

BRIEF COMMUNICATION

Extent of cross-fertilization in *Orobanche cumana* Wallr.M.I. RODRÍGUEZ-OJEDA¹, J.M. FERNÁNDEZ-MARTÍNEZ², L. VELASCO², and B. PÉREZ-VICH^{2*}*Koiposol Semillas S.A. Avda. San Francisco Javier 24-7, E-41018 Sevilla, Spain¹**Instituto de Agricultura Sostenible (IAS-CSIC), Alameda del Obispo s/n, E-14004 Córdoba, Spain²***Abstract**

Sunflower broomrape (*Orobanche cumana* Wallr.) is considered a self-fertilizing species, but there is no indication as to whether it is strictly self-fertilized or that it presents some extent of cross-fertilization. The objective of this research was to measure the rate of cross-fertilization in *O. cumana* using an unpigmented recessive mutant as a visual marker. A pot and a field experiment in which single unpigmented plants were surrounded by a large number of pigmented plants were conducted. Occurrence of F₁ hybrids, readily distinguishable from unpigmented plants in the progenies of unpigmented plants provided a direct measurement of the cross-fertilization rate. Progenies of unpigmented plants contained 21.5 % of F₁ hybrids in the pot experiment and 28.8 % in the field experiment. The results revealed that *O. cumana* is a partially allogamous species, which has great relevance for understanding the genetic structure and dynamics of populations and, ultimately, race evolution in this parasitic plant.

Additional key words: allogamy, anthocyaninless, autogamy, sunflower broomrape, unpigmented plants.

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant that constrains sunflower production in many countries around the world (Škorić *et al.* 2010). A particularity of *O. cumana* parasitisation of sunflower is a clear differentiation of parasitic races and a host-parasite interaction that generally fits the gene-for-gene model which does not occur in other crop/*Orobanche* associations (Pérez-de-Luque *et al.* 2009). In recent years, the appearance of new *O. cumana* races has jeopardized sunflower production in large areas of Spain, Romania, Turkey, Ukraine, and Russia (Fernández-Martínez *et al.* 2009). Understanding race evolution in *O. cumana* would require a previous knowledge of the genetic structure and dynamics of *O. cumana* populations.

The mating system is a major determinant of the genetic structure of populations (Lloyd and Schoen 1992, Charlesworth 2003). Most *Orobanche* species are pollinated by insects, particularly bumblebees and bees, but some species are self-fertilizing (Musselman *et al.* 1981, Kreutz 1995). Their mating systems are commonly correlated with flower morphology; whereas some self-fertilizing species such as *O. cumana* develop bent tubular corollas with small lower lips that may not

facilitate pollinator landing, other species like *O. crenata* Forssk. and *O. foetida* Poir. have large lower lips that serve as landing platforms for pollinating insects (Satovic *et al.* 2009). A molecular study on intra- and inter-population genetic variation in eight European populations of *O. cumana* parasitizing sunflower identifies low intra-population and large inter-population genetic variations which pointed to a self-fertilizing mating system (Gagne *et al.* 1998).

Despite the above mentioned studies, which suggest that *O. cumana* is primarily a self-fertilizing species, no studies to measure the rate of cross-fertilization have been undertaken. The objective of this research was to measure the rate of cross-fertilization in *O. cumana* using an unpigmented recessive mutant as a visual marker.

Two experiments to evaluate the cross-pollination rate were conducted in Córdoba, Spain in 2008, one of them in pots and the other one in a field. *O. cumana* lines EK-12, developed by inbreeding a population collected in Écija, Spain, and EK-A1, developed by inbreeding a single unpigmented plant lacking anthocyanin (Rodríguez-Ojeda *et al.* 2011), were used. The unpigmented plant trait is determined by partially recessive alleles at a single locus in such a way that

Received 5 June 2012, accepted 15 October 2012.

Acknowledgements: The research was funded by Fundación Ramón Areces, Madrid, Spain. The contribution of Dr. Enrique Quesada Moraga, entomologist from the University of Córdoba, Spain, to taxonomic classification of pollinators is gratefully acknowledged.

* Corresponding author; fax: (+34) 957 499252, e-mail: bperez@ias.csic.es

heterozygotes $P_g p_g$ show greenish stems that are easily distinguishable from homozygotes $P_g P_g$ with bluish-violet stems and $p_g p_g$, with yellow stems (Rodríguez-Ojeda *et al.* 2011).

Seeds of the confectionery sunflower (*Helianthus annuus* L.) line B-117, susceptible to all tested *O. cumana* populations, were planted in small pots (7 × 7 × 7 cm) containing a mixture of sand and peat (1:1, v/v). The mixture (approximately 330 cm³) had been previously carefully mixed with 25 mg of broomrape seeds of either EK-12 or EK-A1 lines to obtain a homogeneously infested substrate. In total, 498 pots were inoculated with EK-12 and 140 pots with EK-A1. The plants were kept in a growth chamber for 15 - 20 d at day/night temperatures of 25/18 °C, air humidity of 55 %, and a 14-h photoperiod with irradiance of 300 μmol m⁻² s⁻¹. After this time, about half of the plants were used for the pot experiment and the other half were used for the field experiment. Additionally, 96 pots containing soil inoculated with EK-12 and 96 pots containing soil inoculated with EK-A1 were prepared as described above for seed multiplication.

In the pot experiment, the plants were transplanted into 3500-cm³ plastic pots filled with uninfested sand-silt-peat (2:1:1; v/v/v) mixture. Pots inoculated with EK-12 and EK-A1 were arranged in five rows of 35 pots each in such a way that every EK-A1 pot was surrounded by eight EK-12 pots. The pots were maintained under open air conditions, *i.e.*, they were placed on the ground with no mesh cover. Shoots of EK-A1 were removed periodically before flowering, usually at the stage of 3 - 4 cm height to leave one single shoot per pot in order to avoid cross-fertilization between neighbouring unpigmented plants. Shoots of EK-12 were not removed. The number of EK-12 shoots per pot was recorded. Sunflower plants used for seed multiplication of lines EK-12 and EK-A1 were transplanted into plastic pots as described above and maintained under open air conditions in an area separated from the outcrossing experiment. Individual plants of EK-12 and EK-A1 were bagged before flowering (Rodríguez-Ojeda *et al.* 2010).

In the field experiment, the sunflower plants were transplanted into the field at the experimental farm of the Institute for Sustainable Agriculture, Córdoba, in 17 rows 5 m long with a distance of 1 m between rows and 25 cm between plants in the row. Plants grown in soil inoculated with EK-12 and EK-A1 were arranged in a similar pattern to that in the pot experiment, *i.e.*, sunflower plants inoculated with EK-A1 were surrounded by eight plants inoculated with EK-12. Shoots of EK-A1 were removed periodically as described for the pot experiment to leave one single shoot per sunflower plant. Shoots of EK-12 were not removed. The number of EK-12 shoots per sunflower plant was recorded.

Plants of EK-A1 from both the pot and field experiments were bagged around 2 weeks after the end of flowering to avoid seed losses. At maturity, the bags were cut and the seeds of each plant were threshed separately. Seeds from 15 EK-A1 plants, seven from the pot experi-

ment and eight from the field experiment, produced sufficient amounts of seed for evaluation of progenies. For this purpose, seed from each progeny was used to inoculate soil as described above, and this soil was used for filling 18 small pots in which seeds of sunflower line B117 were sown. Eighteen pots containing soil inoculated with seeds of EK-12 and 18 pots containing soil inoculated with seeds of EK-A1 from the previous-year seed multiplication were prepared as a control. The plants were transplanted into larger pots with the same soil and maintained under open air conditions. After broomrape emergence, the number of shoots with yellow or greenish stems was recorded periodically. Plants of EK-12 and EK-A1 were used as a visual control. They bred true for plant colour, *i.e.*, all plants of EK-12 were pigmented (bluish-violet stems) and all plants of EK-A1 were unpigmented (yellow stems). The rate of cross-fertilization was calculated as the percentage of broomrape shoots with greenish stems in the progenies of EK-A1 plants. It is important to note that plant pigmentation has no effect on parasitism as revealed in a previous study (Rodríguez-Ojeda *et al.* 2011). A χ^2 -test for homogeneity was conducted within each experiment (Steel and Torrie 1980).

Table 1. Number of yellow and greenish broomrape shoots and percentage of greenish ones obtained in the evaluation of the progenies of individual yellow-stemmed plants of line EK-A1 under open-pollination conditions in pot and field experiments.

Exp.	EK-A1	Number of progenies yellow	greenish	[%]	χ^2 (P)
Pots	1	21	7	25.0	26.2 (<0.01)
	2	40	21	34.4	
	3	65	34	34.3	
	4	218	41	15.8	
	5	36	14	28.0	
	6	27	5	15.6	
	7	109	19	14.8	
	total	516	141	21.5	
Field	1	34	14	29.2	13.6 (0.06)
	2	16	3	15.8	
	3	56	11	16.4	
	4	18	5	21.7	
	5	36	24	40.0	
	6	27	11	28.9	
	7	14	6	30.0	
	8	27	18	40.0	
	total	228	92	28.8	

The average number of bluish-violet shoots per sunflower plant in the pot experiment was 17.7 ± 13.9 (mean \pm SD). Evaluation of progenies of seven unpigmented plants revealed that between 14.8 and 34.4 % of the progeny shoots had greenish stems, *i.e.*, they were F₁ plants derived from hybrid seeds produced by cross-fertilization with pollen of bluish-violet plants (Table 1). The progenies were not homogeneous

($\chi^2 = 26.2$, $P < 0.01$) for the cross-fertilization rate. The sum of the seven progenies resulted in 516 yellow shoots (S_1 plants) and 141 greenish shoots (F_1 plants) indicating an average occurrence of 21.5 % cross-fertilization in the conditions of the pot experiment.

The average number of bluish-violet broomrape shoots per sunflower plant was 21.1 ± 14.7 in the field experiment. Evaluation of stem colour of the progenies of 8 unpigmented mutants revealed that between 15.8 and 40.0 % of the progeny shoots had greenish stems (Table 1). Homogeneity of the progenies ($\chi^2 = 13.6$, $P = 0.06$) was indicated. The sum of the 8 progenies resulted in 228 yellow shoots and 92 greenish shoots indicating an average cross-fertilization of 28.8 % in the field experiment.

These results suggest that *O. cumana* is a partially allogamous species. The observed rates of cross-fertilization were only applicable to the plant material, experimental design, and environments under which the experiments were conducted, as a number of studies have suggested that there is both temporal and spatial variation in the rate of cross-pollination in partially allogamous plants (Suso and Moreno 1999). However, the results of this research clearly indicate that *O. cumana* is not a strictly self-pollinated species and some extent of cross-fertilization should be expected. Previous studies based on flower morphology (Satovic *et al.* 2009) and genetic structure of populations (Gagne *et al.* 1989) suggest that *O. cumana* is a self-pollinating species. There was a large variation for the rate of cross-fertilization among progenies in the two environments of this study, particularly in the pot experiment. Spatial heterogeneity for the rate of cross-fertilization has been reported in other partially allogamous species such as safflower (McPherson *et al.* 2009).

Even though the objective of the research was not to determine the agents of cross-pollination in *O. cumana*, which, on the other hand, would have required specific additional experiments, we observed the presence of insects visiting *O. cumana* flowers. They corresponded to small to moderate-sized specimens of *Hymenoptera* with a length from around 3 to 9 mm. Fig. 1 shows a small specimen (5 mm length) of the *Halictidae* family captured inside a flower of an unpigmented *O. cumana* plant in the course of the field experiment. It is important to note that species of *Halictidae* are known as pollinators as well as pollen feeders (Armbruster and Baldwin 1998). Nevertheless, it could not be checked whether putative pollinators carried *O. cumana* pollen. Full demonstration of the role of insects on *O. cumana* cross pollination will require additional insect capture and confirmation that

they actually carry pollen of this species.

Flowering at the host species and the parasite overlapped under the conditions of the experiments. Sunflowers are well known as excellent plants for attracting pollinators (Jones and Gillett 2005). The direction in which sunflower attractiveness to pollinators may have influenced the results of the present research is unknown. Studies in the host-parasite system *Centaurea scabiosa-Orobancha elatior* found that the bumblebee pollinator *Bombus pascuorum* only rarely moved between inflorescences of the host and the parasite and therefore the presence of one plant was unlikely to be facilitating pollination in the other (Ollerton *et al.* 2007). But, on the other hand, competition between the host and the parasite species for pollinators may be important if flowering overlap and pollinators are a limiting resource (Ollerton *et al.* 2007).



Fig. 1. Putative pollinator of the *Halictidae* family (*Hymenoptera*) captured inside a flower of an unpigmented *Orobancha cumana* plant.

Investigating genetic diversity within and among populations is useful for understanding the evolutionary potential of a species. The mating system greatly influences the amount and partitioning of genetic diversity within and among populations (Lloyd and Schoen 1992, Charlesworth 2003). The existence of a significant rate of outcrossing in *O. cumana* implies the possibility of gene flow between plants and subsequently genetic recombination of avirulence genes which might contribute to the complex racial status of this species.

References

- Armbruster, W.S., Baldwin, B.G.: Switch from specialized to generalized pollination. - *Nature* **394**: 632, 1998.
- Charlesworth, D: Effects of inbreeding on the genetic diversity of populations. - *Phil. Trans. roy. Soc. B* **358**: 1051-1070, 2003.
- Fernández-Martínez, J.M., Domínguez, J., Pérez-Vich, B., Velasco, L.: Current research strategies for sunflower broomrape control in Spain. - *Helia* **32**: 73-84, 2009.

- Gagne, G., Roeckel-Drevet, P., Grezes-Besset, B., Shindrova, P., Ivanov, P., Grand-Ravel, C., Vear, F., Tourvieille de Labrouhe, D., Charmet, G., Nicolas, P.: Study of the variability and evolution of *Orobanche cumana* populations infesting sunflower in different European countries. - *Theor. appl. Genet.* **96**: 1216-1222, 1998.
- Jones, G.A., Gillett, J.L.: Intercropping with sunflowers to attract beneficial insects in organic agriculture. - *Florida Entomol.* **88**: 91-96, 2005.
- Kreutz, C.A.J.: *Orobanche*: the European Broomrape Species. I. Central and Northern Europe. - Stichting Naturpublicaties Limburg, Maastricht 1995.
- Lloyd, D.G., Schoen, D.J.: Self- and cross-fertilization in plants. I. Functional dimensions. - *Int. J. Plant Sci.* **153**: 358-369, 1992.
- McPherson, M.A., Good, A.G., Topinka, A.K.C., Yang, R.C., McKenzie, R.H., Cathcart, R.J., Christianson, J.A., Strobeck, C., Hall, L.M.: Pollen-mediated gene flow from transgenic safflower (*Carthamus tinctorius* L.) intended for plant molecular farming to conventional safflower. - *Environ. Biosafety Res.* **8**: 19-32, 2009.
- Musselman, L.J., Parker, C., Dixon, N.: Notes on the autogamy and flower structure in agronomically important species of *Striga* (Scrophulariaceae) and *Orobanche* (Orobanchaceae). - *Beitr. Biol. Pflanz.* **56**: 329-343, 1981.
- Ollerton, J., Stott, A., Allnut, E., Shove, S., Taylor, C., Lamborn, E.: Pollination niche overlap between a parasitic plant and its host. - *Oecologia* **151**: 473-85, 2007.
- Pérez-de-Luque, A., Fondevilla, S., Pérez-Vich, B., Aly, R., Thoiron, S., Simier, P., Castillejo, M.A., Fernández-Martínez, J.M., J Jorrín, J., Rubiales, D., Delavault, P.: Understanding *Orobanche* and *Phelipanche*-host plant interaction and developing resistance. - *Weed Res.* **49** (S1): 8-22, 2009.
- Rodríguez-Ojeda, M.I., Pérez-Vich, B., Alonso, L.C., Fernández-Escobar, J.: The influence of flowering plant isolation on seed production and seed quality in *Orobanche cumana*. - *Weed Res.* **50**: 515-518, 2010.
- Rodríguez-Ojeda, M.I., Velasco, L., Alonso, L.C., Fernández-Escobar, J., Pérez-Vich, B.: Inheritance of the unpigmented plant trait in *Orobanche cumana*. - *Weed Res.* **51**: 151-156, 2011.
- Satovic, Z., Joel, D.M., Rubiales, D., Cubero, J.I., Román, B.: Population genetics in weedy species of *Orobanche*. - *Aust. Plant Pathol.* **38**: 228-234, 2009.
- Škorić, D., Păcureanu-Joița, M., Sava, E. Sunflower breeding for resistance to broomrape (*Orobanche cumana* Wallr.). - *An. INCDA Fundulea* **78**: 63-79, 2010.
- Steel, R.G.D., Torrie, J.H.: *Principles and Procedures of Statistics: a Biometrical Approach*. - McGraw-Hill, New York, 1980.
- Suso, M.J., Moreno, M.T.: Variation in outcrossing rate and genetic structure on six cultivars of *Vicia faba* L. as affected by geographic location and year. - *Plant Breed.* **118**: 347-350, 1999.