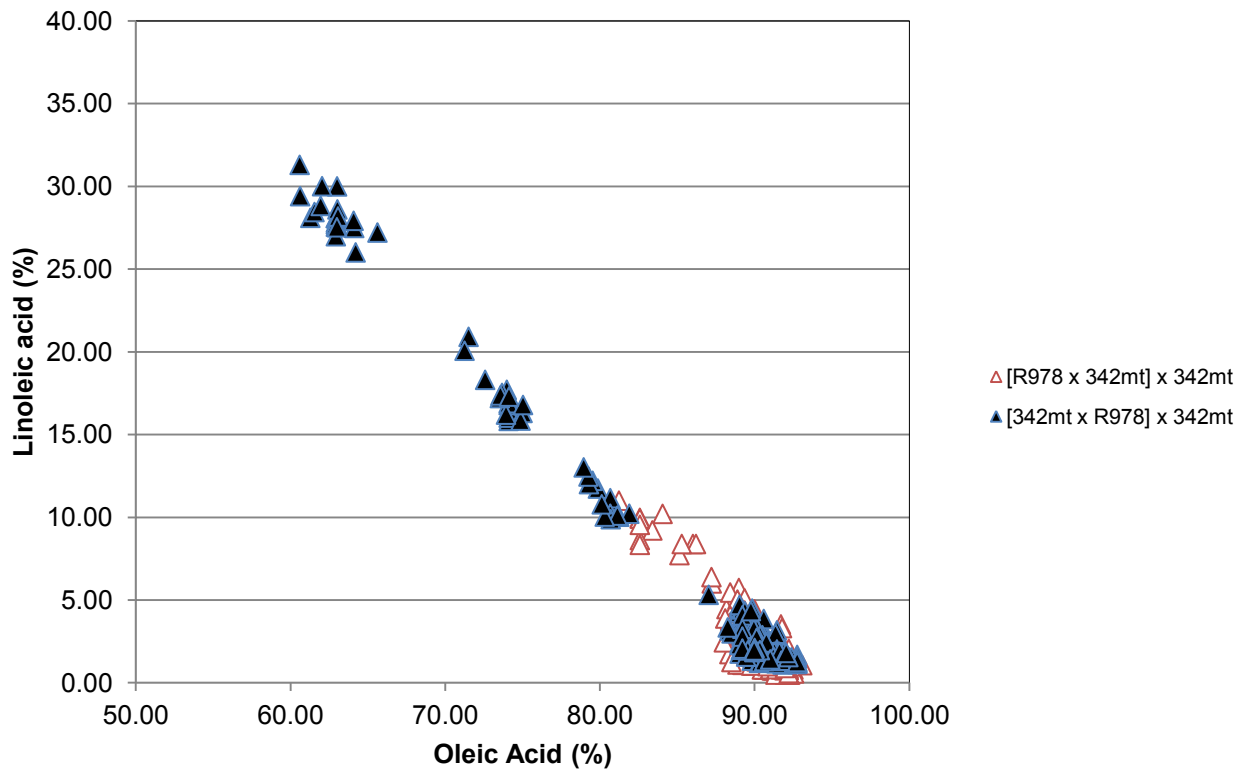
Fig. 15. Oleic linoleic acids relationship in the F_2 seeds from the cross 342mt x R978 and R978 x 342mt.
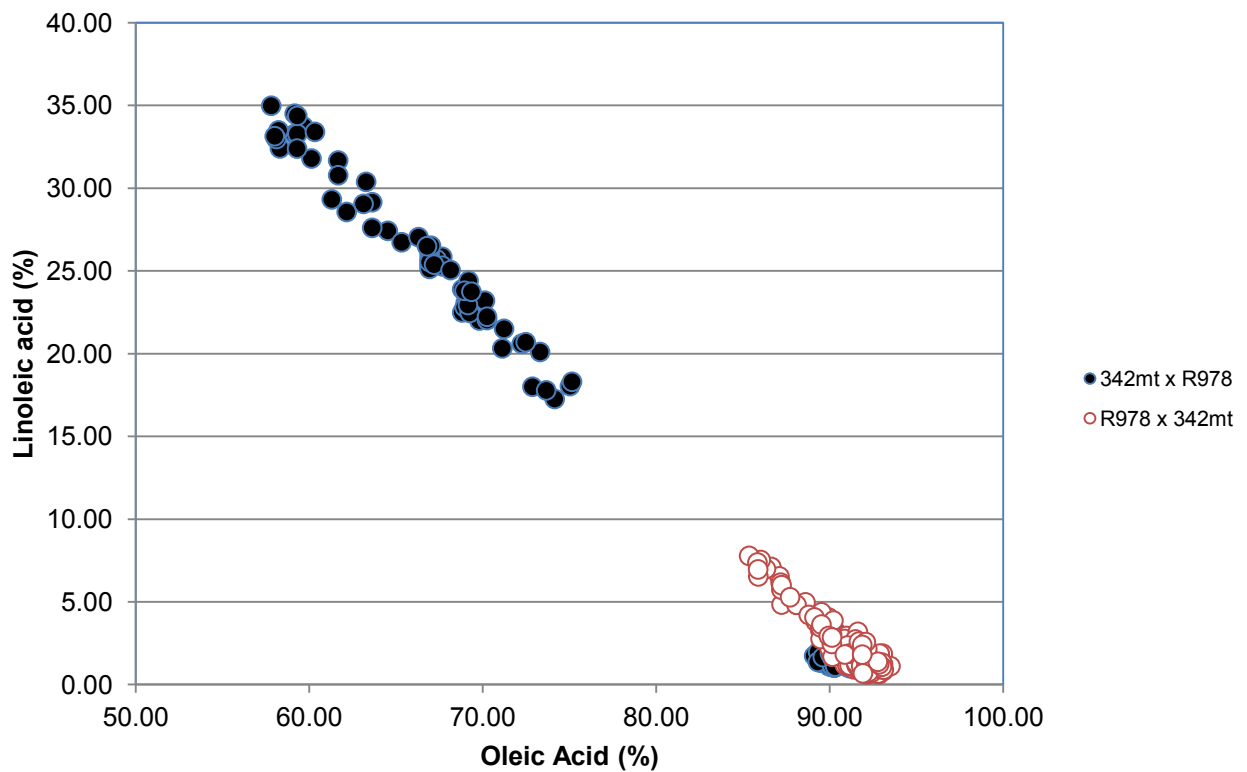
3.4   DISCUSSION

3.4.1 Experiment 1

Line R978 was insensitive to environment conditions and had the same composition through sowing date. On the other hand, fatty acids composition in line 342mt varies through sowing date: oleic acid content increases with temperature (Table 7 and Table 8). Temperature was the major environmental factor that varies among sowing date. The other factors that may modify fatty acids composition, such as water (Roche *et al.*, 2006) and nitrogen (Zheljazkov *et al.*, 2009), were controlled. Different genetic background and modifier genes could cause differences between high oleic inbred lines (Lacombe *et al.*, 2004). Thus, some high oleic genotypes showed a response to temperature (Izquierdo and Aguirrezábal, 2008) while others did not (Lacombe and Bervillé, 2000; Lagravére *et al.*, 2000).

It seems that difference in oleic acid content at maturity is due to a diverse pattern of fatty acid accumulation and Microsomal Oleate Desaturase (MOD) activity and, therefore, to different metabolism in high oleic mutants (Fig. 12). Different fatty acid metabolisms in high oleic hybrids were already found by Lagravére *et al.* (2004). At 13 DAF, line R978 showed an intermediate linoleic content among those reported by Lagravére *et al.* (2004). These Authors reported a linoleic acid percentage of 21.17% and 0.50% in two HO hybrids. According to Martínez-Rivas *et al.* (2001) and Lagravére *et al.* (2004), two desaturase systems are involved in the biosynthesis of unsaturated fatty acids. The first one is a constitutive system present in the whole plant, and the second is specifically devoted to storage metabolism in the achenes. During the first part of the seed filling period, the constitutive desaturase could be responsible for a low accumulation of linoleic acid in all kinds of hybrids. The specific desaturase is then involved in the accumulation of high quantities of linoleic acid in the standard hybrids, but its activity is lacking in high oleic varieties and thus oleic acid accumulates. Data showed that 342mt and R978 displayed OAC of 91% and 85%, respectively, and they are responding differently to environment: We are therefore allowed to hypothetize that they carry 2 different constitutive systems for MOD. In 342mt there is a MOD constitutive system involved in the accumulation of linoleic acid more active or active longer than in R978. This is confirmed by the low temperature effect. At 13 DAF, R978 did not show any variation in oleic-linoleic ratio, while 342mt showed a strong response to low temperature (Fig. 13). The low temperature-induced variations in lipid composition at 13 DAF are different between sowing date in line 342mt and 342mt x R978 hybrid (Fig. 13). This could be related to the environmental temperature that may modify the activity of the enzyme (Garcés *et al.*, 1992) and to a "memory effect" of early temperature regime on the fatty acid desaturation mechanism (Izquierdo *et al.*,

2002). Furthermore, Schlueter *et al.* (2007) found many oleate desaturase genes in soybean and they reported that some of these genes show temperature-dependent changes in transcript accumulation in developing pod. A mechanism comparable with soybean may be proposed in sunflower.

Absence of temperature sensitivity from 20 DAF is due to the age of seeds (Garcés *et al.*, 1992).

Temperature response during the seed filling phase was shown due to a maternal effect. The maternal effect, small but significant, on oleic and linoleic acid contents, was detected in reciprocal hybrids plants. The difference was found on response to low temperature at 13 DAF that we suppose depends on the constitutive desaturase. Within the second sowing date, the environment could mask the maternal effect and, therefore, reciprocal hybrids are equal. When parents displaying small differences in fatty acid composition are crossed, the environment could easily mask any maternal effects (Gilsinger *et al.*, 2010). Different response magnitude between 342mt and its 342mt x R978 hybrid could be related to a different duration of F to PM. It is important to note that the magnitude of the maternal effect could be the result of a number of morphological or physiological processes, all of which could be controlled by genes in the nucleus or cytoplasm (Gilsinger *et al.*, 2010).

3.4.2 Experiment 2

No transgressive segregation for all fatty acids was detected in the reciprocal F_1 seeds. Saturated fatty acids content in tested genotypes was not affected by maternal effect and this was in agreement with Perez-Vich *et al.* (1999), Perez-Vich *et al.* (2002) and Velasco *et al.* (2007). There is a small number of studies on saturated fatty acids content inheritance and the traits appear to be complex (Vick *et al.*, 2004).

No significant differences in fatty acids composition was detected in the reciprocal F_1 seed hybrid generation in the first and in the second sowing date. There was a significant difference in stearic and oleic acids content mean values between reciprocal F_1 seeds in the third sowing date. The difference in stearic acid content between reciprocal F_1 seeds was not observed between reciprocal F_1 plants (F_2 seeds averaged) and reciprocal backcrosses, indicating the absence of cytoplasmic effects. The differences between reciprocal F_1 seeds may be due to maternal phenotypic and may be related to differences in phenological development of the parental plants (Table 9). The difference in oleic acid content between reciprocal F_1 seeds in the third sowing date was due to a decrease in stearic acid content. The reciprocal F_1 seeds had the same linoleic acid content and ODS activity and those was similar to R978 parent line in each cross. Thus, oleic acid content in seeds of the

cross 342mt x R978 was not affected by ODS index or linoleic acid content but it was affected by stearic acid content.

With regard to the maternal effect on oleic acid content, if the trait had been inherited maternally, the F_1 generation oleic acid content would have been equal to the oleic acid content of the female parent used in crosses and the F_2 and BC_1F_1 generations would not show segregation.

In our case, inbred lines were both high oleic but they were quite different in oleic acid content. Oleic acid content of the reciprocal F_1 seeds was the same in the two date of sowing and this observation seems to indicate a complete influence of the embryo genotype on the trait. This was in agreement with Miller *et al.* (1987), Fernández-Martínez *et al.* (1989) and Varés *et al.* (2000). On the other hand, F_1 generation was tested in three sowing dates but only in one year and one location. There was not significant difference in the accumulation of GDD from flowering to 25 daf. Therefore, it is possible to propose three hypotheses: i) hybrid genotype and thus dominance of the hypothetical heterozygous genotype, ii) the environment could have masked the phenotypic expression of some genes and thus transgressive segregation in F_1 generation was not detected and/or iii) a maternal effect masked the F_1 genotype.

As far as hypothesis two is concerned (the environment could have masked the phenotypic expression of some genes), if the environment masked the phenotypic expression of some genes in F_1 seeds each F_1 plant or some F_1 plants would have shown a different segregation ratio in F_2 seeds. All F_1 plants (replicates) had the same segregation ratio so hypothesis two was rejected. Inbred lines tested were bred true for the high oleic trait. Results showed that different genotypes gave a HO phenotype (see below) and this was in agreement with Fernández-Martínez *et al.* (1989) and Velasco *et al.* (2000). On the other hand, hypothesis three was partial fulfilled. F_1 seeds of the cross 342mt x R978 showed a higher oleic acid content than the female parent and so a different phenotype from the female parent. F_1 seeds of the cross R978 x 342mt had an equal oleic acid content to the female parent. Linoleic acid content and ODS showed the same trends. The reciprocal F_1 seeds had a different O/L ratio. F_1 seeds from the cross 342mt x R978 had a higher O/L ratio than the female parent line while reciprocal F_1 seed had an equal O/L ratio to the female parent. Thus, hypothesis three was not rejected.

To verify the hypothesis of maternal effects, single seeds were analyzed from the reciprocal F_2 and BC_1F_1 generation. Single seeds analyzed in these generations showed segregation (Table 16) only in one reciprocal F_2 and BC_1F_1 with female parent line and in the first sowing date (lowest temperature). Sowing date altered fatty acids composition in F_2 seeds and in backcross generations

when inbred line 342mt was used as hybrid female parental line. Variation in fatty acids composition among sowing date was due to a different segregating pattern in seeds and not to an overall variation in seed fatty acids composition. Probably some of the genes that controlled HO trait have no phenotypic expression at higher temperatures (Velasco *et al.*, 2000) which was the case for the second sowing date (Table 14). If oleic acid content was controlled by maternal effects, each seed of these generations would have had oleic acid contents similar to the female parent with no segregation. When inbred line R978 was used as hybrid female parental line no segregation was detected in any of the tested generation and sowing date. A maternal effect was detected on high oleic trait in the high oleic mutant. From a breeding point of view, the female parent choice is fundamental to select HO hybrids that have no variation in oleic acid content across environments. Cultivated HO genotypes are hybrids and the harvest is given by F_2 seeds generation. Thus, the maternal effect could be used to select hybrids insensible to environmental conditions and thus having a high and equal content of oleic acid across the environments (years and locations).

Segregation ratio fitted in F_2 and in backcross with female parent 342mt suggested that the high oleic trait was controlled by at least three loci (Fernández-Martínez *et al.*, 1989; Lacombe *et al.*, 2001; Lacombe *et al.*, 2004). In this experiment, tested genotype was homozygous for the Pervenets allele (HO inbred line derived from Pervenets population) and thus segregation was caused by other factors. Those factors may be present or not in HO inbred lines, masked by maternal effects, and so discordant conclusions concerning the genetic of HO trait were reported in literature (see Lacombe and Bervillé, 2000).

As a hypothesis, considering that the parents breed true for the trait and with two different genotypes, an additional major gene and some modifier genes, other than Pervenet allele (OL$_1$), could be acting on oleic acid content. A second major gene, designated as OL*s*, when is homozygous for the recessive allele (3:1 segregation ratio fitted in F_2 generation and no detected in backcross with R978), could be acting on Pervenets allele and decrease oleic acid content in combination with a modifier gene. The result with hybrid plants suggested that the variation in oleic acid content of the homozygous inbred line 342mt was recessive since it did not appear in the hybrid seeds and in the backcross with R978. Furthermore, in experiment 1 only in inbred line 342mt oleic acid content was affected by low temperature. Our results suggested that response to low temperature was caused by this major gene. In mid-oleic sunflower types the presence of a gene has been reported that, when is homozygous for the recessive allele, causes instability in oleic acid content and whose expression was affected by modifiers (Triboï-Blondel *et al.*, 2000). Lagravére *et al.* (2000) suggested that hybrids with low oleic acid potentials could be more sensitive to

environmental conditions such as temperature, while hybrids with a higher oleic acid content genetic potential were insensitive to temperature conditions.

The different response of the HO genotypes to environmental conditions could be related to this gene that modifies the potential in oleic acid content.

The results with reciprocal F_2 (R978 x 342mt) and backcrosses suggested a maternal effect on this gene and/or modifier genes. Maternal effect on oleic acid content in HO sunflower was reported only by Varés *et al.* (2002). It is expected that oleic acid content of the backcross [342mt x R978] x 342mt would be equal to [R978 x 342mt] x 342mt because the only factor that was different between these reciprocal crosses was the cytoplasm of the maternal plants. This expectation was not satisfied in the first sowing date. On the other hand, in the second sowing date reciprocal backcrosses were equal in oleic acid content. The F_2 segregating generation seeds from the cross R978 x 342mt had an equal oleic acid content through sowing date while the reciprocal population showed variation. Thus, a cytoplasmic or a nuclear x cytoplasmic effect on oleic acid content was hypothesized. It appears that the cytoplasm is involved in the maternal effects for oleic acid content because the mean of the F_2 seeds produced on the F_1 plants of reciprocal cross were significantly different. Information on cytoplasmic effects would be useful to the breeder in the selection of male and female parents. We could not exclude that observed differences among tested genotypes were caused by other types of maternal effects. We tested only F_1 and F_2 generations from two HO genotypes, that displayed few differences, in one location. No information was obtained about the persistence of reciprocal differences in later generations. If cytoplasmic effects are present, significant differences should be observed among reciprocal crosses in each successive self-pollinated generation (F_3 or BC_1F_2 and latter). If cytoplasmic × nuclear genetic effects exist, those effects may dissipate or appear in each successive self-pollinated generation, depending on the interaction. It is also possible that different cytoplasmic effects affecting the same trait could mask each other, only to reappear in later generations (Gilsinger *et al.*, 2010).

Continuous variation in oleic / linoleic acids content (Fig. 14) and the frequency of the recombinant type in F_2 and backcross generation for ODS classes (Table 17) suggested that the third factor could be a combination of modifier genes, each having a small effect on the trait (Fernandez *et al.*, 1999; Lacombe *et al.*, 2001; Lacombe *et al.*, 2002), that modify oleic acid content only when Ols was homozygous for the recessive allele. Each modifier gene has small quantitative effects on the level of expression of Ols, but in combination they cause a greater variability. This result is in agreement with reports of accumulation of genes with a minor effect on oleic acid inheritance in sunflower (Urie, 1985; Fernendez-Martinez *et al.*, 1989; 1990).

The third element could be one or more quantitative trait loci. The segregation ratio (3:1) in backcross generation with female parent line suggested two hypotheses. The first hypothesis was that it could be a gene complex (Fernandez *et al.*, 1999) and so genes were inherited together. The second hypothesis was epistasis. An epistatic combination for modifier alleles was suggested by Lacombe *et al.* (2002) and a QTL x QTL epistatic interaction was reported by Shuppert *et al.* (2003).

The following genotypes on the basis of the ODS index were postulated for inbred lines:

1) 342mt: $OL_1OL_1OL_sOL_sOL_mOL_m$

2) R978: $OL_1OL_1OL_sOL_sOL_MOL_M$

Where OL_1 is the Pervenets Allele; OL_S is a major gene that, when is homozygous for the recessive allele (Ol_sOl_s), decreases oleic acid content and OLm are modifier genes. OLs and/or OLm expression is modify by maternal effects.

The difference among HO inbred lines depends on maternal effects and segregation of the alleles. We suppose that there were both dominant alleles of these nuclear modifier genes in line R978 as a consequence of maternal effects. The different behavior of these genes under different environmental conditions and in different cytoplasm make HO trait a complex one. Therefore further experiments not only under controlled environments with different temperatures and in several locations under different field natural conditions but also with F_3 and BC_1F_2 generations are needed for a better understanding of the genetic system controlling oleic acid levels and how it is influenced by temperature.

3.5 CONCLUSION

High oleic inbred lines with different genetic background are not equal for oleic acid content and they show a different interaction genotype by sowing date. Inbred line 342mt was sensitive to temperature, while inbred line R978 was insensitive to temperature.

An oleate desaturase was active in the early seed filling phase in both lines. 342mt showed greater ODS activity than R978. All these differences depend on different nuclear genes and on maternal effect. High oleic trait was controlled by at least three loci and influenced by maternal effect, so different genotypes could show a HO phenotype. Variation in fatty acids composition among sowing date in segregating generation was due to a different segregating pattern in seeds and not to an overall variation in seeds fatty acids composition. When a second major gene, designated as OLs, is homozygous for the recessive allele, oleic acid content was affected by temperature only when 342mt was used as hybrids female parent. It was observed that the cytoplasmic effect might have an important role in the genetic control of these traits and thus it could be used in breeding. Maternal effect could modify phenotypic expression of some genes and so the 90% threshold in oleic acid content, a goal in a breeding program to select high oleic hybrids for industrial use. No studies have yet been reported on the cytoplasmic effect on the inheritance of oleic acid in HO sunflower.

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4   Inheritance of the High Oleic Trait in a High x High Oleic cross

ABSTRACT

The high oleic sunflower was developed based on the mutant Pervenets. Research on genetic control of the high oleic trait led to several hypotheses on the number of genes (major genes, modifier genes and suppressors), on their dominance relationships and maternal influence on the trait. Our approach to study the inheritance of the HO trait was based on the cross between HO inbred lines only. Therefore, *Ol* (or Pervenets Allele) was homozygous. All variation observed across generations was due to other genetic factors. Two inbred lines were crossed and each successive plant generation (F_1, F_2 and partially F_3) was analyzed. Parental line, F_1 plants and F_2 plants were tested in two years: 2010 and 2011. A classical approach based on segregation pattern fitting and a quantitative genetic approach based on the generation means were performed. Parental lines and F_1 plants showed a HO stable phenotype. On the other hand, in the F_2 plant generation some individual seeds appeared showing a MO or LO phenotype (oleic acid content <55%). In the seeds collected from one plant a longitudinal gradient from embryo to upper cotyledons was found. A phenotypical maternal effect on the trait was suggested. A 13:3 segregation ratio (non recombinant − recombinant plant) fitted well the data. The hypothesis of dominant epistasis or suppression or duplicate genes was supported and thus the hypothesis that two genes, in addition to the Pervenets allele, were involved in HO trait control. We hypothesize that the recessive gene was another FAD-2 gene or some kind of suppressor. Using the F_3 seed families as the unit of measurement, the two-gene model failed. A third element seems involved in HO control. According to segregation pattern and analysis in some F3 plants we suppose that this element was a combination of modifier gene that acts on major genes. With these results some indications for breeding activity are given.

Key Words: HO phenotype, Segregating Population, Fatty Acids, Half-seed Technique

4.1   INTRODUCTION

The development of a high oleic sunflower mutant was first reported by the Russian researcher K.I. Soldatov (1976). He developed the first stable high oleic acid open-pollinated cultivar "Pervenets" through chemical mutagenesis with dimethyl sulfate (DMS) and further selections for high oleic acid content. Pervenets was used worldwide as a High Oleic source in breeding programs to produce High Oleic (HO) lines and commercial hybrids with an oleic acid content over 80%. In practice now, all HO lines derive from the mutant population Pervenets and they display seed oil with the oleic acid content over 80%. However, the HO genotype carries not only the Pervenets mutation but also different factors that affect OAC (Urie, 1985; Miller *et al.*, 1987; Lacombe *et al.*, 2001; Demurin and Borisenko, 2011).

Many genetic approaches have been developed to study the HO mutation derived from Pervenets. Several indications on number of genes that controlled HO are reported in different publications: one, two, three or five genes acting on HO trait. Fick (1984) reported a single dominant gene with partially dominant gene action; Urie (1985) reported a fully dominant gene action by a single dominant gene and detected the presence of modifiers. One *Ol* gene with incomplete penetrance determined by genotypic epistatic factors of reversion was reported by Demurin and Škoric (1996) and Demurin (2003). Miller *et al.* (1987) found two genes acting on HO trait: a partially dominant major gene *Ol* and a modifier gene *ml* acting recessively. Fernandez *et al.* (1999) suggested that the *ol* gene was recessive and the modifier gene Ml was dominant. According to Lacombe *et al.* (2001) the HO trait was directed by two loci: High oleic locus oleHOS and suppressor locus *Sup*. Other studies reported three complementary genes Ol_1, Ol_2 and Ol_3 (Fernández-Martinez *et al.*, 1989) or five genes Ol_1, Ol_2, Ol_3, Ol_4 and Ol_5 (Velasco *et al.*, 2000).

Various levels of dominance were reported in different publications. The level of dominance of high oleic may depend on genetic background of inbred lines used in the crosses. These genetic factors might be modifier genes and/or suppressor/s.

The presence of several modifier genes in inbred lines was demonstrated (Miller *et al.*, 1987, Fernández-Martínez *et al.*, 1989; Fernandez *et al.*, 1999). Modifiers exert a dramatic effect upon the expression of alleles they control (Velasco *et al.*, 2012). No indication on the number of modifier genes (Perez-Vich *et al.*, 2002) and their interaction with *Ol* (Pervenets allele) is reported in literature. The action of a suppressor of HO phenotype was first proposed by Lacombe *et al.* (2001). Demurin (2003) and Demurin and Borisenko (2011) reported the existence of suppression (epistatic

suppressor) in normal and HO genotypes. Thus, suppression seems to be common in several sunflower genotypes. For this reasons, HO appear to be a complex trait.

The presence of modifiers hinders breeding for oil quality traits. Accordingly, identification of modifiers in the initial breeding material is mandatory and germplasm containing them must be discarded to avoid unnecessary problems during the final steps of breeding (Velasco *et al.*, 2012).

Our approach to study inheritance of the HO trait was based on the cross between HO inbred lines. HO x HO crosses have been used by seed industry to select Mid-oleic types (Vick and Miller, 2002).

It has been hypothesized, on the basis of the phenotype, that in a High Oleic x High Oleic cross, *Ol* (or Pervenets Allele) was homozygous. All variation observed across generations was due to other genetic and environmental factors. Two HO inbred lines were crossed and each successive plant generation (F_1, F_2 and partially F_3) was analyzed.

The aims of the work were to: (i) study the inheritance of oleic acid content and (ii) detect if there are other genetic factors, in addition to Pervenets allele, affecting oleic acid percentage in a High Oleic x High Oleic cross.

4.2   MATERIALS AND METHODS

4.2.1 Plant Material

The sunflower (*Helianthus annuus* L.) seeds used in this work were from two self-pollinated F_1 plants derived from the cross 342cms x R978 (HO x HO). Line 342 is a selection, made at University of Udine, derived by Ha 342 USDA, and it is a cytoplasmic male sterile line with a single head. Line R978, selected by University of Udine, is a fully-branched type and it is a male fertility restorer. All plant materials were High Oleic.

The F_2 populations plants were grown and each plant was self-pollinated to obtain the F_3 seeds. The F_3 families, each derived from an individual F_2 plant, were planted at Udine in 2011. F_3 seed families derived from seven F_2 plants, namely family A, B, C, 2871/20, S, T, Z. The seven families were: 1 family with all individuals that showed a high oleic phenotype (C), 3 families with individuals that showed an intermediate high oleic content (A, B; Z) and 3 families with individuals that showed a low oleic acid content (2871/20, S, T).

4.2.2 Field trials

Parental lines, F_1 plants and segregating populations were grown during 2010 and 2011 at the experimental field of University of Udine, Azienda Agraria Universitaria "A. Servadei" (46°04'N, 13°22'E, altitude 109 m), in North-East Italy. For the parental lines and F_1 plants, the experiment was designed as a complete randomization scheme, with three replications. The date of sowing was 19th April in 2010 and 18th May 2011. Plants were thinned after seedling emergence from 10 to 7.5 plants m^{-2}. The seeds were sown at a spacing of 0.75 m between rows. Plants were thinned after seedling emergence from 10 to 7.5 plants m^{-2}. Nitrogen was applied at 100 kg ha^{-1}. Weeds and diseases were controlled, and regular watering throughout the experiment ensured that plants were not subjected to water deficit during the entire growth period. All plants were covered with paper bags, at the R4 stage (Schneiter and Miller, 1981), to prevent cross-fertilization.

4.2.3 Sampling

Plants were harvested at physiological maturity (R9 phase; Schneiter and Miller, 1981). Seeds were dried in an oven at 30 °C for 48-72 h. Seeds from head outer rings (1-6), with the same developmental timing, were separated. 100 seeds were counted and dehulled. Kernels were ground to a fine powder using a coffee grinder. 200 mg of kernel powder were weighed to performer fatty acid analysis.

A screening on 15 F_2 seeds from each of the F_1 plants was performed to identify the presence or absence of segregation. A screening on 12 F_3 seeds from each of the F_2 plants was performed to identify the presence or absence of segregation (Perez-Vich *et al.*, 1999) for a high C18:1 content.

When segregation was found, to fit a segregation ratio 48 additional F_3 seeds from whole sunflower head were analyzed from those plants showing segregation for C18 : 1 content.

A screening on 15 F_4 seeds from each of the F_3 plants was performed.

4.2.4 Fatty Acid determination

Single seeds were dehulled and grounded. Lipids were extracted in 1 mL of *n*-hexane. Fatty acids were converted in Fatty Acid Methyl Esters (FAMEs) by transesterification with a methanolic potassium hydroxide solution (2N). The fatty acid composition of seeds from each generation was analyzed by gas chromatography and every fatty acid was expressed as a percentage of the total fatty acids detected in the oil. The gas chromatograph was fitted with a 60 m HP-88 capillary column (Agilent Technologies, USA). Helium was used as carrier gas, and the injector, detector and oven temperatures were 230, 250 and 200 °C, respectively. 5 µL of sample were injected manually in split mode. Different FAMEs were identified by comparison with known standards.

4.2.5 Half-Seed Technique

Half-seed technique was used on F_3 seeds. Seven F_3 seed families (derived from the self-pollinated F_2 plants grown during 2010) were grown in 2011. A total of 30 F_3 half-seeds were analyzed from each F_2 plants. Each individual seed was cut with the razor into two part: upper cotyledons and embryo (embryo + lower cotyledons). Upper cotyledons (the top of the seed) were analyzed for fatty acid composition by gas chromatography. The bottom (embryo + lower cotyledons) of the seed could be embryo cultured to produce seedlings for transplanting. The embryo was put on paper soaked with water in a Petri dishes to germinate. After germination, seedling was transferred to a pot filled with potting compost in the greenhouse for acclimations. After 15 days, when plants were 10 cm tall, they were transplanted in the field.

4.2.6 Seed Spatial Analysis

Individual seeds from F_2 plant were cut with the razor into two parts, upper cotyledons and embryo (embryo + lower cotyledons), which were analyzed separately.

4.2.7 Statistical Analysis

Means were calculated for all fatty acids in the parental, in F_1 plant generations and in F_2 plant generations and compared using the t-test (independent t-test; two-tailed). Chi-squared tests were used to determine the goodness-of-fit of observed segregations to expected genetic ratios. A chi-squared test for heterogeneity was also performed to examine whether different populations from the same type of cross displayed similar genetic behavior.

4.2.8 Quantitative Genetic Analysis

Estimates of the genetic parameters were obtained with the variance of parents P1 (VP1) and P2 (VP2), F1 (VF1) and F2 (VF2) generations. Variance components were estimated as described by Allard (2001) using the following equations:

Phenotypic variance (VP) = VF2

Environmental variance (VE) = 1/4 (2VF1+VP1+VP2)

Genetic variance (Vg) = VP - VE.

4.2.9 Broad-sense heritability (h_b^2)

Broad-sense heritability is the proportion of phenotypic variance that is attributable to differences among individuals in their genotype. It measures how much of the difference among individuals is attributable to differences in their genes. It was calculated as:

$$h_b^2 = Var(G)/Var(P)$$

where Var (G) indicates Genotypic Variance and Var(P) indicates Phenotypic Variance.

4.3   RESULTS

In 2010, seeds from the hybrid plants had an average oleic acid content equal to 90.3%, i.e. similar to the content of the male parent R978 (91.3%). Female parent showed an average oleic acid content of 85.0%. The F_1 plants (F_2 seed) did not show variation in the OAC. Seeds from the F_2 plants (average F_3 seeds) had an oleic acid content averaged to 84.5% (Table 18).

In 2011, seeds from the hybrid plants had on average an oleic acid content equal to 90.2%, no different from male parent R978 (91.4%). Female parent showed an average oleic acid content of 85.5%. The F_1 plants (F_2 seed) did not show segregation. Seeds from the F_2 plants (average F_3 seeds) had an oleic acid content of 88.4% (Table 18).

Table 18. Fatty acid composition in F_2 population as plant mean in 2010 and 2011.

Fatty acid	2010			2011		
	Mean (%)	Min	Max	Mean (%)	Min	Max
Palmitic	3.96	3.19	6.65	3.68	2.87	5.08
Stearic	3.26	2.32	5.37	3.00	1.75	4.17
Oleic	84.52	58.14	92.56	88.42	60.10	93.38
Linoleic	8.26	1.06	39.17	4.91	0.77	31.27

The two segregating populations were equal for saturated (Palmitic acid t-Test= 1.84, $p>0.05$; Stearic t-Test= 1.71, $p>0.05$) and unsaturated fatty acids content (Oleic acid t-Test= 1.91, $p>0.05$; Linoleic acid t-Test= 1.72, $p>0.05$). The two sample means come from the same population.

Thirty-two F_2 plants were male-fertile and 15 F_2 plants were male-sterile, which fitted the expected 3:1 (male-fertile:male-sterile) ratio (χ^2= 1.20, $p = 0.55$) in 2010. Forty-four F_2 plants were male-fertile and 22 F_2 plants were male-sterile, which fitted the expected 3:1 (male-fertile:male-sterile) ratio (χ^2= 3.00, $p = 0.22$) in 2010.

The OAC threshold for classification of F_2 plants (means of F_3 seeds) should be located at 80% that was the minimum oleic acid content of the female parent. In the context of this threshold, 25% and 19% of the F_2 plants could be considered recombinants in 2010 and in 2011 respectively (Table 19).

Table 19. Absolute frequency in phenotypic class based on oleic acid content expressed as mean plant.

Phenotypic class - Oleic Acid Content	Year 2010	Year 2011
<60%	2	0
61-70%	1	4
71-80%	5	4
81-90%	12	10
> 90%	12	24
Total Plants	32	42

A segregation ratio of 3:1 (non-recombinant F_2 plant – recombinant F_2 plant) was fitted (polled χ^2 0.3348 - p 0.85). Heterogeneity analysis (χ^2 2.5279 - p 0.28) indicated that the two segregating populations were equal for segregating ratio. One gene model fitted well the data.

Environmental effects need to be considered (see Chapter 2 and 3). We found that environmental conditions, in particular temperature, modify the segregation pattern in F_2 seed generation (see Chapter 3). To detect segregation in F_2 plants, 12 individual seeds were analyzed. If segregation was found, the remaining 48 individual seeds were analyzed from whole capitulum to compute a segregation ratio. A different frequency in oleic phenotypic classes was found in analysis of seeds from outer rings and whole capitulum (Table 20; Table 23; Table 24).

Table 20. Seed fatty acid composition from individual F_3 seed analysis in 2010 and 2011.

Fatty acid	2010			2011		
	Population mean (%)	Min	Max	Population mean	Min	Max
Palmitic	3.91	2.72	9.76	3.66	2.01	7.60
Stearic	3.41	1.84	5.72	2.85	0.92	4.78
Oleic	81.51	20.63	93.61	88.64	35.72	93.98
Linoleic	11.18	0.77	67.57	4.85	0.81	54.40

The average of seed fatty acids composition obtained from 100 seed analysis was similar to average of seed fatty acids composition obtained from individual seed analysis in 2010 and 2011 (Table 20). Range was different for oleic and linoleic acid content (Table 20).

Some F_3 seeds with a Low Oleic phenotype appeared. Considering as threshold for LO phenotypic classification the level of oleic acid in the Pervenets mutant (Soldatov, 1976) and previous classification (Low, Intermediate and High Oleic) reported in literature (Miller *et al.*, 1987; Fernández-Martínez *et al.*, 1989; Lacombe *et al.*, 2001), and that in our experiment no individual was found in oleic phenotypical class 56-60%, a 55% of oleic acid content was selected as threshold

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to identify low oleic phenotype (Table 22). The variation in oleic acid content from 90% to 65% could be related to modifiers (Lacombe *et al.*, 2001) but a content in oleic acid lower than 55% or LO phenotype would be related to other genetic factors.

Table 21. Number of recombinant and non-recombinant plants on single seed basis. Recombinants were plants that showed some seeds with a LO phenotype.

Year	2010	2011
F2 Plants Harvested	32	42
F2 Plants "Recombinants"	6	8
F2 Plants "Non Recombinants"	26	34

When seeds were analyzed as individual seed, plants that showed F_3 recombinant seeds were 19% in 2010 and in 2011 (Table 21) and so another segregation ratio was fitted in F_2 plants.

19% of the F_2 plants in each year had some seeds with a content in oleic acid lower than 55%. In other words, seeds with a Low Oleic phenotype appear in the F_3 seed generation of the cross 342cms x R978. A segregation ratio of 13:3 (non-recombinant F_2 plant – recombinant F_2 plant) was fitted (polled χ^2 0.0014 - *p* 0.97). Heterogeneity analysis (χ^2 0.0010 - *p* 0.97) indicated that the two segregating population were equal for segregating ratio. A two-gene model with dominant epistasis or suppression hypothesis fitted well the data. Two genes act on oleic acid level in F_2 plants.

Table 22. Oleic acid content - phenotypical class. 12 individual seeds per plant were analyzed. In 2010, some plants (3 plants) did not have enough seeds for 12 individual determinations.

Phenotypic class Oleic Acid Content	2010		2011		Total	
	Seed number	Frequency	Seed number	Frequency	Seed number	Frequency
<55%	11	3.1%	28	5.6%	39	5%
61-80%	20	5.6%	32	6.3%	52	6%
80-90%	55	15.4%	52	10.3%	107	12%
> 90%	272	76.0%	392	77.8%	664	77%
Total	358		504		862	

If the analysis ended here, the two-gene model fitted well the data. However, instead of using families as the unit of measurement, an analysis was done using the individual F_3 seed phenotype. The result was far from the expected 13:3 segregation ratio under the "two-gene model" and consequently this model was rejected.

The frequency of individuals that showed a content in oleic acid lower than 55% over the population was 3.1% and 5.6% in 2010 and 2011 respectively. The difference between the two could be related to a distortion in F_2 plants (different population size, unknown phenotype in male sterile plant, environmental condition at the flowering, etc.) or more simply to F_1 plant to F_1 plant variability. However, the frequency was in agreement with a three-gene model with suppression (expected frequency of 1.6% in F_2 seeds population). On the other hand, modifiers could be acting increasing or decreasing oleic acid content. If considering all individuals with a content in oleic acid lower than 80%, the hypothesis of two complementary genes could be not rejected. Inferring on intermediate phenotype was complex.

Considering only the recombinant plants (6 plants in 2010 and 8 plants in 2011) that originated offspring with a LO phenotype, the frequency of of these individuals (F_3 seeds) was 30% and 16% on the total of F_3 seeds analyzed (48 seeds per plant) from these plants in 2010 and 2011 respectively (Table 23 and Table 24). If LO phenotype was due to two complementary genes than it would have a frequency of 6.25% (15:1 ratio suppression) or 18.75% (13:3 ratio dominant epistasis) in F_2 generation. Thus a 12.50% or 37.5% frequency was expected in F_3 generation. No F_2 plants showed all progeny with a LO phenotype.

To test the hypothesis of dominant epistasis or suppression, the families of F_3 seeds were analyzed separately. Different segregation ratios were fitted (Table 25 and Table 26). We analyzed an F_3 generation, so different segregation patterns were expected. A pooled analysis revealed that a 3:1 (polled χ^2 2.2667 - p 0.26) and a 13:3 (polled χ^2 2.4114 - p 0.30) segregation pattern fitted well the data in 2010 and 2011 respectively. Heterogeneity test applied for all plants with the same hypothesis for ratio test (3:1 and 13:3) was inconsistent (3:1, heterogeneity χ^2 44.2222 - $p < 0.001$; 13:3, heterogeneity χ^2 28.1527 - $p < 0.001$). Thus, the phenotypes of the offspring were due to a maternal phenotypic effect (genotype of the maternal plant) but also to the genotype of seed (embryo).

Individual F_2 plants were different with respect to the segregation pattern. In 2010, 2 plants showed a 1:1 High to Low Phenotype segregation ratio, 2 plants a 3:1 segregation ratio, 1 a 13:3 and 1 15:1 (Table 25). In 2011 4 plants showed a 15:1, 3 plants showed a 13:3 ratio and 1 plant showed a 1:1 ratio (Table 26). It was obvious that individual F_2 plants were different with respect to the segregation patterns: an F_3 seed generation was analyzed. It was not obvious that the relative frequency of plants for each segregation ratio fitted varied from first to second year. In other words, we observed a different relative abundance of plants for each segregation pattern fitted.

Table 23. Analysis of 48 individual seeds from whole head in year 2010.

Phenotypic class – Oleic Acid Content	Plant						Total
	1	2	3	4	5	6	
<55%	22	8	27	12	12	3	84
61-80%	14	9	16	8	0	2	49
80-90%	12	31	5	4	0	0	52
> 90%	0	0	0	24	36	43	103
Total	48	48	48	48	48	48	288

Generally, in Mendelian segregation, it is expected that in F_2 generation the half of all individuals are heterozygous at a certain locus. In the next generation, it is expected that about half of progeny shows a segregation pattern similar to F_2 generation. We considered only the plants that showed recombination, thus we suppose that the first gene was in homozygous form for the recessive allele. The second and the third elements could be in heterozygous or homozygous form. So, only plants that had the second and third gene in heterozygous form originated the same segregation pattern expected in F_2 generation. With the hypothesis of dominant epistasis it was expected that 25% of the plants showed the same segregation pattern (13:3 HO to LO) expected in F_2 generation, that 12.5% of the plants showed a 3:1 Low to High segregation pattern and that 6.25% of the plants showed a LO phenotype. A 1:1 segregation ratio was not explainable in an F_3 generation. No 3:1 Low oleic to High oleic segregation pattern was fitted. The observed plants with a 13:3 ratio were higher than expected in each year. For the same reasons (with different ratios), the suppression hypothesis was not supported.

The different segregation ratio obtained among plants (recombinant plant by year) and the different abundance in the Mid Oleic phenotypic class (among years) suggested that the third element could be a gene-complex or a polygene that mimics segregation patterns produced by Mendelian segregation. Different segregating ratio across years and a continuous variation in oleic acid content in those plants suggested that the third element was a combination of modifier genes.

Table 24. Analysis of 48 individual seeds (bulk) from whole head in year 2011.

Phenotypic class – Oleic Acid Content	Plant								Total
	1	2	3	4	5	6	7	8	
<55%	4	8	4	4	4	20	4	8	56
61-80%	4	16	0	4	0	0	4	4	32
80-90%	12	16	16	4	4	12	0	0	64
> 90%	28	8	4	36	40	16	40	36	208
Total	48	48	24	48	48	48	48	48	360

Table 25. Chi-square analysis for goodness of fit of segregation ratio observed in F_3 seeds (only from F_2 plant recombinant). Year 2010.

Plant	Segregating Ratio	Phenotypic Class	Observed	Expected	χ^2	Probability
1	1:1	High	26	24	0.3357	0.85
		Low	22	24		
2	13:3	High	40	39	0.1500	0.93
		Low	8	9		
3	1:1	High	21	24	0.7619	0.68
		Low	27	24		
4	3:1	High	36	36	0.0000	1.00
		Low	12	12		
5	3:1	High	36	36	0.0000	1.00
		Low	12	12		
6	15:1	High	45	45	0.0000	1.00
		Low	3	3		

Table 26. Chi-square analysis for goodness of fit of segregation ratio observed in F_3 seeds (only from F_2 plant recombinant). Year 2011.

Plant	Segregating Ratio	Phenotypic Class	Observed	Expected	χ^2	Probability
1	15:1	High	44	45	0.3556	0.84
		Low	4	3		
2	13:3	High	40	39	0.1368	0.93
		Low	8	9		
3	13:3	High	20	20	0.0684	0.97
		Low	4	5		
4	15:1	High	44	45	0.3556	0.84
		Low	4	3		
5	15:1	High	44	45	0.3556	0.84
		Low	4	3		
6	1:1	High	28	24	1.3333	0.51
		Low	20	24		
7	15:1	High	44	45	0.3556	0.84
		Low	4	3		
8	13:3	High	40	39	0.1368	0.93
		Low	8	9		

4.3.1 Quantitative approach

Several hypotheses could be formulated with a qualitative approach but none could be tested appropriately because we used equal HO parent lines and we suspected a distortion in the segregation pattern in the F_2 plant generations (use of cms parent and small-sized segregating population).

Thus a quantitative approach to analyzing the High Oleic trait over generations was performed. The first step was fitting a simple model (additive – dominance) with three parameters: *m*, *d* and *h*, where *m* was the mean that was mid-parents value (P1+P2/2) or hybrid value (Hayman, 1960), *d* were the additive effects and *h* were the dominance effects. A chi-square goodness of fit with one degree of freedom (number of generations – model parameter) was performed (from Table 27 to Table 30). A three parameter additive – dominance model described oleic acid content in each year while it did not describe linoleic acid content in the generations (year 2010, Table 29). So oleic acid content was not affected by epistatic effect (Table 27 and Table 28). With respect to linoleic acid content, failure to fit the non-epistatic model (*m*, *d* and *h*) is a definite indication of epistasis in the general sense (Hayman, 1958) or linkage (Table 29). Hybrid's deviation from mid-parents value in oleic and linoleic acid content and its reduction in the next generation indicated that dominance was present.

The gene action was additive when |h/d|=0, incompletely dominant when 0<|h/d|<1 and dominant when |h/d|=1.

Broad sense hereditably was 0.93 and 0.92 for oleic and linoleic acid respectively in both years.

Table 27. Oleic acid content in year 2010.

Generation	Observed	Expected	Diff.	Parameter		h/d
P_1	85.17	86.2	-1.1	m	88.3	0.68
P_2	91.40	92.4	-1.1	d	-3.1	
F_1	90.39	87.2	3.2	h	-2.1	
F_2	84.52	86.2	-1.7	χ^2 0.17 (p=0.68)		
				m=mid parent value		

Table 28. Oleic acid content in year 2011.

Generation	Observed	Expected	Diff	Parameter		h/d
P_1	85.57	86.4	-0.9	m	88.5	0.60
P_2	91.37	92.2	-0.9	d	-2.9	
F_1	90.21	87.6	2.6	h	-1.8	
F_2	88.42	86.6	1.8	χ^2 0.13 (p=0.71)		
				m=mid parent value		

Table 29. Linoleic acid content in year 2010.

Generation	Observed	Expected	Diff	Parameter		h/d
P_1	7.5	7.7	-0.2	m	5.3	1
P_2	2.7	2.9	-0.2	d	-2.4	
F_1	2.8	2.8	0.0	h	-2.4	
F_2	8.3	3.5	4.8	χ^2 6.6 (p<0.01)		
				m=mid parent value		

Table 30. Linoleic acid content in year 2011.

Generation	Observed	Expected	Diff	Parameter		h/d
P_1	6.4	6.6	-0.2	m	4.1	0.20
P_2	1.8	2.0	-0.2	d	2.3	
F_1	3.1	3.9	-0.8	h	-0.5	
F_2	4.9	5.1	-0.2	χ^2 0.23 (p=0.63)		
				m=mid parent value		

Fig. 16. Oleic and linoleic acid contents in whole seed and half-seed (plant 4, year 2010).
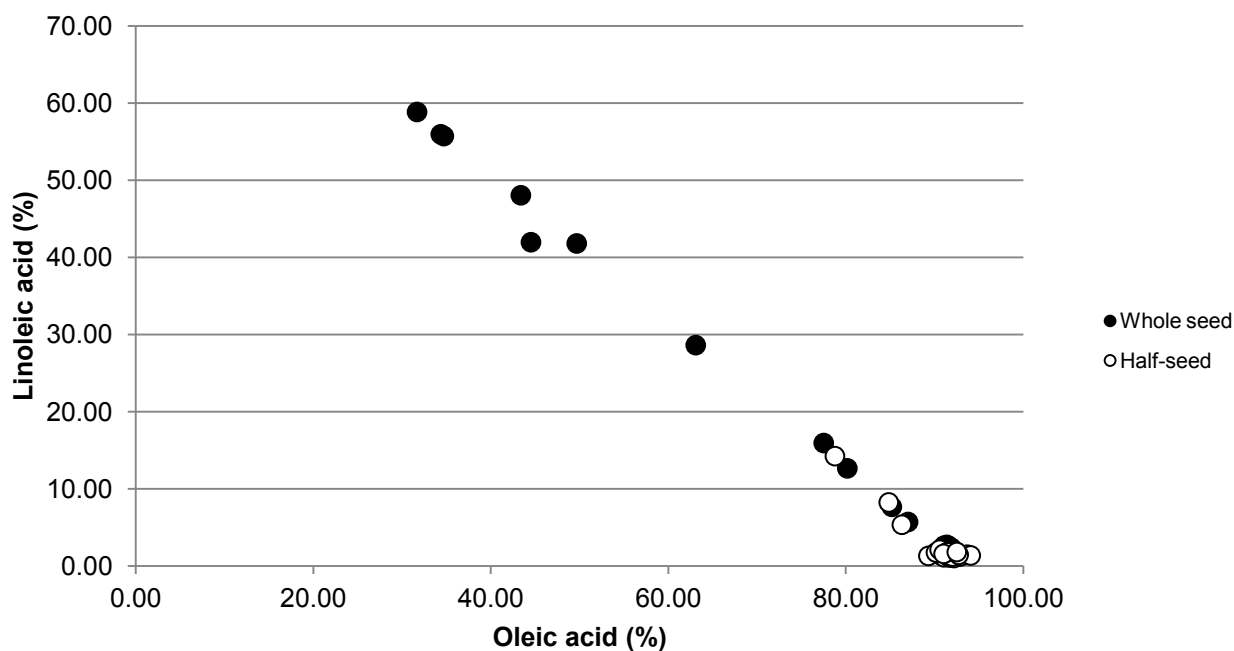
4.3.2 Longitudinal gradient in seed

Half-seed technique revealed that in F_3 seeds from one F_2 plant a spatial gradient in seed occurred (Fig. 16). Oleic and linoleic acid contents in whole seed and in half-seed were different. Analysis of embryo + lower cotyledons and upper cotyledons separately showed in seeds from one individual (plant 4 of 2010) a longitudinal gradient in oleic and linoleic fatty acid content with an increase of 12.97% and a decrease of 12.14% in oleic acid and linoleic acid content respectively from embryo + lower cotyledons to upper cotyledons. Variation in oleic acid content was related to linoleic acid content (Table 31).

Table 31. Average (and range) in oleic and linoleic acid content in the seeds parts of plant 4.

Number of seeds	Oleic acid (%)		Linoleic acid (%)	
	Embryo + lower cotyledons	Upper cotyledons	Embryo + lower cotyledons	Upper cotyledons
25	78.30 (42.01-82.88)	91.26 (71.30-92.69)	14.09 (5.80-41.17)	1.95 (1.24-16.25)

4.3.3 F3 family S analysis

Analysis of F_3 plants on single seed basis showed that in one family, called S family, three F_3 plants showed only LO progeny. Continuous variation in oleic and linoleic acid content (Fig. 17) suggested that a combination of modifier genes affected unsaturated fatty acids in this family. We hypothesize that modifier genes were present and act on oleic or linoleic acid level. Half-seed analysis showed that S12 and S17 had a LO phenotype (35% and 48% in oleic acid content respectively) while S13 had an intermediate oleic acid content (63% in oleic acid content).

Fig. 17. Oleic linoleic acids content in three F_3 plants (F_4 seeds) from S family.
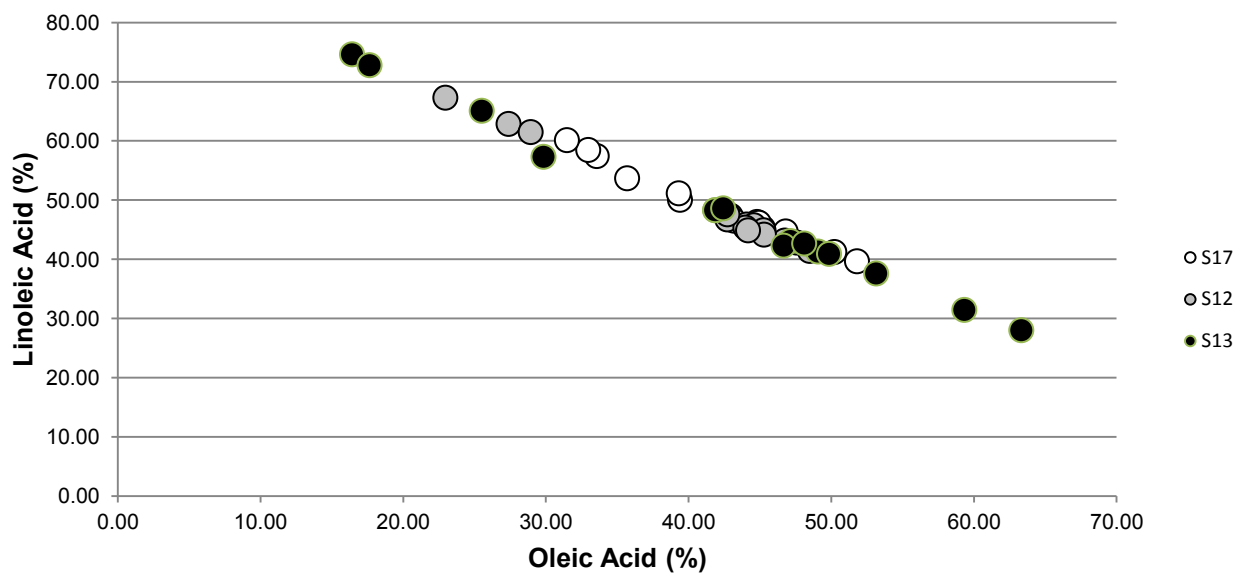
4.4   DISCUSSION

Classification of the F_3 individual seeds in oleic phenotypic classes was difficult: the distribution is continuous and we cannot choose a threshold between the low and the high oleic classes. This was the main problem in our approach to studying the high oleic trait. Several factors could modify oleic and linoleic acids content in the high oleic mutants and modifier genes could originate a high variability in oleic acid content across generations (Lacombe *et al.*, 2001). Phenotypic expression of modifier genes could be affected by temperature (Velasco *et al.*, 2000) and, therefore, temperature could modify segregation ratio (Chapter 3). Offspring could be grouped into different phenotypic classes according to their oleic acid content. The choice of class interval in oleic acid content and number of phenotypic classes could modify segregation ratios fitted and thus the conclusions. As a first approach to studying the High Oleic trait in a HO x HO cross a prudential threshold limit (55%) was chosen. We cannot discriminate between MO and HO as effects of major and/or minor genes. Thus, all individuals with an oleic acid content higher than 55% were classified as HO. We speculate that MO phenotypes were the results of several and distinct phenomena. All these remarks argue that the expression of the mutation Pervenets is modified in segregating individuals making trouble to delimit oleic classes.

The F_1 plants (average of F_2 seeds) did not show recombination and transgressive segregation. Oleic and linoleic acids content in seeds from F_1 plants was equal to male parent line in each year. Thus, there was dominance of R978 on 342 inbred line for high oleic trait. In the F_2 plants, segregation and recombination were found and on average oleic acid content was lower than in the F_1 generation in 2010 and showed a slight decrease in 2011. This suggested that the environment, and in particular temperature during the seed filling phase (Izquierdo and Aguirrezábal, 2008), acts on the HO phenotype. The F_2 plants that displayed a content in oleic acid lower than parental inbred lines were found with a frequency of 25% in the first year. This was in agreement with the results of the experiment with reciprocal crosses (see Chapter 3), where we reported the action of a second major locus that modified oleic acid content in one reciprocal cross. In the second year recombinant plants were found with a frequency of 20%. The difference in observed frequency could be related to modifier genes (temperature), small size population, and environment effects on flowering and fertilization.

Different segregation ratio for the OAC across years could be related to temperature during flowering and seed filling phase. We found that temperature modifies the OAC and thus segregation ratio in F_2 generation (Chapter 3).

Frequency could be distorted by the small number of individuals analyzed. Theoretically, if we suppose that three genes act on the High Oleic trait, then the minimum population size would be 64 individuals and this would be replicated (128 individuals each year). We had only 32 and 42 male-fertile plants at harvest in the first and second year respectively. Thus a severe distortion in segregation patterns in F_2 population may have occurred.

From a physiological point of view, all factors affecting the fertilization process will interfere with the inheritance pattern. Environmental factors may also influence pollen number and quality. Depending on self-fertilizing ability of F_1 and F_2 plants, progenies may be more or less distorted for the ratio HO / LO. All these factors certainly accumulate in different progenies, creating complex and variable segregation patterns for the HO trait in various environments (Lacombe and Bervillé, 2000). We analyzed F_2 plants, thus segregation occurred for different traits that could affect seed oil composition such as date of flowering (flowering time), seed filling duration and branching.

However, recombinant types appeared in the F_2 plants generation. This suggested that a maternal phenotypic effect (i.e. mother plant controlled offspring phenotype; see below) occurred on oleic acid content.

Considering only the plants that originated offspring with a content in oleic acid lower than 55%, the frequency of the F_2 plants that had recombinant individual seeds was 19% in each year. A segregating ratio of 13:3 (non-recombinant to recombinant plants) fitted well the data. Data supported the hypothesis of dominant epistasis or suppression; therefore, besides the Pervenets allele, two loci controlled the high oleic trait. Analysis of the segregating pattern in F_3 individual seeds did not support it. If two genes controlled the HO trait then expected frequency of LO phenotype in F_3 seed would be about 12.5% (suppression) or 37.5% (epistasis). The observed frequency was lower (about 3 and 6%, polled 5%) than expected. However, LO phenotype appeared in F_3 generation and it had never been detected in the F_2 seeds. Again, F_2 plants that displayed all seeds with a LO phenotype were not found. This observations suggested that: three genes controlled the trait, it could be cytoplasm x nucleus interaction and/or a phenotypic effect of mother plant on offspring's phenotype.

In the context of the hypothesis, if three genes with suppression controlled HO in our tested genotype, then in F_2 seeds a LO phenotype would have an expected frequency of 1.6%; thus too small a population size was used and the use of cms plant material could have distorted segregation patterns and the evaluation of the F_2 generation (F_2 seeds with LO phenotype and F_2 plants with a LO phenotype were not found). On the other hand, in our experiment with reciprocal mates in F_2

generation we never detected individuals in LO class in 1500 seeds analyzed, but some individuals displayed a oleic acid content in the lower extreme range of the HO class. The minimum value in oleic acid content was 57.8% in the F_2 generation from the cross 342mt x R978.

The absence of recombinant phenotype in F_2 generation suggested that maternal effects occurred. A cytoplasm x nucleus interaction could mask LO phenotype in F_2 generation and so LO phenotype reappears in the next generation. A cytoplasmic effect was found on oleic and linoleic acids content in this plant material (see Chapter 3). In our experiment with reciprocal we never detected LO individuals in F_2 seeds. We speculate that cytoplasm affected the expression of the major gene Ols in male-sterile cytoplasm (see Chapter 3). Modifier genes have no phenotypic effect in absence of alteration in this major gene. In the case of cytoplasm x nucleus interaction, it is expected that in F_2 generation the recombinant phenotype appears.

We could not exclude that mother plant influenced the phenotype of the offspring in some other way. A longitudinal gradient of oleic acid content was found in the F_3 seed family collected from plant 4 (2010) and thus a signal from the mother plant to seeds could be hypothesized. In F_1 plant generation, all individual are heterozygous, so in F_2 plant the gene action was revealed. A longitudinal gradient of oleic acid content has been reported in sunflower (Fernández-Moya *et al.*, 2003; Demurin *et al.*, 2008). A maternal phenotypic effect on the phenotype of offspring might explain why segregation was not detected in the F_2 seed generation. In high stearic sunflower, Fernández-Moya *et al.* (2003) reported a mother plant control on the high stearic trait in the high-stearic temperature-dependent mutant line CAS-14. These authors speculated that the lower expression of the stearate desaturase (high content in stearic acid at high temperature) was due to the existence of a thermosensitive element in the fatty acid biosynthesis regulatory cascade originating from the maternal plant during capitulum and seed development. Gilsinger *et al.* (2010) found in soybean that offspring's phenotype was affected by maternal plant. Maternal effects between reciprocal crosses dissipated when soybean seeds were grown in vitro, while significant differences between the parents were maintained. This is evidence that factors translocated from the maternal plant may be causing the maternal effect. In the early studies on high oleic inheritance a maternal influence was reported (Miller *et al.*, 1987). In the same way, maternal influence was reported on high linoleic trait (Simpson *et al.*, 1989). We suggest that the effects of Ol*s* and of this recessive gene are additive because in the reciprocal F_2 and backcrosses the minimum in oleic acid content was 57% while in F_2 plants the minimum was 21 and 36% in 2010 and 2011, respectively.

Lacombe *et al.* (2001), in a LO x HO cross, reported that the High Oleic trait is due to two independent loci: the locus carrying Pervenets allele and another locus. One allele at this other locus

may suppress the effect of Pervenets allele on the HO trait. They observed a 1:3 [LO]:[HO] segregation ratio in the F_2 generation (F_2 seed and F_1 plant) and this was in agreement with a single gene with two alleles. On the other hand, they found that in Recombinant Inbred Lines a second locus directed the HO trait. They suggested that this second locus directing the HO trait did not carry the supHOAC allele in the F_2. Our results were in agreement. We did not found recombination in F_2 but in F_3. So a phenotypic effect of mother plant on offspring may explain why in the F_2 seed generation no recombination was found, but it appeared in F_3 seeds generation.

The quantitative approach indicated that oleic acid content was affected by additive and dominance gene action. Dominance was involved because oleic acid level in F_1 generation was higher than Mid-parent value and the mean in oleic acid content decreased in the F_2 generation. The parameters *d* and *h* were the same in the two years and the *h*/*d* ratio suggested that gene action was incompletely dominant. So the hypothesis that a second major gene controlled the high oleic trait was supported. We suggested that this gene was Ol*s* (Chapter 3) that it competes for the final product namely oleic acid. It could be a *Ml* modifier gene (Miller *et al.*, 1987) or a second major gene (Fernández-Martínez *et al.*, 1989; Velasco *et al.*, 2000). Linoleic acid content was affected by epistatic interaction because failure to fit the non-epistatic model (*m*, *d* and *h*) is a definite indication of epistasis (in the general sense; Hayman, 1958). In high oleic sunflower an epistatic combination has already been suggested for modifier alleles affecting oleic acid content (Lacombe *et al.*, 2002). In HO sunflower a significant epistatic interaction has been reported between QTL, and it was hypothesized to be modifier genes of Ol (Perez-Vich *et al.*, 2002; Schuppert *et al.*, 2003). Perez-Vich *et al.* (2000a) reported that the loci controlling the high-C16:0 trait exerted an epistatic effect over the loci responsible for the high- C18:0 trait. A similar effect could be proposed for the loci controlling oleic and linoleic acid in HO sunflower. In peanut epistasis in the general sense was suggested between several loci that control fatty acid composition (Isleib *et al.*, 2006). The parameter *d* had the same value in both years but it changed sign and *h* had a different value. So d/h value indicating a variation in degree of dominance: from dominant (2010) to incompletely dominant (2011). This suggested that the lack of evidence for epistasis in the second year is due to a change in the action of the gene. We supposed that variation between 2010 and 2011 was due to the presence of genotype by environment interaction.

The hypothesis of the action of two or three genes was supported. We suggested that modifier genes act on the Pervenets Allele and on another gene that influenced linoleic acid content. In other words, modifier genes act on the Pervenets Allele and/or on Ol*s*. A recessive gene, designated as Ol*l*, acts on these genes and it causes a maternal phenotypic effect. At this point, Ol*s* could be a FAD2 gene

independently from the oleoyl-PC desaturase locus (in our case this locus was homozygous for the Pervenets allele). In sunflower three-different FAD2 (Fatty Acids Desaturase) were identified (Martinez-Rivas *et al.*, 2001). Different FAD2 genes were found in soybean (Heppard *et al.*, 1996; Tang *et al.*, 2005; Byfield and Upchurch, 2007; Schlueter *et al.*, 2007; Bachlava *et al.*, 2008; Bachlava *et al.*, 2009; Upchurch and Ramirez, 2010) and in peanut (Jung *et al.*, 2000a). In peanut, when the high oleic variety F435 was used in crosses with normal type, the F_2 segregation ratio of normal oleate to high oleate progeny was 3:1 or 15:1 depending on the normal oleate varieties used in the crosses (Moore and Knauft 1989; Isleib *et al.* 1996). Jung *et al.* (2000b) reported that a mutation in ahFAD2A and a significant reduction in levels of the ahFAD2B transcript together cause the high oleate phenotype in peanut varieties. The normal oleate peanut varieties were different for ahFAD2A alleles. In addition to those two genetic loci, some modifiers or additional epistatic interactions may be occurring (López *et al.*, 2001; Barkley *et al.*, 2011). Recently, García-Moreno *et al.* (2012) demonstrated that unstable expression of high gamma-tocopherol content in sunflower seeds was due to interaction between duplicated loci that revert the high gamma-tocopherol phenotype to intermediate-low gamma-tocopherol values. It is known that duplicate genes provide alternative genetic determination of a specific phenotype (Griffiths *et al.*, 2000). It could mimic a segregation pattern typical of epistasis or suppression. The presence of phenotypes that are extreme relative to those of either parental line could be related to complementary action of additive alleles that are dispersed between the parental lines (parental lines are fixed for sets of alleles that have opposing effects within lines). Thus, the same phenotype is due to different genetic and physiological process.

On the basis of phenotype, without molecular analysis, we cannot discriminate between epistasis or suppression or duplicate genes action, so we can make only several hypothesis. Investigating the genetic nature of this phenomenon could be a topic of interest for further studies on HO trait. However, some hypothesis can be made.

We speculate that MO phenotypes were due to different phenomena: (i) modifier genes that act on the Pervenets Allele and/or Ols and (ii) Ol*l* that acts on Ols and/or on modifier genes. These two phenomena give the same phenotype in the progeny, suggesting an overlap in their distribution. Thus, different genotypes were indistinguishable on the basis of phenotype. For instance, in the mid-oleic mutant soybean line M23, there was significant variation among the olol individuals and their distribution overlapped that of the OlOl and Olol individuals, which indicated that modifying genes had an important influence on the trait (Alt *et.*, 2005). In sunflower, different MO inbred lines

seem to possess different modifier genes affecting oleic acid concentration, or differ in their interaction with the genetic factors influencing the oleic level (Miller and Vick, 2002).

In the context of the hypothesis of the action of two major genes on oleic acid content, the 13:3 ratio fitted in F_2 plants generation was due to dominant epistasis or suppression. The first element could be Ol*s* that it acts on the Pervenets allele reducing the oleic acid content. Ol*s* could be the "instability" gene reported by Triboï-Blondel *et al.* (2000). Ol*s* could codify for another FAD or acting on oleic /linoleic ratio in some other way. A partially recessive gene with maternal influence that modifies linoleic acid content (higher-than-normal and stable) was reported by Simpson *et al.* (1989). The second element could be Ol*l* that it causes a maternal phenotypic effect. Ol*l* is epistatic on Ol*s*. The effects of Ol*s* and Ol*l* are additive. These two elements are subjected to the combined action of modifier genes.

Demurin and Borisenko (2011) reported that the normal line RIL100 contained a high oleic mutation Ol in hypostatic condition (a suppressor masked HO phenotype). Inheritance of the high oleic mutation in the crosses of VK508 (HO) with suppressors (LO lines that carried a suppressor) in F2 fitted a digenic model of epistatic action of Sup over Ol in the ratio of 13 normal (LO): 3 mutant (HO). L26 (HO) line showed resistance to suppressor with complete dominance of Ol mutation in the F1 and monogenic inheritance (3:1 of HO to normal) in the F2 when LG 26 was crossed with suppressor-carrying lines. Suppression seems to be common in several sunflower genotypes. In our case, the suppressor of HO phenotype could be a gene that codifies for another FAD2.

Continuous distributions in oleic and linoleic acids content indicated polygenic inheritance (Fig. 17). In addition to those two major genes, we suppose that a polygene or a gene-complex (Fernandez *et al.*, 1999) acts on the oleic and linoleic acid content. This polygene or gene-complex mimics the segregation patterns produced by Mendelian segregation but originating in a non-Mendelian manner. This third element acts on the other elements in the same way, increasing or decreasing oleic and linoleic acid content. We suppose that this element was a combination of modifier genes originating different phenotypes.

The occurrence of different segregation patterns (3:1 High to Low in 2010 or 13:3 in 2011) in pooled analysis and on single recombinant plants basis may depend on the environment (temperature) and/or by a different configuration of modifier genes. The occurrence of different segregation patterns depending on allelic configuration of modifier genes has been previously

reported in sunflower for high oleic acid content (Velasco *et al.*, 2000) and gamma tocopherol (García-Moreno *et al.*, 2012).

The recombination observed in the two populations indicated that 342cms (and isogenic line 342mt) and R978 differed for some of the alleles that condition their high oleic content. Previous results indicated that 342mt carried the recessive allele of Ol*s*. 342mt and 342cms were isogenic inbred lines. Thus, 342cms led the recessive allele of Ol*s*. The recessive gene Ol*l* was carried by R978 inbred line because in the cross 342mt x R978 no LO phenotype appeared in F_2 and BC_1F_1 generations (Chapter 3) and 342 cms was HO stable. If the Ol*l* gene was carried by 342 inbred lines, then 342 inbred would not be stable (HO, mid – oleic and LO phenotype). At present, there is no indication on the number of the *Ol* (Pervenets allele) modifier genes and their interaction with *Ol*. Fernandez *et al.* (1999) used R978 inbred line as HO parent line in a cross with the LO inbred line HA89 and they fitted a 15:1 segregation ratio in F_2 generation (15 Low : 1 High) and suggested that *Ol* (Pervenets Allele) was recessive and ML was a gene complex or a polygene dominant on the Pervenets allele. So the hypothesis that some negative elements for a High Oleic content was carried out in R978 cannot be rejected. They used in backcross R978 as mother plant and found some LO individuals in the backcross seed generation. This suggests that the recessive plant control was led by the inbred line R978. In addition, we detect a cytoplasmic effect on some genetic factors (Ol*s*) that affected oleic acid content. Thus, a different selection pressure on these genes between HO inbred lines was probable.

With these results we postulate that epistasis or duplicate genes are the most probable types of gene interaction that originated the LO phenotype rather than suppression in narrow sense.

In this work, we demonstrated that a suppressor of HO phenotype or a other negative element for oleic acid content was carried by a HO stable inbred line. Therefore other factors affected the HO phenotype. The Pervenets allele was essential for HO phenotype but not always sufficient as reported by several Authors for producing high oleic acid concentrations (Soldatov 1976; Urie 1985; Miller *et al.,* 1987; Fernandez-Martínez *et al.,* 1989; Fernandez *et al.,* 1999; Lacombe and Bervillé 2001; Lacombe *et al.*, 2002; Pérez-Vich *et al.,* 2002; Varès *et al.,* 2002, Lacombe *et al.,* 2004).

From a breeding point of view, selection in the early segregating generations (with a high degree of heterozygosis) on single seed basis or on single plant basis was not recommended for the High Oleic trait. Dominance is involved, thus plants that are heterozygous for the HO trait would mask the segregating seeds that have a recessive genotype. If the genotype of the maternal plant controls phenotypic expression of fatty acid composition in the developing seeds, selection progress on a

single seed basis may be reduced because selection would be based on the phenotype resulting from the genotype of the maternal plant and not on the genotype of the seed. As the level of inbreeding increases, the impact of this type of maternal effect would decrease because the genotype of the seed is more likely to match the genotype of the maternal parent on which it developed (Gilsinger *et al.*, 2010). Thus, selection on a single seed basis would start in F_4-F_5 plant generation (with a higher level of inbreeding). When the half-seed technique is used, the longitudinal gradient of oleic acid content in the seed would be taken into account (i.e. a recombinant type could be classified HO and so genotype with a low oleic potential kept during selection).

With regard to selection on single plant basis, the other problem during selection could be cytoplasmic effects or cytoplasm x nucleus interactions. In the latter case, unfavorable genetic factors could appear phenotypically in later self-pollinated generations or in crosses between inbred lines for hybrids production and evaluation. Thus, selection on single plant basis would start in later self-pollinated (F_5-F_6) generations with a high level of inbreeding and several generations would be evaluated (to detect if negative effects appear or disappear). On the other hand, cytoplasm could mask unfavorable genetic factors and so cytoplasmic effects could be used to select hybrids insensible to environmental condition.

To select hybrid with a HO content and stable across environments, a strategy could be the development of tester lines from high x high crosses. Use of these inbred lines in Test-crosses could allow to eliminate these negative genetic effects from HO breeding material. This genetic material could be used to detect if more mutations were carried in Pervenets. Crosses between HO have never been performed systematically. Systematic crosses between HO lines and complementary tests could demonstrate if more mutations were carried in Pervenets. This knowledge would permit to improve breeding procedures.

From a physiological and genetic point of view, understanding the nature and behavior of these other elements and their influence on the Pervenets mutation could be a topic of interest and it can be used to develop marker-assisted selection. Suppression, epistasis or duplicate genes give the same segregation ratio but at molecular level there are many different possible mechanisms.

4.5   CONCLUSIONS

The HO trait is a complex trait modified by different genetic elements. The Pervenets allele carrying the whole functionnal MOD gene and direct repeat of exon1 and part of intron1 is necessary but not always sufficient for producing high oleic acid content in the seed oil. In the F_3 seed generation several individuals with a low oleic phenotype appeared. Other genetic factors (we suppose two major genes and modifier gene) and gene interaction affected oleic and linoleic acids content in HO mutants. HO inbred lines, that displayed a stable HO phenotype, can lead a suppressor of HO phenotype. Our results suggested that inbred lines differed for some of the alleles that condition their high oleic content. 342cms inbred line leads a recessive factor, designated as Ol*s*, and R978 leads a recessive allele, designated as Ol*l*, that originated a maternal phenotypic effect and affected Ol*s* and/or Pervenets allele expression in the next generations. Modifier genes could originate a high variability in oleic acid content across generations. MO phenotype is originated by modifier genes and we suggest that it is the results of two different phenomena. MO phenotype originates by the action of modifier genes on the Pervenets allele and/or on Ol*s* and MO phenotype originates by the action of Ol*l* on Ol*s* and/or modifier genes. Thus, an overlapping in MO phenotype in the F_3 generation between these two phenomena is supposed. Phenotypic data alone do not allow us to discriminate the nature of gene interaction (epistasis, suppression or duplicate genes). However, the results suggested that epistasis or duplicate genes are the most probable type of gene interaction. Breeding for the HO trait on single seed basis would be avoided in the early generations (with a high degree of heterozygous) because several types of maternal effect seem to be involved in the control of oleic and linoleic acid content. LO recombinant inbred plant from cross HO x HO could be used as tester to detected these negative elements in stable HO inbred lines.

Further studies both in the field and with molecular approaches are needed to elucidate the nature of HO phenotype suppression, the number of suppressors, the number of modifier genes and their interaction with the other genetic elements.

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Tesi di dottorato di Claudio Ferfuia, discussa presso l'Università degli Studi di Udine

5   General Conclusions

This work has several limitations. Only two HO inbred lines were used, in one location (Udine) and in three years. A limited number of generations were analyzed. With this in mind, the conclusions are valid for these sunflower genotypes and in Udine environmental conditions.

Fatty acid composition in high oleic sunflower depends mainly on genotype. However, the environment could modify oleic acid content in some HO genotypes but the effects are smaller than in standard type sunflower (an oleic acid percentage increase of 3% from 400 to 500 Growing Degree Days accumulated). Nevertheless, environmental effects are significant to prevent to achieve the 90% threshold in oleic acid content. The genetic variability found among high oleic genotypes in response to temperature could be related to modifier genes and to maternal effects. In segregating generations, temperature seems to affect phenotypic expression of several genes. Some differences are detected in the period in which temperature has maximum effect on fatty acid composition and this suggests that temperature could affect different processes.

The HO trait is complex. HO genotypes, that displayed a stable HO phenotype, with a different genetic background have a different behavior and they differed for some of the alleles that condition their high oleic content. The Pervenets allele, carrying the whole functional MOD gene and direct repeat of exon1 and part of intron1, is essential for HO phenotype but not sufficient for producing high oleic acid content in the seed oil.

Three factors, in addition to Pervenets allele, seem to modify oleic acid content: two major genes and a combination of modifiers with maternal effects.

Maternal effects affected oleic and linoleic acids content: a cytoplasmic and a phenotypic maternal effect seem to affect unsaturated fatty acid content in HO genotypes. Oleic/linoleic ratio was affected by low temperature only in one inbred line. Furthermore, experiments with hybrids made in both directions suggest that variation in oleic acid content in response to low temperature was inherited from mother plant. Its effects depend on cytoplasms (either PET1 or Normal) and on nuclear alleles. These nuclear alleles are Ols, Oll and Olm. Ols acts on the Pervenets allele reducing the oleic acid content and it could codify for another FAD or acting on oleic /linoleic ratio in some other way. The second element, designated as Oll, when it is heterozygous or homozygous for the dominant allele, originated a maternal phenotypic effect and affected Ols and/or Pervenets allele expression in the next generations (Oll is epistatic on Ols). Olm is a combination of modifier genes (a polygene or a gene-complex), each having a small effect on the trait, that modify oleic acid content only when Ols was homozygous for the recessive allele. Each modifier gene has small quantitative effects on the level of expression of Ols, but in combination they cause a greater

variability. Ol*l* could acts on modifier genes: Ol*m* modify oleic acid content only when Ol*l* was homozygous for the recessive allele.

Interestingly, only in Normal cytoplasm recombinant phenotypes were found in the F_2 generation, while in PET1 cytoplasm they did not occur. A recombinant class of Mid-Oleic type appeared. A gene, designated as Ol_s, when it is homozygous for the recessive allele (Ol_sOl_s) decreases oleic acid content. Expression of this gene seems to be affected by cytoplasm (no expression in PET1 cytoplasm), by modifier genes, and by temperature. We have suggested that this gene could be another FAD2 gene normally not expressed in the seed, but that may be expressed.

In F_3 seeds, LO phenotype appeared. It was not detected in F_2 generation. It is suggested that the HO trait was influenced also by phenotypic maternal effect. A recessive gene, designated as $Ol_{l,}$ influenced oleic acid content, acting on Ol_s and/or on modifier genes. This is in agreement with early studies on inheritance of the HO trait.

The approach to studying inheritance of oleic acid content based on cross HO x HO has partially failed. Offspring with a Mid-Oleic phenotype appear and they complicate the interpretation of the results. An alternative approach could be to study oleic acid inheritance under controlled environment with different temperatures. To detect modifier genes (number) an approach could be the study of the Near Isogenic Lines (NILs), which are genetically identical, except for one or a few loci, obtained from backcross generations. More information could be obtained on modifiers by NILs rather than Recombinant Inbred Lines (RILs).

On the basis of phenotypic observation, with a limited number of generations, only several hypotheses could be formulated. Genotypic and phenotypic data are needed to understand the nature of these elements. However, the hypotheses could include epistasis, suppression or duplicate genes. The results suggested that epistasis or duplicate genes were the most probable type of gene interaction. Thus, the cross between HO stable inbred lines could originate offspring with a low oleic phenotype. This is another important aspect in improving HO varieties based on a breeding method including such interactions.

To obtain environment-insensitive hybrids, selection should be based on inbred lines that do not show any phenotypic variation in oleic acid content across years and locations. It was observed that the cytoplasmic (PET1) effect might have an important role in the genetic control of these traits. Maternal effect could modify phenotypic expression of some genes and so the 90% threshold in oleic acid content, a goal in a breeding program to select high oleic hybrids for industrial use. Cytoplasmic effect could be used by breeders to obtain stable HO hybrids, insensitive to

environment. Selection for increased oleic acid composition on single seed basis and on half-seed technique in early self-pollinated generations should be avoided. Selection on single seed basis or on single plant basis should be made with increasing of inbreeding level. Inbred lines with a LO phenotype developed from HO x HO crosses could be used as tester line to select, against negative factors, new HO lines. To obtain hybrids with a content in oleic acid higher than 93-95% it is necessary to select inbred lines with a low content in saturated fatty acids.

Further works would be focused on physiological and genetic aspects to elucidate gene action and metabolic pathways involved in high oleic acid content.

ACKNOWLEDGEMENTS