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DIFFERENTIATION OF THE PHYTOPATHOGENIC FUNGI INVOLVED IN SEPTORIA LEAF SPOT COMPLEX BY CLASSICAL AND MOLECULAR METHODS

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Abstract: Three fungal species (*Parastagonospora nodorum, Parastagonospora avenae* f. sp. *triticea and Zymoseptoria tritici*) are involved in septoria leaf spot complex. The successful identification of the pathogens by symptoms, morphological and cultural characteristics is difficult, especially the differentiation between the first two species. Internal transcribed spacer (ITS) is the most commonly sequenced DNA region in molecular studies of fungi and is recommended as a universal barcode for this group of eukaryotic organisms. The purpose of present investigation was to confirm the affiliation of some isolates from this complex to the corresponding pathogenic species through the application of classical and molecular methods. The typical symptoms caused by the respective fungi were described and illustrated. Details of the morphological and cultural characteristics of the isolates were provided. *In vitro* test for mycelial interactions between isolates of *P. nodorum* and *P. avenae* f. sp. *triticea* was carried out by the dual culture method. The ITS regions of rDNA of 12 monoconidial isolates obtained from *Triticum durum* and *T. aestivum* were sequenced and analyzed by alignment to ITS sequences of standards in GenBank (NCBI database). Seven of isolates showed 99% similarities with *P. nodorum* in the ITS region of nuclear rDNA. Two other isolates were 100% identical to *Phaeosphaeria avenaria* f. sp. *triticea* (old synonym of the sexual morph of *P. avenae* f. sp. *triticea*). One isolate was referred to *P. avenae*, which determined its phylogenetic distance from the other isolates of the same group. The sequences of two other isolates overlapped 100% with *Z. tritici* standards in NCBI. Phylogenetic analysis showed distribution of isolates in 2 groups – *P. nodorum/P. avenae* involved in one group and *Z.*

tritici in another, which confirmed the genetic relatedness along with the phenotypical similarity of the first two species. The obtained results proved that combining classical and molecular methods would be necessary for the correct differentiation of phytopathogenic fungi included in the septoria leaf spot complex.

INTRODUCTION

Septoria diseases have significant impact on wheat production in many countries of the world (Figueroa *et al.,* 2018). Their increased economic importance is due to intensive wheat production, susceptibility of cultivars, changes in cultivation practices, increased use of nitrogen fertilizers etc. (Krupinsky, 1999).

The term septoria diseases historically referred to diseases caused by three fungal pathogens of the genus S*eptoria: S. nodorum, S. avenae* f. sp. *triticea* and *S. tritici*. Later, the first two species were renamed *Stagonospora* based on the length: width ratio of their conidia. It was assumed that the conidia in the genus *Septoria* might be 10 times longer than broad in contrast to those in the genus *Stagonospora* (Cunfer and Ueng, 1999). Several years ago some changes were introduced in the taxonomy of *S. tritici.* A new genus *Zymoseptoria* gen. nov. was introduced and several *Septoria* species found on wheat hosts, including *S. tritici,* were affiliated to it (Quaedvlieg *et al.,* 2011). Later, the both species of *Stagonospora* (*S. nodorum* and *S. avenae* f. sp. *triticea*) were assigned to a new genus *Parastagonospora* (Quaedvlieg *et al.,* 2013).

These three pathogens co-exist together in the wheat management systems in Bulgaria and incite similar leaf symptoms (Rodeva *et al.,* 2014). The purpose of this investigation was to make a differentiation of the phytopathogenic fungi involved in septoria leaf spot complex on the basis of phenotypic and genotypic characteristics using classical and molecular methods.

MATERIALS AND METHODS

The leaf samples of wheat (*Triticum aestivum* L. and *T. durum* Desf.) were collected from commercial and experimental fields. The diagnostics of septoria leaf spotting started on the basis of symptoms. The isolation of pathogens was made by transfer of separate pycnidia on potato dextrose agar (PDA). The monoconidial isolates were obtained by pouring a small amount of diluted suspension onto the surface of agar plate containing 2% water agar and separating single germinated conidia after 24 hours. The inoculum needed to study the growth and sporulation of the fungi was obtained by cultivation of the isolates on PDA in dark at 22°C. For the morphological and cultural characterization the isolates were cultivated on PDA in thermostat under 12 h-alternating day-night light conditions at 22°C. *In vitro* test for mycelial interactions was carried out with isolates identified as *Parastagonospora nodorum* and *P. avenae* f. sp. *triticea* by applying the dual culture method. Each isolate was grown on PDA for 5 days before pairing. Mycelial disks (5 mm in diameter) were cut from the periphery of actively growing colonies and placed apart on PDA in 9-cm petri dishes, one pairing per dish. Isolates were paired in all possible combinations. Each strain was also grown individually for growth rate comparisons. All treatments were replicated three times and incubated in dark at temperature of 22°C. Mycelial interactions were recorded

15 days after inoculation. Pairings were scored as compatible when the two isolates merged to form one colony with no distinct interaction zone and as incompatible when they failed to grow together and a thin mycelial-free space remained between them.

Twelve monoconidial isolates (Table 1) were selected for phylogenetic analysis. The production of fungal biomass was made on PDA at 22 ± 2°C for 10 days. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was performed with the universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990), using the following PCR program: 96ºC – 2 min, followed by 35 cycles of 96ºC – 1 min, 55ºC – 1 min, 72ºC – 2 min and final elongation at 72ºC – 10 min. The reactions were performed using PuReTaqTM Ready-To-GoTM PCR beads (GE Healthcare Life Sciences), according to the manufacturer's instructions. The ITS regions of rDNA of the isolates were sequenced and analyzed by alignment with database from the National Center for Biotechnology Information (NCBI) – Bethesda, USA, using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed applying the Mega6 program (Tamura *et al.,* 2013).

Table 1 Isolates of *Parastagonospora nodorum* (Pan), *Parastagonospora avena*e f. sp. *triticea* (Pat) and *Zymoseptoria tritici* (Ztr) used for phylogenetic analysis

No	Isolate designation	Host plant cultivar	Location
I. *Parastagonospora nodorum* (syn. *Stagonospora nodorum; Septoria nodorum*)			
1.	Pan1	Td* Pescadou	IPPG** - Sofia
2.	Pan3	Td Zvezditsa	IPPG - Sofia
3.	Pan4	Td Deyana	IPPG - Sofia
4.	Pan5	Td Victoria	IPPG - Sofia
5.	Pan6	Td Zvezditsa	IPPG - Sofia
6.	Pan7	Td Meridiano	IPPG - Sofia
7.	Pan9	Td Pescadou	IPPG - Sofia
II. *Parastagonospora avenae* f.sp. *triticea* (syn. *Stagonospora; Septoria*)			
8.	Pat1	Td Victoria	Td Victoria
9.	Pat3	Ta Sadovo1	Ta Sadovo1
10.	Pat4	Ta 58-2	Ta 58-2
III. *Zymoseptoria tritici* (syn. *Septoria tritici; Mycosphaerella graminicola*)			
11.	Ztr2	Ta Enola	Commercial field – Knezha
12.	Ztr4	Td Yukon	IPPG - Sofia

*Td – *Triticum durum*; Ta – *Triticum aestivum*

**IPPG – Institute of Plant Physiology and Genetics

RESULTS AND DISCUSSION

The spots caused by *Z. tritici* on young wheat plants in the spring first appeared on the lowest leaves, especially those that were in contact with the soil. They were watery, grey-green to light brown, with yellow halo and contained a small number of pycnidia (**Fig. 1a**).

On the leaves of adult plants, the lesions were limited by the leaf nerves, narrow, elongated, rectangular, with rounded edges. Their length ranged from 5 to 15 mm. The disease progress in the susceptible wheat varieties led to spot coalescing, formation of large necrotic area and development of great number of pycnidia, very often arranged in rows (**Fig. 1b**).

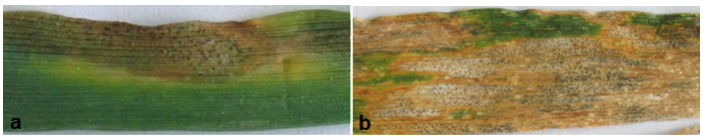

Fig. 1 Symptoms caused by *Zymoseptoria tritici* on wheat leaves:
a. on young plants early in the spring; b. on adult plants.

The early symptoms incited by *P. nodorum* were relatively small, oval to lenticular, initially chlorotic, later light brown with no or a few pycnidia (**Fig. 2a**). The spots on the adult plants were elongated oval with pointed ends, reddish-brown with a fading center where a lot of scattered pycnidia occurred. The lesion often coalesced (**Fig. 2b**).

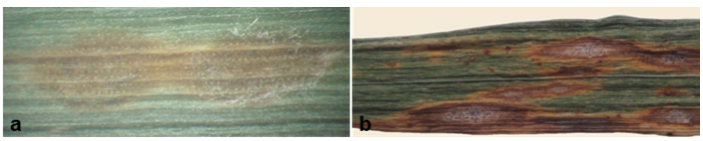

Fig. 2 Symptoms caused by *Parastagonospora nodorum* on wheat leaves:
a. on young plants early in the spring; b. on adult plants.

On young leaves *P. avenae* f. sp. *triticea* caused oblong, irregular, grayish-brownish watery spots with dark brown edges. They bore scattered single, spherical, dark brown pycnidia (**Fig. 3a**). Typical symptoms, appearing later in the growing season were oval-rhombic, ovate, lenticular, brown spots, sometimes surrounded by a dark brown border, with slightly pointed ends and fading center bearing many scattered pycnidia. The spots enlarged and merge into extensive necrosis (**Fig. 3b**).

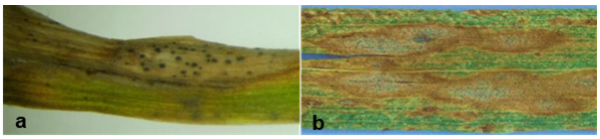

Fig. 3 Symptoms caused by *Parastagonospora avenae* f. sp. *triticea* on wheat leaves: a. on young plants early in the spring; b. on adult plants.

Colonies of *Z. tritici* on PDA consisted of a large number of secondary conidia, which have a pink color in mass during the first 2 weeks of cultivation (**Fig. 4a**). With age, the conidia began to germinate and formed a dark, thick, mycelial mass with a stroma-like surface. The colonies grew very slowly. They were compact, dome-raised, with a deeply contoured surface, where difficult to distinguish pycnidia appeared extruding the conidial jelly in the form of pale-white, well-noticeable drops (**Fig. 4b**).

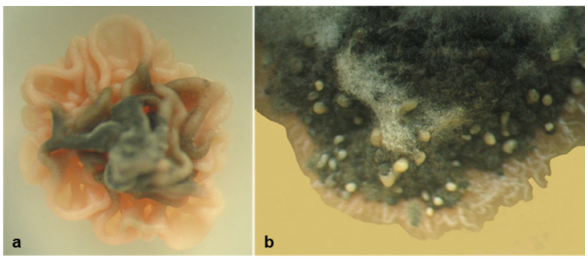

Fig. 4 Colony morphology of *Zymoseptoria tritici* on potato-dextrose agar: a. after 14 days of cultivation; b. after 30 days of cultivation.

On PDA *P. nodorum* had a slower growth than *P. avenae* f. sp. *triticea*. The colonies of both fungi had a correct round shape. From above, those of *P. nodorum* had a white periphery and a gray central part, and of *P. avenae* f. sp. *triticea* – a cream-pink color and a pronounced concentric zonality (**Fig. 5a**). The reverse side of *P. nodorum* colonies had a reddish-brown color and that of *P. avenae* f. sp. *triticea* – dark pink-red (**Fig. 5b**). With age, the *P. nodorum* colonies darkened. On the above side, their color changed in gray-greenish with violet hue, and on the reverse side – in dark gray-black. The pigment released in the culture medium was yellowish-brown in the beginning, later became light brown.

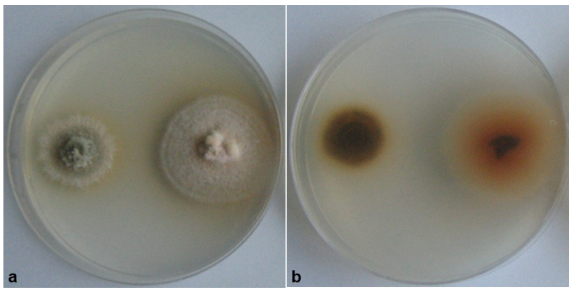

Fig. 5 . Colony morphology of *Parastagonospora nodorum* (on the left) and *Parastagonospora avenae* f. sp. *triticea* (on the right) on potato dextrose agar after 10 days cultivation: a. above; b. reverse.

All isolates included in the *in vitro* test for mycelial interactions displayed self-compatibility on PDA 15 days after inoculation. Pairings grew together and formed a confluent colony. Mycelial pairing between the isolates of *P. nodorum* and *P. avenae* f. sp. *triticea* showed that initially, the two colonies grew evenly each to other on PDA. After 15 days of cultivation, an incompatible reaction was observed, in which there was a pronounced area of mutual suppression of about 5 mm (**Fig. 6)**. Both pathogens emitted a red brown pigment in the culture medium, more intensely *P. avenae* f. sp. *triticea*.

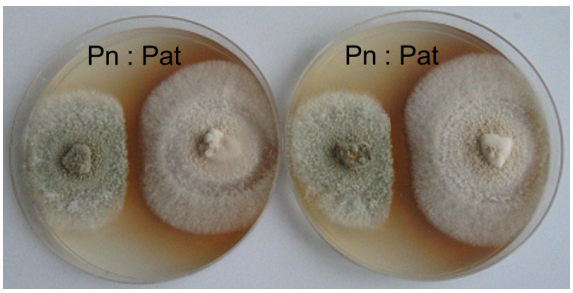

Fig. 6 Mycelial incompatibility of *Parastagonospora nodorum* (Pn) and *Parastagonospora avenae* f. sp. *triticea* (Pat) on potato-dextrose agar after 15 days cultivation by the dual culture method.

In *Z. tritici*, two types of pycnidia were found: normal size (diameter 85-190 μm) containing only macroconidia or mixed macro- and microconidia and smaller (diameter 65-120 μm) in which only microconidia were found. The micropycnidia were formed more often on the leaf sheaths. On the leaves occurred mostly normal size pycnidia with hyaline, thin-walled, filamentous, smooth, straight or curved, guttulate macroconidia, with 3-5 unclear septae, 30-70 x 1.0-2.5 μm (**Fig. 7a**).

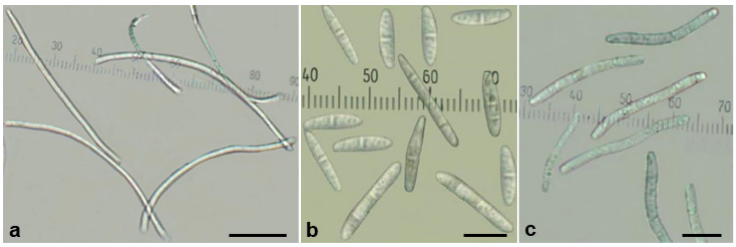

Fig. 7 Conidial morphology of the three studied species:
a. *Zymoseptoria tritici* (scale = 20 μm); b. *Parastagonospora nodorum* (scale = 10 μm); c. *Parastagonospora avenae* f. sp. *triticea* (scale = 10 μm).

Parastagonospora nodorum could be distinguished from *P. avenae* f. sp. *triticea* to a certain extent by the conidial morphology. Conidia of *P. nodorum* were shorter (very rarely more than 25 μm) and had 0 to 3 well noticeable septae (**Fig 7b**). *Parastagonospora avenae* f. sp. *triticea* was characterized by a larger conidial size, the presence of droplets and a greater number of unclear septae in them (**Fig. 7c**).

Sequence analysis and the comparison of studied isolates with database of NCBI showed that 7 of them (Pan1, Pan3, Pan4, Pan5, Pan6, Pan7 and Pan9) had 99% similarities with *P. nodorum* in the ITS region of nuclear rDNA. Two of the sequenced isolates (Pat1 and Pat3) were 100% identical to *Phaeosphaeria avenaria* f. sp. *triticea* (old synonym of the sexual morph of *P. avenae* f. sp. *triticea*). Isolate Pat4 showed 98% homology with *P. avenae*, which determined its phylogenetic distance from other isolates of the same group. The sequences of Ztr2 and Ztr4 isolates overlapped 100% with *Z. tritici* standards in NCBI (**Fig. 8**).

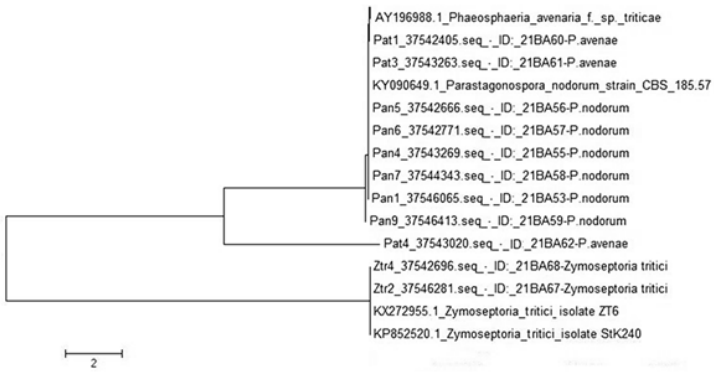

Fig. 8 . Phylogenetic analysis of isolates belonging to *Parastagonospora nodorum*, *Parastagonospora avenae* f. sp. *triticea* and *Zymoseptoria tritici.*

Zymoseptoria tritici is one of the most important fungal pathogens on wheat in Europe (Eyal, 1981; 1999; Kema *et al.,* 2008). The disease it causes could be distinguished from the other two septoria diseases on the basis of symptoms – the size and shape of the spots and the distribution of pycnidia in them. The phenotypic characteristics as morphology of the colonies and conidia are also very useful for differentiation of the fungus. Previous investigations revealed that the ITS region of *Z. tritici* differed from those of *Parastagonospora* species. The sequence similarity was low (ca 70%) as compared to within the *Parastagonospora* species (ca 95-98%) (Ueng *et al.,* 1998).

Both *Parastagonospora* species: *P. nodorum* and *P. avenae* f. sp. *triticea* cause two symptomatically similar leaf diseases. *P. nodorum* also incites glume blotch on wheat and related cereals and occurs widely around the world (Eyal,

1999; Solomon, 2006). *Parastagonospora nodorum* and *P. avenae* f. sp. *triticea* are morphologically similar. To a certain extent, they can be distinguished by the color of the colonies and the smaller dimensions of the conidia of *P. nodorum* (Richardson and Noble, 1970; Obst, 1972; Krüger and Hoffmann, 1978; Rodeva, 1989), although there is a certain range in which the sizes of the conidia of these two species overlap.

PCR is the most important and sensitive technique presently available for the detection of plant pathogens. Main advantages of PCR techniques include high sensitivity, specificity and reliability (Capote *et al.,* 2012). Now, PCR is widely used for plant pathogen detection as well. For the pathogen diagnostics, PCR technology requires specific primers to initiate the DNA replication process, which could limit the practical applicability and at the same time is costly, but with no doubt this approach gives highly specific results (Aslam *et al.,* 2017). A PCR-RFLP assay was designed to distinguish *P. nodorum* from *P. avenae* f. sp. *triticea*, which revealed that approximately 4% of the isolates of both pathogens showed evidence of hybridization (McDonald *et al.,* 2012). According to the data of phylogenetic analysis obtained in the present study *P. nodorum* and *P. avenae* f. sp. *triticea* were distributed in one group. It was found that the isolates of *P. nodorum* showed a significantly lower degree of genetic variation than the isolates of *P. avenae* (Ueng and Chen, 1994). Our results also revealed that the studied *P. nodorum* isolates were closely related. The isolates of *P. avenae* f. sp. *triticea* displayed higher genetic variation. Pat4 split the phylogeny and was distant from Pat lineage. *P. avenae* has two formae specialis, *P. avenae* f. sp. *triticea* (Pat) with a broader host range, infecting wheat, barley, rye and several common grasses, and *P. avenae* f. sp. *avenaria* (Paa), which incites a leaf disease on oat (Ueng and Chen, 1994). With sequence similarities in the noncoding ITS region of nuclear rDNA, the partial glyceraldehyde-3-phosphate dehydrogenase (gpd) gene fragment containing the intron 4, and the full-length β-glucosidase (bgl1) gene, five *Phaeosphaeria* isolates from oat were molecularly determined to be Paa and two oat isolates from Poland appeared to be Pat (Reszka *et al.,* 2005). These results suggested that classification of two *Phaeosphaeria avenaria* formae speciales based on host specificity should be re-evaluated. In our investigation Pat4 could belong to Paa although it was isolated from wheat (*T. aestivum*).

ITS is the most commonly sequenced DNA region in molecular studies of fungi and is recommended as a universal barcode for this group of eukaryotic organisms (Begerow *et al.,* 2010; Schoch *et al.,* 2012). In this investigation the affiliation of 12 isolates was determined on the basis of disease symptoms and phenotypic characters and confirmed by phylogenetic analysis.

CONCLUSIONS

The successful identification of the pathogens in the septoria leaf spotting complex occurring on bread and durum wheat in Bulgaria on the basis of symptoms, morphological and cultural characteristics was difficult, especially distinguishing *P. nodorum* from *P. avenae* f. sp. *triticea*.

Phylogenetic analysis showed distribution of isolates in 2 groups - *P. nodorum / P. avenae* and *Z. tritici*, which confirmed the genetic relatedness along with phenotypical similarity of the first two species. The obtained results proved that combining classical and molecular methods was necessary for the correct differentiation of phytopathogenic fungi included in the septoria leaf spot complex.

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CONFLICT OF INTEREST

The authors of the present manuscript declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENTS

The collection of leaf samples was implemented by S.N., Z.S. and R.R. The isolation, morphological and cultural characterization of the isolates, in vitro test for mycelial interactions as well as the color photographs of symptoms and colony and conidial morphology was made at the Institute of Plant Physiology and Genetics (IPPG) – Sofia by S.N., Z.S. and R.R. Molecular investigation was carried out at AgroBioInstitute (ABI) – Sofia by S.S. and P.H. All authors contributed to the analysis of the results and to the writing of the manuscript. R.R., S.S. and P.H. have seen and approved the final version of the manuscript being submitted.

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