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OCCURRENCE OF *Neotyphodium* AND *Epichloë* FUNGI IN MEADOW FESCUE AND RED FESCUE IN POLAND AND SCREENING OF ENDOPHYTE ISOLATES AS POTENTIAL BIOLOGICAL CONTROL AGENTS

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Abstract. *Neotyphodium* and *Epichloë* endophytes often influence beneficially on the host plant. Thus, could be used as a biological factor enhancing grass growth and resistance to stress factors. A detailed study was made to: (i) determine the infestation of genotypes of meadow fescue and red fescue, which occur in natural grass communities, in Poland by the *Neotyphodium*/*Epichloë* endophytes, (ii) define the level of ergovaline, a toxic alkaloid produced by active associations, (iii) determine the activity of antagonistic distinguishing isolates of endophytic fungi against selected microorganisms. There were analysed 204 genotypes of meadow fescue and 171 genotypes of red fescue. Mean frequency of the infection with endophytes was 74.5% and 64.3% respectively. Ergovaline was produced by 77% of E+ meadow fescue genotypes and 80.9% of red fescue genotypes. Mean content of the alkaloid was 0.202 and 0.151 $\mu g \cdot g\ DM^{-1}$ respectively. FpII30, FpII67, FpII77, FpII168 and FrII82 demonstrated a high ability for protection of the host grasses from all the tested pathogens. These isolates due to high antimicrobial activity and a lack of ergovaline production have a great potential for biological control of grasses.

Key words: Endophyte, ergovaline, dual-culture, biological controlof grasses

INTRODUCTION

Under natural conditions, grasses very often form symbiotic associations with fungi as well as with bacteria. The associations of the greatest importance include relationships with endophytic fungi [Bacon and de Battista 1991]. Those fungi infect the roots and culms, usually developing intercellular in the plant tissue, with no symptoms triggered. The endophytes of the greatest importance, infesting grass roots, are considered endomycorrhizal fungi of genus *Glomus* [Smith and Read 1997]. In the grass culms, however, we can find three different groups of endophytic fungi: e-endophytes,

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p-endophytes as well as a-endophytes. The last group is represented by *Acremonium* genus fungi, e.g. *A chilense, A. strictum, A. alternatum* of the *Simplex* section. They are very frequently isolated, however, their role is not quite known. Most probably their importance is inconsiderable. The second group of p-endophytes is made up of fungi referred to as *Phialophora*-like and *Gliocladium*-like [Siegel et al. 1995]. They infest, respectively, grasses of genus *Festuca* as well as *Lolium perenne*. They often occur in cosymbiosis with e-endophytes. Their effect on the plant and other symbiotic organisms has been investigated inconsiderably. The most numerous group of endophytes developing in the aboveground part of grasses is made up of e-endophytes representing family *Clavicipitaceae* (Sordariomycetes) [Marcinkowska 2010].

At present 58 species of those endophytes are differentiated between: grouped in 7 genera: *Atkinsonella, Balansia, Epichloë, Myriogenospora, Nigrocornus* and *Parepichloë*, reproducing generatively (sexual reproduction) as well as *Neotyphodium,* reproducing vegetatively only (asexual reproduction) [Glenn et al. 1996, White and Reddy 1998, Cheplick and Faeth 2009]. A characteristic feature of those fungi is their vertical method of reproduction. It involves the mycelium getting grown over to kernel primordia and spreading with them only. Exceptionally, some *Epichloë* species can, under some conditions, create stroma with ascospores, on the host plant inflorescence, thanks to which they reproduce horizontally [Schardl 2001]. Those endophytes can infest more than 290 grass species. The most intensive research is currently performed into *Epichloë* spp. and *Neotyphodium* spp., mostly due to the fact that they infest the economically most crucial grass genera *Festuca* and *Lolium*. The importance of endophytes comes mostly from the host plant producing alkaloids toxic to farm animals [Thompson and Stuedemann 1993]. On the other hand, those endophytes can have a positive effect on the plant. That effect is multidirectional and very complex. The greatest advantage for the plant resulting from the presence of the endophyte is seen from its greater resistance to stress [Faeth et al. 2001]. The infested plants (E+) show a well-developed root system and the roots are longer and smaller in diameter, thanks to which the plant can take water from deeper soil layers [Latch et al. 1985, Malinowski et al. 1999]. It was also found that stomas in E+ plants close under drought earlier and faster than in plants E− [Elmi and West 1995]. Endophytes decrease also the sensitivity of the plant to darkening and increase its competitiveness in the environment [Lewis 2004]. They inhibit germination and growth of other plants, e.g. weeds, secreting compounds allelopatic in nature into soil [Vázquez de Aldana et al. 2012].

Besides, the plants infested by endophytes get better adapted to the environments poorer in minerals [Lewis 2004]. Although endophytes do not get developed in the roots only in the aboveground part, however, they show a great effect on chemical transformations which occur there and on the rhizosphere. Partially, it also accounts for a greater resistance of plants E+ to infection by some harmful nematodes [Elmi et al. 2000]. There was also found a higher degree of resistance of the plants with endophyte on the feeding and egg laying by insects [Johnson et al. 1985] and the infection by pathogens [Clarke et al. 2006, Wäli et al. 2006]. The above facts call for a search for the isolates of *Neotyphodium*/*Epichloë* which could be used as a biological factor enhancing grass growth and resistance to stress factors. The condition, however, is a lack of the abilities of selecting isolates to produce toxic alkaloids in the plant. With that in mind,

a detailed study was made to: (i) determine the infestation of genotypes of meadow fescue and red fescue, which occur in natural grass communities, in Poland by the *Neotyphodium*/*Epichloë* endophytes, (ii) define the level of ergovaline, a toxic alkaloid produced by active associations, (iii) determine the activity of antagonistic distinguishing isolates of endophytic fungi against selected microorganisms under laboratory and pot conditions.

MATERIALS AND METHODS

Detection of *Neotyphodium* and *Epichloë* endophytes. There were analysed genotypes of meadow fescue and red fescue collected from different natural habitats in 13 provinces, in Poland, during expeditions conducted in the years 2004–2010. The collection of plant genotypes was maintained at the Mochełek Experiment Station, University of Technology and Life Sciences, Bydgoszcz, Poland. Endophytic fungi representing genera *Neotyphodium* and *Epichloë* were detected with use of Phytoscreen™ *Neotyphodium* Immunoblot Kits (AGRINOSTICS, Ltd. Co., Watkinsville, GA, U.S.).

Three tillers were cut off the plant and one 2 mm long cross section was taken from each tiller base. Cross sections were placed on the nitrocellulose membrane lying in a special container on the sponge and filter paper wetted with extraction buffer. The membranes were incubated for 18 h at 4°C and then dried for 15 min at 70°C. In the next step the membrane was blocked with BWW reagent and pooled monoclonal antibodies (MAB 4H2, 5C7 and 15D7) were added. After one-hour incubation the membrane was washed with BWW reagent and second specific antibodies (RAM – Rabbit anti-mouse antibody) were added. The membrane was washed again after incubation (1 h) and the protein-A with an alkaline phosphatase enzyme conjugate was added to complete the stacking effect. Then, after 30-min-long incubation, the chromogen solution (TRIS+Napthol+Fast Red Chromogen) was added. After about 15–20 min of incubation in dark a pink color developed wherever the membrane bounded endophyte specific proteins.

The method of leaf sheaths staining with rose Bengal [Saha et al. 1988] was additionally used in questionable situations. The inner epidermis of leaf sheath was peeled out and treated with a drop of the staining solution. Then, after about 1 min, the epidermis pieces were examined microscopically at 100–400× magnification. The presence of twisted unbranched intercellular hyphae proved infection of the genotype by the endophyte.

Analyses of ergovaline content in plants. Meadow fescue and red fescue genotypes marked as infected by N/E endophytes at the first stage of our study were screened for ergovaline presence in order to eliminate the genotypes with a high potential of this alkaloid production. In order to analyze the ergovaline content in plant material, the pot experiments were carried out as follows: plants were split into single tillers, planted into pots filled with sterilized peat substrate (Profi-Substrate, Gramoflor, Germany) and placed in a climate chamber at 22°C under a day/night cycle of 15h/9h and watered as needed for 6 weeks. Then four well developed plants of each genotype were selected, checked for endophyte presence (Phytoscreen™ Immunoblot Kit, staining

with rose Bengal), cut down and placed into the climatic chamber at 21–22°C under a day/night cycle of 15 h / 9 h and watered as needed for about 4 weeks. After that time the above-ground parts of the plants were harvested at full bloom, lyophilized, powdered in a laboratory mill, and taken for HPLC analyses. The chemical analyses of ergovaline content were carried out according to the modified method of Rottinghaus et al. [1991] described by Pańka [2011].

Isolation of *Neotyphodium* and *Epichloë* endophytes. Based on the ability of grass/endophyte associations to produce toxic ergovaline, there were selected genotypes with a low level (> 0.02 $\mu g \cdot g^{-1}$) of the alkaloid or not producing it at all. Potentially promising endophyte strains were isolated from selected genotypes. Ten pieces of stem, each 20-mm long, sampled from tiller bases of each genotype were divided into four 5-mm-long subsections and surface-sterilized in 75% ethanol for 2 min and in 10% sodium hypochlorite for 1 min. Then each subsection was rinsed three times for 2 min in sterile water, cut into small pieces which were placed on PDA (potato dextrose agar) medium containing 10 $\mu l \cdot cm^{-3}$ of dihydrostreptomycin sulfate and 10 $\mu l \cdot cm^{-3}$ of penicillin and incubated for 3–4 weeks at 22°C in darkness. Endophyte colonies were transferred onto the PDA medium and used for establishing dual culture assays.

Antagonistic properties of endophytes *in vitro*. Dual-culture assays were carried out according to the modified method by Christensen [1996]. The isolated strains of N/E endophytes were inoculated onto the centre of Petri dishes containing the PDA medium as 5-mm-diameter agar discs overgrown with endophytic mycelium. The plates were incubated in the dark at 25°C until the colonies reached about 10–15 mm in diameter. Then two 5-mm-diameter PDA discs overgrown with the test fungus were placed on the dishes with endophyte colony on the same diameter near the edges. The plates were incubated up to 14 days at 25°C in darkness. The control dishes contained only discs of the test fungus placed at the opposite ends of the dish. There were five reps of each endophyte/test fungus combination. The measurements of the growth inhibition zone width were taken when the two colonies in the control combination met in the centre of the Petri dish (fast-growing fungi) or after 14 days of incubation (slow-growing fungi). An average width of the growth inhibition zone calculated from 5 reps indicated the level of antagonistic properties of the endophyte strain.

The antagonistic activity of N/E endophytes was studied towards the common pathogens of grasses [Prończuk 2000]: *Bipolaris sorokiniana, Drechslera dictyoides*, *D. siccans, Fusarium avenaceum, F. culmorum, F. equiseti, F. poae, Microdochium nivale*, *Rhizoctonia solani*, and *Trichoderma viride*. All the test fungi were isolated earlier from roots, tillers, and leaves of the grasses.

Pot experiment. The strains of N/E endophytes selected to dual-culture assays were also screened for their potential to protect the host plant against pathogens *in vivo*. Pot experiment was established in the same way as described above for the analyses of ergovaline content. Endophyte-free plants for control combinations were derived from E+ clones by applying propiconazole (Bumper 250 EC, Makhteshim Chemical Works, Beer-Sheva, Israel) to plants in hydroponic culture in the greenhouse. Four well-developed healthy plants of each E+ and E– genotype were selected, checked for endo-

phyte presence [Phytoscreen™ Immunoblot Kit, staining with rose Bengal], placed into climatic chamber at 22°C under a day/night cycle of 15h/9h and inoculated with test pathogens: *Bipolaris sorokiniana, Drechslera* sp., *Fusarium poae, Rhizoctonia solani*. The inoculation material consisted of spores and hyphae fragments suspended in distilled water ($2.0 \cdot 10^6$ cfu per mL). The plants were inoculated with pathogens infection material using foliar spray. The plants in the control combination were sprayed with distilled water only. The pots were placed randomly into plastic containers and covered with a transparent foil cover, creating a tent that maintained high humidity and an almost continuous state of free moisture. The plants were watered as needed and misted twice a day. Ten days after inoculation the degree of tillers infestation was evaluated based on a five-degree scale where 0° stood for a lack of infection symptoms, 1° – single spots, 1–10% leaf area with infection symptoms, 2° – from 11 to 30% of the leaf-area infection, poor wilting, 3° – from 31 to 60% of the area with infection symptoms, clear wilting, 4° – above 60% of the area with disease symptoms, heavy wilting. The experiment was conducted in 4 reps (a single pot constitutes one rep). Thirty leaves (10 tillers × 3 leaves per tiller) per replication were sampled and scored. The degrees were transformed into disease index values (DI in %) according to the Townsend and Heuberger formula [Wenzel 1948]. Lower DI values stand for a higher plant resistance to infection by the pathogen. To confirm the identity of the causal agent, the pathogen was isolated from diseased leaves on the PDA medium.

The data were analyzed using the statistical package SAS 9.3. (SAS Institute, 2006––2010). The ANOVA model included meadow or red fescue genotype, endophyte status, and their interaction as a fixed factor in completely randomized design with four replicates, and the post ANOVA separation of means using Tukey's HSD test at $P < 0.05$ was used.

RESULTS

Occurrence of *Neotyphodium*/*Epichloë* endophytes in Poland. There were gathered 204 samples of meadow fescue and 171 samples of red fescue plants originated from different uncultivated habitats (tab. 1, 2). The level of infection was high. *Neotyphodium uncinatum* was detected in 152 plants of meadow fescue. Mean frequency of the infection of this species with endophyte was 74.5%. The lowest level (50%) was observed in the Kujawsko-Pomorskie province and the highest (85.7%) – in the Podlaskie province. A lower level of infection by an endophyte was observed in red fescue genotypes. The frequency of colonization of this grass species reached 64.3% and ranged between 33.3% in the Łódzkie province to 84.6% in the Dolnośląskie province.

Taking parts of Poland into consideration, a higher (81%) occurrence of endophyte infected meadow fescue ecotypes was noted in northern provinces, a lower infection (74.2%) in southern provinces and the lowest (62.1%) – in the central part of Poland. A similar occurrence was found for red fescue. A higher colonization (75%) was observed in northern part of Poland, lower (62.7%) – in southern Poland and the lowest (50%) – in central provinces.

We also found the Phytoscreen™ Immunoblot Kit to be a very efficient tool of N/E endophytes detection in plant material. However, in case of weak associations when an endophyte hyphae is scarce and grows slowly inside a plant tissue, the reliability of the result may be questionable and need to be confirmed by traditional staining with rose Bengal or aniline blue. There was a considerable number of such associations in our study.

Ergovaline production. Most of the grass/endophyte relationships studied were able to produce toxic ergovaline. We found about three quarters of meadow fescue/endophyte associations producing this alkaloid and over four fifths associations of red fescue contained the toxic metabolite (tab. 1, 2). In three provinces (Opolskie, Świętokrzyskie, Wielkopolskie) 100% of meadow fescue associations produced ergovaline and the lowest detected amounts of the alkaloid: 0.031, 0.021 and 0.011 μg·g DM^{-1} were recorded in those provinces, respectively. The lowest percentage (62.5%) of metabolite producing associations occurred in the Lubuskie and Podlaskie provinces. Thirty five meadow fescue associations did not contain any detectable amount of ergovaline and 32 produced the alkaloid in the amounts not exceeding 0.02 μg·g DM^{-1} (tab. 1).

Table 1. Occurrence of *Neotyphodium uncinatum* and ergovaline content in meadow fescue genotypes collected in different regions of Poland

Voivodeship	Number of collected genotypes	Endophyte infected genotypes/ Percentage	Ergovaline producing associations/ Percentage	Ergovaline content (μg·g DM^{-1})	
				mean	range (min.–max.)
Dolnośląskie	12	10/83.3	8/80.0	0.164	0.0–0.512
Kujawsko-Pomorskie	8	4/50.0	3/75.0	0.332	0.0–0.554
Lubuskie	11	8/72.7	5/62.5	0.076	0.0–0.282
Łódzkie	11	6/54.5	5/83.3	0.084	0.0–0.267
Małopolskie	31	24/77.4	18/75.0	0.139	0.0–3.675
Opolskie	10	7/70.0	7/100	0.198	0.031–0.523
Podkarpackie	15	10/66.7	8/80.0	0.174	0.0–0.342
Podlaskie	28	24/85.7	15/62.5	0.114	0.0–3.340
Pomorskie	25	20/80.0	16/80.0	0.295	0.0–1.324
Śląskie	14	11/78.6	8/72.7	0.269	0.0–1.323
Świętokrzyskie	11	7/63.6	7/100	0.342	0.021–1.089
Warmińsko-Mazurskie	21	16/76.2	12/75.0	0.367	0.0–2.347
Wielkopolskie	7	5/71.4	5/100	0.076	0.011–0.332
Total	204	152/74.5	117/77,0	0.202	

Ergovaline production in red fescue associations was lower when averaged for the provinces, as compared to meadow fescue and it was 0.151, 0.202 μg·g DM^{-1} respectively. The highest level of the metabolite (2.347 μg·g DM^{-1}) was observed in the Warmińsko-Mazurskie province. The mean content of the alkaloid ranged from 0.021 (Łódzkie) to 0.375 μg·g DM^{-1} (Warmińsko-Mazurskie). Twenty one red fescue/endophyte associations did not produce ergovaline. The low level of the toxin, <0.02 μg·g DM^{-1} was detected in 28 associations (tab. 2).

Table 2. Occurrence of *Epichloë festucae* and ergovaline content in red fescue genotypes collected in different regions of Poland

Voivodeship	Number of collected genotypes	Endophyte infected genotypes/ Percentage	Ergovaline producing associations/ Percentage	Ergovaline content (μg·g DM^{-1})	
				mean	range (min.–max.)
Dolnośląskie	13	11/84.6	9/81.8	0.243	0.0–1.013
Kujawsko-Pomorskie	7	4/57.1	4/100	0.133	0.021–0.211
Lubuskie	10	6/60.0	6/100	0.143	0.015–0.311
Łódzkie	9	3/33.3	3/100	0.021	0.011–0.134
Małopolskie	25	16/64.0	15/93.7	0.135	0.0–0.243
Opolskie	7	3/42.9	3/100	0.097	0.021–0.110
Podkarpackie	16	10/62.5	7/70.0	0.199	0.0–0.454
Podlaskie	20	14/70.0	12/85.7	0.254	0.0–0.876
Pomorskie	16	12/75.0	10/83.3	0.112	0.0–1.211
Śląskie	11	5/45.5	3/60.0	0.067	0.0–0.123
Świętokrzyskie	9	6/66.7	5/83.3	0.145	0.0–0.389
Warmińsko-Mazurskie	20	16/80.0	10/62.5	0.375	0.0–2.356
Wielkopolskie	8	4/50.0	2/50.0	0.037	0.0–0.061
Total	171	110/64.3	89/80.9	0.151	

Endophyte infected genotypes, both meadow and red fescue, collected in the northern part of Poland contained the highest mean amount of ergovaline: 0.259 and 0.247 μg·g DM^{-1}, respectively; followed by southern Poland: 0.214, 0.148 μg·g DM^{-1}, while the central part reported the lowest concentration: 0.142, 0.084 μg·g DM^{-1}, respectively.

Dual-culture assays. Based on the ergovaline content in the grass genotypes studied and on our own observations, we selected 10 meadow fescue genotypes and 7 red fescue genotypes for endophyte isolation, *in vitro* tests and for the pot experiment. We have found a substantial number of associations to be weak, although they did not produce ergovaline. Endophytic symbiont grew slowly inside a plant tissue, mycelium was sparse and not all the plant tillers were infected. Such symbiotic relationships were eliminated from further tests.

We observed the highest antibiotic activity (>7.0 mm) for FpII30, FpII67, FpII98 and FpII168 *N. uncinatum* endophyte isolates originated from meadow fescue and for FrII82, FrII134 *E. festucae* isolates originated from red fescue genotypes (tab. 3). Most often and to the highest extent the growth of the following test fungi was inhibited by the endophyte isolates: *B. sorokiniana, D. dictyoides, F. avenaceum, F. equiseti, R. solani* (for meadow fescue genotypes) and *B. sorokiniana, D. dictyoides, D. siccans, F. avenaceum, R. solani* (for red fescue genotypes). *Trichoderma viride*, known as an antagonistic fungus for many pathogens, was inhibited least by all the endophyte isolates tested.

Table 3. Inhibition of growth of test fungi by *Neotyphodium uncinatum* and *Epichloë festucae* isolates *in vitro*

	No.	Code of association	Ergovaline content	Mean growth inhibition zone of test fungi (mm)										Mean
				test fungi										
				B. s.[1]	*D. d.*	*D. s.*	*F. a.*	*F. c.*	*F. e.*	*F. p.*	*M. n.*	*R. s.*	*T. v.*	
Meadow fescue	1.	FpII7	0.010	18.4	6.7	5.7	7.8	7.5	8.5	4.5	0.0	0.0	2.0	6.1
	2.	FpII30	0.0	24.2	7.4	9.3	6.9	5.7	10.2	6.5	5.9	10.5	1.0	8.8
	3.	FpII34	0.0	10.3	8.9	5.3	8.9	2.8	6.9	5.3	6.6	3.5	0.0	5.9
	4.	FpII56	0.0	12.5	10.6	4.8	4.3	4.6	9.7	0.0	5.8	5.6	1.4	5.9
	5.	FpII67	0.016	18.6	12.4	9.2	12.5	9.5	12.7	4.2	3.8	18.5	0.0	10.1
	6.	FpII77	0.0	10.4	7.5	5.8	6.7	2.2	6.8	5.4	0.0	2.4	0.0	4.7
	7.	FpII98	0.0	20.9	5.8	6.5	7.8	8.7	9.8	2.2	3.5	7.7	2.2	7.5
	8.	FpII120	0.0	17.5	6.7	5.3	2.6	9.7	6.7	3.2	0.0	8.7	1.2	6.2
	9.	FpII167	0.012	18.2	8.8	5.6	7.5	0.0	8.3	0.0	2.5	0.0	1.5	5.2
	10.	FpII168	0.0	22.3	13.5	10.4	8.4	7.5	9.2	6.4	7.5	12.6	0.0	9.8
	Mean			17.3	8.8	6.8	7.3	5.8	8.9	3.8	3.6	7.0	0.9	
Red fescue	1	FrII45	0.0	9.6	5.5	3.4	6.6	3.1	4.3	6.7	4.6	0.6	1.0	4.5
	2	FrII67	0.012	14.5	3.4	4.3	0.0	0.0	5.4	7.3	6.3	3.1	0.0	4.4
	3	FrII69	0.0	13.8	4.2	5.5	0.0	8.3	0.0	7.4	0.0	2.3	0.0	4.2
	4	FrII78	0.013	15.7	1.6	10.4	2.4	7.4	3.5	6.6	3.5	3.5	0.8	5.5
	5	FrII82	0.0	20.3	8.6	9.6	6.4	6.7	8.9	7.3	7.9	10.6	2.0	8.8
	6	FrII134	0.0	18.4	8.9	7.5	7.8	6.5	5.7	5.7	4.3	9.8	1.0	7.6
	7	FrII152	0.0	4.6	9.0	0.0	9.7	5.6	0.0	4.6	0.0	4.5	1.6	4.0
	Mean			13.8	5.9	13.8	5.9	5.8	4.7	5.4	4.0	6.5	3.8	

[1] Test fungi: *B.s .– Bipolaris sorokiniana, D.d. – Drechslera dictyoides, D.s. – D. siccans, F.a. – Fusarium avenaceum, F.c. – F. culmorum, F.e. – F. equiseti, F.p. – F. poae, M.n. –Microdochium nivale, R.s. – Rhizoctonia solani, T.v. – Trichoderma viride.*

Table 4. Susceptibility (Mean DI in %) of endophyte infected (E+) and non-infected (E-) meadow fescue genotypes to infection by test fungi

	Endophyte status	Meadow fescue/Endophyte association										Mean
		FpII7	FpII30	FpII34	FpII56	FpII67	FpII77	FpII98	FpII120	FpII167	FpII168	
Bipolaris sorokiniana	E+	20.0aAB	15.1aA	20.5aAB	24.6aB	13.4aA	14.2aA	22.7aB	24.4aB	25.7aB	14.9aA	19.5a
	E-	28.1bABCD	45.4bE	27.4bABCD	23.6aAB	33.6bD	33.1bCD	21.3aA	30.8bBCD	25.9aABC	24.3bAB	29.3b
	mean	24.0ABC	30.3D	24.0ABC	24.1ABC	23.5ABC	23.7ABC	22.0AB	27.6CD	25.8BCD	19.6A	
Drechslera sp.	E+	25.6a	9.9a	22.8a	15.2a	6.8a	7.4a	9.2a	39.3a	31.7a	14.9a	18.3a
	E-	30.2bA	58.9bF	31.6bAB	40.7bCDE	45.8bE	39.5bCDE	38.2bBCD	39.3aCDE	38.1bBCD	37.9bBC	40.0b
	mean	27.9A	34.4B	27.2A	27.9A	26.3A	23.4A	23.7A	39.3B	34.9B	26.4A	
Fusarium poae	E+	51.2aF	31.5aBC	41.5aDE	38.3aCD	26.2aAB	29.4aABC	48.3aEF	65.6aG	45.4aDEF	20.9aA	39.8a
	E-	56.3aCD	82.1bF	48.9bBC	38.8aA	79.8bEF	59.3bD	44.5aAB	70.9aE	50.1aBCD	54.6bCD	58.5b
	mean	53.8EF	56.8F	45.2B	38.5AB	53.0DEF	44.3ABC	46.4CD	68.2G	47.8CDE	37.7A	
Rhizoctonia solani	E+	51.0aDE	17.9aA	45.7aCD	35.8aB	21.1aA	45.2aCD	54.9aE	44.2aBCD	42.2aBC	24.1aA	38.2a
	E-	50.0aBC	66.9bE	48.0aB	39.1aA	65.5bDE	66.0bDE	57.7aCD	47.9aB	44.4aAB	45.8bAB	53.1b
	mean	50.5DE	42.4BC	46.8CD	37.5AB	43.3BC	55.6E	56.3E	46.1CD	43.3BC	35.0A	

* Means marked with different capital letters in rows and lower case letters in columns differ significantly at $P < 0.05$

Table 5. Susceptibility (Mean DI in %) of endophyte infected (E+) and non-infected (E-) red fescue genotypes to infection by test fungi

	Endophyte status	Red fescue/Endophyte association							Mean
		FrII45	FrII67	FrII69	FrII78	FrII82	FrII134	FrII152	
Bipolaris sorokiniana	E+	34.2aC	44.6aD	22.4aB	34.8aC	13.4aA	46.1aD	41.9aCD	33.9a
	E-	36.3aB	44.8aC	22.1aA	33.5aB	45.8bCD	44.8aC	52.0bD	39.9b
	mean	35.2B	44.7C	22.3A	34.1B	29.6B	45.4C	47.0C	
Drechslera sp.	E+	37.0aD	32.6aCD	29.1aBCD	25.0aABC	19.8aA	23.9aA	35.0aD	28.9a
	E-	36.4aA	36.0aA	34.3aA	34.7bA	59.0bB	34.9bA	55.1bB	41.5b
	mean	36,7BC	34,3ABC	31,7AB	29,8A	39,4CD	29,4A	45,0D	
Fusarium poae	E+	25.6aA	36.9aB	39.8aBC	46.0aC	25.8aA	35.7aB	24.2aA	33.4a
	E-	29.9aAB	38.2aB	42.7aCD	52.9bE	46.8bDE	38.0aBC	26.4aA	39.3b
	mean	27.7A	37.5B	41.3BC	49.5C	36.3B	36.8B	25.3A	
Rhizoctonia solani	E+	21.0aAB	49.1aD	25.7aAB	36.8aC	19.3aA	55.7aD	29.0aBC	33.8a
	E-	25.1aA	55.2bD	44.7bBC	42.9bB	56.5bD	53.4aCD	35.8aB	44.8b
	mean	23.0A	52.1D	35.2BC	39.8C	37.9BC	54.6D	32.4B	

* Means marked with different capital letters in rows and lower case letters in columns differ significantly at $P < 0.05$

Pot experiment. All the meadow fescue and red fescue associations were susceptible to infection by the pathogens tested in a pot experiment (tab. 4, 5). However, the extent of susceptibility was different for individual genotypes and also differed depending on the pathogen. A significant influence of the endophyte on the host plant resistance was also observed when averaged over plant genotypes, however, not for all the individuals.

It was FpII30E meadow fescue genotype which was least resistant to infection by all the pathogens. The most severe symptoms were noted for this genotype in combination with *F. poae*. The highest decrease in diseases severity due to endophyte presence was observed also for FpII30. The high protective influence of the endophyte against all the pathogens tested was also noted for the following genotypes: FpII67, FpII77 and FpII168. The endophyte of FpII34 genotype guaranteed a resistance to the host against 3 pathogens: *B. sorokiniana, Drechslera* sp. and *F. poae*. The other genotypes were protected against two or one pathogen only (tab. 4).

The protective influence of the endophyte on red fescue genotypes was weaker and less frequent, as compared to meadow fescue genotypes. The only exception was FrII82, association with complete and very high protection of the endophyte against all the pathogens tested. Endophyte also increased resistance of FrII78 genotype against three pathogens: *Drechslera* sp., *F. poae* and *R. solani*, however, not against *B. sorokiniana*. Other red fescue genotypes were protected by the endophyte against two pathogens (FrII152), one pathogen (FrII67, FrII134) or they did not have any protective influence at all (FrII45). *Drechslera* sp. and *R. solani* were the pathogens most frequently inhibited by the endophytes of red fescue (tab. 5).

DISCUSSION

Meadow fescue and red fescue are grass species of high economic importance in European countries. The first one is generally used for pasture and foraging and the second is one of the key turf grass species. Like many other grasses, meadow and red fescues can be infected by endophytic microorganisms [Guillaumin et al. 2001, Schardl 2001, Lembicz et al. 2010, Gundel et al. 2011, Wiewióra 2011, Żurek et al. 2012], with the most important ones being fungi of genera *Neotyphodium* and *Epichloë*. Leuchtmann [1992] reports on 290 grass species being infected by N/E endophytes and about 20–30% of all grasses may be the hosts of these fungi. In a French study 237 species of European grasses were examined for the endophyte presence and 22 were found to be infected [Leyronas and Raynal 2001]. Also Zabalgogeazcoa et al. [2003] report on the frequency of endophyte infected grass species in semiarid grassland ecosystems in western Spain to account for 22%. *Lolium* and *Festuca* species are often noted to show a high infection. Our results confirm a high level of endophyte infections of these species. We have detected mycelium of the endophyte in 74.5% genotypes of meadow fescue and in 64.3% of red fescue genotypes. The identical results for meadow fescue were recorded by Wiewióra [2011]. However, the author detected endophyte in red fescue to account for 40.8%. In a study by Lewis [1994], colonization of fescues was very high, as 77% of sampled sites for red fescue and 100% for meadow and tall fescue

contained an endophyte. Range of colonization was also high: 0–100% (red fescue) and 96–100% (meadow and tall fescue).

The higher the infection level of plants by N/E fungi, the more dangerous the grass may be for animals due to the toxins produced. Most of endophytic fungi are known to produce toxic alkaloids in infected plants, as claimed by Leuchtmann [1992]. Although, natural grass communities in Europe are often infected by N/E endophytes, there is no clear evidence on the threat posed to animals from these toxins, which must be due to a high botanical diversity of permanent pastures in Poland and other European countries [Zabalgogeazcoa and Bony 2005]. Thus the effect of dilution of toxins produced only by few species in a grassland community may occur. In our study, the endophytes of meadow and red fescues produced quite high amounts of toxic ergovaline when averaged. Additionally, the percentage of the alkaloid producing isolates was also high: 77% for meadow fescue and 80.9% for red fescue. In a study by Wiewióra [2011], the number of ergovaline producing isolates was lower: 61.2% and 32.8%, respectively. The author reported also a lower mean and maximum content of the alkaloid in both grasses. Higher amounts of ergovaline were detected by other authors. The following maximum contents were recorded for individual grass species: perennial ryegrass – 1.71 $\mu g \cdot g^{-1}$ [Żurek et al. 2010a, 2010b], 4.65 $\mu g \cdot g^{-1}$ [Cagaš et al. 1999]; tall fescue – 33.0 $\mu g \cdot g^{-1}$ [Dahl Jensen et al. 2007]; meadow fescue –2.297 $\mu g \cdot g^{-1}$ [Podkówka et al. 2011], 4.836 $\mu g \cdot g^{-1}$ [Pańka et al. 2011]; red fescue – 0.54 $\mu g \cdot g^{-1}$ [Żurek et al. 2010a, 2010b], 1.40 $\mu g \cdot g^{-1}$ [Leuchtmann 1992].

The production of ergovaline may vary in a wide range. The level of the alkaloid depends especially on the genotype of the host plant, the endophyte genotype and environmental conditions [Vázquez de Aldana et al. 2010]. Animal toxicoses can occur when the ergovaline content ranged from 0.2 to 0.4 $\mu g \cdot g^{-1}$ in fresh forage [Bony and Delatour 2001]. The toxic effect may dramatically increase when ergovaline producing cultivars are cultivated as artificial monospecies stands [Kim et al. 2007]. Generally, the high frequency of grass species infected by endophytes may indicate a selective pressure favoring the infected over non-infected grasses due to ergovaline toxic effects on herbivores and insects [Leuchtmann 1992]. However, from the agronomic point of view, ergovaline production is undesirable, but other alkaloids (e.g. peramine, janithrems, lolines), serving protection of the host against insect pests, nematodes and pathogens should be synthesized. Although, quite high antibiotic activity of N/E endophytes *in vitro* is well known and documented [Christensen 1996, Pańka 2005, 2008] the influence of an endophyte *in situ* is not often visible to the same extent and *vice versa* [Christensen 1996, Wäli et al. 2006]. In our study, isolate FrII134 showed high activity in dual culture assay, however, it did not exhibit this activity in the pot experiment. Another endophyte isolate FpII77 demonstrated high protective effect to the host in the pot experiment but it was not distinguished *in vitro*. Hence, selecting endophytes as potential biological agents, one should not only rely on *in vitro* tests. The differences between laboratory and pot experiments indicates the existence of other mechanisms responsible for protective influence of endophytes against pathogens, such as the production of phenolic compounds, PR proteins and volatile organic compounds [Pańka et al. 2013a].

Some N/E endophyte isolates can exhibit a very high level of protection of the host plant against pathogens. In our study, 4 meadow fescue and 1 red fescue endophytes increased resistance of the host plant against all the pathogens tested. The literature data confirm a beneficial effect of endophytes on the resistance of the plants to many pathogens, such as *Fusarium oxysporum* causing damping-off in Arizona fescue [Reddy and Faeth 2010], *Sclerotinia homeocarpa* triggering dollar spot disease [Clarke et al. 2006], *Rhizoctonia zeae* infecting tall fescue [Gwinn and Gavin 1992, Pańka et al. 2013b], *Fusarium poae* infecting perennial ryegrass [Pańka et al. 2013a]. There is also evidence that endophytes have no effect on or even decrease plants resistance to pathogens [Burpee and Bouton 1993, Schmidt 1994, Wäli et al. 2006, Pańka et al. 2011, Pańka et al. 2013a, 2013b]. With that in mind, the selection of the N/E endophytes for biological protection of grasses calls for a detailed study of each individual association in order to prove the beneficial influence of the symbiont on the host plant against biotic and abiotic factors.

Resistance to abiotic stresses of the endophyte isolates selected in our study will be further researched.

CONCLUSIONS

1. The colonization of meadow fescue and red fescue genotypes collected in Poland by N/E endophytes is very high and it accounts for 74.5% and 64.3%, respectively.

2. A high percentage of endophytes living in associations with meadow fescue and red fescue in Poland show an ability to produce ergovaline; sometimes to a very high extent.

3. FpII30, FpII67, FpII98 and FpII168 endophytes isolated from meadow fescue and FrII82, FrII134 from red fescue exhibit high antifungal activity against *B. sorokiniana, D. dictyoides, D. siccans, F. avenaceum, F. equiseti, R. solani in vitro.*

4. FpII30, FpII67, FpII77 and FpII168 endophytes isolated from meadow fescue and FrII82 from red fescue demonstrate a high ability for protection of the host grasses from infection by *B. sorokiniana, Drechslera* sp., *Fusarium poae* and *Rhizoctonia solani.*

5. Selected endophyte isolates FpII30, FpII67, FpII77, FpII168 and FrII82 due to high antimicrobial activity and a lack of ergovaline production have a great potential for biological control of grasses and will be studied further. After additional tests, they could be used as biological factors for improving the utility value of new grass varieties and their resistance to stress factors.

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WYSTĘPOWANIE GRZYBÓW Z RODZAJU *Neotyphodium* I *Epichloë* W KOSTRZEWIE ŁĄKOWEJ I KOSTRZEWIE CZERWONEJ W POLSCE ORAZ SELEKCJA ENDOFITÓW JAKO POTENCJALNYCH CZYNNIKÓW BIOLOGICZNEJ OCHRONY TRAW

Streszczenie. Endofity z rodzaju *Neotyphodium* i *Epichloë* często wpływają pozytywnie na roślinę żywicielską, dlatego mogą być wykorzystane jako biologiczny czynnik poprawiający wzrost traw oraz ich odporność na czynniki stresowe. Szczegółowe badania podjęto w celu: (i) określenia zasiedlenia genotypów kostrzewy łąkowej i kostrzewy czerwonej występujących w naturalnych zbiorowiskach trawiastych w Polsce przez endofity z rodzaju *Neotyphodium*/*Epichloë*, (ii) określenia poziomu ergowaliny, toksycznego alka-

loidu produkowanego przez aktywne asocjacje, (iii) określenia aktywności antagonistycznej wyróżniających się izolatów grzybów endofitycznych w stosunku do wybranych mikroorganizmów. Badano 204 genotypy kostrzewy łąkowej i 171 genotypów kostrzewy czerwonej. Średnie zasiedlenie badanych gatunków traw przez endofity wynosiło odpowiednio 74,5% i 64,3%. Ergowalinę produkowało 77% zasiedlonych genotypów kostrzewy łąkowej i 80,9% genotypów kostrzewy czerwonej. Średnia zawartość alkaloidu wynosiła odpowiednio 0,202 i 0,151 $\mu g \cdot g\ DM^{-1}$. Izolaty FpII30, FpII67, FpII77, FpII168 i FrII82 wysoce skutecznie chroniły roślinę żywicielską przed wszystkimi testowymi patogenami. Izolaty te, ze względu na swoją dużą aktywność antygrzybową oraz brak zdolności produkowania ergowaliny mogą zostać wykorzystane do biologicznej ochrony traw.

Słowa kluczowe: Endofit, ergowalina, testy płytkowe, biologiczna ochrona traw

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