Ana Laura Martínez*, Freda Anderson, Facundo Quiroz, Antonio Garayalde, Ignacio Erreguerena, Lorena Armando, Norma Huguet and Alicia Carrera

Methodologies for *Plasmopara halstedii* Research

https://doi.org/10.1515/helia-2019-0013
Received July 19, 2019; accepted August 28, 2019

Abstract: The objective of this work was to find practical procedures to overcome methodological drawbacks encountered during studies on sunflower downy mildew. Techniques for recovering living isolates of *Plasmopara halstedii* from the field and for the preservation of infected leaf samples for further molecular analysis were developed. A Polymerase Chain Reaction (PCR)-based test for the detection of *P. halstedii* in sunflower leaves and a method to remove azoxystrobin from fungicide-treated seeds are proposed. *In situ*-inoculations of pre-germinated seeds allowed the recovery of living isolates from the field. Three sample-preservation methods were evaluated (silica, heating and lyophilization) resulting in high yield and quality of the DNA extract. It was detected the presence of the pathogen in symptomless leaves through PCR using molecular markers based on expressed

***Corresponding author: Ana Laura Martínez,** Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Bahía Blanca CP 8000, Argentina, E-mail: almartinez@cerzos-conicet.edu.ar
Freda Anderson, Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Bahía Blanca CP 8000, Argentina, E-mail: anderson@criba.edu.ar
Facundo Quiroz, Instituto Nacional de Tecnología Agropecuaria (INTA) Estación Experimental Agropecuaria Balcarce, Buenos Aires CP 7620, Argentina, E-mail: quiroz.facundo@inta.gob.ar
Antonio Garayalde, Department of Mathematics, Universidad Nacional del Sur, Bahía Blanca CP 8000, Argentina, E-mail: agarayalde@criba.edu.ar
Ignacio Erreguerena, Instituto Nacional de Tecnología Agropecuaria (INTA) Estación Experimental Agropecuaria Balcarce, Buenos Aires CP 7620, Argentina, E-mail: erreguerena.ignacio@inta.gob.ar
Lorena Armando, Department of Agronomy, Universidad Nacional del Sur, Bahía Blanca CP 8000, Argentina, E-mail: larmando@criba.edu.ar
Norma Huguet, Sunflower pathology services (Phytopathologist–private consultant), Rosario CP 2000, Argentina, E-mail: nhuguet@normahuguet.com.ar
Alicia Carrera, Department of Agronomy, Universidad Nacional del Sur, Bahía Blanca CP 8000, Argentina, E-mail: acarrera@criba.edu.ar

sequence tags. A treatment using sodium hypochlorite is recommended for the removal of azoxystrobin from fungicide treated seeds.

Keywords: downy mildew, sunflower, inoculation, DNA isolation, molecular detection, azoxystrobin

Introduction

Sunflower downy mildew caused by the oomycete *Plasmopara halstedii* (Farl.) Berl. & De Toni (Hall, 1989) is one of the most destructive diseases of sunflower around the world (Gascuel *et al.*, 2015). Use of resistance genes in sunflower or pathogen-free seeds are highly recommended to avoid pathogen attack (Gulya *et al.*, 2005). The universally accepted nomenclature system used for race characterization of *P. halstedii* is based on a set of sunflower inbred differential lines carrying different profiles of major resistance genes (Gulya *et al.*, 1991). Breeders employ more than 20 of these genes to protect sunflower against downy mildew, but several of them have been overcome by newly emerged races with increasing virulence, as a result of the rapid adaptability and evolution of the pathogen. Consequently, breeders require the identification of additional and more durable resistances (Pecrix *et al.*, 2018). The finding of which needs ample germplasm screening with high quality inoculum from evolving pathogen sources. In the same way, continuous and comprehensive research concerning race composition of *P. halstedii* is needed to efficiently fight this pathogen (Viranyi *et al.*, 2015). Due to the biotrophic nature of this organism, such studies require specific knowledge on management of samples collected during surveys to ensure the recovery of living isolates for race determination and appropriate leaf tissue preservation for further molecular studies. It was noticed at early stages of our investigations that the use of the methods currently in practice for collecting and preserving isolates (citas) were not effective enough in ensuring a good quality of the samples that arrived back at the laboratory, especially in regard to the viability of the isolates, taking into account the long distances covered during field trips in Argentina under the hot and dry weather conditions, characteristic of the season in which sunflower is grown. Hence, how to best collect inoculum and preserve samples from epiphytotics in the field was investigated in this work.

Inspection of fields is an essential tool for the visual estimation of downy mildew incidence, particularly when performed during mid-season surveys (stage V8-V12 to R5) (Gulya *et al.*, 2013). However, it has been shown that evaluations

based on the appearance of typical symptoms may underestimate downy mildew incidence due to the occurrence of latent infections (Cohen and Sackston, 1974). Such infections are significant in the epidemiology of *P. halstedii* since systemically infected asymptomatic plants may contaminate the soil with oospores by sporulation on roots and, in addition, produce infected seed that contribute to the widespread dissemination of the disease. For this reason, counting with a molecular test to rapidly confirm the pathogen's presence in a cropping field, is highly convenient. With this aim, we developed a PCR-based protocol for *P. halstedii* detection that was tested on leaves from non-sporulating plants. .

Another relevant aspect of controlled inoculations is that this procedure is often hindered by the fact that the seed samples to be screened have previously been treated with fungicide. Commercial formulations employ metalaxyl, and more recently azoxystrobin (Trojanová *et al.*, 2017), as active ingredients. To our knowledge, no literature reporting treatments for fungicide removal is available. Considering previous work that demonstrated the capacity of different substances to dissolve azoxystrobin (FAO, 2018), different treatments for fungicide removal were assayed to evaluate if treated seed could still be used in inoculation experiments.

Materials and methods

Collection of inoculum at field

Eighteen sunflower cropping fields were surveyed during season 2017 in Santa Fe Province, Argentina. Leaves with conspicuous signs of sporulation were collected and sporangia were washed off the leaf blades (one per plant) by forcibly applying distilled water while brushing with a soft paintbrush. Suspensions recovered in this way were poured into Petri dishes containing 25 pre-germinated seeds each (kept for 48 h on wet filter paper at 25 °C in darkness) and used as inoculum for *in situ* inoculations. The inoculum was left in contact with the seeds for 12 h under a 8 °C–15 °C temperature range in a portable refrigeration box, after which it was discarded. The inoculated seeds were transported to the laboratory within the following 72 h at the same temperature conditions. The isolates from different locations were stored separately, so that each Petri dish contained one isolate. Once in the laboratory, inoculated seedlings were maintained under controlled growth conditions (14 h light, 23 °C) (Gulya *et al.*, 1991) for inoculum increase or for further race characterization. Incidence of downy mildew was evaluated as the percentage of sporulating plantlets.

Conservation methods for molecular experiments

Samples of healthy and diseased leaves were also collected in the field from individual sunflower plants. In order to preserve the samples for DNA extraction, the leaves were: i) stored in bags containing silica-gel where they remained for a week (SG), or ii) put in bags and transported in a portable refrigeration box (temperature 8 °C to 15 °C) to the laboratory where they were dried at 60 °C for 48 hours (H) or frozen at −80 °C and lyophilized (L). Total DNA (belonging to the pathogen and sunflower) isolation was performed following a CTAB protocol with four replicas for each drying method (Hoisington *et al.*, 1992).

Molecular detection of *P. halstedii* in sunflower tissue

Two silica-dried sunflower leaves (with/without sporulation signs) taken from a singleinfected sunflower plant in the field, were separately ground with a mortar. Artificial mixtures with increasing ratios of tissue bearing sporulation were prepared, with three repetitions of each combination (Table 1). DNA was extracted from all the samples through the CTAB protocol (Hoisington *et al.*, 1992). Five markers *(Pha39, Pha42, Pha 43, Pha 74, Pha 106)* based on Expressed Sequence Tags (EST) of *P. halstedii* (Giresse *et al.*, 2007) were amplified using polymerase chain reaction (PCR) as follows: a final volume of 25 µL containing 20 ng of genomic DNA, 2.5 mM $MgCl_2$, 1.5 mM dNTPs, 8 pmol/µL of each primer, and 1 U Taq DNA polymerase in reaction buffer 1X. The amplification program consisted of 38 cycles and annealing at 57 °C, following (Giresse *et al.*, 2007). The amplicons were separated by 1.5 % agarose gel in TAE buffer 1X 80 V and were visualized by ethidium bromide dyeing and photographed under ultraviolet light. Internal amplification controls were included: DNA from *P. halstedii* sporangia (positive control), DNA from a non-infected sunflower leaf (negative control) and a sample without DNA

Table 1: Artificial mixtures (M0 % to M100 %) of leaf-powder with different proportions of tissue bearing sporulation.

	Sporulation levels				
	M0 %	**M20 %**	**M50 %**	**M80 %**	**M100 %**
Foliar tissue bearing no sporulation (mg)	50	40	25	10	0
Foliar tissue bearing sporulation (mg)	0	10	25	40	50

(blank). Additionally, to evaluate whether the same target genomic regions were amplified in all the samples, isolates previously characterized by race (710, 730 and 770) were included.

To test if the resulting fragments corresponded to the target pathogen genes, PCR products were purified with a Wizard ®SV Gel and PCR Clean up System kit and then sequenced (Macrogen Inc., Seoul, South Korea). Resulting nucleotide sequences were queried against the Genbank database (https://www.ncbi.nlm.nih.gov/genbank/), and compared to the sequences of the race-characterized isolates. To evaluate its reproducibility, the whole experiment was performed on 25 samples collected across different producing regions in Argentina.

In order to determine the sensitivity of the detection protocol, two dried sunflower leaves (with or without sporulation signs) taken from a singleinfected sunflower plant in the field, were separately ground with a mortar. Artificial mixtures with increasing ratios of tissue bearing sporulation were prepared, with three repetitions of each combination (Table 1). DNA was extracted from all the samples through the CTAB protocol and amplified by PCR as described above. Amplicons were analyzed by agarose gel.

The accuracy of the molecular test for detecting *P. halstedii* was additionally evaluated. Seedlings from the susceptible sunflower line HA89 were inoculated using the Whole Seedling Immersion protocol (WSI) (Gulya *et al.*, 1991) with a calibrated suspension of 30.000 sporangia/ml of fresh inoculum belonging to race 710. Inoculated seedlings were then grown at 22 °C and a 14 h light photoperiod. After induction (48 h at 17 °C in complete darkness), seedlings with absence of sporulation were selected for DNA extraction, PCR and histological analyses, to investigate the presence of the pathogen. For the latter, hand longitudinal sections of hypocotyls were performed with a razor blade. Some of the sections were mounted in aniline blue (0.05 %) and others in distilled water for inspection under an optical microscope to determine the presence/absence of the pathogen.

Fungicide removal from sunflower seeds

Seeds from a downy mildew-susceptible sunflower hybrid (ACA 861) treated with an azoxystrobin based fungicide, DYNASTY ®10FS SEMILLERO (metil(E)-2-(2-[6-(2-cianofenoxi) pirimidin-4-iloxi] fenil) 22-3-metoxiacrilate, 10 % w/v) specially designed for downy mildew control, were exposed to the following pure solvents: methanol (M), acetone (A) and dichloromethane (D), 10 seeds per treatment, with two replicas; an additional treatment with sodium hypochlorite (80 Cl g/L) at 50 % v/v (SH) was also evaluated taking into account the accessibility and low cost of this

product. A sample consisting of 10 seeds not treated with fungicide was included as control and underwent the same procedures, except that distilled water replaced the chemical treatment. A sample of 10 fungicide-coated seeds that was no exposed to any washing treatment was included as negative control. For fungicide-removal treatments the seeds were exposed for two periods, T1: 3 minutes and T2: 6 minutes, except for Sodium Hypochlorite in which the exposure periods were T1: 20 minutes and T2: 40 minutes. After completing the process, seeds were washed three times with distilled water, left in distilled water overnight and dehulled the following day. Seedlings were inoculated using the WSI protocol (Gulya *et al.*, 1991). All treated seeds were sown in a pot containing perlite and placed in a growth chamber (14 h light, 23 °C) for 12 days. After induction, the success in removing the fungicide from seeds was evaluated in terms of the proportion of plantlets showing sporulation expressed as percentage (incidence of downy mildew). The experiment was performed twice. The differences in responses to chemical removal treatments and to the time of exposure were evaluated through analysis of variance (two-way ANOVA) under a completely randomized design. Factors were chemical removal treatments (SH, M, A, D) and exposure periods (T1, T2). The mean values of incidence (%) were compared using the Fisher's Least Significant Difference test (0.05).

Results

As a result of the *in-situ* inoculations, over 80 % of the seedlings inoculated with each of the 18 isolates showed sporulation on cotyledons. Table 2 shows the results of DNA extraction and Figure 1 shows an agarose gel of genomic DNA from the infected leaf tissue collected in the field and preserved according to three different drying methods tested.

Table 2: Ratio of absorbance at 260 and 280 nanometers and DNA concentration obtained from leaves infected with downy mildew using three preservation methods (SG: silica-gel; H: heater drying and L: lyophilization).

Preservation method	DNA concentration (ng/uL)	$A_{260/280}$
SG	2773	1.92
H	1560	1.84
L	1634	1.73

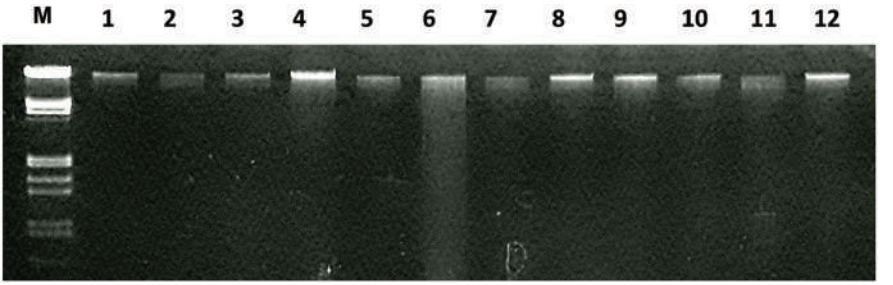

Figure 1: Genomic DNA in agarose gel of infected sunflower samples dried by the methods tested. Lane 1 a 4: samples dried by silica gel (SG), 5 to 8: samples dried at 60 °C (H), 9 to 12: lyophilized samples (L). M: ladder 100 bp.

BLASTn analysis in the Genbank database against the correspondent accessions (Giresse *et al.*, 2007) confirmed that all the PCR products tested belonged to codifying DNA of *P. halstedii*, including the controls of races 710, 730, and 770 (Table 3). The whole experiment was repeated on 25 samples of infected leaves collected from different fields across the country, which included *in situ* inoculations, DNA extractions, PCR amplifications and sequencing, all with similar results.

Table 3: Parameters obtained through BLASTn alignments against National Center for Biotechnology Information database for the nucleotide sequences corresponding to amplifications of ESTs markers.

ESTs markers	GenBank accession	Fragment size (bp)	Score	E-value	Identity (%)	Query coverage (%)
Pha 39	CB174648	237	219	$3{,}00e^{-61}$	100 %	70
Pha 42	CB174650	256	148	$2{,}00e^{-40}$	96.7 %	74
Pha 43	CB174680	279	326	$9{,}00e^{-94}$	96 %	72
Pha 74	CB174642	366	520	$3{,}00e^{-152}$	98 %	74
Pha 106	CB174676	298	433	$3{,}00e^{-126}$	97 %	80

All the samples consisting of a mixture of asymptomatic and symptomatic tissue, from M0 % to M100 %, amplified fragments for the specific *P. halstedii* EST based-marker *Pha 42* (Figure 2). The histological examination of an inoculated plantlet that showed no sporulation exhibited multiple hyphae and well–developed haustoria in host cells. In the same way, the amplification of *Pha74* on DNA from this asymptomatic plantlet resulted in amplicons of the expected size (Figure 3).

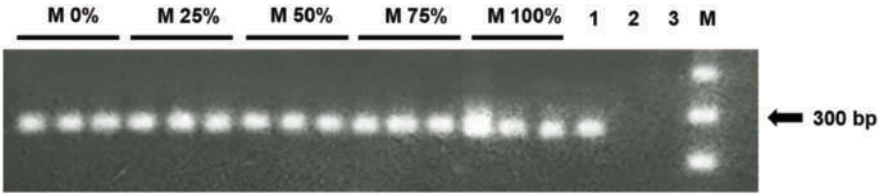

Figure 2: Amplification products of *Pha42* marker from mixtures with different proportions of tissue bearing sporulation (M0 % to M100 %). Three replications for each mixture. Controls: 1: *P. halstedii* DNA; 2: healthy sunflower DNA; 3: without DNA . M: 100 bp ladder.

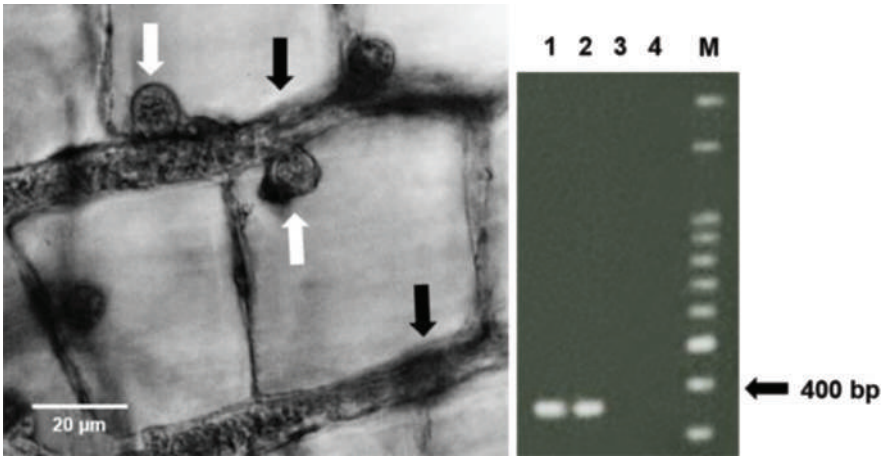

Figure 3: Left: Detail of a histological longitudinal section mounted in water of a downy mildew-infected hypocotyl from a symptomless seedling. White arrows point at haustoria, black arrows at hyphae. Right: Amplification products of *Pha74* primer pair. 1: DNA from artificially inoculated seedlings; 2: *P. halstedii* DNA; 3: healthy sunflower DNA. 4: negative control without DNA. M: 100 bp ladder.

All the different fungicide removal treatments showed infected plantlets (Figure 4). Significant differences were found between treatments and time of exposure for the mean values of incidence ($p = 0.014$ and $p = 0.025$ respectively). Sodium hypochlorite, methanol and acetone treatments were statistically equal in azoxystrobin removal, reaching incidence mean values of 97 %, 97 % and 85 % respectively; whereas the dichloromethane wash only rendered 56 % of plants with sporulation (Fisher's LSD test $\alpha = 0.05$). Times of exposure were significantly different, T1 reaching a higher mean incidence value than T2 (0.94 vs 0.79). The controls consisting of fungicide treated and non-trated seeds that were inoculated, gave 0 % and 100 % of sporulating plantlets, respectively.

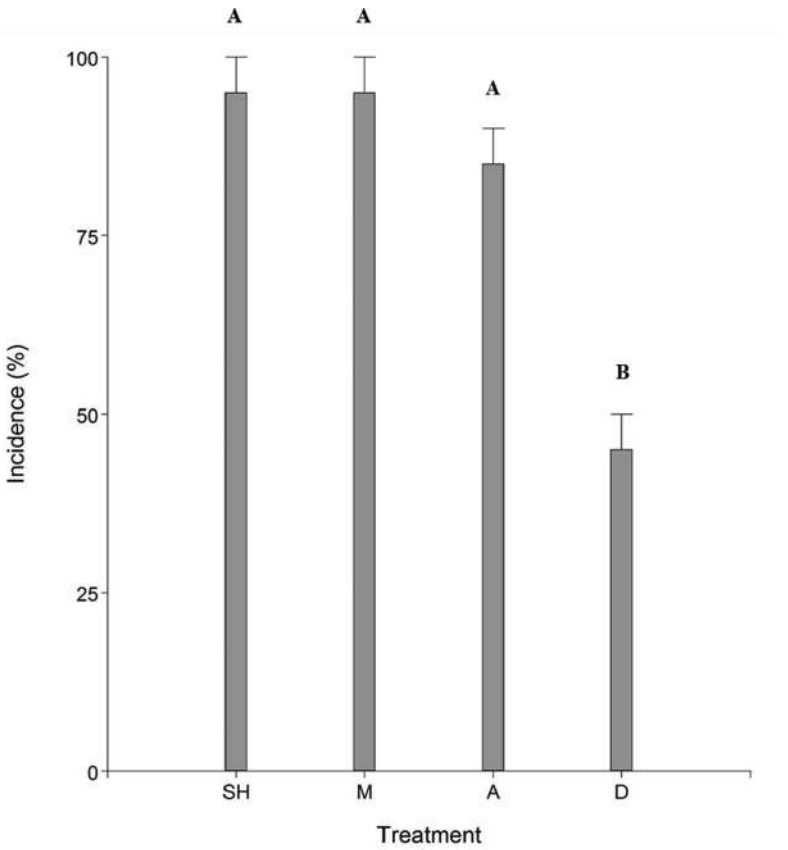

Figure 4: Mean incidence of downy mildew after azoxystrobin-washing treatments (20 seeds per treatment) from the two exposure periods to chemicals. SH: sodium hypochlorite, M: methanol, A: acetone, D: dichloromethane.

Discussion

Previous research recommends the collection and immediate transport to the laboratory of sunflower leaf samples with sporulation or, alternatively, the induction of sporulation from collected leaves with mildew symptoms to obtain inoculum for further testing (Gulya *et al.*, 1991). However, comprehensive programs to control the disease normally involve extensive sampling of fields which are usually far from the laboratories where the research is conducted. This, in addition to the high temperatures that occur during the sunflower crop season,

seriously attempts against storage conditions so that the collected zoosporangia samples easily deteriorate during transport. The results obtained here suggest that *in situ* inoculation avoids the loss of zoosporangia infectivity as a consequence of the transport period from the field to the laboratory, providing a high final infection percentage.

It is relevant to consider the nature of the isolates recovered with this methodology. The genetic variability of *P. halstedii* is such that an affected individual plant in the field may bear sporulation of different races (Spring and Zipper, 2016). Isolates from only one race are needed when looking for race-specific resistance within a sunflower collection (Niks *et al.*, 2019). A complete genetic homogeneity can be achieved by using pure isolates derived from single-zoospore inoculations (Spring *et al.*, 1998). In contrast, isolates recovered from *in situ*-inoculations such as the ones performed here will most likely be genetically heterogeneous and thus better reflect the original variability of the pathogen in the field (Molinero-Ruiz *et al.*, 2002). Hence, the use of the *in situ*-inoculations proposed in this work would be more appropriate for evaluating complete resistance on different sunflower genotypes. It was possible to obtain significant yields of DNA, which were enough for multiple amplification reactions by all the preservation methods tested. Besides, considering that a nucleic acid sample of high purity is expected to show a A260/A280 ratio ranging 1.8–2.0, the three preservation methods rendered high quality DNA. In addition, the agarose gel demonstrated that the genomic DNA showed no degradation. Notwithstanding, the silica-gel drying method would be the most convenient one because it allows the desiccation process of plant tissues to start as soon as they are collected. Although the obtained DNA extract contained both pathogen and sunflower DNA, this inconvenient was overcome by using molecular markers for *P. halstedii* genome. It is acknowledged that with this protocol the concentration of *P. halstedii* DNA in the total DNA extract is uncertain.

Amplification of ESTs marker *Pha74* in samples with no sporulation (M0 %) was explained by the systemic growth of the pathogen, which was also confirmed by the microscopic examination of cotyledon sections. The set of twelve ESTs-based molecular markers presented by Giresse et. al, in 2007 was originally conceived for the molecular characterization of isolates of the pathogen and their genotyping from sporulating lesions, but their application is now extended to the detection of the pathogen from DNA extracted from leaves regardless of the presence or not of sporulation.

The possibility of cross-amplification of the ESTs-based primers was discarded by Giresse and co-workers since the primers were originally tested on three closely related Oomycetes species: *Bremia lactucae* (lettuce downy

mildew), *Phytophthora infestans*, (potato late blight) and *Plasmopara viticola* (grapevine downy mildew) and no amplification occurred in any of these species.

The test proposed here to assess the occurrence of systemic growth of the pathogen in asymptomatic plants, could help determine the presence of the pathogen in sunflower samples collected directly from fields, even at early symptomless stages. The test offers an easily visualized output that is supported on nucleotide sequence data and can be performed by laboratories with regular infrastructure since it is cost-effective. This test could be used as a complement of the visual estimations of downy mildew incidence, particularly on asymptomatic plants or on those carrying latent infections, to determine the extent of the patches of infected plants, which is relevant in commercial seed production. Monitoring downy mildew incidence could thus combine visual estimation at the optimal growing stages and molecular detection techniques for phytosanitary characterization of seed crops.

It was demonstrated that sodium hypochlorite, methanol and acetone were similarly effective fungicide-removals. In view of this, the use of sodium hypochlorite is recommended since it is an accessible product and a disinfectant of seed surfaces with no effect on germination rates. Besides, the shortest time of exposure to washing (20 minutes) was enough to remove the fungicide from the pericarps. The ability of seeds to germinate was not affected by the solvents used in this essay since 100 % of the seeds in all treatments germinated normally. Toxic effects of some solvents on germination of sunflower seeds have only been observed after much longer immersion periods than the exposure time used in our study (Lewis *et al.*, 1979).

Conclusions

In-situ inoculation of pre-germinated seeds is a suitable method for transporting and preserving *P. halstedii* living inoculum collected during field surveys. The silica-gel conservation method for transporting samples for DNA isolation is practical and provides high DNA yield and quality for further PCR reactions. The ESTs-based molecular markers are capable of detecting the pathogen in sunflower leaves with no external symptoms of mildew, becoming a *P. halstedii* diagnosis by conventional PCR. Sodium hypochlorite can remove the azoxystrobin-based fungicide from sunflower seeds, allowing fungicide-treated seeds to be used for downy mildew inoculation essays.

Acknowledgements: To Dr Thomas J. Gulya (USDA-ARS) for his support and helpful advice on planning the experiments. Mention of commercial products or trade names in this article is entirely for the purpose of providing specific information and does not imply any recommendation or endorsement.

References

Cohen, Y., Sackston, W.E., 1974. Seed infection and latent infection of sunflowers by Plasmopara halstedii. Canadian Journal of Botany 52(1): 231–238.

FAO. Specifications and Evaluations for Agricultural Pesticides. Azoxystrobin. Accessed April 2018.

Gascuel, Q., Martinez, Y., Boniface, M.C., Vear, F., Pichon, M., Godiard, L., 2015. The sunflower downy mildew pathogen Plasmopara halstedii. Molecular Plant Pathology 16(2): 109–122. DOI:10.1111/mpp.12164.

Giresse, X., Tourvieille de Labrouhe, D., Richard-Cervera, S., Delmotte, F., 2007. Twelve polymorphic expressed sequence tags-derived markers for Plasmopara halstedii, the causal agent of sunflower downy mildew. Molecular Ecology Notes 7(6): 1363–1365. DOI:10.1111/j.1471-8286.2007.01887.x.

Gulya, T., Kandel, H., McMullen, M., Knodel, J., Berglund, D., Mathew, F., … Markell, S., 2013. Prevalence and incidence of sunflower downy mildew in North Dakota between 2001 and 2011. Plant Health Progress 14(1): 20. DOI:10.1094/PHP-2013-0522-01-RS.

Gulya, T.J., 2005. Evaluation of wild annual Helianthus species for resistance to downy mildew and Sclerotinia stalk rot. *In*: Proc 27th Sunflower Res forum, Fargo, N.D., pp. 12–13.

Gulya, T.J., Miller, J.F., Viranyi, F., Sackston, W.E., 1991. Proposed internationally standardized methods for race identification of Plasmopara halstedii. Helia 14(15): 11–20.

Hall, G., 1989. CMI descriptions of pathogenic fungi and bacteria, Set 98, Nos. 971-980. Mycopathologia 106(3): 183–211.

Hoisington, D., Gonzalez de Leon, D., Khairallah, M., 1992. Laboratory protocols: CIMMYT applied molecular genetics laboratory (No. 14756).

Lewis, J.A., Papavizas, G.C., O'Neill, N.R., 1979. Effect of seed immersion in organic solvents on germinability. The Journal of Agricultural Science 92(3): 563–570. DOI:10.1017/S0021859600053806.

Molinero-Ruiz, M.L., Domínguez, J., Melero-Vara, J.M., 2002. Races of isolates of Plasmopara halstedii from Spain and studies on their virulence. Plant Disease 86(7): 736–740.

Niks, R.E., Parlevliet, J.E., Lindhout, P., Bai, Y., 2019. Breeding Crops with Resistance to Diseases and Pests. The Netherlands: Wageningen Academic Publishers.

Pecrix, Y., Penouilh-Suzette, C., Muños, S., Vear, F., Godiard, L., 2018. Ten broad spectrum resistances to downy mildew physically mapped on the sunflower genome. Frontiers in Plant Science 9: 1780. DOI:10.1111/tpj.14157.

Spring, O., Rozynek, B., Zipper, R., 1998. Single spore infections with sunflower downy mildew. Journal of Phytopathology 146(11–12): 577–579.

Spring, O., Zipper, R., 2016. Asexual recombinants of Plasmopara halstedii pathotypes from dual infection of sunflower. PloS One 11(12). DOI:10.1371/journal.pone.0167015.

Trojanová, Z., Sedlářová, M., Gulya, T.J., Lebeda, A., 2017. Methodology of virulence screening and race characterization of Plasmopara halstedii, and resistance evaluation in sunflower–a review. Plant Pathology 66(2): 171–185. DOI:10.1111/ppa.12722.

Viranyi, F., Gulya, T.J., Tourvieille, D.L., 2015. Recent changes in the pathogenic variability of Plasmopara halstedii (sunflower downy mildew) populations from different continents. Helia 38(63): 149–162. DOI:10.1515/helia-2015-0009.

Resumen

Metodologías para investigaciones en *Plasmopara halstedii*

Mildiu causado por *Plasmopara halstedii* es una enfermedad importante del cultivo de girasol. El presente estudio se centró en poner a punto metodologías aplicables a investigaciones en relación a la recuperación de aislamientos vivos desde epifitias a campo, la preservación de muestras foliares colectadas en lotes infectados para análisis moleculares, el desarrollo de un diagnóstico rápido por PCR para la detección de mildiu en hojas de girasol y un método para remover azoxystrobina, el principio activo de un fungicida especifico de mildiu, para utilizar semilla curada en posteriores ensayos de inoculación. Para la recuperación de aislamientos vivos, se propuso la inoculación *in situ*. Se testearon tres métodos de conservación de muestras foliares (silica-gel, secado en estufa y liofilizado) en términos de concentración y calidad del ADN. Se utilizó PCR con marcadores basados en secuencias expresadas (ESTs) para detectar el patógeno en muestras de hoja. Se emplearon hipoclorito de sodio, metanol, acetona y diclorometano para remover azoxistrobina de semillas tratadas. Se obtuvo inóculo viable con altas tasas de infección por medio de la inoculación *in situ* propuesta y ADN de alta concentración y calidad con los métodos de conservación testeados. Los marcadores ESTs detectaron el patógeno en muestras de plantas asintomáticas con alta sensibilidad. Se encontraron diferencias significativas entre los tratamientos utilizados, siendo hipoclorito de sodio, metanol y acetona 40 % más eficientes que diclorometano.

Résumé

Méthodologies de recherche sur *Plasmopara halstedii*

Le mildiou de *Plasmopara halstedii* est une maladie importante du tournesol. La présente étude porte sur le développement de méthodologies applicables à la recherche concernant la récupération d'isolements vivants d'épaves sur le terrain, la conservation d'échantillons foliaires prélevés dans des lots infectés à des fins d'analyse moléculaire, la mise au point d'un diagnostic rapide par PCR pour la

détection de mildiou dans les feuilles de tournesol et méthode d'élimination de l'azoxystrobine, l'ingrédient actif d'un fongicide midiu spécifique, destiné à l'utilisation de semences traitées lors de tests d'inoculation ultérieurs. Pour la récupération des isolats vivants, une inoculation *in situ* a été proposée. Trois méthodes de conservation des échantillons de feuilles (gel de silice, séchage au four et lyophilisation) ont été testées en termes de quantité et de qualité d'ADN. La PCR avec des marqueurs basés sur des séquences exprimées (EST) a été utilisée pour détecter l'agent pathogène dans des échantillons de feuilles. De l'hypochlorite de sodium, du méthanol, de l'acétone et du dichlorométhane ont été utilisés pour éliminer l'azoxystrobine des semences traitées. Un inoculum viable a été obtenu avec des taux d'infection élevés grâce à l'inoculation in situ proposée et à un ADN de grande quantité et de haute qualité grâce aux méthodes de conservation testées. Les marqueurs EST ont détecté l'agent pathogène dans des échantillons de plantes asymptomatiques. Des différences significatives ont été observées entre les traitements utilisés: hypochlorite de sodium, méthanol et acétone 40 % plus efficace que le dichlorométhane.