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ORIGINAL ARTICLE

Eyespot resistance of winter wheat breeding lines evaluated with marker-assisted selection and inoculation tests at the seedling and adult plant stages

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Abstract

Eyespot is one of the most important fungal diseases of the stem base of wheat (*Triticum aestivum* L.). The presented study clearly demonstrated that the *Pch1* gene was the main effective source for reducing the eyespot disease score in the analyzed winter wheat lines. Nevertheless, *Pch1* was present only in 8–9% of the investigated lines. Using an isoenzymatic marker and molecular markers, the presence of the *Pch1* gene and lack of the *Pch2* gene was identified in six lines. Two lines, SMH 9409 and DL 358/13/4, were polymorphic in an isoenzymatic marker study. In the remaining three lines, C 3373/11-1, KBH 15.15 and KBP 1416, the *Pch1* gene was identified only with the use of an isoenzymatic marker. Both genes *Pch1* and *Pch2*, as well as the resistant variety Rendezvous, were found in three lines: DD 248/12, KBP 15.2 and STH 4431. In line DD 708/13, the presence of the *Pch1* and *Pch2* genes was identified, where the association between the *Pch1* and the locus of the *Xorw5* marker was broken. It was shown that the presence or absence of *Pch1* and *Pch2* genes did not significantly affect the grain yield (from the plot), although the yield was highest in the presence of both genes. A significant effect of the presence of the *Pch1* gene on thousand kernel weight (TKW) was observed. Lines with the *Pch1* gene showed significantly higher TKW values than lines without both genes or with the *Pch2* gene only.

Keywords: eyespot, marker-assisted selection, *Oculimacula*, resistance, wheat

Introduction

Eyespot (strawbreaker or foot rot) is one of the most important fungal diseases of the stem base of wheat (*Triticum aestivum* L.) and other cereals like barley, rye and triticale. It is most damaging in regions with damp, mild autumns and winters. Because of the good environmental conditions for infection, winter cereals are more sensitive to eyespot than spring cereals (Murray 2010). Eyespot is caused by two necrotrophic fungi, *Oculimacula yallundae* (syn. *Tapesia yallunde,* Walwork and Spooner) Crous and W. Gams and *O. acuformis* (syn. *T. acuformis* Boerema, R. Pieters and Hamers) (Crous *et al.* 2003). The pathogen is soilborne and the fungal infection reduces nutrient transport at the stem base, resulting in lodging which can lead to significant yield reduction (Lucas *et al.* 2000). Severe cases of the disease can reduce yield by up to 40–50% in susceptible varieties (Murray 2010; Zanke *et al.* 2017). The occurrence of eyespot leads to reduced tiller number, premature death of stems, reduced kernel number per head and thousand kernel weight (TKW) (Scott and Hollins 1974; Murray and Bruehl 1986; Janczewska 1991; Murray 2010).

There are two main genetically characterized sources of resistance identified in commercial wheat varieties. The single major gene *Pch1* is the most effective and prominent eyespot resistance gene. The *Pch1* gene was transferred to hexaploid wheat from the *Aegilops ventricosa* (Zhuk.) Chennav. ($2n = 4x = 28$, $D^vD^vM^vM^v$) (Worland *et al.* 1988; Mena *et al.* 1992). The transferred sequence with the *Pch1* gene has been mapped to the distal end of chromosome arm 7DL (Jahier *et al.* 1978; McMillin *et al.* 1986; Worland *et al.* 1988). Selection for *Pch1* relied on the presence of allele *Ep-D1b* of a co-segregating endopeptidase as well as the presence of molecular markers localized near the *Pch1* loci (McMillin *et al.* 1986). Some of the Single Sequence Repeats (SSRs) markers i.e. *Xorw1* and *Xorw5* are accurate in predicting the presence or absence of the *Pch1* gene (Leonard *et al.* 2008) but it is difficult to distinguish the allelic variation using basic horizontal electrophoresis methods on an agarose gel. Nevertheless, PCR-based sequence-tagged-site (STS) marker *Xorw1*, derived from an oligopeptidase B encoding wheat expressed-sequence-tag (EST), showed complete linkage with *Ep-D1* (Leonard *et al.* 2008). Other *Pch1*-linked markers i.e. *Xgwm37, Xbarc76, XustSSR2001-7DL*, *Xwmc14*, *Xbarc97* and *Xcfd175*, flank the *Pch1* locus. The closest one is *Xust2001-7DL* (3.9 cM) with easily identified allelic variations (Santra *et al.* 2006). *Pch1* has been widely used in both Europe and the USA to increase eyespot resistance and it is present in several hexaploid wheat varieties (Santra *et al.* 2006). However, its presence in commercial wheat varieties in Europe has been limited because of the undesirable genes derived from *Ae. ventricosa*. Despite the effectiveness of *Pch1* against eyespot, a significant reduction of yield and TKW have been observed in the absence of the disease (Koen *et al.* 2002).

The second *Pch2* gene, originated from French variety Cappelle Desprez (Vincent *et al.* 1952; de la Peña *et al.* 1996) has less influence on the resistance to eyespot and has been mapped to the long arm of chromosome 7A (Chapman *et al.* 2008). It determines immunity at the seedling stage; however, it is less effective in preventing pathogenic fungi from the *Oculimacula* genus than the *Pch1* gene (Burt *et al.* 2014). For this reason, the *Pch2* gene can be treated as an additional source of wheat resistance to eyespot. *Pch2* has been mapped between molecular markers *Xwmc346* and *Xcfa2040*, and it is closely associated with *Xwmc525* (Chapman *et al.* 2008). It is worth mentioning that *Pch1* and *Pch2* are not homeoloci (Pasquariello *et al.* 2017). In the Capelle Desprez variety, high resistance to the adult plant stage has also been observed, which is conditioned by a quantitative trait loci (QTL) located on chromosome 5A (Muranty *et al.* 2002; Burt 2010).

Breeding for resistance is an environmentally friendly and sustainable strategy to overcome eyespot disease. The VPM-1 (*Ae. ventricosa* × *T. persicum* × × v. Marne) was the first breeding line resistant to eyespot (with *Pch1*) (Maia 1967) which was used as a base for breeders to create resistant varieties like Rendezvous. Since wheat varieties carrying *Pch1* can be affected by significant grain yield losses caused by eyespot infestation, there is a need to enhance the resistance of wheat. One of the strategies is pyramidization of *Pch1* and *Pch2* genes. Pyramiding entails stacking up both genes in one variety for stronger and more durable resistance. The main objectives of this study were: (1) to select breeding lines of winter wheat with the expression of *Pch1* and *Pch2* genes; (2) to identify sources of resistance to eyespot in winter wheat breeding lines with a diverse genetic basis selected in the field inoculation test; (3) to evaluate the effect of the presence of the resistance genes on yield components.

Materials and Methods

Plant material

Lines of winter wheat (*Triticum aestivum* L.), derived from Polish breeding companies and obtained from various breeding programs, were studied for eyespot resistance. One hundred and seventy lines and three varieties of wheat: Kilimanjaro, Artist and Patras (without *Pch1*) as susceptibility control and variety Rendezvous as a resistance control were analyzed. *Pch1* and

Table 1. Molecular markers associated with *Pch1* and *Pch2* genes used in the study

Gene	Marker name	Primer sequence 5′ → 3′	Location on chromosomes
Pch1	*Xust2001-7DL*	Forward CATCGTGTGGCCAACTTGTT Reverse TTCCTCGTGTCTAGTGTCTC	7D
Pch1	*Xorw1*	Forward CTATTACATGAAATCTTATTCTCC Reverse CAGCAGTAACGAGAATGTGG	7D
Pch1	*Xorw5*	Forward GCATCCTCGCCTTCATGC Reverse CGACCATCTCGACCACAGG	7D
Pch2	*Xwmc525*	Forward GTTTGACGTGTTTGCTGCTTAC Reverse CTACGGATAATGATTGCTGGCT	7A

Pch2 genes were identified by endopeptidase assay and SSR markers (Tab. 1).

Endopeptidase assay

Five plants from each line were examined in endopeptidase and SSR marker analysis. The enzyme was extracted by grinding two-week-old leaves using a plexiglass bar in 10 μl of 0.025 M glycyl-glycine buffer (pH 7.4; SIGMA). To load samples, paper strips were soaked in the enzyme extract for each line and inserted into the gel. The 10% starch (SIGMA) gel was run at 4°C at 200 V. After electrophoresis, the gel was incubated in the dark at 37°C for 30 min with 0.5% solution of low melting agarose containing 2.56 mg Fast Black K Salt (SIGMA) and 1.12 mg N-α-benzoyl-DL-arginine-LB-naphthylamide (BANA; SIGMA) in 0.1 M Trizma maleate (SIGMA) – NaOH (Santra *et al*. 2006).

SSR marker analysis

The identification of SSR markers was made using the same leaf tissue that was used for the endopeptidase assay. Leaves of two-week-old seedlings were placed in Eppendorf tubes (2 ml) and frozen (−80°C) for storage. Total genomic DNA was extracted using GeneMATRIX Plant and Funghi DNA Purification Kit (EURx Ltd.) according to the manufacturer's instructions. Isolated DNA was stored at 4°C. Three SSR markers i.e. *Xust2001-7DL*, *Xorw1* and *Xorw5* associated with the locus *Pch1*, which confers resistance to eyespot, were used (Tab. 1). Additionally, the presence of *Xwmc525* marker associated with the *Pch2* gene was identified. The PCR profile consisted of denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 53–62°C (appropriate for each pair of primers) for 30 s and 72°C for 1 min, followed by final extension for 10 min at 72°C and a soak temperature of 4°C. The products of amplification were separated using 3% agarose (SIGMA) gel (1X TBE buffer, 5 h at 100 V) stained with Midori Green (Nippon Genetics Europe), visualized and photographed with UVIsave HD 5 imaging system (Uvidoc Cambridge).

Phenotyping for the *Pch1* controlled eyespot resistance – field experiment

A field experiment was carried out in 2017 in Kopaszewo – Danko Plant Breeding Station near Poznań, Poland (52°13'16"N, 16°41'30"E). The experiment was carried out in four replications using 50 plants per replication. The plant material was inoculated by spraying at the BBCH 31–32 growth stage with a fresh conidial-mycelium suspension of *O. acuformis* and *O. yallundae* (1 : 1 ratio, 4×10^6 spores · ml^{-1}) in April. Plants with eyespot symptoms were evaluated at maturity at BBCH 71–92. The evaluation was performed on 20 plants from each replicate of each wheat line and the control. The percent of infected leaf sheaths was determined and the leaf sheath infection index (*K*-index) was calculated. The level of the leaf sheath infection was measured using a I – IV scale: I – no symptoms; II – less than 50% of leaf sheath surface infected; III – over 50% of leaf sheath surface infected; IV – 100% of leaf sheath surface infected, rotten tissue. The results were presented as a mean from each replication according to the *K*-index which was calculated according to the formula (Kwiatek *et al*. 2012):

$$K = \frac{[n(\text{II}) \times 0.25] + [n(\text{III}) \times 0.75] + n(\text{IV})}{n(\text{I} + \text{II} + \text{III})},$$

where: n – the number of evaluated stalks.

Phenotyping for *Pch2* controlled seedling eyespot resistance – phytotron inoculation test

Plants of each genotype were planted in pots with a diameter of 5.5 cm in autoclaved soil for germination (Archut-Fruhstorfer 5 Erde, Hawita). In a phytotron chamber (IPG, PAS), wheat lines were grown for 21 days under controlled conditions of light and temperature (10°C and 10 h of day-light). Inoculation was performed using the brushing method with a fresh conidial-mycelium suspension of *O. acuformis* and *O. yallundae* (1 : 1 ratio, 4×10^{-6} spores · ml^{-1}). During the 6 following weeks plants were watered to obtain high humidity appropriate for the infection of the pathogen. Next, the plants were evaluated for symptoms of infection with *Oculimacula* species. Coleoptiles and leaf sheaths were subsequently unveiled and the infection score was determined according to the scale which reflects the number of infected leaf sheaths of wheat (minor changes, no penetration to the axial area) or complete penetration (major changes, probable penetration to the axial area), where: 0 = seedling uninfected, 1 = coleoptile infected, 2 = coleoptile heavily infected, 3 = first sheath infected, 4 = first sheath completely penetrated (Fig. 1).

Analysis of yield structure parameters

To verify if the presence of resistance genes affects the yield level, analysis of yield size from the plot (grain yield) and the TKW of the tested lines of wheat, was performed. Grain was collected from non-inoculated plots. The experiment was done in 2017 in Nagradowice – Poznań Plant Breeding Station near Poznań, Poland (52°19′14″N, 17°8′54″E). Wheat lines were sown in three replications in 10 m^2 plots. Grain was harvested using a cereal

Fig. 1. Seedling infection scores in wheat after inoculation with pathogenic species from *Oculimacula* sp.: 0 = seedling uninfected, 1 = coleoptile infected, 2 = coleoptile heavily infected, 3 = first sheath infected, 4 = first sheath completely penetrated

plot harvester. Yield and TKW were measured at 15% moisture of grain.

The effect of resistance genes on the above yield parameters was analyzed using analysis of variance procedure (ANOVA) of Microsoft® Excel 2016/XLSTAT© Ecology (Version 2018.5 58166, Addinsoft, Inc., Brooklyn, NY, USA). Grain yield and TKW were dependent variables and explanatory variables were *Pch1* (0 = lack of gene; 1 = presence of gene) and *Pch2* (0, 1). The effect of the presence of a single gene as well as the interaction were analyzed. Means were compared using Fisher's LSD test.

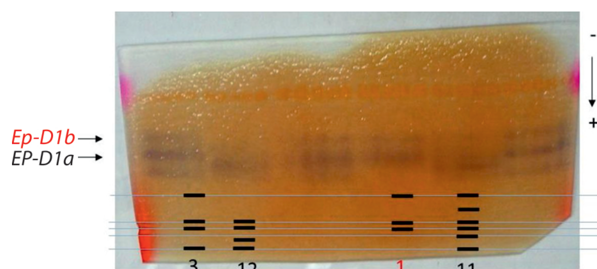

Fig. 2. Selected classes of zymograms in wheat lines (*Ep-D1b* – Type 1) – electrophoresis on the starch gel

Results and Discussion

To verify the resistance to eyespot caused by fungi of the genus *Oculimacula* sp., identification of the *Pch1* and *Pch2* genes combined with inoculation tests were performed. *Pch1* resistance gene was introgressed to bread wheat from *Ae. ventricosa* (Maia 1967; Jahier *et al.* 1978). It was reported that *Pch1* locus co-segregated with endopeptidase gene allele *Ep-D1b* (McMillin *et al.* 1986; Koebner *et al.* 1988) with no recombination observed between *Ep-D1* and *Pch1* (Worland *et al.* 1988). The expression of the *Pch1* gene effectively blocks the development of the pathogen, thus it has been incorporated into several American and European varieties of hexaploid wheat (Santra *et al.* 2006).

For the identification of *Pch1*, the isoenzymatic marker *Ep-D1b* and three SSR markers were used i.e. *Xust2001-7DL*, *Xorw1* and *Xorw5* (7DL) (Tab. 1). Analyses of isoenzymes, performed in these studies, revealed zymograms that could be attributed to 11 out of 13 classes, observed previously for winter wheat lines by the members of the Institute of Plant Genetics (data unpublished) (Fig. 2, Tab. 2). Type 1,

Table 2. Classes of zymograms observed in analyzed winter wheat lines

Genotypes	Band	Type of banding pattern												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Ep-D1b	upper	–	–	–		–	–		–		–	–		
	middle	–	–	–	–	–		–	–		=			=
	lower	–		–	–		–	–		–	–	–	–	–
Ep-D1a	upper		–		–	–						–	–	
	lower			–		–	–	–	–	–	–	–	–	–
No. of lines		16	1	85	1	22	0	12	21	6	0	6	3	1

One line – one band; two lines – two bands; type 1 – genotypes with *Ep-D1b*; type 2–13 – genotypes without *Ep-D1b*

obtained for control variety Rendezvous and 15 analyzed lines (8.6% of lines), was characterized by three bands for *Ep-D1b*, which identified lines resistant to infection by *O. yallundae* and *O. acuformis*. It was reported that the presence of bands characteristic for *Ep-D1b* with the absence of bands for *Ep-D1a*, ensure resistance to eyespot (Santra *et al*. 2006). Remaining lines exhibited Type 2 to Type 13 patterns, which are specified in Table 2. In the presented studies, none of the lines represented Type 6 and 10 of zymograms (Tab. 2). Among analyzed lines, three of them were polymorphic. However, two lines represented a banding pattern typical for resistant plants. Most of the analyzed lines revealed Type 3 pattern (48.9% of lines). Subsequent types of zymograms, with regard to the number of lines, constitute Types 5 and 8 (Tab. 2).

Marker *XustSSR2001-7DL* is closely linked to the *Ep-D1* and lies approximately 2 cM from this locus (Groenewald *et al.* 2003). Santra *et al.* (2006) found marker *XustSSR2001-7DL* to be 90% accurate in predicting the correct phenotype. Therefore, in this study, we used two more recent markers *Xorw1* and *Xorw5*, which are more accurate in identifying the presence of a *Pch1* gene because they are completely linked to *Ep-D1* and *Pch1* (Leonard *et al*. 2008). Effectiveness of the markers selected for the identification of the *Pch1* gene in winter wheat varieties in the presented study had already been reported by other researchers as well as in our previous research (Groenewald *et al*. 2003; Santra *et al*. 2006; Kwiatek *et al*. 2012; Burt *et al*. 2014; Kwiatek *et al.* 2015, 2016).

According to Groenewald *et al.* (2003), two amplification products of *XustSSR2001-7DL* were characterized, with bands which were 240 bp or 220 bp in size (Fig. 3). As a result of the PCR reaction performed with DNA of the control variety of wheat Rendezvous, a product of 240 bp in size was obtained. For the analyzed lines single bands of 220 bp or 240 bp were observed. In 12 lines (6.9%) bands of the same size as for the Rendezvous (240 bp), characteristic for the resistant plants, were obtained (Fig. 3, Tab. 3).

Two amplification products of *Xorw5* marker – 140 bp and null (lack of band) were characterized. The control variety Rendezvous revealed null product and the same pattern was observed for 11 analyzed lines of winter wheat (Fig. 4, Tab. 3).

Four amplification products were characterized for *Xorw1* marker: 140, 150, 160 and 170 bp in size. For the resistant variety Rendezvous, the 160 bp band was obtained. Bands of the same size were present in 12 analyzed lines of winter wheat (Fig. 5, Tab. 3). Amplification products obtained for the resistant lines with *Xorw1* and *Xorw5* markers were in accordance with the report of Leonard *et al.* (2008).

The presence of the *Pch2* gene was identified using closely associated *Xwmc525* marker (Chapman *et al.* 2008). This gene determines resistance mainly at the seedling stage. An amplification product of 180 bp was found in resistant variety Rendezvous (Fig. 6). Such

Table 3. Lines of winter wheat in which markers for the *Pch1* and *Pch2* genes were identified

No.	Line	*Pch1* markers and amplification products [bp]				*Pch2* marker and amplification products [bp]
		EpD1b	*Xorw1*	*Xust2001*	*Xorw5*	*Xwmc525*
Rv	Rendezvous	1	160	240	null	180
1	DD248/12	1	160	240	null	180
2	KBP 15.2	1	160	240	null	180
3	STH 4431	1	160	240	null	180
4	NAD 14016	1	160	240	null	150
5	AND 4023/14	1	160	240	null	150
6	AND4019/14	1	160	240	null	150
7	KBP 15.12	1	160	240	null	150
8	KBP 296/48	1	160	240	null	150
9	POB 1216	1	160	240	null	150
10	SMH 9409	1 and 3	160	240	null	150
11	DL 358/13/4	1 and 3	160	240	140	150
12	DD 708/13	1	160	240	140	180
13	C 3373/11-1	1	140 + 150 + 170	220	140	150
14	KBH 15.15	1	140 + 150 + 170	220	140	150
15	KBP 1416	1	140 + 140 + 170	220	null	150

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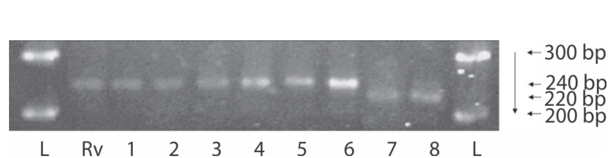

Fig. 3. Amplification products of marker *Xust2001-7DL* (240 bp – the presence of *Pch1*, 220 bp – lack of *Pch1*). Rv – Rendezvous, 1–6 – the presence of *Pch1*, 7–8 – lack of *Pch1*, L – DNA ladder

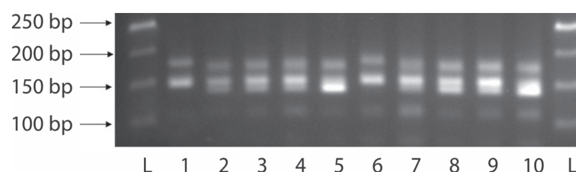

Fig. 4. Amplification products of marker *Xorw5* (null; lack of band – the presence of *Pch1*, 140 bp – lack of *Pch1*). 1 – Rendezvous, 6 – the presence of *Pch1*, 2–5 and 7–10 – lack of *Pch1*, L – DNA ladder

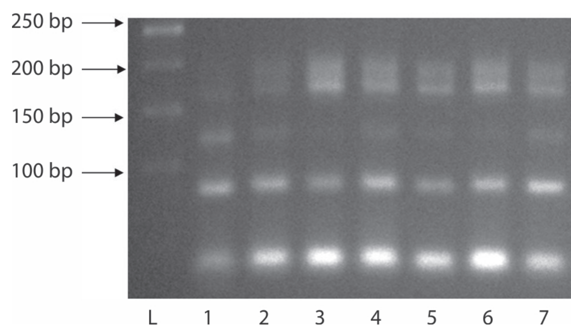

Fig. 5. Amplification products of marker *Xorw1* (160 bp – the presence of *Pch1*, 140 bp – lack of *Pch1*). 1 – Rendezvous, 2–7 – the presence of *Pch1*, L – DNA ladder

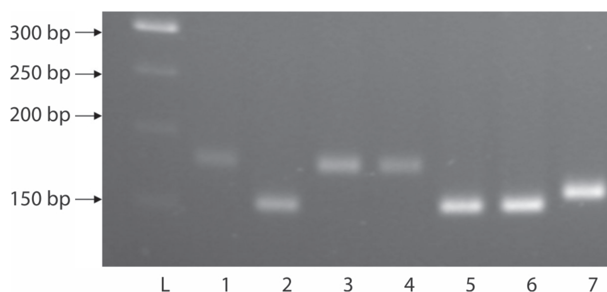

Fig. 6. Amplification products of marker *Xwmc525* (180 bp – presence of *Pch2*, 150 and 160 bp – lack of *Pch2*). 1 – Rendezvous, 3–4 – the presence of *Pch2*, 2 and 5–7 – lack of *Pch2*, L – DNA ladder

amplification products were also found in 68 analyzed wheat lines. Furthermore, recent research showed that *Pch1* and *Pch2* are not homeoloci (Pasquirello *et al.* 2017).

In order to verify the *Pch1* gene effectiveness, a field inoculation test combined with the use of phenotypic markers was carried out to evaluate the eyespot infection level of winter wheat lines at the adult plant stage, after artificial inoculation with the suspension of mycelia and spores of two *Oculimacula* species.

The lines bearing the *Pch1* gene were characterized by the lowest *K*-index (mean 0.54; 0–2) and the lowest percentage of leaf sheath infection (mean 1.3%; 0–4). For the lines in which the *Pch2* gene was present, the *K*-index was high – 5.7 and the percentage of infected leaf sheaths was also high – 24.80%. The lines in which both *Pch1* and *Pch2* genes were present showed a *K*-index of 0.77 and a low percentage of infected leaf sheaths – 5%. Lines with neither *Pch1* nor *Pch2* genes, had the highest infection rate *K* = 6.2 and the highest average percentage of infected leaf sheaths (29.20%) (Tab. 4). Both parameters (*K*-index and % of infected leaf sheaths) characterizing the level of infection of the tested lines with *Oculimacul*a species, showed a high degree of correlation ($R^2 = 0.94$).

Verification of *Pch2* gene expression was examined in a seedling inoculation test performed in a phytotron chamber. The lines of winter wheat, tested for the seedling infection by *Oculimacula* fungi, were assigned to five classes of infection (Fig. 1):

- Class I included variety Rendezvous without symptoms of infection;
- Class II characterized lines with a rate of infection in the range of 0.1–1.0 (60 lines);

Table 4. Results of seedling inoculation test (phytotron chamber) and field inoculation test for lines with or without *Pch1* and *Pch2* genes

Resistance genes	Seedling test infection		Field test			
			infection index *K*		% of infected leaf sheaths	
	mean	range	mean	range	mean	range
Pch1 (10, 7)*	1.20 ab**	0–3	0.54 b	0–2	1.30 c	0–4
Pch2 (60, 59)	1.40 ab	0–4	5.70 a	0–10.5	24.80 b	0–54
Pch1 + *Pch2* (4, 4)	0.80 b	0–3	0.77 b	0.15–4	5.00 c	1–15
Lack of genes (90, 86)	1.70 a	0–4	6.20 a	0–12.5	29.20 a	0–52

*number of lines in seedling and field test, respectively; **means marked with the same letter are not significantly different at p = 0.05 (analysis of variance, Fisher's test)

- Class III characterized plants with a rate of infection in the range of 1.1–2.0 (53 lines);
- Class IV – 34 lines with a rate of infection in the range of 2.1–3.0;
- Class V – included 14 with a rate of infection in the range of 3.1–4.0.

The lowest infection of seedlings was recorded in lines with both *Pch1* and *Pch2* genes, on average 0.8 (from 0–3). Lines with the *Pch1* gene only showed higher infection – on average 1.20 and similar lines with the *Pch2* gene (mean 1.40). Lines lacking the *Pch1* and *Pch2* genes showed the highest seedling infection, on average 1.70 (from 0–6) (Tab. 4).

In order to show whether the presence of *Pch1* and *Pch2* genes, conferring resistance to eyespot in the investigated winter wheat lines, is important for the yield structure components, analysis of variance (ANOVA) of grain yield and TKW was performed. It was shown that the presence of *Pch1* and *Pch2* genes or their absence did not significantly affect the grain yield (from the plot), although the yield was highest in the presence of both genes (Fig. 7).

However, a significant influence of the *Pch1* gene on the TKW was observed. Lines with the *Pch1* gene showed significantly higher values of TKW than lines without both genes or with the *Pch2* gene only (Figs 8 and 9). The weight of thousand kernels increased if the *Pch1* gene was present and additionally increased when the presence of both *Pch1* and *Pch2* genes was identified (Figs 8 and 9). Koen *et al.* (2002) found that out of 17 near-isogenic eyespot resistant lines only three had reduced yield or TKW as compared with susceptible parents without the *Pch1* gene.

Our results clearly demonstrated that *Pch1* was the main effective source for reducing the eyespot disease score in the analyzed winter wheat lines. Nevertheless, *Pch1* was present only in 6.9% of the investigated lines.

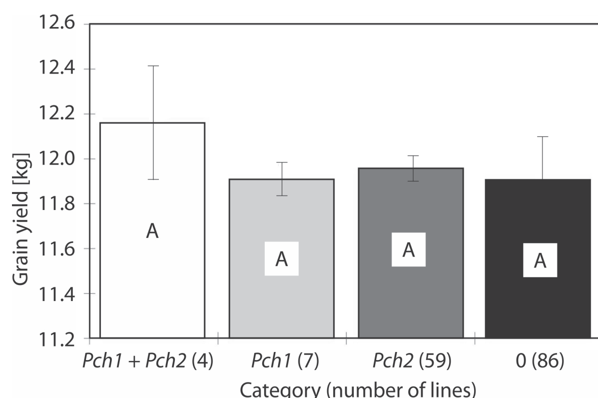

Fig. 7. Comparison of the average grain yield for groups of lines having the *Pch1* gene, *Pch2* gene, *Pch1* + *Pch2* and those lacking the *Pch1* and *Pch2* genes (0). Differences between values marked with the same letter are not significant (analysis of variance, Fisher's test, p = 0.05). The error bars indicate the standard errors

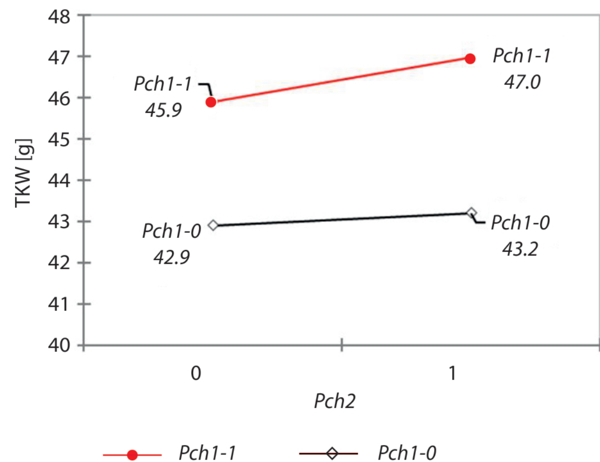

Fig. 8. Comparison of the thousand kernel weight (TKW) for groups of lines having the *Pch1* gene (*Pch1-1*), the *Pch2* gene (*Pch2-1*), *Pch1* + *Pch2* (*Pch1-1* and *Pch2-1*) and those lacking the *Pch1* and *Pch2* genes (*Pch1-0* and *Pch2-0*). Standard errors in brackets

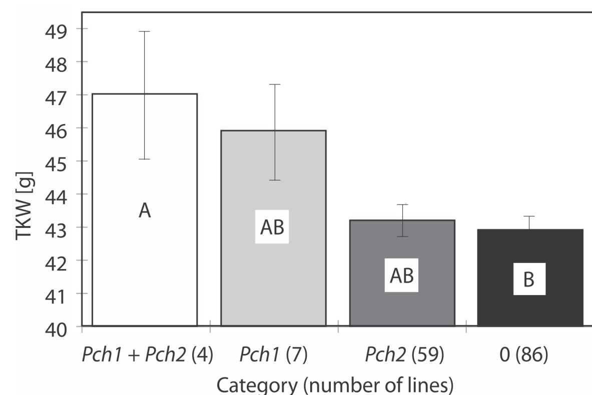

Fig. 9. Comparison of the thousand kernel weight (TKW) for groups of lines that have the *Pch1* gene, *Pch2* gene, *Pch1* + + *Pch2* genes and those lacking the *Pch1* and *Pch2* genes (0). Differences between values marked with the same letter are not significant (analysis of variance, Fisher's test, p = 0.05). The error bars indicate the standard errors

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