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Neotyphodium / *Epichloë* ENDOPHYTES OF PERENNIAL RYEGRASS, MEADOW FESCUE AND RED FESCUE CULTIVARS CULTIVATED IN POLAND

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Abstract. Grasses are very often infected by endophytic fungi of the *Neotyphodium* and *Epichloë* genera. They can produce metabolites toxic to livestock as well as beneficially affect grasses. Thus, endophytes can be used for plants improvement. These facts stimulated a more detailed investigation aimed at providing more insight into the problem of these endophytes in Poland. The aims of the research were as follows: (i) to determine the levels of colonization of Polish-grown cultivars of perennial ryegrass, meadow fescue, and red fescue with endophytic fungi of the genera *Neotyphodium* and *Epichloë*; (ii) to determine the potential hazards associated with the toxins produced by the active associations; (iii) to isolate viable endophytes of the genera *Neotyphodium* and *Epichloë* from active associations; and (iv) to determine the levels of antagonistic activity of the obtained endophyte isolates against selected microorganisms, in particular serious grass pathogens, *in vitro*. The staining method was used for endophytes detection in grass seed collection. Endophytes were isolated on PDA medium and characterized. Antagonistic activity of N/E isolates and seed extracts were determined in dual culture assays and on microscope slides respectively. Ergovaline and lolitrem B content were assayed. There were 242 samples of seeds of 50 cultivars of grasses collected in Poland, including 124 samples of 20 cultivars of perennial ryegrass, 61 samples of 10 cultivars of meadow fescue, and 57 samples of 20 cultivars of red fescue. Endophytic fungi were only detected in 33 seed samples of 5 perennial ryegrass cultivars and in 15 samples of 2 meadow fescue cultivars. The levels of seed colonization of these species reached maximum 8 and 90%, respectively. No colonization by endophytes was found in seeds of red fescue. The viability of endophytic mycelium in the seeds of perennial ryegrass and meadow fescue was very low. Endophytic fungi were isolated only from 3 and 4 samples of these grasses respectively. In laboratory conditions, endophytic fungi showed stronger antagonistic properties at 25°C. The highest and most frequent growth inhibition was observed for *M. nivale, Rhizoctonia solani, Fusarium avenaceum* and *F. equiseti.* The obligate pathogens *B. graminis*, *P. coronata*, and *P. graminis* ssp. *graminicola* were not sensitive to water and alcohol extracts of E+ seeds. Isolates Fp28, Fp40, and, to a lesser degree, Fp37, originating from meadow fescue, were characterized by the strongest antagonistic properties. Moreover,

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they did not produce (Fp28 and Fp37), or produced only small amounts (Fp40) of, ergovaline, which is toxic to animals. 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.

Key words: *Lolium perenne, Festuca pratensis,* pathogens, ergovaline, lolitrem

INTRODUCTION

Grasses are plants which in their evolution have developed a number of symbiotic associations. These include endo- and ectomycorrhizal relationships and mutualistic associations with endophytic fungi [Bacon and De Battista 1991, Smith and Read 1997]. Endophytes only grow systemically inside of tillers, penetrating plant tissues intercellularly without causing any symptoms that would indicate their presence. Among the best-studied grass endophytes are fungi of the family *Clavicipitaceae* (Sordariomycetes) [Marcinkowska 2010], within which seven genera are currently recognized [White 1994, White and Reddy 1998]. Morgan-Jones and Gams [1982] have additionally erected the section *Albo-lanosa*, placing in it the genus *Acremonium*, which is an imperfect, anamorphic state of *Epichloë* spp. that colonizes grasses of the subfamily *Pooideae* [Siegel 1993]. This genus also contains numerous saprotrophs. To separate the endophytic *Acremonium* spp. (e-endophytes) from the remaining species, Glenn et al. [1996] have introduced a new genus – *Neotyphodium*. The most important endophytes from this genus are: *Neotyphodium lolii* (Latch, Christensen, and Samuels) Glenn, Bacon and Hanlin which infects perennial ryegrass (*Lolium perenne* L.), *N. uncinatum* (Gams, Petrini and Schmidt) Glenn, Bacon and Hanlin the symbiont of meadow fescue (*Festuca pratensis* Huds.) and *N. coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin – the tall fescue (*Festuca arundinaceae* Schreb.) endophyte. Beside those already mentioned, another important endophytic species is *Epichloë festucae*, which occurs mainly in red fescue [Schardl 2001]. With respect to development and effect on the host plant, it closely resembles fungi of the genus *Neotyphodium*.

Interest in endophytes results primarily from the fact that they produce in the colonized plant, substances which are toxic to livestock. Among the best-characterized of those toxins are ergovaline, ergovalinine, ergotamine and lolitrem B, which may cause diseases, e.g., fescue toxicosis, ryegrass staggers, that can even lead to an animal's death [Heeswijck van and Mc Donald 1992, Porter and Thompson 1992, Thompson and Stuedemann 1993]. These diseases are well known especially in the United States, Australia, and New Zealand, where they can annually cause large losses in livestock production, estimated at nearly a billion dollars per year [Hoveland 2000]. In Europe, according to the current state of knowledge, endophytic fungi do not cause such large losses. Apart from their negative impact, endophytic fungi can also exert a beneficial effect on grasses. They stimulate growth and development of the host plant, affect its tillering, contribute to its dark green color, and enhance its persistence and competitiveness relative to uncolonized grasses, especially in adverse environmental conditions.

Prończuk and Prończuk [2000], in a study comparing the general esthetic aspect of American cultivars of red fescue, Jamestown (without an endophyte) and Jamestown II (infected with an endophyte), to those of the European cultivars Nimba and Lifalla, found Jamestown II to be superior to the remaining ones in the winter and spring seasons. The positive effects of endophytes are mainly a consequence of the presence of certain chemical compounds produced under their influence in the plant. The best-characterized of these are peramine and loline compounds. It is those substances that are believed to be the major elements responsible for enhanced resistance of host plants to various biotic and abiotic stress factors. Peramine deters various pests from feeding on colonized plants [Johnson et al. 1985]. Loline compounds, on the other hand, are credited with multidirectional action that, among others, reduces feeding by pests and take part in drought stress tolerance mechanisms [Bush et al. 1993]. In grassland communities not used for pasture or forage, there is a very positive aspect to the presence of endophytes. In particular, in intensively used turf-type grasses, e.g., sports grounds, symbiosis of grasses with endophytic fungi is highly desirable. Positive effects can also be observed in the presence of these fungi in plantations of grasses cultivated for seed and forage, which are often attacked by numerous pathogens and pests. Use of endophytes could reduce the costs associated with plant care and protection. However, their application, especially in forage grass cultivation, is hindered by the threat they pose to farm animals. These facts have stimulated a more detailed investigation aimed at providing more insight into the problem of endophytes of the genera *Neotyphodium/Epichloë* in Poland. Previous studies in this area, conducted in the Department of Phytopathology and Molecular Mycology of the University of Technology and Life Sciences in Bydgoszcz, and in other research centers in Poland show that further investigations are necessary [Pańka and Łukanowski 2000, Pańka and Sadowski 2002, Wiewióra et al. 2006, 2007]. Therefore, a study was conducted with the following aims: (i) to determine the levels of colonization of Polish-grown cultivars of perennial ryegrass, meadow fescue, and red fescue with endophytic fungi of the genera *Neotyphodium* and *Epichloë*; (ii) to determine the potential hazards associated with the toxins produced by the active associations; (iii) to isolate viable endophytes of the genera *Neotyphodium* and *Epichloë* from active associations; and (iv) to determine the levels of antagonistic activity of the obtained endophyte isolates against selected microorganisms, in particular serious grass pathogens, *in vitro*.

MATERIALS AND METHODS

Detection and isolation of *Neotyphodium* and *Epichloë* endophytes. To collect the largest possible number of seed samples of the cultivars of perennial ryegrass, meadow fescue, and red fescue registered in Poland, seeds were obtained from breeders, grass seed production and trading companies, and Seed Testing Laboratories of Voivodeship Inspectorates of Plant Health and Seed Inspection in the years 2004–2009. Mycelium of endophytes from the genera *Neotyphodium* and *Epichloë* were detected by staining the seeds with rose Bengal [Saha et al. 1988]. The analysis was performed using 100 seeds each from the different batches. The stained samples were examined for

the presence of endophyte mycelium under a microscope at 200–400× magnification after previous crushing of the caryopses on a glass slide.

To isolate endophytes from the plant material, 20 seeds from each colonized samples were collected and sown in pots with chemically sterilized peat substrate (Profi-Substrate, Gramoflor, Germany). After 4 weeks of growth in a climate chamber under a day/night cycle of 15 h/9 h, the tillers were sampled and tested for the presence of endophytes using rose Bengal staining. Four, the most external but living leaf sheaths were used. The inner epidermis was peel out and treated with drop of staining solution and after about 1 min examined microspocically (100–400×). The presence of characteristically twisted, unbranched, intercellular hyphae proved that endophyte is alive and can be isolated from the host plant. Isolations were made as follows. Ten, 20-mm-long pieces of tiller bases from each pot were sampled and divided into 4 5-mm-long parts. Then, sterilized in 75% ethanol for 2 min and 10% sodium hypochlorite for 1 min, rinsed in sterile water three times for 2 min. After sterilization, each part was further divided into small pieces which were placed on plates with PDA medium (Potato Dextrose Agar, Difco, supplemented with 10 $\mu l \cdot cm^{-3}$ of streptomycin and 10 $\mu l \cdot cm^{-3}$ of penicillin) and incubated for 3–4 weeks at 22°C in darkness.

Characteristics of endophyte isolates. The basic macro- and microscopic characteristics of the obtained isolates of endophytic fungi were determined, and the rate of their linear growth was investigated. Observations were carried out in cultures growing in Petri dishes on PDA medium at pH 6.5. Disks of PDA, 5 mm in diameter, overgrown with endophyte mycelium were inoculated in the center of the plates. Then, they were incubated in a thermostat at 24°C for 28 days without exposure to light. After that time, the colony growth rate was estimated by measuring the cultures in two perpendicular directions. Also analyzed were the color and structure of the mycelium and the presence and size of conidia. The experiment was done in 5 replicates, with one plate in each. Data of growth rate of endophyte isolates were subjected to analysis of variance using PROC GLM in SAS® 9.2. (SAS® Institute, Inc., Cary, NC, USA). The model included seven endophyte isolates with 5 replications in a completely randomized design. Means were separated using Tukey's HSD test at α level of 0.05.

Antagonistic properties of endophytes *in vitro*. Dual-culture assays. Plate tests were carried out according to the modified method of Christensen [1996]. Isolates of endophytic fungi were inoculated onto 20 Petri plates containing PDA medium. After about 3–4 weeks of mycelial growth, 5-mm-diameter agar discs overgrown with endophytic mycelium were cut out. The disks were transferred to the center of the Petri plates containing PDA medium. The plates were incubated at 25°C in the dark until the colony diameter reached about 10–15 mm. After that time, antagonistic activity of the endophytes against test fungi was determined by placing 5-mm-diameter PDA discs overgrown with hyphae of the test fungus on each plate. The disks were taken from the margin of an actively growing colony and were placed at opposite ends of the plate, close to its edge. Experiments were done in 5 replicates (plates) for each test organism. In a control treatment, the test organism without the endophyte was placed on the plate. The plates were incubated in the dark for 14 days at 10°C and 25°C. Zones of inhibited growth of the test microorganisms were measured when fungal colonies growing on the control plate met in the central part of the plate or after 14 days of incubation. The an-

tagonistic activity of the endophyte isolates was investigated towards to test microorganisms: *Bipolaris sorokiniana, Drechslera dictyoides*, *D. siccans, Fusarium avenaceum, F. culmorum, F. equiseti, F. poae, Microdochium nivale*, *Rhizoctonia solani* and *Trichoderma viride*. The fungi had been isolated earlier from fragments of roots, tillers, and leaves of grasses collected from experimental plots of the Research Station of the University of Technology and Life Sciences, in Mochełek. Growth inhibition data were subjected to analysis of variance using PROC GLM in SAS® 9.2. (SAS® Institute, Inc., Cary, NC, USA). The model included seven endophyte isolates and ten test microorganisms with 5 replications in a completely randomized design. Means were separated using Tukey's HSD test at α level of 0.05.

Tests on extracts. The antagonistic properties of the isolated endophytes against obligate parasites as well as other pathogens tested earlier on Petri plates were additionally investigated in a series of glass slide tests according to the method of Holzmann-Wirth et al. [2000]. The effect of water and methanol extracts of seed samples colonized (E+) with living endophyte mycelium on germination of spores or hyphae fragments of the test fungi was analyzed. The experiments were carried out as follows: lyophilized seed samples were ground in a laboratory mill, then 1 g of the material was collected and 20 ml of an extraction solution, methanol (95%) or distilled water was added to it. Extraction was carried out for 1 h on an orbital shaker (120 rpm). Next, the extracts were filtered through filter paper, evaporated and redissolved in 2 ml of the extraction solution. Next, 50 µl of the obtained solution was withdrawn, placed on a glass slide, and evaporated to dryness. Ten µl each of the suspensions of spores and/or hyphae fragments of the test fungi was placed on and next to (control) the resulting residue, and then icubated in a humid chamber for 12 h at room temperature. After that time, the degree of inhibition of germination and growth of spores and/or hyphae was analyzed under a microscope.

Analyses of toxins in plants. In order to analyze the toxins in plant material, pot experiments were carried out as follows: 5 seeds from each sample colonized by the endophyte were sown into pots filled with sterilized peat substrate (Profi-Substrate, Gramoflor, Germany). The pots were placed in a climate chamber at 22°C under a day/night cycle of 15h/9h and watered as needed for 6–8 weeks. After about 4 weeks of growth, the plants were sampled and tested for the presence of endophytes using rose Bengal staining Saha et al. [1988]. Uncolonized plants (E-) were removed from the pots. The above-ground parts of the plants were harvested at full bloom, lyophilized, powdered in a laboratory mill, and then taken for HPLC analyses. Seed samples were also lyophilized and milled.

Ergovaline analysis. Chemical analyses were carried out according to modified Rottinghaus et al. [1991] method as follows. Five cm^3 20% acetic acid and 1 µg ergotamine as an internal standard were added to 0.2 g dry samples and intensively stirred using a vortex stirrer. The samples were then placed for 12 h at ca. 4 ^{0}C and stirred every hour. After that time, the samples were centrifuged, and 3 cm^3 of the extract was applied to a solid phase extraction column containing 300 mg of C18 packing material. Before use, the columns were preconditioned by washing with 3 cm^3 of methanol followed by 4 cm^3 of deionized water. After passing the extract through the column, the latter was rinsed with 2 cm^3 of acetic acid previously used for extraction, and then with

4 cm^3 of deionized water. The toxins adsorbed in the packing material were eluted using three portions (1 cm^3 total volume) of ammonia solution (0.04%) in methanol. After stirring, the samples were immediately analyzed using a Perkin-Elmer Series 200 high-performance liquid chromatography system, equipped with a fluorescence detector. The excitation wavelength was set at λ_{ex} = 235 nm, and the emission wavelength was λ_{em} = 415 nm. Chromatographic separation was performed on an Alltech Komasil C18 column (150 mm). Elution was carried out isocratically using methanol and 0.1 $M \cdot dm^3$ ammonium acetate at a volume ratio of 3:1. Ergovaline concentration was calculated on the basis of peak area, taking into account recovery, determined separately for each sample on the basis of the concentration of the internal standard, ergotamine. Because pure ergovaline was not available as a standard for the preparation of the standard curve for quantitative calculation, ergotamine, a compound of a very similar structure, was used.

Lolitrem B analysis. Chemical analyses were carried out according to modified Gallagher's [1985] method as follows. A weighed portion of lyophilized and milled plant material (0.5 g) was transferred to 12-ml sealed glass vials. 8 ml of a chloroform:methanol solution (2:1) was added, and gentle stirring was applied for 18 h. Next, the sample was centrifuged (5 min., 2500 rpm), and 1 ml of the extract was withdrawn and dried under a stream of nitrogen

Clean-up was performed on Sep-Pak Silica columns pre-conditioned with 5 ml dichloromethane. The dried extract was loaded onto the Sep-Pak with four rinses of 0.5 ml dichloromethane The column was rinsed with 2 ml dichloromethane/acetonitrile solution (80:20), and the eluate was discarded. The sample was eluted into a glass vial using 6 ml of the dichloromethane/acetonitrile solution (80:20) and solvent was evaporated under a stream of nitrogen. Samples subsequently were dissolved in 500 µl of mobile phase (dichloromethane:acetonitrile 80:20 v/v), capped and stored until injections onto the HPLC column.

Chromatographic analysis was conducted with a liquid chromatography system comprising a HP 1050 pump, a HP fluorescence detector, and Chemstation software for data acquisition and processing. Compounds were separated on an Econosphere Silica 5U column (250 × 4.6 mm, 5 µm at room temperature 25+/-4ºC). The mobile phase was dichloromethane:ACN solution (85:15) run at a flow rate of 1.4 $ml \cdot min^{-1}$. Fluorometric detection was performed at λ_{Ex} = 268 nm and λ_{Em} = 440 nm. Injection volume was 20 µl. Because lolitrem B was not available as a standard, the concentration of this compound in the samples was expressed in units of peak area in a chromatogram.

RESULTS

Seeds infection by endophytic fungi. There were 242 samples of seeds of 50 varieties of grasses collected in Poland, including 124 samples of 20 cultivars of perennial ryegrass, 61 samples of 10 cultivars of meadow fescue, and 57 samples of 20 cultivars of red fescue (tab. 1). Endophytic fungi were only detected in the seeds of perennial ryegrass and meadow fescue. Characteristically twisted, intercellular, sparingly branched hyphae were observed in the aleurone layer of the kernels. No colonization by

Table 1. Occurrence of *Neotyphodium lolii*, *N. uncinatum* and *Epichloë festucae* in perennial ryegrass, meadow fescue and red fescue cultivars respectively

Tabela 1. Zasiedlenie odmian życicy trwałej, kostrzewy łąkowej i kostrzewy czerwonej przez odpowiednio *Neotyphodium lolii*, *N. uncinatum* i *Epichloë festucae*

Cultivar Odmiana	Number of examined seed lots Liczba przetestowanych prób nasion	Number of infected seed lots/and percentage of their infection Liczba prób nasion zasiedlonych/i procent ich zasiedlenia
Perennial ryegrass – Życica trwała		
Anna	3	0
Argona	9	5/6; 4; 3; 2; 1
Arka	4	0
Barball	3	0
Barcredo	3	0
Baristra	4	0
Barrage	4	0
Barplus	4	0
Gazon	12	8/5; 4; 4; 4; 3; 3; 1; 1
Ilirka	3	0
Maja	6	0
Mara	5	0
Naki	18	11/6; 5; 5; 4; 4; 4; 3; 3; 1; 1; 1
Niga	3	0
Nira	8	0
Rela	4	4/8; 8; 7; 6
Sabor	2	0
Solen	12	5/4; 4; 2; 1; 1
Stadion	14	0
Talgo	3	0
Meadow fescue – Kostrzewa łąkowa		
Artema	5	0
Aureus	4	0
Cykada	12	0
Justa	9	7/90; 89; 85; 80; 70; 53; 46
Mewa	6	0
Pasja	10	8/52; 50; 46; 43; 40; 36; 35; 24
Skawa	5	0
Skiba	3	0
Skra	5	0
Wanda	2	0
Red fescue – Kostrzewa czerwona		
Adio	2	0
Areta	2	0
Atra	1	0
Barcrown	2	0
Bargena	2	0
Bargreen	2	0
Barma	2	0
Barnica	2	0
Barskol	2	0
Bargaret	1	0
Baroyal	2	0
Brudzyńska	6	0
Jagna	1	0
Kos	11	0
Kristina	1	0
Leo	5	0
Lucinda	1	0
Nimba	4	0
Olivia	2	0
Reda	6	0

endophytes was found in the samples of red fescue. The mycelium of *N. lolii* was identified in 33 samples of 5 cultivars of perennial ryegrass Argona, Gazon, Naki, Rela, and Solen. The levels of colonization of those samples were very low and did not exceed 6, 5, 6, 8, and 4%, respectively. Relatively highest colonization levels were observed in samples of the variety Rela – from 6 to 8%. In the case of the remaining varieties, mean percent of colonization was not higher than 3.4% ('Naki'). Endophyte mycelium was isolated from three samples of the varieties Naki (Lp61), Rela (Lp89), and Solen (Lp95) (tab. 1).

Neotyphodium uncinatum, the endophyte of meadow fescue, was found in 15 samples of 2 cultivars, Pasja and Justa. The level of colonization was high and ranged from 24 to 52% for the former and 46 to 90% for the latter. Mean percent of colonization was 40.8 for Pasja and 73.3 for Justa. Endophytes were successfully isolated in 4 cases, two from the cultivar Justa (Fp22 and Fp28) and two from the cultivar Pasja (Fp37 and Fp40). No endophytic mycelium was detected in the remaining 8 cultivars of meadow fescue (tab. 1).

Isolations of fungi of the genera *Neotyphodium* and *Epichloë* are among the most difficult ones. Their hyphae are found inside plant tissues and are characterized by very slow growth. This is why other, usually faster-growing microorganisms, often hinder successful isolation of an endophyte. The key factors here are the way and time of surface sterilisation, which depend to a large degree on the size of the plant material being disinfected. Too long disinfection times eliminate microorganisms along with the endophyte, and too short disinfection times create a high risk of contamination by other microorganisms. Moreover, the individual properties of each isolate, related to the rate and type of plant tissue colonization and the isolate's growth and development, affect the success of isolation. This often necessitates multiple repetitions of isolations.

Characteristics of isolated endophytes *in vitro*. In the first instance, the macro- and microscopic characteristics of the obtained isolates were determined (tab. 2). The endophyte colonies were very similar to one another within one species. Isolates of *N. lolii* formed slightly raised colonies with a wrinkled surface and with a slightly fluffy white mycelium. Their growth rate was significantly the weakest and ranged from 0.33 to 0.41 mm a day. The presence of small conidia was observed. The colonies of *N. uncinatum* were also elevated and wrinkled, but the mycelium had a more felty structure and was white to cream-white. They grew significantly faster, especially Fp22 and Fp40, at a mean rate of 0.61 mm a day and did not produce spores on PDA medium.

Large differences were observed in the response of the test microorganisms to the presence of the endophytic fungi *in vitro* (tab. 3). At 10°C, all *Neotyphodium* spp. isolates inhibited, to varying degrees, growth of *F. equiseti*, *M. nivale*, and *R. solani*. Growth of the largest number of microorganisms was inhibited by isolates Fp22 and Fp40 of meadow fescue. The largest mean inhibition zones were recorded on plates with *B. sorokiniana* and *R. solani*. Significantly least affected by the endophytes was the development of *T. viride* and *F. poae*. Isolate Fp40 had significantly the strongest antagonistic properties.

At 25°C, a temperature which is more conducive to the development of most microorganisms, the researched endophytic fungi showed a greater activity. They all inhibited the growth of *M. nivale, R. solani*, *F. avenaceum*, and *F. equiseti*. The endophytes

Table 2. Characteristics of *Neotyphodium lolii* and *N. uncinatum* cultures isolated from perennial ryegrass and meadow fescue cultivars respectively
Tabela 2. Charakterystyka kultur *Neotyphodium lolii* i *N. uncinatum* wyizolowanych z odmian życicy trwałej i kostrzewy łąkowej

Endophyte isolate Izolat endofita	Growth rate, mm per day Tempo wzrostu, mm/dzień	Size of spores, µm Wymiary zarodników, µm	Endophyte colony appearance Wygląd kolonii endofita
N. lolii – Lp61	0.33 A*	4–7 × 2–3	raised and wrinkled, slightly fluffy and white mycelium wzniesiona i pomarszczona, grzybnia lekko puszysta, biała
N. lolii – Lp89	0.38 AB	4–7 × 2–3	slightly raised and wrinkled; slightly fluffy, white mycelium lekko wzniesiona i pomarszczona, grzybnia lekko puszysta, biała
N. lolii – Lp95	0.41 B	4–7 × 2–3	slightly raised and wrinkled; slightly fluffy, white mycelium lekko wzniesiona i pomarszczona, grzybnia lekko puszysta, biała
N. uncinatum – Fp22	0.65 D	brak	slightly raised and wrinkled; felty, cream-white mycelium lekko wzniesiona i pomarszczona, grzybnia filcowata, biało-kremowa
N. uncinatum – Fp28	0.54 C	brak	raised and slightly wrinkled; felty, cream-white mycelium wzniesiona i lekko pomarszczona, grzybnia filcowata, biało-kremowa
N. uncinatum – Fp37	0.55 C	brak	raised and slightly wrinkled; felty, cream-white mycelium wzniesiona i lekko pomarszczona, grzybnia filcowata, biało-kremowa
N. uncinatum – Fp40	0.68 D	brak	slightly raised and wrinkled; felty, white mycelium lekko wzniesiona i pomarszczona, grzybnia filcowata, biała

* Means marked by the same letters do not differ significantly at $\alpha = 0.05$ according to Tukey test
* Wartości oznaczone tą samą literą nie różnią się istotnie przy poziomie istotności $\alpha = 0{,}05$ według testu Tukeya

Table 3. Inhibition of growth of test fungi by *Neotyphodium lolii* and *N. uncinatum* isolates in 10°C and 25°C (dual culture assays)
Tabela 3. Inhibicyjny wpływ izolatów *Neotyphodium lolii* i *N. uncinatum* na wzrost grzybów testowych w temp. 10 i 25°C (doświadczenia płytkowe)

Grzyby testowe Test fungi	Mean growth inhibition zone of test fungi, mm Średnia szerokość strefy zahamowania wzrostu grzybów testowych, mm															
	temperature – temperatura 10°C								temperature – temperatura 25°C							
	Lp61	Lp89	Lp95	Fp22	Fp28	Fp37	Fp40	mean średnia	Lp61	Lp89	Lp95	Fp22	Fp28	Fp37	Fp40	mean średnia
Bipolaris sorokiniana	0	0	18.5	22.1	19.3	24.0	26.3	15.7 H*	0	0	15.6	20.6	20.3	14.3	15.6	12.3 I
Drechslera dictyoides	0	1.2	3.2	6.0	0	5.3	6.5	3.2 D	0	0	5.5	7.5	0	8.7	6.6	4.0 D
D. siccans	2.3	0	0	0	0	4.5	6.6	1.9 C	0	0	0	0	2.3	5.6	6.0	2.0 A
Fusarium avenaceum	0	2.0	2.3	10.2	9.6	3.2	0	3.9 E	5.6	6.8	5.8	12.2	14.3	5.9	5.7	8.0 E
F. culmorum	0	0	0	2.5	5.6	0	2.0	1.4 B	0	0	2.3	5.6	5.9	2.3	5.5	3.1 C
F. equiseti	2.3	2.1	1.0	6.8	9.8	10.5	9.8	6.0 F	6.8	8.7	5.8	8.7	12.3	12.6	10.2	9.3 F
F. poae	1.0	0	0	2.2	0	0	2.8	0.9 A	3.2	0	0	6.5	0	0	5.5	2.2 B
Microdochium nivale	2.2	1.3	5.2	2.4	8.9	10.2	12.3	6.1 F	6.5	9.8	7.5	12.1	12.2	14.3	14.2	10.9 H
Rhizoctonia solani	5.3	5.6	9.8	3.6	8.7	2.4	15.3	7.2 G	6.6	9.0	12.2	8.8	8.6	9.2	18.6	10.4 G
Trichoderma viride	1.0	2.0	0	1.1	0	0	0	0.6 A	2.0	4.8	0	6.8	0	0	0	1.9 A
Mean – Średnia	1.4 A	1.4 A	3.9 B	5.6 C	6.2 D	6.0 D	8.2 E		3.1 A	3.9 B	5.5 C	8.9 F	7.6 E	7.3 D	8.8 F	

* Means marked by the same letters do not differ significantly at $\alpha = 0.05$ according to Tukey test
* Wartości oznaczone tą samą literą nie różnią się istotnie przy poziomie istotności $\alpha = 0{,}05$. według testu Tukeya

Table 4. Effect of water and ethanol extracts made from endophyte infected seeds on spores germination of test fungi (microscope slide tests)
Tabela 4. Wpływ wodnych i alkoholowych ekstraktów z nasion zasiedlonych przez endofity na kiełkowanie zarodników grzybów testowych (doświadczenie na szkiełkach mikroskopowych)

Test fungi Grzyby testowe	*Neotyphodium lolii*								*Neotyphodium uncinatum*					
	Lp61		Lp89		Lp95		Fp22		Fp28		Fp37		Fp40	
	W[1]	E	W	E	W	E	W	E	W	E	W	E	W	E
Bipolaris sorokiniana	+[2]	+	-	-	-	-	+	+	+	+	+	+	-	-
Blumeria graminis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Drechslera dictyoides	-	-	-	-	-	-	+	+	+	+	+	+	+	+
D. siccans	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Fusarium avenaceum	+	+	+	+	+	+	-	+	-	+	-	+	+	+
F. culmorum	+	+	+	+	+	+	-	+	-	+	-	+	+	+
F. equiseti	+	+	+	+	+	+	-	+	-	+	-	+	+	+
F. poae	+	+	+	+	+	+	-	+	-	+	-	+	+	+
Microdochium nivale	+	+	+	+	+	+	-	+	-	+	-	+	+	+
Puccinia coronata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. graminis ssp. *graminicola*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trichoderma viride	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] W – water extract – wyciąg wodny; E – ethanol extract – wyciąg alkoholowy
[2] + inhibition of spores germination – hamowanie kiełkowania zarodników, lack of inhibition of spores germination – brak działania inhibicyjnego

which most frequently affected the development of the test fungi included Fp22, Fp40, and Fp37, while Fp22, Fp28 and Fp40 were characterized by significantly the strongest antagonistic properties. *Trichoderma viride* and *D. siccans* were significantly the least inhibited of all the test fungi at the higher temperature.

Effect of water and alcohol extracts of colonized seed samples on spore germination of the test fungi was differentiated (tab. 4). No inhibition of spore germination by the seed extracts was observed in the fungi *B. graminis*, *P. coronata*, *P. graminis* ssp. *graminicola*, and *T. viride* . Spore germination of pathogens from the genus *Drechslera* was not inhibited by extracts of colonized seeds of perennial ryegrass. Those extracts only slightly inhibited the spores of *B. sorokiniana*. A vast majority of the extracts showed inhibitory properties against fungal spores of the genera *Fusarium* and *M. nivale*. Only in three cases (Fp22, Fp28, and Fp37), was no effect of water extracts recorded. Germination of spores of *B. sorokiniana* and pathogens of the genus *Drechslera* was, in most cases, inhibited by extracts of colonized seeds of meadow fescue.

Toxins production. Large differences were observed in the contents of the analyzed toxins (tab. 5). In 2 samples of meadow fescue, no ergovaline was detected, either in seeds or in plants. The remaining two samples contained small quantities of this toxin (0.184 to 0.851 µg/g DM the sample). Higher ergovaline concentrations were always recorded in seeds.

Table 5. Ergovaline and lolitrem B content in seeds and plants of perennial ryegrass and meadow fescue infected with tested isolates of *Neotyphodium lolii* and *N. uncinatum*

Tabela 5. Zawartość ergowaliny i lolitremu B w nasionach i roślinach życicy trwałej i kostrzewy łąkowej zasiedlonych przez badane izolaty *Neotyphodium lolii* i *N. uncinatum*

Tested association Badana asocjacja	Ergovaline, µg·g DM^{-1} Ergowalina, µg·g SM^{-1}		Lolitrem B, LU·s·g DM^{-1} Lolitrem B, LU·s·g SM^{-1}	
	seeds – nasiona	plants – rośliny	seeds – nasiona	plants – rośliny
L. perenne/N. lolii – Lp61	-	-	48	40
L. perenne/N. lolii – Lp89	-	-	1519	56
L. perenne/N. lolii – Lp95	-	-	985	392
F. pratensis/N. uncinatum – Fp22	0.851	0.184	-	-
F. pratensis/N. uncinatum – Fp28	0	0	-	-
F. pratensis/N. uncinatum – Fp37	0	0	-	-
F. pratensis/N. uncinatum – Fp40	0.371	0.239	-	-

Lolitrem B was found in all of the analyzed perennial ryegress samples, originating from both seeds and plants. The levels of concentration of this toxin fluctuated widely. Higher lolitrem B concentrations were always recorded in samples from seeds. In the case of sample Lp89, a 27-fold higher concentration was observed comparing to Lp61. Relatively small amounts of this alkaloid were detected in plants. The sample with isolate Lp61 contained the lowest concentration of lolitrem B.

DISCUSSION

The main way of dispersion of N/E endophytes and the only one for N is transfer inside kernels. However, low persistence of mycelium in storaged seeds and high sensitivity to the adverse conditions of traditional seed storage often lead to the loss of viability of endophytes [Siegel et al. 1985, Rolston et al. 1986]. Wheatley [2005] reports that storage of grass seeds in ambient conditions reduces the viability of endophytic mycelium to zero after approx. 18–24 months. Reduction of air humidity to approx. 20–30% and temperature to several degrees above 0°C may considerably extend the viability of endophytes, at the same time ensuring good germination of seeds [Clement et al. 2004, Moyer 2004]. Frequent turnover of seed material, on the other hand, is a cause of a relatively fast decrease in percent colonization by endophytic fungi. This is especially characteristic of certified sowing material from registered cultivars of different grass species and of fields that have been cultivated for a short time [Cappelli and Buonaurio 2001]. In the present investigations, colonization of varieties of the tested grass species was also relatively low. Endophytes were detected in 33 out of 124 perennial ryegrass seed samples (26.8%), and colonization of these samples did not exceed 8%. In case of meadow fescue – 24.6% of the samples contained an endophyte; however, some of those samples showed a very high level of colonization (up to 90%). No endophytic fungi were detected in red fescue. In a study by Pfannmöller et al. [1994), colonization of fescues was also very low, as only 17 out of the 153 samples tested contained an endophyte. The largest number of colonized seeds (9) and the highest degree of colonization (70%) were observed in seed samples of meadow fescue. By contrast, only 4 samples of tall fescue out of the 33 samples tested were found to be colonized by *N. coenophialum*, with maximum colonization of 32%. Riccioni et al. [1994], obtained similar results when studying colonization of varieties of tall fescue in Italy. They noted the presence of endophytes in 4 out of the 21 varieties tested, with the variety Titan, of USA origin, showing the highest colonization (57%). Results of our previous research [Pańka and Łukanowski 2001, Pańka and Sadowski 2002] on colonization of cultivars of perennial ryegrass also confirm the present findings concerning the low abundance of endophytes in the cultivars tested. Considerably higher colonization percentages are observed in natural grass communities and in grass fields which have been cultivated for many years [Faeth et al. 2001].

The present study showed a differential inhibitory effect of the tested isolates of endophytic fungi on mycelial growth and germination of spores of numerous pathogens. A similar diversity of antagonistic effect was observed by Riccioni et al. [1994]. In a study of the effect of *A. coenophialum* and other *Acremonium* spp. isolated from tall fescue on the growth of *Drechslera erytrospila* and *D. graminea* on PDA medium, they recorded both a moderate inhibitory effect (a 10 to 20-mm growth inhibition zone) as well as a complete lack of influence. Similarly, in experiments by Christensen [1996], *N. coenophialum* inhibited mycelial growth of *Drechslera erythrospila* and *Rhizoctonia solani* both to a small degree and at a level of over 15 mm. This is also confirmed by the results of our previous studies [Pańka 2008]. In the present study, a slight effect was observed of 3 isolates of *Neotyphodium* spp. on the growth of *T. viride*, a well-known anatagonist of many pathogens. The remaining isolates did not inhibit the growth of this

fungus, which is a very positive result. White and Cole [1985] also observed a slight inhibitory effect of *N. coenophialum* on the growth of *Trichoderma harzianum*. In the present study plate experiments were, in many cases, corroborated by the experiment with extracts. Also Holzmann-Wirth et al. [2000] observed antibiotic activity of *N. lolii* in plate tests and in tests with extracts. Unfortunately, the obligate pathogens *B. graminis*, *P. coronata,* and *P. graminis* ssp. *graminicola* turned out not to be sensitive to any of the extracts tested. In both types of experiments, the isolates of *N. uncinatum* showed more activity. Importantly, two of them, Fp28 and Fp37, did not produce ergovaline in a pot experiment. They also showed a higher growth rate compared to *N. lolii*. It points to the potentially higher possibilities of colonizing host plants with these isolates, and, hence, their greater usefulness in biological protection of grasses. The relatively high *in vitro* activity of the investigated isolates of *Neotyphodium* spp. suggests that their action may also be visible under field conditions. One, however, should be aware of the fact that in natural conditions, there is an additional effect of many external factors which may largely modify the ultimate reaction of an association to biotic stress [Christensen 1996, Wäli et al. 2006].

CONCLUSIONS

1. Cultivars of perennial ryegrass and meadow fescue available on the Polish market in the recent years showed very low levels of colonization by endophytic fungi of the *Neotyphodium* genus. Red fescue cultivars were not infected by endophytic fungi.

2. The viability of endophytic mycelium in the seed material of perennial ryegrass and meadow fescue cultivars was very low and did not exceed 26,6%.

3. Endophytic fungi showed stronger antagonistic properties at higher temperature – 25°C than at 10°C in vitro.

4. *Microdochium nivale, Rhizoctonia solani, Fusarium avenaceum* and *F. equiseti* were inhibited by *Neotyphodium* spp. at the highest degree and the most frequently.

5. Water and alcohol extracts of E+ seeds did not affect the obligate pathogens *B. graminis*, *P. coronata*, and *P. graminis* ssp. *graminicola*.

6. *Neotyphodium uncinatum* isolates: Fp28, Fp40, and, to a lesser degree, Fp37, originating from meadow fescue, showed the higher potential as biological control agents. They were characterized by the strongest antagonistic properties, and did not produce (Fp28 and Fp37), or produced only small amounts (Fp40) of, ergovaline. After additional tests, they could be used as biological factors for improving the utility value of new grass cultivars and their resistance to stress factors.

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ENDOFITY KOMPLEKSU *Neotyphodium* / *Epichloë* ZASIEDLAJĄCE UPRAWIANE W POLSCE ODMIANY ŻYCICY TRWAŁEJ, KOSTRZEWY ŁĄKOWEJ I KOSTRZEWY CZERWONEJ

Streszczenie. Celem przeprowadzonych badań było (i) określenie poziomu zasiedlenia uprawianych w Polsce odmian życicy trwałej, kostrzewy łąkowej i kostrzewy czerwonej, przez endofity z rodzaju *Neotyphodium* i *Epichloë*, (ii) wyizolowanie endofitów z zasiedlonych roślin oraz określenie ich antagonistycznego oddziaływania w stosunku do wybranych mikroorganizmów *in vitro*, (iii) określenie potencjalnego zagrożenia ze strony toksyn produkowanych przez aktywne asocjacje. Endofity wykrywano metodą barwienia, izolowano na pożywkę PDA i charakteryzowano. Następnie analizowano antagonistyczne właściwości uzyskanych izolatów w testach płytkowych oraz ekstraktów z nasion na szkiełkach mikroskopowych. Oznaczano także poziom ergowaliny oraz lolitremu B. Zebrano na terenie Polski 242 próby nasion życicy trwałej (124), kostrzewy łąkowej (61) i kostrzewy czerwonej (57). Grzyby endofityczne wykryto w 33 próbach życicy trwałej i 15 próbach kostrzewy łąkowej, a ich poziom zasiedlenia osiągnął odpowiednio 8 i 90%. Endofity wyizolowano z 3 prób pierwszego gatunku i 4 prób drugiego. Nie wykryto endofitów w próbach nasion kostrzewy czerwonej. Grzyby endofityczne wykazały silniejszy antagonistyczny wpływ w temp. 25°C. Najczęściej, i w największym stopniu hamowały one wzrost *M. nivale, Rhizoctonia solani*, *Fusarium avenaceum* i *F. equiseti*. Patogeny obligatoryjne *Blumeria graminis*, *Puccinia coronata* i *P. graminis* ssp. *graminicola* były niewrażliwe na wodne i alkoholowe ekstrakty z nasion E+. Najsilniejszymi właściwościami antagonistycznymi charakteryzowały się izolaty Fp28, Fp40 i w mniejszym stopniu Fp37 pochodzące z kostrzewy łąkowej. Ponadto nie produkowały one (Fp28, Fp37) lub tylko niewielkie ilości (Fp40) toksycznej dla zwierząt ergowaliny. Po dodatkowych testach mogą one zostać wykorzystane jako biologiczny czynnik poprawiający wartość użytkową i odporność na czynniki stresowe nowych odmian traw.

Słowa kluczowe: *Lolium perenne, Festuca pratensis,* patogeny, ergowalina, lolitrem

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