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The effect of fungicides used in the protection of forest tree seedlings on the growth of ectomycorrhizal fungi

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Fungitoxical activity of ten fungicides most commonly used in the phytopathological protection of forest nurseries was studied, using the *in vitro* screening method. The fungitoxical activity was studied against five species of ectomycorrhizal fungi (seven strains). The resulting growth inhibition of fungi species and strains tested was presented in terms of fungitoxicity classes of the preparations used. The highest total fungitoxicity against the mycelia of fungi taxa tested was found for Euparen, Bravo, Dithane M-45 and Ridomil. The weakest fungitoxical effect was observed for Topsin M and Bayleton. The least susceptible for the action of the fungicides studied were mycelia of *Suillus luteus*, while the most susceptible were those of *Hebeloma crustuliniforme* and *Laccaria laccata*. The study results are useful for the selection of fungi strains proper for the artificial mycorrhization of seedlings.

Key words: ectomycorrhizal fungi, mycorrhization of tree seedlings, fungicides, class of fungitoxicity.

INTRODUCTION

All seedlings destined for the afforestation of so called difficult grounds: proper waste lands, post agricultural abandoned grounds, terrain heavily polluted by industrial air pollution, post-industrial terrain, forest area subject in the past to the destructive fires, they all have to be supplied with the specific, well developed mycorrhizas. If there are no proper mycorrhizas or – if there is present ectendomycorrhiza, the seedlings suffer and die which leads, in the consequence, to the failure of forest cultures; to the necessity of costly additional planting (partial or complete) using properly mycorrhized seedlings.

Also, the artificial mycorrhization is needed in the case of growing seedlings in containerized nurseries with the use of different soil substrates and particularly so — using the barren substrates (K o w a l s k i et al. 1994; P a c h l e w s k i 1993).

In Poland a program of forest tree seedlings mycorrhization has been initiated following an original technology; it covered, initially, the mycorrhization of Scots pine, the most important forest forming species. Scots pine seedlings constitute more than 60% of the yearly output of State Forests nurseries. At a commercial scale, the practical implementation of mycorrhization has started in a large, modern forest nursery located at the Rudy Raciborskie Forest District (Katowice Regional Directorate of State Forests). This has been under the scientific supervision of Professor Stefan Kowalski. The present paper is part of auxiliary investigation completed within the above-mentioned program.

The seedlings meant for artificial mycorrhization may be subject, both in the stage of mycorrhization and during their subsequent cultivation in the nursery, to fungal diseases. The most commonly observed diseases at the stage of seedlings are: seedling damping-off (*Fusarium oxysporum*, *Rhizoctonia solani* and many other fungi species), the spring needle-blight of Scots pine (*Lophodermium seditiosum*), the gray mould (*Botrytis cinerea*), twigs dying off (*Gremmeniella abietina* and other fungi), drying of twig tops (*Diplodia pinea*) and Scots pine twisting rust (*Melampsora pinitorqua*) (G r z y w a c z 1993a).

So far, no clear results were obtained in the study on the side effect of the application of commercial fungicides used in the prophylactics and therapy of seedling diseases for the mycorrhizal fungi. It was found, on the one hand, that the use of fungicides in the concentrations recommended by the producers did not inhibit the creation of mycorrhizas, on the contrary — they stimulate their occurrence in some cases. It has been found, however, in a number of papers, that fungicides influence definitely negatively both mycorrhizal fungi and mycorrhizas themselves, particularly in very young seedlings; moreover, they may lead to a complete disappearance of the mycorrhizas specific for Scots pine, fork-like ramifications of roots endings. Some papers referring the negative effect of fungicides on the creation of mycorrhizas state that the effect stopped at the end of the second growing period and no more disturbance occurred in the subsequent growth of the young trees. R u d a w s k a (1993) presented in her reviews and analyses the state-of -the -art of the response of mycorrhizal fungi and mycorrhizas to the action of pesticides. The actually observed discrepancy of the results has been likely due to differences in the conditions in which both the *in vitro* and *in vivo* studies were performed: different tree species, differences in fungicides used and their concentrations, varying mycorrhizal fungi, soil biology etc.

While selecting fungi species and strains to be used for the artificial mycorrhization of forest tree seedlings one has to consider the differences in the physiological condition of ectomycorrhizal fungi: their readiness to create mycorrhiza, the rate of creation of the mycorrhizal contact, the degree of mycorrhization of the root system, the thickness of the fungal mantle, the width and range of Hartig net, their influence on the host-organism growth and development; their antagonistic activity against selected pathogens of trees' root systems, their susceptibility to varying soil reaction (pH) and to the content of heavy metals in the soil and, finally, their susceptibility to the pesticides used (K o w a l s k i et al. 1989; K o w a l s k i et al. 1996).

This paper has been aimed at the preliminary assessment of susceptibility of fungi strains to be inoculum for the artificial mycorrhization to the action of fungicides most often used in the control of pathogenic fungi present in the cultures of Scots pine.

MATERIAL AND METHODS

The range and degree of fungitoxical activity are the most important parameters of any fungicide. These parameters allow for the determination of the fungicide's usability for the protection of plants. The values of the parameters are being determined during the first, initial stage of study, while assessing new active substances or new area of their use. Such an assessment is performed based on the fungicide's impact on the radial growth of mycelium put on an artificial substrate. For the needs of the present paper, the Melin-Norkans medium (MMN) has been used. After sterilization and cooling down the 60°C, the prescribed amount of fungicides solution was added to the medium. The range of fungicides concentrations used was chosen so that the lowest effective dosage could be determined such that inhibits fully (100%) the growth of mycelium, that is ED_{100} as well as a number of intermediate dosages enabling the determination of the ED_{50} value, that is such a concentration that produces a 50% inhibition of mycelium growth as compared to the control mycelium (growing on the pure medium, with no fungicide used). The following concentrations of commercial fungicides were used (in ppm): 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000 and 10 000. Every treatment was used in five replications, using the Petri dishes of 6 cm in diameter and containing 5 ml of medium each. This scheme was , also, used in the control. After the medium became solidified, it was inoculated with mycelium at the center of the dish. As inoculum, fragments of 2-week old fungus cultures were used of dimension 3 × 3 mm. All the handling was performed in the inoculation chamber, after adequate sterilization of atmospheric air and microbiological tools.

The dishes, after inoculation, were placed in the dark, in thermostats at $22 \pm 2^{\circ}C$ air temperature and $80 \pm 5\%$ of relative air humidity. The mycelium diameter was recorded after three weeks of growth. The colony diameter recording was performed from beneath the bottom side of the dish within 1 mm (two perpendicular measurements). Then, the mean diameter of colony was calculated (using the five single measurements) in all the treatments and these data served as a basis for determination of the so called per cent of inhibition index following the formula:

$$i = \frac{k - a}{k} 100\%$$

where:
i — per cent of inhibition of mycelium growth,
k — control mycelium colony diameter,
a — diameter of mycelium grown in a medium treated with determined concentration of fungicide.

Finally, the assessment of fungitoxicity of preparations under study was expressed in fungitoxicity classes (Table 1). The classes of fungitoxicity were described in terms of Arabic numbers and not in Roman numbers — just to be easily recognizable from the classes of toxicity as used against humans and homoiothermic animals, the latter are commonly expressed in the Roman numbers. In the classification system proposed, the seven classes are determined, with their limits set according to the geometrical sequence of step 10 (G r z y w a c z 1987).

The susceptibility towards fungicides was studied of the following ectomycorrhizal fungi: *Amanita muscaria* (L.: Fr.) Pers., 3677 (*Amanitaceae*), *Hebeloma crustuliniforme* (Bull.) Quel. W33, W40, W53 (*Cortinariaceae*),

T a b l e 1
Classes of fungitoxicity of chemical preparations as determined following the screaning method of AG

Class	Description of fungitoxicity towards the test species of fungus	ED_{50} value in ppm
1	extremaly toxic	< 0,1
2	very toxic	0,1 – 1
3	toxic	1,1 – 10
4	moderately toxic	11 – 100
5	little toxic	101 – 1000
6	very little toxic	1001 – 10000
7	non toxic	> 10000

Laccaria laccata (Scop.: Fr.) Berk. et Br., 49195 (*Tricholomataceae*), *Lactarius rufus* (Scop.: Fr.) Fr., 3673 (*Russulaceae*), *Suillus luteus* (L.) S.F. Gray, W 9 (*Boletaceae*). Seven fungi strains belonging to 5 species were tested altogether. They all were taken from the collection of pure cultures of the Department of Forest Phytopathology, the Agricultural Academy in Kraków. We obtained them thanks the courtesy of Prof. Dr hab. Stefan Kowalski.

Ten commercial fungicides were used out of the most commonly used in forest nurseries (G r z y w a c z 1993b): Bayleton 5 WP (triadimefon), Bravo 500 SC (chlorothalonil), Captan 50 WP (captan), Carpene 65 WP (dodine), Dithane M-45 (mancozeb), Euparen 50 WP (dichlofluanid), Ridomil MZ 72-WP (mancozeb + metalaxyl), Rubigan 12 EC (fenarimol), Topsin M 70 WP (thiophanate methyl), Funaben T (carbendazim + thiram). The Bravo and Topsin M preparations belong to class V of toxicity towards humans and homoiothermic animals, all the remaining preparations – belong to class IV. The abbreviations given at the preparations commercial names mean: WP – powder for the production of water suspense; SC – concentrate of solution; EC – concentrate for the production of water emulsion.

In total, 4200 Petri dishes filled with medium were used in the study of ten fungicides used in 11 concentrations and the control; in the study seven strains of fungi were tested, and each experimental treatment was repeated five times.

RESULTS

The fungicides studied in this work ranged in their direct action against mycelium of the ectomycorrhizal fungi species tested from very toxic down the class of little toxic. The mean class of toxicity was 3.23 and in particular cases it ranged from 1.86 to 4.86 (Table 2). The most toxic preparations proved: Euparen, Bravo, Dithane and Ridomil. The least toxic towards the tested fungi species were: Topsin M and Bayleton. The differentiation in toxicity class of particular preparations was noticeably high: from class 1, that is $ED_{50} < 0.1$ ppm after the use of Bravo towards *Hebeloma crustuliniforme* and *Laccaria laccata*; up to class 6, that is ED_{50} between 5000 and 10 000, after the use of Topsin M towards the mycelium of *Amanita muscaria*. If so, the latter should be, in theory, recommended for practical implementation in the protection of artificially mycorrhized seedlings in forest tree nurseries against fungal diseases – because of their minimum toxicity against the mycorrhizal fungi. The term 'in theory' was used as the study was only performed in the laboratory and using the screening method. To recommend the preparations unconditionally, also the *in vivo* studies need to be completed, in the field conditions of forest nurseries: a whole cycle of seedlings spraying with the preparations needs to be performed.

Table 2
Classes of fungitoxicity of chemical preparations studied towards the ectomycorrhizal species tested

Fungicides	Test fungi							Mean class of fungitoxicity
	A. muscaria	H. crust. W33	H. crust. W40	H. crust. W53	L. laccata	L. rufus	S. luteus	
1. Bayleton	4	4	3	4	5	4	4	4,00
2. Bravo	2	1	1	1	1	4	4	2,00
3. Captan	4	3	4	3	4	4	4	3,71
4. Carpene	3	4	5	3	3	3	5	3,71
5. Dithane	3	3	3	2	3	3	3	2,86
6. Euparen	2	3	1	1	1	2	1	1,86
7. Ridomil	3	4	2	3	2	3	3	2,86
8. Rubigan	2	3	4	3	3	3	5	3,29
9. Topsin M	6	4	5	4	5	5	5	4,86
10. Funaben T	3	4	4	3	2	3	3	3,14
Mean class	3,2	3,3	3,2	2,7	2,9	3,4	3,9	3,23

Particular ectomycorrhizal fungi tested showed a varying susceptibility for the action of fungicides used. The least susceptible proved to be *S. luteus*, *L. rufus* and *H. crustuliniforme* (strain W 33). The most susceptible for the impact of the fungicides were the strains of *H. crustuliniforme* W 53 and *L. laccata*. The intraspecific differentiation in susceptibility against fungicides was found in particular strains of *H. crustuliniforme*. This fact confirms the necessity to perform such tests for the selection of the most useful strains and species for the needs of seedlings mycorrhization, such taxa that would suit possibly best the nursery environment and the cultivation technologies used.

DISCUSSION

The majority of traditional fungicides belong to the group of the pre-infection action type (superficial, protectional activity). They are characteristic of usually little *in vitro* fungitoxicity (B o r e c k i 1996). To this group belong, among others, the preparations containing compounds of copper, sulfur, simple aromatic compounds, phtalimide and thiocarbaminide compounds. Out of the fungicides tested in this paper, the following preparations belong to this group: Dithane M-45 and Ridomil (both containing mancozeb) as well as Funaben T

(containing, among others, thiram) and Captan (phtalimide compounds). Except Captan, all the remaining above mentioned fungicides proved to be rather highly toxic against fungi *in vitro* but the final assessment of their usability for the protection of mycorrhized seedlings has to be done in the field — in the nurseries, through spraying the seedlings and studying the effect of the treatment on mycorrhizas.

On the other hand, Bayleton belonging to the triazole compounds, rather weakly affects fungi in their catabolic phase of development. Only in the anabolic phase, when the fungus initiates the parasitic contact with the host plant and it uptakes nutrients intensively, the process of inhibition of fungus biosynthesis accelerates the mechanism of which is in the arresting demethylation of lanosterol at the carbon atom in position C_{14} (the P-450 cytochrome function becomes blocked) — (B o r e c k i 1996). As a consequence, inhibited becomes the process of transformation of ergosterol into the growing substances and the accumulation of lipids in the cells of fungus. This is why the little toxicity of Bayleton could have been result of the used method of study: the screening method; the fungicide may prove more toxic for the ectomycorrhizal fungi when used directly.

Considering the mechanism of action of particular groups of fungicides it appears that the assessment results of their toxicity towards the mycorrhizal fungi as performed with the use of *in vitro* screening method may be different from their actual toxicity as determined using the *in vivo* method in forest nurseries. Therefore, it is recommended that in the future study on the selection of fungi to be used in the artificial mycorrhization of seedlings and for the elimination of those fungicides most toxic towards this group of fungi, direct field tests should be performed. A program of such investigations, with a whole cycle of observations planned, has been prepared at the area of the Forest Research Station of the Warsaw Agricultural University in Rogów.

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Wpływ fungicydów stosowanych w ochronie sadzonek drzew leśnych przed chorobami na wzrost grzybni gatunków tworzących z nimi ektomikoryzy

S t r e s z c z e n i e

Zbadano metodą skriningową *in vitro* aktywność grzybobójczą 10 najczęściej stosowanych fungicydów w ochronie szkółek leśnych przed chorobami w stosunku do grzybni 5 gatunków ektomikoryzowych (7 szczepów): *Amanita muscaria*, *Hebeloma crustuliniforme* W33, W49, W53, *Laccaria laccata*, *Lactarius rufus* i *Suillus luteus*. Inhibicję wzrostu grzybni przedstawiono w postaci klas fungitoksyczności według propozycji AG. Największą toksyczność w stosunku do grzybni testowych gatunków wykazywał Euparen, Bravo, Dithane M-45 i Ridomil, najmniejszą zaś Topsin M i Bayleton. Najmniej wrażliwa na stosowane w szkółkach leśnych fungicydy była grzybnia *S. luteus*, a najbardziej *H. crustuliniforme* W53 i *L. laccata*. Przeprowadzone badania służą selekcji szczepów grzybni do sztucznej mikoryzacji sadzonek drzew leśnych. Uzyskane wyniki przedyskutowano z uwzględnieniem mechanizmu działania na grzybnię środków czynnych zawartych w fungicydach. Okazuje się, że toksyczność ustalona metodą pożywkową *in vitro* może być inna od faktycznej w stosunku do grzybów mikoryzowych, oznaczonej po bezpośrednim opryskiwaniu mikoryzowych sadzonek. Zaplanowano dalsze badania w testach polowych na jedno- i dwuletnich sadzonkach sosny zwyczajnej.