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INFLUENCE OF SOME CHEMICALS ON THE VIABILITY OF *PHOMOPSIS VITICOLA* SACC. SPORES

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Abstract: The effect of nine fungicides and chitosan on the viability of *Phomopsis viticola* Sacc. spores occurring abundantly in pycnidia on carnation leaves, was tested in the laboratory conditions. The chemicals selected represent different mode of action on the pathogen and they were recognized as very effective in limiting *P. viticola* development within many viticultural regions. Fungicides were tested at six various concentrations of active ingredients, i.e. 10, 50, 100, 200, 500 mg. a.i./cm^3 and at the concentrations that were recommended by the manufacturers. It was found that chlorothalonil, flusilazol and thiophanate – methyl caused the decay of all *P. viticola* spores in each studied concentration. After using mancozeb, cyprodinil, azoxystrobin and chitosan a strong inhibition of spore viability were also observed, especially at the concentrations of 100 mg of a.i. on cm^3 and above. The other fungicides seem to be less effective compared with the above-metioned ones. Basing on the present studies and abundant information from literature, some of the most effective chemicals which should be tested on grapevine plantations in Poland were indicated.

Key words: *Phomopsis viticola*, fungicides, chitosan, grapevine

INTRODUCTION

Phomopsis viticola belongs to the serious pathogens of grapevine cultivated in many regions of the world (Kozar and Berezovskaja 1980; Cavanni et al. 1987; Hewitt and Pearson 1988; Simon 1993; Merrin et al. 1995; Scheper et al. 1997). In Poland the presence of this fungus was noted for the first time on grapevine cultivated under foil tunnel conditions (Machowicz-Stefaniak 1993). Nowadays, the cultures of *P. viticola* are commonly obtained from propagation material originated from many plantations and nurseries in our country (Ewa Król – unpublished). In Polish conditions *P. viticola* caused disease symptoms only on grapevine shoots this resulted in introducing the name "bark necrosis of grapevine" (Machowicz-Stefa-

niak and Kuropatwa 1993). One of the strategies for grapevine protection against this pathogen is to reduce *P. viticola* inoculum on canes, both during the vegetative and dormant periods, which also results in the protection against fungus distribution (Hewitt and Pearson 1988; Simon 1993; Merrin et al. 1995; Castillo-Pando et al. 1997; Scheper et al. 1997). The program of Grapevine Protection in Poland considers the control of powdery mildew [*Plasmopara viticola* (Berk. Et Curt.) Berl. et de Toni], downy mildew [*Uncinula necator* (Schwein.)Burill.] and grey mould (*Botrytis cinerea* Pers.). It is known from literature that some fungicides applied against the above – mentioned pathogenes may also control *P.viticola* (Maček and Zgur 1989; Kuropatwa 1994) but other chemicals, especially promising in controlling this pathogen are mentioned (Hewitt and Pearson 1988; Castillo-Pando et al. 1997; Scheper et al. 1997; Mostert et al. 2000). This information suggests a possibility of the effect of some fungicides on the viability of spores and ability to resume the mycelial growth of native strains of *P. viticola.* One of the methods of fungicidal toxicity evaluation is determination of their influence on the linear growth of colonies and sporulation on a medium with fungicides (Borecki 1984). This method may be useful for fungal species which sporulated easily and quickly on the artificial medium but in the case of *P. viticola* which sporulated late and with difficulty, the evaluation of chemicals in this way is impossible. It seems that stimulation of the pathogen to abundant sporulation before an accurate evaluation of the influence of fungicides on spore viability is the best solution, and it was adopted in the present paper.

The purpose of the present work was to indicate some of the most effective chemicals which should be evaluated on grapevine cultivated in Polish conditions.

MATERIAL AND METHODS

Three strains of *P. viticola* isolated earlier from canes with symptoms of bark necrosis, 9 fungicides and chitosan were chosen for the experiment (Table 1). The studies were carried out in laboratory according to the method described in detail by Castillo-Pando et al. (1997). In order to obtain abundant sporulation of *P. viticola* the fungal cultures were grown on 1/4 strength potato dextrose agar (PDA, bioMerieux) amended with pieces of carnation leaves (Fig. 1) in definite conditions (Castillo-Pando et al. 1997). For each fungicide studied, the suspensions in 100 ml of water were made in 250 ml Erlenmayer's flasks. Six different concentrations of

Table 1. List of examined fungicides

Fungicides	Concentration of a.i. (%)	Producer
Alliette 80 WP	80% fosetyl – Al	Aventis CropScience S.A. – France
Amistar 250 SC	250 g/l azoxystrobin	Syngenta Limited – Gread Britain
Biochicol 020 PC	20% chitosan	Gumitex Poli-Farm Sp.z.o.o. – Łowicz. Poland
Bravo 500 SC	500g/l chlorothalonil	Syngenta Limited – Gread Britain
Chorus 75 WG	75% cyprodinil	Novartis Crop Protection AG – Switzerland
Dithane M-45 80 WP	80% mancozeb	Dow AgroSciences Sp. z o.o. – Poland
Folpan 80 WG	80% folpet	Makhteshim – Agan. Israel
Punch 400 EC	400g/l flusilazol	Du Pont de Nemours
Topsin M 70 WP	70% tiophanate – methyl	Nippon Soda Company Limited – Japan
Zato 50 WG	50% trifloxystrobin	Novartis Crop Protection AG – Switzerland

active ingredient were used, i.e: 10, 50, 100, 200, 500 mg a.i./cm^3 and the concentrations that were recommended by the manufactures, i.e.: fosetyl-Al 1600 mg a.i./cm^3, chitosan 4000 mg a.i./cm^3, chlorothalonil 1500 mg a.i./cm^3, cyprodinil 300 mg a.i/cm^3, folpet 1600 mg a.i./cm^3 , flusilazol 100 mg a.i./cm^3, mancozeb 2400 mg a.i /cm^3, tiophanate-methyl 700 mg a.i/cm^3, azoxystrobin 250 mg a.i./cm^3, trifloxystrobin 150 mg a.i./cm^3. Four pieces of carnation leaf carrying pycnidia were immersed in flasks with the studied suspensions. The carnation leaves dipped in sterile, distillated water constituted control. The studied material was placed in water bath shaker type 357 at temperature 20–22°C. Four replications were used for each chemical, strain of *P. viticola* and concentration. The carnation leaves were drained in sterile blotting-paper and transferred onto PDA medium in Petri dishes. Estimation of the effectiveness of fungicides was carried out after 72 h based on the number of decayed spores and ability of the pathogen of resuming the mycelial growth around carnation leaves. From each treatment of the experiment and from each replication 100 alpha spores originated from various pycnidia were observed (total 400 spores) and then the percentage of decayed spores were calucated. The results were analysed using the analysis of variance and Tukey's confidence interval. Moreover, an approximate dose of ED_{50} for *P. viticola* was determined and fungitoxic or fungistatic effects of the chemicals were estimated. In the case of the fungistatic influence dishes with the studied material were kept in thermostat for 3 weeks in order to observe possible changes in the morphology of mycelium and spores.

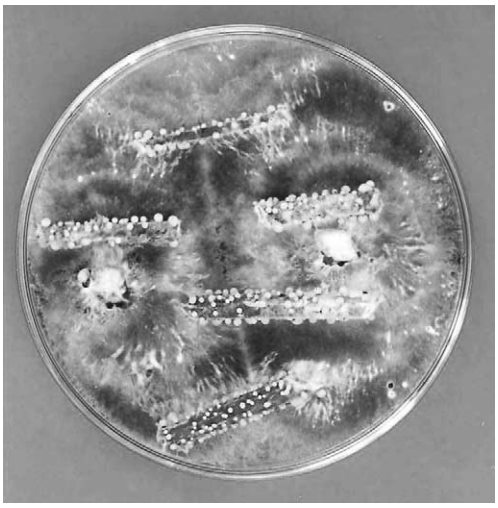

Fig. 1. Abundant sporulation of *P. viticola* on the pieces of carnation leaf

RESULTS

The influence of the tested chemicals on the viability of *P. viticola* spores was differentiated. Chlorothalonil, flusilazol, tiophanate – methyl were toxic to the pathogen because they killed 100% of spores in all concentrations and no mycelial growth was observed. The destroyed alpha spores were strongly narrowed or rounded out with abundant granulation in the thickened cytoplasm (Fig. 2). These spores usually agglomerated in groups of several or a few. Spores of beta type were also strongly deformed, usually narrowed and excessively curved (Fig. 2). In the case of the above – metioned preparations their ED_{50} for *P. viticola* was lower than 10 mg a.i./cm^3. It was observed that after application of other chemicals their efficiency in reducing the pathogen spore viability generally increased with increasing concentration. However, the majority of chemicals appeared to be highly effective

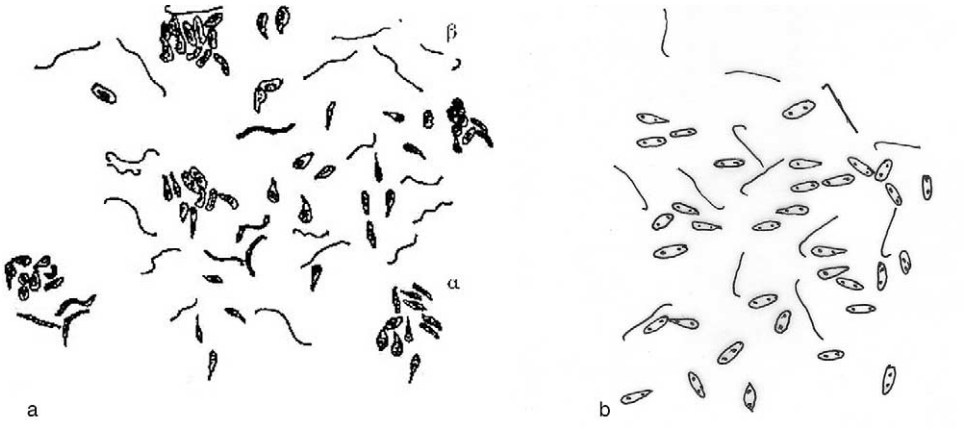

Fig. 2. Destruction of *P. viticola* spores growing in the presence of 10 mg of tiophanate-methyl/cm^3 (a) and appearance of spores in the control (b) (400× magnification)

at concentrations to 200 mg a.i./cm^3 and increasing the active ingredient concentration had no essential effect on the number of destroyed spores. After application of azoxystrobin and chitosan in the concentration of 10 mg a.i./cm^3, 78.8% and 55.8% of the killed spores were found, respectively, which allowed to estimate that ED_{50} for *P. viticola* could be obtained at low concentrations. At the concentration of 100 mg a.i./cm^3 and above, these chemicals inhibited the spore viability equally effectively as chlorothalonil, flusilazol and tiophanate – methyl because percent of the destroyed spores was not significantly different after using all of the five mentioned chemicals (Table 2). However, in spite of strongly inhibited spore viability, azoxystrobin and chitosan slightly limited the mycelial growth of pathogen since at all studied concentrations of active ingredient, the growth of *P. viticola* hyphae was

Table 2. The effect of chemicals on viability of *P. viticola* spores

Chemicals	Percentage of killed spores depending on concentration of a.i. in μg/cm^3					
	10 P_1P_2	50 P_1P_2	100 P_1P_2	200 P_1P_2	500 P_1P_2	CR P_1P_2
chlorothalonil	100 aA	100 aA	100 aA	100 aA	100 aA	100 aA
flusilazol	100 aA	100 aA	100 aA	100 aA	100 aA	100 aA
thiophanate – methyl	100 aA	100 aA	100 aA	100 aA	100 aA	100 aA
cyprodinil	44.5 cdA	58.8 cB	84.3 aC	93 aD	100 aE	100 aE
mancozeb	29.3 deA	81.3 bB	98.3 aC	100 aC	100 aC	100 aC
azoxystrobin	78.8 bA	85.8 bB	88.8 aB	97.8 aC	98.5 aC	98.3 aC
chitosan	55.8 cA	74.3 bcB	83.5 aC	86 aC	91.8 aD	95.8 abD
fosetyl – Al	12.8 efA	22.6 dB	27.5 bD	37.8 bC	48 cD	71.3 bE
folpet	5.5 fA	12.8 dfB	14 bcB	15 cB	65.5 bC	75.3 bD
trifloxystrobin	8.5 efA	10.3 dfAB	14.5 bcBC	16.8 cC	22.5 cD	14.8 cBC
control	0 fA	0 fA	0 cA	0 cA	0 dA	0 cA

CR – concentration recommended by manufacturers
P_1 – differences depending on chemicals at given concentration – small letters $LSD_{0.05}=20.9$
P_2 – differences depending on concentration of given chemical – capital letters $LSD_{0.05}=5.34$
Means followed by the same letter do not differ significantly

observed around the pieces of carnation leaves. A thorough microscope observation showed that these fungal hyphae were often thicker and filled with granulation and the cytoplasm detached from the cell walls. After 3 weeks of cultivation on the *P. viticola* mycelium only scarce pycnidia with a lot of β conidia were formed. Cyprodinil and mancozeb caused the decay of 50% of pathogen spores in concentrations of above 50 mg a.i./cm^3 .The former caused the death of all pathogen conidia at the concentration of 500 mg a.i./cm^3 and the one recommended by the manufacturers and the letter at the concentrations 200 mg a.i./cm^3, 500 mg a.i./cm^3 and the recommended one. Moreover, these chemicals used at concentrations below 200 mg a.i./cm^3 considerably inhibited the growth of *P. viticola* mycelium because for 3 weeks of the observations the colonies around the carnation leaves had a small diameter, hyphae were strongly deformed, filled with granulation and along them, a big, round swellings resembling chlamydospores were observed (Fig. 3). Pycnidia were formed occasionally. After the application of fosetyl – Al, folpet, trifloxystrobin, rich mycelial growth was found in all studied concentrations. In spite of intensive growth of the colonies numerous pathogen hyphae were swollen, strongly shortened and with thickened cytoplasm, especially in concentrations above 100 mg a.i./cm^3. Besides beta spores dominated in the secreted pycnidia (Fig. 4). More-

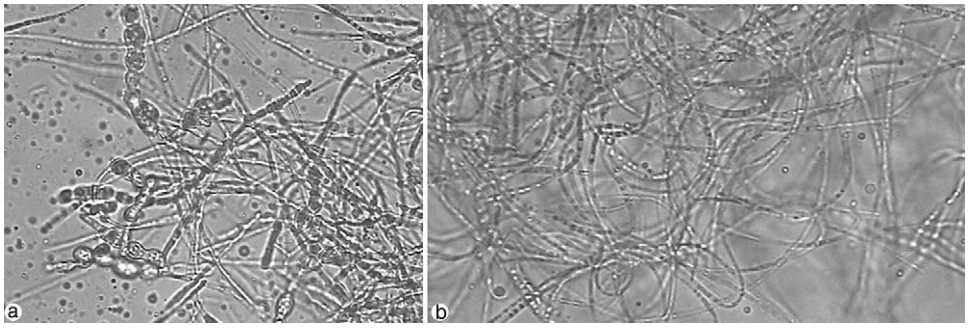

Fig. 3. Deformation of *P. viticola* hyphae after application of 200 mg of mancozeb/cm^3 (a) and appearance of hyphae in the control (b) (400× magnification)

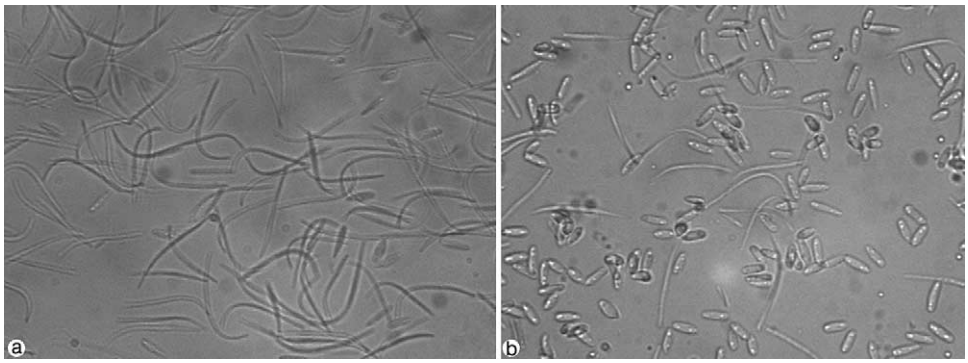

Fig. 4. Sporulation of *P. viticola* after application of chemicals (a) and in the control (b) (400× magnification)

Table 3. The effect of chemicals on *P. viticola*

Chemicals	Concentration in mg of a.i./cm^3					
	10	50	100	200	500	CR
chlorothalonil	–	–	–	–	–	–
flusilazol	–	–	–	–	–	–
thiophanate – methyl	–	–	–	–	–	–
cyprodinil	+	+	+	+	+	–
mancozeb	+	+	+	–	–	–
azoxystrobin	+	+	+	+	+	+
chitosan	+	+	+	+	+	+
fosetyl – Al	+	+	+	+	+	+
folpet	+	+	+	+	+	+
trifloxystrobin	+	+	+	+	+	+

CR – concentration recommended by manufacturers
– fungitoxic effect
+ fungistatic effect

over, folpet destroyed 50% of *P. viticola* spores at concentrations only above 500 mg a.i./cm^3 while fosetyl– Al at the concentration recommended by the manufacturers, i.e. 1600 mg a.i./cm. It was observed that chlorothalonil, flusilazol, tiophanate – methyl were fungitoxic towards *P. viticola* in all studied concentrations (Table 3). Mancozeb was fungitoxic at concentrations 200, 500 mg a.i./cm^3 and the one recommended by the manufacturer, whereas cyprodinil only at the concentration recommended. The other chemicals were fungistatic towards *P. viticola* (Table 3).

DISCUSSION

The studies indicated that the majority of chemicals might, in different degree, inhibit the viability of *P. viticola* spores.

It seems that among three chemicals which completely reduced viability of the pathogen spores chlorothalonil may have a wider application in *P. viticola* control in Polish conditions. It results both from common usage of chlorothalonil in programmes of grapevine protection against various phytopathogenes in the world (Hewitt and Pearson 1988; Berezovskaja and Volosina 1987; Kozar and Berezovskaja 1988) and from its ability to inhibit the spore viability of native *P. vitcola* strains.

A high effectiveness of tiophanate – methyl shown in the present experiment confirmed the previous information about inhibition of spore viability and mycelial growth of *P. viticola* by benzimidazole fungicides, both in vitro (Maček and Zgur 1989; Kuropatwa 1994; Todorova and Katerova 1995; Castillo-Pando et al. 1997) and in vivo (Bourbos and Skoudridakis 1991; Berezovskaja and Volosina 1997; Kozar and Berezovskaja 1988). It is suggested that high effectiveness of fungicides from this chemical group results from their ability of translocation into the plant tissues and destruction of the pathogens during the time of infection and incubation (Castillo-Pando et al.1997; Schüepp and Siegfried 1988). However, the application of thiophanate – methyl should be limited to one treatment during the vegetative period because of the possibility to develop the resistance to these chemicals of wide range of fungal species (Czerniakowski and Czerniakowski 1999).

Similarly, the development of resistance within population of phytopathogenes to sterol biosynthesis inhibitors and anilinopyrimidine fungicides (Lattore 2002; Baroffio et al. 2003) may result in limitation of flusilazol and cyprodinil usage in spite of their effectiveness in suppressing *P. viticola* spore viability.

High percentage of decayed spores of *P. viticola* which was observed in the present study after application of mancozeb, and fungitoxic effect of this preparation in concentrations above 100 mg a.i./cm^3 indicate its effectiveness in inhibiting the viability of spores and development of pathogen mycelium, which confirms the results of other authors (Maček and Zgur 1989; Kuropatwa 1994; Castillo-Pando et al. 1997). Preventive effect of mancozeb on a wide range of pathogens contributes to frequent application of this preparation in many vineyards in the world (Cavanni et al. 1987; Scheper et al. 1997; Egger 2000). It is concluded that common usage of mancozeb against downy mildew in Polish conditions may cause simultaneous suppression of *P. viticola* growth.

Strong reduction of *P. viticola* spore viability by azoxystrobin supported the findings of other authors who showed high effectiveness of this chemical in inhibition of the development of this pathegen both in the laboratory and under field conditions (Castillo-Pando et al. 1997; Politi 1998; Isakovic 1999; Mostert et al. 2000). Considering the fact that strobilurin fungicides give excellent protection against various pathogens, they demonstrate preventive and curative properties, systemic translocation and lasting effect in plant tissues, they have selective effect on beneficial insects and can destroy some fungal species which are resistant to other chemicals (Politi 1998; Czerniakowski and Czerniakowski 1999; Isakovic 1999; Mostert et al. 2000), it is concluded that this chemical should be tested on grapevine plantations in Poland.

Similarly, a strong inhibition of spore viability by chitosan, even if small concentrations of active ingredient were used, suggested that chitosan could become attractive in grapevine protection and should be tested under field conditions. Considering that chitosan, besides its direct activity against various pathogens, is able to induce series of defence reaction in plants (Pospieszny and Struszczyk 1994; Pospieszny 1997), one should expect that its effectiveness in vivo will be higher than in vitro. Morever, a lack of harmfulness of chitosan to living organisms (Pospieszny and Struszczyk 1994; Pospieszny 1997) gives the possibility to use chitosan in ecological and integrated pest management programmes of plant protection, which seems to be especially useful in an environmentaly friendly view of grapevine protection. This fact is in agreement with the opinion of other researches who reported that preparation Elewa based on chitosan, successfully limited growth of some pathogens, including *P. viticola* (Schilder et al. 2002).

It seems that weaker limitation of viability of *P. viticola* spores after fosetyl-Al and folpet application confirms the need for more frequent spraying during vegetative period or using these preparations in a mixture with other fungicides, which increases their effectiveness (Janilloux et al. 1987; Bourbos and Skoudridakis 1991; Bugaret 1993).

Moreover, it is commonly known that fosetyl – Al weakly or not at all affects the pathogens in vitro but stimulates defense reaction of plant tissues this considerably increases its effectiveness directly on the plants (Borecki 1984; Janilloux et al. 1987).

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POLISH SUMMARY

ODDZIAŁYWANIE WYBRANYCH PREPARATÓW NA ŻYWOTNOŚĆ ZARODNIKÓW *PHOMOPSIS VITICOLA* SACC.

W warunkach laboratoryjnych przebadano wpływ 9 fungicydów i chitozanu na żywotność zarodników *P.viticola* masowo tworzących się w piknidiach na liściach goździka. Wybrane do testowania związki chemiczne reprezentowały różne mechanizmy oddziaływania na patogena i uznane zostały za efektywne w ograniczaniu rozwoju *P. viticola* w wielu rejonach uprawy winorośli. Dla każdego fungicydu zastosowano 6 stężeń substancji aktywnej tj.: 10, 50, 100, 200, 500 mg/cm^3 oraz stężenie zalecane w praktyce. Okazało się, że chlorotalonil, flusilazol i tiofanat metylowy powodowały obumarcie wszystkich zarodników *P. viticola* w każdym badanym stężeniu. Silne hamowanie żywotności piknospor zaobserwowano także po zastosowaniu mankozebu, cyprodinilu, azoksystrobiny i chitozanu, szczególnie w stężeniach 100 mg/cm^3 i wyższych. Pozostałe związki chemiczne uznano za mniej efektywne w hamowaniu żywotności zarodników niż preparaty wymienione powyżej. Biorąc pod uwagę wyniki obecnych badań oraz liczne informacje z literatury wskazano najefektywniejsze fungicydy, które należało by przetestować w warunkach uprawy winorośli w Polsce.