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Mycobiota in Needles and Shoots of *Pinus nigra* following Infection by *Dothistroma septosporum*

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With 1 Figure

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Summary

KOWALSKI T. & DROŻYŃSKA K. 2011. Mycobiota in needles and shoots of *Pinus nigra* following infection by *Dothistroma septosporum*. – Phyton (Horn, Austria) 51 (2): 277–287, with 1 figure.

A 25-year-old *Pinus nigra* ARN. seed plantation in Miechów Forest District, in the south of Poland, was studied. The *P. nigra* had shown symptoms of red band needle blight caused by *Dothistroma septosporum* (DOROG.) MORELET (*Mycosphaerellaceae*) since 1997. This disease leads to premature needle death and defoliation followed by dying of twigs and branches. The aims of the study were: 1. to recognize the extent to which needle infection by *D. septosporum* affects the structure of the needle endophyte community and colonization by other fungi which may contribute to needle loss, and 2. to determine whether stress caused by *D. septosporum* infection over many years facilitates latent infection of shoots by other potential pathogens, including contributors to bark and cambium necroses. Mycological analyses were done on three groups of *P. nigra* needles, i.e. A type needles (living, symptomless), B type (living, with necrotic bands typical of *D. septosporum* infection) and C type (prematurely dead as a result of primary infection by *D. septosporum*), and on living, 1-4-year-old shoots. Needles and shoots were colonized by 47 and 38 fungal species, respectively. *Anthostomella formosa, Cenangium ferruginosum, Cyclaneusma minus, Lophodermium pinastri, Sclerophoma pythiophila* and *Verticicladium trifidum* colonized more than 10% of needles. Fungi occurred at sig-

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nificantly different frequencies in needles with different health status. *Cystodendron* sp., *Fusicoccum* sp., *Geniculosporium serpens, Mollisia cinerea, Pezicula eucrita, Phialocephala* cf. *dimorphospora, Phomopsis occulta, Sirodothis* sp.1 and *Therrya pini* colonized more than 10% of shoots. Among the well-known causal agents of diseases of shoots, *C. ferruginosum* was recorded only sporadically, *Gremmeniella abietina* and *Sphaeropsis sapinea* were not isolated at all. The significance and importance of individual fungi in induction of diseases of needles and of shoot dieback are discussed.

Zusammenfassung

KOWALSKI T. & DROŻYŃSKA K. 2011. Mycobiota in needles and shoots of *Pinus nigra* following infection by *Dothistroma septosporum*. [Mykobionten in Nadeln und Trieben von *Pinus nigra* nach Infektion mit *Dothistroma septosporum*]. – Phyton (Horn, Austria) 51 (2): 277–287, mit 1 Abbildung.

Die Untersuchungen wurden in einer 25 Jahre alte *Pinus nigra* ARN. Samenzucht-Anlage im Forstgebiet von Miechów, Südpolen durchgeführt. Seit 1997 zeigte *P. nigra* Symptome der Rotband-Nadelbleiche, die durch *Dothistroma septosporum* (DOROG.) MORELET (*Mycosphaerellaceae*) hervorgerufen wird. Diese Krankheit führt zu einem vorzeitigen Absterben und Abfallen der Nadeln und einem darauf folgendem Absterben der Zweige und Äste. Der Zweck der Untersuchung war: 1. Zu erkennen, inwieweit eine Infektion mit *D. septosporum* die Zusammensetzung der endophytischen Nadelbewohner beeinflusst und die Ansiedelung anderer Pilze, die zum Nadelabfall führen, begünstigt und 2. ob der jahrelange Stress durch die *D. septosporum* Infektion latente Krankheiten der Triebe durch potentielle Pathogene, die mitverantwortlich für Borken- und Kambium-Nekrosen sind, erleichtert. Die mykologischen Untersuchungen wurden an drei verschiedenen Nadeltypen durchgeführt: A-Typ-Nadeln (lebend, ohne Symptome), B-Typ-Nadeln (lebend mit roten Bändern, typisch für *D. septosporum*-Infektionen) und C-Typ-Nadeln (vorzeitig abgestorben als Folge der Infektion mit *D. septosporum*) und an 1 bis 4 jährigen lebenden Trieben. Nadeln und Triebe waren mit 47 bzw. 38 Pilzarten besiedelt. *Anthostomella formosa, Cenangium ferruginosum, Cyclaneusma minus, Lophodermium pinastri, Sclerophoma pythiophila* und *Verticicladium trifidum* besiedelten mehr als 10% der Nadeln. Die Pilzdichte war abhängig vom Gesundheitszustand der Nadeln. *Cystodendron* sp., *Fusicoccum* sp., *Geniculosporium serpens, Mollisia cinerea, Pezicula eucrita, Phialocephala* cf. *dimorphospora, Phomopsis occulta, Sirodothis* sp.1 und *Therrya pini* besiedelten mehr als 10% der Triebe. Unter den gut bekannten Auslösern von Triebkrankheiten wurde *C. ferruginosum* nur sporadisch beobachtet, *Gremmeniella abietina* und *Sphaeropsis sapinea* wurden überhaupt nicht gefunden. Bedeutung und Wichtigkeit der einzelnen Pilzarten für das Auslösen von Nadelkrankheiten und Triebsterben werden diskutiert.

Introduction

Pinus nigra ARN. is not native to Poland. It was introduced mostly in the industrial regions. Its survival rate in certain regions was not high because of its susceptibility to fungal pathogens. The most common and important pathogen was *Gremmeniella abietina* (LAG.) MORELET, *Helotiaceae* (KOWALSKI 1990, KOWALSKI & DOMAŃSKI 1983).

Red band needle blight caused by *Dothistroma septosporum* (DOROG.) MORELET (*Mycosphaerellaceae*) was observed in Poland for the first time in 1990 (KOWALSKI & JANKOWIAK 1998). In Poland, as in other countries (BUTIN 1984, KIRISITS 2010), *P. nigra* proved to be very susceptible to infection by the fungus. In some stands all trees show symptoms of severe infection, including discoloration of needles leading to their premature dying and defoliation followed by dying of branches, particularly in the lower crown (KOWALSKI & al. 1998). Studies on genetic diversity of Polish isolates of *D. septosporum* are currently in progress (KRAJ & KOWALSKI unpubl.).

The aims of the studies presented in this paper were: 1. to recognize the extent to which needle infection by *D. septosporum* affects the structure of the needle endophyte community and colonization by other fungi which may contribute to needle loss, and 2. to determine whether stress caused by *D. septosporum* infection over many years facilitates latent infection of twigs by other potential pathogens, including contributors to bark and cambium necroses.

Material and Methods

The study was made in a seed plantation of *P. nigra* in Miechów Forest District (N 50 12 47,7; E 20 01 37,7), 20 km north-east of Krakow. The plantation was established in highland forest in 1986 when 8096 trees were planted at 3 x 3 m spacing in an area of 6.72 ha. Each year since 1997 the plantation has shown symptoms of *D. septosporum* red band needle blight including premature dying and defoliation followed by dying of branches (KOWALSKI & al. 1998, KOPEĆ 2006).

In 2008, thirty trees were randomly chosen in various parts of the plantation. One branch with symptoms of red band needle blight (discoloration of needles and partial defoliation) was collected from the lower crown of each tree. The samples were taken to the laboratory, stored at 5 °C and processed within 24 h. Needles and branches 1–4 years old were selected for study. One needle representing each of the four age groups (1-, 2-, 3- and 4-year-old) and each of three health status categories, i.e. A type needles (living, symptomless), B type needles (living, with necrotic bands typical of *D. septosporum* infection) and C type needles (prematurely dead as a result of primary infection by *D. septosporum*), were selected for mycological analyses. After removal of all needles, shoots were washed in tap water using a brush and divided into four pieces according to age (1-, 2-, 3- and 4-year-old pieces). Needles and shoots were surface-sterilized by soaking in 96% ethanol for 60 s, then in a solution of sodium hypochlorite (approx. 8% of available chlorine) for 5 min and finally in 96% ethanol for 30 s. After drying in layers of blotting paper, each needle was cut into six parts and each part was placed in a Petri dish containing 2% malt extract agar (MEA, Difco). Six pieces (3 x 2 mm each) of the outer + inner bark were sampled from each shoot from each of the four age groups (1-, 2-, 3- and 4-year-old). There were 120 needles of each type (A, B and C), totaling 360 needles (2160 pieces) and 120 shoots (720 pieces) for analysis. Cultures were incubated at 20 °C in darkness and examined initially every 3-4 d, then weekly for 3 months. Colonies growing from pieces of needles or shoots were subcultured and identified on the basis of their morphology.

The frequency of occurrence of individual fungal species was expressed as the percentage of needles or shoots (bark fragments) colonized. The percentage of shoot pieces (1-, 2-, 3- or 4-year-old) colonized by each individual fungus was calculated to show its distribution in the shoot.

Statistical analysis was completed for the most frequent species of fungi applying the Chi-squared test (STANISZ 1998).

Results

A total 1821 fungal isolates was obtained from all *P. nigra* needles analysed. The largest number of isolates was obtained from the C type needles and the smallest from A type needles (Table 1). The average number of isolates obtained from one needle was in the range 3.4-7.1, depending on needle age (Fig. 1). Needles were colonized by 47 species of fungi. Most diversity was recorded in the C type needles and least diversity in A type needles (Table 1). The majority of fungi belonged to Ascomycota. Only four ascomycete species, *Anthostomella formosa, A. pedemontana, Cyclaneusma minus* and *Sordaria fimicola*, formed teleomorphs in vitro. Others formed anamorphs only. Two taxa represented Basidiomycota.

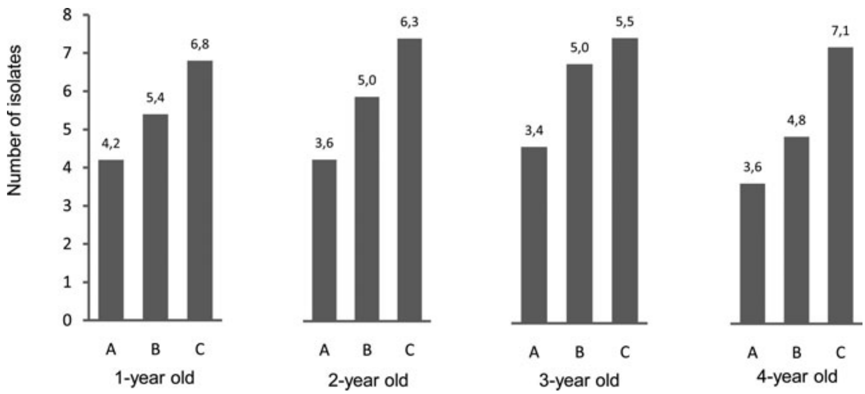

Fig. 1. Average number of fungal isolates obtained from each 1-4-year-old-needles of *P. nigra* (A – living symptomless, B-living with necrotic spots, C – dead needles).

Each of the species *Anthostomella formosa, Cenangium ferruginosum, Cyclaneusma minus, Lophodermium pinastri, Sclerophoma pythiophila* and *Verticicladium trifidum* colonized more than 10% of *P. nigra* needles. The remaining fungi colonized the needles more rarely (Table 1).

Among the most common fungi only *V. trifidum* colonized the different types of needles with similar frequencies (Table 1). Frequency of occurrence of other fungi depended on needle type. *Anthostomella formosa* was the most frequent in A type needles. This fungus was 1.5 times less frequent in B type needles and 14-times less frequent in C type needles. The

Table 1. Fungi isolated from *Pinus nigra* needles differing in health status.

Different letters indicate significant differences according to the chi-squared test (p = 0.05).

Fungi	Colonised needles (%)			
	living symptomless (type A)	living with necrotic spots (type B)	dead (type C)	total
Alternaria alternata (FR.) KEISSLER		1.7	7.5	3.1
Anthostomella formosa KIRSCHST.	70.0 c	44.2 b	5.0 d	39.7 a
Anthostomella pedemontana FERR. & SACC.	6.7	5.0	1.7	4.4
Apiospora montagnei SACC.	1.7	3.3	3.3	2.8
Aureobasidium pullulans (DE BARY) ARNAUD	1.7	5.8	1.7	3.1
Basidiomycetes		1.7		0.6
Cenangium acuum COOKE & PECK		0.8		0.3
Cenangium ferruginosum FR.: FR.	25.8 a	64.2 c	51.7 e	47.2 b
Ceuthospora pinastri (FR.) HÖHN.	0.8	0.8	6.7	2.8
Cladosporium cladosporioides (FRESEN.) DE VRIES	3.3	3.3	2.5	3.1
Cladosporium herbarum (PERS.) LINK & S. F. GRAY			0.8	0.3
Coniochaeta ligniaria (GREV.) MASSEE		0.8		0.3
Coniothyrium fuckelii SACC.		5.0	8.3	4.4
Coniothyrium sp.		2.5	10.0	4.2
Cyclaneusma minus (BUTIN) DICOSMO, PEREDO & MINTER	6.7 d	32.5 a	33.3 a	24.2 c
Cystodendron sp.			0.8	0.3
Didymosphaeria igniaria BOOTH	0.8			0.3
Epicoccum nigrum LINK		1.7	9.2	3.6
Exophiala sp.	0.8			0.3
Fusarium sp.			0.8	0.3
Geniculosporium serpens CHESTERS & GREENHALGH	4.2	3.3	5.0	4.2
Hypoxylon cf. *udum* (PERS.) FR.	0.8	0.8	1.7	1.1
Lecytophora hoffmannii (VAN BEYMA) W. GAMS & MCGINNIS	3.3	3.3	1.7	2.8
Lophodermium pinastri (SCHRAD. & HOOK) CHEV.	0.8 g	9.2 df	72.5 c	27.5 c
Lophodermium seditiosum MINTER, STALEY & MILLAR	2.5	4.2	5.0	3.3
Mollisia cinerea (BATSCH: FR.) KARST.	0.8			0.3
Pestalotiopsis funerea DESM.	0.8	0.8	1.7	1.1
Phialophora melinii (NANNF.) CONANT	0.8	0.8		0.6
Phoma pinastrella SACC.	1.7	1.7	0.8	1.4
Phomopsis occulta TRAV.			0.8	0.3
Rhizoctonia solani KÜHN.		4.2	1.7	1.9
Sclerophoma pythiophila (CORDA) HÖHN.	1.7 g	25.0 a	13.3 f	13.3 d
Sordaria fimicola (ROBERGE ex DESM.) CES. & DE NOT.	8.3		4.2	4.2
Sporormia intermedia AUERSW.	5.8	3.3	3.3	4.2
Sporothrix sp.		1.7	0.8	0.8
Torula sp.		1.7	1.7	1.1
Trimmatostroma cf. *abietis* BUTIN & PEHL		0.8	0.8	0.6
Verticicladium trifidum PREUSS	11.7 df	10.0 df	9.2 df	10.3 d
Xylaria sp.	0.8	0.8	2.5	1.4
Not sporulating fungi	13.3	35.0	24.2	24.2
Number of needle (needle pieces) analysed	120 (720)	120 (720)	120 (720)	360 (2160)
Number of isolates (fungal species)	446 (30)	604 (36)	771 (40)	1821 (47)

frequency of occurrence of other common fungi was greater in B type needles than in A type needles. *Lophodermium pinastri* was 8 times more frequent in C type needles than in B type needles. Differences in frequency of *Cyclaneusma minus* in B type and C type needles were not statistically significant. *Cenangium ferruginosum* and *S. pythiophila* were more frequent in B type needles than in C type needles (Table 1). *Alternaria alternata, Ceuthospora pinastri, Coniothyrium* spp. and *Epicoccum nigrum* were more frequent in C type needles (Table 1).

Table 2. Fungi isolated from living, 1-4-year-old shoots of *Pinus nigra*.

Fungi	Shoot pieces number (%)	Shoots number (%)
Asterosporium asterospermum (PERS.) S. HUGHES	1 (0.1)	1 (0.8)
Cenangium ferruginosum FR.: FR.	1 (0.1)	1 (0.8)
Coniochaeta ligniaria (GREV.) MASSEE	12 (1.7)	6 (5.0)
Coniothyrium fuckelii SACC.	2 (0.3)	2 (1.7)
Crumenulopsis pinicola (REBENT.) J. W. GROVES	16 (2.2)	8 (6.7)
Cystodendron sp.	73 (10.1)	39 (32.5)
Fusicoccum sp.	21 (6.5)	24 (20.0)
Geniculosporium serpens CHESTERS & GREENH.	21 (2.9)	14 (11.7)
Lecytophora hoffmannii (VAN BEYMA) W. GAMS & MCGINNIS	9 (1.3)	8 (6.7)
Mollisia cinerea (BATSCH: FR.) KARST.	83 (11.5)	37 (30.8)
Mollisia spp. (4 species)	30 (4.2)	23 (19.2)
Pezicula eucrita P. KARST.	105 (14.6)	58 (48.3)
Pezicula cinnamomea (DC.) SACC.	3 (0.4)	1 (0.8)
Phialocephala cf. *dimorphospora* W. B. KENDR.	37 (5.1)	26 (21.7)
Phialophora spp. (2 species)	24 (3.3)	12 (10.0)
Phomopsis conorum (SACC.) DIED.	29 (4.0)	11 (9.2)
Phomopsis occulta TRAV.	38 (5.3)	15 (12.5)
Rhizoctonia solani KÜHN	1 (0.1)	1 (0.8)
Sclerophoma pythiophila (CORDA) HÖHN.	2 (0.30)	1 (0.8)
Scoleconectria cucurbitula (TODE) C. BOOTH	1 (0.1)	1 (0.8)
Sirodothis sp. 1	169 (23.5)	59 (49.2)
Sirodothis sp. 2	3 (0.4)	2 (1.7)
Sordaria fimicola (ROB. ex DESM.) CES. & DE NOT.	4 (0.6)	2 (1.7)
Sporormia intermedia AUERSW.	2 (0.3)	1 (0.8)
Therrya pini (ALB. & SCHWEIN.) HÖHN.	41 (5.7)	21 (17.5)
Trimmatostroma cf. *abietis* BUTIN & PEHL	5 (0.7)	3 (2.5)
Verticicladium trifidum PREUSS	5 (0.7)	2 (1.7)
Xylaria sp.	7 (1.0)	4 (3.3)
Not sporulating fungi (4 species)	6 (0.8)	6 (5.0)
Number of shoots /shoot pieces analysed	720	120

A total of 779 isolates of fungi representing 38 taxa was isolated from shoots of *P. nigra* (Table 2). More than 10% of shoots were colonized by *Cystodendron* sp., *Fusicoccum* sp., *Geniculosporium serpens, Mollisia ci-*

nerea, Pezicula eucrita, Phialocephala cf. *dimorphospora, Phomopsis occulta, Sirodothis* sp.1 and *Therrya pini* (Table 2). Data on the distribution of fungi in shoot pieces of different age groups show that three fungal species were the most common, i.e. *Sirodothis* sp.1 (23.5% of pieces colonized), *P. eucrita* (14.6%) and *Cystodendron* sp. (10.1%). Distribution of the nineteen fungal taxa was very limited. They colonized less than 1% of shoots pieces (Table 2).

The most frequent species of fungi all colonized *P. nigra* shoots irrespective of their age (Table 3). Some differences in their frequencies of occurrence were observed, however. Two-year-old shoots were mostly colonized by *Pezicula eucrita*, and shoots in other age groups (I, II and IV) mostly by *Sirodothis* sp.1. The frequencies of *Cystodendron* sp., *Geniculosporium serpens* and *Mollisia cinerea* increased as the shoots aged. *Fusicoccum* sp., *Phomopsis occulta* and *Therrya pini* were more frequent in the younger shoots (Table 3).

Table 3. Occurrence of fungi in living shoots of *Pinus nigra*, depending on shoot age class (for fungi with frequency >10 %).

Fungi	1-year-old number (%)	2-years-old number (%)	3-years-old number (%)	4-years-old number (%)
Cystodendron sp.	5 (16.7)	8 (26.7)	10 (33.3)	16 (53.3)
Geniculosporium serpens	1 (3.3)	12 (40.0)	4 (13.3)	7 (23.3)
Fusicoccum sp.	9 (30.0)	7 (23.3)	7 (23.3)	5 (16.7)
Mollisia cinerea	5 (16.7)	6 (20.0)	13 (43.3)	13 (43.3)
Pezicula eucrita	15 (50.0)	17 (56.7)	13 (43.3)	13 (43.3)
Phialocephala cf. *dimorphospora*	6 (20.0)	5 (16.7)	10 (33.3)	5 (16.7)
Sirodothis sp. 1	17 (56.7)	13 (43.3)	17 (56.7)	19 (63.3)
Therrya pini	8 (26.7)	6 (20.0)	2 (6.7)	5 (16.7)
Phomopsis occulta	5 (16.7)	5 (16.7)	4 (13.3)	1 (3.3)
Number of shoots analysed	30	30	30	30
Number of fungal isolates	185	192	193	209

Discussion

The health status of *P. nigra* needles from the seed plantation in Miechów Forest District was variable. There were needles that were living and symptomless, living but with local, necrotic bands typical of *D. septosporum* infection, and prematurely dead as a result of *D. septosporum* infection.

Fungi that colonize living and symptomless needles may be considered as endophytes (Petrini 1991). The data presented show that the spectrum of fungal endophytes in *P. nigra* needles may include 30 taxa. The diversity of fungal endophytes was much greater than that of endophytes in needles of *P. nigra* growing in a zone heavily polluted by industrial emissions (KOWALSKI & ZYCH 2002, JURC & al. 1995) or in needles of *P. mugo* growing in the Alps (SIEBER & al. 1999). It was, however, much less than the spectrum of endophytes in needles of *P. sylvestris* in Poland (KOWALSKI & STAŃCZYKIEWICZ 2000).

The data presented show that the *P. nigra* needles are subject to latent infection by a specific community of fungi. Members of the fungal community may occur with different frequency depending on local conditions (JURC & al. 1995, KOWALSKI & ZYCH 2002, ZAMORA & al. 2008). Living and symptomless needles in Miechów, situated in the south of Poland, were colonized mostly by *A. formosa* followed by *C. ferruginosum* and *V. trifidum*. In other locations in Poland the most common were *C. ferruginosum* (KOWALSKI & ZYCH 2002) and in Slovenia *C. ferruginosum* or *Cyclaneusma niveum* (JURC & al. 1995). ZAMORA & al. 2008 showed that tissue health decisively affects the frequency and distribution of certain fungal species. This is supported by the present results, i.e. earlier infection of *P. nigra* needles by *D. septosporum* predisposed them to infection by *C. ferruginosum, C. minus, L. pinastri* and *S. pythiophila*, the frequencies of which increased as needles became diseased. Some of the above-mentioned species may advance the decay and decomposition of tissues begun by *D. septosporum*. *Cyclaneusma minus* is a well-known causal agent of Cyclaneusma needle cast in *P. sylvestris*. This disease may occur even with no earlier primary infection by other pathogens (KOWALSKI 1988, BUTIN 1996). *Sclerophoma pythiophila* is a fungus that often accompanies other pathogens of needles and twigs in pines and other conifers damaged by biotic and abiotic factors. It often appears as a saprotroph or weak pathogen (KOWALSKI & DOMAŃSKI 1983, ZAMORA & al. 2008). *Lophodermium pinastri* is an endophyte that increases in frequency continuously as needles age. Its endophytic activity is followed by its function as a primary saprotroph with significant contribution to needle decomposition (KOWALSKI & STAŃCZYKIEWICZ 2000, DECKERT & al. 2001, ORTIZ-GARCIA & al. 2003, ZAMORA & al. 2008). *Cenangium ferruginosum* is usually regarded as an opportunistic pathogen that kills the bark of shoots weakened by environment, insects and other pathogens. Its pathogenic significance in needles has not, however, been recognized and defined (BUTIN 1996, SANTAMARÍA & al. 2006, ZAMORA & al. 2008). Instead, this fungus may eliminate from its vicinity other serious pathogens of *P. sylvestris* needles, including *Lophodermium seditiosum*, as shown in paired-cultures in vitro (KOWALSKI & LANG 1983). Needles infected by *D. septosporum* were not usually infected by other, more virulent, fungal pathogens of needles. The serious pathogen of pine needles, *L. seditiosum*, occurred only sporadically.

Formation of fruit-bodies by *G. abietina, S. sapinea* and *C. ferruginosum* has been observed on dying shoots of *P. nigra* in the seed plantation in

Miechów very often (KOWALSKI unpubl.). The data show that the first two species were absent in living shoots with needles seriously infected by *D. septosporum*. The endophytic activity of *G. abietina* and *S. sapinea* followed by an increased pathogenic activity of both fungi on trees subjected to environmental stress is well-known (SIEPMANN 1975, STANOSZ & al. 1997). This indicates that shoots of *P. nigra* in the seed plantation in Miechów, under conditions favorable for pathogens, are infected by *G. abietina* and *S. sapinea* by air-borne or rain-splashed spores. Both pathogens lead to dieback of shoots with no earlier latent phase.

Cenangium ferruginosum, on the other hand, was present in living shoots of *P. nigra*, although only sporadically. The possibility of invasion into shoots from needles in which *C. ferruginosum* often lives endophytically cannot be excluded. Such a possibility in *P. sylvestris* and *P. nigra* was pointed out by KOWALSKI & BUDNIK 1977 and JURC & al. 2000.

Fungi known to be causal agents of bark and cambium necrosis that occurs particularly under environmental stress were also recorded in living shoots of *P. nigra*. These include *Pezicula eucrita* and *Phomopsis occulta* (KOWALSKI 1990). Some fungi recorded in shoots may contribute to natural pruning (BUTIN & KOWALSKI 1990, 1992, MINTER 1996).

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