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CHITOSAN ACTIVITY IN AN INHIBITION OF *IN VITRO* GROWTH OF *PHOMOPSIS VITICOLA* AND PROTECTION OF GRAPEVINE CANES AGAINST THE PATHOGEN

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Abstract

The effectiveness of Biochicol 020 PC, based on chitosan, in inhibiting *Phomopsis viticola* development was tested under laboratory conditions and on stored grapevine canes. Chitosan inhibited linear growth of *P. viticola* colonies to smaller extent than azoxystrobin and mancozeb, which were used for comparison. However, canes dipped in chitosan solutions and inoculated with the pathogen showed reduction of incidence and severity of grapevine bark necrosis, as compared to control, and to fungicides. Besides, the higher chitosan concentration the greater reduction of *P. viticola* development, both in *in vitro* and *in planta*.

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

Introduction

Phomopsis cane and leaf spot, known in Poland as bark necrosis of grapevine, is a widespread disease in many viticultural regions in the world (Cavanni et al. 1987, Hewitt and Pearson 1988, Machowicz-Stefaniak and Kuropatwa 1993, Merrin et al. 1995, Scheper et al. 1997). Necrotic lesions on shoots, rachises and leaves are the most common symptoms of the disease (Machowicz-Stefaniak and Kuropatwa 1993, Kuropatwa 1994, Mostert et al. 2000). During the dormant season infected canes may become bleached and numerous pycnidia are produced. *Phomopsis viticola* overwinters as mycelium in grapevine canes and buds or as pycnidia partly immersed in diseased tissue (Cavanni et al. 1987, Hewitt and Pearson 1988, Simon 1993, Castillo-Pando et al. 1997).

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One of the best control practices is chemical treatment of dormant canes before storage, which results in reduction of inoculum level and spread of the pathogen. The fungicides are used most frequently, but recently a big interest in environment friendly products for grapevine control has been observed (Castillo-Pando et al. 1997, Scheper et al. 1997, Schilder et al. 2002). Some literature information point at chitosan possible attractiveness in grapevine protection, especially against powdery mildew (*Plasmopara viticola*), downy mildew (*Uncinula necator*) and grey mould (*Botrytis cinerea*) (Schilder et al. 2002). Besides direct activity against various pathogens, it is able to induce a series of defense reactions in plants and it is harmless to living organisms (Pospieszny and Struszczyk 1994, Pospieszny 1997). These features give the possibility to use chitosan in ecological and integrated pest management programmes of grapevine protection (Schilder et al. 2002).

Since literature dealing with chitosan application for protection of grapevine against *P. viticola* is very scanty, the study was undertaken in order to evaluate its influence on the development of the pathogen.

Materials and methods

Three strains of *P. viticola* isolated earlier from canes with symptoms of bark necrosis, Biochicol 020 PC (2% of chitosan), and Amistar 250 SC (250 ml azoxystrobin per 1 l) and Dithane M-45 80 WP (80% of mancozeb) as standard fungicides were chosen for the *in vitro* and *in vivo* experiments.

Evaluation of chitosan-induced inhibition of linear *Phomopsis viticola* growth on malt extract agar

The studies were carried out in laboratory on poor malt extract agar (bioMerioux), according to the method described by Pięta et al. (1998). The active ingredients of tested chemicals were added to the sterilized medium cooled to 40°C at concentrations tested in the earlier paper (Król 2005), i.e.: 10, 50, 100, 200, 500 μg/cm^3 and doses recommended by manufacturers, i.e. chitosan 4000 μg/cm^3, azoxystrobin 250 μg/cm^3, mancozeb 2400 μg/cm^3. Next, the 3-mm-diameter discs overgrown with mycelium and taken from a 10-day-old culture of *P. viticola* were transferred onto agar medium with chemicals. For control inoculum of *P. viticola* was placed on malt extract agar without chemicals. Four replicates were used for each concentration of chemicals and for each strain of *P. viticola* studied. The Petri dishes were incubated at 24°C in dark. Linear growth of *P. viticola* colonies was estimated after four and eight days, based on the mean value from two measurements of colony diameter performed crosswise. Next, the diameter of *P. viticola* colonies on medium with chemicals was compared to the growth on pure medium. The results were statistically analysed using analysis of variance and Tukey's confidence intervals.

Evaluation of chitosan ability to protect grapevine canes from *Phomopsis viticola*

Grapevine canes of cultivars 'Agat Donskij', 'Iza', 'Prim', RF-16 and Biochicol 020 PC at concentrations 0.5% and 1.0%, Amistar 250 SC, Dithane M-45 80 WP (both in concentration 1.0%) and three strains of *P. viticola* were studied. The effectiveness of treatment with chitosan against canes colonization and infection, and next growth of *P. viticola* hyphae was investigated on 15–20 cm long sections of grapevine canes (grafts), according to Król (2004). The canes were dipped in chemicals, next after 4 h – in the inoculum (10^6 spores or/and hyphal fragments per 1 ml), and stored four months in a cellar under conditions described in detail by Król (2004). The canes dipped only in inoculum of each strain of *P. viticola* constituted positive control. 30 canes were used for each cultivar, concentration of chemicals and strain of the pathogen. The total number of studied grafts was 120 for one chemical (4 cultivars × 30) and 150 for one cultivar [(4 chemicals + control) × 30].

The efficiency of chitosan was estimated on the basis of the number of successful infections, i.e. presence of necrosis or pathogen's pycnidia on the canes protected by chitosan and reisolation of *P. viticola*. For this purpose the plant material was tested in laboratory using artificial culture method (Machowicz-Stefaniak and Kuropatwa 1993). The results obtained were statistically analysed and compared with data obtained from combinations with fungicides.

Results

The diameter of four-day-old colonies of *P. viticola* decreased with increasing chitosan concentration, being significantly smaller than the diameter of control colonies at all the concentrations studied (Table 1). After eight days the diameter of the colonies, growing only at concentration of 500 μg of chitosan per 1 cm^3 and concentration recommended by manufacturers was significantly smaller than the diameter of control colonies (Table 2). At the concentrations below 500 μg of chitosan per 1 cm^3 the diameter of the colonies growing in combinations with chitosan and in control combination did not differ significantly (Table 2). Moreover, at concentrations of chitosan below 500 μg/cm^3 no visible, macroscopic changes in the morphology of *P. viticola* colonies were observed. Like in the control combination the mycelium of the studied pathogen was thick, flat to floccose, mainly cream-white with beige-brown colouration. Only in concentrations 500 μg/cm^3 and recommended by manufacturers, colonies of *P. viticola* were pale-white and of looser structure. Microscope observation proved that the hyphae at these concentrations were often strongly deformed, filled with granulation and big, round, chlamydospore-like swellings were observed along them (Phot. 1). At the same time strong inhibition of *P. viticola* growth at all studied concentrations was observed on medium with mancozeb and azoxystrobin (Tables 1, 2). After four

Table 1

Diameter (mm) and inhibition (%, in brackets) of four-day-old colonies of *Phomopsis viticola* on malt extract medium containing tested chemicals (mean values for three strains) at different concentrations of active ingredient

Chemical	10 μg/cm^3	50 μg/cm^3	100 μg/cm^3	200 μg/cm^3	500 μg/cm^3	CR
Chitosan	48.8 c A (3.9)	47.8 c A (5.9)	45.3 c B (10.8)	44.5 b BC (12.4)	42.3 b C (16.7)	29.5 b D (41.9)
Azoxystrobin	5.5 b A (49.8)	20.3 b B (60)	10.5 b C (79.3)	0.5 a D (99)	0 a D (100)	0 a D (100)
Mancozeb	13.0 a A (74.4)	0 a B (100)	0 a B (100)	0 a B (100)	0 a B (100)	0 a B (100)
Control	50.8 d A (0)	50.8 d A (0)	50.8 d A (0)	50.8 c A (0)	50.8 c A (0)	50.8 c A (0)

CR – concentration recommended by manufacturers.
Small letters – differences depending on chemical at given concentration, $LSD_{0.05} = 1.72$.
Capital letters – differences depending on concentration at given chemical, $LSD_{0.05} = 2.35$.
Means differ significantly if they are not marked with the same letter.

Table 2

Diameter (mm) and inhibition (%, in brackets) of eight-day-old colonies of *Phomopsis viticola* on malt extract medium containing tested chemicals (mean values for three strains) at different concentrations of active ingredient

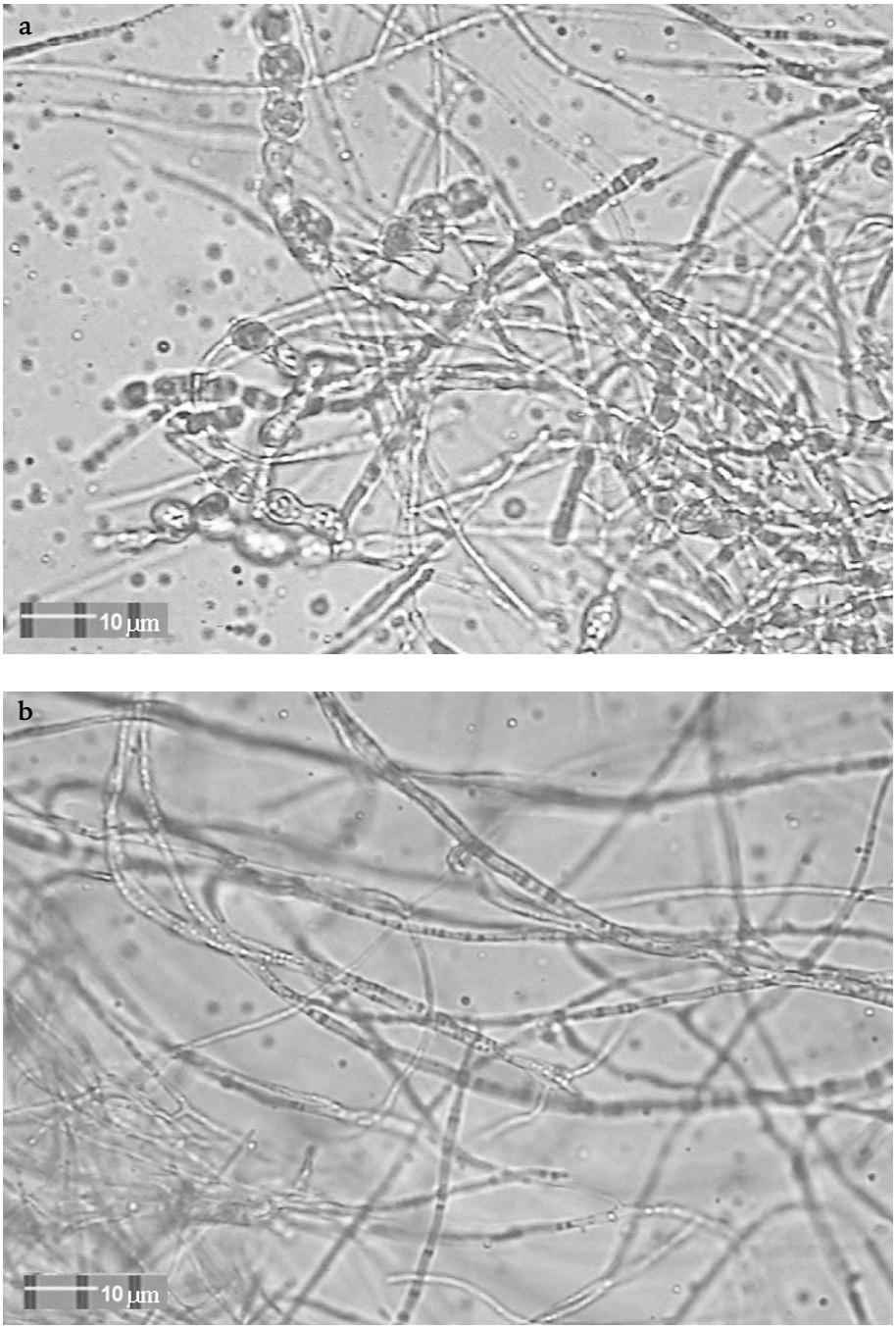
Chemical	10 μg/cm^3	50 μg/cm^3	100 μg/cm^3	200 μg/cm^3	500 μg/cm^3	CR
Chitosan	90 c A (0)	90 c A (0)	90 c A (0)	90 c A (0)	77 d B (14.4)	56 d C (37.8)
Azoxystrobin	71.8 b A (20.2)	53.5 b B (40.6)	49.5 b C (45)	41.8 b D (53.6)	4.5 b F (95)	31.8 b E (64.7)
Mancozeb	50 a A (44.4)	1.8 a B (98)	0 a C (100)	0 a C (100)	0 a C (100)	0 a C (100)
Control	90 c A (0)	90 c A (0)	90 c A (0)	90 c A (0)	90 c A (0)	90 c A (0)

Explanations – see Table 1.

days of incubation the former compound completely inhibited the colonies growth at concentrations 50 μg of a.i. per 1 cm^3 and above, whereas the latter – at 500 μg of a.i. per 1 cm^3 and at the concentration recommended by manufacturers (Table 1). After eight days only mancozeb was able to completely inhibit *P. viticola* colonies growth at 100 μg of a.i. per 1 cm^3 and above, and was fungitoxic to the pathogen at these concentrations (Table 2).

All treatments of grapevine canes reduced the incidence of necrosis and the formation of pathogen pycnidia, as compared with control (Table 3).

Biochicol 020 PC at 1.0% was more effective than at 0.5%, as 9.2% and 18.3% positive infections were observed, respectively (Table 3). The effectiveness of Biochicol 020 PC at 1.0% and Dithane M-45 80 WP was similar, and the percentage of canes with disease symptoms did not differ significantly. Amistar 250 SC



Phot. 1. Deformation of *Phomopsis viticola* hyphae after application of chitosan (a) and the appearance of hyphae in control (b) (photo by E. Zalewska)

Table 3

Number of positive infections (from 30 in each combination) by *Phomopsis viticola* on the canes protected with chitosan and standard products (mean values for three strains)

Chemical	'Agat Donskij'	'Iza'	'Prim'	RF-16	Total
Biochicol 020 PC 0.5%	4	6	5	7	22 c (18.3%)
Biochicol 020 PC 1.0%	2	3	2	4	11 b (9.2%)
Amistar 250 SC 1.0%	0	1	0	3	4 a (3.3%)
Dithane M-45 80 WP 1%	1	1	3	3	8 b (6.7%)
Positive control	23	26	26	27	102 d (85.0%)
Total	30 (20%)	37 (24.7%)	36 (24%)	77 (51.3%)	

Means differ significantly if they are not marked with the same letter.
$LSD_{0.05} = 3.85$.

seemed to protect grapevine canes significantly better than the other preparations studied (Table 3). Besides, the highest number of positive infection was found on RF-16 cultivar and the lowest on 'Agat Donskij' (Table 3). Mycological analysis showed that from the canes protected with chemicals, cultures of *P. viticola* were reisolated only from external tissue, whereas from control ones they were reisolated both from external and internal tissue.

Discussion

The studies showed that the influence of chitosan on the linear growth of *P. viticola* colonies depended on the concentration of active ingredient and time of incubation (Tables 1, 2).

Much information from literature suggested that chitosan effectively inhibited the growth and development of the majority of pathogenic fungi species under laboratory conditions (Allan and Hadwiger 1979, Pospieszny and Struszczyk 1994, Pięta et al. 1998, Pastucha 2001). However, poor inhibition of *P. viticola* colonies growth, observed in the present studies, confirmed the literature data, that direct activity of chitosan is modified by various factors, among others fungus species or strain, but the mechanisms of this phenomenon is unknown so far (Allan and Hadwiger 1979, Pospieszny 1997, Wojdyła and Orlikowski 1997, Maćkowiak and Pospieszny 2002). Although chitosan poorly inhibited the growth of *P. viticola* mycelium, it was able to effectively limit the spores viability of this pathogen (Król 2005) and protect grapevine canes from infection. Therefore, it seems that chitosan has a good antisporulation properties but probably for limiting colony growth a long lasting contact of chitosan and pathogen is necessary, as Pospieszny (1997) claims. The reduction of necrosis symptoms on grapevine canes by Biochicol 020 PC in concentration of 1% with similar effectiveness as Dithane M-45 80 WP indicated that the chemical can become an alternative to traditional

fungicides in grapevine canes protection against *P. viticola*. Considering that chitosan, besides its direct activity against various pathogens, is able to induce synthesis of a wide spectrum of defensive chemicals in plant (Pospieszny and Struszczyk 1994, Pospieszny 1997, Maćkowiak and Pospieszny 2002) and may contribute to reducing necrosis and colonization of grapevine canes by *P. viticola*, it is concluded that Biochicol 020 PC seems promising in grapevine control against the pathogen.

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