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EARLY PCR DETECTION OF THE *MYCOSPHAERELLA GRAMINICOLA* IN THE LEAVES OF WINTER WHEAT IN POLAND

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ABSTRACT

The causal agent of *Septoria tritici blotch (STB)* is the fungus *Mycosphaerella graminicola*. Polymerase chain reaction (PCR) was used for the early detection of *M. graminicola* genetic material in 160 samples of winter wheat leaves from Poland, collected in the spring and autumn of 2012. Difference was noted between the number of detections in autumn and spring. Species-specific PCR for *M. graminicola* amplified the expected DNA fragment in 21.43% of 2012 spring samples and in 11.11% of 2012 autumn samples. The highest number of *M. graminicola*-positive samples was identified in eastern and north-western Poland. The frequency of *M. graminicola* detections was highest where wheat followed winter rape and winter wheat, as opposed to maize. Detection of *M. graminicola* DNA was lower mainly in leaves of wheat that was sown later. The results show that the PCR assay is a reliable method for the early detection of the presence of *M. graminicola* DNA in the leaves of wheat.

Key words: *Mycosphaerella graminicola*; detection, winter wheat; species-specific PCR.

INTRODUCTION

Mycosphaerella graminicola (Fuckel) Schroeter (anamorph *Septoria tritici* Rob. Ex 20 Desm.) causes Septoria tritici blotch (STB), currently the major foliar disease of winter wheat worldwide (Bearchell et al., 2005). When the flag leaf and the two leaves below become infected, severe yield losses occur (Robert et al., 2005). STB can be the cause of yield losses of up to 30-40% (King et al., 1983; Eyal et al., 1987).

First symptoms of STB are usually detected on lower leaves. The first phase of STB is the production of ascospores in autumn, serving as a source of inoculum for the following spring (Sanderson, 1972). The symptoms are yellowish or chlorotic flecks and necrotic blotches containing varying density of small black pycnidia in lesions, the most reliable sign for the disease identification in the field (Kema et al., 1996). Asexual and sexual reproduction cycles of this fungus are known. Asexual pycnidiospores are transported from plant to plant via rain splash. Dispersal of ascospores produced by the sexual stage is mediated by wind, which suggests their high potential for long-distance travel (Zhan et al., 2003).

The experiments were carried out to apply the agar plate technique in the identification of *M. graminicola*. However, this fungus was easily overgrown by fast-growing saprophytes of wheat and this method is now considered as not practical for routine testing (Brokenshire, 1975; Consolo et al., 2009). In addition, methods based on the isolation into pure culture, obtaining monosporous isolates and microscopy are laborious and not sufficiently effective (Abramova et al., 2008). However, using PCR and ELISA test for pre-symptomatic detection of *M. graminicola* can improve the disease control by planning better-timed fungicide applications and enhancing risk assessment models (Lovell et al., 1997; Kendall et al., 1998).

Our objective was to determine the presence of *M. graminicola* in two early pre-symptomatic periods in samples from different regions of Poland. By comparing the results obtained with the selected factors

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(wheat variety, geographic region, date of sowing and previous crop), we hope to find important interactions that might lead to more profitable disease control.

MATERIAL AND METHODS

Sampling

Samples of winter wheat plants were collected in the spring and autumn of 2012 from random fields located in Poland (number of fields – 70 in spring, 90 in autumn). Samples were collected during the tillering and just before the stem elongation stage in spring 2012, while in autumn 2012 during 3-4 leaves unfolded stage. The number of plants collected varied slightly at each site, ranging from 10 to 20 plants, depending on the size of the field. Samples were divided into following four groups according to geographic and climatic regions: eastern, central, south-western and north-western Poland. Growers were provided with a questionnaire to supply information on the previous crop, sowing date and wheat variety. Each sample was taken to the laboratory where it was stored at -20ºC.

DNA extraction

The plant tissue from about 10-15 leaves per each site was used for DNA isolation. DNA was extracted from 30- to 40-mg subsamples of wheat leaves that were transferred to 2 ml Eppendorf tubes and pestled in liquid nitrogen until a fine powder. DNA was obtained using Plant and Fungi DNA Purification Kit (EURx, Poland), according to the manufacturer's instructions. DNA purity and concentration was determined spectrophotometrically (NanoDrop, ThermoScientific). Until analysis, DNA samples were stored at -20ºC.

PCR identification of *M. graminicola*

Species-specific PCR primers were used for the identification of *M. graminicola*. Among 4 primer pairs designed by Consolo et al. (2009), we have selected primer set A, consisting of forward primer 5'-CCAAAAAACACTGCATCTCTGCG-3' and reverse primer 5'-CGTGAACTCCGCGGCGA GACGTG-3', derived from the internal ITS region of *M. graminicola*. Selected primer set amplified a fragment of 336 bp. Reactions were run in 25 µl volume using 2 x PCR Master Mix (Thermo Scientific Fermentas, Lithuania) with 20 pmol of each primer and 20 ng of DNA on a SensoQuestLabcycler (SensoQuest GmbH, Germany). Thermal cycling conditions were as follows: an initial step at 95ºC for 3 min and 40 cycles at 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s followed by 72ºC for 8 min.

Amplification products were separated by electrophoresis in 1.5% (wt/vol) agarose gels stained with ethidium bromide in 1× TBE 1.5 h at 120 V. Then DNA bands were visualised using a GelDoc 2000 gel documentation system (BioRad, USA), and sizes of the PCR products were determined by comparison against the migration of GeneRuler 100 bp plus DNA Ladder (Thermo Scientific Fermentas, Lithuania).

Data analysis

Data analysis was carried out to (i) determine whether the PCR application could be used for the identification of pre-symptomatic *M. graminicola*, and (ii) to assess whether the incidence of pre-symptomatic *M. graminicola* is dependent on one of the factors, i.e., geographic location, date of sowing, wheat variety and date of sampling. Charts were plotted using Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

Several PCR-based assays have been developed for *M. graminicola* detection (Beck and Ligon, 1995; Fraaije et al. 1999, 2001). However, Guo et al. (2006) found that PCR amplification with above-mentioned assays resulted in the amplification of non-specific bands derived from other wheat pathogens. PCR-based method developed in the study of Consolo et al. (2009) provides an efficient strategy for the detection of *M. graminicola* that was tested with many fungal isolates and wheat seed lots. Primer set A designed by these authors successfully amplified the target DNA sequence from as little material as 0.5 pg, and therefore, it was selected for this study.

Table 1 shows the incidence of *M. graminicola* DNA identified by the PCR technique in the eastern, central, south-western and north-western regions of Poland. Species-specific PCR for *M. graminicola* amplified the expected DNA fragment in 21.43% of 2012 spring samples and in 11.11% of 2012 autumn samples of winter wheat. As expected, the difference between the detection of *M. graminicola* in autumn and spring was evident. Shaner and Buechley (1995) reported that the symptoms of *M. graminicola* presence in winter wheat appeared usually early in the spring. Our results suggest that the presence of *M. graminicola* DNA on leaves could also be detected in autumn. Ascospores may constitute the main source of *M. graminicola* genetic material during this season, and probably are the main cause of the primary infections in autumn (Zhan et al., 2001).

Table 1. Incidence of *M. graminicola* detected by the diagnostic polymerase chain reaction technique in different sampling regions (n = 160)

Region	Date of sampling		Total (%)
	Spring 2012 (%)	Autumn 2012 (%)	
Eastern Poland	4/14 (28.57)	2/14 (14.29)	6/28 (21.43)
North-western Poland	4/21 (19.05)	5/23 (21.74)	9/44 (20.45)
	3/13 (23.07)	1/18 (5.56)	4/31 (12.90)
South-western Poland	4/22 (18.18)	2/35 (5.71)	6/57 (10.53)
Total	15/70 (21.43)	10/90 (11.11)	25/160 (15.63)

(Data are presented as number of positive samples/all samples, %.)

The highest number of *M. graminicola*-positive samples was identified in the eastern (21.43%) and north-western (20.45%) regions of Poland. These results confirm that in general the severity of fungal diseases is higher in eastern Poland (Walczak et al., 2009).

According to the questionnaire results, 56 winter wheat varieties were present, none of which differed significantly in the number of *M. graminicola* detections (data not shown). However, it has been shown that the variety of wheat could be a very important factor determining the prevalence of fungal pathogen (Sebei and Harrabi, 2008). Wheat cultivars and cultivar mixtures are also important factors influencing disease progress. It should be noted that this effect could be strongly dependent on the weather conditions (Cowger and Mundt, 2002). Most of wheat cultivars resistant to STB were evaluated with single-genotype isolates of *M. graminicola*. It is known that on the field wheat cultivar is exposed to mixtures of isolates (Brown et al., 2001). It is important to evaluate the correlation between wheat cultivars with *M. graminicola* in different locations and during different years.

A limited range of forecrops was present in the surveyed samples, mainly composed of winter rape, maize or winter wheat. Previous crop was the factor associated with the occurrence of *M. graminicola* in wheat leaves (Table 2). The frequency of *M. graminicola* detections was highest in the instances where wheat followed winter rape or winter wheat. It is known that the higher number of fungal infections of winter wheat is positively correlated with cereal forecrops (Sawińska et al., 2006).

Table 2. M. graminicola incidence in winter wheat in relation to previous crop (n = 126)

Previous crop	Date of sampling		Total (%)
	Spring 2012 (%)	Autumn 2012 (%)	
Winter rape	10/24 (41.66%)	4/44 (9.09%)	14/68 (20.59%)
Maize	0/10 (0.0%)	1/8 (12.5%)	1/18 (5.55%)
Winter wheat	2/18 (11.11%)	5/22 (22.72%)	7/40 (17.5%)

(Data are presented as number of positive samples/all samples, %.)

Furthermore, the percentage of *M. graminicola* incidence depended on the date of sowing (Figure 1). The percentage of samples with *M. graminicola* detection was lower mainly in leaves of wheat that was sown

later. However, plants sown later are smaller, and therefore, closer to the inoculum sources, which could increase the risk of disease progression (Shaw, 1991).

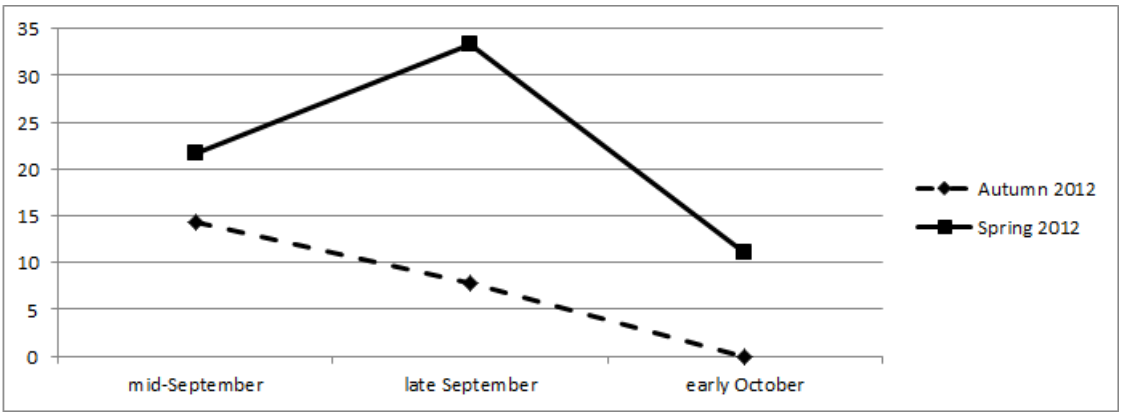

Figure 1. The percentage of *M. graminicola* incidence in leaves of winter wheat sown in different months (early - 1st decade of the month, mid - 2nd decade, late - 3rd decade)
Earlier and later dates of sowing were disregarded due to the low number of samples.

CONCLUSIONS

In the present work, species-specific PCR method was used for *M. graminicola* detection in wheat leaves from eastern, central, south-western and north-western Poland. The higher number of *M. graminicola* detections in spring in comparison to autumn was evident. The highest number of *M. graminicola*-positive samples was also identified in the eastern and north-western regions of Poland. None of wheat varieties differed significantly in the number of *M. graminicola* detections. Effect of the previous crop was observed, the frequency of *M. graminicola* detections was highest in the instances where wheat followed winter rape or winter wheat. *M. graminicola* detections was also lowest on plants that were sown later. The obtained findings indicated that the climate, previous crop and date of sowing could influence the risk of *M. graminicola* infection progression.

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