Specialisation of *Rhynchosporium secalis* (Oud.) J.J. Davis Infecting Barley and Rye

Ludmila LEBEDEVA[1] and Ludvík TVARŮŽEK[2]

[1]All-Russian Institute for Plant Protection, St-Petersburg-Pushkin, Russia;
[2]Agricultural Research Institute Kroměříž, Ltd., Kroměříž, Czech Republic

Abstract

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Fifty-five isolates of *Rhynchosporium secalis* from *Hordeum vulgare* and 34 isolates from *Secale cereale* were compared for growth on different nutrient media, effect of temperature on growth and morphology of colonies. The pathogenicity of the isolates was assessed on 10 rye varieties, 10 triticale varieties and the susceptible barley variety Gambrinus. The triticale varieties differed in the number of rye chromosomes in the genome. Isozymes of *R. secalis* isolated from infected leaves of barley and rye were compared. The RAPD-PCR method was used for comparison of isolates on DNA-markers. The analysis indicated two specialised forms of the fungus; each of them able to develop only on its original host.

Keywords: *Rhynchosporium secalis*; barley; rye; specialisation

Diseases rank among factors that reduce grain yield and quality of cereals. One of them is leaf scald of barley and rye caused by the imperfect fungus *Rhynchosporium secalis* (Oud.) J.J. Davis. Yield loss in rye and barley due to this disease can amount to more than 40% (McDonald *et al*. 1999; Xi *et al*. 2000).

In nature, *R. secalis* is able to infect not only cultivated barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.), but also wild barley *(Hordeum murinum* L.), various species of wheat (*Triticum monococcum* L., *T. durum* Desf., *T. aestivum* L.), triticale (× *Triticosecale* Wittm.), oats (*Avena sativa* L.), wild grass species: couch-grass (*Agropyron repens* L.), wild oat (*Avena fatua* L.), smooth brome (*Bromus inermis* Leys.), sonchus (*Sonchus oleraceus L.*), spreading millet (*Milium effusum* L.) and others.

Available literature data on specialisation of the pathogen are contradictory and do not allow unambiguous conclusions as for infection transfer from one crop to the other.

Bartels (1928) cross-infected rye, barley, wheat, oats and couch-grass with a suspension of *R. secalis* obtained from these plants. He demonstrated that the fungus was an "omnivorous" parasite, i.e. it does not have any specialised forms. Caldwell (1937) and Schein (1958) arrived at the same conclusion.

Canadian researchers, having studied 75 isolates of *R. secalis* collected from barley leaves in 23 countries, assume that *R. secalis* is a strictly

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specialised pathogen and the isolates taken from barley and rye are able to infect only the corresponding crop (Jackson *et al.* 1978). Similar results were obtained by Robinson *et al*. (1996). *R. secalis* isolates taken from barley, rye and couch-grass in Finland were used for analyses. The isolates from barley did not infect rye, those from couch-grass did not infect barley and rye, and the isolates from rye were pathogenic to rye only.

Jørgensen and Smedegaard-Petersen (1995) and Cromey (2002), who performed cross-infection of rye and barley with the isolates from rye, barley and wild grass species, concluded that *R. secalis* had strictly specialised forms.

Such contradictory literature data on the presence or absence of specialised forms of *R. secalis* called for further investigations to elucidate the specialisation of *R. secalis*. Outcomes of these investigations would be necessary to identify a source of infection, and for deciding on measures of protection against the pathogen and for alternation of crops in crop rotations.

Thus, the purpose of this work was to study the specialisation of *Rhynchosporium secalis* infecting rye and barley. Isolates of *R. secalis* obtained from rye and barley were compared for morphological-culturing traits, isozyme polymorphism, DNA structure and pathogenicity on barley, rye and triticale.

MATERIAL AND METHODS

Leaves of barley and rye plants with scald symptoms were collected in the northwestern region of Russia and in southern Moravia of the Czech Republic during the summer of 2003. Infected leaves were taken at DC 51 (Tottman & Broad 1987); at the tube-phase of plants; we used the hierarchical sampling scheme (McDonald *et al*. 1999).

Following surface sterilisation (15 s in 90% ethanol and 80 s in 1% NaOCl, then rinsing in sterile water), leaf segments of barley and rye were placed on potato-dextrose agar (PDA) in Petri dishes. Leaf segments were incubated in a polythermostat at 18 ± 1°C in darkness for 2 weeks until pink, when the sporulating colonies were visible. In this manner, 54 barley isolates and 34 rye isolates of *R. secalis* were obtained. Single-spore cultures were made and subcultures were incubated at 18 ± 1°C for 2–3 weeks.

The growth rate of the pathogen was studied on different nutrient media (water agar, Czapek agar, carrot-dextrose agar, oat agar, potato-dextrose agar (PDA), and PDA with addition of 0.1% of yeast extract) and in the range of temperatures from 10 to 22°C. The changes in size of the colonies were determined after 14 and 21 days.

Pathogenicity of *R. secalis* isolates was assessed on seedlings at DC 13. Plant seeds were sown in pots containing nutrient-supplemented peat and placed in a greenhouse at 18–20°C under natural daylight. Ten rye varieties, 10 triticale varieties and the susceptible barley variety Gambrinus were used in the experiment. The triticale varieties differed in the origin and number of rye chromosomes in the genome. We used the mixture of isolates originated from rye and from barley. The isolates were taken from Russian and the CZ populations randomly (Table 1).

Spore suspensions were prepared by scraping 2–3 week old cultures after adding 10 ml of distilled water. Suspensions were adjusted to 10^6 spores/ml, and one drop of surfactant (Tween 20) per 26 ml suspension was added before inoculation. 10 ml of inoculum was sprayed with a hand sprayer.

Table 1. Mycelial growth rate of *Rhynchosporium secalis* isolates from barley and rye on different nutrient media

Nutrient media	Average mycelial growth rate			
	rye isolates		barley isolates	
	after 12 days (mm)	(mm/day)	after 12 days (mm)	(mm/day)
Water agar	2.7 ± 0.37	0.2 ± 0.03	3.7 ± 0.42	0.2 ± 0.06
Czapek agar	4.2 ± 0.03	0.3 ± 0.00	6.7 ± 0.03	0.5 ± 0.04
Carrot-dextrose agar	10.7 ± 0.11	0.8 ± 0.00	13.3 ± 0.09	0.9 ± 0.08
Oat agar	11.4 ± 0.02	0.8 ± 0.01	12.6 ± 0.01	0.9 ± 0.01
PDA	14.4 ± 0.43	1.0 ± 0.03	16.4 ± 0.34	1.2 ± 0.03
PDA with 0.1% of yeast extract	14.7 ± 0.38	1.0 ± 0.03	16.8 ± 0.08	1.2 ± 0.32

Inoculated plants were covered with moistened polyethylene bags (relative air humidity = 100%). High humidity was maintained for 48 h. Scald symptoms became evident after about 3 weeks, when the seedlings had produced 4–6 leaves (DC 14–16).

Seedlings were scored for scald symptoms according to the five-point disease rating scale of Jackson and Webster (1976), where 0 = no visible symptoms; 1 = small lesions on leaf margins; 2 = small lesions on lamina; 3 = large coalescing lesions on leaf; 4 = total leaf collapse. Scores 1 and 2 were classified as low infection responses (LIR), with 3 and 4 as high infection responses (HIR).

The rye and barley isolates of *R. secalis* assayed for isozymes were grown on PDA. 30 mg of mycelium was scraped off the surface with a metal spatula and crushed in a small plastic tube (V = 10 ml) with a little acid-washed sand. Three or four drops of extraction buffer (0.5M sucrose, 0.1% ascorbic acid, 0.1% cysteine hydrochloride in tris-citrate buffer, pH 8-7, based on the recipe of Sako and Stachmann (1972)), were added. The samples were centrifuged at 12 000 g/min with cooling for 15 min. The resulting supernatants were used immediately for electrophoresis.

Slab polyacrylamide gels (365 mm × 100 mm × 1.5 mm), each with 96 samples, were run vertically with a buffer system. A solution of tris-glycine (pH 8.3) was used as the electrode buffer. A constant current of 100 mA at a temperature of 10°C during 2.5 h was maintained. Electrophoresis of the samples was carried out until the tracker dye (bromphenol blue) had migrated 40 mm from the sample slots in direction from cathode to anode.

Method of ensymes detection and determination. The following isozymes were used in the analysis: α-esterase (6% w/v), β-esterase (6% w/v), diaforase (6% w/v), aspartate aminotransferase (7.5% w/v) (Wehling 1986) and superoxide dismutase (7.5% w/v) (Beauchaump & Fridovich 1971). 58 isolates obtained from barley and 30 isolates – from rye were run on every gel. A minimum of three replicates, each obtained from separate cultures and run on different gels, were estimated for each isolate. A relative mobility (*Rf*) value was assigned to each band of enzyme activity detected. The most cathodes band of isolate was assigned an *Rf* value of 100. Relative mobility of all other bands was calculated by dividing the distance migrated by 100. For each isolate, presence or absence of a band of enzyme activity for each enzyme used in this study defined an electrophoretic phenotype.

The results of isozyme analysis were processed using the Neighbour-Joining method, software package Phylip 6.0 (Felsenstein 1989) which allows to construct phylogenetic trees showing the degree of genetic relationship of barley and rye isolates of *R. secalis*.

The RAPD-PCR method was used for comparison of barley and rye isolates of *R. secalis* on DNA-markers. Fresh fungal mycelium (10–12 days old) of each isolate was scraped off the medium in Petri dishes and transferred to a 1.5 ml tube. Total DNA was extracted by adding 600 µl of extraction buffer [50mM Tris-HCl (pH 7.8), 50mM EDTA, 150mM NaCl, 2.5% N-lauroyl sarcosine, 500mM 2-mercaptoethanol, 600 g proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml] at 65°C for 4 h with frequent mixing. The NaCl concentration of the solution was adjusted to 1M, and an equal volume of chloroform-octanol mixture (24:1) was added and left for 15 min at room temperature. After centrifugation at 12 000 g for 2 min, the aqueous phase was transferred into a new tube (procedure was repeated twice) and the DNA was precipitated with isopropyl alcohol (0.6 volume) for a few minutes at 21°C. The precipitate was rinsed twice with 70% ethanol, dried, and dissolved in TE buffer (1mM Tris-HCl (pH 7.8), 0.1mM EDTA). The final DNA concentration was 50 to 100 µg/ml.

For PCR amplification of fungal DNA, one casual primer – OPA-01 was chosen from following primers studied: OPA-01, OPA6, OPA7, OPA12, OPA14, OPA15 (with length 10 oligonucleotides), which was obtained from Operon Technologies, Inc. (Alameda, CA).

RESULTS AND DISCUSSION

Growth on nutrient media

It is known that a slow growth rate on all nutrient media is characteristic for *R. secalis* (Čoja 1998; Konovalova *et al.* 1999). At isolation of the fungus from infected leaves of both barley and rye, the first signs of growth were observed after 12–14 days. On some media, the growth rate of barley isolates differed from that of rye isolates (Table 1).

The highest average growth rate of mycelium was on PDA and PDA with yeast extract. It was found that the growth rate of barley isolates on some

nutrient media differs from that of rye isolates; barley isolates grew faster than rye isolates.

Effect of temperature on *R. secalis* growth

We demonstrated that rye and barley isolates responsed differently to the culturing temperature in nutrient medium. It is well-known that the optimum temperature for barley isolates is 18-20°C (Čoja 1998; Salamati & Magnus 1997; Konovalova *et al.* 1999). The fastest growth of rye isolates was recorded at temperatures of 14–18°C (Table 2). A temperature of 20°C and higher suppressed the growth of rye isolates and was followed by death of the fungus. In contrast, such a temperature was not critical for the isolates from barley.

Table 2. Effect of temperature on the growth of mycelium of *R. secalis* isolates from rye

Temperature (°C)	Average mycelial growth rate after 12 days (mm)	Average increase of mycelia for 7 days
10–12	12.8 ± 0.50	0.8 ± 0.04
12–14	13.4 ± 0.44	0.9 ± 0.03
14–16	14.7 ± 0.38	1.1 ± 0.03
16–18	14.3 ± 0.35	1.0 ± 0.03
20–22	7.6 ± 0.43	0.4 ± 0.04

Morphology of *R. secalis* colonies

The colonies of *R. secalis* sampled from both rye and barley were round with even edges, longitudinal or transverse radial wrinkles, and a yeast-like tapering centre.

However, the comparative analysis of *R. secalis* isolates from barley and rye revealed substantial differences in colour of colonies. All barley isolates collected in Russia and the Czech Republic were classified according to colour into five groups: black, dark brown, light brown, creamy and pink, although most isolates (82%) were dark coloured.

The isolates from rye were classified into seven groups: black, brown, black-brown, black and white, pink-brown, creamy-brown, creamy-pink-brown. Each group included about the same number of isolates.

Growth and colour type of the mycelium of the pathogen infecting barley have been described by many researchers. Čoja (1998) described six colour types for mycelium of barley isolates: dark pink, yellow, orange, light brown, dark brown, and black. McDermott *et al.* (1989) reported that colonies of *R. secalis* developed either a black or a light creamy colour, which remained stable through repeated transfers to fresh PDA medium. We found five colour types for barley isolates of *R. secalis* and documented that the rye isolates had two more colour types that were absent in barley isolates.

Analysis of isozymes of *R. secalis*

The comparative analysis of six isozyme spectra of *R. secalis* isolates taken from infected leaves of barley and rye showed not only their variability but also their differences in each examined enzyme.

The following isozymes were used for the analysis: superoxide dismutase (SOD), diafarase (DIA), α-esterase (α-EST), β-esterase (β-EST), and aspartate aminotransferase (AAT).

The SOD zymogram for the isolates from rye detected three fractions with various electrophoretic mobility (*Rf* = 0.45, 0.52 and 0.55), while each isolate contained only one of the fractions (Figure 1a).

SOD for barley isolates of *R. secalis* had one stable activity zone of the enzyme, *Rf* = 0.15, and five bands were distinct at various mobility, *Rf* = 0.17, *Rf* = 0.19, *Rf* = 0.23, *Rf* = 0.27 and *Rf* = 0.30. These bands were present in various combinations and at various frequencies (Figure 1a). The presence of the stable activity zone (*Rf* = 0.15) in all examined barley isolates of *R. secalis* allowed to assume that it was typical for the fungus infecting barley. At the same time, this band was absent in the pathogen infecting rye.

The results of *α*-EST zymogram for the isolates taken from rye showed four basic bands: two in the middle part of the zymogram (*Rf* = 0.52 and 0.55) and two in the bottom part (*Rf* = 0.84 and 0.87), with the middle bands being coloured more than the bottom ones. Most isolates differed from each other in mobility and colouration intensity of the bottom doublets. The two bottom mobility bands were characteristic for all analysed rye isolates. In 48% of the isolates no bands were found in the middle part of the zymogram, 16% of the isolates showed one band at *Rf* = 0.55, 12% had one band

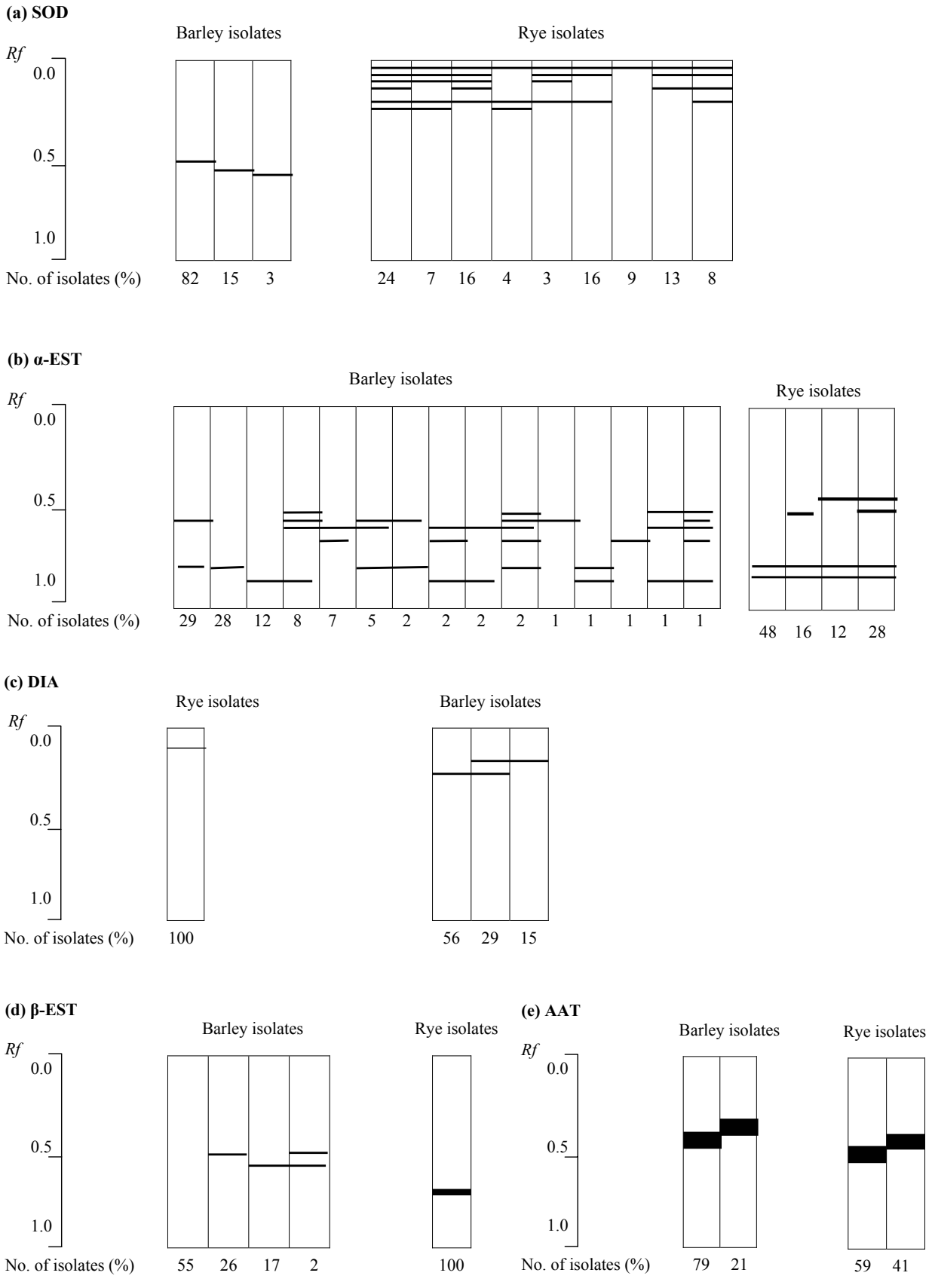

Figure 1. Comparison of isozyme patterns of *R. secalis* isolates from barley and rye

at *Rf* = 0.52, and in 28% of the isolates all four bands were detected.

The zymogram of α-EST for barley isolates was polymorphic and had six fractions with various frequencies of occurrences. Of them, four zones had the identical mobility as in rye isolates: *Rf* = 0.52 and 0.55 and *Rf* = 0.84 and 0.87. Two other fractions were characteristic for barley isolates only at *Rf* = 0.61 and *Rf* = 0.48. None of the isolates showed all six bands (Figure 1b).

Comparison of *α*-EST patterns for barley and rye isolates revealed that all rye isolates had two stable bands at *Rf* = 0.84 and 0.87. In barley isolates, only one of the two activity bands was present. Only 1% of barley isolates had both bands.

DIA for barley isolates was represented by one monomorphic band at an extremely weak zone of enzymatic activity. The isolates from rye were polymorphic in this enzyme (Figure 1c).

β-EST patterns from isolates from barley were polymorphic, and those from rye monomorphic (Figure 1d).

In *R. secalis* isolates from rye, at most two types of wide dark coloured bands were detected at the AAT – a fast one at *Rf* = 0.47–0.55 and a slow one at *Rf* = 0.40–0.48. The first band occurred in 59% of the isolates, and the second in 41% of them.

Likewise, two types of wide dark coloured bands were found in the barley isolates. However, in contrast to the rye isolates, the two bands were less mobile and had *Rf* = 0.29–0.40 (21% of all isolates) and *Rf* = 0.33–0.42 (79% of isolates) (Figure 1e).

The comparative analysis of *R. secalis* isolates according to the mobility of each of the studied

I (top cluster) – isolates from barley
II (bottom cluster) – isolates from rye
N – quantity of *R. secalis* isolates

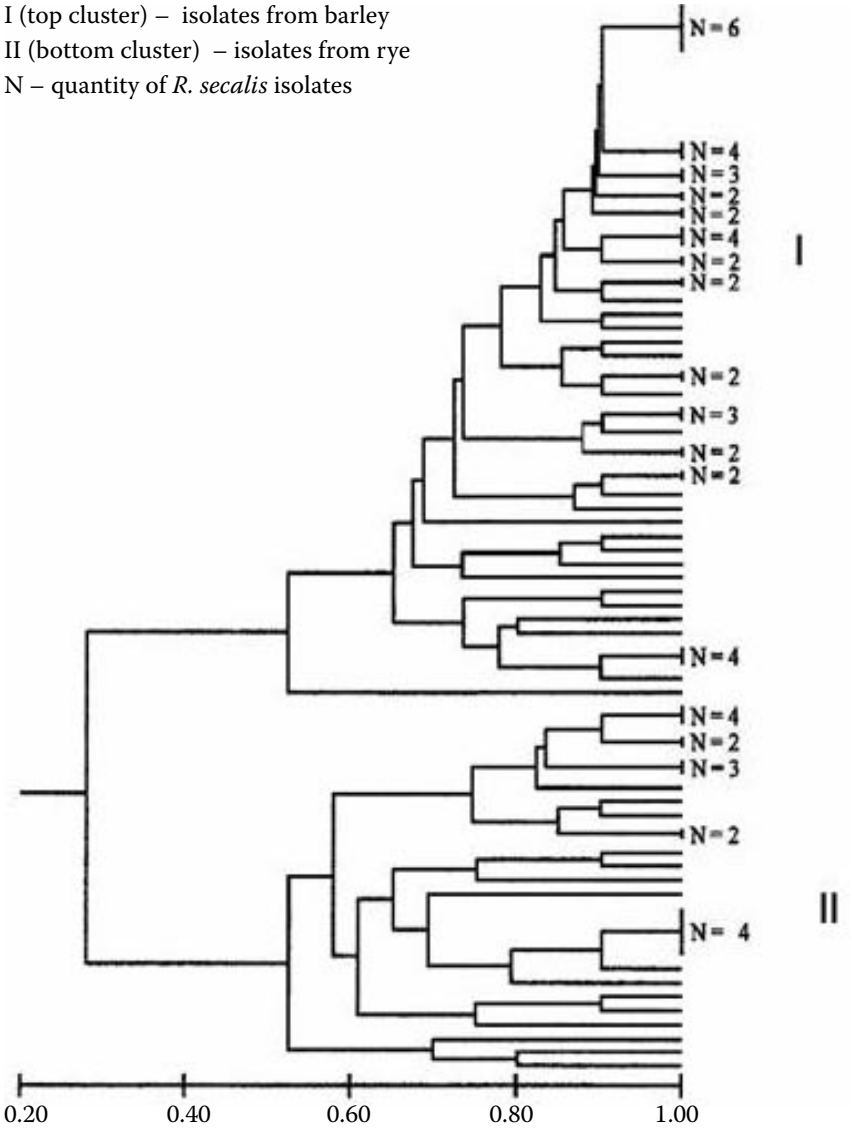

Figure 2. Phenogram of isozyme polymorphism similarities of R. *secalis* isolates taken from infected leaves of barley and rye

enzymes showed their differences in construction of a phenogram (Figure 2). The phenogram, constructed using a binary matrix based on six isozymes, reflects the genetic relation of the isolates taken from barley and rye. All examined isolates were divided into two clusters: top and bottom. The first (top) cluster included most isolates from barley, and the second (bottom) one those from rye. Based on these data, it can be stated that the isolates from rye and those from barley differ considerably in the studied isozymes.

Characterisation of *R. secalis* isolates using DNA markers

The analysis of rye and barley isolates of *R. secalis* based on DNA markers using RAPD-PCR with a casual primer (OPA-01) also revealed differences between the two species.

Two DNA polymorphisms (700 and 500 bp) were found in the isolates of *R. secalis* from rye in contrast to the isolates from barley (Figure 3). Considering the fact that one casual primer and an insufficient number of rye isolates were tested, only preliminary conclusions can be inferred about a genetic dissimilarity of these groups of isolates.

Analysis of pathogenicity of *R. secalis* isolates

The analysis of pathogenicity of *R. secalis* isolates from infected rye and barley leaves that was carried out on the susceptible barley variety Gambrinus (CI 13533), 10 rye varieties, 10 triticale varieties differing in a level of ploidy revealed a strict specialisation of the pathogen (Table 3).

Isolates of *R. secalis* from barley were pathogenic on the barley variety Gambrinus only, while the varieties of rye and triticale were resistant and did not show any visible symptoms of infection. The isolates from winter rye infected rye varieties only, whereas the barley variety was resistant. Triticale varieties, regardless of their differences in the genome, were also resistant to these isolates.

However, it was noted that the mixed spore suspension from barley isolates caused minor lesions on the rye varieties Matador and Picasso. Though the lesions were rather like typical symptoms of the disease, no fungus spores were found on these leaf segments when investigated by microscope, and the pathogen was not detected by re-isolation. It is evident that the development of the fungus was suppressed. Thus, we suppose that this limited development was associated with its strict specialisation. On the other hand, it is possible that

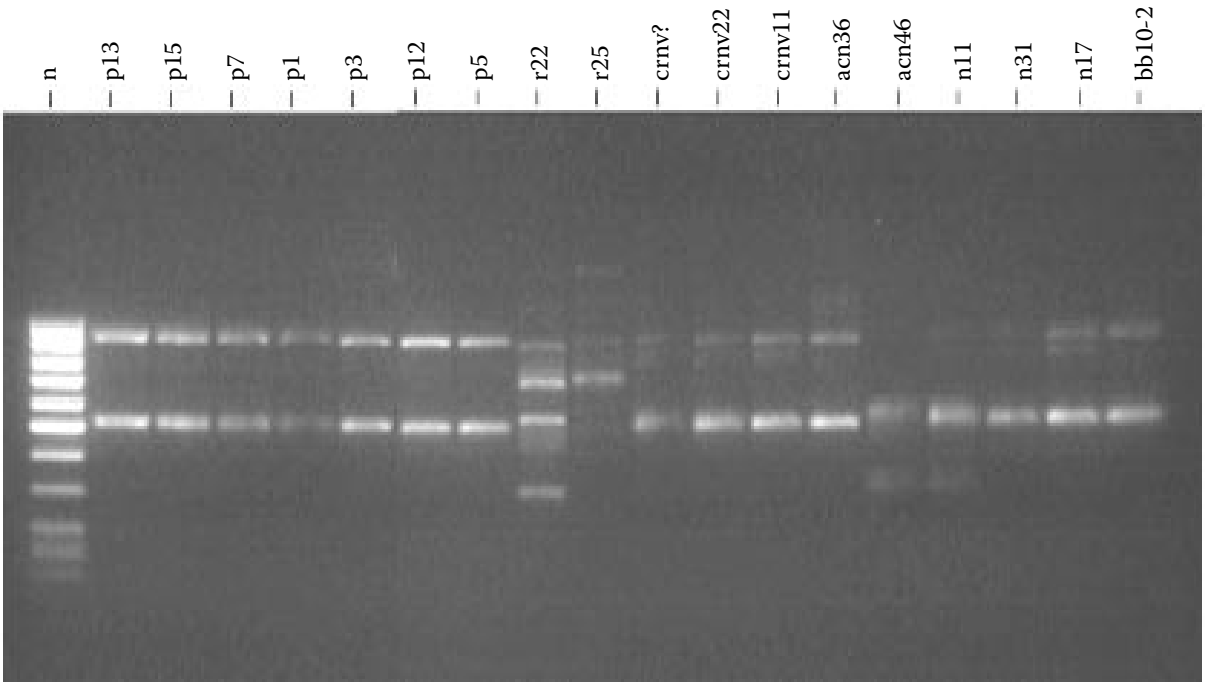

Figure 3. Random amplified polymorphic DNA (RAPD) analysis showing different amplification patterns for isolates of *R. secalis* from rye (r22 and r23) and from barley

Table 3. Pathogenicity of *R. secalis* isolates from barley and rye

Infected plants	Reaction to infection		
	barley isolates		rye isolates from Russia
	from the Czech Republic	from Russia	
Barley variety			
Gambrinus	S	S	R
Triticale varieties			
Prat 67 (4*x*)	R	R	R
Prat 68 (4*x*)	R	R	R
Prat 3 (8*x*)	R	R	R
Prao 5/2 (8*x*)	R	R	R
Prao (8*x*)	R	R	R
Lasko (6*x*)	R	R	R
LT363/75 (6*x*)	R	R	R
CZR 630 (6*x*)	R	R	R
KS 126 (6*x*)	R	R	R
UH92/73 (6*x*)	R	R	R
Rye varieties			
Albedo	R	R	S
Apart (HY)	R	R	S
Aventino	R	R	S
Danko	R	R	S
Fernando (HY)	R	R	S
Locarno (HY)	R	R	S
Matador	R	R	S
Picasso (HY)	R	R	S
Rapid (HY)	R	R	S
Selgo	R	R	S

R = resistant; S = susceptible

we did not succeed in detection of barley isolates virulent on the used rye and triticale varieties.

The comparative analysis of *R. secalis* isolates taken from infected leaves of rye and barley, based on morphological-culturing traits, isozyme spectra, DNA structure and pathogenicity, indicated two specialised forms of the fungus, each of them able to develop only on its original host.

We believe that work in this direction should be continued, as its results are important for both revealing the source of the infection and subsequently for carrying out appropriate control measures against the pathogen, and for considering possible changes of crops in a crop rotation.

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Corresponding author:

Dr. Ing. Ludvík Tvarůžek, Zemědělský výzkumný ústav Kroměříž, s.r.o., Havlíčkova 2787, 767 01 Kroměříž, Česká republika

Tel: + 420 573 317 138, fax: + 420 573 339 725, e-mail: tvaruzek@vukrom.cz