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Effects of Scots pine (*Pinus sylvestris*) natural selection in old foci of *Heterobasidion annosum* root rot

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Abstract: *Heterobasidion annosum* (Fr.) Bref. is a fungal pathogen causing root rot – one of the most economically important diseases in coniferous stands in Europe, Asia and North America. A major objective of this study was to determine if the offspring of self-sown trees from natural regeneration in old foci of root rot is more resistant to infection by this pathogen than the offspring of plus trees. In experiments conducted in the greenhouse above 700 seedlings grown from seeds collected from 60 trees in 6 old foci of the disease (research plots) and 480 seedlings grown from seeds collected in 4 seed orchards were used. The pathogen was represented by 7 different fungal strains. Plus trees, whose grafts are used to establish seed orchards, were the selected elite of forest trees, but their offspring after inoculation with *H. annosum* had significantly worse results than the seedlings developed from the seeds collected in old foci of the disease (research plots). The greatest differences were observed in seedling dry weight. In inoculated seedlings from seed orchards it was 14% lower than in the control, while the dry weight of inoculated seedlings from research plots did not differ significantly from that of non-inoculated seedlings.

Additional key words: forest pathology, forest silviculture, farmland afforestation, resistance, natural selection

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Introduction

Heterobasidion annosum (Fr.) Bref. is a fungal pathogen causing root rot. Beside *Armillaria* root rot, it is one of the most economically important diseases of forest trees in Poland. This disease is well known all over Europe but also in Asia and North America. The pathogen has a wide range of host plant species. It is found in over 200 species of trees and shrubs. The greatest damages, of economic importance, are caused by *H. annosum* in coniferous stands, mostly of the genus *Pinus* (Korhonen and Stenlid 1998; Sierota et al. 2002; Werner and Łakomy 2002a; Mańka 2005; Bendel and Rigling 2008; Scire et al. 2008).

H. annosum spreads in the environment by basidiospores, dikaryotic conidia and mycelium in roots. In suitable conditions, in each of the 3 stages of its development, the pathogen can infect and colonize effectively the root system and initiate the disease (Sierota 1995). Generally, there are 2 possible routes of infection: through wounds and through feeding roots. The first route is observed mostly in older forest stands. Mańka (2005) assigns to this category also the colonization of stumps of recently felled trees. In forest nurseries and young forest plantations, mostly thin roots are infected (Korhonen 1978; Sierota 1995).

H. annosum, because of its exceptional adaptations, enabling the infection of live roots and their wood decay, is sometimes classified as a facultative biotroph (Haars and Hüttermann 1980; Hüttermann et al. 1980). However, in the forest environment it is often outcompeted by saprotrophic and symbiotic microorganisms (Sierota 1995; Napierała-Filipiak 2000). Thus particularly favourable conditions for development of this disease are found in forest stands planted on former fields. The development of *H. annosum* in such conditions is favoured by a higher bioavailability of nitrogen and phosphorus, as well as the lack of natural competitors in the soil. This disease in young pine stands established on former arable lands causes death of a large number of trees. A particularly high number of infections is observed in the plantations that are the second generation of forest on former fields.

Many of the studies conducted in the last 20 years, concerned with the mechanism of resistance coniferous trees to annosum root rot, were conducted on seedlings in forest nurseries and young forest plantations. In the last decade, those investigations started to include also genetic analyses of the DNA of both the pathogen and its host plant. Research on spruce (Asiegbu et al. 1993, 1994, 1995, 1998; Heneen et al. 1994a, 1994b) and pine (Werner 1991a, 1991b, 1993; Werner and Idzikowska 2001) suggests that young conifers may be good materials for research on plant resistance.

It seems that the damages caused by annosum root rot in young forest plantations and later in young forest stands could be reduced if forest management practice involved planting of more resistant seedlings, particularly on former arable lands. Earlier research, conducted in vitro (Werner 1991b), suggests that a higher resistance is observed in, e.g., the offspring of the trees that remained in forest areas where root rot killed many other trees.

Napierała-Filipiak (2000) studied the limiting effect of mycorrhizal fungi on infections of Scots pine seedlings by *H. annosum*. The offspring of 10 young pine trees, self-sown within an old focus of annosum root rot, showed a higher resistance to infection by *H. annosum* in greenhouse conditions than the offspring of trees from a seed orchard in Bolewice (Poland). Pine seedlings developed from seeds of trees of the second generation formed also larger root systems and more easily initiated symbiotic relations with mycorrhizal fungi.

The major aim of this study was to compare the resistance of the offspring of plus trees and the offspring of trees that were naturally reproduced in old foci of the disease. Plus trees, whose grafts are a source of seeds in seed orchards, are distinguished by particularly favourable features in respect of forest management (Giertych 1989). Seedlings developed from seeds from seed orchards are commonly regarded as the best materials for forest regeneration, and are more and more widely used in forest practice.

Material and methods

Plant material

In this study we compared 2 main groups of pine (*P. sylvestris*) seedlings:

1) seedlings grown from seeds collected from self-sown trees (60) in old foci of the disease (6 research plots described in Table 1);
2) seedlings grown from seeds collected in 4 seed orchards (Poland, forest districts: Syców, Łopuchówko, Oborniki Śląskie, and Gniezno), with unknown resistance to the pathogen.

Initially, we surveyed many older pine stands (aged 50–100 years), planted on former fields, where annosum root rot was observed in the past., followed by natural regeneration of forest stands within the old foci of this disease. Finally, for this study, we selected 6 plots (Table 1) that fulfilled the following criteria:

– forest stand dominated by Scots pine, aged at least 50 years;
– earlier occurrence of annosum root rot;
– presence of self-sown pine trees (from natural regeneration) that produce seed cones every year.

Within the selected plots, we recorded major characteristics of the older trees and the younger (self sown) generation, i.e. estimated tree age, height and diameter, and health status.

In winter, in each of the selected plots, we collected seed cones from 10 trees (60 trees in total) de-

Table 1. Geographic location and description of our research plots in old foci of the disease

Plots no.	Forest ranger	Subcompartment	Latitude	Longitude	Forest site type	Average age (years)	
						Old trees	Trees from natural regeneration
1.	Żółno	51c	54°09’02”	17°47’35”	fresh coniferous forest	90	30
2.	Murzynówko	43a	52°09’24”	17°24’38”	fresh mixed broadleaved forest	65	25
3.	Mokrz	92c	52°45’07”	16°16’19”	fresh coniferous forest	70	20
4.	Zwola (plot 1)	142k	52°07’04”	17°08’32”	fresh mixed coniferous forest	55	15
5.	Zwola (plot 2)	117g	52°06’39”	17°09’37”	fresh coniferous forest	75	20
6.	Cieciorka	204a and 204k	53°56’58”	18°11’30”	fresh coniferous forest	100	20

veloped in the gaps that resulted from felling of trees damaged by annosum root rot in the past. From each tree, we collected about 10 seed cones. Seeds extracted from the cones originating from the same plot were pooled.

Seeds originating from 4 seed orchards were randomly selected from the general reserves of seeds collected from individual plantations by forest workers.

Thus, in total, 10 pooled samples from 10 provenances (4 seed orchards and 6 research plots) were used in our experiments.

Pathogen

In describing experiment, seedlings were inoculated by 7 strains of *H. annosum*, which differed in pathogenicity and were labelled as Borówiec, 02/126P, 0/118P, 97067P, 203013P, 203015P and 203041P. This strains was described and identified as P intersterility group by Werner and Łakomy (2002b).

Inoculation experiment in the greenhouse

Pine seeds from the 10 provenances were sown in a mixture of peat and perlite. One-month-old seedlings were transplanted to growing trays (15 seedlings per tray) filled with a mixture of peat and perlite (1:2, v/v). Each provenance were represented by 8 growing trays (120 seedlings). One trays for one strains of pathogen and one trays with non-inoculated seedlings (control). In the initial period of growth, before inoculation, the seedlings were fertilized with a complete nutrient medium (Ingestad 1979) once a week.

After 6 months of culture, seedlings were inoculated with *H. annosum* (represented by 7 strains) grown on pine sawdust soaked with a liquid medium according to Pachlewski (1983). Around each seedling we placed sawdust overgrown with hyphae of the pathogen. To accelerate the infection, roots of the studied plants were scarified with a scalpel. After 3 months, the plants were inoculated again, by pouring around each seedling a suspension containing spores and hyphae of the same strain of *H. annosum*. The control for each provenance consisted of non-inoculated seedlings.

After 18 months from the first inoculation, the seedlings were gently pulled out of the substrate, washed, dried for 72 h at 60°C, and weighed.

Statistic analysis

Analysis of variance (ANOVA), and Student's test were conducted using statistical analysis software Statistica version 8.0 (StatSoft Polska Inc., USA). Data in % were transformed before the analysis according to formula of Bliss (Snedecor and Cochran 1976).

Results

Seedling provenance, type provenance (seed orchards, research plots), infection and interaction between them significantly affected many of the analysed characters (Table 2). Seedlings inoculated with *H. annosum* from seed orchards in Syców and Łopuchówko, as well as seedlings from plot Zwola-2, had significantly lower dry weight of needles and stems, as compared to the control (Table 2). Inoculation of seedlings deriving from seed orchards significantly decreased the dry weight of their aboveground parts, as compared to the control (Table 2, Figs 1A and 1B). Inoculation with *H. annosum* stimulated the plants to develop more roots. Significant differences from the control are found in seedlings deriving from research plots (Fig. 1C), particularly in plants from Mokrz and Zwola-2 (Table 2). As compared to the control, a significantly lower dry weight ratio of aboveground parts to roots was found in most of the 10 provenances. In inoculated seedlings developed from seeds collected from our research plots located in forest districts Żółno and Murzynówko no significant differences were observed only (Table 2). Overall, the seedlings from seed orchards had significantly lower values of this ratio, as compared to the control (Fig. 1E).

Seed origin significantly influenced the differences between dry weight of inoculated seedlings and non-inoculated seedlings (Table 2). Inoculated seedlings from seed orchards in Gniezno, Syców, and Łopuchówko had a significantly lower dry weight than the control group. Inoculation of the seedlings deriving from our research plots did not affect significantly their weight. An exception was the group of seedlings originating from Zwola-2. Figure 1D shows mean values for seed orchards and research plots. As compared to the control, dry weight of inoculated seedlings from seed orchards was significantly lower (Student's *t*-test, $P<0.05$). The average weight of these seedlings amounted to 86% of the control value. In the group deriving from our research plots, the corresponding value was 96%. These differences between groups were statistically significant (Student's *t*-test, $P<0.05$, percentage of the control value were analysed).

We found no significant differences in survival rate of pine seedlings from the 10 provenances, both inoculated and not inoculated with *H. annosum*, because 100% of seedlings survived in all the analysed experimental variants.

Discussion

One of the major objectives of this study was to determine if the offspring of self-sown trees in old foci of annosum root rot is more resistant to infection by *H. annosum* than the offspring of plus trees, i.e. theo-

Table 2. Mean growth of seedlings of various provenances, grown in greenhouse conditions and inoculated (I) or not inoculated (C) by *Heterobasidion annosum*. Values in brackets – standard deviation. Significance of differences between the control and inoculated seedlings (Student's *t*-test): * $P<0.05$; ** $P<0.01$

Provenance	Type of provenance	Dry weight (g)								Dry weight ratio of aboveground parts to roots	
		needles		stems		roots		whole seedlings			
		C	I	C	I	C	I	C	I	C	I
Gniezno	SO	1.17	0.95	0.83	0.70	0.58	0.55	2.58*	2.20	3.46*	2.85
		(0.462)	(0.404)	(0.357)	(0.332)	(0.324)	(0.262)	(1.099)	(0.954)	(1.351)	(0.954)
Oborniki Śląskie	SO	1.21	1.13	0.81	0.75	0.51	0.54	2.53	2.42	3.97*	3.50
		(0.262)	(0.338)	(0.216)	(0.240)	(0.152)	(0.193)	0.621)	(0.726)	(1.019)	(0.873)
Syców	SO	1.38*	1.03	0.99*	0.75	0.48	0.52	2.85*	2.30	4.92**	3.42
		(0.520)	(0.430)	(0.462)	(0.342)	(0.311)	(0.222)	(1.302)	(0.970)	(1.511)	(0.869)
Łopuchówko	SO	1.39*	1.12	1.00*	0.77	0.49	0.56	2.88*	2.45	4.86**	3.39
		(0.389)	(0.406)	(0.261)	(0.301)	(0.139)	(0.225)	(0.736)	(0.906)	(1.531)	(0.670)
Żółno	RP	1.23	1.17	0.75	0.71	0.63	0.62	2.61	2.50	3.11	3.05
		(0.247)	(0.322)	(0.209)	(0.220)	(0.174)	(0.221)	(0.588)	(0.701)	(0.643)	(0.829)
Murzynówko	RP	1.16	1.11	0.70	0.76	0.50	0.57	2.36	2.44	3.70	3.28
		(0.475)	(0.378)	(0.337)	(0.276)	(0.195)	(0.199)	(0.979)	(0.829)	(0.750)	(1.268)
Mokrz	RP	1.10	1.05	0.80	0.75	0.50*	0.60	2.40	2.40	3.81*	3.11
		(0.333)	(0.329)	(0.397)	(0.229)	(0.150)	(0.274)	(0.744)	(0.761)	(0.573)	(0.790)
Zwola 1	RP	1.20	1.05	0.89	0.80	0.59	0.66	2.68	2.51	3.57*	2.81
		(0.330)	(0.320)	(0.278)	(0.240)	(0.163)	(0.189)	(0.681)	(0.693)	(0.872)	(0.671)
Zwola 2	RP	1.27**	0.96	0.92*	0.75	0.49**	0.65	2.68*	2.36	4.44**	2.62
		(0.223)	(0.247)	(0.207)	(0.187)	(0.123)	(0.170)	(0.495)	(0.534)	(0.870)	(0.626)
Cieciorka	RP	1.02	0.92	0.60	0.54	0.47	0.49	2.09	1.95	3.45*	3.00
		(0.333)	(0.241)	(0.222)	(0.168)	(0.129)	(0.173)	(0.632)	(0.552)	(0.697)	(0.652)
seed orchards	SO	1.29*	1.16	0.91*	0.74	0.51	0.54	2.71*	2.44	4.30*	3.29
		(0.420)	(0.399)	(0.346)	(0.304)	(0.242)	(0.225)	(0.943)	(0.874)	(1.492)	(0.817)
research plots	RP	1.16*	1.04	0.78	0.72	0.53*	0.60	2.47	2.36	3.68*	2.98
		(0.326)	(0.321)	(0.271)	(0.238)	(0.162)	(0.214)	(0.695)	(0.692)	(0.816)	(0.866)
ANOVA	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Type of provenance (TP)	1	4.14	**0.0421**	10.87	**0.0010**	3.56	0.0596	2.39	0.1225	76.22	**<0.0001**
Infection (I)	1	28.86	**0.0000**	21.08	**<0.0001**	5.94	**0.0150**	11.35	**0.0008**	147.78	**<0.0001**
TP x I	1	3.22	0.0728	4.89	**0.0272**	1.15	0.2843	3.57	0.0592	15.21	**0.0001**
Provenance (type of prov.)	8	6.91	**<0.0001**	9.82	**<0.0001**	5.89	**<0.0001**	6.35	**<0.0001**	7.67	**<0.0001**
df terror		1185		1185		1185		1185		1185	

SO – seed orchards, RP – research plots

retically the best material used for afforestation and reforestation. It must be remembered that plus trees, whose grafts are used to establish seed orchards, are undoubtedly the elite of forest trees. As a result of strict selection, they are distinguished by a very high quality, fast growth, and very good health. Thus the seedlings deriving from seed orchards are the offspring of the elite. By contrast, the seedlings deriving from our research plots are the offspring of typical 'average trees'. Despite this, in our experiments, the seedlings deriving from seed orchards, after inoculation with *H. annosum*, had significantly worse results than the seedlings representing naturally regenerated populations in old foci of the disease (research plots). The greatest differences were observed in seedling dry weight, which in inoculated seedlings from seed orchards was 14% lower than in the control. In contrast, the dry weight of inoculated seedlings from research plots did not differ significantly from that of non-inoculated seedlings.

It is noteworthy that seedlings in the greenhouse grew in much better conditions than in the wild. The substrate (peat + perlite) warranted a proper soil structure, the liquid medium (Ingestad 1979) supplied proper concentrations of nutrients, and regular watering ensured optimum soil moisture content and humidity, while the heated greenhouse provided a suitable air temperature and lack of freezing stress.

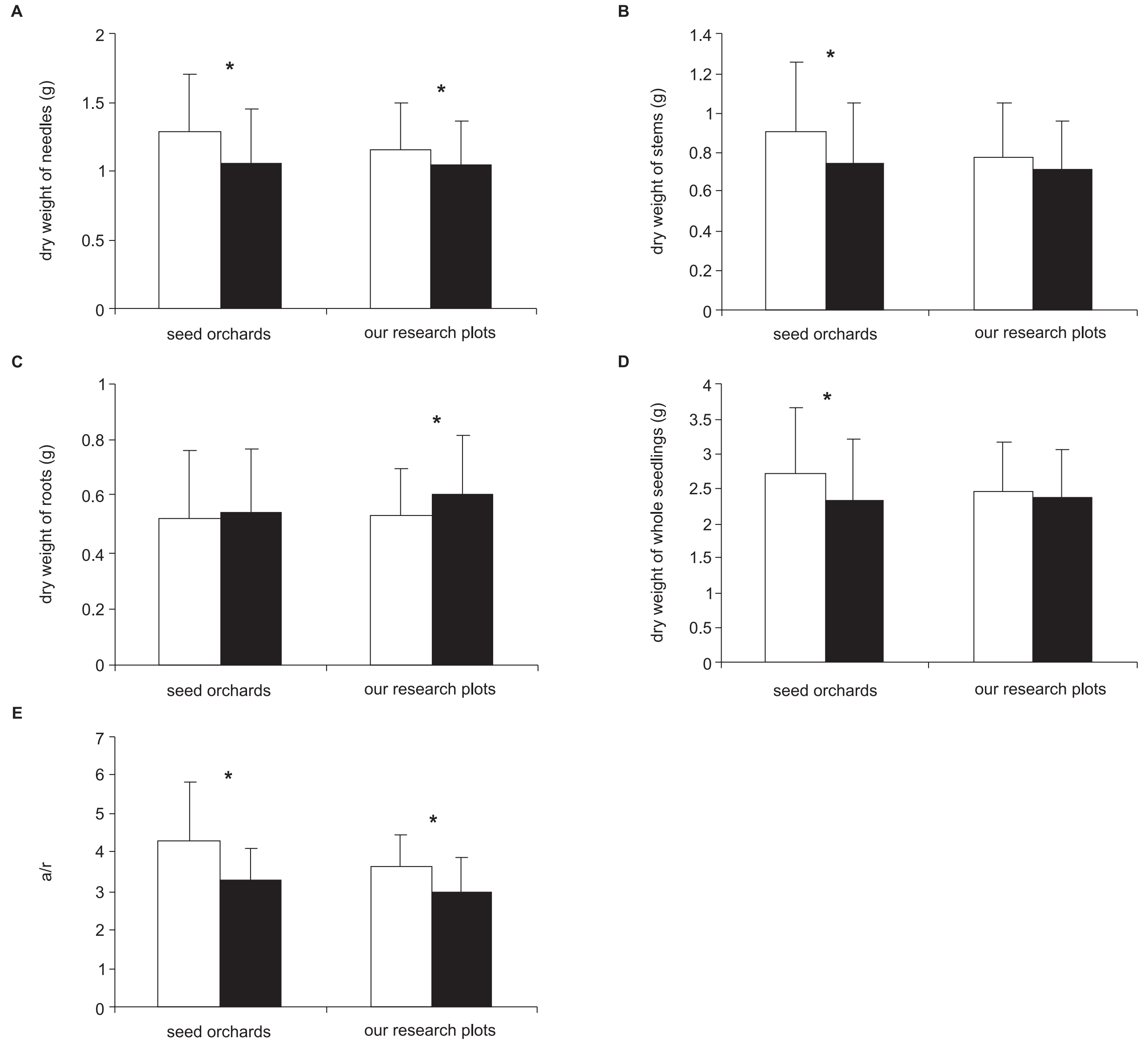

Fig. 1. Mean growth of seedlings representing seed orchards and research plots, grown in greenhouse conditions and not inoculated (white bars) or inoculated (dark bars) by *Heterobasidion annosum*: (A) dry weight of needles, (B) dry weight of stems, (C) dry weight of roots, (D) dry weight of whole plants, (E) dry weight ratio of aboveground parts to roots. Significance of differences between the control and inoculated seedlings (Student's *t*-test): * $P<0.05$; ** $P<0.01$

Good growth conditions are evidenced by the intensive seedling growth and development. Such conditions are certainly not typical of the harsh environment of coniferous woodland. Soil drought, in particular, is commonly observed there, because of the permeable substrate. Many studies have shown that a strong stress experienced by a host plant usually favours pathogen growth (e.g. Werner 1991a).

Generally, the course of infection of pine seedlings and their reactions did not deviate from those reported earlier by Werner (1991a, 2001) as well as Werner and Idzikowska (2001). Differences between the group of resistant and sensitive trees were less conspicuous here than those described by the authors mentioned above. This probably results from the diversity and variation of the plant material used in this study. In above cited studies tree selection based on individuals rather than populations. In pine seedlings of both groups, gradation of resistance can be observed (high values of standard deviation in table 2). This is typical for horizontal resistance, conditioned by many genes responsible for many characters, which are usually not directly linked with resistance to the analysed causative factor but make the disease progress much more difficult (Wellendorf and Thomsen 2008; Eckhard et al. 2009). According to Werner (1991a), maternal trees are clearly subject to selection for resistance at the sites where highly pathogenic strains of *H. annosum* exist. The selection leads to the death of sensitive trees before they start to flower abundantly. In the forest stands where less aggressive strains of the fungus are found, the resistance structure of the offspring and the mother trees can be very similar. Our study plots probably represent an average range of pathogenicity of *H. annosum* and consequently also average selection of maternal trees.

The increase in dry weight of roots, noticed mostly in inoculated seedlings from our research plots, was probably associated with the observed resistance reaction: formation of new roots. This is consistent with the findings of Werner (1991a) and Napierała-Filipiak (2000).

When selecting our research plots, we used 3 criteria described in Material and Methods. It was not easy to find plots meeting all the criteria. Natural regeneration was a major problem. The light-demanding pine, is self sown in the forest interior less often than shade-tolerant species. Even if this happens, it usually grows slowly there and does not produce seed cones or does it relatively late. More important reason for the small number of self-sown populations of such trees is their frequent removal during management activities in older forest stands. A widely held view is (or at least used to be until recently) that the self-sown seedlings of the studied species have a low breeding value for reproduction of the forest stand. Such self-sown seedlings only rarely cover evenly and sufficiently densely the whole area. Their supplementation by additional planting of nursery-grown seedlings, after removal of canopy trees, leads to formation of an uneven canopy layer in the future, which generally decreases to a large extent the quality of the future forest stand and the produced timber. Self-sown pine seedlings were not earlier taken into account as a source of seeds.

Proving that the offspring of trees resulting from natural regeneration in old foci of the disease has a greater, genetically conditioned resistance to annosum root rot, would enable assigning some forest stands and plots a special status and their special treatment. This, in turn, would lead to an increased number of self-sown pine populations meeting the criteria described above.

It seems that the methods applied in our experiments agree with the suggestions made by Werner (1991a), who warned against resistance assessments based on only results of infection of mature trees and recommended experiments using a close-to-natural route of infection.

It is difficult to determine unambiguously how high the increase in resistance will be if we use for reforestation and afforestation the seedlings developed from seeds collected from selected, more resistant trees. In this study, the results of infection with annosum root rot was assessed within a relatively short time after infection. In the wild, where the pathogen affects plants for much longer life periods, differences in the resistance of pine trees from various provenances should be more conspicuous.

Seeds of the trees resulting from natural regeneration in old foci of the disease were products of open pollination. The pollen used in this process probably only partly originated from trees resistant to annosum root rot. If only such trees were crossed, then the increase in resistance should be significantly higher. This is (indirectly) suggested, e.g., by reasearch of Arnerup et al. (2010) concerning *Picea abies* and *H. parviporum* – a pathogen closely related to *H. annosum*. Thus it seems advisable to establish an experimental seed orchard based on grafts of resistant trees. The great individual variation in the resistance of the offspring of maternal trees in both studied groups also confirms that we should search further for resistance characters at the level of individuals, as suggested earlier by Dimitri (1982) and Werner (1991a).

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