Development of a test to diagnose the presence of sunflower downy mildew (*Plasmopara halstedii*) in seed samples

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Summary:

Plasmopara halstedii, an obligate parasite that causes sunflower downy mildew, can be transmitted by seed. Demonstration of seed dissemination through biological tests is not always easy and is time consuming, so we attempted to develop a molecular diagnostic. First, three methods of DNA extraction from sunflower seed were compared and it was found that their efficiencies varied by a factor of 8.5. Primers specific to sunflower downy mildew are available and they made it possible to amplify by PCR a fragment specific to *P. halstedii*, which can be observed on agarose gel after staining with ethidium bromide. Using the most efficient extraction method, the *P. halstedii* fragment can be detected directly after PCR when DNA was extracted from seed frozen soon after harvest, whereas no visible PCR amplification occurs when both others extraction methods were used. If the DNA are transferred by the Southern technique onto a membrane which is then hybridized with the specific fragment labelled with P^{32}, sensitivity and frequency of detection are increased, but it would be useful to develop a non-radioactive method to use as a diagnostic.

Résumé :

L'agent du mildiou du tournesol (*Plasmopara halstedii* (Farl) Berl et de Toni) est un parasite obligatoire, qui peut se conserver dans les semences. Sa détection biologique n'étant pas très fiable, nous essayons de mettre au point un test de diagnostic moléculaire de sa présence dans les graines. Nous avons tout d'abord comparé 3 méthodes d'extraction de l'ADN des semences. Le rendement de l'extraction a été augmenté d'un facteur 8,5. Nous disposons d'amorces permettant l'amplification spécifique du mildiou du tournesol. Grâce à celles-ci, nous pouvons amplifier par PCR un fragment spécifique de *P. halstedii*, observable sur gel, après coloration par le bromure d'éthidium. L'une des méthodes d'extraction paraît plus performante que les autres. En effet, par cette méthode, le fragment spécifique peut être détecté directement après PCR, lorsque l'ADN a été extrait de graines infectées congelées après récolte, alors qu'aucune amplification n'est visible sur gel lorsque les deux autres techniques ont été employées. Si les acides nucléiques sont transférés par Southern sur une membrane et que cette membrane est hybridée avec le fragment spécifique marqué au ^{32}P, alors la sensibilité et la fréquence de détection sont augmentées, mais cette technique utilisant la radioactivité doit être améliorée dans un but de mise au point d'un kit de diagnostic.

Introduction :

The obligate parasite *Plasmopara halstedii* (Farl) Berl and De Toni is an Oomycete, that causes downy mildew of sunflower (*Helianthus annuus* L.). This pathogen is conserved, in the form of oospores, over several years in soil crop debris. Primary infections are produced by zoospores at the young roots of sunflowers after germination of these oospores. The primary infection may cause dwarfing or even seedling death. Sporulation on leaves gives rise to a second generation of zoospores, that can cause secondary, aerial infections. Leppik (1962) suggested that sunflower downy mildew could be seed-borne. Recently, optical and electron microscopy studies have confirmed that the fungus can be found in the form both of mycelium and oospores between the hull and the kernel, on, or close to, the seed coat (Meliala and Tourvieille, 1998*).* The fact that *P.halstedii* could be conserved in seed has suggested that the disease may have been introduced into some countries from infected seed (Leppik, 1962; Zimmer, 1971; Döken, 1989).

In the last few years, new races of downy mildew have been identified in the USA (Gulya *et al.,* 1991), in Spain (Melero-Vera *et al*., 1996) in France (Roeckel-Drevet *et al.*, 1997), in South Africa (Viljoen *et al*., 1997), in Iran (Zad and Rahmanpour, 1997) and in Italy (Zazzerini and Tosi, 1998). Some of these races may have come from mutations of existing races, others may have been introduced on infected seed. The main control of *P. halstedii* on the sunflower crop comes from genetically resistant varieties. However, not all varieties grown, for example in France, are resistant to all the races known in the country. The other control method has been to use a seed treatment with the fungicide, metalaxyl, but, since 1995 a considerable number of downy mildew isolates not susceptible to this treatment have been identified. In France, more than 50% of the isolates analysed were of this type (Albourie *et al.*, 1998). In the USA, T. Gulya (personal communication) reported the same problem. This development of new races worldwide is preoccupying for the crop and official authorities (in France, SPV, GEVES) would appreciate a method to check the absence of downy mildew in seed to be exported or imported.

To detect the presence of a fungus in seed, a sample can be sown and the plants observed to see whether they show symptoms. However, this method is long (several weeks) and uncertain because only a small proportion of seed harvested on a downy mildew infected plant produces diseased plants, while the fungus may remain in the soil or on the hull and be able to infect other plants or crops in other years. Thus, a sure and rapid diagnostic test would be very useful. The approach described here used RAPD, with amplification of DNA fragments specific either to the species or to a particular race (Dobrowski and O'Brien, 1993; Murillo *et al*., 1998). Cloning and sequencing of these fragments were realised in the aim of producing SCAR primers, which could then be used to amplify a single fragment unique to *Plasmopara halstedii*.

Materials and Methods

Sunflower seed samples:

The samples were obtained from CETIOM, Rustica Prograin Genetique and INRA.

Samples GM 99.1 to 99.6 came from seed lots that had been dried and stored for 18 months at 4°C. The sample GM 99.7 was not dried, but frozen from fresh. Samples GM 99.26 and 99.30 were checked for presence of *Plasmopara halstedii* and stored at 4°C for a few weeks.

Determination of SCAR primers:

The DNA from five French races (100=1, 300=D, 700=C, 703=B, 710=A) was used for RAPD amplification with the primer OPJ15 (Operon Technologies Inc. (CA, USA) kit). A fragment polymorphic for race 100 was cloned and sequenced. From this sequence, two primers were synthesised (8MREV and 8MFW) which gave amplification of a fragment specific to *Plasmopara halstedii* (Roeckel-Drevet *et al*., 1999).

DNA extraction techniques:

Three techniques were studied: (1) the method of Dellaporta *et al*. (1983) with minor modifications; (2) the method using the DNA extraction kit for plants and fungi from Amersham (RPN 8511) (Amersham International plc, England); (3) a combined method using some of the techniques of the two preceding methods.

Amplification:

The reaction mixture contained 100 µM of each dNTP, 2,5 µl of 10X reaction buffer (100 mM Tris HCl, 15 mM MgCl2, 500 mM KCl, pH 8.3), 0.2 µM of each primer, 1,5 U of Taq DNA Polymerase (Appligene Oncor). After denaturation for 3 min at 94°C, 25 amplification cycles (94°C-1 min, 50°C-30 s, 72°C-2 min) were carried out then completed by 7 min at 72°C.

Detection of the amplified fragment:

The amplification product was placed on a 1.4% agarose gel and subjected to electrophoresis. The gel was then observed under UV, after staining with ethidium bromide. If no fragment was visible, the gel was transferred onto a nylon membrane, that was then put in contact with a radioactive probe complementary with the sequence of the fragment required. Hybridisation was shown by autoradiography. This technique improved the limit of detection by a factor of 1000 (Roeckel-Drevet *et al*., 1999).

Results

Comparison with the infected seedling test:

DNA extractions were made from sunflower seedlings infected with the usual seedling test and with or without sporulation symptoms on cotyledons. In both cases, PCR showed the presence of the downy mildew specific DNA fragment.

Comparison of three types of DNA extraction from seed samples:

The extractions were made using 500 mg seed samples.

Method	1-Dellaporta	2- Amersham kit	3-combined method
mean yield (µg DNA/mg seed) with seed samples GM 99.1 to 99.6	0.82	1.47	-
mean yield (µg DNA/mg seed) with seed samples : GT 99.8, GM 99.7, GM 99.26, GM 99.30	-	1.69	5.35

The method incorporating parts of the other two gave the best results. It was used to extract DNA from 15 seed samples, the contamination of which had previously been checked by optical microscopy. The average yield obtained was 7.1 µg DNA/ mg seed weight. The extraction efficiency was thus increased by a factor of 8.5 between method 1 and method 3.

PCR results and hybridisation:

The PCR was made with 50 ng DNA extracted from seed.

Seed sample	Extraction technique	DNA yield µg DNA/mg seed	PCR	PCR + hybridisation
GM 99.1	kit	1.43	-	+
GM 99.2	kit	0.94	-	-
GM 99.3	kit	2.2	-	-
GM 99.4	kit	1.77	-	-
GM 99.5	kit	1.35	-	++
GM 99.6	kit	1.11	-	++
GM 99.7	kit mixed	1.90 5.35	- ++	++ +++
GT 99.8 (control)	mixed	2.35	-	-
GM 99.26	mixed	7.91	-	++
GM 99.30	mixed	5.8	-	+

- : no signal detected, + : detectable signal, ++ : well visible signal, +++ : strong signal

The table shows that detection can be possible directly after PCR amplification (GM 99.7), and that the strength of signal is not correlated with the DNA content of the base solution obtained after extraction and purification. It may also be noted that, although all the samples tested came from sunflower plants infected with downy mildew, the fungus was not always detected, even after hybridisation with a radio-active probe.

Discussion

The results of the extraction tests show considerable improvement in the quantity of DNA extracted from seed samples using a method developed partly from that of Dellaporta and partly from the Amersham kit. It should be noted that an increase in extraction yield does not necessarily give an increase in the proportion of fungal DNA extracted. There was no correlation between the DNA yield in the extracts and the strength of the *Plasmopara halstedii* signal. We can suppose that PCR inhibiting compounds are not eliminated equally between the different methods. In order to verify this hypothesis, it will be necessary to use an internal control for the PCR reaction, based on the amplification of a sunflower fragment. We can also suppose that the efficiency of fungal DNA extraction is different between the methods tested. The separation of the hulls from the kernels could be an improvement of the technique as it would probably increase the proportion of fungal DNA in the extract. However, we think that the most important factor, in this fungal DNA recovery, is the quality of the material before extraction. Indeed, in this study, a positive signal of the presence of *P. halstedii* DNA was obtained directly from PCR only with the sample GM 99.7, extracted by the combined method. This sample had the particularity of having been a sample of fresh seed which was frozen very shortly after harvest, without drying. All the other seed samples were dried normally and kept at 4°C for several weeks to several months. It can be suggested that freezing made it possible to maintain the fungal mycelium in an extractable state. This mycelium is delicate and any shock will cause its breakdown; with seed drying, the mycelium may lose its nuclei and so little DNA remains. The mycelial walls of the fungus could still be detected by immunological type reactions, from specific antigens for *P. halstedii* but this type of detection would not make it possible to determine the risk of introducing viable downy mildew into a field. For that, it will be necessary to investigate what quantity of fungal DNA in seed corresponds with appearance of downy mildew symptoms in a biological test, to decide whether the detection after hybridisation is of use.

Conclusions

Yield of DNA from extraction has been improved by a factor of 8.5, but it will be necessary to increase the proportion of fungal DNA in the total extract. This could be done using the hulls only. The main problem will be concerning seed storage, as seed is normally dried at 4°C and then stocked dry. For downy mildew diagnosis directly by PCR, it would be necessary to freeze seed directly after harvest. This might be possible for local seed productions destined for export, but the diagnostic could not be made on imported seed. Thus, further work is necessary to improve detection on normally conserved seed. At present, the test requires use of radio-activity and it would be useful to simplify this procedure so that downy mildew could be diagnosed in any seed sample.

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