EVALUATION OF AN INTERSPECIFIC SUNFLOWER POPULATION ISSUED FROM THE PERENNIAL SPECIES *H. occidentalis* ssp. *plantagineus* FOR RESISTANCE TO *Diaporthe helianthi* AND *Sclerotinia sclerotiorum* IN RELATION WITH PHENOTYPIC AND MOLECULAR TRAITS.

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Résumé

L'introgression à partir des espèces sauvages pérennes du genre *Helianthus* permet d'élargir la base de sélection du tournesol. Nous avons construit une population interspécifique entre le tournesol cultivé et l'espèce sauvage pérenne *Helianthus occidentalis* ssp. *plantagineus*. Trois générations de brassage et de sélection douce ont été réalisées pour favoriser la recombinaison entre génomes et améliorer la population. A partir de 512 plantes autofécondées, 51 S1 ont été choisies en fonction de caractères phénotypiques (hauteur, ramification, teneur en huile, poids de 1000 grains) et de marqueurs RAPD spécifiques du parent sauvage. Les S1 ont été brassées en polycross pour évaluation de la résistance au Phomopsis et au Sclerotinia en infection semi-naturelle dans un réseau multilocal. La population interspécifique manifeste une résistance améliorée au Sclerotinia. Le résultats obtenus pour la résistance au Phomopsis permettent d'approcher le niveau du témoin résistant Agrisol. Des fragments RAPD sauvages sont associés respectivement à ces deux résistances. L'utilisation directe de marqueurs de résistance d'origine sauvage devrait donc permettre d'améliorer le niveau de résistance de la population ainsi que d'autres caractères d'intérêt agronomique.

Summary

Introgression of genetic factors from the perennial wild *Helianthus* species into cultivated sunflower is used to enlarge its genetic basis and to improve agronomic value. An interspecific population involving the cultivated sunflower (*H. annuus*) and the wild perennial species *H. occidentalis* ssp. *plantagineus*, was constructed. It was submitted to 3 generations of inter-crossing to favour genomic recombination and bred to improve *per se* value, through maternal pedigree. Out of 512 selfed plants, fifty one S0 plants were chosen on phenotypic traits (plant height, branching, earliness, oil content, seed size and seed yield) and presence of RAPD molecular markers specific of the parental wild species. After intercrossing, the corresponding S1 polycross progenies were produced and evaluated for *Diaporthe helianthi* (Phomopsis) and S*clerotinia sclerotiorum* resistance, in multilocal trials performed in semi-natural infestation conditions.
Results indicated that head resistance to S*clerotinia sclerotiorum* was transferred into the interspecific population. A significant genetic variation associated positively with specific RAPD fragments from the wild parent was underlined. Regarding Phomopsis resistance, we found some progenies with resistance levels comparable to the resistant control AGRISOL. Molecular data suggest that Phomopsis and Sclerotinia resistance originated from the wild species used in the initial cross.
A relationship was established between disease resistance level or other phenotypic traits and molecular markers. The interest of increasing the frequency of individuals with RAPD fragments specific of the wild perennial species and associated with favourable traits is discussed.

Key words

Sunflower, *Helianthus* ssp *occidentalis*, interspecific hybridization, disease resistance, RAPD markers.

INTRODUCTION

Wild perennial species of section A*trorubentes* represent a large genetic potential to improve the cultivated sunflower for many traits. Screening for broomrape resistance, Ruso et al (1996), have shown that most of the 26 perennial species were immune to the populations of Orobanche. Similarly, resistance to Phomopsis has been reported in *H. tuberosus* (Langar *et al.* 1997) and in interspecific crosses derived from *H. tuberosus* or *H.* x *laetiflorus* (Degener *et al* 1999). Gene transfer in crosses between cultivated sunflower (*H. annuus*) and wild perennial *Helianthus* species have been shown to be not frequent (Cazaux *et al.* 1996), difficult to manage, but efficient to improve disease resistance and several other traits in sunflower. In previous studies on the molecular taxonomy of *Helianthus* genus, Sossey *et al.* (1998, 1999) using RAPD markers indicated that four basic genomes can explain the organization of this genus: **P** genome being common to all perennial species, **A** genome unique to section *Atrorubentes*, **H** genome unique to section *Helianthus* and **C** genome common to all sections. So, availability of such genome-specific RAPD markers provided powerful tools for recognizing genomes which constitute the genus. A simple method could be used to characterize these fragments and to mark the introgressed traits in interspecific progenies. Marker introgressed zones of wild species were assumed to be good "candidates" in order to identify segments carrying the resistance to diseases issued from wild species.

Our goal was to enlarge the genetic basis of cultivated sunflower through introgression of favourable factors originated from the perennial species *H. occidentalis ssp. plantagineus*. In this purpose, an interspecific population was constructed between this wild species and the sunflower. After several generations of intercrossing performed in order to favour genetic recombination, S0 individuals were selected on the basis of both phenotypic traits and RAPD markers specific of the wild parent. S1 polycross seeds were then produced to screen favourable factors to *Sclerotinia sclerotiorum* and to *Diaporthe helianthi* (Phomopsis).

This paper reports the effect of increasing RAPD fragments specific of the wild species, in the population individuals, and the probability of isolation of new favourable introgressed agronomic traits. Our conclusion is that the screening of interspecific progenies requires complementary molecular tools to efficiently eliminate poor material and isolate introgressed plants, before evaluation tests which are costly.

MATERIAL AND METHODS

Plant material

The inbred line *H. annuus* L. Cms-ISS56 used as maternal parent and pollinated with *H. occidentalis* ssp *plantagineus* MPHE 231 produced few interspecific F1 hybrid seeds. One F1 plant backcrossed with *H. annuus* RHA274 inbred line, generated backcross (BC'1) seeds. Three of issued BC'1 plants were used either (1) to pollinate Cms-HA89 or Cms-125 female inbred lines or (2) to receive pollen in a cultivated nursery. All cultivated lines used were susceptible to Phomopsis and Sclerotinia. The BC'1 open pollinated (33 individuals) and the BC'2 (120 individuals) were interpollinated under isolation cage to generate the 85OCC interspecific population. This population was open pollinated through maternal pedigree and after 3 inter-crosses in isolation, we planted a sample of about 1000 plants. Each pedigree was represented by 30 to 60 plants. A low selection pressure (about 30 % eliminated), was applied to improve the main agronomic traits.

Molecular markers

Total DNA for RAPD analyses was isolated from mature leaf tissue according to the method described by Dellaporta et al, (1983). We considered primers amplifying any fragment of the section *Atrorubentes* as indicated Sossey et *al.* (1998, 1999). So, we used nine primers (A05, A11, A12, A14, B15, B18, C04, C15 and C16) corresponding to 17 amplified fragments in *H. occidentalis* sp *plantagineus* MPHE 231 or in perennial *Helianthus* species. In order to reduce the number of RAPD analyses requested (512 per pool), we decided to construct balanced bulks of eight plants. So, these plants were distributed in a cube of 8*8*8 numbers. In this way, all the individuals of one pool will be controlled through 192 amplifications, instead of 512 ones. Since we looked for introgressed RAPD fragments expected to be rare in the pool according to our previous work on *Helianthus* genome (Cazaux *et al.* 1996), it is likely that one fragment has no chance to be present in all the individuals of one bulk. We therefore expected that one introgressed plant should display the RAPD fragment in three bulks (internal positive control) whereas the other bulks did not display it (internal negative control). As

external controls, we used the *H. annuus* FS20-6-2 inbred line and *H. occidentalis* ssp *plantagineus* MPHE 231 to verify the absence or the presence of the introgressed fragment, respectively.

Characterisation of S0 plants and production of S1 polycross

One thousand plants were grown in the experimental field and we characterized a sample of 512 plants for both phenotypic traits and RAPD markers. The following agromorphological traits were recorded: sowing-flowering duration, male fertility, presence of anthocyans on floral disk, plant height, branching intensity (0-3 scale), self fertility, 1000 seed weight and seed oil content. Besides, RAPD characterization was performed, as indicated above, on 512 plants at bud stage. Fifty-one plants were selected mainly for presence of specific perennial or specific *H. occidentalis* ssp *plantagineus* MPHE 231 RAPD markers and -to a less extent- against undesirable traits of the wild (self-incompatibility, extreme branching). All the sampled plants were selfed and the 51 S1 families were increased in isolation to produce the S1 polycross seeds required for agronomic and disease resistance evaluation.

Polycross S1 progenies evaluation for Phomopsis and Sclerotinia resistance

Phomopsis and Sclerotinia resistance was evaluated in field trials with favourable climatic conditions for contamination. Phomopsis inoculum was carried through spreading contaminated stems on the soil and plant infestation was reinforced by supplying weekly irrigation. The percentage of plants with necrotic lesions was then recorded during maturation phase and compared to cultivated controls RIGASOL and AGRISOL for resistant hybrids, or DK3790 and VIKI for susceptible ones.

Sclerotinia evaluation was performed in sclerotia-contaminated fields, with irrigation during the flowering-maturation period. The percentage of plants with necrotic lesions on head was recorded and compared to the cultivated controls FANTASOL (tolerant) and NATIL (susceptible). S1 polycross families were tested in trials, each with two replications of 25 plants and in two locations.

RESULTS

1) Relation between phenotypic traits and RAPD markers in S0 population.

We found 17 RAPD fragments introgressed from the perennial species, in the 85OCC population. These markers displayed relations with different genomes from *Helianthus* genus:

- A05_350, A14_300, A14_900, C15_1000 and C16_700 markers appeared characteristic of the **P** genome (Sossey-Alaoui *et al*., 1998 and 1999),
- A05_650, A05_1400, A14_1400, B07_600, B18_1100 and C04 markers are specific of *H. occidentalis* ssp *plantagineus* MPHE 231,
- A11_1000, C16_800 are specific of the **A** genome (Sossey-Alaoui *et al*., 1998 and 1999) as well as B07_350 (unpublished),
- A12_400 and A12_500, A12_800 are specific of the **C** genome (Sossey-Alaoui *et al*., 1998 and 1999).

The introgression in the 85OCC population of *H. occidentalis* MPHE 231 fragments was limited, since only 25.2 % of the studied plants in the population carry at less one introgressed fragment. A total of 17 RAPD fragments of wild origin were recovered in 99 plants bearing one fragment (among a sample of 512 S0 plants), 21 plants yielded 2 fragments, 8 yielded 3 fragments and 1 yielded 4 fragments. We found a relative large amount of statistical association between markers (Table 1) *i.e.* 8 significant associations with Fisher exact test ($p < 0.05$). No repulsion between markers was detected. Moreover, fragments A05_650, A05_1400, A11_1000, A12_400, A14_300, A14_900, C15_1000, C16_700 and C16_800 appear mutually directly or indirectly associated.

Some significant associations were observed between marker occurrence and qualitative trait (Table 2). A05_350 was strictly associated ($P=0.026$) to cytoplasm male sterility restorer status, A05_1400 was also strongly associated ($p=0.034$) to this status and C04 was strictly linked to anthocyan on head ($P < 0.000$).

Few relations were observed between marker occurrence and quantitative characters (Table 3). We observe that:

- Earliness is positively associated to C16_800 RAPD marker ($P = 0.048$),
- Plant height reduction is also positively linked to A11_1000 ($P = 0.000$),
- Branching intensity is negatively related to the presence of A12_800 marker ($P=0.036$) and
- Seed size reduction (thousand seed weight) is linked to A12_500 RAPD marker ($P=0.040$).

2) Evaluation of the polycross S1 progenies for disease resistance and relation with RAPD markers

Sclerotinia resistance: compared to the resistant control FANTASOL, all fifty one polycross S1 progenies in the interspecific population 85OCC were statistically more susceptible (Table 5). However, some of them (29, 30, 31 and 47) appeared better for Sclerotinia head attack than the susceptible control NATIL. These data clearly suggest that 85OCC population is susceptible to Sclerotinia head attack, nevertheless some resistance factors could be detected within and between families.

Phomopsis resistance: evaluation of percentage of stem attack in field trials indicated that 26 % of the polycross S1 progenies were statistically equivalent to the most resistant cultivated control AGRISOL, whereas all the progenies were better than the most susceptible control DK3790. These results suggest that 85OCC population carry efficient resistance genes to Phomopsis.

Some significant relations between S1 polycross disease resistance and RAPD markers were observed (Table 4):

- Sclerotinia resistance on head was found positively related to A05_650 (P=0.033) and to A12_500 (P= 0.021) RAPD markers.
- Phomopsis resistance was found positively related to A11_1000 (P=0.047) and A14_300 (P=0.050) RAPD markers. Moreover, a significant relation was detected between the number of introgressed markers (from the perennial species) and the rate of resistance to Phomopsis ($P < 0.002$).

DISCUSSION AND CONCLUSION

The introgression in the 85OCC interspecific population of *H. Occidentalis* ssp. *plantagineus* fragments was rather limited, since we observe that only 129 plants (on a total of 512) carry at less one such DNA fragment. Data suggest that strong elimination of wild genome occurred during first backcross generation since the phenotype of the derived BC population was close to cultivated. The light selection pressure applied during the following cycles to favour seed set and reduce undesirable traits (branching, height, oil content, seed size) likely generated an interspecific population with few fragments inherited from the wild parent. However, a striking relation in the partial grouping of 9 fragments out of 17 (table 1) suggest a possibility of transmission of a big fragment than structural rearrangements and selection drive. After several generations of inter-crossing, the interspecific 85OCC population exhibit great genetic variability for many traits inherited from the wild parent: (self sterility, branching, seed size, oil content). In order to favour emergency of useful wild traits in the population, a selection to increase the plants with *H. occidentalis* ssp. *plantagineus* specific RAPD markers was applied, so that the frequency of introgressed fragment per plant increased from 0.33 in the original to 1.11 in the sampled population. As a result, the evaluation performed on the derived S1 progenies, indicated that on the 17 RAPD markers examined, 8 are significantly related to the wild traits (anthocyans, restoration, earliness, plant height, branching) and 4 are positively associated to Phomopsis or Sclerotinia resistance. As suggested by Skoric (1985) and Vranceanu *et al.* (1993) the genetic control of Phomopsis and Sclerotinia resistance involves most likely several genes and another useful conclusion to be drawn, is that even if this introgressed population is not outstanding for Sclerotinia tolerance, specific factors of resistance could be extracted from this population with the help of molecular markers. The molecular data obtained clearly suggest that disease resistance be directly issued from the perennial species *H. occidentalis* ssp. *plantagineus*.

Our experience with perennial species indicate that interspecific hybrid plants of the first generation exhibit generally strong sterility (due to genomic divergence and subsequent chromosomal abnormalities) which makes difficult their use in breeding programs. In our

strategy of perennial variability utilisation we suggest a preliminary recombination step associated with low selection pressure to favour inter-genomic rearrangements and to decrease frequency of undesirable traits (such as sterility, branching, low oil content). In a second step, the identification of specific fragment introgressed in interspecific populations has proved to be useful to increase the frequency of wild traits in the interspecific population and to enhance the probability to detect useful associations with agronomic characters.
This strategy could be compared with Advanced Backcross QTL strategy proposed by Tanksley *et al*. (1996). In the present study, inter-genomic recombination was favoured as well as the detection of recessive genes. This population strategy will possibly yield individuals with longer homozygous segments for the donor wild parent which will result in linkage drag maintain.

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Table 1: Significant relation between marker occurrence (Fisher exact test probability).

Fragments	A05 _350	A05 _650	A05 _1400	A11 _1000	A12 _400	A12 _500	A12 _800	A14 _300	A14 _900	A14 _1400	B07 _350	B07 _600	B18 _1100	C04	C15 _1000	C16 _700	C16 _800
A05_ 350																	
A05_ 650																	
A05_ 1400		.004															
A11_ 1000																	
A12_ 400				.039													
A12_ 500																	
A12_ 800																	
A14_ 300																	
A14_ 900			.037					.008									
A14_ 1400																	
B07_ 350																	
B07_ 600																	
B18_ 1100																	
C04																	
C15_ 1000			.040														
C16_ 700		.023		.016													
C16_ 800					.003												

Table 2. Relation between RAPD fragments and qualitative traits.

Trait:	Female		Hermaphrodite		
RAPD fragment	Number +	Number -	Number +	Number -	Fisher exact test Prob
A05 350	0	132	13	367	0.026
A05 1400	1	131	18	362	0.034

Trait:	Anthocyan on disk flower		Yellow disk flower		
RAPD fragment	Number +	Number -	Number +	Number -	Fisher exact test Prob
C04	9	168	0	335	0.000

Table 3. Relation between RAPD fragments and quantitative traits.

Trait	Plant height					1000 kernel weight				
RAPD	Nb +	Mean +	Nb -	Mean -	Prob.	Nb +	Mean +	Nb -	Mean -	Prob.
A11 1000	2	55	506	120	0.000	-	-	-	-	-
A12 500	-	-	-	-	-	8	39.6	243	51.5	0.038

Trait	Days to flowering					Branching intensity				
RAPD	Nb +	Mean +	Nb-	Mean -	Prob	Nb +	Mean +	Nb-	Mean -	Prob
A12 800	-	-	-	-	-	15	1.07	496	1.78	0.036
C16 800	5	62.6	505	67.8	0.048	-	-	-	-	-

Table 4. Relation between RAPD fragments and disease resistance.

Trait	Sclerotinia					Phomopsis				
RAPD	Nb +	Mean +	Nb -	Mean -	Prob	Nb +	Mean	Nb -	Mean -	Prob
A05 650	2	37.3	47	53.3	0.026	-	-	-	-	-
A11 1000	-	-	-	-	-	2	6.93	47	15.4	0.040
A12 500	5	42.5	44	53.7	0.016	-	-	-	-	-
A14 300	-	-	-	-	-	3	8.17	46	15.5	0.031

Table 5. Evaluation of the S1 progenies for resistance to Phomopsis and Sclerotinia.

S1	Phomopsis	Sclero	**S1**	Phomopsis	Sclero	**S1**	Phomopsis	Sclero
1	9.90	59.36	**18**	13.79	39.81	**35**	18.01	64.22
2	9.89	47.49	**19**	13.41	58.40	**36**	12.69	54.94
3	4.84	73.39	**20**	22.52	55.03	**37**	7.58	38.53
4	10.95	46.55	**21**	1.33	60.79	**38**	1.54	41.86
5	19.13	69.17	**22**	10.73	64.51	**39**	8.99	47.92
6	6.33	72.88	**23**	6.68	43.22	**40**	4.28	31.53
7	5.99	77.71	**24**	10.68	65.42	**41**	12.39	62.97
8	12.60	56.58	**25**	21.02	45.40	**42**	13.14	71.61
9	18.70	77.77	**26**	10.31	39.40	**43**	14.95	50.42
10	8.76	65.15	**27**	9.18	67.00	**44**	8.64	59.69
11	11.37	78.33	**28**	10.65	47.19	**46**	14.44	57.21
12	18.35	54.40	**29**	6.13	25.31	**47**	12.67	32.99
13	13.11	53.82	**30**	4.10	21.57	**48**	20.02	64.24
14	22.80	76.08	**31**	17.68	21.53	**49**	15.00	58.51
15	16.83	72.10	**32**	10.42	55.00	**50**	26.39	64.66
16	8.47	67.65	**33**	5.52	40.63	**51**	10.23	53.48
17	10.28	46.36	**34**	3.31	42.37	-	-	-

Phomopsis controls : AGRISOL= 3.14 %, RIGASOL= 19.43 %; DK3790= 37.92 %; VIKI = 14.59 %
LSD = 5.62 %

Sclerotinia controls : FANTASOL= 8.07 %; NATIL = 40.41 %
LSD= 6.68 %.