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USE OF SCAR-PCR IN DIAGNOSTICS OF STEM BASE PATHOGENS OF THE *Rhizoctonia* AND *Oculimacula* GENUS

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A b s t r a c t

The aim of the paper is to compare the efficacy of SCAR-PCR assay and conventional diagnostic technique (visual assessment, isolation on PDA medium) in the identification of fungi from the genera *Rhizoctonia* and *Oculimacula* from winter triticale, rye, and barley during the shooting stage. The usefulness of molecular diagnosis of fungal pathogens in crop plants has been demonstrated. The application of SCAR--PCR assay allowed early detection of the following pathogens: *O. yallundae*, *O. acuformis, R. cerealis* and *R. solani*, in plant tissues. This method was particularly effective in detection of *R. solani*. The research showed the usefulness of PCR markers for early detection of fungal pathogens, even if symptoms were not visible. Using the PCR technique, especially in combination with conventional methods, substantially increases the precision and effectiveness of disease diagnostics.

Key words: PCR assay, sharp eyespot, eyespot, diagnostics, barley, triticale, rye

INTRODUCTION

Foot diseases are one of the most important problems in cereal production. Among them, eyespot caused by *Oculimacula yallundae* (formerly *Tapesia yallundae*) and *O. acuformis* (formerly *T. acuformis*) is regarded as the most dangerous disease in temperate climate (C r o u s et al. 2003; R a y et al. 2006; C h a p m a n et al. 2008). It can be found on many cereal species, including wheat, barley, oats, triticale, and rye. These cereals can also be infected by another stem base pathogen – *Rhizoctonia cerealis*, the main causal agent of sharp eyespot. This disease causes lower yield losses than eyespot (C r o m e y et al. 2002). Due to the fact that fungicides are effective only against eyespot, it becomes increasingly important. More and more symptoms of sharp eyespot are observed under field conditions (L e m a ń c z y k , 2010a). Unfortunately, the symptoms of these two diseases are similar and they can be sometimes confused.

Visual diagnosis of stem base diseases is very difficult, especially when the infection is caused by more than one pathogen. In such cases, one can easily make a mistake, especially in the early stages of the disease. In addition, symptoms may be obscured by the second pathogen. Traditionally, confirmation of visual assessments concerning the presence of the causal agent of stem base diseases is based on the isolation and identification of pathogens on artificial nutrient media. However, saprotrophic organisms are numerously isolated from diseased tissues, because of the slower growth of typical pathogens obtained using such type of identification method (B a t e m a n and K w a ś n a , 1999).

Molecular methods based on polymerase chain reaction (PCR), especially SCAR-PCR, may be very helpful in the diagnosis of plant diseases. Analysis of molecular markers using a SCAR (Sequence Characterized Amplified Region) allows one to obtain a species-specific amplicon (N i c h o l s o n et al. 1997; Ł u k a n o w s k i et al. 2009). The use of species-specific primers allows the identification of the pathogen, often before the appearance of disease symptoms, also directly in infected tissues without their isolation (N i c h o l s o n and P a r r y , 1996). Early detection of the pest allows for quick application of the relevant plant protection product. It is extremely important because fungicide treatments against stem base diseases are recommended to be applied in the early stages of plant development, when the plant often does not have any visible sign of disease. It should be emphasized that

the fungicides effectively control eyespot, but not sharp eyespot. For this reason, it is very important to accurately determine the threats posed by various pathogens in order to conduct fungicide treatment only when it is really necessary.

SCAR is a fast and relatively inexpensive technique. However, it is used mainly in the identification of pathogens of wheat, but there is little information regarding its application in the case of other cereals. The aim of the study was to examine the usefulness of the SCAR-PCR technique, in comparison with traditional methods in the diagnosis of stem base pathogens (from the genus *Rhizoctonia* and *Oculimacula*) of winter forms of rye, triticale and barley, in the initial stage of shooting.

MATERIALS AND METHODS

Survey and visual assessment

Plants of winter rye (*Secale cereale* L.), cultivar Dańkowskie Złote, were sampled from production fields located in Mochełek (17°51'E, 53°13'N), whereas winter triticale (*×Triticosecale* Wittm. ex A.Camus), cultivar Gniewko, and winter barley (*Hordeum vulgare* L.), cultivar Traminer, were collected from fields in Chrząstowo (17°33'E, 53°10'N). In the laboratory, plants were thoroughly and carefully rinsed off the soil and divided into groups according to the type of symptoms:

R – stems with pale brown, spindle-shaped, clearly defined spots with a dark edge, typical symptoms for sharp eyespot,

O – stems with brown, spindle-shaped spots, typical symptoms of eyespot,

F – stems with brown streaks, lines or leaking, symptoms typical of *Fusarium* foot rot,

H – healthy stems.

In 2008, 32 plants of barley, 29 of triticale, 31 of rye, and in 2007 35 plants of rye were chosen for further analysis. Samples were collected in the initial shooting stage – GS 31 (Zadoks et al. 1974). The choice of this phase was caused by a commonly recommended time of eyespot control. Isolation of fungi was conducted on artificial media and confirmation of their presence in plant tissues was done with PCR assay (Doyle and Doyle, 1990; Nicholson and Parry, 1996).

Isolation of fungi and identification by morphology

Ten 2mm fragments of the stem base were cut from each sample of every studied cereal species. In the case of visible disease symptoms, the fragments were taken from the places where changes were most pronounced. From macroscopically healthy stems, the pieces were cut from the tillering node.

The fragments were washed for 45 minutes in running tap water, separately for each plant, surface sterilized in 1% $AgNO_3$ solution for 15 seconds and washed three times for 1 min. in sterile distilled water. Five stem base fragments were put onto Petri dishes with Potato Dextrose Agar (PDA) enriched with streptomycin (50mg streptomycin per 1L of medium). The samples were incubated at 22°C and transferred onto agar slants. When the mycelium appeared on the medium surface, the fragments were taken from the plates, frozen (-30°C) and used for further molecular analysis.

Fungal isolates were identified with mycological keys. Particular species of the *Rhizoctonia* genus were distinguished using the technique of staining fungal hyphae (Bandoni, 1979). Moreover, the linear growth rate of mycelium was determined on PDA medium. Identification of *O. yallundae* and *O. acuformis* was based on the available papers (Creighton, 1989; Crous et al. 2003; Korbas, 2008).

Extraction of DNA and molecular identification

Total DNA isolation was carried out according to the modified protocol described by Doyle and Doyle (1990). The same fragments, which had been previously placed on PDA medium, were molecularly tested for the presence of *R. cerealis* and *R. solani*, *O. yallundae*, and *O. acuformis*. They were pre-homogenized and placed in 1.5 ml Eppendorf tubes. Then, they were crushed in liquid nitrogen for 5 min. into fine powder and poured with 600µl of extraction buffer (0.24 ml 5% CTAB, 0.17ml 5M NaCl, 0.02ml 5M EDTA, 0.06ml 1M tris-HCl pH 8, 1.2µl β-mercaptoetanol, 0.12g PVP, 0.12 ml H_2O miliQ). The tubes were incubated in water bath at 65°C for 40 min., and their content was mixed by inverting every ten minutes. After cooling, 300µl of chlorophorm and isoamyl alcohol mixture (24:1 v/v) and 300µl of phenol solution with a pH of 8.0 was added to every tube and vortexed. The tubes were centrifuged (12000 RPM, 10 min.) and the upper phase (supernatant) was transferred into a new tube and mixed with 600µl of chlorophorm and isoamyl alcohol mixture (24:1 v/v) by inverting the tubes for 3 minutes. After centrifugation, the supernatant was transferred to a new tube with 50µl 5M NaOH and 700µl of frozen 95% ethanol was added to precipitate DNA. It was washed twice with 300µl of frozen 70% ethanol, centrifuged, ethanol was discarded and DNA was left to dry. Efficiency of DNA isolation was measured by its concentration determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,

Inc. USA). DNA concentration used for PCR assay was 150 ng×µl^{-1}.

The PCR reactions were carried out using a Taq PCR Core Kit (QIAGEN Inc.) in a total volume of 12.5µl. Each reaction mixture contained 3.9µl H_2O, 2.5µl 5× buffer Q, 1.25µl 10× PCR buffer, 0.5µl $MgCl_2$, 0.25µl dNTP mix, 0.75 primer I (Table 1), primer 0.75 II, 0.1µl *Taq* polymerase, 2.5µl DNA. The PCR amplification was carried out in a thermocycler (Eppendorf Mastercycler ep gradient, Germany) in 30 cycles using cycling conditions presented in Table 2.

The PCR products were checked by electrophoresis of 5 µl of product in a 1.4% agarose gel containing ethidium bromide to stain the DNA. The gel was visualized immediately after the run using an ultraviolet light box. Pictures were taken with a digital camera using the program Biocapt (Vilbert Lourmat, France). Perfect™ 100 bp DNA Ladder (Eurx, Poland) was used as a molecular size standard for PCR products.

To avoid false-negative results caused by too small amount of PCR product invisible on the agarose gel, a second series of reactions was carried out, using the amplification product of the previous reactions as a template.

Table 1.
List of SCAR primers used for detection of plant pathogens

Pathogen	Primer	Primer sequence (5' – 3')	Fragment size	Reference
R. cerealis	Rc2F	5'-AAAACTGGCAACCCTTGGTG-3'	800 bp	Nicholson and Parry, 1996
	Rc2R	5'-TAACTCACCACTCCAGCC-3'		
R. solani	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	550 bp	Johanson et al., 1998
	GMRS-3	5'-AGTGGAACCAAGCATAACACT-3'		
O. yallundae (type W)	TyV5F	5'-CGC CCA GGT AGA GAT TCA GG-3'	600 bp	Nicholson et al., 1997
	TyV5R	5'-GGG AGA AAA CGA CGA GAT GC-3'		
O. acuformis (type R)	Ta05F	5'-GTA TCG GAC GGA GAT CCA GC-3'	330 bp	Nicholson et al., 1997
	Ta05R	5'-TTG CTC AGT GCA TTG TCG G-3'		

Table 2.
PCR conditions used in the study

Step	Temperature [°C]	Pathogen	Time (min)
Initial denaturation	94		3
Denaturation	94		1
Annealing	58.5	*R. cerealis*	1
	58.5	*R. solani*	1
	60.4	*O. yallundae*	1
	60.6	*O. acuformis*	1
Elongation	72		2
Final extension	72		5
Cooling	4		-

RESULTS

Survey and visual assessment

Visual plant health assessments conducted at shooting stage (GS 31) revealed relatively low severity of diseases being researched. Sharp eyespot symptoms were noted only on rye. In 2007, they were observed on 0.5% of the plants, and in 2008 on 1.0% (Table 3). Eyespot was visible on 0.5-3.5% of the plants. During the ripening (GS 77), there were also no symptoms of sharp eyespot on barley, whereas on the other cereal species they were observed on 4.0-6.5% of the plants. Barley also showed a low number of eyespot symptoms (1.5%), while they were often noted on rye, especially in 2008 (32%), and triticale (9.5%).

Table 3.
Share of stems (in %) with sharp eyespot and eyespot symptoms calculated on the basis of macroscopic assessment

Species	Year	Sharp eyespot		Eyespot	
		GS 31	GS 77	GS 31	GS 77
Rye	2007	0.5	4.0	0.5	7.5
	2008	1.0	5.0	3.5	32
Triticale	2008	0.0	6.5	3.0	9.5
Barley	2008	0.0	0.0	1.0	1.5

Isolation of fungi and morphological identification

Saprotrophic fungi were mostly isolated both from stem bases with lesions as well as from those with no visible disease symptoms (Table 4-7). These fungi were mostly represented by the genus *Penicillium* and *Trichoderma*. A single stem base was usually colonised by fungi representing various species or genera. Occasionally, pathogenic fungi were also obtained, among them *Fusarium avenaceum*, *F. culmorum*, *F. solani* and *F. merismoides*, which was also isolated from visually healthy plants.

Table 4.
Identification of the genus *Rhizoctonia* and *Oculimacula* causing rye stem base diseases with PCR assay, isolation on PDA and visual assessment, in 2007 (GS 31)

No. of stem	Visual assessment	PCR detection				Isolation on PDA medium				
		Rc[5]	Rs[6]	Oy[7]	Oa[8]	Rc	Rs	Oy	Oa	Others[11]
1	R[1]	–[9]/–[10]	–/+	–/–	–/–	–	–	–	–	P[12](3)
2	R	–/–	–/–	–/–	–/–	–	–	–	–	Fc[13](1), T[14](3)
3	O[2]	–/–	+/+	–/–	–/–	–	–	–	–	–
4	O	–/–	–/–	–/–	–/–	–	–	–	–	T(2)
5	F[3]	–/–	–/–	–/–	–/–	–	–	–	–	Fc(3), M[15](1)
6	F	–/–	–/–	–/–	–/–	–	–	–	–	Fc(1)
7	H[4]	–/–	+/+	–/–	–/–	–	–	–	–	–
8	H	–/–	+/+	–/–	–/–	–	–	–	–	–
9	H	–/–	–/+	–/–	–/–	–	–	–	–	P(3)
10	H	–/–	–/+	–/–	–/–	–	–	–	–	Fa[16](1), P(2)
11	H	–/–	+/+	–/–	–/–	–	–	–	–	T(1)
12	H	–/–	+/+	–/–	–/–	–	–	–	–	–
13	H	–/–	–/–	–/–	–/–	–	–	–	–	–
14	H	–/–	–/–	–/–	–/–	–	–	–	–	–
15	H	–/–	–/–	–/–	–/–	–	–	–	–	P(2)
16	H	–/–	+/+	–/–	–/–	–	–	–	–	–
17	H	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
18	H	–/–	–/–	–/–	–/–	–	–	–	–	–
19	H	–/–	–/–	–/–	–/–	–	–	–	–	An[17](1), P(2)
20	H	–/–	–/–	–/–	–/–	–	–	–	–	P(2)
21	H	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
22	H	–/–	–/–	–/–	–/–	–	–	–	–	P(4)
23	H	–/–	–/–	–/–	–/–	–	–	–	–	–
24	H	–/–	–/–	–/–	–/–	–	–	–	–	–
25	H	–/–	–/+	–/–	–/–	–	–	–	–	P(3)
26	H	–/–	–/–	–/–	–/–	–	–	–	–	T(2)
27	H	–/–	–/–	–/–	–/–	–	–	–	–	P(3)
28	H	–/–	–/+	–/–	–/–	–	–	–	–	–
29	H	–/–	–/+	–/–	–/–	–	–	–	–	Aa[18](1), Fc(1), Py[19](1)
30	H	–/–	–/–	–/–	–/–	–	–	–	–	T(2)
31	H	–/–	–/–	–/–	–/–	–	–	–	–	Fa(1)
32	H	–/–	–/–	–/–	–/–	–	–	–	–	–
33	H	–/–	+/+	–/–	–/–	–	–	–	–	An(1), P(3)
34	H	–/–	+/+	–/–	–/–	–	–	–	–	T(4)
35	H	–/–	+/+	–/–	–/–	–	–	–	–	P(2), T(3)

[1] R – sharp eyespot; [2] O – eyespot; [3] F – *Fusarium* foot rot; [4] H – healthy; [5] Rc – *R. cerealis*; [6] Rs – *R. solani*; [7] Oy – *O. yallundae*; [8] Oa – *O. acuformis*; [9] – positive result (+) or negative result (–) of first PCR reaction; [10] – positive result (+) or negative result (–) of second PCR reaction; [11] other fungi (in bracket number of isolates): [12] P – *Penicillium* sp.; [13] Fc – *Fusarium culmorum*; [14] T – *Trichoderma* sp.; [15] M – *Mucor* sp.; [16] Fa – *Fusarium avenaceum*; [17] An – *Aspergillus niger*; [18] Aa – *Alternaria alternata*; [19] Py – *Pythium* sp.

Table 5.
Identification of the genus *Rhizoctonia* and *Oculimacula* causing rye stem base diseases with PCR assay, isolation on PDA and visual assessment, in 2008 (GS 31)

No. of stem	Visual assessment	PCR detection				Isolation on PDA medium				
		Rc[5]	Rs[6]	Oy[7]	Oa[8]	Rc	Rs	Oy	Oa	Others[11]
1	R[1]	–[9]/+[10]	–/+	–/–	–/–	–	–	–	–	P[12](1), T[13](1)
2	R	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
3	R	–/+	–/+	–/–	–/–	–	–	–	–	T(1)
4	R	–/+	–/+	–/–	–/–	–	–	–	–	P(3)
5	O[2]	–/–	–/+	–/–	–/+	–	–	–	(4)	Mm[14](1), P(1)
6	O	–/–	–/+	+/–	–/–	–	–	–	–	Ap[15](1), P(2), Ph[16](1)
7	O	–/–	–/+	–/+	–/–	–	–	(1)	–	P(1)
8	O	–/–	–/+	–/+	–/–	–	–	(3)	–	Mr[17](1), P(5), T(2)
9	O	–/–	–/+	+/–	–/–	–	–	(1)	–	P(2), T(1)
10	O	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
11	O	–/–	–/+	–/–	–/–	–	–	–	–	P(3)
12	O	–/–	–/+	–/–	–/–	–	–	–	–	–
13	O	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
14	O	–/–	–/–	–/+	–/–	–	–	–	–	–
15	O	–/–	–/–	–/–	–/–	–	–	–	–	Fc[18](1), T(1)
16	O	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
17	F[3]	–/–	–/+	–/–	–/–	–	–	–	–	–
18	F	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
19	F	–/–	–/+	–/–	–/–	–	–	–	–	Mr(4), T(1)
20	F	–/–	+/+	–/–	–/–	–	–	(2)	–	P(4), T(1)
21	O	–/–	+/+	–/–	–/–	–	–	(3)	–	Mm(1), P(1)
22	F	–/–	+/+	–/–	–/–	–	–	–	–	P(2)
23	F	–/–	+/+	–/–	–/–	–	–	–	–	Af[19](1)
24	H[4]	–/–	+/+	–/–	–/–	–	–	–	–	An[20](4)
25	H	–/–	+/+	–/–	–/–	–	–	–	–	–
26	H	–/–	+/+	–/–	–/–	–	–	–	–	–
27	H	–/–	+/+	–/–	–/–	–	–	–	–	P(4), Ph(1)
28	H	–/–	–/+	–/–	–/–	–	–	–	–	P(2)
29	H	–/–	–/+	–/–	–/–	–	–	–	–	Fc(1), P(3)
30	H	–/–	–/+	–/–	–/–	–	–	–	–	Af(1), P(1)
31	H	–/–	–/–	–/+	–/–	–	–	–	–	An(1), P(1), T(1)

[1] R – sharp eyespot; [2] O – eyespot; [3] F – *Fusarium* foot rot; [4] H – healthy [5] Rc – *R. cerealis*; [6] Rs – *R. solani*; [7] Oy – *O. yallundae*; [8] Oa – *O. acuformis*; [9] – positive result (+) or negative result (–) of first PCR reaction; [10] – positive result (+) or negative result (–) of second PCR reaction; [11] other fungi (in bracket number of isolates): [12] P – *Penicillium* sp.; [13] T – *Trichoderma* sp.; [14] Mm – *Mucor mucedo*; [15] Ap – *Arthinium phaeospermum*; [16] Ph – *Phoma* sp.; [17] Mr – *Myrothecium roridum*; [18] Fc – *F. culmorum*; [19] Af – *Aspergillus fumigatus*; [20] An – *A. niger*

In 2008 only *O. yallundae* was isolated from stem bases of rye (Table 4, 5). This species was found in 4 plants with brown, spindle-shaped spots, and in one plant with brown streaks. It should be noted here that this fungus was not isolated from all fragments obtained after cutting the individual stem bases. It was often accompanied by saprotrophic fungi.

O. yallundae was isolated on PDA medium from 6 barley stems with disease symptoms (Table 6). *O. acuformis* and fungi from the *Rhizoctonia* genus were not obtained.

Among the fungi from the *Rhizoctonia* genus, only one *R. solani* isolate was obtained on PDA medium from the stem of triticale (Table 7). There was also isolated *O. yallundae* from 4 plants of this cereal.

Molecular identification with PCR assay

PCR assay (primers ITS1 and GMRS-3) revealed the presence of *R. solani* in rye stem bases, giving the expected amplification product of 550 bp. In 2007, after the first PCR series, this species was found in 9 stems and after the second series of PCR assay, which used the amplification product of the previous PCR assay as a template, it was found in 14 stems (Table 4).

In 2008, molecular assays revealed the presence of the following pathogens: *R. solani* in 28 rye stems, including those with no disease symptoms; *R. cerealis* in 3 plants with sharp eyespot symptoms; *O. yallundae* in 6 plants, and *O. acuformis* in one plant. It should be noted that these results in most cases corresponded to the identification based on visual assessments of disease symptoms (Table 5).

The presence of *O. yallundae* in 6 plants of barley and *R. solani* in 28 plants was detected with PCR assay (Table 6). PCR assays confirmed only the presence of *R. solani* in triticale (Table 7).

Table 6.
Identification of the genus *Rhizoctonia* and *Oculimacula* causing barley stem base diseases with PCR assay, isolation on PDA and visual assessment (GS 31)

No. of stem	Visual assessment	PCR detection				Isolation on PDA medium				
		Rc[5]	Rs[6]	Oy[7]	Oa[8]	Rc	Rs	Oy	Oa	Others[11]
1	O[1]	–[8]/–[9]	+/+	–/+	–/–	–	–	–	–	T[11](1)
2	O	–/–	–/+	–/–	–/–	–	–	–	–	P[12](1), Th[13](1)
3	O	–/–	–/+	–/–	–/–	–	–	–	–	T (1)
4	O	–/–	–/+	+/+	–/–	–	–	(2)	–	An[14](1), As[15](1), Ph[16](2)
5	O	–/–	–/+	–/–	–/–	–	–	(3)	–	Ch[17](1), P(3)
6	O	–/–	–/+	–/–	–/–	–	–	(3)	–	P(2)
7	O	–/–	–/+	–/–	–/–	–	–	–	–	An(1)
8	O	–/–	–/+	–/–	–/–	–	–	(4)	–	Gr[18](1), P(3), T(1)
9	O	–/–	–/+	+/–	–/–	–	–	(3)	–	P(1)
10	O	–/–	–/+	–/–	–/–	–	–	–	–	–
11	O	–/–	+/+	–/–	–/–	–	–	–	–	An (1), T(1)
12	O	–/–	–/+	+/+	–/–	–	–	(6)	–	P(1)
13	F[2]	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
14	F	–/–	–/+	–/–	–/–	–	–	–	–	M[19](1), P(4), T(1)
15	F	–/–	–/+	–/–	–/–	–	–	–	–	P(1), Tp[20](1), Tv[21](3)
16	F	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
17	F	–/–	–/+	–/–	–/–	–	–	–	–	P(1), T(1)
18	F	–/–	–/+	–/–	–/–	–	–	–	–	T(1), M(1)
19	F	–/–	–/+	–/–	–/–	–	–	–	–	Ch(1), P(5)
20	F	–/–	+/+	–/–	–/–	–	–	–	–	T(1)
21	F	–/–	–/+	–/–	–/–	–	–	–	–	P(3)
22	F	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
23	F	–/–	+/+	–/–	–/–	–	–	–	–	T(1)
24	H[3]	–/–	–/–	–/–	–/–	–	–	–	–	P(5)
25	H	–/–	–/–	–/–	–/–	–	–	–	–	Rh[22](1)
26	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
27	H	–/–	–/–	–/–	–/–	–	–	–	–	Rh(1)
28	H	–/–	+/+	–/–	–/–	–	–	–	–	T(1)
29	H	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
30	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1), T(1)
31	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
32	H	–/–	–/–	–/–	–/–	–	–	–	–	P(1)

[1] O – eyespot; [2] F – *Fusarium* foot rot; [3] H – healthy; [4] Rc – *R. cerealis*; [5] Rs – *R. solani*; [6] Oy – *O. yallundae*; [7] Oa – *O. acuformis*; [8] – positive result (+) or negative result (–) of first PCR reaction; [9] – positive result (+) or negative result (–) of second PCR reaction; [10] other fungi (in bracket number of isolates): [11] T – *Trichoderma* sp.; [12] P – *Penicillium* sp.; [13] Th – *Trichoderma haruatum*; [14] An – *A. niger*; [15] As – *Acremonium strictum*; [16] Ph – *Phoma* sp.; [17] Ch – *C. herbarum*; [18] Gr – *Gymnoascus reesii*; [19] Mm – *Mucor mucedo*; [20] Tp – *Trichoderma polysporum*; [21] Tv – *Trichoderma viride*; [22] Rh – *Rhizopus* sp.

Comparison of visual disease assessment, PCR assay, and isolation on PDA

The three analyzed methods were able to confirm only the presence of eyespot on 3 plants of barley and 4 plants of rye assessed in 2008 (Table 8). The occurrence of sharp eyespot was confirmed with a visual and molecular technique, but *R. cerealis* was not isolated on PDA medium. This resume does not include *R. solani*, since this species occurred on most plants, regardless of symptoms. In addition, during the maturity stage, there was no significant increase in the number of plants with sharp eyespot, which might suggest that this species was not the causal agent of this disease.

Table 7.
Identification of the genus *Rhizoctonia* and *Oculimacula* causing triticale stem base diseases with PCR assay, isolation on PDA and visual assessment (GS 31)

No. of stem	Visual assessment	PCR detection				Isolation on PDA medium				
		Rc[5]	Rs[6]	Oy[7]	Oa[8]	Rc	Rs	Oy	Oa	Others[11]
1	O[1]	–[8]/–[9]	–/+	–/–	–/–	–	–	–	–	T[11](1)
2	O	–/–	–/+	–/–	–/–	–	–	–	–	Ab[12](1), Ch[13](2), P[14](2)
3	O	–/–	–/+	–/–	–/–	–	–	(3)	–	Aa[15](3), Ch(1)
4	O	–/–	–/+	–/–	–/–	–	–	–	–	Ab(1), Ap[16](1), P(2)
5	O	–/–	–/+	–/–	–/–	–	–	–	–	Fm[17](3), Fs[18](2)
6	O	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
7	O	–/–	–/–	–/–	–/–	–	–	(1)	–	Aa(2), P(2)
8	O	–/–	–/–	–/–	–/–	–	(1)	–	–	P(1), T(1)
9	O	–/–	–/–	–/–	–/–	–	–	–	–	P(4), T(1)
10	O	–/–	–/–	–/–	–/–	–	–	–	–	Fm(2), Fs(3), P(2)
11	O	–/–	–/–	–/–	–/–	–	–	–	–	T(1)
12	O	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
13	F[2]	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
14	F	–/–	–/+	–/–	–/–	–	–	(1)	–	Rh[19](1)
15	F	–/–	–/+	–/–	–/–	–	–	–	–	P(4)
16	F	–/–	–/–	–/–	–/–	–	–	(1)	–	P(1), T(1)
17	F	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
18	F	–/–	–/–	–/–	–/–	–	–	–	–	T(1)
19	F	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
20	F	–/–	–/+	–/–	–/–	–	–	–	–	P(1), T(1)
21	F	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
22	H[3]	–/–	–/–	–/–	–/–	–	–	–	–	T(1)
23	H	–/–	–/+	–/–	–/–	–	–	–	–	An[20](1), Fs(1), V[21](1)
24	H	–/–	–/+	–/–	–/–	–	–	–	–	P(2), T(1)
25	H	–/–	–/–	–/–	–/–	–	–	–	–	P(1)
26	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
27	H	–/–	–/+	–/–	–/–	–	–	–	–	T(1)
28	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1)
29	H	–/–	–/+	–/–	–/–	–	–	–	–	P(1), T(1)

[1] O – eyespot; [2] F – *Fusarium* foot rot; [3] H – healthy; [4] Rc – *R. cerealis*; [5] Rs – *R. solani*; [6] Oy – *O. yallundae*; [7] Oa – *O. acuformis*; [8] – positive result (+) or negative result (–) of first PCR reaction; [9] – positive result (+) or negative result (–) of second PCR reaction; [10] other fungi (in bracket number of isolates): [11] T – *Trichoderma* sp.; [12] Ab – *Aureobasidium bolleyi*; [13] Ch – *Cladosporium herbarum*; [14] P – *Penicillium* sp.; [15] Aa – *A. alternata*; [16] Ap – *Aureobasidium pullulans*; [17] Fm – *Fusarium merismoides*; [18] Fs – *Fusarium solani*; [19] Rh – *Rhizopus* sp.; [20] An – *A.* niger; [21] V – *Verticillium* sp.

Table 8.
Comparison of the number of positively diagnosed plants with visual disease assessment, PCR assay and isolation on PDA

Species	Year	Sharp eyespot (*R. cerealis*)				Eyespot (*O. yallundae*, *O. acuformis*)			
		Visual[1]	PCR[2]	PDA[3]	All[4]	Visual	PCR	PDA	All
Rye	2007	2	0	0	0	1	0	0	0
	2008	4	3	0	0	13	7	6	4
Triticale	2008	0	0	0	0	12	0	5	0
Barley	2008	0	0	0	0	12	4	6	3

[1] – visual assessment of disease symptoms; [2] diagnosis of disease factor occurrence with PCR assay; [3] – identification of disease factor on PDA; [4] positive result of disease factor diagnosis using all three techniques

DISCUSSION

The usefulness of SCAR-PCR assay for the determination of the presence of *R. solani*, *R. cerealis*, *O. yallundae,* and *O. acuformis* in the individual cereal stems was confirmed. The detection was successful regardless of disease symptoms, also in the case when the traditional method that involves isolation on microbiological media did not show their presence.

Mainly saprotrophic fungi were isolated on PDA, and only occasionally *O. yallundae*, *O. acuformis,* and *R. solani* were found. *Rhizoctonia cerealis* and *Oculimacula* spp., as pathogens specialized in cereal infection, grow relatively slowly on artificial media and are often overgrown by fast growing saprotrophic fungi and *Fusarium* spp. (Bateman and Kwaśna, 1999). According to Creighton (1989), fungi from the genus *Oculimacula* should not be identified as particular species on the basis of morphological features without additional tests, i.e. pathogenicity and molecular studies.

According to data presented by other authors, diagnostics carried out by traditional techniques are not always able to be confirmed with molecular assays, and vice versa (Nicholson et al. 1997; Turner et al. 1999). Nicholson and Parry (1996) report, however, that there is a correlation between the results of PCR, conventional isolation on artificial media, and visual assessment. Using methods based on PCR, especially in combination with conventional methods used in phytopathology, substantially increases the precision and effectiveness of diagnostics of plant diseases. Matusinsky et al. (2008), using species-specific SCAR primers, also confirmed the presence of *R. cerealis* in plant tissues of cereals. Nicholson et al. (2002) and Ray et al. (2004) indicated that the amount of DNA of *R. cerealis* in wheat tissue increased in particular growth stages of plants. This pathogen usually occurs in later development stages, only occasionally in earlier ones, and sometimes it is not observed at all.

Most of the papers on the use of PCR technique in plant pathogen diagnostics concern the molecular confirmation of pathogen identity previously isolated on artificial media (Irzykowska et al. 2005; Korbas, 2008). In this study, the presence of the examined fungi in DNA samples derived from plants was also confirmed. Previously, SCAR primers were successfully used in identification of *R. cerealis* and *R. solani* obtained from winter triticale (Lemańczyk, 2010a), as well as from the spring form of barley, wheat, and triticale (Lemańczyk, 2010b). Their use in our study proved to be particularly effective for identifying *R. solani* in plant tissues. The fungus was found in tissues exhibiting different symptoms, as well as in symptomless plants. The presence of *R. cerealis* was confirmed only in three plants, despite the fact that symptoms of sharp eyespot were noted also on other plants. This could result from a low concentration of fungal DNA preventing its detection. However, Nicholson and Parry (1996) report that a positive result is not obtained only for concentrations lower than 1 pg of pure DNA of *R. cerealis*. According to them, the sensitivity level is one of the most important factors in detection of pathogens. Amplification performance depends largely on purification of genomic DNA from PCR inhibitors such as polysaccharides, phenolic compounds and enzymatic proteins reducing the efficiency of reaction (Wilson, 1997).

PCR technique is considered very reliable (Nicholson and Parry, 1996), but SCAR primers do not always guarantee correct results. Łukanowski et al. (2009) reported the possibility of changes in sequences of genomes to which primers hybridize. This indicates that there is a risk of obtaining false-negative results that may lead to erroneous conclusions. Therefore, a negative PCR result should be treated with some caution, and in case of doubt, if possible, should be retested using a different pair of primers or analysis of ITS regions (internal transcribed spacer).

Differences in length and sequence of ITS-1 and ITS-2 regions were used to identify isolates of *R. cerealis* and *R. solani* (Irzykowska et al. 2005). ITS sequence analysis is a method used for, e.g., the determination of phylogenetic relationships between species. It allows one to identify *Rhizoctonia* isolates belonging to particular anastomosis groups within species in this genus (Sharon et al. 2006). Sequencing of ITS regions resulted in designing primer pairs specific for W and R pathotypes (*O. yallundae* and *O. acuformis*) (Poupard et al. 1993).

In this research, we were not able to confirm the presence of *O. yallundae* and *O. acuformis* in all plants with disease symptoms typical for these pathogens. Łapiński et al. (2008) molecularly detected the presence of these fungi in rye, triticale during tillering and later growth stages, while there were no results for barley. It has been reported that DNA content of these pathogens in tissues increases during plant growth (Ray et al. 2004; Walsh et al. 2005).

Oculimacula acuformis was found only in one plant. According to Głazek (2009), this species was more numerous on wheat fields in south-central Poland as compared to *O. yallundae*, which explains the late appearance of disease symptoms, however, plants for analysis were sampled during ripening. Korbas (2008) reported that *O. yallundae* predominates at shooting stage, whereas in later phases the difference in proportions among different species was significantly reduced. This explains the fact that in our study this species also dominated. *O. yallundae* colonizes plant tissue faster than *O. acuformis,* which results from its

rapid linear growth (Nicholson et al. 2002; Korbas 2008). Nicholson et al. (2002) and Ray et al. (2006) reported a higher content of DNA of *O. yallundae* than *O. acuformis* in the earlier growth stages of wheat.

In these studies, there was no simultaneous occurrence of *R. cerealis*, *O. yallundae,* and *O. acuformis* on the same stems. Such a situation was also reported by Nicholson and Parry (1996). Faster-growing *O. yallundae* can inhibit the development of *R. cerealis*. Therefore, in tissues showing symptoms of eyespot, apart from *Oculimacula*, only *R. solani* was noted as a faster growing species belonging to necrotrophic microorganisms (Bateman and Kwaśna, 1999).

CONCLUSIONS

1. The research showed the usefulness of PCR markers for detection of fungal pathogens, even if symptoms were not visible. The application of SCAR-PCR assay allowed early detection of *O. yallundae*, *O. acuformis, R. cerealis,* and *R. solani* in plant tissues.
2. Using the PCR technique, especially in combination with conventional methods, substantially increases the precision and effectiveness of disease diagnosis.

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Zastosowanie SCAR-PCR w diagnostyce patogenów podstawy źdźbła zbóż z rodzajów *Rhizoctonia* i *Oculimacula*

Streszczenie

Porównywano zastosowanie metody SCAR-PCR i konwencjonalnej (wizualna ocena, izolacja na pożywce PDA) w identyfikacji grzybów z rodzajów *Rhizoctonia* i *Oculimacula* na formach ozimych pszenżyta, żyta i jęczmienia, pobieranych w fazie strzelania w źdźbło. Wykazano przydatność diagnostyki molekularnej patogenów grzybowych roślin w uprawach polowych. Zastosowanie metody SCAR-PCR umożliwiło wczesne wykrycie patogenów: *O. yallundae*, *O. acuformis*, *R. cerealis* i *R. solani* w tkankach roślinnych. Detekcja molekularna okazała się szczególnie skuteczna w wykrywaniu obecności *R. solani*. Badania wskazują na użyteczność stosowania markerów PCR do wczesnego wykrywania patogenów grzybowych, również w przypadku braku objawów chorobowych. Wykorzystanie techniki PCR zwłaszcza w połączeniu z klasycznymi metodami znacząco podnosi precyzję i skuteczność diagnostyki chorób roślin.