

## Weedy sunflowers in France: Prevalence and first inferences on their origin

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### ABSTRACT

Sunflower is not a native of Europe. Weedy forms morphologically close to American wild *Helianthus annuus* have, however, been observed in France since 2004. In order to describe the situation and test hypotheses regarding their origin and evolution, we surveyed the infestation of sunflower fields by weedy sunflower in the Lauragais region (South of France) from 2005 to 2007, and described more precisely five weedy populations using morphological, agronomic and genetic descriptors. Weedy sunflowers affected around 15% of sunflower fields and caused yield losses that reached 50% in strongly infested patches. The five weedy populations surveyed were composed of a wide diversity of morphotypes showing an association of wild and domesticated traits in proportions that differed between populations. The genetic diversity was stronger in weedy populations than in volunteer populations and than in a pool of representative conventional and ornamental varieties. A wide diversity of original alleles was detected and their frequency varied between populations. All weedy plants carried the PET1-cytoplasm conferring male-sterility. These results suggest that weedy sunflowers may have arisen through the hybridization of cultivated and wild sunflower, either during the creation of improved inbred lines or during the seed production process; precursors of the weeds would have then been introduced through the seed lots. Until now, the only available methods to control these weeds are mechanical weeding at the very beginning of the infestation of a field. Studies are underway to get a better understanding of the formation and the spread of weedy populations in the agro-ecosystem.

**Key words:** genetic diversity – microsatellites – weeds – wild hybridization.

### INTRODUCTION

Wild *Helianthus annuus* is a native of North America and does not occur naturally in Europe. However, weedy forms, showing morphological traits of wild sunflower (e.g. branching, anthocyanins in stem and disk, seed shattering) have been observed for decades in and around agricultural fields in Spain, Italy and Central Europe (Faure et al., 2002; Holec et al., 2005). They can compete with crops and are thus potentially problematic for cultivation. In North America, where weedy sunflowers also occur, they can decrease substantially yields of corn and soybean (Kane and Rieseberg, 2008). Moreover, the potential intercrossing between cultivated and weedy sunflower raises the question of the transfer of advantageous crop traits to the weeds (Mercer et al., 2007), which can contribute to the evolution of more aggressive weeds (case of Johnsongrass; Morrell et al., 2005). In 2004, patches of weedy sunflower within sunflower fields were officially observed for the first time in France, in the East of Toulouse. Interviews with farmers and a first survey revealed that they occurred in a consequent number of fields and in some cases for more than 10 years (Muller et al., 2006). However, they seemed to occur almost only within sunflower fields, and no other crops were strongly concerned. The main questions raised by these observations were: (i) what is the origin of the French weedy populations: are they issued from the reversion of the crop to weedy forms, through the evolution of volunteers, or from the introduction of original wild forms or crop-wild hybrids? (ii) What are the prevalence and dynamics of weedy sunflower in the agro-ecosystem in France? (iii) What impact do these weedy forms have on sunflower cultivation and how can they be controlled? To start answering these questions and make a first description of weedy sunflowers in France, we conducted studies across the agro-ecosystem and within cultivated fields, using morphological traits, and molecular markers.

### MATERIALS AND METHODS

During summers 2005, 2006 and 2007, we followed the same roads every year over an area of approximately 1200 km<sup>2</sup> in the East of Toulouse, a region called Lauragais. Along these roads we

observed every sunflower field and scored the infestation level on the following scale: 0 (no weedy sunflower), 1 (less than 10 plants), 2 (a few patches) and 3 (very infested).

In summer 2006, we surveyed in detail 5 sunflower fields. These fields were selected at the beginning of July, as they showed a strong infestation with weeds (level 3). In 4 of these fields, an experiment was conducted to estimate the impact of weeds on yield. Weeds were manually destroyed on 6 rows of cultivated sunflower (weeded - 30m<sup>2</sup>) and the density of weeds in an adjacent area of the same size was estimated (infested). At the end of season, crop heads were manually harvested on the weeded and the infested plots, on 24m<sup>2</sup> only to avoid border effect. The seed production, seed weight and oil content of the crop were determined.

On 18 and 25<sup>th</sup> July, during the flowering time period of the weeds, 30 branched weeds were randomly chosen on each of the 5 fields. The following traits were scored: male-sterility and anthocyanin pigmentation of petiole, disk and stem. One head per plant was bagged before the opening of the first flower. At the end of August, bagged heads were collected, and on each of the surveyed plants, at least one open-pollinated head was collected for DNA analysis. The number of seeds produced by the bagged head was determined in the laboratory. A plant was scored as self-incompatible if less than 5 seeds had been produced, and as self-compatible in the other case.

In spring 2006, 6 open-pollinated seeds (achenes) per maternal plant were placed on filter paper and soaked in tap water. After 3 days at 25°C, the number of germinated seeds was counted. If no seed had germinated in a maternal family, we removed the seed coat and allowed two more days to germinate. We scored the need to remove the seed coat to obtain germination of at least one seed per maternal plant.

As a whole, 6 traits had been scored. For each trait and each population, the frequency of the wild type (e.g. anthocyanins in 3 locations, self-incompatibility and seed-coat removing) was computed. For male-sterility, we computed the frequency of male-sterile plants. The significance of differences in frequency across the set of populations was tested with a chi-square test on contingency tables. The correlation between traits within each population was investigated with exact test on contingency table. All tests were performed with SAS software (SAS Institute, Cary, NC, USA).

For molecular analyses, in addition to the 5 weedy populations described above (one open-pollinated seed per maternal family), we also included (i) two natural volunteer populations, one located in the Lauragais (FR006) and the other in the Gard (FR001). These populations occurred in fallows and exhibited only domesticated traits. Sampling was made of open-pollinated seeds taken from distinct maternal plants (9 for FR006, 44 for FR001). (ii) 18 conventional F<sub>1</sub>-hybrid varieties historically or currently grown in the Lauragais region (iii) 6 ornamental varieties. Between 3 and 5 seeds per variety were analyzed. DNA was isolated from 100mg of young leaves with the standard protocol of Dneasy kit (Qiagen). Thirteen microsatellite markers from Tang et al. (2002) were used. The amplification reaction consisted of 50ng DNA, 4pmol of unlabelled reverse primer, 2pmol of forward primer, fluorescently labelled with NED, HEX or FAM, 1x reaction buffer, 2 mM MgCl<sub>2</sub>, 200μM dNTP, 0.15U Taq DNA polymerase, in a total volume of 25μL. The amplification method was as follows: 95°C for 2 minutes, 36 cycles of 94°C for 30s, Tx for 30s (Tx is initially 63°C and decreases of 1°C per cycle for the 6 first cycles, until it reaches 57°C), and 72°C for 45s; followed by a final extension for 20 minutes at 72°C. Amplification products were analyzed on an ABI 3130xl Genetic analyzer. The GENEMAPPER (Applied Biosystems) software was used to score the genotypes.

Standard statistics of genetic diversity were computed on groups of populations and per population: the number of detected alleles (*A*), unbiased genetic diversity *H<sub>e</sub>* (Nei, 1987) and allelic richness (number of alleles standardized for the same sample size for each population, Petit et al. 1998), using the program FSTAT (Goudet, 2001). For each natural population (weeds and volunteers), the frequency of original alleles (e.g. absent from the conventional varieties) was computed.

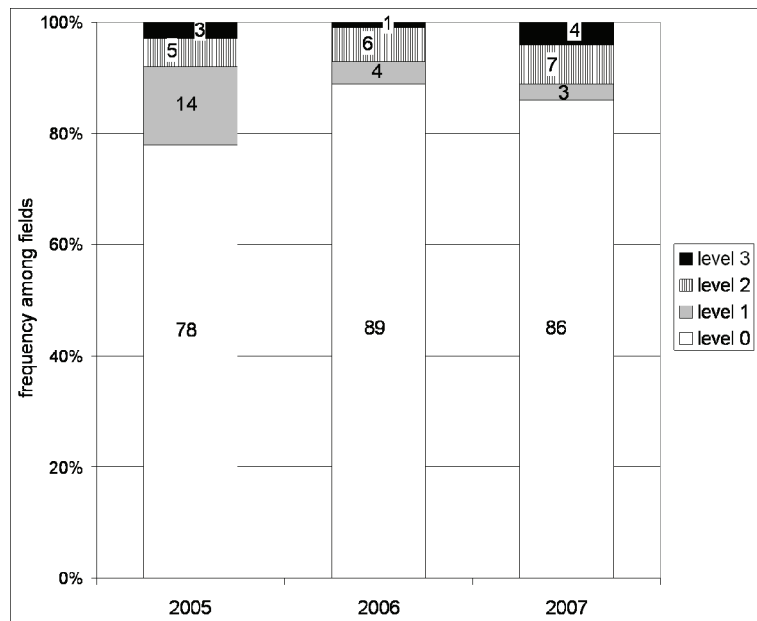
Additionally, we used a polymerase chain reaction-based marker system that identifies sunflower plants carrying the mitochondrial DNA conferring male-sterility (PET1). PET1 is found in all male-sterile plants used for F<sub>1</sub>-hybrid seed production and thus in all F<sub>1</sub>-hybrid plants; it has been introduced from *H. petiolaris* and is theoretically absent from wild *H. annuus* (Rieseberg et al., 1994). We applied the protocol described in Rieseberg et al. (1994).

## RESULTS

### *Field surveys over 3 years*

In the Lauragais region, the frequency of sunflower fields infested with weedy sunflower varies between 22% in 2005 to 11% in 2006 (Fig. 1.). As the standard rotation in Lauragais is sunflower-wheat, comparison between 2005 and 2007 deals roughly with the same sunflower fields. However in 2005, the

frequency of fields with a low level of infestation seems to be an overestimation. Indeed, in this first year of the survey, sporadic volunteers may have been confused with weedy sunflower. Considering this, the prevalence of infested fields seems to have been stable. Between 1 and 4% of the fields were strongly infested. In these cases, the density of weeds could reach 15 plants/m<sup>2</sup> over some patches (Table 1).



**Fig. 1.** Frequency of fields showing the different levels of infestation. The total number of fields surveyed for 2005, 2006 and 2007 were, respectively, 231, 227 and 196.

#### Impact on yield

High densities of weedy sunflowers greatly decreased crop yield (Table 1): Losses can reach more than 50%. These losses are due to a reduction in seed number and seed weight. But the seed oil content was unaffected. Visually, weedy sunflowers largely overtopped the crop. In strongly infested fields, harvest became impossible and some farmers have given up sunflower cultivation.

**Table 1.** Measures of the impact of weeds on sunflower cultivation.

Population	Weed density infested plot (plants/m <sup>2</sup> )	Yield infested plot (t/ha)	Yield weeded plot (t/ha)	Sw <sup>1</sup> Infested plot (g)	Sw <sup>1</sup> weeded plot (g)
Baziège	12	1.79	2.74	50.9	59.6
Gardouch	12	1.41	2.73	43.8	49.8
Fourquevaux	13	1.04	2.59	30.5	44.4
Odars	15	1.72	2.99	44.7	58.1

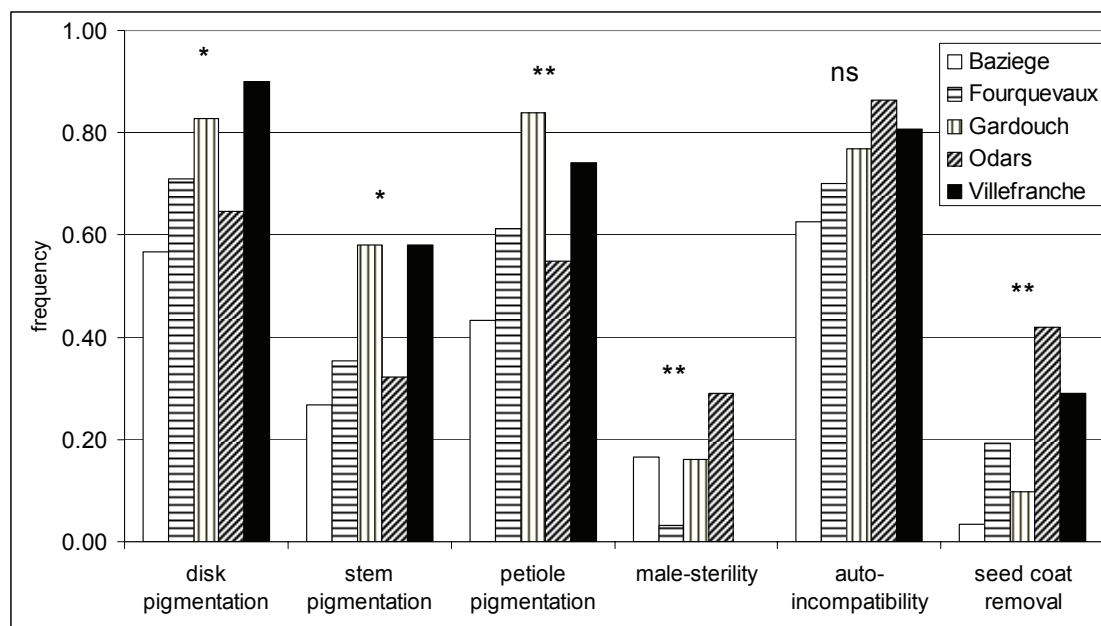
<sup>1</sup>Sw: weight of 1000 achenes

#### Morphological traits

The weedy forms are morphologically clearly different from the volunteers issued from the segregation of hybrid-F<sub>1</sub> varieties. Our observations confirmed that they exhibit traits typical of wild forms of *H. annuus*: pigmentation of disk, stems and petioles, self-incompatibility, and seed dormancy (Fig. 2). A trait more typical of a cultivated variety, although sometimes observed in wild populations, male-sterility, was also observed at a varying rate in the weedy populations. For 5 out of 6 traits, the frequency varied significantly between populations (chi-square). These variations were correlated between traits, namely Baziège was the population with the lowest frequency of wild-like traits for all traits, whereas Villefranche was the population with most wild phenotypes. This tendency was also observed for less quantifiable traits such as seed shattering, or amount of branching.

Significant correlations between traits were only observed between the scoring of anthocyanins in different locations (stem, petioles and disk). No other correlation was detected, confirming the visual observation that all kinds of combination of traits were present in the fields. Weedy populations are

characterized by a high morphological diversity with plants combining in different proportions domesticated and wild traits, from typical F2 plants to typical wild-like phenotypes.



**Fig. 2.** Frequency of phenotypic traits in the different populations. Significance of frequency differences between populations: \*:  $P < 0.05$  ; \*\*:  $P < 0.01$  ; ns: not significant

The flowering time periods of cultivated and weedy sunflowers overlap, the first weeds starting flowering during the flowering of the crop. But the flowering time period of the weeds extends almost until harvest time.

#### Genetic analyses

The total genetic diversity was much greater over the weedy populations than in all the other groups analyzed: 137 different alleles were detected over 13 loci in the weedy populations, compared to 45 in the volunteer populations, 50 over the conventional varieties and 82 over the ornamental varieties. Many alleles were thus detected in the weeds, that were absent from the varieties. We call these alleles “original alleles” in the following. This pattern of genetic diversity is similar when comparing intrapopulation diversity between weedy and volunteer populations (Table 2).

Original alleles are relatively rare: their frequency within weedy populations varies from 7 to 23% (Table 2). The main alleles detected in the weeds are thus shared with the conventional varieties, and apart from the original alleles, the genetic composition of the weedy populations is rather similar to the pool of conventional varieties analyzed. Interestingly, in Villefranche, the population showing the most originality at the molecular level was the one that also exhibits more wild-like phenotypes, whereas in Baziège, showing the lowest frequency of wild-like phenotypes, it had a genotypic composition closer to that of the cultivated pool (Fig. 2, Table 2).

All weedy plants, conventional variety and volunteers analyzed carry the PET1-cytoplasm. Except one (SunrichF1), all ornamental varieties carried the fertile cytoplasm.

**Table 2.** Genetic diversity statistics within 5 weedy populations and 2 volunteer populations.

Accession	Baziège	Fourquevaux	Gardouch	Odars	Villefranche	FR001	FR006
Sample size	30	31	31	31	31	44	9
$H_e$	0.504	0.568	0.602	0.633	0.680	0.466	0.410
Allelic richness	3.34	3.97	3.89	4.21	4.81	2.36	2.43
Frequency of original alleles	0.072	0.127	0.163	0.165	0.232	0.002	0.009

#### DISCUSSION

This first description of weedy sunflower in France shows that it is a non negligible phenomenon. It affects around 15% of fields in the Lauragais region, and has also been observed in other areas in the South-West and West of France (unpublished results). Weedy plants can reach a locally high density and compete strongly with the crop leading to over 50% of loss of yield. Oleic acid content for high oleic varieties had also been shown to be affected when the field was strongly infested. This decrease was not detected at the single crop head level but on a sample of the whole harvest. It was thus probably due to the mixing of crop and weed seeds during harvest rather than to pollination by weed pollen (unpublished results). Seeds are dormant and can stay in the seed bank for many years (Alexander and Schrag, 2003). Some farmers have given up sunflower cultivation in strongly infested fields. Weedy sunflowers have also been observed in other spring crops such as sorghum, but never in winter crops such as wheat or oilseed rape.

Preliminary observations and interviews of farmers have revealed that the infestation level within a field generally increases from one generation to the other; this is due to the high seed production of weeds and to the fact that seeds are dispersed at maturity and fall readily on the soil compared to the seeds produced by the crop. By contrast, the dispersion of weeds to adjacent fields seems more restricted: quite often, a sharp contrast is observed between a strongly infested field and a neighbouring perfectly clean one. This suggests that seed and pollen flow from field to field is either rare or not sufficiently efficient compared to the spread of a weedy population within a given field. A more thorough survey of the infestation of a network of fields over time is underway: the results after a few years may give precise answers to the potential spread of weedy populations across the agro-ecosystem.

Morphologic and genetic data suggest that the origin of these populations is the hybridization between crops and wild plants. To be specific, as all weedy plants analyzed carry the PET1-cytoplasm, they all have in their maternal lineage a cultivated plant. This hybridization may for instance have occurred in seed-producing fields, when wild *H. annuus* occurs at proximity and can pollinate the female line. As the number of original alleles per locus is high, the diversity of wild forms involved in this origin is probably strong (more than a few plants are probably involved). But crop-wild hybridization also occurs in sunflower breeding, when wild genetic resources are used to introduce valuable traits into cultivated lines. The use of lines insufficiently inbred in the F<sub>1</sub>-variety creation process could have led to varieties containing wild genetic variability. Rare remaining wild traits could have constituted a starting point for later weed evolution in a field. More detailed analyses are required to attempt to discriminate between these hypotheses and to infer if weedy sunflower evolved only once or multiple times within a region and over the different French areas.

As the frequency of cultivated-like alleles is strong, and as the combinations of phenotypic characters are important, a lot of crop-weed intercrossing may have occurred in the history of these populations. Further genetic analyses are planned to precisely estimate the rate of crop-weed hybridization within a field and the rate of pollen and seed dispersal between adjacent or more distant fields. Understanding in more details the process of formation and spread of weedy populations both at the field and at the agro-ecosystem levels is important to be able to predict the risk of establishment of new populations.

Methods to control these weeds are currently very limited. As weedy sunflowers affect cultivated sunflower fields, the methods used to control the weed may have an impact on the crop itself. Varieties resistant to herbicides such as imidazolinone and sulfonylurea are currently used in other countries. These herbicides could be used to eradicate weedy sunflowers in fields cultivated with resistant varieties. However, the risk of crop-weed gene flow and of transfer of the resistance to the weed has to be considered (Massinga et al., 2003). The only method that can now be advised to farmers is thus mechanical weeding: as soon as the first weeds are observed on a field, they have to be destroyed, before they produce seeds and give rise to a bigger population which is more difficult to control.

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