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HALF-SEED ANALYSIS FOR COMPARING LINOLENIC ACID SYNTHESIS BETWEEN HIGH AND LOW OLEIC ACID SUNFLOWER INBRED LINES

S. Tahmasebi-Enferadi*, Z. Rabiei, M. Turi, M. Baldini, and G.P. Vannozzi

Dipartimento Produzione Vegetale e Tecnologie Agrarie,Università di Udine, via delle Scienze 208, 33100 Udine Italy.

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SUMMARY

The modification of fatty acid composition of two sunflowers inbred lines, HA89 and R978, low oleic acid (normal) and high oleic acid (mutant) respectively, in seeds and during the first stages of growth (A_1-B_2) was studied under controlled conditions. Enzymatic mechanisms have great effect on the catabolism of seed stored lipids. Temperature and oxygen regulation influence developing sunflower seeds. For simultaneous study of seed and developing seed, half-seed analysis technique was used. The behavior of the fatty acids during the germination in cotyledon of seed showed the increase of linolenic acid in both lines, demonstrating the activity of linoleic acid desaturase (Δ6-desaturase). But linoleic acid as a substrate for linoleate desaturase increased during all stages of developing only in mutant line that revealed higher activity of oleic acid desaturase (Δ12-desaturase) in transforming oleic acid to linoleic acid in this line, and lower activity of this enzyme in low oleic acid line, the reasons probably being the low availability of substrate of this enzyme in low oleic acid line and the complexity of enzymatic mechanisms. The modification of fatty acids in developing sunflower, depends not only on ambient conditions such as temperature and oxygen regulation as described by many authors, but also on the genotype.

Key words: **half-seed analysis, linolenic acid, linoleic acid desaturase, oleic acid desaturase, sunflower**

INTRODUCTION

During the first phase of development of *Hellianthus annuus* L. seed after seeding, the metabolism of triacylglycerols (TAG) and the conversion of them into new triglycerides and polar lipids like linolenic acid take place as described by Ichihara *et al*. (1980).

* Corresponding author, Phone: +39(0432) 558665, Fax: +39 (0432) 558603, e-mail: thamasebi@uniud.it

It is obvious that the composition of seed reserved lipids does not resemble that of vegetative tissues. In fact there are traceable quantities of linoleic acid (0.1% or trace) in sunflower seed according to Conte *et al.* (1989) whereas the abundant amounts of linolenic acid are found in green tissues of plant, which is associated with cellular and sub-cellular membranes that have been studied by Salisbury and Ross (1988).

The catabolism of storage lipids begins with the action of lipase (Salisbury and Ross, 1988). Most TAG are hydrolyzed in the first 10 days after seeding (DAS) depending on the action of lipase according to Ichihara *et al.* (1980), but desaturation of fatty acids with increasing of unsaturated ratio involved different types of desaturase, as described by many authors. Two forms of desaturase, oleic acid desaturase and linoleic acid desaturase are present at the endoplasmic reticulum in the cytosol (ER fad2 and ER fad3, respectively) and in chloroplasts (Plastid fad6 and Plastid fad7, respectively), which are responsible of the modification of oleic acid to linoleic acid and linoleic acid to linolenic acid as described by Mollers (2002).

Some plant species such as melon, cucumber and sunflower have the capacity of photosynthesis even in the cotyledon (Salisbury and Ross, 1988), which make them able to synthesize linolenic acid. Also, the other factor which affects the mechanism of linolenic acid synthesis is germination temperature, as described by Mazliak *et al.* (1977) and Graces *et al.* (1992).

The monitoring of the synthesis of this tri-unsaturated acid, which is considered an important polar lipid in green tissues (Stumpf, 1976), at different phases of development and in single seeds, was made possible by using the half-seed analysis.

In the half-seed analysis described by Downey and Harvey (1963), the embryo is separated from the rest of the seed; the former is grown and the latter is analyzed. This technique provides information about the synthesis mechanisms and catabolism of fatty acids in single seeds and during the growth of these seeds. Other applications of this technique to several oilseed crops have been reported by (Yermanos, 1968; Roy and Tarr, 1987), and it has been used for investigation of fatty acids (Jonassen and Munk, 1981; Gallopini *et al.*, 1984).

The paper reports the results of studies on the metabolism of the seed storage lipids in the seed and the different stages of development (10,13,16,19 DAS) in combination with half-seed analysis technique and the eventually new lipid polar synthesis in different vegetative tissues in two different sunflowers inbred lines, low oleic acid content (LOAC) and high oleic acid content (HOAC) and comparison between them.

MATERIALS AND METHODS

Two sunflower *(Helianthus annuus L.)* seed inbred lines used in this study were R 978 HOAC, a restorer inbred line selected at the University of Udine from

material coming from Pervenets and kindly supplied by Prof. G.P.Vannozzi, and HA89 the LOAC, a restorer inbred line released by Dr. J. F. Miller at Fargo, ND, USA.

Ten seeds from external regions of a single capitulum of the two inbreds were sampled; seeds surfaces were sterilized according to the method described by Burrus *et al*. (1991). Seeds were then cut horizontally in two parts as described by Conte *et al*. (1986). The portions containing the embryos were planted for germination, under controlled conditions, at constant temperature of 25°C in a growth chamber in test tubes (130 × 25 mm) with a solid medium composed of MS salts (Murashige and Skoog, 1962), light intensity 900 μ. $E.m^{-2}s^{-1}$ and relative humidity of about 40-50%. These were grown till 19 DAS (B_2). The other part was subjected directly to identifying fatty acid composition.

Methyl ester preparation for gas chromatography technique for reserve seeds were done as described by Conte *et al*. (1986) and for analysis of fresh tissues at A_1, A_2, B_1, B_2 phases according to the method described by Conte *et al*. (1989).

The total fatty acid composition was determined with a HRGC Mega 2, Fisons gas chromatography equipped with a split injection system and flame ionization detector (FID) a fused-silica capillary column 30m × 0.32mm i.d. and the percentages of fatty acids were obtained by integrating the peak with Chrom-Card, Fisons Ins. Software.

In this study a two-way ANOVA Completely Randomized Design with 10 replicates was used. The first factor (A) days after seeding (in seed, 10, 13, 16, and 19) and the second factor (B) genotype were constructed using two inbred lines, LOAC and HOAC.

RESULTS AND DISCUSSION

The statistic analysis of the data from the experiments carried out on sunflower revealed that the differences between treatments (DAS), genotypes (low or high oleic acid), and their interaction were significantly different. No significant variation between other mutant lines (high stearic or high palmitic) was observed by Cantisan *et al*. (1999). Stearic acid was not included to have a low interaction between the considered period of growth and genotype (Table 1).

In Table 2, the two inbred lines were considered together. The changes in palmitic acid content were not significantly different. The changes in oleic acid content were not significantly different in the last phase, but the content was significantly decreased in the first and second phase. The changes of linoleic acid were significantly different at all phases with an abnormal rhythm, and the changes of linolenic

acid were significantly different at the first and second phases, during A_2-B_1 were not significant, and they were again significant in the last phase.

Table 1: Means squares of the analyzed characters obtained from adopted ANOVA model

Fatty acid content (%)	C16: 0	C18: 0	C18: 1	C18: 2	C18: 3
Treatment (A)	***	**	***	***	***
Genotype (B)	***	****	***	***	***
Interaction (A × B)	***	*	***	***	***

, * Significant at the P£ 0.05, and P £ 0.01 levels, respectively.

Table 2: Joint statistic analysis of two different sunflowers inbred lines during 19 DAS

Days after seeding	Fatty acid content (%)				
	C16: 0	C18: 0	C18: 1	C18: 2	C18: 3
Seed	4.63 b	2.39 ab	64.37 a	28.53 b	0 d
10	4.93 b	2.89 a	59.88 b	30.98 a	1.59 c
13	3.48 b	2.43 ab	56.04 c	28.87 b	6.62 b
16	5.14 b	1.96 b	55.19 c	32.46 a	6.28 b
19	10.30 a	2.41 ab	53.52 c	30.75 a	9.05 a

Means followed by the same letter are not significantly different at 1% level as indicated by Student –Newman-Keuls Test.

Finally, it was revealed that these two inbred lines had completely different metabolisms of fatty acids, not only in seed, but during all stages of growth, even in linolenic acid taking into account that its amount was zero at seed in both lines (Conte *et al.*, 1989) (Table 3).

Table 3: Statistic analysis of the differences in fatty acids metabolism of two different sunflowers inbred lines during 19 DAS

Line	Fatty acid content (%)				
	C16: 0	C18: 0	C18: 1	C18: 2	C18: 3
R 973	2.92 b	2.12 b	81.58 a	11.52 b	1.41 b
HA 89	8.47 a	2.71 a	34.02 b	49.12 a	8.01 a

Means followed by the same letter are not significantly different at 1% level as indicated by Student –Newman-Keuls Test.

Modification of fatty acids in low oleic acid (normal) and high oleic acid (mutant) line during 19 days after seeding

In LOAC line, the evaluation of palmitic acid in the seed and in the period till 16 DAS did not show any significant difference. During next 3 days there was an accelerated increase of this acid, possibly because of the increase of photosynthesis activity in B_2 (Figure 1a).

In HOAC line, palmitic acid changes resembled the changes of low oleic acid line to some extent. The changes can divided in three stages, the first stage from seeding to 10 DAS, when the content remained to some extent constant, the second

stage between 10 and16 DAS, when it decreased, and the last stage when accumulation of this acid took place again (Figure 1a).

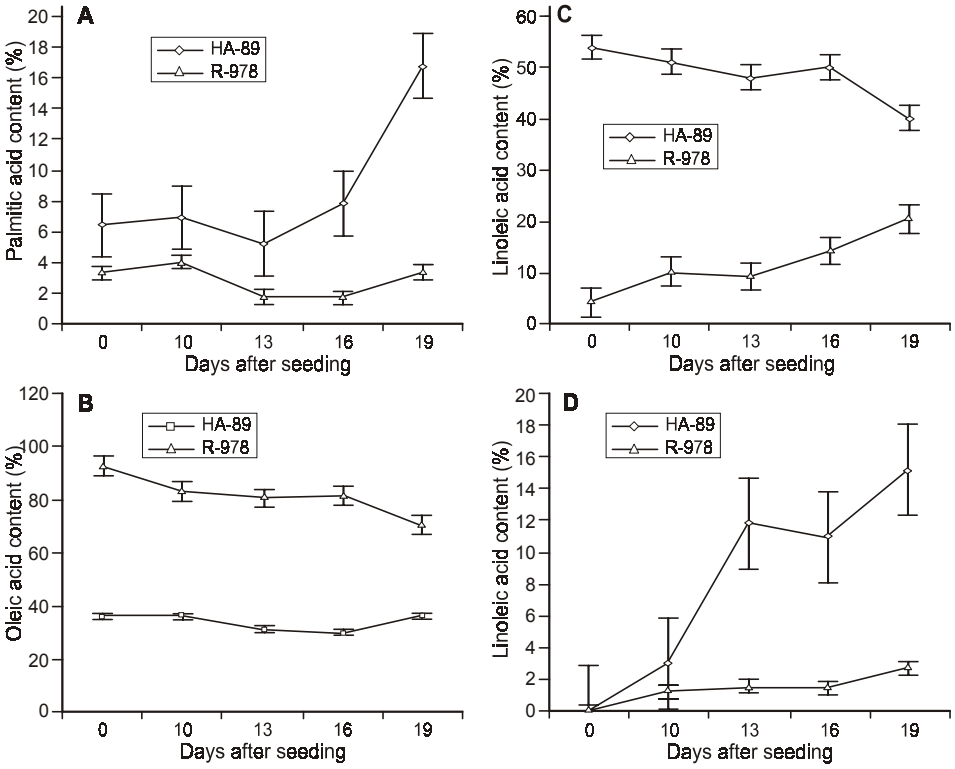

Figure 1: Modification of fatty acids in two different sunflower inbred lines, HA 89 and R 973 during 19 DAS, palmitic acid (a), oleic acid (b), linoleic acid (c), linolenic acid (d).

In LOAC line, stearic acid content remained more or less constant in all phases. This acid can transform to oleic acid by the action of Δ9 desaturase as described by Arao and Yamada (1994).

The behavior of stearic acid in HOAC line was completely like that in LOAC, and there was no significant difference in any phase of development of sunflower seed. It implies that the line (low oleic or high oleic acid) had no effect on the metabolism of this acid in any way during the experimental period (A_1-B_2).

In LOAC line, oleic acid content, as a monounsaturated acid and a substrate for oleate desaturase in seed, was 36% and it remained constant during first 10 days after seeding, but then it gradually decreased to 31% on the 13th day (5% reduction) as a result of oleate desaturase action as described by Mollers (2002). Linoleic acid content revealed a 6% reduction exactly in the same phase (seeding to 13 DAS), as a result of linoleate desaturase, so that both of them had the actual linolenic acid content of 11%. Oleic and linoleic acid content remained constant between 13 and 16 DAS and this state continued for oleic acid till the 19th DAS, which is an unknown

phenomenon. According to other researchers, temperature and oxygen regulate the activity of desaturase enzymes (Martinez-Rivas *et al.*, 2000) or it is done by light intensity (Rafael *et al.*, 1991). For presenting controlled conditions, abnormal activity of oleate desaturase can imply the complexity of enzymatic activity (Figure 1b, c).

The decrease of linoleic acid kept up with the increase of linolenic acid from the 16th to 19th DAS, which was predictable and normal (Figure 1c, d). Although linolenic acid content in seed is zero, its synthesis starts at 10 DAS. It is important to mention that the biosynthesis of this acid in plants like melon and cucumber may take place even at the first stages of cotyledon (Salisbury and Ross, 1988). Its content increased between 10 and 13 DAS, it remained constant between 13 and 16 DAS, and in B_2 it increased to 14%. This polyunsaturated fatty acid can be found especially in green tissues, which perform photosynthesis (Figure 1d).

In HOAC line, oleic acid catabolism during the first 10 DAS was 8.6%, in accordance with 7.6% increase of linoleic acid and 1.24% increase of linolenic acid (Figure 1b, c, d). Oleic, linoleic, and linolenic acids remained constant during the following 6 days and the changes of these fatty acids were not significant. But, a decrease in oleic acid, concomitantly with the increase in linoleic and linolenic acids, took place in the last phase (B_2).

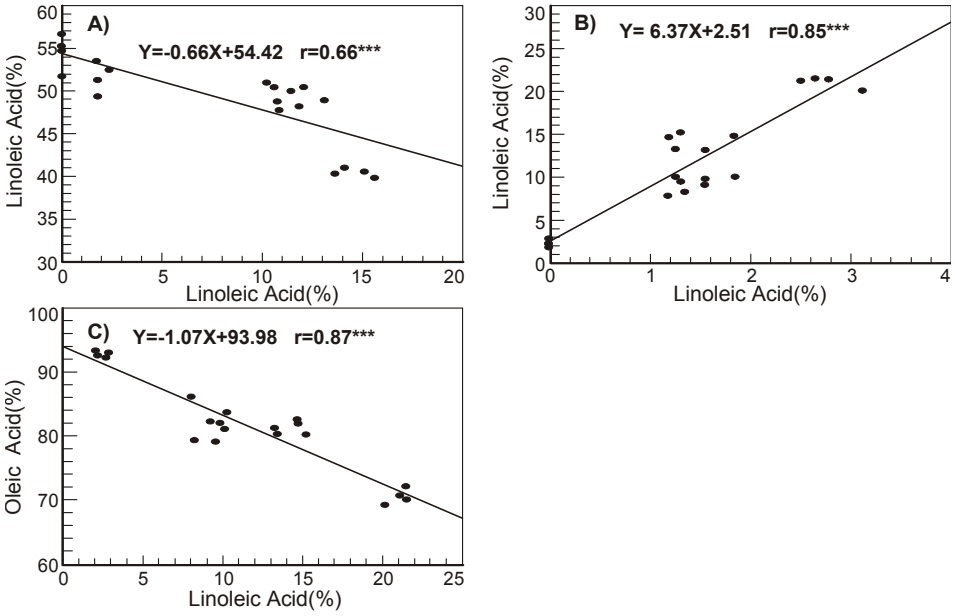

Figure 2: Correlations between linoleic acid and linolenic acid in low oleic acid hybrid (A), in high oleic acid hybrid (B), and between oleic acid and linoleic acid in high oleic hybrid.

Study on the relationships between these fatty acids showed a negative correlation (r=0.66***) between linoleic and linolenic acid in low oleic acid line (Figure 2A) and a significantly negative correlation (r=0.87***) between oleic acid and linoleic

acid in high oleic acid line (Figure 2C) and a significantly positive correlation (r=0.85***) between linoleic acid and linolenic acid high oleic acid line (Figure 2B).

CONCLUSION

It was concluded that the changes of palmitic, linoleic and linolenic acids in the cotyledon in both lines were significant, which could be related to oleic acid and linoleic acid desaturases activity.

The changes of oleic acid in the cotyledon were significant only in the mutant line. The low evolution of oleic acid in LOAC line revealed the lack of oleate desaturase activity compared with the mutant line. Study on the relationships between fatty acids showed a negative correlation between linoleic and linolenic acid content in low oleic acid line ($r=0.66^{***}$), that it demonstrated the decrease of linoleic acid by the action of linoleic acid desaturase lead to the increase of linolenic acid, whereas in mutant line the correlation is positive ($r=0.85^{***}$). This difference was explained in Figure 2C, in which the decrease of oleic acid (by the action of oleate desaturase) kept up with the synthesis of linoleic acid that made abundant linoleic acid, showing a complexity of enzymatic mechanism.

The modification of fatty acids in developing sunflower depends not only on external factors like temperature and oxygen, as described by many authors, but also on genotype.

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ANÁLISIS REALIZADO UTILIZANDO LA MITAD DE LA SEMILLA PARA LA COMPARACIÓN DE LA SÍNTESIS DEL ÁCIDO LINOLÉNICO EN LAS LÍNEAS CONSANGUÍNEAS DE GIRASOL CON ALTO Y BAJO CONTENIDO DE ÁCIDO OLEICO

RESUMEN

En el trabajo, en las condiciones controladas, se modificó el contenido de ácidos grasos de dos líneas consanguínea de girasol (HA89, línea normal con bajo contenido de ácido oleico, y R978, línea mutante con alto contenido de ácido oleico) en la semilla y en las fases tempranas de crecimiento (A_1-B_2). Los mecanismos enzimáticos tienen gran influencia en el catabolismo de lípidos almacenados en la semilla. La temperatura y regulación de oxígeno, influyeron en el desarrollo de la semilla de girasol. Para una simultánea investigación de la semilla, antes y durante el desarrollo, fue utilizada la técnica de análisis de una mitad de la semilla. El comportamiento de los ácidos grasos durante la germinación en el cotiledón de la semilla, fue caracterizado por el aumento del contenido del ácido linolénico en ambas líneas, lo que indica a la actividad de desaturasa del ácido linólico (Δ6-desaturasa). Pero, el contenido del ácido linólico como substrato para desaturasa linoleata, iba creciendo en todas las fases de desarrollo, solamente en la línea mutante, lo que indica una mayor actividad de desaturasa del ácido oleico (Δ12-desaturasa) en transformación del ácido oleico en ácido linólico en esta línea, tanto como en menor actividad de este enzima en la línea con bajo contenido de ácido oleico, probablemente como consecuencia de baja accesibilidad del substrato de este enzima en la línea de bajo contenido de ácido oleico tanto como la complejidad de los mecanismos enzimáticos. La modificación de los ácidos grasos en girasol en desarrollo, depende no sólo de las condiciones del medio ambiente, como son la temperatura y regulación de oxígeno, lo que han descrito muchos autores, sino también de genotipo.

ANALYSE DE GRAINES À DEMI POUR COMPARER LES SYNTHÈSES ENTRE LES ACIDES OLÉIQUES ET LINOLÉIQUES DANS LES LIGNES CULTIVÉES DE TOURNESOL

RÉSUMÉ

Dans les conditions contrôlées la modification de composition d'acides gras de deux lignes cultivées de tournesol, la ligne (normale) HA89, d'un contenu faible d'acide oléique et la ligne (mutante) R978, d'un contenu élevé d'acide oléique a été étudiée dans les graines pendant la première phase de croissance (A_1-B_2). Les mécanismes d'enzyme ont un effet important sur le catabolisme de lipides stockés dans les graines. La température et la régulation d'oxygène ont eu une influence sur le développement de graines de tournesol. Pour une recherche simultanée de graines, avant et pendant le développement, l'analyse de graines à demi était appliquée. Le comportement d'acides gras pendant la germination dans le cotylédon de graines a démontré une augmentation d'acides linoléiques chez les deux lignes, montrant une activité de formation d'acides linoléiques non saturés (Δ6-désaturase). Cependant le contenu d'acides linoléiques comme substrat de formation d'acides linoléiques non saturés a augmenté pendant toutes les phases de développement seulement chez les lignes mutantes, ce qui prouve une plus grande activité de formation d'acides oléiques non saturés (Δ12-désaturase) dans la transformation d'acides oléiques en linoléiques dans cette ligne et une plus petite activité de cet enzyme chez les lignes d'un niveau faible d'acides oléiques, probablement, c'est la conséquence d'une accessibilité faible de substrat de cet enzyme chez les lignes d'un niveau faible d'acides oléiques et aussi bien la complexité de mécanismes d'enzyme. La modification d'acides gras pendant la croissance des tournesols dépend, pas seulement, des conditions d'ambiance comme température et régulation d'oxygène, décrits par plusieurs auteurs, mais aussi bien des génotypes.