Differentiation of Cereal Eyespot Fungi using Morphologic Characteristics and PCR Diagnostic Tests*

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Abstract

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Stem parts of winter wheat infected with eyespot caused by *Pseudocercosporella herpotrichoides* were collected at various locations in the Czech Republic in 1995–1998. Pure colonies of the fungi were cultivated on potato-dextrose agar, visually classified into the teleomorphs and tested for sensitivity to MBC-fungicides. Selected isolates of *Tapesia yallundae* and *T. acuformis* were examined using RAPD markers. Of the total number of 1014 isolates collected, 70% were assessed as W-type (*T. yallundae*) and 30% as R-type (*T. acuformis*). The frequencies of *T. yallundae/T. acuformis* in each of the 4 years were 73/27, 78/22, 65/35 and 66/34%. That the isolates belonged to different pathotypes was proved by RAPD analysis on ten isolates that had been visually identified as the W-type and on ten of the R-type. Among five tested primers, only 5′ GATGACCG 3′ provided polymorphic RAPD products at minimum non-specific amplification. The genetic similarity was assessed by Dice's coefficient of similarity which identified two characteristic groups corresponding to particular pathotypes.

Key words: eyespot; *Pseudocercosporella herpotrichoides*; *Tapesia yallundae*; *T. acuformis*; benomyl resistance; DNA; Polymerase Chain Reaction; Random Amplified Polymorphic DNA; fingerprinting

Eyespot, caused by *Pseudocercosporella herpotrichoides*, is a serious disease of winter wheat that frequently occurs in the Czech Republic. The pathogen survives on crop debris which serves as an inoculum reservoir for future crops. Agronomic factors can greatly influence disease incidence. Many results suggest important effects of crop rotation, sowing date, growth regulator use, intensive cultural practices and climatic conditions on the abundance of eyespot (COLBACH *et al.* 1997). Eyespot lesions cause yield loss when there is substantial girdling of the stem. The stem is softened and thus predisposed to lodging (JONES 1994). Short-stem cultivars do not lodge, but the disease reduces grain yield (BENADA *et al.* 1981).

Two forms of the causal agent of eyespot, the anamorph state of *P. herpotrichoides*, are recognized. They differ in morphological characters and host range. Isolates of the W-type (or N-type) are pathogenic on wheat but cause little disease on rye, whereas R-type (or L-type) isolates are generally equally pathogenic on both hosts (NICHOLSON *et al.* 1997).

The teleomorph of *P. herpotrichoides*, *Tapesia yallundae*, was first identified by WALLWORK (1987). Recent studies, based on the failure of isolates from the two groups to intercross, have indicated that W- and R-type isolates represent different biological species (DYER *et al.* 1996). It has also been proposed that the two groups (W- and R-type) be referred to as *T. yallundae* and *T. acuformis*, respectively, in accordance with the nomenclature suggested by ROBBERTSE *et al.* (1995). Field populations taken from infected stems usually contain both species in various ratios.

A great variability is observed between the two species, especially for pathogenicity and fungicide sensitivity. *T. acuformis* is less sensitive than *T. yallundae* to triazoles, although resistance of *T. yallundae* to this fungicide group has been observed, and isolates resistant to prochloraz and MBC-generating fungicides have been found in both species (CAVELIER *et al.* 1992).

The visual diagnosis of stem base diseases is problematic because all foot rot components can be indistinguishable at early and late growth stages. Symptom assessment

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also fails to account for the relative contribution to tissue browning by the different species mentioned above. Eyespot, sharp eyespot and fusarium can be easily confused (POLLEY & TURNER 1995).

Until recently, no methods have been available for identification and quantification of individual fungal species present within plant tissue. Molecular techniques being developed will assist in the study of a disease complex as they utilize fungal species-specific DNA markers. The sensitivity of such methods for disease detection can be enhanced by polymerase chain reaction (PCR) technology, which allows small quantities of fungal DNA to be amplified for subsequent analysis (BARDSLEY *et al.* 1998). These assays have been developed for specific detection of several fungal pathogens involved in the stem base disease complex of cereals, including *T. yallundae* and *T. acuformis*.

This paper describes part of the studies aimed at assessing the characteristics of the eyespot populations in the main regions with cereal crops, their sensitivity to benzimidazole fungicides and the use of PCR diagnostics to identify these fungal pathogens *in vitro*.

MATERIAL AND METHODS

A four-step assessment procedure was used:

Step I: During the 4 years from 1995 to 1998, stem bases of winter wheat showing symptoms of eyespot infection were collected at 57 locations across the Czech Republic (Table 1). The sites were chosen to cover the main cereal growing areas, and in 171 fields altogether 1014 samples were collected. The samples were collected during grain filling (growth stage 83–85 using BBCH growth scale).

Step II: A 1.5–2 cm piece of the stem base of the main shoot or first tiller was cut from each plant, placed in a paper bag, labelled according to symptoms and stored at –20°C until required. The pieces were surface-sterilized in 1% sodium hypochloride (Savo) for 4–5 minutes, rinsed in sterile distilled water and partially dried in a sterile air flow (BATEMAN 1993).

Each straw piece was cut in half longitudinally. One half, containing a lesion, was divided into four pieces that were then placed on potato-dextrose agar containing antibiotics, at six pieces per Petri dish (CREIGHTON & BATEMAN 1988). The dishes were placed in a dark chamber at 20°C for 3–4 weeks.

T. yallundae (W-type) and *T. acuformis* (R-type) colonies were identified by the colour produced in the agar; green-black for *T. yallundae* and pale brown or pink for *T. acuformis*, and by different morphologies of their colonies. Isolates with faster growing colonies and smooth margins belong to *T. yallundae*, while slower growing and feathery-edged colonies belong to *T. acuformis*, that generally grow at about half the rate of *T. yallundae* isolates. However, some isolates do not match the criteria of colony morphology.

The identification was confirmed by the presence of conidia after dishes had been placed under near-ultraviolet light. Identification of other fungi was normally also possible. *Fusarium* spp. were identified by fast growth and their characteristic conidia. *Rhizoctonia cerealis* was recorded rarely. Frequently, symptoms of more than one disease occurred on the same stem.

Step III: The response of all isolates to 2.0 ppm carbendazim was evaluated. Amended potato-dextrose agar was prepared by adding 4 mg of the product Bavistin containing 50% of carbendazim to 1 l sterile destilled water. Four replicate plates of each isolate were incubated at 21°C for 4 weeks. Morphology was recorded after 3 weeks. Colony growth was determined by measuring two diameters at right angles after 4 weeks.

Growth of the fungus on the medium containing carbendazim indicated MBC-resistance. The same method was used for chosen resistant isolates. These were cultivated on potato-dextrose agar with 5 and 10 mg carbendazim (10 or 20 mg Bavistin per liter).

Step IV: Pure cultures of *T. yallundae* and *T. acuformis* (20 isolates, 10 of each) were assessed using PCR. DNA was isolated from cultivated fungal mycelium using a modified CTAB method described by SAGHAI-MAROOF *et al.* (1984). RNA was enzymatically eliminated by RNasis A. A phenolic method was used for purification of DNA. For DNA quantification (to determine concentration), a UV spectrophotometric method was used. Quality of the isolated DNA was verified by means of electrophoretic separation in a 1% agar gel in horizontal electrophoresis.

Composition of the RAPD reaction was optimized by means of diallele combinations (concentration of the template DNA, primer, $MgCl_2$, thermally stable DNA polymerase) and temperature and time profile of the amplification. Out of five primers (eight bases) one primer (5'GATGACCG 3') producing genotype-specific products was selected.

By optimizing the concentration of individual components, a complete mix for the 25 µl RAPD reaction was developed with the following individual reaction components: 10mM Tris-HCl (pH 8.8), 2.5mM $MgCl_2$, 50mM KCl, 0.08% Nonidet P40, 200mM dNTP, 40 ng template DNA, 20 ng primer 5' GATGACCG 3', 0.7 U *Thermus aquaticus* DNA recombinable polymerase (MBI Fermentas), and 30 µl mineral oil.

For DNA amplification the thermocycler Cyclogene (Techne, Great Britain) was used. For RAPD, the following temperature and time profiles were applied: 1× (93.0°C, 120 s), 35× (92.0°C, 60 s – 36.0°C, 60 s – 72.0°C, 60 s), 1× (72.0°C, 420 s).

RAPD markers were separated by vertical electrophoresis in TBE buffer. 5% polyacrylamide gel was used (SAMBROOK *et al.* 1989). The separation ran for 160 min at a constant voltage of 190 V. Gels were stained by Ag^+ ions according to CAETANO-ANOLLÉS & GRESSHOFF (1994).

A complex evaluation of variability among the genotypes of *T. yallundae* and *T. acuformis* by means of all

Table 1. Locations in the Czech Republic where isolates were collected in 1995–1998 years (total number of isolates *T. yallundae*)

Location	Total	*T. yallundae* [%]	Isolates resistant to MBC [%]	Total	*T. yallundae* [%]	Isolates resistant to MBC [%]
	1995			1996		
Brno	22	50	0			
Jeseník	13	62	0			
Jihlava	24	46	8			
Kroměříž	118	94	4	161	96	11
Nový Jičín	25	84	4			
Pelhřimov	26	8	88	20	0	100
Strukov	12	83	0			
Svitavy	10	90	0			
Total	250					
Hustopeče				14	57	21
Kujavy				10	80	30
Loštice I				12	100	8
Loštice II				10	40	30
Mohelnice				11	0	100
Olomouc				13	92	15
Štěpánkovice				13	69	15
Větřkovice				24	54	8
CC I (Kroměříž I)				11	91	91
CC II (Kroměříž II)				11	100	0
Total				310		
	1997			1998		
Chlumec	12	100	0			
Kroměříž	54	91	13	13	92	0
Litomyšl	11	18	91			
Nový Jičín	14	100	7			
Pelhřimov	11	18	91	6	58	38
Polná	18	28	6	5	40	20
Říčany	14	29	14	4	0	0
Tábor	20	0	0	6	33	16
V. Bitýška	10	0	0	1	100	0
V. Chýška I	17	29	35			
V. Chýška II	11	55	9			
Vranov	11	100	0			
Vyškov	11	91	0			
Znojmo	15	93	13			
CC I (Kroměříž I)	26	100	92			
CC II (Kroměříž II)	25	84	4			
Total	280					
Otice				22	100	5
Kobeřice				8	75	75
Třeboň				5	40	40
V. Bíteš				5	60	0
Mikolajice				14	7	0
Leskovec				3	100	0
Hadec				5	40	40
V. Bitýška II				15	73	66
V. Mýto				5	20	20
Polná II				7	85	14
Kutná Hora				4	25	25
Kroměříž-Boka				2	50	50
Kateřinky				5	100	40
Humpolec				10	0	0
Košín u Tábora				5	100	0
Kutná Hora II				7	100	0
Pelhřimov II				4	0	0
Total				174		

RAPD markers was performed on the GelManager for Windows computer program. For evaluation of electrophoreograms with this program (Dice's coefficients of similarity, cluster analysis), a procedure described by JACKMAN (1994) was used.

RESULTS

Based on the morphology of the colonies on potato-dextrose agar, the isolates could be classified into two types (Fig. 1): fast and even growth with green-black coloured medium (W-type conforming with *T. yallundae*), or slow and feathery growth with pale brown or pink coloured medium (R-type conforming with *T. acuformis*).

Of the 1014 isolates which were collected from 1995 to 1998, 70% were assessed as W-type (*T. yallundae*) and 30% as R-type (*T. acuformis*). The frequencies of *T. yallundae* in individual years were 73, 78, 65, and 66%. The frequencies of *T. acuformis* for the same time were 27, 22, 35, and 34% (Fig. 2).

Of the isolates 24% were resistant to carbendazim; they were found at 20 the locations. Over 30% of the resistant isolates were detected at seven locations. *T. acuformis* isolates exhibited resistance more frequently (Fig. 2).

For sensitive isolates, a mean EC_{50} value for carbendazim was 0.0203 mg/l in 1995 and 0.0324 in 1997. Growth of some resistant isolates was not inhibited at concentrations of carbendazim up to 10 mg/l (Table 2).

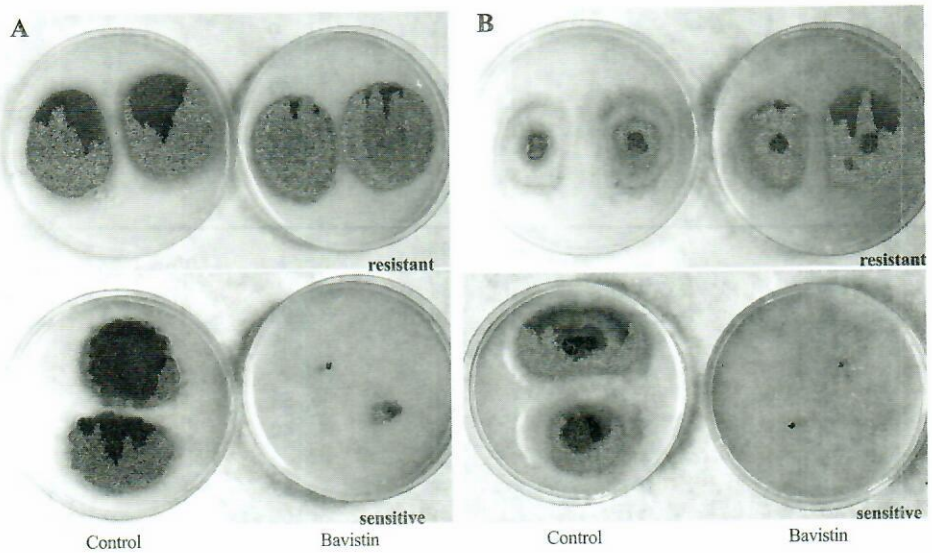

Fig. 1. *Tapesia yallundae* (A) and *Tapesia acuformis* (B) on potato dextrose agar (Left: columns A and B – control, right: columns with carbendazim[Bavistin])

Table 2. ANOVA – Effect of isolate and concentration of fungicide on size of colonies

Source of variability	dF	Mean square	*F*	Significance
Izolates	10	20.355	6.549	**
Concentration	3	71.174	22.903	**
Residue	30	3.107	–	–
Total	43	–	–	–

Variant	Size of colonies	Significance**	Variant	Size of colonies	Significance**
pel 26	14.75	A	pel 2	19.00	BCDE
pel 4	16.50	AB	pel 6	20.25	CDE
pel 7	17.25	ABC	pel 24	21.00	DE
pel 10	17.50	ABC	nj 2	21.50	DE
pel 20	18.25	BCD	pel 3	22.00	E
pel 25	18.25	BCD			
Control	16.09	A	carbendazim (5 mg/l)	20.54	B
Carbendazim (1 mg/l)	17.09	A	carbendazim (10 mg/l)	21.27	B

** $P \geq 0.01$

Table 3. Genetic similarity (%) between *Pseudocercosporella herpotrichoides* pathotypes using Dice's coefficient of comparison

	R10	R12	R13	R14	R15	R9	R1	R2	R3	R11	R18	R20	W5	W10	W12	W14	W15	W16	W17	W18	W1	W2	W3	W4
R10	100																							
R12	100	100																						
R13	100	93	100																					
R14	93	87	100	100																				
R15	93	87	100	93	100																			
R9	80	80	80	80	80	100																		
R1	73	80	80	80	87	80	100																	
R2	73	67	67	73	87	80	100	100																
R3	73	87	80	80	93	87	80	87	100															
R11	93	87	87	87	93	73	80	80	93	100														
R18	62	69	69	62	76	76	76	83	76	76	100													
R20	76	76	76	76	76	62	83	69	76	76	100	100												
W5	62	62	69	62	62	69	55	69	76	83	50	43	100											
W10	62	69	69	62	62	55	55	62	83	69	50	36	93	100										
W12	69	62	69	62	62	62	69	62	69	62	50	43	93	100	100									
W14	69	62	62	69	55	55	62	48	62	69	43	36	86	86	93	100								
W15	60	60	60	60	60	67	60	47	53	53	62	55	69	83	76	76	100							
W16	60	67	60	53	60	67	67	53	53	53	62	55	69	76	69	83	100	100						
W17	67	67	60	67	60	67	67	53	53	53	55	48	69	83	76	76	100	100	100					
W18	67	67	67	60	63	67	67	60	60	53	55	48	76	76	76	76	100	100	100	100				
W1	67	67	67	63	60	67	67	67	60	60	55	48	69	76	69	76	100	100	100	100	100			
W2	60	67	53	60	53	67	53	47	47	47	55	48	69	76	76	69	93	93	93	93	93	93		
W3	60	67	53	60	53	67	60	53	47	47	55	48	69	69	69	76	93	93	93	93	93	100	100	
W4	60	60	53	47	53	60	53	47	53	47	48	48	62	69	69	76	87	93	93	93	93	100	100	100
	R10	R12	R13	R14	R15	R9	R1	R2	R3	R11	R18	R20	W5	W10	W12	W14	W15	W16	W17	W18	W1	W2	W3	W4

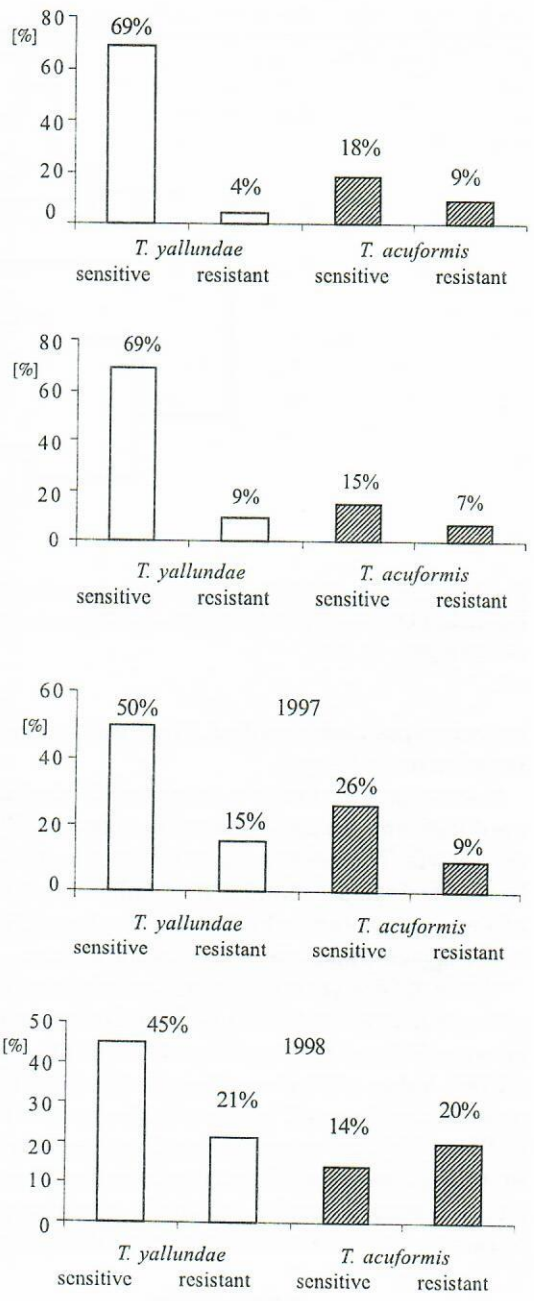

Fig. 2. Eyespot populations in the Czech Republic (in % of isolates collected in 1995–1998)

That isolates which had been visually identified as W-type or R-type did indeed belong to different and genetically distinct pathotypes was proved by RAPD analysis. Only 5′ GATGACCG 3′ provided polymorphic RAPD products at minimum non-specific amplification. These products were subjected to a computer image analysis – densitometric evaluation. Genetic similarity was assessed on the basis of comparison of "bands" position according to Dice (Table 3, Fig. 3). Two characteristic groups corresponding to particu-

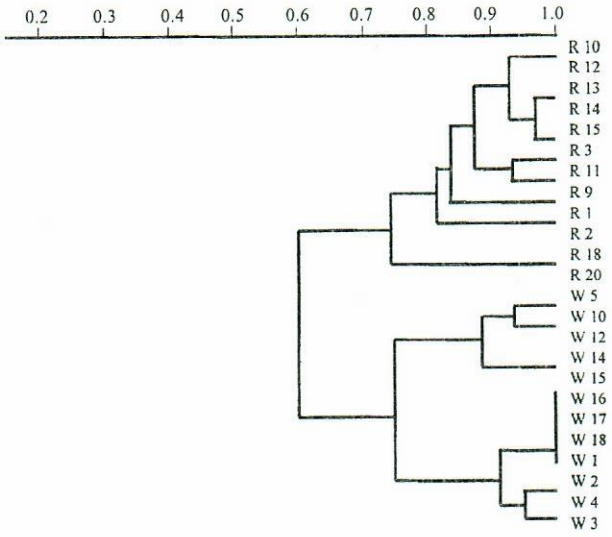

Fig. 3. Dendrogram for different pathotypes according to Dice's coefficient of comparison R10–R20 are R-types and W5–W3 are W-types

lar pathotypes were identified. There was greater genetic variation in the W-type.

In some isolates, two or more mycelial colonies developed that were visually distinct in colour, consistency and growth. They were cultured separately and analyzed by RAPD. Figure 4 shows that visually distinct colonies of the same isolate exhibited electrophoretic patterns which agreed with those of the original isolate.

Values of Dice's coefficients of similarity were computed for pathotypes of the W-type (*T. yallundae*) and ranged between 62.0 and 100%, with a coefficient of variation of 13.99%. Values of Dice's coefficients of similarity for pathotypes of the R-type (*T. acuformis*) also ranged between 62.0 and 100%, and the coefficient of variation was 11.98%. In the *T. yallundae* and *T. acuformis* species 12 and 6 isolate pairs were detected, respectively, which have a value of Dice's coefficient of 100% (refer to Table 3, Fig. 3).

DISCUSSION

The information presented in this paper confirms that eyespot is now a disease which frequently occurs in the Czech Republic in winter wheat crops. BENADA *et al.* (1981) reported the first occurrence of eyespot in 1975, and since then disease incidence and severity have increased along with the expanding area of cultivation of winter wheat. Similar to neighbouring countries, the frequency of disease occurrence depends on farming practices, the varieties grown and weather conditions of individual years (VÁŇOVÁ & MARKOVÁ 1989). At present, about 22% of the crop is treated with fungicides in regions with intense winter wheat growing, but of the crop of the whole country only about 5% is treated (POLIŠENSKÁ 1998). Mostly fungicides with carbendazim as active ingredient are applied. Prochloraz is used to control snow mould (*Fusarium nivale*). Recently, combinations of a triazole fungicide with carbendazim have been preferred.

The occurrence of fungicide-resistant strains of *P. herpotrichoides* was investigated from 1995 to 1998. In individual years, 250, 310, 280 and 174 isolates were taken and examined. Resistance to MBC-fungicides was found in 13% of the isolates in 1995, and in 16, 24 and 41% of those from the following years (Fig. 2). If more than 30% of resistant isolates in a field can be considered as the threshold for inefficacy of a fungicide, this case was found in one field in 1995, in five fields in 1996, in four in 1997 and seven fields in 1998. Such a low occurrence of resistant isolates until now is probably due to cropping systems where the application of benzimidazole fungicides once in 2–3 years constitutes a low selection pressure. In the Czech Republic, no practical resistance, i.e., the occurrence of resistant strains along with failure of fungicide efficacy, has been observed under field conditions till now. The situation in countries of Western Europe (CAVELIER *et al.* 1992) is different because fungicides have been applied at higher levels, more frequently and for a longer time.

In axenic culture, isolates of *P. herpotrichoides* were classified as two distinct morphological types, described as fast-even and slow-feathery (HOLLINS *et al.* 1985). In morphology and pathogenicity the fast-even isolates conformed with the published descriptions of the W-type (*T. yallundae*), while slow-feathery isolates conformed with the descriptions of R-types (*T. acuformis*). The frequency of *T. yallundae* and *T. acuformis* species in isolates collected in the fields infected by eyespot in the Czech Republic were on the average 70.5% for *T. yallundae* and 29.5% for *T. acuformis*. DANIELS *et al.* (1991) showed that the mode of infection of wheat seedlings differed between the two types. In addition, field experiments have indicated that the epidemiology of the two types might also differ. These differences were not apparent in the samples taken at GS 30-31. The differences are greatest in the period of stem lesion establishment. When the fungus is spreading from the innermost leaf sheaths onto the stem is an important stage in the development of eyespot epidemics. At this stage, the W-type apparently grows faster than R-types; later the growth of R-types is faster (GOULDS & FITT 1988). Great variability is also observed between the two species in their sensitivity to fungicides. *T. acuformis* is less susceptible than *T. yallundae* to triazoles; however, resistance of *T. yallundae* to this fungicide group was observed, and isolates resistant to prochloraz were found in both species (CAVELIER *et al.* 1992). An increasing proportion of the R-type in the eyespot population might lower the damage by eyespot.

The epidemiological differences between the types of *Tapesia* spp. during the establishment phase of stem lesions suggest that accurate forecasts of eyespot incidence on leaf sheaths cannot be obtained by visual diagnosis.

Diagnosis of eyespot and other diseases (sharp eyespot, foot rot) is difficult, and identification at the species level is only possible by isolation of the pathogens from the plant into axenic culture. A number of techniques have been developed to study eyespot. The rapid and accurate differentiation of W and R pathotypes is possible with the PCR assay. In the present study we have used RAPDs to identify markers specific to the two species and used these to generate PCR primers.

The same method was used for example by NICHOLSON *et al.* (1997), but in this research the RAP markers were separated with another type of electrophoresis – vertical polyacrylamide electrophoresis. Our study verified that RAPD methods are applicable for identification of two species – *T. yallundae* and *T. acuformis*. It was found further that the staining of electrophoretic patterns with Ag^+ ions is very sensitive. The genetic analysis and staining method enabled the detection of intraspecies variability. The quality of the electrophoretic patterns is much better than that of esterase zymograms, as published by MOREAU and MARAITE (1996). Our methods for fingerprinting the genus *Tapesia* are also less toxic than the use of radioactive labelled probes reported by NICHOLSON *et al.* (1994).

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Souhrn

VÁŇOVÁ M., POLIŠENSKÁ I., VEJL P., SKUPINOVÁ S. (2000): **Detekce a identifikace původce stéblolamu podle morfologických znaků a pomocí metod polymerázové řetězové reakce.** Plant Protect. Sci., **36**: 57–64.

V letech 1995–1998 byly prováděny sběry bazálních částí stébla pšenice ozimé infikovaných stéblolamem působeným houbou *Pseudocercosporella herpotrichoides*. Byl hledán optimální termín sběru, tj. takový stupeň rozvoje patogena na hostiteli, který umožní co nejsnažší získání čistých izolátů a co nejmenší kontaminaci ostatními houbami. Nejvhodnější byla doba v mléčně voskové zralosti, kdy houba prorůstá z listových pochev na stéblo. Houba byla kultivována na bramboro-dextrosovém agaru a podle morfologických znaků, které popsali HOLINS *et al.* (1985), byly izoláty rozděleny do dvou skupin, které se lišily barvou a strukturou mycelia a kolonie vytvářející mycelium. Patotypy se lišily rychlostí růstu a velikostí kolonie. Tyto dvě morfologicky odlišné formy anamorfního stadia houby *Pseudocercosporella herpotrichoides* byly označeny jako *Tapesia yallundae* (W-patotyp) a *T. acuformis* (R-patotyp) (DYER *et al.* 1996). Z celkového počtu 1 014 izolátů bylo 70 % určeno jako *Tapesia yallundae* (W-typy) a 30 % jako *Tapesia acuformis* (R-typy). Četnost *T. yallundae*/*T. acuformis* v každém ze čtyř sledovaných let byla 73/27, 78/22, 65/35, 66/34 %. Izoláty byly dále kultivovány na bramboro-dextrosovém agaru s přídavkem 2,0 ppm carbendazimu. V jednotlivých letech byla četnost rezistentních typů *T. yallundae* 4, 9, 15, 21 % a *T. acuformis* 9, 7, 9 a 20 %. Vybrané rezistentní izoláty byly dále kultivovány při dávkách 1, 5 a 10 mg/l carbendazimu. Byly zjištěny statisticky průkazné rozdíly mezi izoláty i koncentracemi přípravku. Morfologicky odlišné izoláty byly diagnostikovány užitím metod polymerázové řetězové reakce. Z pěti testovaných primerů pouze 5′ GATGACCG 3′ umožňoval identifikaci izolátů jednotlivých druhů. Byly vybrány konkrétní RAPD produkty – zóny, které umožnily identifikovat morgologicky odlišné izoláty. Genetická podobnost byla stanovena Diceho podobnostními koeficienty, které identifikovaly dvě charakteristické odlišné skupiny.

Klíčová slova: stéblolam; *Pseudocercosporella herpotrichoides; Tapesia yallundae*; *T. acuformis*; benomyl; rezistence; DNA; polymerázová řetězová reakce; RAPD; fingerprinting

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