

**Molecular and phenotypic characterisation
of French isolates
of *Diaporthe helianthi* / *Phomopsis helianthi* Munt.-Cvet.**

**Anne Viguié, Véronique Says-Lesage, Pascal Walser, Jeanne Tourvieille,
Patricia Drevet, Paul Nicolas, Félicity Vear, Denis Tourvieille de Labrouhe.**

I.N.R.A., G.R.E.A.T, Station d'Amélioration des Plantes et de Pathologie végétale, Domaine
de Crouelle, 63039 Clermont Ferrand cedex 2, France. E-mail : tourvie@clermont.inra.fr

Summary:

Among a French collection of 150 *Diaporthe helianthi* / *Phomopsis helianthi* Munt.-Cvet. isolates collected on sunflower since 1995, 4 were chosen for their different characteristics according to their geographic origin and the part of the infected plant where they were sampled (leaf, petiole, capitulum). Phenotypic and molecular characterisation studies were conducted on these isolates. Phenotypic studies corresponded to their linear and ponderal growths, their morphology in culture on agar medium and their aggressiveness on sunflower leaf and stem through artificial contaminations in the field. Molecular characterisation concerned on one hand the whole genome (AFLP) and on the other hand the ITS sequences. The 4 isolates present phenotypic differences in their growth *in vitro*, their morphology (ability to differentiate perithecia) and their aggressiveness on sunflower. Similar variability was also observed in AFLP analyses of the *D./P. helianthi* genome. This fungus appears to have a genome of variable size. In contrast, ITS sequences (concerning only a part of the genome) did not show differences between the 4 isolates, confirming that they do belong to probably the same species.

Résumé :

Au sein d'une collection de 150 isolats français prélevés sur tournesol depuis 1995 et appartenant à l'espèce *Diaporthe helianthi* / *Phomopsis helianthi* Munt.-Cvet., 4 isolats ont été choisis selon leurs caractéristiques diverses concernant leur origine géographique et leur site de prélèvement sur la plante (feuille, pétiole, capitule). Des études de caractérisation phénotypique et moléculaire ont été menées sur ces isolats. Les études phénotypiques ont concerné les croissances linéaire et pondérale des isolats, leur morphologie en culture sur milieu gélosé, et leur agressivité sur feuille et tige de tournesol suite à des contaminations artificielles en champ. La caractérisation au niveau moléculaire a porté d'une part sur le génome entier (AFLP) et d'autre part sur les séquences de leur portion génomique ITS. Les 4 isolats présentent des différences au niveau phénotypique, dans leur croissance *in vitro*, leur morphologie (aptitude à différencier des périthèces), leur agressivité sur tournesol. Cette variabilité a été retrouvée au niveau moléculaire lors des analyses en AFLP. Nous faisons l'hypothèse que ce champignon présente un génome de taille variable. Par contre, les séquençages d'ITS (qui ne concernent donc qu'une partie du génome) n'ont pas révélé de différences entre les 4 isolats, confirmant, sans doute, qu'ils appartiennent bien à la même espèce.

Introduction

The fungus *Diaporthe helianthi/Phomopsis helianthi* (Muntanola-Cvetkovic et al., 1981) causes stem canker on sunflowers, with economic yield losses in many parts of Europe. The species *D.helianthi* shows considerable variability. First studies were made on Yugoslavian isolates (Muntanola-Cvetkovic et al., 1981; Mihaljcevic & Muntanola-Cvetkovic, 1985a and b), but then other studies, using isolates from several European countries, have also shown differences, mainly according to *in vitro* culture characteristics (Laville, 1986; Vukojevic et al., 1995). However, the importance of *D./P.helianthi* resulting from the losses provoked in the field, studies of its biology and variability cannot be restricted to *in vitro* experiments. Pathological tests to determine differences in aggressiveness between isolates are necessary (Viguié et al., 1999). In addition, for such a highly variable pathogen, molecular analyses can help to determine profiles characterising certain types of isolate of one species.

This study presents a comparison of the phenotypic and molecular variability of four isolates. The pathological study included tests on sunflower leaves and petioles.

Materials and Methods

Fungal isolates:

Isolates 95004, 95045, 95049 and 95099 were collected from sunflowers in 1995 and identified as belonging to the species *D./P.helianthi*. They were chosen among a collection of 150 isolates of this species, constituted in 1995 (INRA, 1996), for their differences in geographical origin and plant part from which they were collected (leaf, petiole, capitulum).

Characterisation methods:

1.Phenotype observations: The isolates were grown on 1% malt, 1.5% agar medium at $23\pm 1^{\circ}\text{C}$ in the dark. For each isolate, thirteen 9mm Petri dishes were inoculated. The diameter of fungal cultures was measured after 6 days' incubation. The cultures were maintained after this observation in order to follow changes in their morphology: their macroscopic appearance was noted and also any fruiting bodies (microscopic observation). These observations were made in 1996 and again in 1998, when, in addition to treatments maintained in the dark, additional replications were given 12h white light/24h. In another experiment, the isolates were grown in sterile 250ml flasks containing 0.1% malt, 0.5% agar medium at 23°C , with a natural photoperiod of 13h/24h. There were 5 replications for each isolate. Culture weight was measured after 35 days incubation, by filtering and drying of the mycelium which had developed (Mohammed, 1987).

Two pathological tests were carried out on adult sunflower plants grown in the field or under netting tunnels (Tourvieille et al, 1986). The leaf test (Bertrand and Tourvieille, 1987) was made on the hybrid varieties VIKI (Maïsadour, susceptible) and Agrisol (Pau Semences, resistant). The infections were made at the star bud stage on the last two leaves to have reached maximum size. For each isolate, five plants of each genotype were infected. The length of the lesion along the main vein for the tip of the leaf was measured after 10 days. The petiole test (Bertrand and Tourvieille, 1987) was carried out on the susceptible INRA line, 2603. Infections were made just before flowering on one petiole per plant and 2 replications of five plants for each isolate. The length of lesion on the main stem was measured 15 days after infection.

2. *Molecular characterisation*: The four isolates chosen were part of a series of 52 whose variability was studied by AFLP (Says-Lesage et al, in prep). AFLP amplification was made using EcoR1 primer+ACA and MseI primer +CTA from the Amersham-Life Technologies large genome kit. Amplification of ITS region was carried out with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' AND ITS4 (5'-TCCTCCGCTTATTGATATG C-3'). The reaction mixture contained 100µl of each dNTP, 2.5µl of 10X reaction buffer (100 mM Tris HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3), 0.2µM of each primer, 1.5U of Taq DNA Polymerase (Appligen Oncor). After denaturation for 3 min at 94°C, 25 cycles of amplification (94°C – 1 min, 50°C – 30 sec, 72°C – 2 min) were made, followed by an elongation for 7 min at 72°C. The amplification product was cloned in a pGEM-T vector (Promega) and then sequenced. The sequences were aligned by Clustal W 1.7 (Genome Net Clustalw Server). Enzymatic restriction was obtained with HaeIII, Nde II, Hinf I and Rsa I. Three enzymatic units were used for 250 ng DNA, and enzymatic digestion was obtained after 2h at 37°C.

Results

Phenotypic characteristics:

The four isolates showed differences for all the characters measured: linear growth, weight and aggressiveness (Table 1). Lesion lengths on leaves varied from less than 3mm (isolate 95049) to more than 30mm (isolate 95045). On stems they varied from 75mm (isolate 95049) to 126mm (isolate 95044). Variations were also noted in the frequency with which the isolates produced perithecia and ascospores.

Table 1: Phenotypic characteristics of 4 French isolates of *Diaporthe helianthi* / *Phomopsis helianthi*.

Isolates	Origin : Department / latitude	Plant origin	Growth		Aggressiveness ⁽¹⁾ on leaf after 10 days		Aggressiveness ⁽¹⁾ on stem
			linear ⁽²⁾ (1998)	weight ⁽³⁾	Genotype Viki	Genotype Agrisol	After 15 days Genotype 2603 (Sus.)
95004	31 / 43°30'	leaf	38	154	22.0	12.0	126.0
95045	82 / 44°	capitula	50	158	32.5	31.0	108.5
95049	18 / 47°	petiole	39	217	2.5	2.0	75.0
95099	63 / 45°47'	leaf	53	198	25.0	14.0	108.5

⁽¹⁾aggressiveness: lesion lengths in mm.

⁽²⁾colony diameter in mm, after 6 days of growth on malt 1% malt 1.5% agar medium at 23±1°C in the dark.

⁽³⁾weight (mg) after 35 days culture on 1% malt 0.5% agar medium at 23°C (13h light/24h).

During the 1996 experiments, isolates 95045, 95049 and 95099 produced pycnidia and stylospores during in vitro culture, whereas isolate 95004 produced very few pycnidia. Isolates 95004 and 95045 produced no perithecia in the conditions tested in 1996, whereas isolates 95049 and 95099 produced perithecia and ascospores.

During the 1998 experiments, isolates 95004 and 95099 grew abundant white mycelium, above the culture medium, but produced neither pycnidia nor perithecia. Isolate 95045 produced pycnidia but no perithecia during four months growth. Isolate 95049 was the only

one to produce perithecia. Some of the observations made in 1998 are illustrated in Figures 1 and 2.

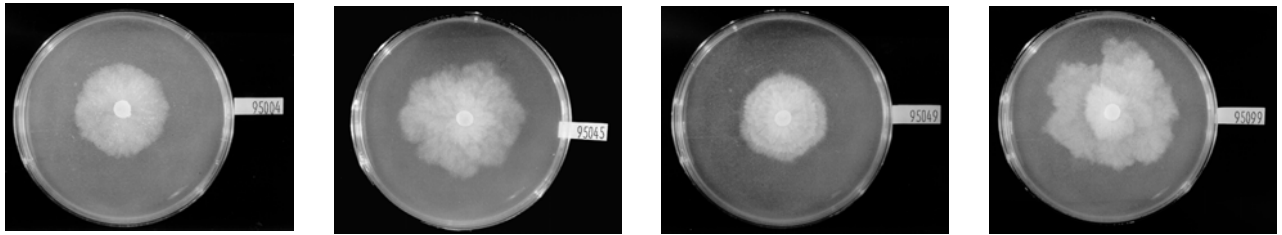


Figure 1: Comparison of colony diameters for *Diaporthe helianthi* / *Phomopsis helianthi* isolates 95004, 95045, 95049 and 95099, after 6 days growth on 1% malt 1.5% agar medium at $23\pm 1^\circ\text{C}$ in the dark.

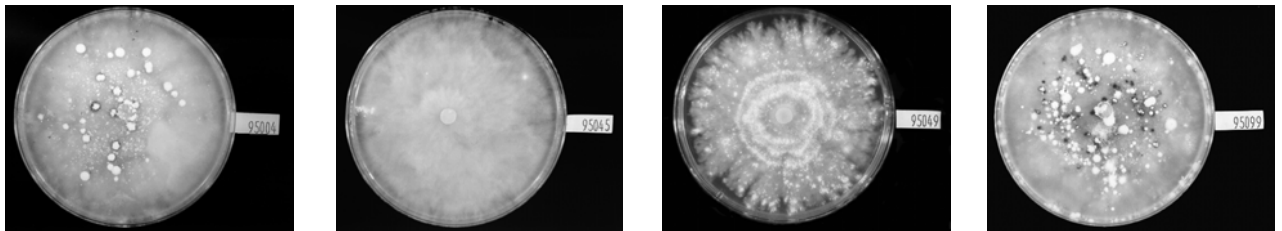


Figure 2: Macroscopic appearance of *Diaporthe helianthi* / *Phomopsis helianthi* isolates 95004, 95045, 95049 and 95099, cultures on 1% malt 1.5% agar medium, after 1 month growth at $23\pm 1^\circ\text{C}$ in the dark.

2. Molecular characteristics:

The number of fragments obtained by AFLP varied from 4 to 111 among the 52 isolates studied at first. The four isolates studied in detail were representative of this variability: 95004 had 33 fragments; 95045: 48 fragments; 95049: 111 fragments and 95099: 10 fragments.

Amplification of ITS region of the 4 isolates gave a fragment of about 580bp (Figure 3). Sequencing of the fragment containing ITS 1, the 5.8S and ITS 2, showed that it is made up of 577bp for isolates 95004 and 95099 and of 578bp for 95045 and 95049. Ordering of these sequences indicates homology of 98 to 99%. Restriction by four enzymes gave the same fragments for all four isolates, with one exception (95099/Nde II) but some of them were too small to be visible on agarose gel after staining with ethidium bromide (Figure 4). In all cases, the ITS showed: 6 restriction sites for Hae III at positions: 100, 165, 429, 461, 474, 478; 3 restriction sites for Hinf I at positions: 275, 283 and 375; 4 restriction sites for Nde II at positions: 45, 354, 399 and 551 except for isolate 95099 for which site 551 was lacking. There were no restriction sites for Rsa I. The fragments obtained with the three enzymes had 4, 13, 32, 65, 99, 100 and 265 bp with HaeIII; 8, 92, 202 and 275 bp with Hinf I; 45, 309, 45, 152 and 26bp with Nde II and 45, 309, 45 and 178bp for isolate 95099.

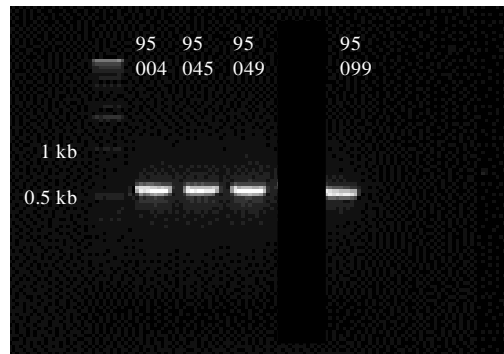


Figure 3: Amplification profile of ITS of DNA of 4 isolates of *Diaporthe helianthi* / *Phomopsis helianthi* (isolates 95004, 95045, 95049 and 95099).

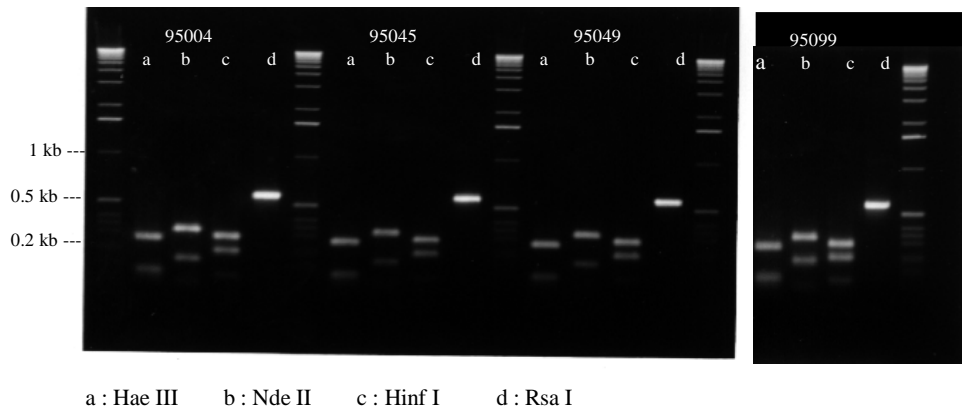


Figure 4: Enzymatic restriction patterns on the ITS sequence of DNA from 4 isolates of *Diaporthe helianthi* / *Phomopsis helianthi* (isolates 95004, 95045, 95049 and 95099) with the enzymes Hae III, Nde II, Hinf I et Rsa I.

Conclusion

The four *P/D.helianthi* isolates showed differences in their phenotype, *in vitro* growth, morphology and aggressiveness on sunflower leaves and stems. The same type of results were obtained by Viguié et al (1999) on a wider range of French isolates. Growth rate on agar medium was not correlated with aggressiveness on sunflower plants, so the two observations can be considered as complementary in the characterisation of *P/D.helianthi* isolates.

Comparable variability was found in the AFLP analyses on the whole genome. The 4 isolates gave very different AFLP. It can be suggested that the wide variability shown in the species *P./D.helianthi* is due to variability in genome size, probably resulting from chromosome rearrangements as has already been shown in many other fungal species (Zolan,1995). In contrast, ITS sequences (which concern only part of the genome) showed only 1 to 2% differences between the four isolates, indicating that they do belong to the same species.

This study appears to be the first to be made associating *in vitro* techniques (phenotypic and molecular studies) and *in vivo* measurements of aggressiveness of *P./D.helianthi*. It confirmed the wide variability which exists among French isolates, and suggests that it would be of interest to make similar studies with a wide range of isolates from different countries to obtain an overall knowledge of the diversity within this species.

RÉFÉRENCES

- BERTRAND F., TOURVIEILLE de LABROUHE D., 1987. Phomopsis du tournesol : test de sélection. Inf. Tech. CETIOM, **98**(I), 12-18.
- INRA, 1996. Caractérisation et génétique des résistances au phomopsis. Rapt. Activité INRA / Promosol 1995, Promosol, 20 Rue Bachaumont, 75002 – Paris, France, 119-120.
- LAVILLE J., 1986. Cahier Technique Tournesol CETIOM, 20-24.
- MOHAMMED C., 1987. Etude comparée des 5 espèces européennes d'*Armillaria* appartenant au complexe *mellea*. Thèse de Doctorat, 208p.
- MIHALJCEVIC M., MUNTAÑOLA-CVETKOVIC M., 1985a. Responses of sunflower plants to different *Phomopsis* isolates. I. From sunflower. Proc. 11th Int. Sunflower Conf., Mar del Plata, Argentine, 10-13 Mars, 413-418.
- MIHALJCEVIC M., MUNTAÑOLA-CVETKOVIC M., 1985b. Responses of sunflower plants to different *Phomopsis* isolates. II. From weeds. Proc. 11th Intern. Sunflower Conf., Mar del Plata, Argentine, 10-13 Mars, 419-424.
- MUNTAÑOLA-CVETKOVIC M., MIHALJCEVIC M., PETROV M., 1981. On the identity of the causative agent of a serious *Phomopsis-Diaporthe* disease in sunflower plants. Nova Hedwigia, **34**, 417-435.
- NEI M. et LI W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. Proceedings of the National Academy of Sciences of the U.S.A., **79**, 5269-5273.
- SAYS-LESAGE V., ROECKEL-DREVET P., TOURVIEILLE de LABROUHE D., NICOLAS P., 1997. Etude de la variabilité moléculaire du phomopsis du tournesol (*Diaporthe helianthi* / *Phomopsis helianthi*) sur une même parcelle. Premières Rencontres de Mycologie-Phytopathologie, Aussois, France, 5-9 Octobre, 217.
- SAYS-LESAGE V., ROECKEL-DREVET P., TOURVIEILLE J., WALSER P., TOURVIEILLE de LABROUHE D., NICOLAS P., 1999. Variabilité moléculaire intraspécifique de *Diaporthe* / *Phomopsis helianthi* à l'échelle d'une parcelle et d'un pays. *In prep.*
- TOURVIEILLE de LABROUHE D., VEAR F., HABOUZIT J., 1986. Culture du tournesol sous tunnel en filet avec humectation contrôlée pour l'étude du *Sclerotinia sclerotiorum*. Inf. Tech. CETIOM, **96**, 20-28.
- VIGUIE A., 1999. Etude du pathosystème *Phomopsis helianthi* / *Diaporthe helianthi* Munt.-Cvet. – tournesol (*Helianthus annuus* L.). Thèse de Doctorat, Université Blaise Pascal, Clermont-Ferrand, France, 84p.
- VIGUIE A., VEAR F., TOURVIEILLE de LABROUHE D., 1999. Interactions between French isolates of *Phomopsis* / *Diaporthe helianthi* Munt.-Cvet. *et al.* and sunflower (*Helianthus annuus* L.) genotypes. European Journal of Plant Pathology, **105**, 693-702.
- VUKOJEVIC J., FRANIC-MIHAILOVIC D., MIHALJCEVIC M., 1995. *In vitro* production of perithecia of *Diaporthe helianthi*. Mycopathologia, **132**, 21-25.
- ZOLAN M.E., 1995. Chromosome Length Polymorphism in Fungi. Microbiological Reviews, **59**(4), 686-698.