University of Technology and Life Sciences, Bydgoszcz, Poland

OCCURRENCE OF *NEOTYPHODIUM LOLII* AND ITS ANTIFUNGAL PROPERTIES

D. Pańka

Abstract

The objectives of the research were to evaluate (i) colonization by *Neotyphodium lolii* in a collection of perennial ryegrass (*Lolium perenne*) ecotypes, and (ii) antagonistic properties of *N. lolii* towards selected fungi *in vitro*. Mycelium of *N. lolii* was detected in 10 out of 22 ecotypes of *L. perenne*. Two methods of detection, staining with the rose bengal and morphological analysis, and amplification of DNA by PCR were equally successful. Four isolates of *N. lolii* (G5, G13, G20 and P18) inhibited growth of all test fungi by similar amounts at 10°C and 25°C. The widest growth-inhibition zones were observed in combinations with *Bipolaris sorokiniana*, *Rhizoctonia cerealis* and *R. solani*. *Fusarium equiseti* was the fungus most often inhibited.

Key words: *Neotyphodium lolii,* endophytes, perennial ryegrass, ecotypes, antagonism

Introduction

Endophytic fungi in the genus *Neotyphodium*, and their *Epichloë* teleomorphs from the family *Clavicipitaceae*, often form symbiotic relationships with numerous species of grasses. They grow in the intercellular spaces of all the above-ground parts of the host plant including flowers and seeds. The presence of hyphae in the seeds helps the fungi to spread effectively in the natural habitat. These endophytic fungi are unable to grow outside the host-plant tissues and so are entirely dependent on the host. The relationship results in the fungus having a strong effect in protecting the host plant from the influence of unfavourable external conditions. This is shown particularly by stimulation of the plant's growth, development, reproduction and decreased susceptibility to drought (West 1994, Ravel et al. 1997, Malinowski and Belesky 2000, Hesse et al. 2002, 2004). In addition, plants colonized by endophytes are less acceptable to grazing by animals and often are more

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resistant to feeding by pests and infection by pathogens (Johnson et al. 1985, Siegel et al. 1985, Lewis 1996). Such effects of endophytes result mainly from the presence of fungal metabolites in the plant tissues or chemical compounds produced by the host plant under influence of the fungus. The positive effects of endophytic fungi on host plants warrant studies on the application of the fungi in biological control of the main pathogens of grasses (Bouton and Easton 2005, Brilman 2005).

The main objectives of the present research were (i) to study the occurrence of *Neotyphodium lolii* in collections of perennial ryegrass (*Lolium perenne*) ecotypes, and (ii) to evaluate the antagonistic properties of Polish isolates of *N. lolii* towards selected fungi *in vitro*.

Materials and methods

Plants of perennial ryegrass used in the study originated from various regions of Poland and were maintained in the ecotype collection at the Mochełek Research Station, University of Technology and Life Sciences, Bydgoszcz, Poland. The collection consists of 22 ecotypes of *L. perenne* (Table 1).

Detection and isolation

Neotyphodium lolii was detected in plants using morphological and molecular methods. The former was based on morphology of hyphae stained with the rose bengal according to Saha et al. (1988). Soft leaf sheaths, not yet dried out, were sampled from the lowest parts of five shoots of each *L. perenne* ecotype. The inner epidermis of each leaf sheath was treated with a drop of the rose bengal and after 1 min observed microscopically (100–400×). The presence of characteristically twisted hyphae of *N. lolii* growing in the intercellular spaces of the epidermis suggested the presence of the endophyte. The presence of fungal hyphae in at least one specimen indicated its occurrence in the ecotype. This was confirmed by additional detection using the molecular method of Dombrowski et al. (2006). DNA was extracted from the plant tissues using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA was amplified by PCR with *Taq* PCR Core Kit (Qiagen, Hilden, Germany) and primers IS-RS-5' (5'GAGCCCCTGATTTCGTAC-3') and IS-NS-3' (5'TTGAAGTAGACACTCATACGCTC-3'), which amplify a region of the intron of the *tub*2 (tubulin 2) gene specific for *Neotyphodium* spp. Each 12.5 µl PCR mixture consisted of 3.9 µl H_2O, 2.5 µl 5× buffer Q, 1.25 µl 10× buffer, 0.5 µl $MgCl_2$ (final concentration 1 mM/µl), 0.25 µl dNTP mix (final concentration 200 µM/µl each), 0.75 µl primer IS-RS-5' (0.6 pM/µl), 0.75 µl primer IS-NS-3' (0.6 pM/µl), 0.1 µl *Taq* polymerase (0.5 unit), 2.5 µl DNA (final concentration 20 ng/µl). The PCR amplification was run on a Uno II thermocycler (Biometra, Germany). Cycling conditions were: an initial denaturation at 94°C for 1 min, followed by 18 cycles of 94°C for 25 s, 73°C for 25 s, and 72°C for 3 min, followed by 32 cycles of

Table 1

Ecotypes of perennial ryegrass (*Lolium perenne*), their origin and the presence of *Neotyphodium lolii* in plants

No.	Code of the ecotype	Origin of the ecotype	Presence of the endophyte	
			staining method	PCR assay
1	G3	Oleszna Podgórska, Dolnośląskie province	–	–
2	G5	Głuszyca Górna, Dolnośląskie province	+	+
3	G7	Dziwiszów, Dolnośląskie province	+	+
4	G10	Świdnik, Dolnośląskie province	+	+
5	G13	Raszów, Dolnośląskie province	+	+
6	G16	Wojbórz, Dolnośląskie province	–	–
7	G20	Szelejewo Górne, Dolnośląskie province	+	+
8	G21	Łężyce, Dolnośląskie province	–	–
9	G23	Złotno, Dolnośląskie province	–	–
10	G25	Boguszyn, Opolskie province	–	–
11	G26	Racibórz, Śląskie province	–	–
12	G37	Chyżne, Małopolskie province	–	–
13	G41	Podczerwone, Małopolskie province	–	–
14	G43	Nowy Targ, Małopolskie province	+	+
15	G52	Myślenice, Małopolskie province	–	–
16	P13	Promiski, Podlaskie province	+	+
17	P14	Promiski, Podlaskie province	–	–
18	P18	Berżniki, Podlaskie province	+	+
19	P28	Leszczewo, Podlaskie province	+	+
20	P33	Leszkiemie, Podlaskie province	+	+
21	P34	Kociołki, Warmińsko-mazurskie province	–	–
22	P38	Banie, Warmińsko-mazurskie province	–	–

94°C for 25 s, 58°C for 1 min, and 72°C for 2 min. After cycling, final elongation was at 73°C for 15 min. The PCR products were checked by electrophoresis of 5 μl of product in 1% agarose gel containing ethidium bromide (0.2 μg/ml) to stain the DNA. Pictures were taken with a digital camera using the program Biocapt (Vilbert Lourmat, France).

Ten pieces of stem, each 20 mm long, sampled from the lower parts of 10 plants from each *L. perenne* ecotype were used for isolation of *N. lolii* in culture. Each piece of stem was divided into four subsections which were surface sterilized in 75% ethanol for 2 min and in 10% sodium hypochlorite for 1 min, rinsed in the sterile distilled water three times for 2 min. Each was then cut further into five 5 mm^2 pieces which were placed on 2% potato dextrose agar (PDA; 40 g filtered white potatoes, 20 g agar, 100 mg dihydrostreptomycin sulphate, 100 mg penicillin G, 1 l distilled water, pH 7) and incubated for three–four weeks at 22°C in darkness.

Antagonistic properties of *Neotyphodium lolii*

Ten isolates of *N. lolii* obtained from the different *L. perenne* ecotypes were tested against different fungi on 2% PDA. Discs of agar (5 mm diameter) with *N. lolii* mycelium were cut from the edge of a three-week-old culture, placed centrally on the 2% PDA in a 9-cm-diameter Petri dish and incubated for three–four weeks at 22°C in darkness (until colonies reached 10–15 mm diameter). Two discs of agar (5 mm diameter) with mycelium of a test fungus were cut off from the edge of a two–three-week-old culture and placed on the Petri dish with *N. lolii*. They were placed at opposite sides of the Petri dish on the same diameter near the edges. There were five replicates of each *N. lolii* + test fungus combination. In control dishes, only discs of the test fungus were placed on 2% PDA, at both ends of the diameter. Cultures of the paired fungi (*N. lolii* + test fungus) and controls (test fungus only) were incubated up to 14 days at 10°C or 25°C in darkness. Measurements were taken when the two colonies in the control met in the centre of the Petri dish (fast growing fungi) or after 14 days of incubation (slow growing fungi). The width (mm) of the zones between fronts of *N. lolii* colony and two colonies of test fungus was measured (Christensen 1996). An average width was calculated from two measurements on each Petri dish. An average width of inhibition zone calculated from five replicates indicated the scale of inhibition in a combination.

The test fungi were pathogens of *L. perenne*, including *Bipolaris sorokiniana, Fusarium avenaceum, F. equiseti, F. solani, Rhizoctonia solani* and *R. cerealis*, as well as *Trichoderma viride*. All the test fungi had been isolated from roots, stems or leaves of *L. perenne* plants sampled from the experimental plots at Mochełek Research Station.

Results

The morphological and molecular method successfully detected *N. lolii* in the 10 out of 22 *L. perenne* ecotypes collected in Poland. The two methods detected the fungus in the same plants (Table 1). While using staining and microscopical observation of the fungus morphology, the characteristically twisted and unbranched mycelium of the endophyte was observed in the intercellular spaces of the leaf sheath epidermis. While using the molecular approach, PCR amplification of the fungus rDNA with specific primers yielded a product of 370 bp. Its presence, after visualization on agarose gel, indicated colonization by *N. lolii*. Both methods proved to be useful and applicable in practice.

Neotyphodium lolii isolates originating from all ecotypes of *L. perenne* were grown on 2% PDA. In paired cultures *N. lolii* inhibited the growth of all test fungi. The rates of inhibition differed and did not depend on the taxonomic position of the test fungus (Table 2). *Fusarium equiseti* was the most sensitive to *N. lolii* in terms of the frequency of reactions, and the amount of inhibition. Growth of *F. equiseti* was inhibited by nine out of 10 isolates of *N. lolii* at 10°C and 25°C. *Fusarium avenaceum* and *R. solani* were among the fungi most often affected by *N. lolii* at higher tempera-

Table 2

Average width of the inhibition zone between *Neotyphodium lolii* isolates and test fungi at 10 and 25°C (mm)

N. lolii isolate	Test fungus						
	Bipolaris sorokiniana	*Fusarium avenaceum*	*Fusarium equiseti*	*Fusarium solani*	*Rhizoctonia cerealis*	*Rhizoctonia solani*	*Trichoderma viride*
10°C							
G5	26.9	9.8	12.3	17.5	10.7	12.2	8.5
G7	10.2	0.0	4.1	0.0	0.0	0.0	0.0
G10	0.0	0.0	3.2	0.0	2.1	1.4	0.0
G13	25.5	10.1	13.4	12.5	6.4	10.6	6.1
G20	26.8	1.9	3.9	15.4	7.3	12.2	11.8
G43	0.0	0.0	2.1	0.0	0.0	0.0	0.0
P13	10.2	8.3	8.5	3.2	3.3	4.1	2.2
P18	27.0	8.5	10.5	16.8	8.5	14.5	3.6
P28	0.0	0.0	1.3	0.0	0.0	0.0	0.0
P33	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25°C							
G5	24.5	9.7	14.0	9.0	18.5	19.2	10.8
G7	5.4	6.2	5.1	0.0	0.0	0.0	0.0
G10	0.0	2.1	2.2	0.0	3.0	3.0	0.0
G13	20.3	6.2	8.1	10.3	14.7	16.4	5.4
G20	22.0	8.1	9.0	8.1	13.8	17.0	9.3
G43	0.0	0.0	4.4	0.0	0.0	1.5	0.0
P13	8.3	5.4	7.8	4.5	2.1	3.5	1.3
P18	23.4	10.5	8.0	10.1	17.2	20.3	6.8
P28	0.0	0.0	2.5	0.0	0.0	0.0	0.0
P33	0.0	0.0	0.0	0.0	0.0	0.0	0.0

ture. Only four isolates of *N. lolii* (P28, P33, G7 and G43) did not usually affect the growth of both pathogens at 25°C. *Fusarium solani* and *T. viride* were the least sensitive to antifungal activity of *N. lolii* in terms of frequency and amount of inhibition. Their growth was inhibited by only 50% of *N. lolli* isolates at 10°C and 25°C.

The most effective *N. lolii* isolates were the four ones G5, G13, G20 and P18. Their activities towards the test fungi were similar at 10°C and 25°C. The inhibition zone between (i) *Bipolaris sorokiniana* and G5, G13, G20 or P18 always exceeded 20 mm at 10°C and 25°C, (ii) *F. equiseti* and G5 was 12.3 mm at 10°C and 14 mm at 25°C, and (iii) *R. cerealis* and *R. solani,* and G5, G13, G20 or P18 ranged between 13.8 mm and 20.3 mm at 25°C. Other isolates of *N. lolii* (G43 and P28) showed low antifungal activity, and one isolate (P33) was completely inactive.

Discussion

The number of plants from which *N. lolii* was identified (less than 50%) suggests moderate colonization of *L. perenne* in Poland. A similar moderate level of colonization was observed throughout Europe (Lewis 2001). Studies in the 22 European countries indicated an average colonization of 49% of plants and seeds by *N. lolii*. Faeth et al. (2001) and Wäli et al. (2001) reported a greater amount of colonization of plants in natural grasslands and less in grasses and cultivars used in agricultural systems. Similar levels of colonization of natural and cultivated grasses by *N. lolii* in Poland were reported by Pańka and Łukanowski (2001) and Pańka and Sadowski (2002).

Obtained results suggest large differences in the antagonistic properties of isolates of *N. lolii*, which supports the earlier observation of Christensen (1996). Christensen (1996), White and Cole (1985), and Pańka (2005 a, b) showed that other *Neotyphodium* species, as a result of metabolites produced, can also inhibit the growth of pathogens of *L. perenne*. *Neotyphodium coenophialum* and *N. uncinatum* affected the growth of *F. equiseti* and *R. cerealis*. *Neotyphodium lolii* showed moderate and high activity against *Drechslera erythrospila* and *R. zeae*.

The considerable antifungal activity of four of the isolates of *N. lolii* (G5, G13, G20 and P18) indicates the necessity for further research on antagonism by *N. lolii* before possible application of the most effective strains in biological protection of plants against abiotic and biotic stresses. Pot and plot experiments should follow the laboratory study to check the endophyte-pathogen relationship in a natural habitat. Relationships observed *in situ* are not usually as distinct and significant as relationships observed *in vitro* (Christensen 1996, Wäli et al. 2006). In natural habitats the final "shape" of the relationship results from the host-plant's genotype, the symbiont's genotype and the environment (Philips and Schardl 1997, Cheplick and Cho 2003, Popay and Bonos 2005). Finally, the mechanism of introduction of the symbiont into the plant must be developed, and conditions which guarantee the symbiont's continuous presence in plant tissues should be identified. New plant-endophyte associations developed artificially are often stable only for a short time because of the small degree of "compatibility" of the partners (Easton et al. 2001, Bouton et al. 2002).

Conclusions

1. In samples from Poland, less than 50% of ecotypes of *Lolium perenne* were colonized by *Neotyphodium lolii*.
2. *Neotyphodium lolii* showed antagonism towards other fungi.
3. There were differences between isolates of *N. lolii* in their antagonistic properties. High to moderate antifungal activity was observed *in vitro* in less than 50% of *N. lolii* isolates. Other isolates were less active or inactive.
4. Most antifungal activity of *N. lolii* was demonstrated in paired cultures with *Bipolaris sorokiniana*, *Rhizoctonia cerealis* and *R. solani*.

Streszczenie

WYSTĘPOWANIE *NEOTYPHODIUM LOLII* I JEGO ANTAGONIZM W STOSUNKU DO INNYCH GRZYBÓW

Celem przeprowadzonych badań było określenie zasiedlenia kolekcji ekotypów życicy trwałej (*Lolium perenne*) przez *Neotyphodium lolii* oraz właściwości antagonistyczne pozyskanych izolatów endofita w stosunku do wybranych grzybów *in vitro*. Grzybnię *N. lolii* wykryto w 10 ekotypach na 22 przebadane. Obydwie zastosowane metody detekcji – barwienia różem bengalskim i analiza morfologiczna oraz amplifikacji DNA metodą PCR okazały się równie skuteczne. Cztery izolaty endofita (G5, G13, G20, P18) hamowały wzrost wszystkich testowanych mikroorganizmów na zbliżonym poziomie w temperaturze 10 i 25°C. Największe strefy zahamowania wzrostu obserwowano w kombinacjach z *Bipolaris sorokiniana, Rhizoctonia solani* i *R. cerealis*. Najczęściej hamowanym patogenem był *Fusarium equiseti.*

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Author's address:
Dr. Dariusz Pańka, Department of Phytopathology, University of Technology and Life Sciences, ul. Kordeckiego 20, 85-225 Bydgoszcz, Poland, e-mail: panka@utp.edu.pl

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