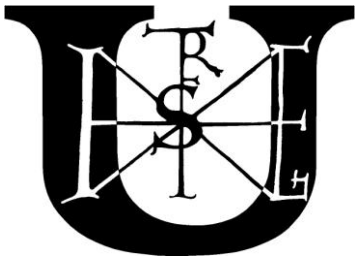

SZENT ISTVÁN UNIVERSITY

PHENOTIPIC AND MOLECULAR GENETIC CHARACTERISATION OF SUNFLOWER INFECTING *PLASMOPARA* POPULATIONS

Theses

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BACKGROUND AND OBJECTIVES

In Hungary, the downy mildew of sunflower, *Plasmopara halstedii* is considered as one of the major yield limiting factor in sunflower production. It has a relatively broad host range within the Asteraceae family, including dangerous weeds, like ragweed (*Ambrosia artemisiifolia* L.), cocklebur (*Xanthium strumarium* L.) and marshelder (*Iva xanthiifolia* Nutt.). Infection starting from the below ground tissues causes systemic infection of the plants, which results in stunting, leaf chlorosis, horizontal head, poor seed set and severe yield reduction. Diseased plants can not recover. A combination of fungicidal seed treatment and genetic resistance may provide with effective protection against the disease.

P. halstedii originated from the American continent and spread into Europe in the early 1940's. It remained pathologically uniform until the introduction of resistant sunflower cultivars. Until now about 30 different pathotypes of the pathogen have been identified worldwide. In Hungary at least five of these exist with certainty. The great variability in the virulence phenotypes of the pathogen are thought to be connected with the selection pressure of the different resistance sources incorporated into the cultivars in production. Another significant change in the pathogen population occurred recently that lead to the appearance and distribution of fungicide tolerant strains against the widely used metalaxyl fungicide. It happened first in France, then in North America, followed by Spain and Germany.

Safe, environmentally friendly and modern sunflower production requires cultivars resistant to the virulence phenotypes of the local pathogen populations. Although virulence characterization of the pathogen populations is based on an internationally accepted methodology using a set of sunflower differential lines with distinct resistance-susceptibility reactions, this technique is time-, space- and labor consuming. It requires quality increase of the differential inbred lines, moreover evaluation process can be hampered due to environmental conditions causing non-typical plant responses.

Recent developments in molecular biology permitted the application of new technologies in plant pathology as well. Beside phenotypic observations molecular characterization became common in population studies. At the molecular level little is known about *P. halstedii*. Based on former RAPD (Randomly Amplified Polymorphic DNA) and RFLP (Restriction Fragment Length Polymorphism) analysis, the pathogen showed low level of polymorphism, despite the increasing number of new virulence phenotypes that have appeared.

The aim of this study was to describe the genetic and molecular variability of *P. halstedii*, the downy mildew pathogen of sunflower, and an attempt to find reliable, molecular based tools for distinguishing newly appeared or evolved virulence phenotypes.

The following tasks were considered:

- Setting up a collection of *P. halstedii* samples originating either from Hungary or from other sources, and identifying their virulence characters for the experiments;
- Characterizing *Plasmopara* sp. isolates collected from cocklebur for their virulence phenotype, morphology, fatty acid composition, metalaxyl sensitivity, cross infectivity and host range;
- Initiating genetically pure single sporangial lines, multiply and characterize their virulence phenotype;
- Conducting comparative studies for variability of *Plasmopara* isolates collected from sunflower and cocklebur by using RAPD, iSSR (inter Simple Sequence Repeat) and S-SAP (Sequence Specific Amplified Polymorphism) analyses;
- Sequencing and characterization of ITS (Internal Transcribed Region) and partial LSU (Large Ribosomal Subunit) for phylogenetics and species delineation;
- Using isozyme pattern analysis by CAE (Cellulose Acetategel Electrophoresis) for population variability assessments.

MATERIAL AND METHODS

Plasmopara isolates and phenotypic characters

Plasmopara samples collected in Hungary from sunflower and cocklebur hosts originated from the culture collection of Szent István University, Crop Protection Institute, while the samples originated from foreign countries were provided from the culture collection of Hohenheim University, Institute of Botany.

Samples were cultured and multiplied on the generally susceptible open pollinated sunflower variety GK-70, except for the isolate collected from cocklebur, which was cultured on the differential line, HA-335. In any case, the whole seedling inoculation technique described by Cohen and Sackston (1973) was used. Genetically pure material was obtained by initiating single sporangial lines applying the technique of Spring et al. (1998). Pathotype (virulence phenotype) identification was made by the methodology described by Tourvieille et al. (2000).

Host range studies of the isolate originated from cocklebur were carried out by cross infection using plants developed from cocklebur fruits collected in the area where the isolate came from. Virulence phenotypes of these isolates were determined by the method of Tourvieille et al. (2000). Metalaxyl sensitivity was evaluated by means of the detached leaf segment method of Rozynek and Spring (2001). Fatty acid composition of the samples originated from cocklebur was analyzed from freshly produced sporangia using the method of Spring and Haas (2002). Fatty acid extraction and composition analysis was made in the Botany Department of Hohenheim University. Fatty acid profiles were compared to the average of *P. halstedii* samples of various geographic origins.

Nucleic acid polymorphism studies

Total **DNA was extracted** from approximately 25-30 mg of *Plasmopara* sporangia and sporangiophores mixture. Their disruption was made by using an IKA ultra turrax machine in order to reduce material loss. DNA was extracted using the Genomic DNA Purification Kit (Fermentas) according to the manufacturer's instructions.

RAPD (Randomly Amplified Polymorphic DNA) **analysis** was conducted on ten isolates representing the five pathotypes common for Hungary. For the RAPD reaction, 66 UBC (University

of British Columbia, Canada) ten base long primers were selected from the 100 and 700 series. The PCR mixture was prepared by using Fermentas products. Reaction was done in Biometra Thermocycler3 machine. Products were visualized under UV illuminator. The presence or absence of product was evaluated by binary codes, which were analyzed by MDS (MultiDimensional Scaling Analysis) method of the SPSS 8.0 program package.

iSSR (inter Simple Sequence Repeat) **analysis** were conducted on field isolates and their single sporangial lines originated either from Hungary, Germany, France and USA. Seven microsatellite primers: $(CAC)_4RC$, $(GTG)_5$, $(GATA)_4$, $(GT)_7YG$, $(GAG)_4RC$, $(GTC)_5$, $(CT)_8T$, and two minisatellite primers: M13 and T3B were used according to Intelmann and Spring (2002).

Identification of LTR region of a *Ty-copia* type retrotransposon was started from a conservative part of the reverse transcriptase gene using the degenerated primers of Flavell et al. (1992). The products were sequenced and evaluated for similarity to retrotransoposon like elements in the NCBI database applying the Blastx search. Chromosome walking was used to reach the LTR region of the transposon. The sequences were aligned using the SeqMan program of DNAStar (Lasergene). LTR-region specific **S-SAP** probe was designed and single sporangial lines from thirty *Plasmopara* isolates of annual, perennial sunflower, as well as from cocklebur were used in the analyses according to Waugh et al. (1997).

ITS-RFLP (ITS-Restriction Fragment Length Polymorphism) was applied to compare the patterns of isolates originated from sunflower and cocklebur. ITS region multiplied in PCR reaction was subject to 11 enzymes restrictions: *Eco*RI, *Hind*III, *BamH*I, *Ava*III, *Hinf*I, *Hin6*I, *Eco*R5, *Mbi*I, *Pst*I, *Sma*I, and *Cfr13*I (Fermentas) according to manufacturer's instructions. The complete sequence description and analysis of ITS of *P. halstedii* was conducted in collaboration with M. Thines according to Thines et al. (2005). The **ITS region** of isolate from cocklebur was described and analyzed as described by Komjáti et al. (2007).

Isoenzyme analyses

Thirty-five single sporangial lines and ten field isolates were involved in this study out of which 41 samples originated from sunflower, three from cocklebur and one from perennial sunflower. The kit of Helena Laboratories (Beaumont, Texas) Super Z-12 set was used for conducting this experiment. Samples were prepared according to Láday et al. (2000). Chemicals used were purchased from Sigma-Aldrich, the Titan III cellulose gels (Helena Laboratories) were

purchased ready to use. Electrophoresis was conducted according to the manual of Hebert and Beaton (1993). All together 16 different enzyme systems were tested using the agar over layer method. Staining recipes were prepared following the manual published by Helena Laboratories (Hebert and Beaton, 1993). Polymorphic enzyme alleles were named based on their relative mobility (RM) from the baselines. Samples were grouped into Multilocus Phenotypes by their combined RM patterns.

RESULTS

Variability in virulence phenotypes of *Plasmopara halstedii* field isolates

Single sporangial lines originating from the 10 field isolates of *P. halstedii* representing the five pathotypes (100, 330, 700, 710 and 730) present in Hungary were found to segregate for virulence character. There were field isolates containing more than one virulence phenotype. Some of the single sporangial clones showed virulence characters as 300 or 310 that were not previously detected in Hungarian pathogen populations.

Phenotypic characterisation of the *Plasmopara* isolates collected from cocklebur (*Xanthium strumarium*)

The X03 isolate collected from *Xanthium* strumarium was found pathogenic to sunflower. It could sporulate on the affected plant tissues. Those plants became stunted, the hypocotyls showed brown discoloration and tissue necrosis. The so-called damping-off symptoms were also observed. Oospores of the pathogen developed in the root tissues of sunflower plants. However, the extent of infection was limited to the cotyledon level, so that no mycelium of the pathogen was detected in the plant tissues above the cotyledon leaves. The typical downy mildew symptom of leaf chlorosis was absent.

Sporangia of the isolate X03 freshly developed on sunflower were successfully used to re-infect its original host, *Xanthium strumarium*, where leaf chlorosis and sporulation also occurred. It means that this isolate completed its life-cycle on both sunflower and cocklebur. Microscopic observations showed that sporangiophores of the isolate were characterized with monopodial branching and the shape of sporangia varied strongly from round, through ovate to elongated or peanut shaped, measuring 25-50 X 20-30 (average 41.1 ± 7.8 x 27.0 ± 4.6) μm. The incubation time prior to zoospore release was 30-45 minutes, the number of zoospores released from one single sporangium varied between 4 and 13, most frequently 6 zoospores were observed (average 6,29 ± 2,0).

When testing for virulence phenotype, this isolate showed an unusual character described with a 717 CLI code, indicating a capacity of cotyledon limited infection on sunflower. X03 was found to be sensitive to metalaxyl fungicide commonly used for sunflower seed treatment against downy mildew.

Its fatty acid profile was statistically different from those *Plasmopara* isolates originating from sunflower.

Molecular characterization of isolates originated from various host plants

Based on **RAPD** analysis the isolates collected from sunflower in Hungary comprised four groups. The first group represented four virulence phenotypes out of the five found in Hungary (330, 700, 710, 730). The second group contained two pathotypes, 100 and 700, as did the third group with pathotypes 330 and 730, whereas one single isolate of 100, collected in 2001, was separated to the fourth group.

The iSSR method resulted in clear, well reproducible patterns for all the isolates originated either from cocklebur, annual or perennial sunflower. A significant genetic distance could be observed between the samples originated from the different hosts. The genetic distance between the samples from the annual or perennial sunflowers was lower than that between the samples from cocklebur. One isolate from cocklebur (X04) grouped together with the samples isolated from sunflower. This X04 isolate regarding its pathogenicity behaviour and virulence phenotype also shared similarity with the samples collected from sunflower. The variability within the groups of isolates from sunflower was low even though it contained samples of various origin, virulence phenotype or differed metalaxyl sensitivity. Although this low variability could indicate some isolate specific patterns but these were found inappropriate for virulence phenotype identification.

The use of degenerated primers for isolating reverse transcriptase gene in *P. halstedii* gave products similar to the relevant genes of *Phytophthora infestans*, a closely related species. LTR retrotransposon was isolated from *P. halstedii* by chromosome walking from the reverse transcriptase gene. **S-SAP** probe was developed on the species specific LTR region. Insertion polymorphism was observed among the samples which could be related to field specific pattern. Although the isolates from perennial sunflower (*H.* × *laetiflorus*) and cocklebur (*X. strumarium*) also contained the species specific probe, their pattern was separated from the isolates collected from sunflower. S-SAP technique allowed differentiating between the isolates from different hosts.

The size of the **ITS** region differed between the isolates from sunflower or cocklebur. The former had a size of app. 2600 bp while the latter had 3225 bp. Both sequences exceeded the expected length for ITS regions. Size increase was due to 1822 bp and 2010 bp long insertions in the ITS2 regions respectively, which showed no homology with known sequences in the NCBI

database. The ITS1 regions shared 92.7% homology between the two origins, while the conservative 5.8S region was 100% identical. Sequence analysis of the insertion in *P. halstedii* indicated that it may contain four 87-91% similar repeated elements, while the insertion of the X03 isolate also contained several repeated elements with varying similarity. The insertions differed in composition and content between the two isolates. ITS sequence indicated clear differences in the genetic background of the two species. X03 isolate formed a monophyletic group with the sunflower infecting *P. halstedii* but differed from it.

As little as 25 mg biomass was sufficient to complete all **cellulose acetate gelectrophoresis** experiments and 31–100 μg sporangia and sporangiophores per reaction were needed to detect enzyme activity. Staining properties of the sixteen isozymes tested varied for stainability, signal intensity and reproducibility. Altogether five isozymes, namely malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), phosphoglucomutase (PGM), acid phosphatase (APH) and glucose-6-phosphate dehydrogenase (G6PDH), revealed polymorphisms among the isolates. Three of these (MDH, IDH and PGM) produced clear and reproducible bands. All the tested isolates in our study were homozygous for the isozymes detected regardless of their origin, suggesting that the pathogen population was rather homogeneous. Based on the results obtained with detectable isozyme loci, four multi-locus phenotypes (MLP) could be distinguished. The majority of these isolates (84%), collected in different sunflower growing regions in Europe and in the USA, belonged to MLP1, whereas MLP2 contained samples from Hungary, Germany and Serbia. The MLP3 contained one single sample originated from perennial sunflower, while MLP4 was formed by the X03 isolate from cocklebur.

NEW FINDINGS

1. Single sporangial lines of *Plasmopara halstedii* field isolates segregated for virulence phenotype as compared to their parent isolates, indicating that it is a rather complex trait of the pathogen population in nature.

2. It was proved for the first time that beside *P. halstedii*, the cocklebur pathogenic *P. angustiterminalis* Novot. is able to infect sunflower, and it completes its asexual and sexual life cycle on sunflower.

3. It was shown that the full length of ITS in *P. halstedii* is approximately 2600 basepairs due to a multiple insertion located in the ITS-2 region. The ITS length of *P. angustiterminalis* were 3225 basepairs, which also contained multiple insertions in the ITS-2 region. The insertions in composition and copy numbers were different between the two species.

4. The presence of *Ty-copia* type LTR retrotransposon in *P. halstedii* was proved for the first time. Its LTR region was successfully used for S-SAP insertion polymorphism studies.

5. Isozyme pattern was described for the first time for *P. halstedii* using the CAE (cellulose acetate gel electrophoresis system). Samples were grouped into for Multilocus Phenotypes. This method underlined the high level of homozygosity of the pathogen.

6. The *P. angustiterminalis* Novot. isolate pathogenic to cocklebur was for the first time characterized by molecular genetic tools as ITS, iSSR and isozyme.

7. It was demonstrated that some sunflower inbred lines resistant to the Hungarian populations of *P. halstedii* could be successfully infected by the *P. angustiterminalis* Novot. isolate collected from cocklebur.

DISCUSSION AND CONCLUSIONS

Light microscopy revealed differences between the *Xanthium* isolate (X03) and the *P. halstedii* isolates from sunflower in several aspects: (i) sporangial morphology, (ii) time of incubation required for zoospore release and, (iii) the number of released zoospores from one single sporangium. The shorter incubation period reduces the time needed under special conditions for infection to take place and it might contribute to the fitness as well as to the aggressivity of the isolate. Although X03 isolate was pathogenic to sunflower, infection was limited to the root-hypocotyl-cotyledon part of the plants and the secondary tissues remained free from the pathogen. This phenomenon questioned the species identification of X03 isolate as being *P. halstedii*, the downy mildew pathogen of sunflower. In fact, based on further investigations, this X03 isolate proved to be different from *P. halstedii*, and was considered to be a member of the species *P. angustiterminalis* Novot. Interestingly, two additional *Plasmopara* isolates (X02, X04) that also originated from cocklebur (*Xanthium strumarium*) showed a high similarity to *P. halstedii* isolates from sunflower suggesting that cocklebur serves as a natural host of both *P. halstedii*, the common downy mildew pathogen of sunflower and *P. angustiterminalis*, a potential pathogen of this crop. Potential mixed infection of cocklebur or volunteer sunflowers by the two different types of *Plasmopara* isolates creates opportunity for genetic interaction between the two species and could lead to the development of new virulence combinations.

The fact that some single sporangial lines of *P. halstedii* initiated from field isolates contained several virulence characters beside the original parental phenotype, moreover new virulent forms appeared which were previously not identified among field isolates in Hungary, indicated that the downy mildew pathogen populations occur in nature as a mixture of virulence phenotypes. This might have significant impact on the survival of the host dependent pathogen by permitting successful attack of the crop plant. In this respect, efficient protection of sunflower against this highly variable pathogen would require new breeding strategies, for example the use of durable resistance beside chemical protection and the combination of these two approaches.

Fatty acid analysis revealed differences between the two types of *Plasmopara* isolates, and this method alone was powerful enough to differentiate the isolates within the species or closely related species. However, further techniques were also applied to confirm the genetic differences among the isolates.

All the molecular tools applied (RAPD, iSSR, S-SAP, and CAE) revealed high level of genetic homogeneity of the samples originated from sunflower despite the versatile virulence phenotypes they represented. In this aspect our results were concordant with the RAPD analysis data obtained only on Hungarian samples and further analyses using versatile molecular tools on a wide range of samples in respect of origin and virulence phenotypes. This might indicate that changes in virulence phenotypes are probably due to minor genetic changes similarly to what was previously observed in case of the highly variable cereal rusts. Although none of the applied techniques provided us with a tool for direct phenotype characterization, each analysis revealed sub-groups within the highly monomorphic samples permitting their further use for population monitoring purposes and tracking the movement of the pathogen in space and time.

Results obtained by iSSR, S-SAP or CAE indicated genetic distances between the isolates collected from either sunflower or other host plants, such as cocklebur or perennial sunflower, the latter having distinct pathogenic and virulence characters. It seems therefore that the tools applied in this study may help to contribute to species description within this economically important group of pathogens forming the *P. halstedii* species complex.

ITS regions which contain conservative and highly variable parts of an organism are thought to have a potential in taxonomic studies at either inter- or intraspecific level. However, in case of the *Plasmopara* samples, the size of ITS region exceeded 3–4 times the corresponding sequences of other species, making the analysis complicated. For multiplying long sequences, fair amount and good quality DNA is needed, which might be difficult to get with host dependent biotroph pathogens like *P. halstedii*, especially with the use of herbaria specimens.

From our results obtained on a partial LSU sequence analysis it could be concluded that this technique was appropriate for species differentiation and it highlighted, at least in our case, clear genetic differences between *P. angustiterminalis* and the *P. halstedii*.

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