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Short communication. Estimation of cross-fertilization rate in safflower (*Carthamus tinctorius* L.)

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Abstract

Safflower is a promising oilseed crop for the production of specialty oils in the Mediterranean area. Oil quality traits such as high oleic acid content, high linoleic acid content, high saturated fatty acid content, or high gamma-tocopherol content have been developed in this crop. The traits are controlled by the genotype of the developing embryo and therefore they are influenced by the presence of foreign pollen. The objective of this research was to study the rate of cross-fertilization in safflower using the high oleic acid trait as a biochemical marker. An experiment in which each high oleic plant was surrounded by 24 low oleic acid plants was conducted over three environments in the same location at Córdoba, Spain. The average rate of cross-fertilization in the three environments was 5.7, 12.1, and 13.2%, though higher frequencies up to 35.9% were detected at the single-plant level and up to 58.3% at the single-head level. The low average outcrossing frequencies identified in this research indicate no need for large isolation distances between conventional cultivars and cultivars with special oil characteristics. However, the occurrence of a significant outcrossing rate should be taken into consideration if transgenic safflower is to be cultivated close to conventional safflower or in areas of distribution of wild *Carthamus* species.

Additional key words: oleic acid; outcrossing; pollen flow.

Resumen

Comunicación corta. Estimación de la tasa de fertilización cruzada en cártamo (*Carthamus tinctorius* L.)

El cártamo es un cultivo oleaginoso con un futuro prometedor para producción de aceites singulares en el área mediterránea. Se han desarrollado en este cultivo caracteres modificados de calidad de aceite como alto contenido en ácido oleico, alto contenido en ácido linoleico, alto contenido en ácidos grasos saturados, o alto contenido en gamma-tocoferol. Estos caracteres están controlados por el genotipo del embrión en desarrollo y por tanto están influenciados por la llegada de polen externo. El objetivo de este trabajo fue el estudio de la frecuencia de fertilización cruzada en cártamo empleando el carácter alto oleico como marcador bioquímico. Se planteó un experimento en tres ambientes en Córdoba, España, en el que cada planta alto oleico estuvo rodeada por 24 plantas bajo oleico. La frecuencia media de fertilización cruzada fue de 5,7, 12,1, and 13,2% en los tres ambientes, si bien se observaron frecuencias de hasta 35,9% a nivel de plantas individuales y hasta 58,3% a nivel de inflorescencias individuales. Los valores relativamente bajos de la frecuencia de fertilización cruzada sugieren que no es necesario establecer grandes distancias de aislamiento entre cultivares convencionales y cultivares con características especiales de aceite. No obstante, la frecuencia de fertilización cruzada observada en este trabajo debe ser tenida en cuenta en caso de cultivo de líneas transgénicas de cártamo en las proximidades de cultivo de cártamo tradicional o en áreas de distribución natural de especies del género *Carthamus*.

Palabras clave adicionales: ácido oleico; fertilización cruzada; flujo de polen.

Safflower (*Carthamus tinctorius* L.) is an ancient crop of the Asteraceae family cultivated mainly for seed oil production as well as for birdseed (Dajue & Mündel, 1996). Nowadays safflower is a minor crop with roughly 800,000 ha cultivated worldwide, about half of them in India (FAOSTAT, 2011). There are two groups of safflower cultivars according to their seed oil quality, characterized by high linoleic acid (70 to 75% of total fatty acids) and high oleic acid content (70 to 75%) (Hamdan *et al.*, 2009a). Safflower seed oils are highly

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valued for food, animal feed and industrial uses such as biofuel production (Singh & Nimbkar, 2007). Safflower has great potential as an oilseed crop for the Mediterranean area, where it performs well under autumn and winter sowing (Fernández-Martínez, 1997).

Safflower is considered a partially allogamous crop with a typical outcrossing rate of less than 10% (Knowles, 1989) predominantly insect-mediated (Pandey & Kumari, 2008), with honey bees being the most common pollinators (Singh & Nimbkar, 2007). However, higher outcrossing rates have been reported. Claasen (1950) observed outcrossing rates between 5 and 40%, whereas rates up to 59% have been reported in India (Singh & Nimbkar, 2007). It is important to note that some estimations of high outcrossing rates are based on plants growing side by side, in which outcrossing may be overestimated due to contact between inflorescences of adjacent plants (Christianson *et al.*, 2008). In this sense, Rudolphi *et al*. (2008) estimated an outcrossing rate between 29.9 and 63.1% within plots at a plant density of 40 plants m^{-2} and an outcrossing rate from 6.5 to 18.1% between plots in a study conducted in Germany. McPherson *et al.* (2009) studied pollen flow at different distances at locations in Canada and Chile. They found outcrossing frequencies between 0.5 and 1.7% at plant distances between 0.3 and 3 m and decreasing frequencies at longer distances, resulting in values lower than 0.05% at distances between 50 and 100 m. Additionally, the authors found that the outcrossing frequency was spatially heterogeneous, indicating asymmetrical pollen distribution.

In partially allogamous crops, the outcrossing rate is influenced by the environmental conditions under which the plants are grown (Becker *et al*., 1992). No studies on the occurrence of natural outcrossing in safflower have been conducted in the Mediterranean area, with great potential for safflower cultivation. Estimation of outcrossing is important for the production of specialty oils with modified fatty acid or tocopherol traits, which are recessive and controlled by the genotype of the developing embryo and consequently influenced by the arrival of foreign pollen (Velasco *et al.*, 2005a). Since such traits are expressed at the F_1 seed level, they can be used as biochemical markers in studies aimed at estimating outcrossing rates. For example, erucic acid content has been extensively used as a biochemical marker for estimating outcrossing rates between lines with contrasting levels of this fatty acid in rapeseed (Rakow & Woods, 1987; Cullen *et al.,* 2008; Leflon *et al.,* 2011). The objective of this research was to estimate the outcrossing rate in safflower in experiments conducted in three environments at the same location in Córdoba, Southern Spain using the monogenic, recessive trait high oleic acid content as a biochemical marker.

The experimental plot to estimate outcrossing rate in safflower consisted of 36 plants of the cultivar CR-6, developed from the cultivar Oleic Leed, with high oleic acid content (75-82%), with each plant surrounded by 24 plants of the cultivar Rancho, with low oleic acid content (20-35%), at a sufficient distance (1.5 × 1 m) to avoid contact between inflorescences of different plants (Fig. 1). Twelve plants of the cultivars Rancho and CR-6 were grown at an adjacent plot and they were bagged before flowering to obtain seed under self-fertilization conditions. High oleic acid content in seeds of CR-6 is monogenic, partially recessive and controlled by the genotype of the developing embryo (Hamdan *et al.*, 2009a). Both lines were selected because they have similar flowering time in the area where the experiments were located. The experiment was conducted in three environments: winter sowing of 2009, winter sowing of 2010, and spring sowing of 2010, in all cases at the experimental farm of CSIC in Córdoba, Southern Spain.

Individual seeds of CR-6 and Rancho were analysed for fatty acid profile by the half-seed technique using the method described by Hamdan *et al.* (2009a) and germinated on moistened filter paper. Seeds were sown in small pots (7 × 7 × 7 cm) and maintained in a growth chamber at 25/18ºC (day/night) for three weeks, then transplanted to the field following the scheme shown in Fig. 1. Transplanting dates were 24 February 2009, 18 January 2010, and 5 April 2010. In 2009 the plot was close to a sunflower plot that was not flowering at the time of safflower flowering. In the first transplanting date of 2010 the plot was close to a *Brassica carinata* multiplication plot under insect-proof cages. In the second transplanting date of 2010 the plot was close to a *Ricinus communis* plot not flowering at the time of safflower flowering. In all cases, no flowering crops were close to the safflower plot nor were other safflower plants growing nearby. Plants of the high oleic acid line CR-6 were harvested at maturity. In 2009, 16 individual heads per CR-6 plant were harvested separately in order to evaluate within-plant variation. In 2010, the heads of each CR-6 plant were bulked.

Twelve seeds per individual head (2009) or 192 seeds per individual plant (2010) were analysed for fatty acid profile (Hamdan *et al.*, 2009a). The rate of cross-fertili-

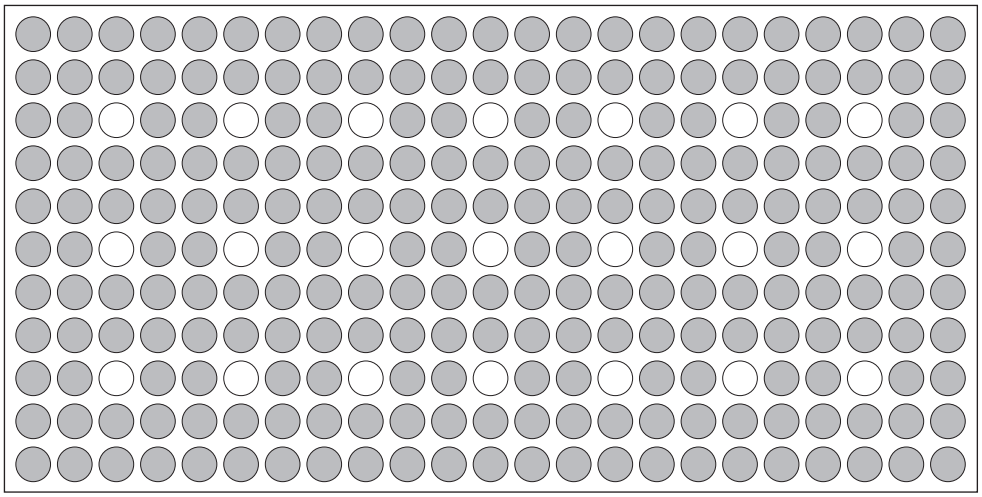

Figure 1. Schematic representation of the experimental design [partial] for studying the cross-fertilization rate in safflower. The experiments consisted of 36 plants of cultivar CR-6 (white circles, high oleic acid cultivar) and 382 plants of cultivar Rancho (grey circles, low oleic acid cultivar), distributed in 38 rows. Rows are separated 1.5 m, with 1-m distance between plants in the row.

zation was calculated as the percentage of low oleic acid seeds (<36%) produced on high-oleic CR-6 plants. This threshold was based on the observed oleic acid content of seeds of CR-6 and Rancho plants produced under self-fertilization as well as on the oleic acid content of seeds produced on CR-6 plants in the outcrossing experiments, which is shown in Fig. 2 for the 2009 experiment. A chi-square test for homogeneity was conducted within each experiment (Steel & Torrie, 1980).

The rate of cross-fertilization in 2009 ranged from 0.0 to 58.3% at the single-head level and from 1.1 to 30.6% at the single-plant level, averaging 5.7%. Only four plants out of 36 showed cross-fertilization above 10%, with values of 10.6, 12.5, 20.8, and 30.6%. Oleic acid content in the plant with the highest cross-fertilization rate averaged 64.2%, compared to 82.7% in self-fertilized plants of CR-6. The rate of cross-fertilization in the winter-sown experiment of 2010 ranged from 0.5 to 35.9%, with an average of 12.1%. In the spring experiment of 2010, cross-fertilization ranged from 1.0 to 34.9%, averaging 13.2%. In both experiments conducted in 2010, most of the plants showed a cross-fertilization rate above 10%, 22 out 36 in the winter experiment and 24 out 36 in the spring experiment. Tests for homogeneity of individual progenies conducted in the three experiments concluded that the progenies were not homogeneous for the rate of cross-fertilization. Chi-square values were 217.6 ($p < 0.01$) in 2009, 64.5 ($p < 0.01$) in the winter of 2010, and 51.1 ($p < 0.01$) in the spring of 2010.

In 2009, some plants of cv. Rancho were affected by wilt disease apparently caused by *Fusarium oxysporum*, which resulted in the lost of 32 plants out of 382 before flowering. Accordingly, there was some variation in the actual number of plants surrounding each CR-6 plant at the time of flowering, ranging from 19 to 24. The correlation coefficient between the rate of cross-fertilization and the number of Rancho plants was not significant ($r = 0.30$, $p > 0.05$), suggesting that the lost of plants in the experiment did not affect the results. No plant losses due to diseases occurred in the 2010 experiments.

The extent of cross-fertilization in safflower depends upon genotypes and environmental conditions (McPherson *et al.*, 2009). The present study was conducted in a typical Mediterranean location using two cultivars adapted to this environment. The results showed average outcrossing rates between 5.7 and 13.2% for a minimum distance of pollinizer plants of 1 m, which are close to the typical levels reported for this species (Knowles, 1989). These are the maximum expected outcrossing frequencies in these conditions, as longer distances are expected to reduce outcrossing frequencies (Rudolphi *et al.*, 2008; McPherson *et al.*, 2009). We also observed large variation between single plants and also between single heads within the same plant, which is in agreement with the results of McPherson *et al.* (2009), who also reported spatial heterogeneity for the outcrossing frequency in safflower.

Safflower is a promising oilseed crop for the Mediterranan area. Several traits have been delevoped for the

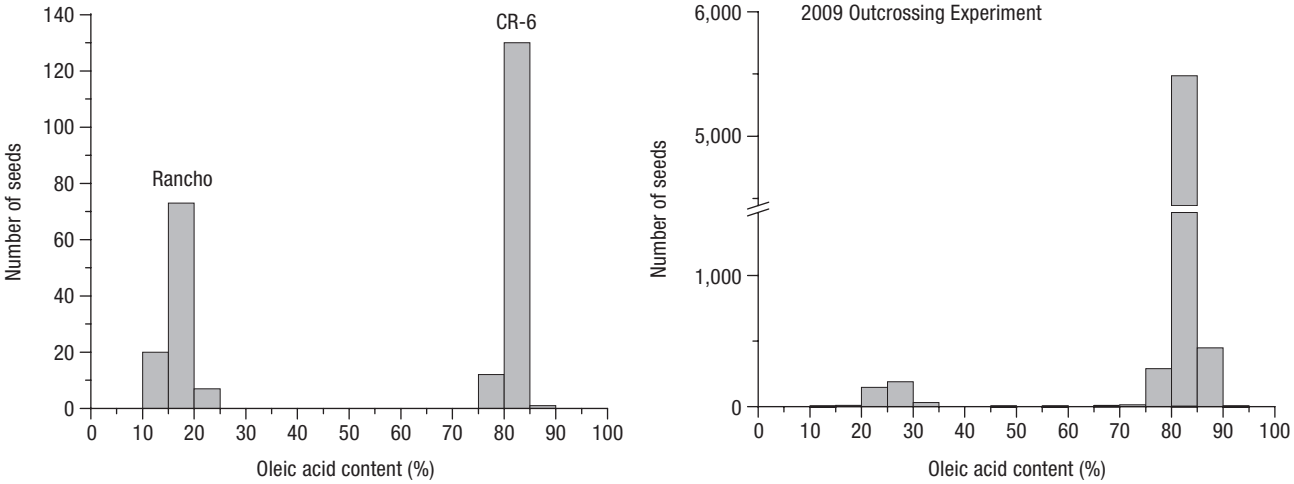

Figure 2. Histograms of oleic acid content (% of total fatty acids) in seeds of cultivars Rancho and CR-6, produced under self-fertilization conditions, and seeds of CR-6 plants from the 2009 outcrossing experiment shown in Fig. 1.

production of specialty safflower oils such as very high linoleic acid content (Futehally & Knowles, 1981), very high oleic acid content (Hamdan *et al.*, 2009a), high saturated fatty acid content (Hamdan *et al.*, 2009b), and high gamma-tocopherol content (Velasco *et al.*, 2005b). In all cases the modified traits are recessive and controlled by the genotype of the developing embryo, therefore being affected by the presence of foreign pollen (Velasco *et al.*, 2005a). Our results indicated low outcrossing frequencies at short distances without contact between adjacent plants. This anticipates no need for large isolation distances between conventional cultivars and cultivars with special oil characteristics, which represents and important advantage in comparison with allogamous crops such as sunflower. However, particular care should be taken for cultivation of transgenic safflower, currently used as a platform for production of high-value proteins (Nykiforuk *et al.,* 2011), in areas of non-transgenic safflower cultivation or wild *Carthamus* species distribution.

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