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Detection of *Plasmopara halstedii* in sunflower seeds: A case study using molecular testing

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ABSTRACT

Plasmopara halstedii (Farl.) Berl. & De Toni causing downy mildew of sunflower is responsible for considerable economic losses worldwide. Because *P. halstedii* can be seed-transmitted, monitoring of seeds for pathogen contamination is important for the sunflower seed trade. The relevance of asymptomatic or latent infections as factors of disease spread have not been studied by molecular techniques. A molecular marker based on a putative effector gene of *P. halstedii* was used to examine the pathogen's presence in asymptomatic sunflowers growing near patches of mildewed plants in naturally infected fields. The method based on conventional PCR was highly sensitive for detection of *P. halstedii* in DNA from whole seeds. By the application of this protocol, we found that all seed samples obtained from symptomatic plants amplified the expected fragment, whereas the diagnostic marker identified the presence of pathogen in one out of 21 asymptomatic plants. Possible uses of this marker to detect downy mildew in seed from asymptomatic plants or for race identification are discussed.

1. Introduction

Sunflower (*Helianthus annuus annuus* L) is the third most important oilseed crop worldwide, after soybean and rapeseed, and is used to produce oil, meal, confectionary products, animal feed (stalks) and a number of industrial applications, e.g., basic component for polymer synthesis, biofuel, emulsifier or lubricants (FAO 2010; Dimitrijevic and Horn 2018). *Plasmopara halstedii* (Farl.) Berl. & De Toni (Hall 1989), is a devastating pathogen causing downy mildew of sunflower. The global impact on yield has been estimated as a 3.5% reduction in commercial seed production, although the yield losses can reach 100% in a contaminated field (Gascuel et al. 2015).

Systemic infection of *P. halstedii* through hypocotyls and roots during sunflower emergence causes symptoms such as dwarfism, stunting, a chlorotic mosaic in the leaves, reduced growth and alterations in secondary metabolism (Spring et al. 1991). When the infected plants reach the reproductive stage, the contaminated seeds are rudimentary and considerable smaller than healthy seeds (Seijas de Varela, 1980). When infections occur after four-leaf stage the resulting plants typically appear healthy but can carry the pathogen in a latent mode; these plants have been reported surrounding patches of diseased sunflowers (Cohen and Sackston 1973; 1974). The symptomless infected plants develop a nearly normal phenotype, although an unnusual angle of the mature capitulum and retarded senescence have been described (Spring, 2001). Plants with latent infection may produce seeds containing quiescent oospores that, in turn, may give rise to more plants with latent infection, accounting for the widespread dissemination of the disease before any plant with typical symptoms could be detected (Sackston, 1981). The transition from local leaf infections to systemic infection in the upper leaves (secondary infections) can result in contaminated seeds of normal appearance, thus increasing the risk of the disease spreading (Spring, 2009).

In this way, the sunflower seed trade may introduce the pathogen to areas free of downy mildew or cause the spread of novel races and/or fungicide-tolerant strains. For this reason, phytosanitary tests designed to certify the absence of *P. halstedii* in traded

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seed are needed (Gascuel et al. 2015). Various methods have been used for the identification of the pathogen, such as specific oligonucleotides, an enzyme test (ELISA) and fatty acid patterns as markers to differentiate infected from healthy sunflower seeds (Viranyi and Spring, 2011). A molecular test for the direct detection of *P. halstedii* in sunflower seeds was developed by Ioos and collaborators, consisting of markers targeting DNA sequences of the large ribosomal subunit *(LSU)* (Ioos et al. 2007). Later, a real time PCR (qPCR)-based test was proposed for *P. halstedii* diagnosis, achieving a detection threshold of one infected seed in 1000 healthy seeds (Ioos et al. 2012). The attempts by Ioos et al. (2007; 2012) and Giresse et al. (2008) to establish a diagnostic test did not become a suitable method in practice yet, mostly due to difficulties in achieving a representative sampling method to detect traces of pathogen in large batches of seeds (Spring, 2019).

The detection of *P. halstedii* is relevant in sunflower shipments that could contain both symptomatic seeds along with normal-sized seeds carrying the pathogen in latent form. Latent infections may lead to asymptomatic plants with little impact on crop yield but with high influence on the dynamics and transmission of the disease by seeds (Spring et al. 2009).

Molecular markers based on *Expressed Sequence Tags (ESTs)* derived from *P. halstedii* genes transcribed during the infection were used in studies on the genetic variability and phylogeny of the pathogen (Giresse et al. 2007; Delmotte et al. 2008). With the aim of studying the occurrence of latent infections transmitting the pathogen to the seeds in naturally infected sunflower fields, a PCR-based test was applied on apparently healthy plants surrounding downy mildew-infected patches. We tested the utility of an EST-based marker corresponding to a gene coding for a putative pathogenicity effector for *P. halstedii* diagnosis.

2. Materials and methods

2.1. Field sampling

During trips to monitor downy mildew in Buenos Aires province, Argentina, three affected sunflower fields were sampled for this study (ASC, MEC, BAL), and their main features are shown in Table 1.

Variability in dwarfism expression was observed, while no signs of secondary infections in the surrounding plants evaluated were evident. At physiological maturity (R-9), heads in each field were collected from symptomatic and from normal sized plants growing close to the downy mildew patches (2 m or less) (Fig. 1). The heads from both types of plants were manually harvested and the seeds were stored separately.

2.2. Sample preparation and DNA extraction

Sunflower heads collected in each location were classified as symptomatic (S) or asymptomatic (A). From each class, three groups composed of seven randomly selected heads were made. Three seeds were collected from each of these seven heads, thereby forming a 21 seed pool as shown in Fig. 2.

The seeds from each pool were reduced to a fine powder by grinding under liquid nitrogen with a mortar and pestle, and three replicates of DNA extractions were performed from the resulting powder. With the aim of analyzing a larger number of individuals, additional samples were created by pooling 200 seeds from each of the seven asymptomatic heads of each location, and DNA isolation was performed.

For the DNA isolation, a CTAB protocol was followed (de León 1994) with modifications consisting of a pretreatment of the achenes with a solution of sodium hydroxide 1% w/v for 10 min to remove pigments and dirt from the hull. This treatment was followed by three rinses with distilled water. Special precautions to avoid cross contamination were taken during DNA isolation procedure. The absorbance ratio of the obtained samples was tested at 260/280 nm in a Nanodrop and the samples were electrophoresed on a 1% agarose gel to evaluate DNA integrity.

2.3. Molecular analysis

The EST-derived marker PHA 42 was selected for molecular analysis (Forward 5′->3′: GGATGTTGCTCGTCAAGTAGC, Reverse 5′->3′: ACGCATCCTACGCATTCAAC) (Giresse et al, 2007). The amplifications were carried out in a final volume of 20 ul, with 10 ng of genomic DNA and 38 cycles at an annealing temperature of 57 °C. A DNA sample from seeds of healthy sunflower plants, a DNA sample obtained from *P. halstedii* fructifications (so called sporulation) and a sample without DNA were included as controls.

To evaluate the specificity of PHA 42, a sample of DNA extract from severely infected seeds available in the laboratory and another DNA sample extracted from *P. halstedii* sporulation were amplified as described above, cloned into the pGEM-T vector (Promega) and sequenced (Macrogen Inc., Seoul, Korea del Sur). The nucleotide sequences obtained were analyzed against the Genbank database using BLASTn (www.ncbi.nlm.nih.gov).

In order to determine the sensitivity threshold of the molecular test, six solutions of DNA containing 10 ng/ul of healthy sunflower seed DNA mixed with decreasing amounts of DNA from severely infected seeds was prepared: D1: 10 ng (ng)/microliter (ul), D2: 0.1 ng/ul, D3: 0.01 ng/ul, D4: 1 picograms (pg) /ul, D5: 0.1pg/ul y D6: 0.05 pg/ul. PCR reactions to amplify the PHA 42 marker were performed as described above using one microliter from each of the D1-D6 solutions as DNA template.

3. Results

The DNA from whole seeds showed no signs of degradation (Fig. 3) and the ratio of absorbance at 260/280 nm ranged 1.8–1.9. The average DNA yield was 500 ng/ul. Amplification of the PHA 42 marker from infected sunflower seed samples and from pathogen sporulation samples resulted in the expected 252 bp amplicon. After cloning and sequencing these samples, the results obtained through the *in silico* analysis are shown in Table 2. Both types of samples amplified the sequence corresponding to *P. halstedii* genome with high values of identity and query coverage. The targeted genomic region corresponded to a putative RxLR effector protein from *P. halstedii*.

All samples obtained from symptomatic plants amplified PHA 42 with the expected band size (Fig. 4). The controls validated that the bands corresponded to the *P. halstedii* genome. No amplifica-

Table 1
Characteristics of the sunflower fields with downy mildew sampled in this study.

Location	Year	Disease incidence (%)	Downy mildew patch size (m^2)	Fungicide seed treatment	Primary use
Hilario Ascasubi (ASC)	2016	<1	3	Apron Gold-Dynasty	Seed
Mechongué (MEC)	2018	5–6	25	Unknown	Oil
Balcarce (BAL)	2017	1–2	10	Apron Gold-Dynasty	Oil

Fig. 1. A = Sunflower field with patches of *P. halstedii*. B = Detail of mildewed plant showing dwarfism, chlorotic mosaic in leaves and small erect head. C = Seeds collected from symptomatic plants (dashed line) and from asymptomatic plants growing near mildewed plants (solid line).

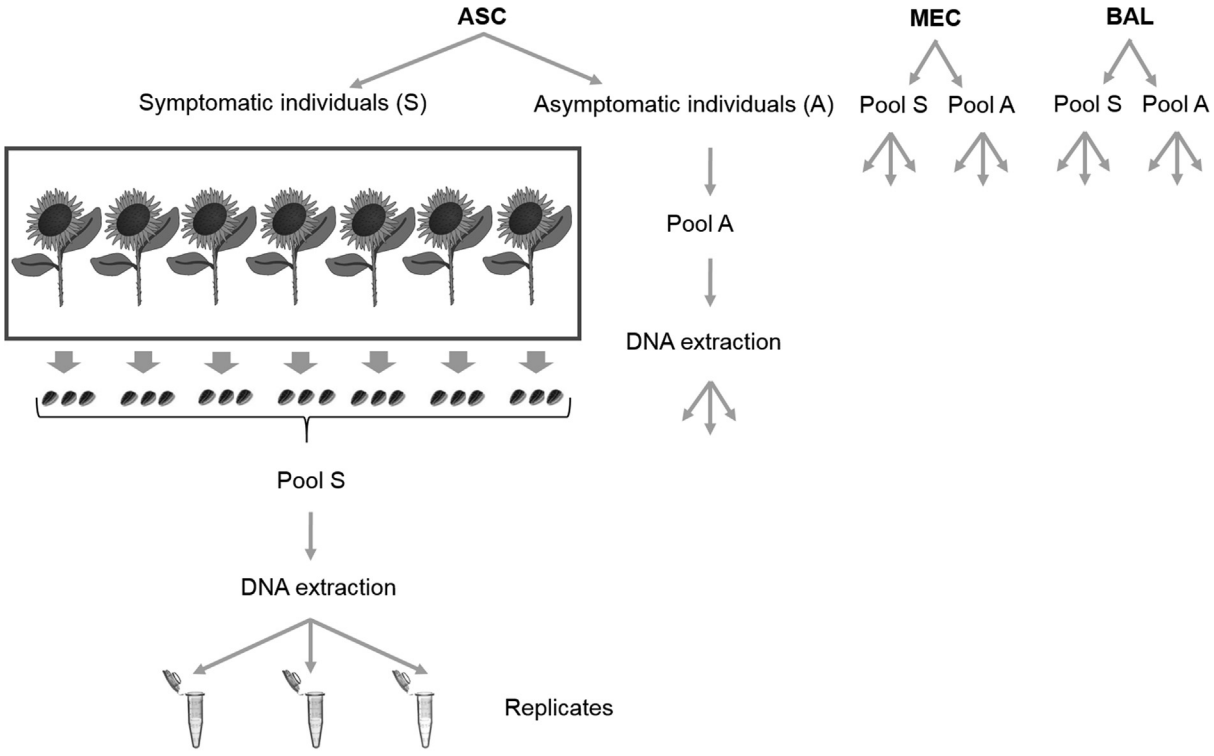

Fig. 2. Schematic representation of seed sample at three mildew infected fields (ASC, MEC, BAL) and DNA extraction design.

tion was observed in any of the 21-seed pool samples collected from asymptomatic individuals. When the presence of *P. halstedii* was investigated on the 200-seed pools obtained from 21 asymptomatic heads, one amplicon was observed and corresponded to an individual collected at the MEC location (Fig. 5).

Fig. 6 shows amplification of PHA 42 in solutions composed of 10 ng/ul of healthy sunflower seeds DNA mixed with decreasing amounts of DNA of infected seeds to evaluate the sensitivity of the method. Amplicons were obtained for the set of dilutions D1 to D5, and D6 showed no amplification. The detection threshold

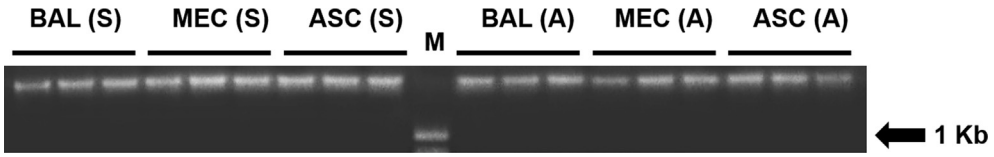

Fig. 3. Agarose electrophoresis with genomic DNA corresponding to the 21-seed pools of symptomatic (S) and asymptomatic (A) individuals collected at three locations BAL, MEC and ASC. M: 100 bp DNA marker. Solid bars indicate three replicates from each 21-seed pool.

Table 2
Characteristic of the sequences obtained from two types of DNA samples: infected seeds and pathogen sporulation. Parameters of the alignment (score, E-value, query cover); fragment size, ID accession in GenBank and description of gene, and the codifying protein.

DNA sample	Significant alignment	Score (bits)	E-value	Query cover (%)	Fragment size (pb)	Accession	Gene	Protein
Symptomatic seeds Sporulation	*Plasmopara halstedii*	466	7e-131	99	252	NW_ 020187567.1	PHALS_12922	Effector RxLR

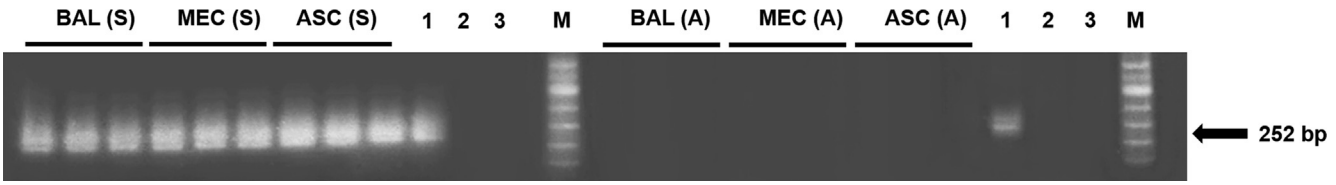

Fig. 4. PCR amplification with PHA 42 primer of symptomatic (S) and asymptomatic individuals (A) collected at locations BAL, MEC and ASC. Solid bars indicate three replicates from each 21-seed pool. 1 = *P. halstedii* DNA, 2 = healthy sunflower seed DNA, 3 = negative control, M = 100 bp DNA marker.

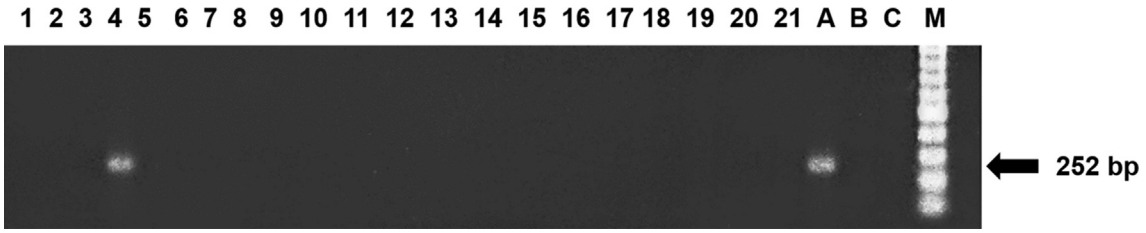

Fig. 5. Agarose gel containing PCR amplified products from PHA 42 marker in 200-seed pools from 21 asymptomatic heads. lanes 1–7 = MEC, 8–14 = ASC, 15–21 = BAL. A = *P. halstedii* DNA, B = healthy sunflower seed DNA, C = negative control, M = 100 bp DNA marker.

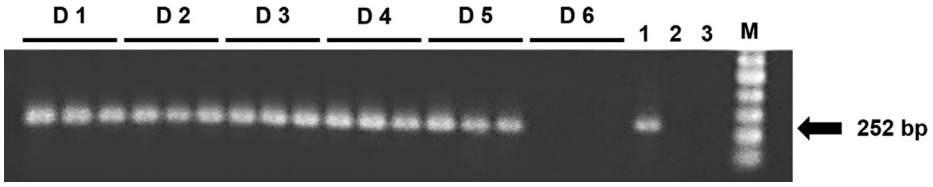

Fig. 6. Sensitivity of PHA 42 marker for detecting *P. halstedii* in mixtures of DNA from pathogen and host. D1-D6 refers to PCR performed on samples containing 10 ng/ul of DNA from healthy sunflower seeds and decreasing amounts of DNA from symptomatic seeds: D1 = 10 ng/ul, D2 = 0,1 ng/ul, D3 = 0.01 ng/ul, D4 = 1 pg/ul, D5 = 0.1 pg/ul, D6 = 0.05 pg/ul. Solid bars indicate PCR replicates. 1 = P. halstedii DNA, 2 = healthy sunflower seed DNA, 3 = negative control, M = 100 bp DNA marker.

was thus established in 0.1 picograms of infected seed DNA per microlitre.

4. Discussion

P. halstedii is a devastating quarantine pathogen for which the transmission to seeds in the field is not completely understood. We achieved specific amplification of the *P. halstedii* PHA 42 marker in DNA from whole seed samples containing DNA from both pathogen and host. These results are relevant for molecular protocols applied on samples containing a high proportion of hull, where polyphenol compounds or some contaminants may cause DNA degradation or PCR failure. The inclusion of the hull fraction in samples is essential because it has been demonstrated that the inner layers of pericarp and seed coat are the main reservoirs of pathogen in seeds collected from sunflower plants systemically infected by *P. halstedii* (Döken, 1989).

All DNA samples obtained from control healthy plants lacked the *P. halstedii* band. The specificity of the marker is likely due to its design based on a *P. halstedii* cDNA (Giresse et al. 2007) that has been characterized as encoding a putative effector protein acting in the cytoplasm of plant cells. Generally, an effector is defined as a protein produced by the pathogen that alters the cellular function of the host, interfering with the plant defense machinery. This family of proteins is strongly associated with virulence of the pathogen: they are secreted by feeding structures, the haustoria, and translocated to the host cell through mechanisms that likely

involve conserved amino acid patterns such as the RxLR (Arg-any amino acid-Leu-Arg) motif (Gascuel et al., 2016a; 2016b). Considering the high specificity of the marker PHA 42, the pathogen's presence was evaluated in seed samples by scoring the amplicons in the agarose gels.

Pooling of seeds from different individuals was thought to increase the likelihood of sampling the pathogen's biomass, even if present in very low proportion. All the replicates of the 21-seed pools of symptomatic individuals resulted in *P. halstedii*-specific amplifications. Amplification was not observed for samples from seed pools derived from asymptomatic plants and this was interpreted as absence of the pathogen. However, two points must be considered: i) it is known that the pathogen is transmitted to seeds in low proportion in secondary or latent infections (Meliala et al. 2000), ii) the number of seeds sampled per head (n = 3) in the initial molecular analysis might be insufficient to recover those infected seeds probably present in low numbers.

When we increased the number of investigated seeds per head (n = 200) we observed one positive result out of 21 asymptomatic individuals studied, with a sensitivity threshold of 0.1 pg/ul. It is worthwhile to note that this threshold could be even lower given that the sample obtained from whole seeds contained *P. halstedii* as well as sunflower DNA. A head of cultivated sunflower has 1000-1500 achenes on average (FAO 2010), so the number of seed sampled in this instance represented a 20 or 18% of the progeny.

Ioos and collaborators developed a detection method based on real time PCR (qPCR) with a detection threshold of one contaminated seed in 1000 healthy seeds (Ioos et al. 2012). In regard to the pathogen detection, this could represent a scenario in which one seed from a symptomatic plant is present in a seed batch. In the present study, however, the detection threshold was determined by mixing different concentrations of DNA from contaminated seeds with a fixed concentration of healthy sunflower DNA. Although the DNA concentration of the pathogen is uncertain in a sample of ground whole seeds, this method was adopted for two reasons: i) in our experience, it is technically challenging to thoroughly grind 1000 seeds and incomplete grinding can result in low quality DNA, and ii) controlled mixtures of DNA could better reflect the real scenario because it includes the possibility of latent infections rather than mixtures containing exclusively healthy and contaminated seeds.

The application of qPCR represented a significant advance in terms of speed, reliability and reproducibility in comparison with conventional PCR (PCRcn) for pathogens diagnosis. However, qPCR is not always synonymous with higher detection sensitivity compared with PCRcn, since the performance of the method depends upon many variables (Bastien et al. 2008). Besides, the diagnostic protocol proposed in this study could be adopted by laboratories with basic equipment since it is rapid, reliable, easy to conduct and cost effective.

The molecular test detected *P. halstedii* in achenes derived from one plant with normal appearance, confirming the potential for transmission of the pathogen by the seeds, in agreement with previous microscopic investigations that proved the presence of downy mildew hyphae in achenes from latent infected plants (Spring, 2001). Interestingly, the sunflower field from which the asymptomatic plant was found to contain the pathogen in the seeds, was most diseased field in comparison with the other sampled fields. More extensive sampling is necessary to confirm this result and to determine the frequency of infected seed production from asymptomatic plants close to plants showing downy mildew symptoms.

In general, seeds from a symptomatic plant are considerable smaller than the asymptomatic ones. Consequently, most of these small seeds would not be retained by the threshing system of the harvesting machine, and thus would be returned to the field. These small seeds would rarely produce new infected plants because their embryos are mostly immature or absent, although the spores present in achenes can remain in the soil and eventually infect the subsequent sunflower crop (Cohen and Sackston 1974). Additionally, we observed a remarkable size gradation within samples from symptomatic heads Consequently, larger-sized infected achenes could be retained by the harvesting machine and contaminate seed lots together with the healthy seeds.

In respect to management of the disease, roguing refers to the act of identifying and removing plants with undesirable characteristics to preserve the quality of the crop being grown (Wright 1980). In fields intended for commercial hybrid seed production, the early roguing of the whole mildewed plants is mandatory in order to avoid disease dispersal. The proposed molecular test could be useful to determine the extent to which asymptomatic infected plants surrounding the diseased patches should be removed.

Finally, a molecular diagnostic for *P. halstedii* race identification would be a useful tool as it would obviate the need to evaluate isolates on a large set of differential lines (Crandall et al 2018). The effector genes are promising markers to correlate the pathogenicity profiles of the strains with polymorphisms in the nucleotide sequences, since these genes have significant roles during infection (Gascuel et al., 2016a; 2016b). Preliminary studies on the sequence variability of PHA 42 in Argentinean *P. halstedii* samples showed new single nucleotide polymorphisms and insertions –deletions (Martínez et al 2019) apart from the ones reported by Giresse et al (2007) in French strains. In a future prospect, the identification of functional polymorphisms in the effector nucleotide sequences could allow not only the identification of the pathogen in a seed sample, but also its classification by pathotype.

5. Conclusions

The molecular test based on PHA 42 marker is efficient for *P. halstedii* detection in whole sunflower seeds. Sunflowers growing near mildewed patches may carry *P. halstedii* in their seeds, becoming a vehicle for the widespread dissemination of the pathogen.

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