Host specificity and genetic diversity of the parasitic plant Phelipanche ramosa on winter oilseed rape in France

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Introduction

Around 4000 angiosperms species are parasitic plants. Among them, broomrapes are obligate parasites which are non-chlorophyllous and thus entirely dependent on their hosts for water, minerals and organic nutrients (holoparasites). *Phelipanche ramosa L. Pomel (syn. Orobanche ramosa)* is by far the most widespread species due to its extremely wide range of host plants from cultivated species to weeds. High infestations lead to severe losses in crop yields, and can even wipe out completely the host.

In France, *P. ramosa* keeps on spreading and parasitizing new host species. New populations have been recorded in the early 2000's, in the western part of France parasitizing winter oilseed rape (WOSR), which thus appears as a new host species. Previous studies based on genetic analysis and cross infections have showed the existence of different types in *P. ramosa* with different host specificity (pathovars). The study of three French populations collected from WOSR, tobacco and hemp fields has revealed inter-population genetic diversity with high adaptation with regards to the host plant (Brault et al. 2007).

However, only few populations (*i.e*. one population per location and per host species) were analyzed, limiting the evaluation of genetic diversity and the understanding of potential adaptation: is the divergence between populations due to geographical distance or to the host specialization? Is the spreading of *P. ramosa* on new species such as *Brassica napus* L. (oilseed rape) due to phenotypic plasticity or to local adaptation (one adaptable entity *vs.* multiple host-specific entities)? These questions are of crucial interest in order to evaluate the adaptability of the parasitic specie. This issue is particularly relevant for *P. ramosa*, which is continuing to spread and to colonize new hosts in France.

In this study the virulence of broomrape populations sampled from hemp and rapeseed fields was studied by cross-infestations. In addition the genetic variability of natural populations of *P. ramosa,* distributed over all regions of France and sampled from several host species, was determined with two cytoplasmic and four microsatellite markers.

Materials and methods

Cross infestation

Two cross-infestation campaigns were carried out. The first one included two natural populations of *P. ramosa* (sampled from hemp and WOSR fields in western France) and two host species (10 varieties of hemp and 2 varieties of WOSR). The second one included the previous *P. ramosa* populations and an additional population sampled on another WOSR field in western France. Four host species were tested in agreement with the main cultivated species challenged with *P. ramosa* in France: WOSR (3 varieties), hemp (3 varieties), sunflower (2 varieties carrying resistance to *Orobanche cumana*) and tobacco (3 varieties). The same protocol was used for both campaigns: 5mg of broomrape seeds were mixed with ground in a 2-liter pot in which one host seed was sown. Ten pots of each association were inoculated and placed in a greenhouse under 14h of light at 20°C (± 3°C). Watering was performed 3 times a week with 3‰ Liquoplant Bleu® (Plantin). Co-cultures were uprooted when emergence of *P. ramosa* were observed in most of pots. Roots were washed with tap-water and broomrape per host plant were counted (from attachment to emergence; see Fer et al. 1997) and their dry-weight measured (DW).

Results didn't fit normal distribution. The non-parametric Kruskal–Wallis test was performed to compare the different associations with the R software (http://www.r-project.org/). Effects of "host species", "variety of host species" and "broomrape population" on "the mean number of broomrape attachments per host plant" and "the mean dry-weight of total broomrape attachments per host plant" were tested.

Genetic analysis

Samples were collected from natural populations distributed over France and 8 host species. *P. ramosa* samples harvested from the cross infestation experiments were also analyzed. Total DNA

was extracted from 10 mg of dried tissue using Nucleospin Plant extraction kit (Macherey Nagel) according to the manufacturer's instructions.

Two intergenic regions exhibiting length polymorphism were amplified: (i) the plastid intergenic region *rpl2* using primers designed from the complete sequence of the plastid genome of *Nicotiana tabacum* and *Solanum lycopersicum* (Kahlau et al. 2006) and (ii) the mitochondrial intergenic region *nad4* using primers designed by Duminil et al. (2002). Primers were designed on conserved regions flanking each gap after a preliminary sequencing of several individuals. Spectrograms were analyzed with Genemapper® 3.7 (Applied Biosystems®) software.

Microsatellite isolation was performed from an enriched library of *P. ramosa* according to the procedure described in Malausa et al. (2011). Primers were designed using Primer3 algorithm (Rozen et al. 2000). Four microsatellites markers were amplified and DNA fragments were fractioned using an ABI PRISM® 3130xl automatic DNA sequencer. Alleles were analyzed and scored with Genemapper® 3.7 (Applied Biosystems®) software.

Results

Cross infestations

Details of P-values are not given. Only significant differences ($P<0.05$) are discussed. Cross infestations revealed differences in host species affinity between the populations of *P. ramosa*. WOSR plants were more parasitized by broomrapes collected from WOSR field (wosr-broomrape) than by broomrape collected from hemp field (table 1). The strongest difference between broomrape populations was observed for hemp. Indeed broomrapes sampled from WOSR field did not develop on any of the 10 tested hemp varieties. These results were confirmed by the second campaign of cross-infestation (table 2): WOSR accessions were more parasitized by wosr-broomrapes than by hemp-broomrapes and wosr-broomrapes did not parasitized hemp.

Table 1: Average number of broomrape attachments on different "host species/broomrape population" associations. These results were obtained for the first campaign, including 100 pots for associations with hemp and 20 pots with WOSR. Numbers in bold are non-cross infestations. Standard deviation is given in parenthesis.

Broomrape population	hemp	WOSR
Wosr-broomrape 1	0.01 (0.10)	**10.65 (5.26)**
Hemp-broomrape	**9.38 (6.25)**	3.60 (1.60)

When cross infestations were carried out between hemp and hemp-broomrapes and WOSR and wosr-broomrapes, the total number of fixations varied according to the variety of the host species (Kruskal-Wallis: $P<2.10^{-3}$ and $P=0.02$ respectively; data not shown). The number of broomrape attachments to sunflower and tobacco differed significantly according to the broomrape population. Tobacco plants were more parasitized by hemp-broomrapes than by wosr-broomrapes contrary to sunflower plants which were more parasitized by wosr-broomrapes than by hemp-broomrapes (table 2). In addition, morphological differences between broomrape populations were observed, resulting in higher total broomrape DW per host plant for wosr-broomrapes than for hemp-broomrape, whatever the host species considered (table 2).

Table 2: Average number of broomrape attachments and total broomrape dry weight within different "host species/broomrape population" associations. These results were obtained for the second campaign of cross infestation, including 30 pots for associations with WOSR, hemp and tobacco and 20 pots for sunflower. Numbers in bold are non-cross infestations. Standard deviation is given in parenthesis.

	WOSR		hemp		tobacco		sunflower	
	Nfix	DW	Nfix	DW	Nfix	DW	Nfix	DW
Wosr-broomrape 1	**6.57** (3.56)	**1.91** (2.16)	0.00 (0.00)	0.00 (0.00)	5.23 2.66)	2.50 (1.5)	3.60 (3.57)	0.13 (0.20)
Wosr-broomrape 2	**12.40** (5.14)	**0.72** (0.38)	0.02 (0.20)	0.00 (0.00)	5.70 (2.53)	1.96 (1.33)	7.04 (8.44)	0.07 (0.05)
Hemp-broomrape	4.54 (3.61)	0.54 (0.36)	**9.6** (5.90)	**0.46** (0.27)	8.37 (4.45)	0.56 (0.27)	1.04 (1.58)	0.02 (0.04)

Genetic analyses

Over the 709 individuals analyzed with cytoplasmic markers, only 2 different haplotypes were identified for both loci. When combining information from both loci, 3 haplotypes were defined (table 3). Populations sampled from the West part of France, including wosr-populations used for cross-infestations, exhibited the same genetic type, whatever the host species (type 1). Only the population sampled from hemp fields on the western region and used for cross infestation was composed by

another genetic type (type 2). No polymorphism was found in these populations. The tobacco-broomrape population harvested from the Southwest part of France was mainly composed by the third genetic type. In East and Southeast parts of the country, broomrape populations sampled from WOSR fields were composed by the three genetic types (table 3). Results did not exhibit specific association of host species and genetic type of broomrape.

Table 3: Distribution of the three genetic *P. ramosa* types defined by the combination of the mitochondrial and the plastidial markers.

	East			West			Southeast			Southwest		
Genetic type	1	2	3	1	2	3	1	2	3	1	2	3
Weeds		2		1								
Hemp	2	13			23							
Cole				20								
WOSR	2	17	3	521			2	18	1			
Geranium				20								
Melon				20								
Tobacco				9						1	0	14
Sunflower				10								
Total	**4**	**32**	**3**	**601**	**23**	**0**	**2**	**18**	**11**	**1**	**0**	**14**

Over the 270 individuals analyzed using four microsatellite loci, only 2 alleles per locus were identified. No heterozygous was found which confirmed the autogamy of *P. ramosa* as hypothetized by Musselman et al. (1981). Alleles were also fixed among populations and only two genotypes could have been defined with these loci: one genotype found in populations from West region of France (except the population sampled from hemp field) and the other one on all other populations analyzed. Combining results from cytoplasmic markers and microsatellites, broomrape populations from the West region of France (except the population sampled from hemp field) exhibited a strong genetic differentiation.

Discussion

Cross-infestation experiments emphasized some differences in virulence of *P. ramosa* populations collected from hemp and WOSR fields. These differences were also supported by genetic analysis with both plastid and microsatellite markers. These results confirmed those of Benharrat et al. (2005) who defined two French pathovars of *P. ramosa*. However, host species is not the only parameter contributing to *P. ramosa* variability.

Geographical location appeared also as a parameter discriminating populations. Western populations have a specific genetic profile shared by populations parasitizing rapeseed, melon, sunflower and weeds. Two hypotheses could explain this geographical specificity: mutations have happened and have spread over the region or an introduction of a new genetic type occurred. On one hand, the absence of heterozygous individuals confirms the autogamy of *P. ramosa* which promotes the fixation of mutations and genetic differentiation of populations. This could support the hypothesis of spread and fixation of one western genotype. On the other hand, intensive WOSR crops in the West part of France from the beginning of the 1980's could also promote accidental introduction of a new genotype. A phylogeographic survey, including populations from the whole area of distribution of *P. ramosa*, could help us to understand the origin of this genotype.

If one genotype is clearly responsible of damages in WOSR fields in the west part of France, two others types of *P. ramosa* in natural populations could also parasitize WOSR crops. Additional cross infestations have to be carried out to better characterize the 3 genetic types. It is also important to notice that the 3 types can coexist within broomrapes populations. Their potential of competition should be evaluate with infestation of mixed-seeds.

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