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GRAMINICOLOUS FUNGI FROM POLAND 2. INTERACTIONS OF INTERNAL FUNGI ISOLATED FROM *PUCCINELLIA DISTANS* AND THEIR SALT TOLERANCE

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Abstract: Internal fungi (endophytes [1]) – *Colletotrichum capsici* (Syd.) Butl. & Bisby and an anamorph of *Epichloë typhina* (Pers.: Fr.) Tul. & C. Tul. [= *Neotyphodium typhinum* (Morgan-Jones & W. Gams) Glenn, C. W. Bacon & Hanlin] – were isolated from sheaths of *Puccinellia distans* (Jacq.) Parl. growing on salinated substrate (NaCl over 4.0 mS/cm^3). Preliminary studies indicated that *Colletotrichum capsici*, causing anthracnose in grass, has higher salt tolerance than the anamorph of *E. typhina*. Biotic series tests showed that the anamorph suppressed *Colletotrichum capsici* growth. The size of the inhibition zone between these fungi *in vitro* suggests that the *E. typhina* anamorph can successfully suppress *C. capsici* infection and growth inside the grass sheaths. Suppression was correlated with the salinity level.

Key words: endophytes, *Epichloë*, *Neotyphodium*, *Colletotrichum*, *in vitro* culture, inhibition zone, halophyte, *Puccinellia distans*, Poland

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INTRODUCTION

Halophytes can be considered as plants well adapted to saline environments such as salt marshes and inland salines. Plants can be colonized by parasitic and saprotrophic fungi inhabiting plant organs above the soil, as well as by internal fungi[1] living inside the same organs and by mycorrhizal fungi inhabiting roots. Kohlmeyer and Gessner (1976), Gessner and Kohlmeyer (1976) and Gessner (1977) recorded both marine and terrestrial filamentous fungi on the aerial parts of salt marsh *Spartina alterniflora* Loisel. Hołownia (1972, 1973) noted three rust species of the genus *Uromyces* (Link) Unger on halophytes growing in Poland.

Some halophytes such as *Suaeda fruticosa* Delile and *Salicornia perennis* Mill. from temperate regions have been studied for their internal fungi associations (Petrini & Fisher 1986; Fisher & Petrini 1987). Some internal fungi are also reported from halophytes of estuarine mangrove forest in India (Suryanarayanan & Kumaresan 2000; Kumaresan & Suryanarayanan 2001). So far the occurrence of internal fungi in halophytic grasses has been almost completely unresearched. Except for information on molecular detection of an anamorph of *Epichloë typhina* (Pers.: Fr.) Tul. & C. Tul. (Lembicz *et al.* 2001), no studies of the occurrence of internal fungi in halophytic grasses have been published. There are many reported investigations of mycorrhiza of halophytes (e.g., Aliasgharzdeh *et al*. 2001). Johnson-Green *et al*. (2001) investigated the influence of salt on mycorrhizal colonization of *Puccinellia nutalliana* (Schult.) Hichc.

According to White (1987), many species of Poaceae could harbor internal fungi. Most of the recent studies have led to the description of new species of *Neotyphodium* Glenn, C. W. Bacon & Hanlin. Unfortunately, many internal fungi remain unidentified. Only a few studies deal with internal fungi of wild species of Poaceae (Naffaa *et al* 1998; Bucheli & Leuchtmann 1996; Saikkonen *et al.* 1999, Brem & Leuchtmann 2001).

[1] Applied to fungi, the general term 'endophyte' is somewhat misleading. I prefer the term 'internal fungi'.

The occurrence of ascomycete fungi on *Puccinellia distans* (Jacq.) Parl. has been described occasionally (Schroeter 1889, 1908; Kochman & Majewski 1973; Majewski 1979; Lembicz 1998; Chlebicki & Lembicz 2001). Chlebicki and Lembicz (2001) noted some fungi growing on stems and inflorescence of this plant. *Colletotrichum capsici* (Syd.) Butl. & Bisby causing anthracnose was the most frequently observed fungus on the host plant. Plant populations growing in anthropogenic habitats were found to be inhabited by nonspecific ubiquists, fungi species related to monocotyledonous plants, and species occurring mainly on grasses. The number of fungus species decreased with increasing salinity of habitats, and saprotrophic fungi were more salt-sensitive than parasitic fungi. Recently Lembicz *et al.* (2001) used PCR technique to find an internal fungus of *Puccinellia distans* that can be considered as anamorph of *Epichloë typhina* [= *Neotyphodium typhinum* (Morgan-Jones & W. Gams) Glenn, C. W. Bacon & Hanlin, formerly *Acremonium typhinum* Morgan-Jones & Gams var. *fasciculatum* White; see Glenn *et al*. 1996].

In Poland, natural inland salines are distributed in the Wielkopolska and Małopolska regions (Wilkoń-Michalska 1963; Trzcińska-Tacik 1988). *Puccinellia distans* subsp. *distans* is a facultative halophyte which colonizes anthropogenic habitats in Central Europe, such as secondary salinated soils along roads and railroads, waste ground and salinated areas rich in nitrogen compounds (Mirek & Trzcińska-Tacik 1981; Dettmar 1993; Jackowiak 1996; Lembicz 1998).

The aim of this study was to identify the fungi present in tissues of host plant organs, and to examine the interrelations between internal fungi as well as their salinity tolerance. The present paper continues those studies on fungi of *Puccinellia distans* (Chlebicki & Lembicz 2001).

MATERIAL AND METHODS

Host plants were collected from anthropogenic habitats at eight localities (Table 1). Fresh plant material was transported in sealed plastic bags and kept refrigerated.

Table 1. Characteristic of analysed habitats with halophyte *Puccinellia distans* and presence of endophytes.

Locality \ Species	*Colletotrichum capsici*	*Neotyphodium typhinum*
Kłodawa	+	
Ciechocinek	+	
Góra	+	
Janikowo	+	+
Jacewo	+	
Wapno	+	+
Pakość	+	
Szarlej	+	

Endophytic fungi were isolated by a standard method (see Leuchtmann & Clay 1988, An *et al.* 1993). Three pieces of sheath *ca* 9 cm long were taken from each plant with sterilized scalpels. All sheaths were surface-sterilized 60 sec in 90% ethanol, followed by 3 min in 5% NaOCl and 30 sec in 90% ethanol. The sheaths were divided into three parts *ca* 2 cm long and then transferred aseptically to Petri dishes 9 cm in diameter containing Ferency medium with oxytetracycline 60 mg/1000 ml. Three dishes per locality were incubated on a laboratory bench at room temperature. After 10 days the fungal isolates were transferred to Petri dishes containing potato dextrose agar (PDA) and incubated 7–30 (45) days, and the fungi growing on them were identified.

All further experiments employed PDA medium, were performed in 6–10 replicates, and used only strains of *Colletotrichum capsici* from Szarlej and an anamorph of *Neotyphodium typhinum* from Wapno.

Some preliminary experiments were done to study the relationships between the growth of single isolates and increasing amounts of salt 0%, 1% and 5% NaCl in the PDA medium. Strains of both fungi were tested. Small pieces (*ca* 10–15 mm^2) of mycelium from the isolate were put in the center of a Petri dish, and their radial growth in each treatment was recorded after 3, 7, 10, 14 and 21 days.

The interrelations between internal fungi were investigated with the biotic series methods of Mańka (1974) and Mańka and Mańka (1995). Small pieces of mycelium (10–15 mm^2) of the two tested fungi were placed on PDA medium 2 cm apart, with the same salt concentrations as in the preliminary experiments. The inhibition zone between the two fungi colonies was taken to

be the distance between the colony edges. The test results were evaluated on the scale given by Mańka (1974) after 10 days and again after 30 days.

Colonies and fungal structures were observed under Nikon SM 1500 and Nikon Labophot 2 microscopes. The colonies were photographed with a Nikon Coolpix 995 camera.

RESULTS

Two species of internal fungi were isolated from leaf sheaths of *Puccinellia distans* (Table 1), but never both from the same leaf sheath. They are characterized as follows:

1. *Colletotrichum capsici* (Syd.) Butl. & Bisby: colonies pale mouse gray in color, reverse at first pale pink with black edges, later completely dark brown, sclerotia absent, conidia falcate, hyaline 20–24(–25) × 3–4 μm; appresoria oval to clavate, brown 10–15 × 8–12 μm. On Ferency agar, colonies grow 0.5 cm after 5 days, 2–4 cm after 7 days; on PDA, colonies grow 8 cm after 16 days and 9 cm after 24 days.

2. *Neotyphodium typhinum* (Morgan-Jones & W. Gams) Glenn, C.W. Bacon & Hanlin: colonies white, cottony to somewhat tufted, vegetative hyphae 1–2 μm wide. On Ferency medium, colonies grow 0.3 cm after 7 days, 1.5 cm after 24 days; on PDA, colonies grow 5 cm after 38 days.

The salt tolerance and sensitivity of the investigated species differed significantly. Growth of *Colletotrichum capsici* was strongly suppressed in medium with 5% salinity (Fig. 1) as compared with its growth at 1% salinity (Table 2). The anamorph of *Neotyphodium typhinum* showed a gradual decline in growth with increasing amounts of NaCl (Fig. 2). The reaction to the duration of salt

Table 2. The colony diameters of *Colletotrichum capsici* and *Epichloë typhina* after 10 days of incubation at different NaCl concentrations in PDA medium [mm].

Species \ Isolate	0%	1% NaCl	5% NaCl
Colletotrichum capsici	68.5	60.6	31.6
Epichloë typhina	27.0	18.33	12.4

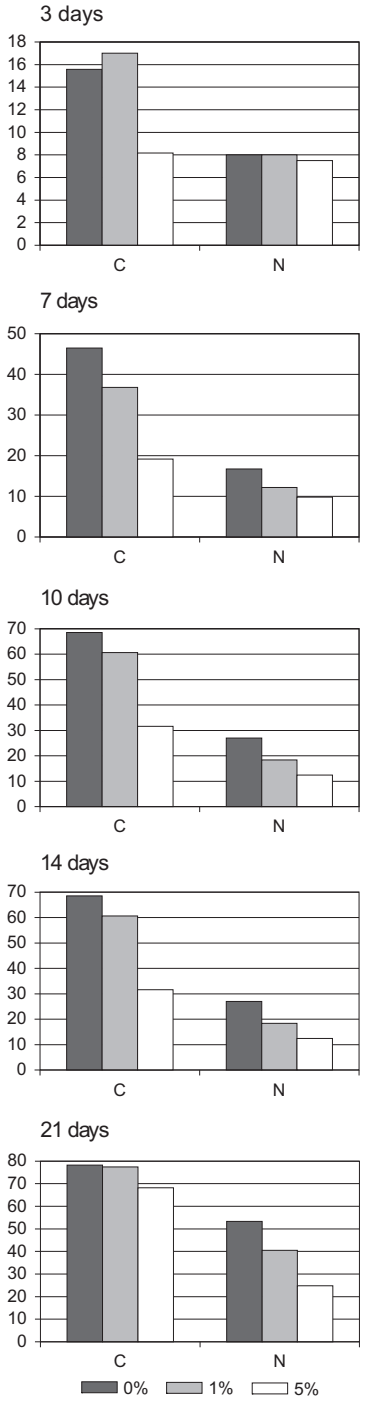

Fig. 1. Diameter [mm] of colonies of *Colletotrichum capsici* (C) and anamorph of *Epichloë typhina* (N) after 3, 7, 14 and 21 days in medium with various NaCl concentrations.

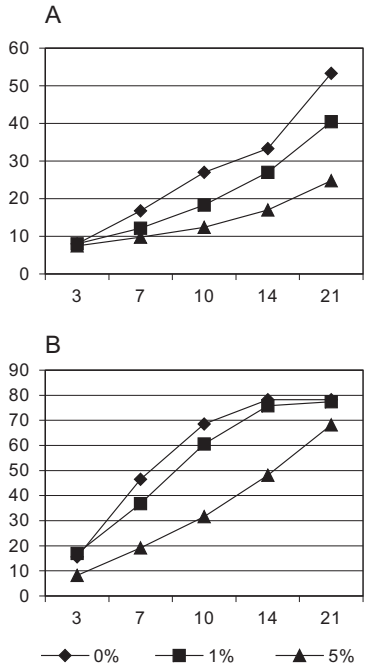

Fig. 2. Relation of growth of *Epichloë typhina* (A) and *Colletotrichum capsici* (B) with increasing amounts of NaCl after 3, 7, 10, 14 and 21 days. X axis – days, Y axis – diameter of fungus colony (mm).

stress also differed between the fungi. The growth of *Neotyphodium typhinum* was increasingly suppressed with the duration of salt stress, inversely than *Colletotrichum capsici* (Fig. 2).

Table 3 presents data on the *in vitro* antagonism between *Colletotrichum capsici* and *Neotyphodium typhinum*, evaluated with biotic tests (Mańka 1974; Mańka & Mańka 1995). The inhibition zone in these tests results from the antagonistic activity of the two investigated fungi growing on the same Petri dish.

Table 3. Evaluation of individual biotic effect (IBE) of anamorph of *Epichloë typhina* on *Colletotrichum capsici* growth. IBE 1 – individual biotic effect and evaluation of inhibition zone (1) according to Mańka (1974). IBE 2 – individual biotic effect and evaluation of inhibition zone (2) according to Mańka and Mańka (1995)

Salt concentration	Surrounding of colony	Inhibition zone (1)	Inhibition zone (2)	Reduction of colony size	IBE 1	IBE 2
0%	0	-5	-22	-1	-6	-23
1%	0	-6	-30	-2	-8	-32
5%	0	-10	-33	-3	-13	-36

The influence of salt concentration on individual biotic effects was also investigated (Fig. 3). The inhibition zone between the investigated fungi varied with the salinity level. The inhibitive reaction was particularly strong at 5% salinity (Table 3); the zone was narrower in pure PDA medium (Table 4) and increased with increasing NaCl. The measured inhibition zones were 3–5 times wider than the median diameter of the grass sheath (2 mm).

Table 4. Distance between colonies' edges of *Colletotrichum capsici* and anamorph of *E. typhina* in various salt (NaCl) concentrations.

Salt concentration	Distance [mm]
0%	4–5
1% NaCl	5–6
5% NaCl	10–11

DISCUSSION

Cabral *et al*. (1993) stated that the term 'endophytic infections' should be used in a broad sense, including any fungi isolated from tissues of a symptomless plant. The anamorph of *Epichloë typhina* is a typical systemic parasitic fungus living mostly inside plant sheaths. However, examination of the growth pattern of *Epichloë/Neotyphodium* hyphae has shown that tissue penetration occurs solely through physical pressure and growth into existing spaces between cells of the host plant (Christensen *et al*. 2002). *Colletotrichum capsici* can be recognized rather as a latent pathogen, whereas the similar species *Colletotrichum dematium* (Pers.: Fr.) Grove is saprotrophic (see Sutton 1980). *Colletotrichum capsici* has been noted frequently on the surface of leaves, sheaths, the base of culms and panicle branches of *Puccinellia distans*, but the fungus has not been found in strongly

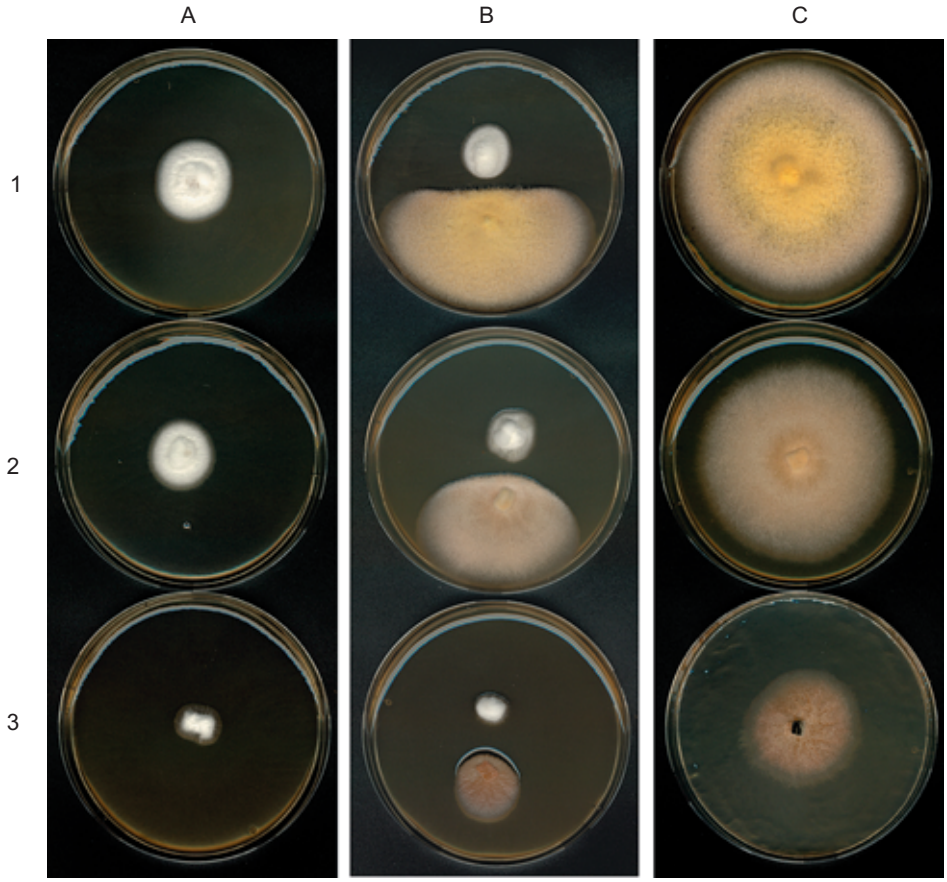

Fig. 3. Biotic test of *Epichloë typhina* versus *Colletotrichum capsici*. A – *Epichloë typhina*, B – dual cultures of both species, C – *Colletotrichum capsici*; 1 – 0% NaCl, 2 – 1% NaCl, 3 – 5% NaCl.

salinated areas in Inowrocław and Ciechocinek, whereas *Neotyphodium typhinum* occurs in moderately as well as in strongly salinated areas (Chlebicki & Lembicz 2001). Only fructifications of *Pleospora herbarum* (Pers.: Fr.) Rabenh., *Puccinia brachypodii* G. Otth var. *poae-nemoralis* (G. Otth) Cummins & H. C. Greene and *Dinemasporium strigosum* (Pers.: Fr.) Sacc. were noted in these strongly salinated places. Pugh and Beeftink (1980) reported that increasing salinity levels adversely affect growth and cellulose decomposition by *Gliocladium roseum*, a species not adapted to saline soil.

Stowell (1995) demonstrated that salt accumulation in the soil causes *Colletotrichum* to produce anthracnose in grasses if salinity is higher than 2.7 dS/m. The soil in the vicinity of *Puccinellia distans* was markedly salinated, over 4.0 dS/m. *Colletotrichum capsici* showed distinct salinity tolerance in artificial cultures *in vitro* with 1% NaCl (Figs 1, 2), but fungal growth was adversely affected by a concentration of 5% NaCl.

When we consider the size of the grass sheaths, the size of the inhibition zone we observed *in vitro* is an important character. Of course, observations *in vitro* may vary a great deal from those *in vivo*, especially as the intercellular compartments of plant living tissues and PDA medium contain completely different substances. However, the very distinct inhibition zone observed between the

investigated fungi suggests that they cannot occur together in the same sheath. Isolation data supported this view. The fungi were isolated separately. The diameter of the sheaths (*ca* 2 mm) does not exceed the size of the inhibition zone. This may be why the mycelia of antagonistic fungi form structures opposite to each other or else complex patterns, as in the case of *Phaeosphaeria fuckelii* (Niessl) Holm and *Colletotrichum dematium* observed *in vivo* in *Calamagrostis* (see Chlebicki 1993). The size of the inhibition zone indicates that *Neotyphodium typhinum* can successfully suppress the invasion and growth of *Colletotrichum capsici* inside grass sheaths. Probably that is why they not occur together in the same grass sheath. An increase in environmental salinity would affect the balance between these species.

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