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DEVELOPMENT OF SUNFLOWER GERMPLASM WITH HIGH DELTA-TOCOPHEROL CONTENT

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SUMMARY

Tocopherols are the main compounds with antioxidant activity in oilseeds. Sunflower seeds contain predominantly alpha-tocopherol, which accounts for more than 90% of the total tocopherols in the seeds. This tocopherol derivative possesses a maximum vitamin E or *in vivo* antioxidant activity, but it exerts a minimum *in vitro* protective action in oils and food containing them. Other tocopherol derivatives such as beta-, gamma-, and delta-tocopherol are more powerful antioxidants than alpha-tocopherol. Accordingly, a partial replacement of alpha-tocopherol in sunflower seeds is an important breeding objective. So far, variants with high gamma tocopherol content (>85%), medium beta-tocopherol content (30% to 50%), and medium delta-tocopherol content (<25%) have been developed. The objective of the present research was to develop sunflower germplasm with novel tocopherol profiles. Seed of four Peredovik accessions of different origins were used for chemical mutagenesis with ethyl methane sulfonate (EMS). Single-seed screening in the M_2 generation resulted in the identification of an M_2 seed with 19% gamma-tocopherol. M_3 seeds exhibited wide segregation for gamma-tocopherol content, from zero to about 85%. Such a wide segregation has not been observed in previously developed high gamma-tocopherol germplasms, which segregate for low and high but not for intermediate levels of gamma-tocopherol. Selection for high gamma-tocopherol content within this mutant produced an $M_{4:5}$ line with stable high concentration of gamma-tocopherol, above 90% of the total tocopherols. The line was designated IAST-1. Crosses between IAST-1 and the line T589, with medium beta-tocopherol content, produced segregants with increased levels of up to 68% delta-tocopherol.

Key words: **antioxidants, high delta-tocopherol content, mutagenesis, sunflower seeds, tocopherols**

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INTRODUCTION

The tocopherols are natural antioxidants that occur as a family of four derivatives named alpha-, beta-, gamma-, and delta-tocopherol. They differ for their *in vitro* and *in vivo* antioxidant activity. *In vitro* action refers to the antioxidant protection of oils, fats and foods containing them, whereas *in vivo* action refers to an antioxidant protective action of lipids in biological systems. The latter is also known as vitamin E activity (Kamal-Eldin and Appelqvist, 1996).

Sunflower seeds contain mainly alpha-tocopherol, which accounts for more than 90% of the total tocopherols. Beta- and gamma-tocopherol can be present in sunflower seeds, usually in amounts below 5% of the total tocopherols (Demurin, 1993; Velasco et al., 2002). Alpha-tocopherol has a maximum vitamin E activity, but its *in vitro* activity is relatively low. Conversely, beta-, gamma-, and delta-tocopherol are more powerful *in vitro* antioxidants (Pongracz et al., 1995). Accordingly, the oxidative stability of sunflower oil is relatively low, especially in the standard oil type with a high concentration of linoleic acid. Because some applications of sunflower oil demand an improved oxidative stability, the modification of the tocopherol profile through a partial substitution of alpha-tocopherol by other tocopherol derivatives with greater *in vitro* antioxidant action is an important breeding goal in this crop (Škorić, 1992).

Breeding efforts in this direction conducted during the past decade have led to the development and characterization of several sources of modified tocopherol profile in sunflower. Demurin (1993) and Demurin et al. (1996) isolated two lines with contrasting tocopherol profiles. The line LG-15, with increased concentration of beta-tocopherol (50% of the total tocopherols), was developed from the open-pollinated variety VNIIMK 8931, whereas the line LG-17, with increased concentration of gamma-tocopherol (95% of the total tocopherols), was isolated from a germplasm entry of the VIR world germplasm collection. Genetic characterization of both lines concluded that the increased levels of beta-tocopherol were produced by recessive alleles at the *Tph1* locus, whereas the increased levels of gamma-tocopherol were the result of recessive alleles at the *Tph2* locus (Demurin et al., 1996). Similarly, Velasco *et al.* (2004a) developed the line T589 with increased levels of beta-tocopherol (>30% of the total tocopherols) from an accession of the open pollinated cultivar Peredovik, as well as the line T2100 with high gamma-tocopherol content (>85% of the total tocopherols) from another accession of Peredovik. Genetic evaluation of both lines confirmed the presence of a recessive allele at a single locus in each of the lines (Velasco and Fernández-Martínez, 2003). A comparative genetic evaluation of LG-15 with T589 and LG-17 with T2100 has not been conducted yet.

Through the utilization of chemical mutagenesis and further recombination of mutants with previously developed germplasm, we have recently developed a sunflower line characterized by a high concentration of delta-tocopherol. The development and isolation of this line is reported in this manuscript.

MATERIALS AND METHODS

The open pollinated cultivar Peredovik, formerly widely cultivated in Spain, was selected for mutagenesis, as previous studies had revealed variability for genes involved in tocopherol biosynthesis in accessions of this cultivar (Velasco et al., 2004a). Four different accessions were chosen and about five hundred seeds from each one were pre-soaked in water for 4 h and then soaked for 2 h with a mutagenic solution 70 mM of ethyl methane sulfonate prepared in a 0.1 M phosphate buffer at pH 7. After mutagenesis, seeds were thoroughly rinsed with running tap water for 16 h to rinse excess EMS and, after drying, they were immediately sown in the field at the experiment farm of Instituto de Agricultura Sostenible, Córdoba, in March 1999. M_1 plants were self-pollinated, using paper bags to prevent uncontrolled pollination. Over one thousand M_1 plants were harvested (1080).

Six M_2 half-seeds per M_1 plant were analyzed for tocopherol profile following the method of Goffman et al. (1999). Half seeds were placed into 10-ml tubes with 2 ml iso-octane. The half seeds were then crushed with a stainless steel rod as fine as possible. The samples were stirred and extracted overnight at room temperature in darkness (extraction time about 16 h). After extraction, the samples were stirred again, centrifuged, and filtered. 5 μl of the extract were analyzed by HPLC using a fluorescence detector at 295 nm excitation and 330 nm emission and iso-octane/ tert-butylmethylether (94:6) as eluent at an isocratic flow rate of 0.8 ml min^{-1}. Chromatographic separation of the tocopherols was performed on a LiChrospher 100 diol column (250 mm $\times$ 3 mm I.D.) with 5-μm spherical particles, connected to a silica guard column (LiChrospher Si 60, 5mm $\times$ 4 mm I.D.).

RESULTS AND DISCUSSION

The progeny of one M_1 plant producing eight M_2 seeds included one seed with 19.2% gamma-tocopherol. This seed was germinated and it produced a viable M_2 plant that yielded 60 M_3 seeds which showed a wide variation for gamma-tocopherol content, from zero to 84.5%, including intermediate values not reported previously in high gamma-tocopherol germplasm (Demurin, 1993; Demurin et al., 1996; Velasco et al., 2004a). A mutant line with more than 90% of the total tocopherols in the gamma-tocopherol form, designated IAST-1, was developed from the segregating M_3 population and reported in more detail elsewhere (Velasco et al., 2004b).

Crosses between plants of IAST-1 and T589, with increased concentration of beta-tocopherol (Velasco et al., 2004a) produced F_1 seeds with standard high alpha-tocopherol content, with less than 2% of both beta- and gamma-tocopherol. The analysis of F_2 seeds from five F_1 plants revealed a wide segregation for tocopherol profile, including an exceptional variation for delta-tocopherol content, from zero to 67.9% delta-tocopherol content (Figure 1). No phenotypic classes for delta-tocopherol content could be distinguished within the F_2 segregation, which did not allow us to infer information on the genetic basis of the unexpected levels of delta-tocopherol. Phenotypic distribution of delta-tocopherol content in F_2 seeds is most clearly observed in Figure 2, which only includes F_2 seeds with more than 2% delta-tocopherol.

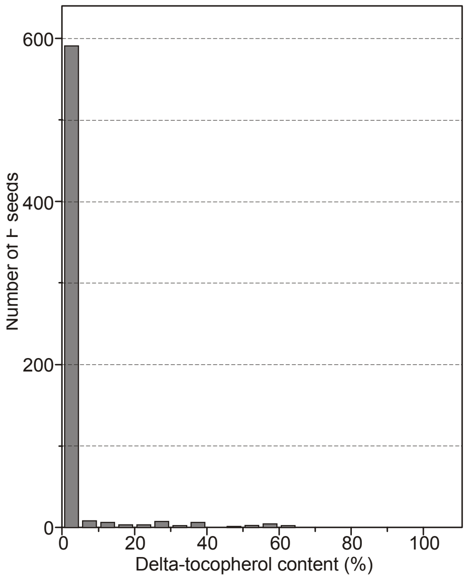

Figure 1: Histogram of delta-tocopherol content (% of the total tocopherols) in F_2 seeds from five F_1 plants of the cross between IAST-1 and T589

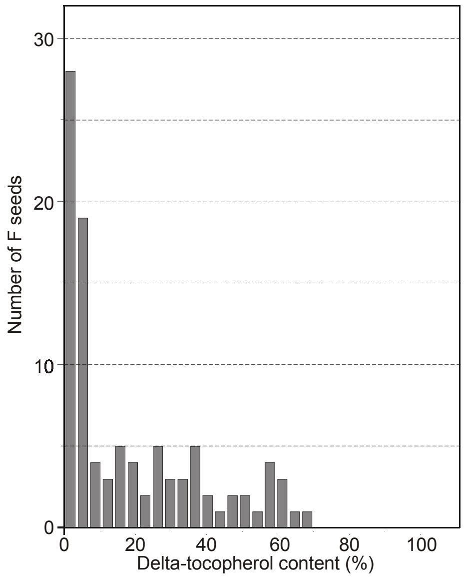

Figure 2: Histogram of delta-tocopherol content (% of the total tocopherols) in F_2 seeds with more than 2% delta-tocopherol from five F_1 plants of the cross between IAST-1 and T589

Half seeds with the highest delta-tocopherol values were germinated and they produced plants that expressed the increased delta-tocopherol trait uniformly, with an average content of 58.2% delta-tocopherol in F_3 seeds. The overall tocopherol profile in F_3 seeds was made up of 4.3% alpha-tocopherol, 3.2% beta-tocopherol, 34.3% gamma-tocopherol, and 58.2% delta-tocopherol. The line was named IAST-4.

In a previous work, Demurin et al. (1996) found that the combined action of mutations at the *Tph1* and *Tph2* genes, associated with increased levels of beta- and gamma-tocopherol, respectively, produced significant amounts of delta-tocopherol, with a maximum content of about 20% of the total tocopherols. The production of about 60% delta-tocopherol in seeds of IAST-4 suggests that this line carries one or several genes different to those reported by Demurin et al. (1996). These genes are probably derived from the high gamma-tocopherol line IAST-1, which was selected from an M_2 population exhibiting a range of variation out of the limits of other germplasms with high levels of gamma-tocopherol reported to date (Velasco et al., 2004b). Nevertheless, further genetic studies are needed for the characterization of the genetic bases of the high delta-tocopherol content of the line IAST-4.

The tocopherol profile of the line IAST-4, characterized by 34.3% gamma-tocopherol and 58.2% delta-tocopherol, is not found within the most common oilseeds, with the sole exception of castor bean. Both gamma- and delta-tocopherol are the tocopherol derivatives with the greatest in vitro antioxidant activity, representing about twice (delta-tocopherol) and three times (gamma-tocopherol) the *in vitro* antioxidant activity of alpha-tocopherol (Pongracz et al., 1995). Current sunflower seed oil, with more than 90% alpha-tocopherol, possesses a low *in vitro* oxidative stability if compared with other seed oils such as soybean, which contains more than 90% of the tocopherols in the gamma- and delta-tocopherol forms (Padley et al., 1994). The novel seed oil of IAST-4 possesses more than 90% of the total tocopherols in the gamma- and delta-tocopherol forms, which will probably determine a much higher oxidative stability than conventional sunflower oil.

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FORMACIÓN DE GERMOPLASMA DE GIRASOL CON ALTO CONTENIDO DE TOCOFEROL DELTA

RESUMEN

Los tocoferoles son los principales compuestos con propiedades antioxidantes en semillas oleaginosas. Las semillas de girasol contienen principalmente alfa-tocoferol, en una proporción superior al 90% del total de tocoferoles. Este tocoferol posee una máxima actividad *in vivo* o actividad de vitamina E, pero ejerce una débil protección *in vitro* de aceites, grasas, y alimentos que los contienen. Otros tocoferoles como beta-, gamma-, y delta-tocoferol presentan mayor eficiencia antioxidante *in vitro*. En consecuencia, la substitución parcial de alfa-tocoferol es un importante objetivo de mejora del girasol. Hasta la fecha, se ha desarrollado material con alto contenido en gamma-tocoferol (>85%), contenido medio en beta-tocoferol (30% a 50%) o delta-tocoferol (hasta 25%). El objetivo de este trabajo fue el desarrollo de germoplasma de girasol con nuevos perfiles de tocoferoles. Para ello se utilizó mutagénesis química con metil sulfonato de etilo (EMS) sobre semillas de cuatro entradas de "Peredovik". Se analizaron medias semillas individuales de la generación M_2, dando lugar a la identificación de una semilla M_2 con 19% de gamma-tocoferol. Las semillas M_3 presentaron una amplia segregación para contenido en gamma-tocoferol, desde cero hasta alrededor de 85%. Una segregación tan amplia para gamma-tocoferol no se ha observado con anterioridad en el germoplasma con altos niveles de este tocoferol desarrollado hasta la fecha, que presenta segregación para niveles altos y bajos pero no para niveles intermedios de gamma-tocoferol. Tras seleccionar para alto contenido en gamma-tocoferol, se obtuvo una línea $M_{4:5}$ estable para alto contenido en gamma-tocoferol, por encima del 90% del total de tocoferoles. La línea se denominó IAST-1. Cruces de IAST-1 con la línea T589, con niveles elevados de beta-tocoferol, dieron como resultado segregantes con niveles elevados de hasta 68% de delta-tocoferol.

FORMATION DE GERMES PLASMIQUES DE TOURNESOL D'UN CONTENU ÉLEVÉ DE DELTA-TOCOPHÉROL

RÉSUMÉ

Les tocophérols sont les principales compositions ayant un effet antioxydant dans les cultures oléagineuses. Dans la graine du tournesol dominent l'alpha-tocophérols contenant 90% du total de tocophérol de cette plante. Ce dérivé de tocophérol a une maximale activité vitaminique ou *in vivo* une activité antioxydant mais *in vitro* un effet minimal, protecteur dans l'huile et la nourriture qui le contient. D'autres dérivés de tocophérol (bêta-, gamma-, et delta-tocophérol) sont plus puissants antioxydants qu'alpha tocophérol. Pour cela le remplacement partiel d'alpha tocophérol dans la graine du tournesol représente un but très important. Dans les recherches récentes les variants de contenu élevé de gamma-tocophérol (>85%), de contenu moyen de bêta-tocophérol (30-50%), et de contenu moyen de delta-tocophérol(<25%) ont été créés. Le but de cette étude était la formation de profiles-tocophérol de germes plasmiques de tournesol. Dans la graine de quatre génotypes "Peredovik" d'origine différente une mutagenèse chimique a été provoquée à l'aide d'éthyle méthane sulfone (EMS). Examinant la graine particulière dans la génération M_2 le résultat a donné une identification de graine contenant 19% de gamma-

tocophérol. La graine de la génération M_3 a démontré une large ségrégation pour le contenu de gamma-tocophérol (de 0 à 85% environ). Une ségrégation aussi large n'a pas été repérée chez les germes plasmiques créés avant concernant le contenu élevé de gamma-tocophérol, pour lesquels il existe une ségrégation concernant le contenu faible et élevé de gamma-tocophérol, mais pas concernant le contenu moyen. La sélection d'un contenu élevé de gamma-tocophérol à l'intérieur de ce mutant a créé une ligne $M_{4;5}$ d'une stable concentration élevée de gamma-tocophérol (au-dessus de 90% du total de tocophérol), qui a été appelée IAST-1. Par le croisement entre les lignes IAST-1 et T589, qui a un contenu moyen de bêta-tocophérol, les "ségrégants" ont été créés d'un contenu de delta-tocophérol élevé jusqu'à 68%.