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A new method to obtain monozoosporangial isolates of sunflower downy mildew (*Plasmopara halstedii*)

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Summary – A new method to obtain monozoosporangial strains was applied on 9 pathotypes of *Plasmopara halstedii* (100, 300, 304, 314, 700, 704, 710, 707 and 714), the parasite causing sunflower downy mildew. Single zoosporangia were isolated from the surface of agar medium and placed on leaf disks on solid Knop medium. The best fungal development was obtained with leaf disks taken from the first pair of sunflower leaves when these measured from 5 to 8cm in length. The percentage success rate in obtaining monozoosporangial strains varied from 1.4 to 7.4% according to race. The percentage of disks showing sporulation depends on spore viability, the physiological state of leaves and the receptivity of the sunflower genotype. This method enabled us to confirm the presence of a new downy mildew pathotype in France: race 707.

***Plasmopara halstedii* / monozoosporangial strain / leaf disk / sunflower / race / isolate**

Résumé – Une nouvelle méthode pour obtenir des souches monozoosporanges a été appliquée sur neuf profils de virulence (100, 300, 304, 314, 700, 704, 710, 707 et 714) de *Plasmopara halstedii*, l'agent du mildiou de tournesol**.** Un seul zoosporange a été isolé à la surface d'un milieu gélosé et déposé sur un disque foliaire placé sur milieu de Knop gélosé. Un meilleur taux de réussite a été obtenu avec des disques foliaires prélevés sur la première paire de feuille lorsqu'elles atteignent 5 à 8 cm. Le taux de souches monosporanges obtenu varie de 1,4 à 7,4 % selon les pathotypes. Le pourcentage de disques sporulés dépend de la viabilité des zoosporanges, de l'état physiologique des feuilles et la réceptivité de génotype du tournesol. Cette méthode nous a permis de confirmer la présence d'un nouveau pathotype sur le territoire français : race 707.

***Plasmopara halstedii* / monozoosporanges / disque foliaire / tournesol / race / isolement**

INTRODUCTION

Sunflower downy mildew is a common disease in many regions where sunflowers (*Helianthus annuus* L.) are grown. The pathogen, *Plasmopara halstedii* (Farlow) Berles & de Toni, is an obligate parasite. The disease affects young plants when the water content of the soil is high and the maximum temperature is between 15 and 18°C. *P. halstedii* is an Oomycete with asexual multiplication by liberation of zoosporangia produced on the under surfaces of sunflower leaves and sexual reproduction giving oospores which are found in crop residues.

P. halstedii shows physiological races (pathotypes) capable of infecting a variable range of sunflower genotypes. The nomenclature of these races is based on the reaction of a series of differential lines (Tourvieille de Labrouhe, 1999). In France, race 100 (European race) has been present in the sunflower crop since 1965. Until 1987, it was the only race identified but in 1988 and 1989, two new races, 710 and 703 appeared (Tourvieille de Labrouhe *et al*., 1991). Since then, prospections each year have demonstrated the existence in France of 3 races in 1993, 5 races in 1994, 6 in 2000, 9 in 2002 and 12 in 2004 (Tourvieille de Labrouhe, 2004). The 6 races identified since 2000 (304, 307, 314, 334, 704 and 714) and purified on specific host differentials had not been described previously in other countries. They appear to result from evolution of the parasite in France.

There are two main reasons why it was useful to obtain monozoosporangial strains of downy mildew. The first is to permit studies of the variability within or between races. A pathotype is generally a population made up of a certain number of fungal genotypes which have in common the same virulence profile. The second use is to demonstrate different pathotypes within a single isolate. An isolate of a pathogen with a complex virulence profile can be a stable mixture of several populations with different virulences and the resistance resulting from the presence of one avirulent pathotype could lead to resistance of the plant host to a virulent strain present in the mixture. An example is provided by an isolate collected in 2004 which showed a new virulence profile: 707. In spite of resistance tests on 29 inbred sunflower lines and 105 hybrid varieties (unpublished data), it had not been possible to determine whether this isolate was truly a new pathotype or whether it was a mixture of several different races.

Research to obtain monospore strains has already been carried out on several pathogen species. For example, Alves-Santos *et al*. (2002) used monoconidial cultures of *Fusarium oxysporium* f. sp. *phaseoli,* which cause haricot bean wilt, to characterise aggressiveness and physiological race specialisation of 11 isolates. Okori *et al*. (2004) studied the variability between isolates of *Cercospora zeae-maydis*, which causes grey leaf rot of maize, also using monoconidial isolates. For the Oomycete, *Phytophthora infestans*, the pathogen of late blight of potatoes, Oliva *et al*. (2002) used monospore cultures to study the characteristics of sexual reproduction. All these reports concern fungi that can easily be multiplied on artificial media. Concerning obligate parasites, Maisonneuve (personal comm.) obtained monospore isolates of *Bremia lactuca*, lettuce downy mildew, after isolation of single spores and deposit on the under surface of young cotyledons (the first pair of leaves just visible) of the variety Cobham-Green, which does not carry any resistance genes. Grief (personal comm.) obtained monozoosporangial strains of *Plasmopara viticola,* vine downy mildew, by isolating single zoosporangia which were placed on the lower surface of young leaves (4-5 leaf stage) of a very susceptible variety.

For sunflower downy mildew, Sackston & Vimard (1988) proposed a method to infect leaf disks: 20 µl of inoculum containing 50 000 zoosporangia per disk, placed on damp blotting paper in Petri dishes. Spring *et al.* (1998) developed a method to produce monozoospore strains. They isolated single zoospores by means of a microlitre syringe and placed them on leaf disks from plants multiplied in a cabinet at 16°C, 80% RH, and 14h photo period. They incubated them in microplates which were kept in the same conditions. We were not able to reproduce these results in our laboratory, so a new isolation method has been developed to isolate single zoosporangia which are used to infect leaf disks to obtain monozoosporangial strains. We used 9 different strains of *P. halstedii* to determine the percentage of sporulated disks with French downy mildew pathotypes and taken leaf disks from young plants grown in a greenhouse (under natural light conditions).

MATERIAL

Fungal isolates: All the strains of *P. halstedii* (Tab. 1) used in this study were collected in France and maintained at INRA, Clermont–Ferrand. The manipulation of this quarantine parasite respected European regulations (No 2003/DRAF/70). Race 100 was isolated in 1966, race 710 in 1988, race 703 in 1889, and race 700 in 1995. Race 707 was isolated in 2004 by the Plant Protection Service during its annual downy mildew survey. The other isolates, DU 1842, DU 1767, DU 1650, DU 1734 and DU 1915 were obtained after 5 years of sunflower mono-culture under insect proof cages infected with races 100 (MIL 001) and 710 (MIL 002). These isolates were characterised for their virulence patterns and were found to be isolates of races 300, 304, 314, 704 and 714 respectively.

Sunflower genotypes: The open pollinated variety Peredovik, of Russian origin and not carrying any known resistance gene was used to produce leaf discs.
The virulence profiles of the *P. halstedii* isolates were determined with the 9 differential inbred sunflower lines which give the international nomenclature of sunflower downy mildew races (Tourvieille de Labrouhe, 1999).

Table 1. List of *Plasmopara halstedii* isolates used in the present study

Reference	*Race*	*Geographical origin*	*Year isolated*	*Collection*
MIL 001	100	France	1966	INRA-CL
DU 1842	300	France	2005	INRA-CL
DU 1767	304	France	2005	INRA-CL
DU 1650	314	France	2005	INRA-CL
MIL 005	700	France	1995	INRA-CL
DU 1734	704	France	2005	INRA-CL
MIL 015	707	France	2004	INRA-CL
MIL 002	710	France	1988	INRA-CL
DU1915	714	France	2005	INRA-CL

INRA-CL = Institut National de la Recherche Agronomique – Clermont-Ferrand (France).

METHODS

Isolate conservation: For long term conservation (Molinero-Demilly *et al*., 2005), the cotyledons of infected seedlings showing a large amount of sporulation were placed in a desiccator containing anhydrous CaCL2 (RH 35%), at 20°C for 24 h (30 h is a maximum). The dried cotyledons were then placed in a container and frozen at – 80°C without any particular precautions.

Characterisation of virulence: The *P. halstedii* isolates were multiplied on a susceptible sunflower variety and then used to infect the 9 differential inbred lines D1 to D9, using the methodology described by Tourvieille de Labrouhe (1999).

Measurement of zoosporangia size: The suspension of zoosporangia was obtained by placing a sporulated cotyledon in 5 ml of physiological serum. The size of 100 zoosporangia per isolate was measured under a light microscope (magnification X400). The area of the zoospores was calculated from an ellipsoid $\pi \times a \times b$, a = 1/2 length, b = 1/2 width.

Production of monozoosporangial isolates:

Preparation of leaf discs:
Seed germination: The sunflower seeds were disinfected with 12% NaOCl solution for 3 minutes, washed with permuted water and placed in Petri dishes containing damp filter paper. They were left to germinate at 20°C in the dark for 48h.
Sowing germinated seed: the germinated seed with 0.5 cm radicals were sown in 7 cm pots containing 60% light peat and 40% dark peat (4 seeds per pot). No nutrients were provided. The pots were maintained in a greenhouse at 18-25°C, RH 50 to 90% and natural light.
Preparation of leaf disks: Leaf disks were cut from the first pair of leaves when they are 5 to 8 cm in length. The leaves were rinsed twice in sterile water to reduce microbial infection. The disks were cut with a 9.1 mm cutter and were placed in 9cm diameter Petri dishes with the lower surface in contact with solid Knop medium (15g agar, 0.5g Ca $(NO_3)_2$, $4H_2O$, 0.125g $MgSO_4$, $7H_2O$, 0.125g KNO_3 and 0.125g KH_2PO_4 / 1L of permuted water), with 6 disks in each dish.

Preparation of the zoosporangia suspension: Inoculum was obtained from infected seedlings of the susceptible variety Peredovik, inoculated by the method of Cohen & Sackston (1973), which, after 12 days incubation, were placed in conditions of saturated humidity for 48h. Each sporulated cotyledon was placed in a tube containing 10 ml of physiological serum (9 g NaCl + 1L permuted water) and shaken. One hundred microlitres of suspension was spread on Petri dishes on the surface of an agar medium (12 g agar / 1L permuted water).

Isolation of zoosporangia: The zoosporangia were collected individually under a reverse microscope (×200) with the cone of a micro-pipette (10 µL) and then placed on a leaf disk in a drop (30 µL) of permuted water.

Incubation of leaf disks: The Petri dishes containing the disks were incubated in a growth chamber (18h light, 16-20°C and 50-60% RH). They were observed every day.

Infection of a germinated seed with a sporulated leaf disk: The disks which developed sporulation were placed individually in an Eppendorf tube with

1 ml of permuted water and one germinated sunflower seed of the variety Peredovik for 4 h. This seed was then planted in a 7 cm diameter pot containing soil-less compost, and maintained in a growth chamber at 18°C, RH 65-90% and a light intensity of 12000 lux [fluorescent tubes Philips 36 W super 80 new generation G 13 (Tourvieille de Labrouche *et al*., 1988). After 14 days incubation, the spores obtained from the infected seedlings could be considered as monozoosporangial isolates. Such isolates were then multiplied on further Peredovik seedlings using the method of Cohen & Sackston (1973).

RESULTS

Production of monozoosporangial isolates: The mean percentage of leaf disks showing sporulation was low (Fig. 1), it varied from 3.4% for isolate DU1842 (profile 300) to 12.2% for isolate DU 1734 (profile 704). All the results are presented in Tab. 2. For all the isolates, the first appearance of symptoms was 5 to 12 days after leaf disk infection.

The proportion of monozoosporangial strains that it was possible to multiply up and maintain varied from 35.8% for isolate MIL 001 (race 100) to 100% for isolate DU 1915 (profile 714) (Tab. 2). The overall percentage of monozoosporangial strains produced from leaf disk infection varied from 1.4 to 7.4%.

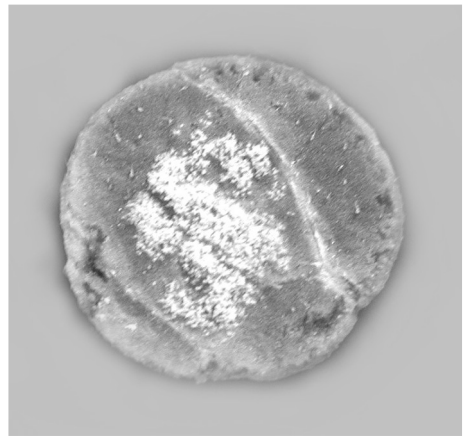

Fig. 1. Sporulation on a sunflower leaf disc, 10 days after infection by a single *Plasmopara halstedii* zoosporangium.

Table 2. Percentages of monozoosporangial strains obtained after infection of sunflower leaf discs by single zoosporangia of different *Plasmopara halstedii* pathotypes

Reference	*Race*	*Nb disks infected*	*Nb disks sporulated*	*% of disks sporulated*	*Nb isolates Multiplied*	*% of isolates maintained*	*% of infections giving monozoosporangial strains*
MIL 001	100	741	28	3.8	10	35.7	1.4
DU 1842	300	350	12	3.4	9	75.0	2.8
DU 1767	304	402	27	6.3	19	70.4	4.7
DU 1650	314	312	16	5.1	11	68.8	3.5
MIL 005	700	240	15	6.3	11	73.3	4.6
DU 1734	704	312	38	12.2	23	60.5	7.4
MIL 015	707	432	18	4.2	15	83.3	3.5
MIL 002	710	405	21	5.2	19	90.5	4.7
DU1915	714	108	6	5.6	6	100.0	5. 6

Characterisation of the virulence profile of monozoosporangial strains: The virulence profiles of monozoosporangial strain obtained from isolate MIL015 are presented in table 3. They all show the same profile (707) as the original isolate, MIL 015.

Analysis of zoosporangium size for the monozoosporangial strains of race 707: Figure 2 shows that there was some variation between the different monozoosporangial strains isolated from MIL 0015: M1 = 304.4 μ^2 ± 15.5, M2 = 464.3 μ^2 ± 54.6. However, this intra-pathotype diversity was less than that between isolates of different races: 251.9 μ^2 ± 11.1 for race 304 (DU 1767) and 555.5 μ^2 ± 60.5 for race 714 (DU 1915). The mean for the 15 monozoosporangial strains, 392.5 μ^2 ± 22.8, was not significantly different from the mean size of zoosporangia produced by the original isolate MIL 015: 375. 1 μ^2 ± 31.9.

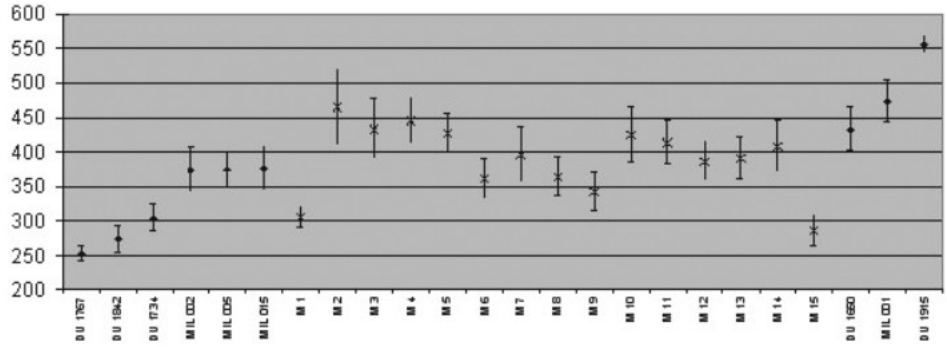

Fig. 2. Size of zoosporangia of different *Plasmopara halstedii* isolates (◆) and 15 (M1-M15) monozoosporangial strains (X) obtained from isolate MIL015 (race 707). Mean of 100 zoosporangia.

Table 3. Characterisation of virulence profiles* of monozoosporangial strains M1 to M15, obtained from the *Plasmopara halstedii* isolate MIL015 on differential sunflower lines.

Differential lines / *Isolate*	*D1*	*D2*	*D3*	*D4*	*D5*	*D6*	*D7*	*D8*	*D9*	*Virulence profile*
MIL015	S	S	S	R	R	R	S	S	S	707
M1 to M15	S	S	S	R	R	R	S	S	S	707

*Tourvieille de Labrouhe (1999). S: Sensible. R: Resistant.

DISCUSSION

The method presented enabled us to obtain monozoosporangial strains of 9 French races of *Plasmopara halstedii* (100, 300, 304, 314, 700, 704, 710, 707 and 714). The rates of production of sporulating leaf disks were not very high

(from 3.4% to 12.2%), but they were similar to those reported by Spring *et al.* (1998) for the same parasite and by Maisonneuve (personal comm.) with *B.lactica*. Spring *et al.* (1998) reported that 5% of leaf disks were infected by a single zoospore and 15% by the zoospores from single zoosporangia. Maisonneuve (personal comm.) obtained, on average, less than one cotyledon sporulated per Petri dish containing 16 to 24 cotyledons, after 10 to 12 days incubation with 16/24h light, 16°C at day and 12°C at night. In contrast, Grief (personal comm.) obtained 30-40% success with *P.viticola,* after only 5 days incubation with 16h light, 22°C and 100% RH.

Spring *et al.* (1998) reported that the proportion of sporulating leaf disks from which it was possible to maintain a strain was 70% for disks infected with a single zoospore and 80% for discs infected by the zoospores from one zoosporangium. In the case of lettuce downy mildew, Maisonneuve (personal comm.) obtained 100% success 10 days after inoculation with a sporulating cotyledon and Grief (personal comm.) obtained the same result with *P.viticola.*

The number of leaf disks which sporulate appears to depend on 3 factors:

Zoosporangia viability: The zoosporangia used for leaf disk infection showed large differences in size: from 14.4 × 12.0 µ to 81.6 × 33.6 µ but it was not possible to define whether all were mature. Meliala (2001) showed that, in general one in three zoosporangia is viable, but this proportion varies between isolates.
The use of physiological serum (NaCl 9%) prolongs the life of zoopores within the zoosporangia by considerably slowing their maturation and thus their liberation. Distribution of 100 µl of suspension over the surface of agar medium in 9 cm Petri dishes makes it quite easy to isolate the number of zoosporangia required for an experiment. However, the zoosporangia need to be separated on the agar and covered with a thin film of water. If there is too much water, or too high concentration of zoosporangia, it is impossible to be sure that only one zoosporangia is placed on each leaf disk.

The physiological state of the sunflower leaves: From which the leaf disks were obtained: observations on plants grown in the greenhouse showed that their conditions of growth affected percentage success of monozoosporangial strain production. *P. halstedii* cannot be maintained on an artificial medium, the Knop medium was used only to improve survival of the sunflower leaf disks. All the sporulating disks came from the first pair of leaves when these measured from 5 to 8 cm in length. Disks from cotyledons or second or third pair of leaves were experimented, but they gave no sporulation. In addition, leaf disks taken from the first pair of leaves on plants grown under high light conditions (spring, summer) give much better results than those from plants grown under poor light (autumn, winter). This is in contrast to Spring *et al.* (1998), who considered that the physiological state of plants was not a factor important in the success of production of monozoosporangial strains. They found that the cotyledons and first leaves of plants aged 14 days, grown in growth chambers at 16°C, 80% RH and 14h light gave the best results, but in our growth chambers, such conditions did not lead to any sporulating leaf disks. It could be that high light intensity, giving increased photosynthesis, leads to a higher concentration of nutriment in leaves when the disks are cut, which would be favourable for fungal growth.
For the leaf disks in Petri dishes, the growth chamber conditions (16-20°C, 50-60% RH and 18h light), produce a very thin film of water on the inside of the Petri dish cover and this appears to give the best micro-climate for downy mildew sporulation on the leaf disks.

Sunflower genotype and *P. halstedii* isolate: The open pollinated variety Peredovik carries no known downy mildew resistance gene but, since it is a population, the plants are not identical. Since the variety shows some partial race non-specific resistance, it is possible that leaf disks from some plants were not very receptive to infection by downy mildew zoospores. This could explain why the levels of infection reported here were not very high. They might be increased if a sunflower genotype with less partial non-specific resistance was used.

In our experiments, the variation in success rate (from 35.7% to 100.0%) may also have been due to differences in the fitness of the different *P. halstedii* isolates. Our observations confirm that there is considerable diversity between monozoosporangial strains for size of zoosporangia. An isolate from the field appears to be a population with some characteristics, such as virulence, in common but others which vary, for example the number of zoospores produced by the zoosporangia, which in turn appears to depend on the size of the latter (Delanoe, 1972). Such characters can be considered to intervene in the fitness of a strain to multiply and become dominant. In our laboratory studies are in progress on the variability concerning fitness of a range of sunflower downy mildew strains.

Production of monozoosporangial strains is a good method to identify and purify a new race. Inoculum collected from plants in the field is made up of thousands of zoosporangia which may come from several different strains or races which infected the same plant. Purification of a race is straightforward if a sunflower genotype with specific susceptibility to the race is available, but if this is not the case, there will always be a doubt. This is particularly true since an avirulent strain in the mixture can cause a defence reaction in the plant which limits or even halts infection by a virulent strain. The production of monozoosporangial strains removes these uncertainties. For example, the monozoosporangial strains confirmed the presence of race 707 in France.

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