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Characterization of the pathogens causing blackleg of oilseed rape in Hungary

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Bianka Bagi

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Doctoral School

name: Doctoral School of Horticultural Sciences

field: Crop Sciences and Horticulture

head: dr. Éva Zámboriné Németh
professor, DSc
Hungarian University of Agriculture and Life Sciences
Institute of Horticulture
Department of Medicinal and Aromatic Plants

supervisors: Dr. Marietta Petróczy
associate professor, PhD
Hungarian University of Agriculture and Life Sciences
Institute of Plant Protection
Department of Plant Pathology

Dr. László Palkovics
professor, DSc
Széchenyi István University
Albert Kázmér Faculty of Mosonmagyaróvár
Department of Plant Sciences

....

Head of Doctoral School .....

Supervisors

1. BACKGROUND AND OBJECTIVES OF THE RESEARCH

Oilseed rape (*Brassica napus* L.) is one of the most important oilseed crops worldwide, and is grown over a large area (Brachaczek et al. 2021). Since the 1980s the introduction of high-yielding hybrids in major rapeseed-growing regions such as Canada, Europe, China, India and Australia has led to a gradual increase in rapeseed production for the food and feed markets. Since the 1990s, however, average yield growth rates in Europe and Australia have started to decline. At that time, protection against biotic factors was inadequate and rapeseed had high levels of erucic acid and glucosinolates, which were unfavourable from both a food and feed point of view (Allender and King, 2010; Zheng et al. 2020).

Although the breeding optimized the levels of erucic acid and glucosinolate, it resulted in reduced resistance to pathogens, including those responsible for blackleg. The decline in resistance has led to an increase in the incidence of pathogens, causing increasing damage to rapeseed growers (Kightley et al. 2012). According to some records, pathogens of blackleg cause significant economic damage worldwide, with losses of more than $900 million annually (Fitt et al. 2008).

The co-presence of the two pathogens responsible for the disease, *Plenodomus lingam* and *Plenodomus biglobosus*, has been confirmed in several European countries so far. *P. lingam* was first described in Hungary in 2006 (Magyar et al. 2006; Szlávik et al. 2006). In 2016, Mitrović et al. isolated *P. biglobosus* from oilseed rape samples collected near Rimski Šančevi, Serbia. From our samples collected in 2018, we identified a few cases of the pathogen, which was previously unknown in Hungary (Bagi et al. 2020).

The objectives of our research were to:

- Mapping the distribution of the pathogens causing blackleg in our country;
- Species-level identification and characterization of *Plenodomus* isolates based on morphological and culture characters;
- Reliable identification of *Plenodomus* species by molecular methods;
- Analysis of the nucleotide sequences of different genomic regions of *Plenodomus* isolates (the entire ITS region, the LSU, the β-tubulin-2 gene, the *rpb2* region and a segment of the *act1* gene) to map the variability of the pathogen population in Hungary and to identify the phylogenetic relationship of the isolates;
- Molecular analysis of other genomic regions (avirulence gene, gene determining mating types);

- *In vitro* susceptibility study of *P. lingam* and *P. biglobosus* isolates to fungicidal agents using a serial dilution poisoned agar plate method.

2. MATERIALS AND METHODS

Collection and storage of plant parts

Between 2017 and 2021, oilseed rape leaves and stems showing symptoms of blackleg were collected from 10 counties near 29 localities in the country, with a total of 502 plant parts collected over the five years. Plant parts showing symptoms were delivered to the university laboratory on the day of collection. In the following, we worked exclusively with leaf and stem fragments on which the asexual fruiting bodies of blackleg, the pycnidia, were formed. The used plant parts were incubated in a humid chamber under natural light at room temperature until the conidia were prebroken (maximum 3 days).

Isolation of pathogens and maintenance of cultures

In many cases, a mass of pycnoconidia erupted from the pycnidia on plant parts placed in the humid chambers. Following the method of Goh (1999), the conidia mass was removed under a stereomicroscope with a glass capillary pulled over a flame and placed in sterile water. The conidia suspension was then vortexed and digested on PDA medium. The petri dishes were incubated at 24 ± 1 °C in the dark, and after 3 days, cultures were established by removing the hyphal tip of the germinating conidia. The isolates were stored for longer periods in test tubes, on slanted agar and on agar plates.

Characterisation and evaluation of morphological features, pathogenicity test

The pycnidia of the two pathogens are very similar in morphological characters (Shoemaker and Brun, 2001; Ghanbarnia et al. 2011). Based on the assessment of the conidial characteristics, the two pathogens cannot be separated and the isolates were identified to species level by molecular methods. The analysis of conidia was performed on 7 randomly selected samples/isolates: the width and length of the conidia were measured to μm and the shape and colour of the conidia were characterised.

During the examination of the features of the isolates, we recorded the shape, colour, edge, patterning, production of pigment, amount of air mycelium formed and the presence of pycnidia. For the isolates for which conidia size was measured, the average daily growth rate of the culture diameter (mm / 24 h) was also determined. Culture characteristics were evaluated on day 28 after inoculation.

To verify the pathogenicity of the isolates, a pathogenicity test was carried out on Koch's postulates using rapeseed plants. The rape plants and their detached leaves were infested.

Molecular studies

For molecular studies, genomic DNA of isolates was extracted using the CTAB (cetyl-trimetil-ammonium-bromide) buffer (Maniatis et al. 1983) and purified using a 24:1 mixture of chloroform and isoamyl alcohol.

Several sections of the genome of *Plenodomus* species have been amplified by polymerase chain reaction (PCR). Species-level identification of isolates was performed by multiplex PCR, using specific primers based on a part of the ITS region. Species-level determination is possible based on target sequence size (Liu et al. 2006). Multiplex PCR was also used to investigate mating types. The forward primers used are mating-type specific (Cozinjsen and Howlett, 2003). To test the *AvrLm4* gene, the method of Van de Wouw and Howlett (2012) was adapted to the Hungarian *P. lingam* isolates. For phylogenetic analysis, the entire ITS region, part of the LSU region, part of the *tub2* gene, part of the *rpb2* region and part of the *act1* gene were examined. We designed our own primers for the *rpb2* region, while for the other regions and genes we used primers published in foreign peer-reviewed journals.

The PCR was verified by gel electrophoresis. Before sequencing, PCR products were purified using High Pure PCR Purification Kit. The concentration of the purified products was checked by spectrophotometer. The sequences were determined by BaseClear B.V. (Leiden, The Netherlands).

The NCBI database BLAST (Basic Local Alignment Search Tool) analysis was used to check, edit and compare the sequences with sequences from the international database (Altschul et al. 1990). The sequences were edited using CLC Sequence Viewer 8 (CLC Bio), corrected where necessary based on the chromatograms, and compared with sequences from isolates from other countries.

For phylogenetic analysis, 26 *P. lingam* and 17 *P. biglobosus* isolates were used. The MEGA11 software package was chosen for sequence analysis and phylogenetic relationships (Tamura et al. 2021). Three separate phylogenetic trees were constructed based on the studied regions. The phylogenetic trees were constructed using Neighbor-Join and Maximum Composite Likelihood (MCL) estimates. To determine the subclade of our isolates, we used ITS regions and the 5.8S rDNA gene as in Mendes-Pereira et al. (2003). For the multi-locus analysis, we used isolates of *P. lingam* 'brassicae' and *P. biglobosus* 'brassicae' for which sequence data of the *tub2* gene, the ITS region, the LSU region and the *rpb2* gene sequences we

also tested are available in the NCBI database. Sequence data for the *act1* gene of these isolates are not available in the database, so a separate phylogenetic tree for the *act1* gene was created using isolates from other NCBI databases.

Testing of fungicidal active substances

To test the fungicidal sensitivity of the isolates, 5 active ingredients (boscalid, fluopiram, azoxystrobin, pyraclostrobin and tebuconazole) were selected. Effect of serial dilution on the growth of mycelia of pathogens was observed using a poisoned agar plate method. The active ingredients were tested at concentrations of 2, 10, 20 and 30 mg/L in four replicates for 5 isolates. We prepared a control containing no solvent and active ingredient and a control containing only solvent. The active ingredients used are not soluble or only slightly soluble in water, therefore isopropanol or methanol was chosen as solvent depending on the active substance. The diameter of the cultures was measured on days 3, 7 and 10.

3. RESULTS

Prevalence of *Plenodomus* species in oilseed rape, distribution of the pathogens

In five years, we isolated 308 times *Plenodomus* species from symptomatic plant parts. Of the isolates, 158 (51.3%) belonged to *P. lingam* and 150 (48.7%) to *P. biglobosus*. Of the 29 sampling location examined, *P. lingam* was isolated from 24 locations and *P. biglobosus* from 18 locations in at least one case. Different proportions of pathogens were isolated at each sampling location.

In 2017, only *P. lingam* was isolates (13), while in 2020, more than 90% of the isolates (40) belonged to *P. biglobosus*.

Symptoms, morphological and culture characteristics of pathogens, pathogenicity test

Leaf spots caused by *P. lingam* and *P. biglobosus* are round in shape with dark margins, several millimetres long, which become lighter in the middle and often erupt. Brown necrotic spots appear on the stems, which later whiten. *P. lingam* causes necrotic spots mainly at the base of the stem, *P. biglobosus* in the upper region. The symptoms caused by the two species are very similar, so they cannot be separated by their leaf and stem symptoms. The necrotic patches on both the leaf and the stem are covered with a mass of pycnidia. The pycnidia we observed were black in colour, roundish and nearly 200-300 μm in size. Conidial mass flows out of the pycnidia in a warm and humid environment, and in large masses it can vary in colour from light brown to burgundy. The conidia of the two pathogens are colourless under microscope, cylindrical,

unicellular, with both ends blunt. The pycnidia and conidia of the two species cannot be distinguished from each other by visual inspection. In the statistical analysis of the conidial dimensions, the conidia of the isolates studied did not differ significantly in either width or length.

Features of *P. lingam* and *P. biglobosus* were evaluated on day 28 after removing. The isolates were grouped into four groups based on the main characteristics. The typical culture of *P. lingam* is greyish-white or dark grey in colour, with a jagged margin, no discolouration of the medium and little air mycelium, while *P. biglobosus* has a characteristic yellowish colouration of the medium and a rich, white air mycelium with blurred margin.

As a result of the pathogenicity test, after 2-3 days at the point of infection, symptoms typical of the pathogens, browning and necrosis of the vessels were observed, and in some cases the formation of pycnidia was also observed.

Molecular analysis of *Plenodomus* isolates

Species-level identification of isolates was based on molecular analysis of ITS regions (ITS1 + 5.8S rDNA + ITS2). The two pathogens can be clearly distinguished from each other by the length of the resulting PCR product. In *P. lingam*, a segment of 331 bp, while in *P. biglobosus*, a segment of 444 bp was generated. On the 308 isolates examined, 158 (51.3%) belonged to *P. lingam* and 150 (48.7%) to *P. biglobosus*.

Sequences of 289 bp in the ITS region of 4 *P. lingam* and 327 bp in the ITS region of 2 *P. biglobosus* were compared with each other and with foreign isolates of the same length from the NCBI database. As a result, *P. lingam* and *P. biglobosus* isolates were found to be 100% reliably separated. When comparing our own *P. lingam* isolates to the reference isolate, 100% identity was observed, while when comparing our own *P. biglobosus* isolates to the reference isolate, 99.7-100% similarity was observed based on BLAST analysis.

For the first time in Hungary, we confirmed the presence of both MAT1-1 and MAT1-2 mating types in *P. lingam* by molecular methods. In the case of MAT1-1, a region of 656 bp, while in the case of MAT1-2, a region of 445 bp was amplified. Among the isolates from Nagylózs and Kétpó we observed isolates of both mating types. From this it can be concluded that *P. lingam* has the potential for sexual reproduction in Hungary.

The *AvrLm4* gene was tested by polymerase chain reaction in a subset of *P. lingam* and *P. biglobosus* isolates. The isolates were derived from different oilseed rape hybrids. In the case of *P. lingam*, the target sequence size was 1127 bp in length, with no amplicon generated in any of the *P. biglobosus* isolates.

The ITS1-5.8S-ITS2 sequence, a region of the LSU, a region of the *tub2* gene, a region of the *rpb2* and a region of the *act1* gene were successfully amplified from 43 isolates. To our knowledge, the sequences of the sections are the first sequence data from Hungary.

In the phylogenetic tree based on the entire ITS1-5.8S-ITS2 region, *P. lingam* and *P. biglobosus* are on separate branches, and the subclades are well separated. The sequences of ITS1-5.8S-ITS2 of the 26 *P. lingam* isolates we tested were fully consistent with the sequence of the same isolate from the United Kingdom. Similarly, the ITS1-5.8S-ITS2 sequences of the 17 *P. biglobosus* isolates were 100% identical to the reference isolate. The analysis clearly shows that all 43 *Plenodomus* isolates belong to the subclades *P. lingam* 'brassicae' and *P. biglobosus* 'brassicae'.

The "multi-locus" analysis showed that more than 98% of the sequences tested were identical to the same sequence in the reference isolates. The studied isolates of *P. lingam* and *P. biglobosus* are separated from each other on the phylogenetic tree, and little variability was observed within the species.

On the tree based on the *act1* gene fragment, *P. lingam* isolates were 99.78-99.89% identical to the nucleotide sequences of the reference isolates, while nucleotide sequences of our *P. biglobosus* isolates were 99.89-100% identical to the same fragment of the reference isolates.

Fungicide sensitivity of *Plenodomus* species

The SDHI fungicide boscalid at concentrations of 10, 20 and 30 mg/L was effective in inhibiting the growth of the tested isolates of both pathogens. At concentration of 2 mg/L, cultures of both pathogens showed little growth over 10 days.

The efficacy of fluopiram, also an SDHI fungicide, varied by species and concentration. Fungicidal effect was observed for all isolates at a concentration of 30 mg/L. At a concentration of 20 mg/L, only minimal mycelial growth was observed in the *P. lingam* isolates tested, while *P. biglobosus* cultures had a larger colony diameter by the end of day 10. The difference between the two pathogens is also observed at concentrations of 10 mg/L and 2 mg/L. In both cases, a larger diameter was measured at the end of day 10 for *P. biglobosus* cultures.

Azoxystrobin, a QoI fungicide, was found to have only fungistatic activity on the majority of isolates, with no fungicidal activity observed at any of the concentrations tested. No significant difference in concentration was observed between the two pathogens.

In a study with pyraclostrobin, also a QoI fungicide, fungicidal activity was observed for all *P. biglobosus* at concentrations of 10, 20 and 30 mg/L, as well as for the *P. lingam* isolate

L280. The L279 isolate also increased at the 30 mg/L concentration. It also showed fungistatic activity at 2 mg/L for all isolates tested.

In the case of tebuconazole, which belongs to the DMI group, different efficacy was observed both by isolate and by concentration. At a concentration of 30 mg/L, a fungicidal effect was observed for all isolates except *P. biglobosus* isolate L308. Even at this concentration, the L308 isolate showed mycelial growth. At the concentration of 20 mg/L, the size of the cultures was almost the same for both species, and at this concentration the active ingredient only produced a fungistatic effect. At concentrations of 10 and 2 mg/L, no significant differences were observed between isolates of the two species.

The solvents at the highest concentrations did not affect the development of *Plenodomus* isolates, so the inhibitory effect can be attributed to the active substances alone.

New Scientific Results

- Following the first identification of *Plenodomus biglobosus* in Hungary (Bagi et al. 2020), we demonstrated its distribution in the country and found that *Plenodomus lingam* and *Plenodomus biglobosus* are a combined cause of leaf spot and stem canker on oilseed rape.
- We characterized at first the variability of the culture characteristics of the *Plenodomus biglobosus* population in Hungary.
- We were the first in Hungary to publish sequence data for the pathogens *Plenodomus lingam* and *Plenodomus biglobosus*: the ITS region, the LSU region, the *tub2* gene, the *rpb2* region and the *act1* gene.
- We were the first to demonstrate the possibility of sexual reproduction in *Plenodomus lingam* in Hungary, and the first to report sequence data on the gene sequence thath determines mating types.
- We were the first in our country to perform *in vitro* susceptibility tests against fungicidal agents on *Plenodomus lingam* and *Plenodomus biglobosus* isolates, showing only fungistatic activity with azoxystrobin, while significant fungicidal activity was observed with boscalid, fluopyram, pyraclostrobin and tebuconazole.

4. DISCUSSIONS AND PROPOSALS

Prevalence of a new pathogen

The emergence of a new pathogen or a change in the genetic composition of a population can call into question the effectiveness of previously applied pest management strategies. Isolated from samples collected in 2018, *P. biglobosus* was described for the first time in Hungary in 2020 on oilseed rape (Bagi et al. 2020). If a new pathogen appears in a country, the next step is to map its spread to help rethink crop protection technologies and plant breeding strategies (Huang et al. 2014). So far, our results suggest that *P. biglobosus* is probably widespread throughout the county. However, based on the presence of pathogens and the frequency of plants infected by them, it is clear that *Plenodomus biglobosus* is more widespread in Hungary than previously thought.

Symptoms, morphological and culture characteristics of pathogens

For the two pathogens, our observed leaf symptoms are consistent with those described by Karolewski et al. (2007) and it can be concluded that the two pathogens cannot be reliably distinguished from each other on the basis of leaf symptoms. The symptoms observed on the stem were consistent with those described by Liu et al. (2014). *P lingam* tends to cause similar necrotising patches in the region near the collar of the stem, while *P. biglobosus* tends to cause similar necrotising patches in the upper part of the stem (Sprague et al. 2007). In the initial phase of our sample collection, we sampled only the stem part of the plants close to the soil, which resulted in the initial isolation of *P. lingam* only. The two pathogens usually appear together on host plants and cause very similar symptoms, and as a consequence, the presence of *P. biglobosus* has not been detected in our country for a long time.

The morphological characters of the pathogens overlap, and atypical characters were also observed in the cultures of some isolates. Based on the literature, the separation and species-level identification of the two pathogens can only be achieved with high confidence using molecular methods (Rouxel et al. 2004), which we confirmed with our results.

Molecular studies

Multiplex PCR was used to rapidly and reliably identify Hungarian Plenodomus isolates to species level based on the method of Liu et al. (2006). We first demonstrated the possibility of sexual reproduction in *P. lingam* by molecularly identifying the mating types MAT1-1 and MAT1-2 (Cozijnsen and Howlett, 2003). The selected primers did not work for the *P. biglobosus* isolates, so new primers need to be designed to further investigate the mating types of the

pathogen. Sexual reproduction allows the pathogen to adapt more rapidly to the host (Parlevliet, 2002), to be able to break through its qualitative resistance (McDonald and Linde, 2002), or to develop resistance to pesticides (Kema et al. 2018).

The *P. lingam* isolates with the *AvrLm4* gene were derived from infected plant parts of different hybrids, suggesting that the resistance of the hybrids does not provide adequate protection against *P. lingam* isolates with the *AvrLm4* gene. We have successfully adapted the method of Van de Wouw and Howlett (2012) to the Hungarian isolates for the *AvrLm4* gene. The genetic composition of the *P. lingam* population is constantly changing. As a consequence, some isolates of the pathogen are able to infect resistant hybrids.

Phylogenetic analysis

Mapping and monitoring the genetic variability of plant pathogens can contribute to better crop protection strategies (Huang et al. 2014). After molecular analysis of the Hungarian *P. lingam* isolates, it became clear that all isolates belong to the 'brassicae' subclade, and despite the morphological diversity of the cultures, they are closely related to each other. Similarly, all of our *P. biglobosus* isolates can be classified in the *P. biglobosus* 'brassicae' subclade. Some researchers believe that the emergence of *P. biglobosus* 'canadensis' in the United Kingdom is responsible for the increase in the importance of *P. biglobosus* (King and West, 2022), and its future emergence in Hungary cannot be ruled out. Similarities in the genetic composition of a pathogen population can be assumed to indicate a reduced ability to adapt to its environment, which may provide important information on the risk of fungicide resistance.

Testing fungicidal active ingredients

In vitro studies with fungicidal agents on *P. biglobosus* were the first in Hungary. Our studies with the two pathogens could form the basis of a broader monitoring effort to optimise crop protection technology.

Our results show that both *P. lingam* and *P. biglobosus* isolates were sensitive to boscalid, which is in agreement with the results of Fajemisin et al. (2022). Based on their results, resistance was not suspected in the Czech isolates.

The *P. lingam* isolates we studied were considered sensitive to fluopyram, whereas Van de Wouw et al. (2021) observed a decrease in sensitivity for some *P. lingam* isolates. In our observations, the *P. biglobosus* isolates tested were also found to be sensitive.

For azoxystrobin, only a fungistatic effect was observed in our study with *P. lingam* and *P. biglobosus* isolates. In the case of azoxystrobin, Mondal et al. (2005) tested *Alternaria*

alternata cultures *in vitro*. Even at concentrations of 100 µg/ml, less than 50% reduction in mycelial growth of the isolates compared to the control was observed. Some observations suggest that this is due to the biological processes of plant pathogenic fungi, which may differ *in vivo* and *in vitro*. Presumably, the *in vitro* tests with *P. lingam* and *P. biglobosus* isolates are not comparable in terms of fungicidal activity within the plant, so *in vivo* experiments are needed to verify this.

P. lingam isolates were found to be sensitive to the active ingredient pyraclostrobin as observed by Mondal et al. (2005), and our results also suggest that *P. biglobosus* isolates are sensitive.

According to Eckert et al. (2010), *P. lingam* isolates were found to be more sensitive to the active ingredient tebuconazole than *P. biglobosus* isolates. The tebuconazole reduced mycelial growth by an average of 50% compared to the control at a concentration of 0.67 mg/L for *P. lingam* and only 1.45 mg/L for *P. biglobosus*. Based on our own experiments, a concentration between 2 mg/L and 10 mg/L would be required for both species to achieve 50% inhibition. Thus, based on our preliminary test, Hungarian isolates of *P. lingam* and *P. biglobosus* appear to be less sensitive to the active ingredient tebuconazole than isolates from the United Kingdom.

Our analysis using active ingredients provides essential information for monitoring future changes in sensitivity. To confirm our results, the same studies would need to be carried out with more definite conclusions about the fungicide susceptibility of the Hungarian *Plenodomus* population. To prevent the development of fungicide resistance, fungicides with different mechanisms of action and different modes of action should continue to be used in combination and/or alternately (Staub, 1991; Brent and Hollomon, 2007).

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5. PUBLICATIONS RELATED TO THE THESIS

1. Peer reviewed scientific papers

   BAGI B., NAGY CS., TÓTH A., PALKOVICS L., PETRÓCZY M. 2020. *Plenodomus biglobosus* on oilseed rape in Hungary. *Phytopathologia Mediterranea* 59 (2): 345-351. IF: 2,020 (2020)

   BAGI B., PALKOVICS L., PETRÓCZY M. 2023. Phylogenetic analysis of *Plenodomus lingam* and *Plenodomus biglobosus* isolates in Hungary. *Journal of Plant Diseases and Protection* – online available – IF: 1,847 (2022)

2. Proofreaded papers

   BAGI B., NAGY CS., TÓTH A., PALKOVICS L., PETRÓCZY M. 2020. Az őszi káposztarepce plenodómuszos betegségének kórokozói. *Növényvédelem* 81 (56): 544-552.

   BAGI B., PALKOVICS L., PETRÓCZY M. 2023. Magyarországi *Plenodomus lingam* és *Plenodomus biglobosus* izolátumok filogenetikai analízise. *Növényvédelem* – in press

3. Conference abstracts

   BAGI B., PETRÓCZY M., NAGY CS., TÓTH A., PALKOVICS L. 2019. Az őszi káposztarepce leptoszfériás betegségének kórokozói. In: HALTRICH A., VARGA Á. (szerk.) 65. Növényvédelmi Tudományos Napok, Budapest, 55.

   BAGI B., JÁKI V., PALKOVICS L., PETRÓCZY M. 2021. A *Plenodomus lingam* és a *Plenodomus biglobosus* génjeinek molekuláris vizsgálata. In: HALTRICH A., VARGA Á. (szerk.) 67. Növényvédelmi Tudományos Napok, Budapest, 61.

   BAGI B., PETRÓCZY M., PALKOVICS L. 2021. Frequency and importance of *Plenodomus* species causing blackleg in Hungary. In: KÖVICS GY., TARCALI G. (szerk.) Tiszántúli Növényvédelmi Fórum (Összefoglalók) – 9th International Plant Protection Symposium at University of Debrecen (Abstracts), Debrecen, 86.