Acta Agrobotanica

DOI: 10.5586/aa.1789

**Publication history**
Received: 2019-09-19
Accepted: 2019-10-21
Published: 2019-12-30

**Handling editor**
Bożena Denisow, Faculty of Environmental Biology, University of Life Sciences in Lublin, Poland

**Authors' contributions**
EDK: research designing, supervising the experiments, writing and editing the manuscript; BAA: conducting experiments, data processing; BZ: helped writing and editing the manuscript; EDZ: data processing, helped writing the manuscript

**Funding**
The project was financed by the Polish Ministry of Science and Higher Education within the framework of the KBN research project: N N310 774940.

**Competing interests**
No competing interests have been declared.



**Citation**
Król ED, Abramczyk BA, Zimowska B, Zalewska ED. Estimation of the antifungal activity of some preparations against *Diaporthe eres* under in vitro conditions. Acta Agrobot. 2019;72(4):1789. https://doi.org/10.5586/aa.1789

ORIGINAL RESEARCH PAPER

# Estimation of the antifungal activity of some preparations against *Diaporthe eres* under in vitro conditions

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## Abstract
Six fungicides from various chemical groups and two natural products, i.e., Biosept Active (grapefruit extract) and Beta-Chikol (chitosan) were tested in vitro against *Diaporthe eres* isolated from the shoots of fruit trees. The preparations were incorporated in PDA medium to provide final fungicide concentrations of 1, 10, and 100 g $cm^{-3}$. Biosept Active concentrations of 0.05%, 0.075%, and 0.1%, and Beta-Chikol concentrations of 1%, 2%, and 2.5%, respectively. The antifungal activity of the preparations was evaluated based on mycelial growth of *D. eres* strains after 4 and 8 days of culture and changes in the morphological structures of the fungus. The highest antifungal activity was registered for thiophanate-methyl at all tested concentrations, followed by thiram, which showed the same activity but only at 100 g $cm^{-3}$. Among the preparations of natural origin, Beta-Chikol was more effective against *D. eres* than Biosept Active. The effects achieved by the former preparation were comparable with those achieved by some of the most effective fungicides tested against *D. eres*.

## Keywords
fruit trees; pathogenic fungi; fungicides; natural products; chitosan; grapefruit extract

## Introduction

*Diaporthe* (anamorph *Phomopsis*) species are widespread and associated with many important plant diseases including necrosis, shoots blight and cancer, decay, wilting, fruit rot, and mummification [1–5]. Following the abolishment of dual nomenclature for fungi, Rossman et al. proposed [6] that the genus name *Diaporthe* should be retained over *Phomopsis* because it was introduced first, represents the majority of species, and therefore has priority. Recently, diseases caused by *Diaporthe* spp. have become an emerging problem orchard worldwide, with serious economic consequences [3,5,7–14]. Of the disease causing species *Diaporthe eres* has been recognized as the main pathogen of woody plants from different botanical families, including Rosaceae, Juglandaceae, Vitaceae, and Ericaceae [6,15,16].

Currently, chemical fungicides are the most common method used to control diseases caused by *Diaporthe* spp. [17–21]. This approach is particularly common against *Diaporthe neoviticola* (*Phomopsis viticola*) because of the substantial damage this pathogen causes to grapevines and the worldwide prevalence and economic importance of this plant [5,17,22–24]. However, excessive use of chemical fungicides has caused serious ecological and environmental damage [25]. Therefore, novel agents with low

toxicity and strong antifungal activity are urgently needed. There are many bioactive compounds in the environment which can be developed into fungicide alternatives [26–29]. These include chitosan and grapefruit extract [23,25,29–36], which exhibit antibacterial activity and biodegradable, nontoxic and capable of directly limit the growth and development of various species of pathogens. These agents can be applied directly before the harvest as they have no currency and prevention date. In addition, they also have the ability to induce a defense response against pathogenes. Due to their unique physical and chemical properties, they are used in agriculture, medicine, food science, industry, and environmental remediation [25,30,31,37–39].

## Material and methods

Three strains of *Diaporthe eres* obtained earlier from diseased shoots of apple (264J), plum (352S), and cherry (322W), six fungicides recommended against various pathogens of fruit trees in Poland, and two natural products registered as biostimulants for plant growth were used in the experiment (Tab. 1).

**Tab. 1** A list of examined preparations.

| Preparations | Concentration of a.i. (%) | Producer |
|---|---|---|
| Discus 500 WG | 500 g/L krezoximmetyl | BASF Polska Sp. z o.o., Poland |
| Kaptan zawiesinowy 50 WP | 50% captan | Organika – Azot, Jaworzno S.A., Poland |
| Miedzian 50 WP | 50% copper oxychloride | Synthos Agro Sp. z o.o., Oświęcim, Poland |
| Sadoplon 75 WP | 75% thiram | |
| Siarkol extra 80 WP | 80% sulfur | CIECH Sarzyna S.A., Poland |
| Topsin M 500 SC | 500 g/L tiophanate-methyl | Nippon Soda, Japan |
| Beta-Chikol | 2% chitosan | Poli-Farm Sp. z o.o., Łowicz, Poland |
| Biosept Active | 33% grapefruit extract | Cintamoni Poland, Majewscy and Koć Sp. j., Piaseczno, Poland |

The strains included in the study were previously identified and tested for pathogenicity and culture requirements [4,40,41]. Based on the RAPD-PCR and comparison of the noncoding sequence of the ITS regions (ITS1, 5.8S rDNA, ITS2) with the sequences available in NCBI database, the studied *Diaporthe* isolates were identified as *Diaporthe eres*, a species complex not described previously on fruit trees in Poland [42].

The studies were carried out in the laboratory according to the method described by Zalewska et al. [34]. The tested fungicides were added to the sterilized PDA medium (BD Difco) cooled to 50°C and at concentrations 1, 10, and 100 g $cm^{-3}$ of active ingredients (a.i.) while the natural products were in three concentrations within the range recommended by the manufacturer, i.e., 0.1%, 0.075%, and 0.05% for Biosept Active (recommended concentration 0.05%–0.1%) and 1%, 2%, and 2.5% for Beta-Chikol (recommended concentration 1%–2.5%).

Mycelial disks of the pathogen (3 mm in diameter) removed from the margins of 10-day-old cultures were transferred to agar media containing the preparations at the tested concentrations; for the control, disks of *D. eres* were placed on agar media without chemicals. Four replicates were used per treatment. For each combination, the inhibition of radial growth compared with untreated control was calculated after 4 and 8 days of incubation at 24°C in the dark according to the Kowalik and Krechnik [43] formula: $I = (C - T)/C \times 100$, where $I$ – inhibition rate of mycelial growth; $C$ – diameter (in millimeters) of the colony on control plate; $T$ – diameter (in millimeters) of colony on the plate with a preparation.

The effectiveness of the preparations was evaluated based on mycelial growth inhibition of *D. eres* strains: each preparation was assigned to one of the four groups of fungicidal activity depending on ED50 values as well as the type of the toxic effect on fungal isolates [44]. Moreover, the macroscopic and microscopic observations were carried out until the thirtieth day of cultivation in order to detect the possible changes in the morphological changes in *D. eres* isolates cultured on the media containing preparations.

## Results

The influence of the studied fungicides on *D. eres* strains was differentiated but all the formulations limited the colony growth of the fungus (Tab. 2–Tab. 7, Fig. 1, Fig. 2, Fig. 5). The diameters of the fungal colonies growing on the media with fungicides were always significantly smaller than those of colonies growing on the control medium, i.e., without the addition of fungicide. Moreover, the diameters of the colonies always decreased with an increasing concentration of the preparation (Tab. 2–Tab. 5).

The highest antifungal activity against *D. eres* was observed for Topsin M 500 SC, at all tested concentrations, followed by Sadoplon 75 WP, which showed the same activity but only at the concentration 100 g $cm^{-3}$ a.i. (Tab. 2–Tab. 5, Fig. 1A,B). In those cases, the mycelial growth of *D. eres* was totally stopped, regardless of the fungal isolate and day of cultivation. However, only Topsin M 500 SC could be included in the first group of fungicidal activity, i.e., as a very strongly fungicidal preparation, since the ED50 against *D. eres* was less than 1 g $cm^{-3}$ a.i. (Tab. 2–Tab. 5, Fig. 1A). High levels of efficiency were recorded when Sadoplon 75 WP was used at the concentrations 1 and 10 g $cm^{-3}$ as well as Discus 500 WG at all tested concentrations. On media containing preparations, the smallest diameters of *D. eres* colonies were observed. After 4 days of cultivation, the colony diameters ranged from 27.3 to 34.3 mm (Sadoplon 75 WP 1 and 10 g $cm^{-3}$), and from 20.5 to 26 mm (Discus 500 WG, all tested concentrations), depending on the concentration. Simultaneously, growth inhibition of the growth of *D. eres* colonies ranged from 29.8% to 44.8% in the presence of Sadoplon 75 WP and from 46.7% to 56.4% on media containing Discus 500 WG (Tab. 2, Tab. 3). After 8 days, the antifungal activity of Sadoplon and Discus preparations slightly decreased since the percentage inhibition of colony growth ranged from 25% to 40.9% (Sadoplon) and from 30.7% to 52.9% (Discus) (Tab. 4, Tab. 5). Based on these results, Discus 500 WG was classified as a strong fungicidal formulation, i.e., belonging to the second group of fungicidal activity, since its ED50 against *Diaporthe* isolates was generally between 1 and 10 g $cm^{-3}$ a.i. but only after 4 days of culture. After 8 days of culture, the fungicidal activity decreased. At that time, Discus 500 WG was among the preparations that exhibited moderate fungicidal activity, i.e., belonged to the third group of fungicidal activity, along with Sadoplon 75 WP and Kaptan 50 WP. ED50 for these fungicides is between 10 and 100 g $cm^{-3}$ (Tab. 3, Tab. 5, Fig. 2A,B).

The lowest antifungal activity was observed for Miedzian 50 WP and Siarkol extra 80 WP at all tested concentrations (Tab. 3, Tab. 5). On media containing these fungicides, the largest diameter of the colonies was observed after 4 and 8 days of culture (Tab. 2, Tab. 4). For this reason, Miedzian 50 WP and Siarkol extra 80 WP were included with fungicides with low efficiency, i.e., the fourth group of fungicidal activity. Their ED50 against for *D. eres* isolates was above 100 g $cm^{-3}$ a.i.

Moreover, it was shown that only Topsin M 500 SC was fungicidal towards *D. eres*, because after transferring the fungus disks from the medium containing this preparation to a clean medium, the studied isolates did not resume the growth.

Macroscopic and microscopic observations showed that the appearance of *D. eres* colonies growing on the media with fungicides at the concentrations 1 and 10 g $cm^{-3}$ was similar to control colonies. Only at the concentration of 100 g $cm^{-3}$, colonies of *D. eres* had a more compact mycelium, especially in the presence of Discus 500 WG which belonged to the second group of fungicidal activity. In those cases, the fungal hyphae were thickened and disintegrated (Fig. 3). As strains of *D. eres* sporulated with difficulty, there was no sporulation of the fungus until the eighth day of the cultivation, both in the presence of fungicides as well as in the control.

**Tab. 2** Effect of fungicides on mycelial growth of *Diaporthe eres* strains after 4 days of cultivation.

| | Diameter of 4-day-old colony in mm depending on a.i. concentration (g cm$^{-3}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Strain 264J | | | Strain 352S | | | Strain 322W | | |
| Fungicide | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 |
| Discus 500 WG | 26.0 $^{aA}$ | 24.0 $^{aB}$ | 22.0 $^{aC}$ | 26.0 $^{aA}$ | 24.0 $^{aB}$ | 22.0 $^{aC}$ | 25.0 $^{aD}$ | 23.8 $^{aB}$ | 20.5 $^{aE}$ |
| Kaptan zaw. 50 WP | 47.0 $^{bdA}$ | 45.0 $^{bB}$ | 17.8 $^{bC}$ | 46.3 $^{bA}$ | 44.0 $^{bD}$ | 16.8 $^{bE}$ | 46.5 $^{bhA}$ | 43.8 $^{bD}$ | 15.8 $^{bF}$ |
| Miedzian 50 WP | 46.0 $^{bA}$ | 41.5 $^{cB}$ | 26.5 $^{cCE}$ | 45.0 $^{bD}$ | 41.3 $^{cB}$ | 26.0 $^{cC}$ | 45.5 $^{bD}$ | 41.5 $^{cB}$ | 27.0 $^{cE}$ |
| Sadoplon 75 WP | 33.8 $^{cAE}$ | 27.3 $^{dB}$ | 0.0 $^{dC}$ | 34.3 $^{cA}$ | 29.0 $^{dD}$ | 0.0 $^{dC}$ | 33.0 $^{cE}$ | 28.0 $^{dB}$ | 0.0 $^{dC}$ |
| Siarkol extra 80 WP | 48.0 $^{dA}$ | 43.0 $^{eB}$ | 23.7 $^{eC}$ | 46.0 $^{bD}$ | 43.0 $^{beB}$ | 23.0 $^{aC}$ | 46.0 $^{bhD}$ | 40.0 $^{eE}$ | 25.0 $^{eF}$ |
| Topsin M 500 SC | 0.0 $^{eA}$ | 0.0 $^{fA}$ | 0.0 $^{dA}$ | 0.0 $^{dA}$ | 0.0 $^{fA}$ | 0.0 $^{eA}$ | 0.0 $^{dA}$ | 0.0 $^{fA}$ | 0.0 $^{dA}$ |
| Control | 49.5 $^{gA}$ | | | 48.8 $^{gA}$ | | | 47 $^{hB}$ | | |

Small letters – differences among fungicides at a given concentration, $p \leq 0.05$ (LSD = 1.48). Capital letters – differences among concentration at a given fungicide, $p \leq 0.05$ (LSD = 0.84). Values marked with the same letter do not differ significantly.

**Tab. 3** Inhibition the colony growth of *Diaporthe eres* strains after 4 days of cultivation.

| | Percent of inhibition in relation to a.i. concentration (g cm$^{-3}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Strain 264J | | | Strain 352S | | | Strain 322W | | |
| Fungicide | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 |
| Discus 500 WG | 47.5 | 51.5 | 55.6 | 46.7 | 50.8 | 55.0 | 46.8 | 49.4 | 56.4 |
| Kaptan zaw. 50 WP | 5.0 | 9.1 | 64.0 | 5.0 | 9.8 | 65.6 | 1.0 | 6.8 | 66.4 |
| Miedzian 50 WP | 7.0 | 16.2 | 46.7 | 7.8 | 15.4 | 46.7 | 3.2 | 11.7 | 42.6 |
| Sadoplon 75 WP | 31.7 | 44.8 | 100.0 | 29.7 | 40.6 | 100.0 | 29.8 | 40.4 | 100.0 |
| Siarkol extra 80 WP | 3.1 | 15.1 | 52.1 | 5.7 | 11.9 | 46.0 | 2.1 | 14.9 | 46.8 |
| Topsin M 500 SC | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

**Tab. 4** Effect of fungicides on mycelial growth of *Diaporthe eres* strains after 8 days of cultivation.

| | Diameter of 8-day-old colony in mm depending on a.i. concentration (g cm$^{-3}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Strain 264J | | | Strain 352S | | | Strain 322W | | |
| Fungicide | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 |
| Discus 500 WG | 61.0 $^{aA}$ | 48.0 $^{aB}$ | 45.5 $^{aC}$ | 60.0 $^{aA}$ | 45.5 $^{aC}$ | 42.0 $^{aD}$ | 58.8 $^{aE}$ | 45.0 $^{aC}$ | 41.0 $^{aD}$ |
| Kaptan zaw. 50 WP | 84.5 $^{bA}$ | 81.0 $^{bB}$ | 35.0 $^{bC}$ | 84.0 $^{bA}$ | 79.8 $^{bD}$ | 35.5 $^{bC}$ | 82.0 $^{bB}$ | 77.5 $^{bE}$ | 36.0 $^{bC}$ |
| Miedzian 50 WP | 86.0 $^{cA}$ | 82.3 $^{cB}$ | 66.5 $^{cC}$ | 85.8 $^{cA}$ | 81.0 $^{bD}$ | 67.3 $^{cC}$ | 85.0 $^{cA}$ | 81.0 $^{cD}$ | 65.5 $^{cC}$ |
| Sadoplon 75 WP | 60.0 $^{aA}$ | 52.0 $^{dB}$ | 0.0 $^{dC}$ | 60.0 $^{aA}$ | 51.8 $^{cB}$ | 0.0 $^{dC}$ | 59.5 $^{dA}$ | 51.5 $^{dB}$ | 0.0 $^{dC}$ |
| Siarkol extra 80 WP | 86.0 $^{cA}$ | 83.0 $^{cB}$ | 58.0 $^{eC}$ | 85.0 $^{bcA}$ | 80.0 $^{bD}$ | 52.0 $^{eE}$ | 86.0 $^{cfA}$ | 78.0 $^{bF}$ | 50.0 $^{eG}$ |
| Topsin M 500 SC | 0.0 $^{dA}$ | 0.0 $^{eA}$ | 0.0 $^{dA}$ | 0.0 $^{dA}$ | 0.0 $^{dA}$ | 0.0 $^{dA}$ | 0.0 $^{eA}$ | 0.0 $^{eA}$ | 0.0 $^{dA}$ |
| Control | 88.0 $^{fA}$ | | | 87.5 $^{fA}$ | | | 87.0 $^{fA}$ | | |

Small letters – differences among fungicides at a given concentration, $p \leq 0.05$ (LSD = 1.08). Capital letters – differences among concentration at a given fungicide, $p \leq 0.05$ (LSD = 1.02). Values marked with the same letter do not differ significantly.

**Tab. 5** Inhibition of colony growth of *Diaporthe eres* strains after 8 days of cultivation.

| | Percent of inhibition in relation to a.i. concentration (g cm$^{-3}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Strain 264J | | | Strain 352S | | | Strain 322W | | |
| Fungicide | 1 | 10 | 100 | 1 | 10 | 100 | 1 | 10 | 100 |
| Discus 500 WG | 30.7 | 45.5 | 48.9 | 31.4 | 48.0 | 52.0 | 46.0 | 48.3 | 52.9 |
| Kaptan zaw. 50 WP | 4.0 | 8.0 | 60.2 | 4.0 | 8.8 | 59.4 | 5.7 | 10.9 | 58.6 |
| Miedzian 50 WP | 2.3 | 6.5 | 24.4 | 2.0 | 7.4 | 23.0 | 1.7 | 6.9 | 24.7 |
| Sadoplon 75 WP | 25.0 | 40.9 | 100.0 | 31.4 | 40.8 | 100.0 | 31.6 | 40.8 | 100.0 |
| Siarkol extra 80 WP | 2.3 | 5.7 | 34.1 | 2.9 | 8.6 | 40.6 | 1.5 | 10.3 | 42.5 |
| Topsin M 500 SC | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

**Tab. 6** Influence of Biosept Active on the growth of *Diaporthe eres* strains.

| Concentration (%) | Strain 264J | Strain 352S | Strain 322W |
|---|---|---|---|
| **Diameter of 4-day-old colonies in mm (inhibition rate)** | | | |
| 0.05 | 24.00 $^{aA}$ (55.6) | 30.00 $^{bA}$ (45.5) | 22.25 $^{cA}$ (58.4) |
| 0.075 | 22.00 $^{aB}$ (59.3) | 24.75 $^{bB}$ (55.0) | 19.25 $^{cB}$ (64.0) |
| 0.1 | 17.50 $^{aC}$ (67.6) | 18.00 $^{aC}$ (67.3) | 11.00 $^{bC}$ (79.4) |
| Control | 53.50 $^{dA}$ | 55.00 $^{dA}$ | 54.00 $^{cA}$ |
| **Diameter of 8-day-old colonies in mm (inhibition rate)** | | | |
| 0.05 | 43.00 $^{aA}$ (47.9) | 41.75 $^{aA}$ (49.4) | 36.25 $^{bB}$ (56.0) |
| 0.075 | 63.75 $^{aA}$ (23.9) | 41.50 $^{bB}$ (50.4) | 37.50 $^{cC}$ (55.4) |
| 0.1 | 40.25 $^{aA}$ (48.9) | 34.50 $^{bB}$ (56.2) | 32.00 $^{cC}$ (59.4) |
| Control | 78.75 $^{dA}$ | 83.75 $^{cB}$ | 82.50 $^{cB}$ |

Small letters – differences among isolates at a given concentration, $p \leq 0.05$ (LSD = 1.55 for 4-day-old colonies and 2.30 for 8-day-old ones). Capital letters – differences among concentration in a given isolate, $p \leq 0.05$ (LSD = 1.22 for 4-day-old colonies and 1.80 for 8-day-old ones). Values marked with the same letter do not differ significantly.

**Tab. 7** Influence of Beta-Chikol on the growth of *Diaporthe eres* strains.

| Concentration (%) | Strain 264J | Strain 352S | Strain 322W |
|---|---|---|---|
| **Diameter of 4-day-old colonies in mm (inhibition rate)** | | | |
| 1 | 7.0 $^{aA}$ (84.1) | 7.0 $^{aA}$ (88.4) | 5.0 $^{bA}$ (88.4) |
| 2 | 5.0 $^{aB}$ (88.6) | 5.0 $^{aB}$ (88.9) | 4.0 $^{bB}$ (64.0) |
| 2.5 | 0.0 $^{aC}$ (100) | 0.0 $^{aC}$ (100) | 0.0 $^{aC}$ (100) |
| Control | 44.0 $^{cA}$ | 45.0 $^{cB}$ | 43.0 $^{bC}$ |
| **Diameter of 8-day-old colonies in mm (inhibition rate)** | | | |
| 1 | 18.0 $^{aA}$ (74.3) | 17.5 $^{aA}$ (75.0) | 15.5 $^{bA}$ (77.5) |
| 2 | 12.0 $^{aB}$ (82.9) | 11.0 $^{bB}$ (84.3) | 9.0 $^{cB}$ (87.0) |
| 2.5 | 9.0 $^{aC}$ (87.1) | 8.0 $^{bC}$ (87.1) | 6.0 $^{cC}$ (91.3) |
| Control | 70.0 $^{cA}$ | 70.0 $^{dA}$ | 69.0 $^{dB}$ |

Small letters – differences among isolates at a given concentration, $p \leq 0.05$ (LSD = 0.85 for 4-day-old colonies and 0.93 for 8-day-old ones). Capital letters – differences among concentration in a given isolate, $p \leq 0.05$ (LSD = 0.67 for 4-day-old colonies and 0.73 for 8-day-old ones). Values marked with the same letter do not differ significantly.

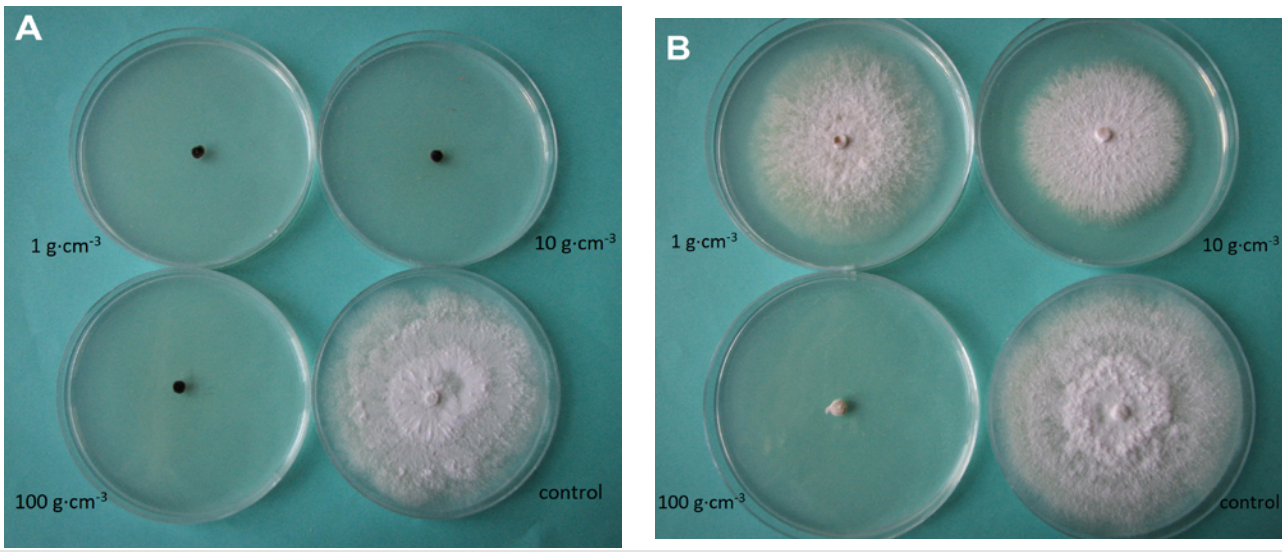


**Fig. 1** Growth of *Diaporthe eres* (strain 264J) on PDA medium containing Topsin M 500 SC (**A**) and Sadoplon 75 WP (**B**) (photo B. Abramczyk).

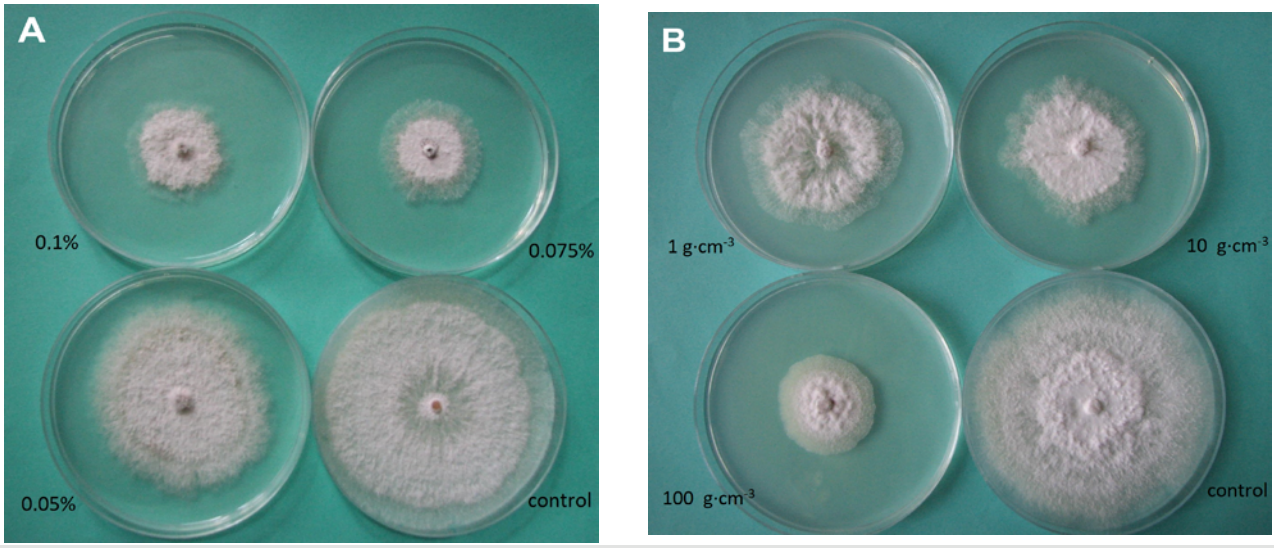


**Fig. 2** Growth of *Diaporthe eres* (strain 264J) on PDA medium containing Discus 500 WG (**A**) and Kaptan 50 WP (**B**) (photo B. Abramczyk).

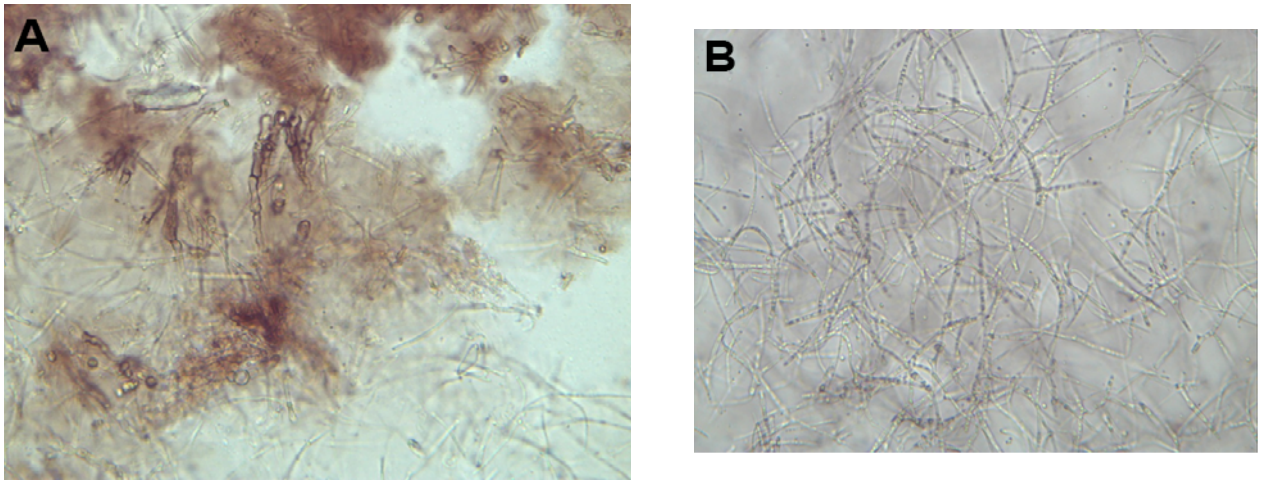


**Fig. 3** Deformation of *Diaporthe eres* hyphae (strain 264J) growing on medium supplemented with Discus 500 WG at the concentration of 10 g $cm^{-3}$ (**A**) and the appearance of hyphae in the control (**B**) (photo B. Abramczyk).

Conidia began to form after about 2–3 weeks, but only in the control combination and on the media containing Miedzian 50 WP and Siarkol extra 80 WP preparations. After 30 days, a few pycnidia were observed in all combinations tested in the experiment. However, on the media containing fungicides the fungus mainly formed beta conidia, while on the control medium alpha conidia predominated (Fig. 4).

Natural products, similar to fungicides, effectively limited the growth of *D. eres* strains. The diameters of 4- and 8-day-old colonies of the tested isolates growing on media containing Biosept Active and Beta-Chikol were significantly smaller than those of the control colonies, regardless of the concentrations and days of incubation (Tab. 6, Tab. 7).

Moreover, it was observed that the smallest diameter of the colonies and simultaneously the strongest inhibition of their growth were noted at the highest concentrations of those products. With the decrease in the concentration, the colony diameters increased and the inhibition rates decreased (Tab. 6, Tab. 7, Fig. 5).

Beta-Chikol was more effective against *D. eres* than Biosept Active. The highest efficiency of the former was noted after 4 days at the concentration 2.5% because no mycelial growth was observed. Only on the surface of the earlier placed mycelial discs, there were bright hyphae of fungus which did not penetrate into the agar medium. However, small colonies with a diameter from 6 to 9 mm appeared around these discs after 8 days of incubation (Tab. 7). Beta-Chikol strongly limited the mycelial growth of *D. eres* strains also at the concentrations of 1% and 2% since the diameters of the fungus colonies ranged from 5 to 7 mm (Beta-Chikol at the concentration 1%) and from 4 to 5 mm (2%) after 4 days, and from 15.5 to 18 mm (1%) and from 9 to 12 mm (2%) after 8 days. Generally, the inhibition rate values for Beta-Chikol were from 64% to 100%, while for Biosept Active from 23.9% to 79.4%, depending on the isolate, the concentration, and the time of incubation (Tab. 6, Tab. 7).

Macroscopic observation showed that *D. eres* colonies growing on the media containing Beta-Chikol and with Biosept Active at the highest concentrations (0.1%) formed a more compact mycelium without characteristic zoning, as compared to the control ones. In microscopic preparations, strongly deformed, thickened, and crumbling hyphae of *D. eres* were observed growing on the media containing the preparations (Fig. 6A), while hyphae of control colonies were thin and long.

Conidia of *D. eres* began to form after 3 weeks in the control combination, while on the media containing the preparations they appeared after 35 days. However, in those cases only fungal colonies with mainly beta conidia were observed, while on the control medium alpha conidia predominated (Fig. 6B).

## Discussion

The growing importance of pathogenic fungi of the genus *Diaporthe* has necessitated the development of effective methods for their control. The first element of an effective antifungal strategy is prevention, implemented through systematic removal of diseased shoots, both during vegetation and during the period of resting of plants, as well as through the use of healthy propagation material. Effective prevention allows reducing the occurrence of disease symptoms at the beginning of the growing season and the number of treatments in the season [17,18,20,33,45,46].

*Diaporthe eres* is still not included among the pathogens in orchard plants protection programs. Therefore, it appears that the information about the possibility of limiting the growth and development of the pathogen could be highly useful.

In the present research, all tested formulations more or less limited the development of the pathogen. This confirms the hypothesis that the protection of fruit trees against various pathogens may also contribute to inhibiting the development of *D. eres*.

However, the highest antifungal activity against this pathogen was registered for Topsin M 500 SC (thiophanate-methyl), at all tested concentrations, followed by Sadoplon 70 WP (thiram), which showed the same activity but only at 100 g $cm^{-3}$. These preparations completely inhibited fungal growth, and thiophanate-methyl was fungicidal. Similarly, Thomidis and Michailides [13] showed that thiophanate-methyl as well as carbendazim and tebuconazole significantly inhibited the growth of *D. eres*,

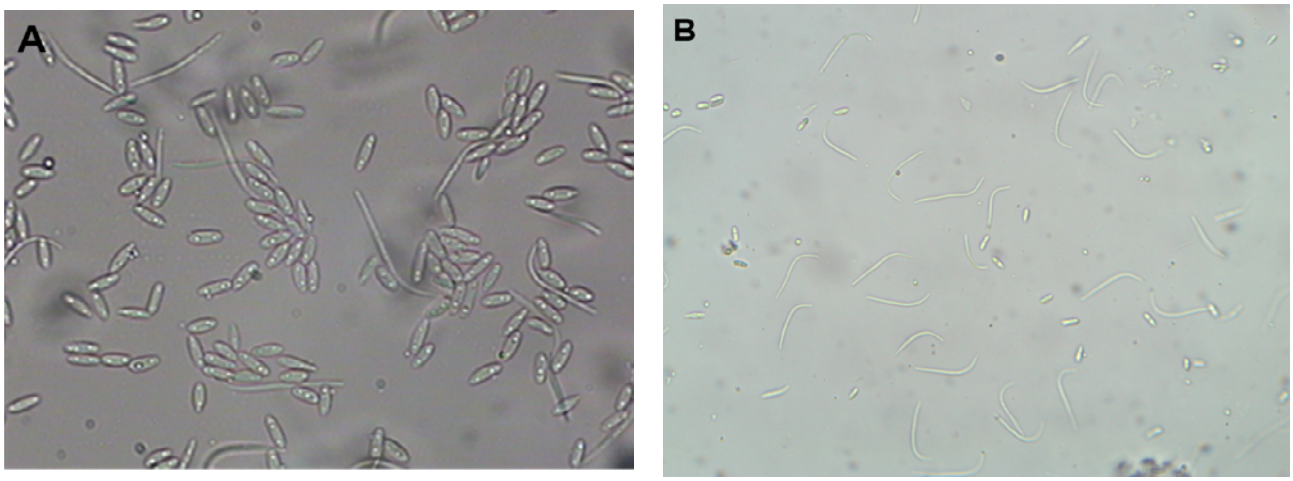


**Fig. 4** Predominance of alpha-type conidia in the control (**A**) and beta-type conidia in cultures growing in the presence of fungicides (**B**) (photo E. Król).

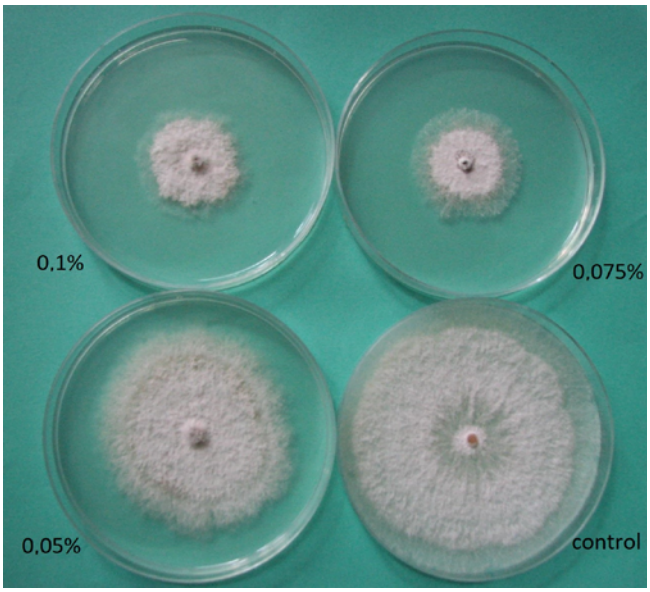


**Fig. 5** Growth of *Diaporthe eres* (strain 322W) on PDA medium containing Biosept Active (photo B. Abramczyk).

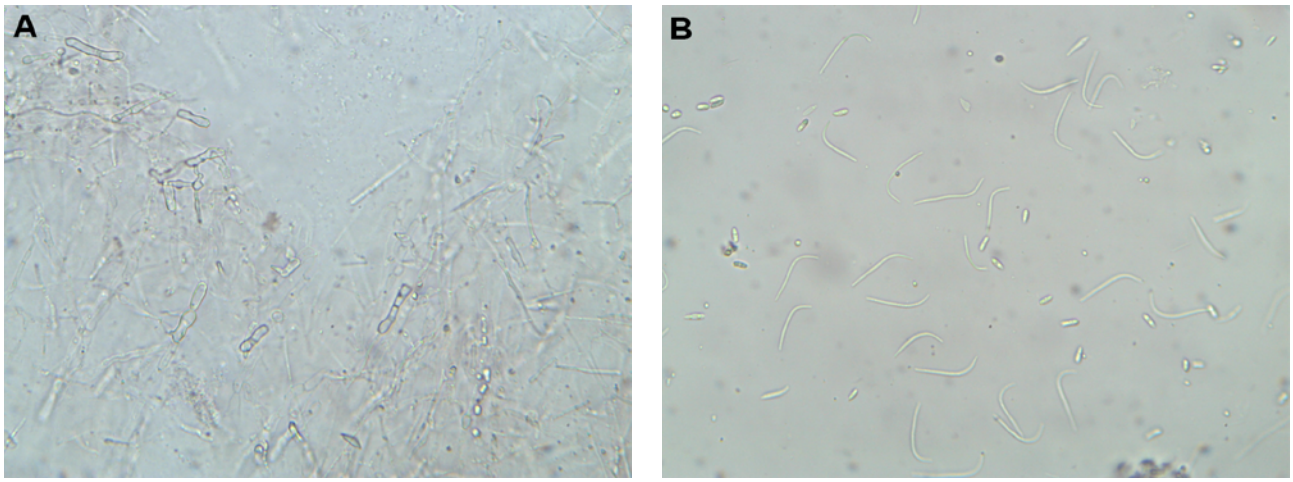


**Fig. 6** Degradation of *Diaporthe eres* hyphae (**A**) and the prevalence of beta-type conidia (**B**) in cultures growing in the presence of Beta-Chikol at the concentration of 2% (photo B. Abramczyk).

whereas iprodione and the mixture of cyprodinil fludioxonil were less effective in inhibiting mycelial growth and disease symptoms.

In the literature, there is very little information on the control of *D. eres* and most studies focus on other species of this genus.

According to some authors, fungicides restricting the development of *P. viticola* are normally recommended for controlling downy mildew (mancozeb), powdery mildew (penconazole, flusilazole, spiroxamine), and bark and wood pathogens (thiophanate-methyl) or those active against a wide range of pathogens (strobilurin fungicides) [17,18,47–49]. Research conducted in Poland showed that the use of both chemical and biological preparations effectively inhibited the growth of *P. viticola* in vitro and in vivo [18,33,47,48,50]. In the world literature, there are only a few studies focusing on the chemical control of pathogens from the genus *Diaporthe* (*Phomopsis*) other than *P. viticola*. This is probably due to their relatively low damage to fruit trees as compared with other pathogens that have attacked orchards for years, causing different diseases.

Lalancette and Robinson [45] reported that seven to eight fungicide sprays during leaf abscission in fall reduced the incidence of cancers caused by *Phomopsis amygdali* in peach trees from 45% to 63%. Some authors reported that copper compounds, chlorothalonil, captan, thiophanate-methyl, azoxystrobin, and myclobutanil were the most effective among the agents used for protection against *P. amygdali*. It was also found that some fungicides used in orchards against pathogens causing brown rot of stone trees and scab peach can also limit the development of *P. amygdali* [13,45,51,52]. In a similar study conducted on almond in Tunisia, Rhouma et al. [19] showed that benomyl, thiophanate-methyl, and carbendazim completely inhibited the growth of mycela and germination of spores of the studied pathogen in vitro and reduced disease symptoms by more than 70% in field conditions. On the other hand, Rohini et al. [20] showed that carbendazim effectively limited the development of *P. vexans* which causes *Phomopsis* leaf blight of brinjal both in the laboratory and in the greenhouse. Despite the high efficacy of thiophanate-methyl, previous studies showed that various isolates of *Phomopsis* spp. acquired resistance to benzimidazoles in field conditions [13,53].

For this reason and considering the appearance of resistant *Diaporthe* strains and the environmental toxicity of fungicides, other options for pathogen control are being sought as alternative to chemical protection. An increase in the number of ecological farms in the last decade is based on natural cultivation methods, i.e., without the use of chemicals to protect plants or the application of mineral fertilization [38].

In recent years, particular attention has been paid to environmentally friendly methods of plant protection. These include the use of knotweed (*Polygonum*), citrus (*Citrus*), or coconut (*Cocos*) extracts and paraffin oil to protect vines against *P. viticola* [23,26]. The protection of plants using natural origin preparations seems to be a very important element in reducing pathogens in orchards and nurseries in European countries due to an obligation to use plant disease management methods.

In the present study, the inhibitor effect of Beta-Chikol on *D. eres* was significantly higher than that of Biosept Active. The same results were obtained by Zalewska et al. [34] with respect to limiting *P. diachenii* growth. Moreover, it follows from the observations that a higher concentration of the active substance contributes to a greater colony growth inhibition.

Similarly, after the application of Biochikol 020 PC which is based on chitosan, *P. viticola* growth was limited and the viability of conidia was decreased. Simultaneously, Biochikol 020 PC protected grapevine canes against *P. viticola* during storage as effectively as did the fungicide Dithane M-45 80 WP (mancozeb) [18,33,37,49].

According to Nascimento et al. [37], chitosan showed a promising inhibitory effect on mycelial growth of the main fungi involved in grapevine wood diseases, including *Phomopsis*. Although different application methods were used, the effects achieved by chitosan were comparable with those obtained with some of the most effective fungicides tested against this pathogen. Moreover, chitosan treated plants showed significantly improved growth (plant height and number of roots) and decreased disease incidence compared with untreated plants.

The obtained results indicate that the direct inhibitory effect of grapefruit extract on *D. eres* was weaker than that of chitosan, despite the presence of biologically active substances are present in the preparation, including endogenous flavonoids that inhibit spore germination, and growth of vegetative hyphae [30,31].

## Conclusions

- Some fungicides used to protect fruit trees against apple scab, powdery mildew, brown rot of stone and pome fruit, cherry leaf spot, cankers, peach leaf curl, or plum pockets can also limit the development of *D. eres*.
- The most promising compound for controlling of *D. eres* was Topsin M 500 SC (thiophanate-methyl) because only this preparation completely inhibited the development of the fungus colony and showed fungicidal activity. Other fungicides, although less effective in inhibiting pathogen colony growth, caused hypha deformation, especially at the highest concentration tested.
- The inhibitory effect of Beta-Chikol towards *D. eres* significantly exceeded that of Biosept Active.
- In the future, chitosan could be an alternative or used to complement conventional fungicides in integrated plant disease management protocols targeting of *D. eres*.

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## Ocena aktywności grzybobójczej wybranych preparatów w stosunku do *Diaporthe eres* w warunkach in vitro


### Streszczenie

Sześć fungicydów z różnych grup chemicznych i dwa preparaty naturalnego pochodzenia tj. Biosept Active (ekstrakt z grejpfruta) i Beta-Chikol (chitosan), przebadano in vitro przeciwko *Diaporthe eres* wyizolowanego z pędów drzew owocowych. Preparaty wprowadzono do pożywki PDA, aby zapewnić końcowe stężenia 1, 10 i 100 g $cm^{-3}$ dla fungicydów i odpowiednio 0,05%, 0,075%, 0,1% i 1%, 2%, 2,5 % w przypadku Biosept Active i Beta-Chikol. Aktywność grzybobójczą preparatów oceniano na podstawie wzrostu grzybni szczepów *D. eres* po 4 i 8 dniach hodowli oraz zmian w strukturach morfologicznych tego grzyba. Najwyższą aktywność grzybobójczą w stosunku do *D. eres* odnotowano dla tiofanatu metylowego, we wszystkich badanych stężeniach, oraz dla tiuramu, ale tylko w stężeniu 100 g $cm^{-3}$. Wśród preparatów naturalnego pochodzenia Beta-Chikol był bardziej skuteczny przeciwko *D. eres* niż Biosept Active. Wyniki uzyskane w przypadku pierwszego preparatu były porównywalne z efektami uzyskanymi przy zastosowaniu niektórych najskuteczniejszych fungicydów testowanych przeciwko *D. eres*.