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Virulence diversity among branched broomrape (*O. ramosa* L.) populations in France

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Abstract – In France, the obligate root parasite *O. ramosa* L. (broomrape) causes significant decreases in yields in several crops such as *Brassica napus* L., *Cannabis sativa* L. and *Nicotiana tabacum* L. Over the past few years, the broad distribution and the continuous expansion of this parasitic plant have been pointed out. The diversity of its host plants and the increase in its locations in France led us to investigate the virulence of some *O. ramosa* populations against their potential host plants. Significant variation in their aggressiveness was observed. These results are therefore consistent with the existence of at least two *O. ramosa* pathovars throughout populations scattered in France. A study by PCR of inter-simple sequence repeat (ISSR) of these two populations allowed us to characterize each of them by specific molecular markers.

broomrape / hemp / oilseed rape / virulence / pathovar

1. INTRODUCTION

Broomrape (*Orobanche*), a root parasite, is devoid of chlorophyll and must develop a specialized structure, a haustorium, to establish connections with the host vascular tissues and to thereby withdraw water, mineral and organic nutrients. Some species of broomrape are able to parasitize a wide range of agricultural crops [5, 9, 23] and among them, *O. ramosa* is one of the most noxious. This is particularly the case in Europe, even if some severe damage has also been reported in other countries such as Australia [23] or Chile [3]. In France, this parasite causes heavy yield losses in several crops, particularly hemp (*Cannabis sativa* L., Cannabinaceae), oilseed rape (*Brassica napus* L., Brassicaceae), and tobacco and tomato (*Nicotiana tabacum* L. and *Lycopersicon esculentum* Mill, Solanaceae). Its presence was reported in 1893 by Lavergne [14] on tomato and hemp; but for some years, severe infestations have been observed in many locations throughout the country, particularly on hemp and oilseed rape. *O. ramosa*'s biological cycle can differ greatly, depending on the host. For instance, the early stage of subterranean development of the parasite (fixation on the host root) occurs in autumn on *Brassica napus* L., but in spring on *Cannabis sativa* L. In spite of the large biological variations in host, soil and climate already underlined, the French *O. ramosa* populations have never been compared for their virulence. Moreover, no information is available concerning the genetic diversity of these *O. ramosa* populations. In order to answer these questions, *O. ramosa* populations collected on

different crops in several locations in France should be better characterized.

Orobanche population diversity has already been studied for *O. aegyptiaca*, *O. cumana* and *O. crenata*. As morphological characters are not reliable for broomrape population comparisons, in recent years molecular markers have been widely used, particularly RAPD markers [4, 22, 25]. Genomic polymorphism analysis has also been carried out thanks to amplification by polymerase chain reaction (PCR) of inter-simple sequence repeat regions (ISSR) [2, 27]. This strategy was retained in the present study, since the RAPD technique produces less reliable and reproducible bands than ISSR amplification [19, 21].

The aim of this study was to compare the virulence of some French *O. ramosa* populations against their potential host (mainly oilseed rape and hemp) and to characterize them by molecular markers.

2. MATERIALS AND METHODS

2.1. Plant material

In France, eight *O. ramosa* L. populations were collected, in 2001, from infested *Buddleja linleyana* F., *Brassica napus* L., *Cannabis sativa* L., *Fagopyrum esculentum* Moench and *Nicotiana tabacum* L. fields (Tab. I). Ten samples (individual plants) per location were studied. The *Brassica napus* varieties (Capitol, Complice, Tradition and Lucia) were obtained from

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Table I. Geographical origin and host plants of *O. ramosa* L. populations.

Population	Origin	Host plant
1	Sarthe	<i>Cannabis sativa</i> L.
2	Charente-Maritime	<i>Nicotiana tabacum</i> L.
3	Aube	<i>Fagopyrum esculentum</i> Moench
4	Maine et Loire	<i>Cannabis sativa</i> L.
5	Vendée	<i>Brassica napus</i> L.
6	Deux-Sèvres	<i>Brassica napus</i> L.
7	Charente-Maritime	<i>Brassica napus</i> L.
8	Deux-Sèvres	<i>Buddleja linleyama</i> F.

CETIOM (Centre Technique Interprofessionnel des Oléagineux Métropolitains, Paris, France) and from SONACOS (Société Nationale de Commercialisation des Semences, Kénitra, Morocco). The *Cannabis sativa* varieties (Felina 34, Fedora 17, D19 and F56) were obtained from FNPC (Fédération Nationale des Producteurs de Chanvre, Sarthe, France).

2.2. Molecular analysis

O. ramosa populations were screened for their molecular markers as follows: total DNA was extracted from 100 mg of frozen tissue (shoot and inflorescence) using the Nucleon Phytopure DNA extraction kit (Amersham, Buckinghamshire, UK).

DNA variations were studied using (CA)₆RG primer, synthesized at ESGS, Cybergene Group, Evry, France. ISSR amplifications were performed in 25 µL of mixture containing 20 ng of template DNA, 100 µM of each dNTP, 4 µM of primer, 2.5 µL of 10X buffer (Appligene, Illkirch, France) and 0.5 U of Taq polymerase (Appligene) in a PCR thermal cycler (Perkin-Elmer Cetus, Norwalk, USA). The ISSR amplification program was as follows: initial denaturation for 1 min at 94°, 35 cycles of 1 min at 94°, 1 min at 50° and 4 min at 72° and a final step for 7 min at 72°. PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide. The replicability of the amplification was checked five times.

2.3. Screening under greenhouse conditions

All experiments were conducted in 1.3-liter pots filled with soil mixture (1: sand, 1: compost, 1: soil), mixed with 10 mg *O. ramosa* seeds collected from oilseed rape or hemp infested fields. The pots were watered and kept in the dark for a week at 20° to allow preconditioning of the *Orobanchae* seeds. Subsequently, three *Brassica napus* or *Cannabis sativa* seeds were sown in the pots. After emergence, seedlings were thinned to one plant per pot. Plants were grown under a 16-hour photoperiod (300 µmol m⁻² s⁻¹ PAR). Host plants were harvested 9 weeks after sowing. *O. ramosa* shoots were counted and the host plant root system washed before screening of all fixed tubercles. All experiments on broomrape infestation were conducted in three pots and replicated three times.

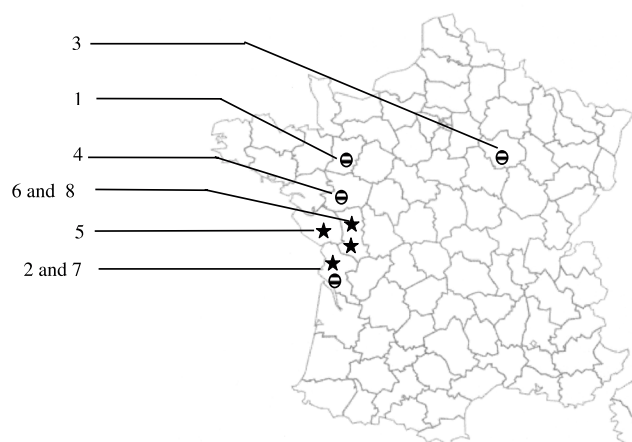


Figure 1. Eight *O. ramosa* populations collected from different locations in France. 1: Sarthe; 2: Charente-Maritime; 3: Aube; 4: Maine et Loire; 5: Vendée; 6: Deux Sèvres; 7: Charente-Maritime; 8: Deux-Sèvres (★: in oilseed rape fields, ⊗: in other crop fields).

3. RESULTS

3.1. Virulence of *O. ramosa* populations collected in France

The virulence of two *O. ramosa* populations was tested against their potential host plants: *C. sativa* and *B. napus*. Seeds of populations A and B were collected in Vendée from an affected oilseed rape field and in Sarthe from an infested hemp field, respectively (Fig. 1). The two locations are 250 km apart.

The resistance of four oilseed rape varieties (Capitol, Complice, Lucia and Tradition) and four hemp varieties (Fedora 17, Felina 34, F 56 and D 19) to *O. ramosa* populations was evaluated by counting *Orobanchae* fixations on host root systems and emerged broomrape. Both oilseed rape and hemp varieties were susceptible to the *O. ramosa* populations collected in oilseed rape fields (population A), but the number of broomrape attachments on root systems was always higher on oilseed rape varieties. For instance, 7 to 22 attachments per plant were counted, as opposed to only 0.3 to 4.9 for the hemp varieties (Fig. 2). All the oilseed varieties, while differently susceptible, supported emerged broomrape, in contrast to hemp varieties: for three varieties out of four, no broomrape shoot emergence was observed. The most susceptible oilseed rape varieties were Capitol and Tradition, whereas the hybrid lines, Complice and Lucia, appeared more resistant. Given the number of attachments and the degree of emergence, this screening indicated that oilseed rape was always more susceptible than hemp to *O. ramosa* population A. Moreover, three hemp varieties out of four supported a significant number of necrosed tubercles, whose number was above those of viable attachments in the case of Fedora 17 and D19. It can therefore be concluded that *O. ramosa* population A was more aggressive for oilseed rape than for hemp.

In contrast, results obtained from infestation by *O. ramosa* population B collected on hemp fields were greatly different, since the number of fixations and emergences were both significantly

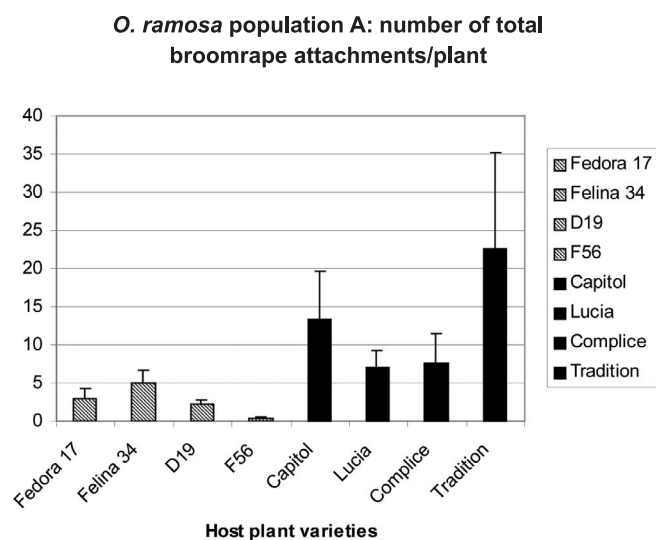


Figure 2. Infestation of four hemp varieties and four oilseed varieties by *O. ramosa* population A. Results represent the mean total number of broomrape fixations per plant \pm SE of three experiments. ■ hemp varieties ■ oilseed varieties.

higher with hemp varieties. For instance, with population B, the susceptibility of hemp varieties varied from 18 to 33 attachments per plant for D19 and Fedora17, respectively (Fig. 3). When the four oilseed varieties were infested, in spite of only 3 fixations per plant, *O. ramosa* completed its life cycle with emerged shoots as was, of course, the case with the hemp varieties.

To summarize, this study showed that, in France, the susceptibility of the two host plant species screened varies according to the *O. ramosa* populations. Thus, oilseed rape varieties appear to be susceptible to the *O. ramosa* population collected in colza fields and more resistant to the *O. ramosa* population originating from hemp fields, and vice versa for the hemp varieties. Based on these results, these two *O. ramosa* populations could be considered as two distinct pathovars (A and B, respectively).

3.2. Identification of the two *O. ramosa* pathovars by molecular markers

The importance of virulence differences between the two *O. ramosa* populations led us to check for morphological features in an attempt to clearly identify them in the fields. However, no reliable morphological trait to differentiate them has been pointed out. To overcome these identification difficulties, specific molecular markers obtained from the ISSR-PCR technique, previously used for an *O. ramosa* Sudan populations study [1] and Orobanche subsection *Minores* species [2] were searched for. The two *O. ramosa* pathovars were studied by analyzing electrophoretic patterns obtained from inter-simple sequence repeat amplification. Therefore eight populations collected from different host plants (Tab. I) in different areas (Fig. 1) were compared. The use of the anchored primer, (CA)₆RG, revealed a genetic polymorphism which allowed the characterization of two groups of populations which each corresponds to a pathovar (A or B). Each of them can be identified

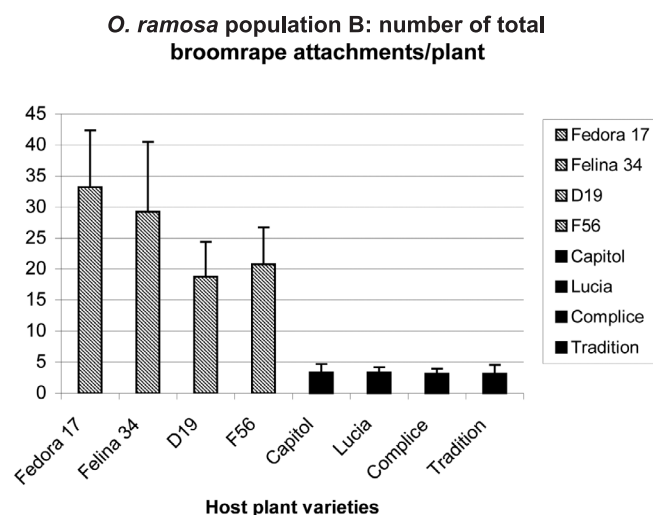


Figure 3. Infestation of four hemp varieties and four oilseed varieties by *O. ramosa* population B. Results represent the mean total number of broomrape fixations per plant \pm SE of three experiments. ■ hemp varieties ■ oilseed varieties.

by specific markers: two specific bands (1200 and 1400 bp) for the pathovar A and two others, 1300 and 1350 bp (Fig. 4), for the pathovar B. The study of ten samples per population revealed a similar electrophoretic pattern for all specimens.

The pathovar B's specific molecular markers (lane 1) (Fig. 4) are present on the electrophoretic pattern of three other *O. ramosa* populations (lanes 2, 3 and 4). These four *O. ramosa* populations (characterized by the same markers) had been collected in localities widely distributed all over the area of sample collection (Fig. 1). They were fixed on three different hosts (*Cannabis sativa* L., *Nicotiana tabacum* L. and *Fagopyrum esculentum* Moench) which belong to the Cannabinaceae, Solanaceae and Polygonaceae families, respectively. Two *O. ramosa* populations (Tab. I) collected on the same host plant, *C. sativa*, in areas (Sarthe and Maine et Loire) more than 100 km apart, exhibited the same electrophoretic profile (Fig. 4).

The four other *O. ramosa* populations were identified by two other specific markers (1200 and 1400 bp). Three of them were collected in *oilseed rape* fields, while the fourth was parasitized *Buddleja linleyana* F. (Tab. I) on the border of oilseed rape fields. Thus, those populations are more closely located westward of the sampling area. In the Charente-Maritime region two populations (Fig. 1) collected less than ten km from each other, on different host plants (tobacco and oilseed rape), were demonstrated to carry different molecular markers, specific to each pathovar (lane 2 and lane 7, Fig. 4).

4. DISCUSSION

In France, *O. ramosa* distribution is broad and in continuous extension. In spite of the wide range of infested crops (tobacco, oilseed rape and hemp), neither molecular characterization nor virulence variation had been undertaken up to now. Thanks to

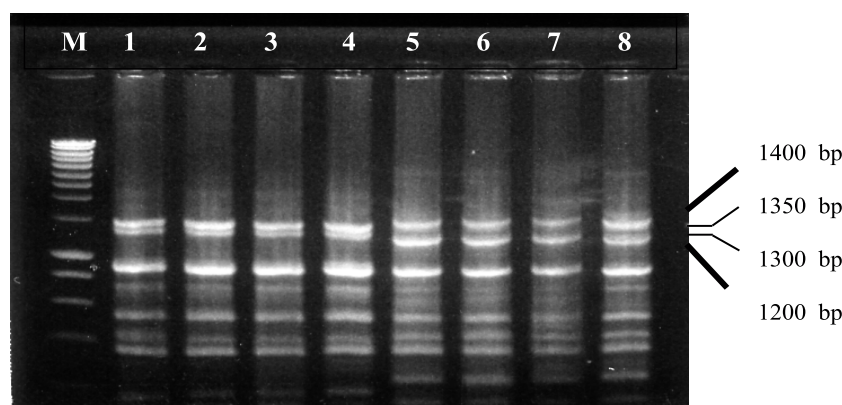


Figure 4. Inter-simple sequence repeat (ISSR) fingerprints of eight French *O. ramosa* populations using the primer (CA)₆RG. M: molecular markers, lane 1 to 8: *O. ramosa* populations fixed on different host plants. Lane 1: *Cannabis sativa* L., lane 2: *Nicotiana tabacum* L., lane 3: *Fagopyrum esculentum* Moench, lane 4: *Cannabis sativa* L., lane 5 to 7: *Brassica napus* L., lane 8: *Buddleja linleyama* F.

— Pathovar A molecular markers
 — Pathovar B molecular markers.

the ISSR method, this study revealed a genetic polymorphism which discriminated the different French populations into two groups. Each of them can be characterized by two specific molecular markers. Our results report that within each group, a distinct pathovar was clearly identified. Such virulence variability among *O. ramosa* populations has until now never been pointed out, even if the existence of “race” [6] has been supposed (but not characterized). Therefore, this raises the question if all the same groups of populations develop the same virulence with respect to their hosts. In this case, the molecular markers identified in this study could be associated with each pathovar. On the contrary, if each population expresses a different pathogenicity, there would thus exist, in France, several other *O. ramosa* pathovars apart from those defined in this study. With the aim of confirming this hypothesis, a significant virulence study of all those populations is currently being carried out.

O. ramosa diversity appears not to be correlated with geographical location. Thus, in the Charente-Maritime region, two populations characterized by distinct molecular markers were collected in two areas less than 10 km apart, whereas *O. ramosa* specimens which exhibit the same ISSR pattern and host spectrum were collected from locations more than 100 km apart (the Sarthe and Maine et Loire regions). These results agree with those of Zeid et al. [30], Roman et al. [26] and Benharrat et al. [2], who compared *O. crenata* and *O. hederiae* populations in Egypt, Spain and Israel, and France, respectively.

In the case of *O. ramosa*, the occurrence of molecular marker diversity does not seem to be strictly linked to the specificity of the host plant since *O. ramosa* belonging to the pathovar B group was collected on various crops such as *C. sativa*, *N. tabacum* and *F. esculentum*, members of three families, the Cannabinaceae, Solanaceae and Polygonaceae, respectively. However, pathovar A group specimens were mostly found to be associated with oilseed rape. In only one case, specimens were collected on another host plant, *Buddleja linleyama* F (Buddlejaceae). The host root system was carefully examined and *O. ramosa* was found connected to its roots. Its localization, on

the border of oilseed rape fields, suggests a contamination by *O. ramosa* seed dissemination. While Musselman and Parker [18] indicated that the host plant influences *O. ramosa* variability, Paran et al. [22] did not observe a similar correlation for *O. aegyptiaca* and *O. crenata*. On the other hand, an ISSR study of *O. hederiae* populations [1] has shown that this monospecific parasitic plant exhibits different genetic patterns according to its geographical location.

The two *O. ramosa* pathovars, characterized by their host plant range, differ, of course, in their pathogenicity against a host. However, a host susceptibility variability exists among the lines and hybrids. Generally speaking, hybrids are more resistant, as also observed by Zehhar et al. [29] who studied interactions between fifteen oilseed varieties and an *O. ramosa* population originating from Sudan. If this population was noted as being less aggressive than the French populations, the same resistance range of three oilseed rape varieties, shared in these two studies, was observed, suggesting that their general resistance level, depending on several genes, remains expressed whatever the pathovar.

The finding of two pathovars among *O. ramosa* collected in France raises the question of how closely they are related; for instance, according to their geographical origin and their date of introduction into this country. The extension of *O. ramosa* in Maine et Loire, on hemp fields, is directly associated with hemp seed importation from Turkey, between 1920 and 1939 [20]. However, the presence of this parasite species has been known in France since at least the XIX century and was described on hemp in 1842 and 1857 by Holandre [10] and Godron [7], respectively. Le Maout and Ducaines [15] observed it in tobacco fields in 1876, while Lavergne [14] announced its presence on tomatoes some years later (1893). The latter also described a broomrape specimen on *Brassica rapa* L. in Lot et Garonne and identified this specimen as *O. speciosa*, already known in Germany on *Brassica napus* L. and *Brassica capitata* L.

In Europe, oilseed rape domestication started in the early Middle Ages, while commercial plantings have been known

since the XVth century. After the Second World War, oilseed rape production increased considerably, but *O. ramosa* extension, on *Brassica napus* fields in France, has only been observed for the last ten years. As *O. ramosa* pathovar A appears to be strictly located where oilseed rape is grown, its extension could coincide with a new event for which two hypotheses could be proposed. This pathovar was introduced with contaminated seeds (for instance, from Turkey, between 1920 and 1939) or it spontaneously burst out after a change in the genetic and phenotypic characteristics of the oilseed rape varieties grown in France. Several changes have occurred over the two last decades in France with the introduction and the intensive culture of lines with low content in glucosinolates which are known to constitute a defence system in cruciferous plants toward pests and diseases. It has been shown that glucosinolates are induced by different defence pathways in *Arabidopsis* ecotypes [16]. Besides, it is known that lines and hybrids are almost exclusively grown in France, instead of populations in most other countries.

It has been shown (Boulet C., pers. comm.) that broomrape pathovar A could parasitize and carry out its life cycle on weeds which are strictly limited in their expansion in oilseed rape areas (*Senecio vulgaris* L., *Ammi majus* L. and *Galium aparine* L.). It is therefore expected that these wild plants have served as “tank” plants, increasing the broomrape seed-bank in the soil, until the oilseed rape introduction in these areas, which led to a burst in *Orobanch*e infestation. Such an observation of an outburst of broomrape has been made with *O. foetida* Poir., in Tunisia [12]. This broomrape was initially known as a parasite of wild plants until its recent development on *Faba bean*, a crop on which *Orobanch*e is more aggressive than on other legumes [13].

If variation in aggressiveness has been demonstrated in different *Orobanch*e species such as *O. cumana* [8, 17, 28] and *O. crenata* [11], no similar study had been carried out for *O. ramosa* prior this work, which revealed large differences in virulence between two French populations. The undoubted identification of a pathovar, particularly virulent and widespread in oilseed rape fields, is of great interest for further studies on *Orobanch*e control.

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