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IN VITRO ACTIVITY OF PREPARATIONS CONTAINING NATURAL SUBSTANCES TOWARDS *BOTRYTIS CINEREA*

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Abstract

The use of natural products for control of fungal diseases of plants is considered an interesting alternative to synthetic fungicides due to their lower impact on the environment. The effect of preparations Biochikol 020 PC, Antifung 20 SL, Bioczos BR and Biosept 33 SL on the linear growth, conidia germination of *Botrytis cinerea* and biotic relations between this fungus and *Trichoderma viride* was assessed. Mycelial growth of *B. cinerea* was strongly inhibited at the maximum concentration (100 ppm) of Antifung and Biosept. All preparations suppressed conidia germination. All the preparations, as opposed to fungicide Topsin M, stimulated suppressive activity of *T. viride* in relation to *B. cinerea*.

Key words: *Botrytis cinerea*, preparations, mycelium growth, conidia germination, biotic relations

Introduction

Grey mould caused by *Botrytis cinerea* is one of the most common plant diseases. The polyphagous pathogen attacks numerous plant species. Protection against grey mould uses both agrotechnical and chemical methods. However, due to common sources of infection and the fungus resistance to anilinpyrimidine, benzimidazole and dichlofluanid fungicides, their efficacy is unsatisfactory (Chapeland et al. 1999, Borecki 2001, Yourman et al. 2001, Leroux et al. 2002, Botrytis... 2004, Myresiotis et al. 2007, Williamson et al. 2007). At the same time consumer pressure on production of healthy and safe food turns attention to potential application of preparations containing natural substances for *B. cinerea* control (Orlikowski et al. 2001).

Plant protection with the preparations registered in Poland within the last 10 years comprise: Biochikol 020 PC containing chitosan ($20 \text{ g}\cdot\text{l}^{-1}$), Antifung 20 SL with 20% of biohumus as the active substance, Bioczoz BR based on garlic pulp and Biosept 33 SL manufactured on the basis of grapefruit extract (33%) (Orlikowski et al. 2002).

The aim of the work was to assess the effect of preparations mentioned above on mycelial growth of *B. cinerea*, its conidia germination and biotic relationship of this fungus towards *Trichoderma viride*.

Material and methods

The research material consisted of fungal isolates obtained from the collection of the Department of Agricultural Environment Protection, University of Agriculture in Cracow: *Botrytis cinerea* and *Trichoderma viride*. The effect of preparations such as Biochikol 020 PC (Gumitex Poli-Farm Łowicz), Antifung 20 SL (Host International Cedry Małe near Gdańsk), Bioczoz BR (Himal Łódź) and Biosept 33 SL (Citamani Poland Piaseczno) was studied at their concentrations of 1, 10, 100 ppm ($\text{mg}\cdot\text{kg}^{-1}$).

The *in vitro* effect of the preparations on *B. cinerea* linear growth was examined with the poisoned medium method (Borecki 1984). Potato dextrose agar (PDA) was prepared with the addition of respective preparations. The media were inoculated with agar disc (5 mm in diameter) overgrown with 2-week-old culture of *B. cinerea*. Control combination consisted of medium without preparations. The results obtained were expressed as the inhibition coefficient of linear fungal growth, calculated according to Abbott's formula (Burgiel 1984).

Germination capacity of *B. cinerea* conidia in the presence of preparations was evaluated with the method described by Burgiel (1984). In water solutions of preparation, a suspension of conidia (sampled from 2-week-old cultures) was prepared. The germination process was stopped by adding a drop of formalin after 48 h of incubation at 21°C . The degree of conidia germination was estimated according to a scale, and the index of conidia germination was calculated according to Burgiel (1984).

The results of the experiments were verified statistically with variance analysis assumed for two-factor experiments (factor A – preparations studied, factor B – concentration of the preparations). Significance of differences was verified with Duncan's test.

The biotic correlations between *B. cinerea* and *T. viride* antagonistic fungus were defined with the biotic series method following Mańka (1974). The analyzed fungi were inoculated at a distance of 2 cm one from another in a central part of Petri plate with PDA medium supplemented with the analyzed preparations at concentrations of 1, 10 or 100 ppm. After 10 days of incubation, each combination was assessed on a scale, regarding three parameters: extent to which one fungal colony was surrounded by the other, inhibition zone and colony diminishing. The highest

mark on the 8-point scale denoted a complete lack of fungal growth. A “+” sign (positive effect) was used in the case of *T. viride* domination, a “-” sign (negative effect) for the domination of *B. cinerea* fungus, and “0” was given if no prevalence of any colony could be observed. The values obtained provided jointly an individual biotic effect (IBE) illustrating the influence of *T. viride* isolate on the growth of *B. cinerea*.

All the above experiments were carried out in four replicates.

Results

The investigated preparations containing natural substances were inhibiting the growth of *B. cinerea* mycelium (Figs. 1 and 2). However, they were acting significantly more weakly than the chemical standard. From among the tested preparations Antifug 20 SL most strongly suppressed mycelial growth. This preparation, even in a concentration of 1 ppm markedly inhibited the mycelium growth. High efficiency of biohumus solution was obtained at 100 ppm, when the inhibition of mycelial growth reached almost 80% and was the highest among the results obtained under the influence of the preparations applied. Also grapefruit extract at a concentration of 100 ppm strongly restrained *B. cinerea* growth limiting it by half. On the other hand, Biochikol 020 PC applied even at the highest concentrations did not block the mycelial growth.

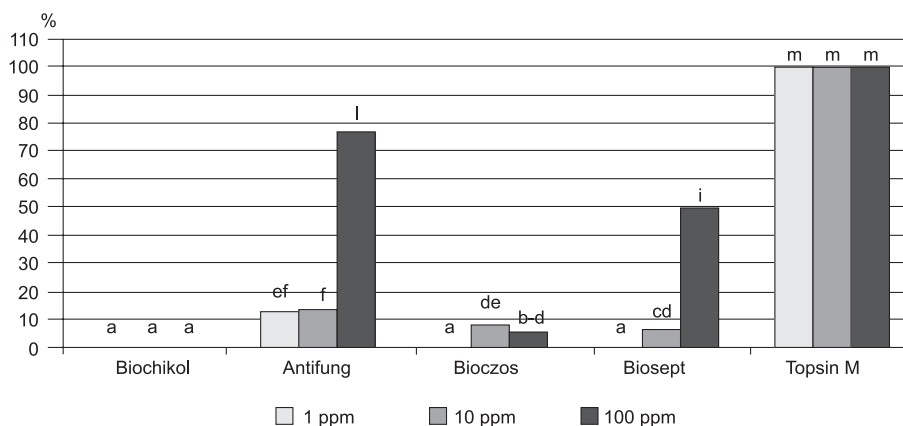


Fig. 1. Effect of preparations on inhibition of mycelium development of *Botrytis cinerea* – percentage of control without preparation (columns marked with different letters differed significantly according to Duncan's test at $p = 0.05$)

All preparations blocked *B. cinerea* conidia germination (Fig. 3). It was found that the value of conidia germination index was declining with increasing concentrations of active substance. Topsin M applied as a chemical standard at the concentrations of 10 and 100 ppm caused that *B. cinerea* conidia did not form germ hyphae at all. Preparations were acting obviously more weakly than a chemical,

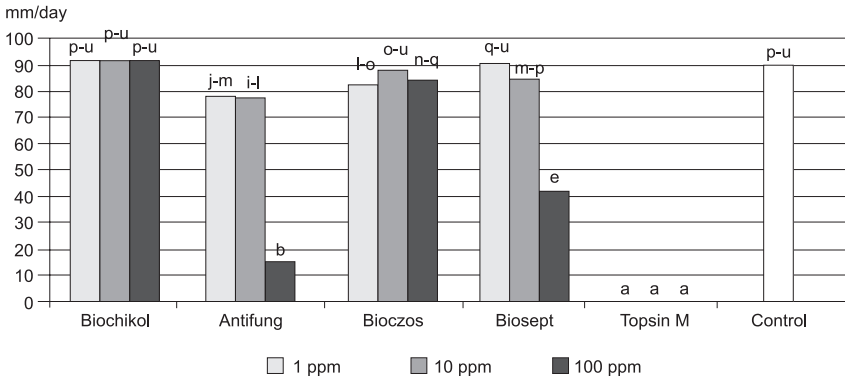


Fig. 2. Growth rate of *Botrytis cinerea* exposed to preparations (columns marked with different letters differed significantly according to Duncan's test at $p = 0.05$)

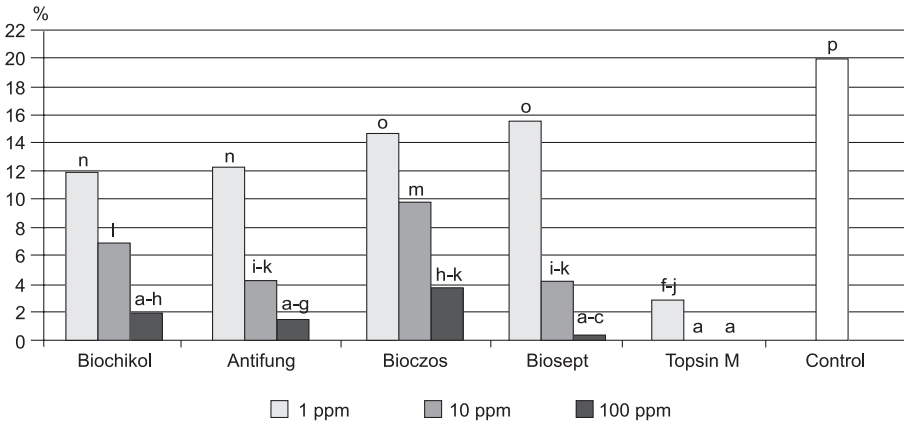


Fig. 3. Effect of preparations on conidial germination of *Botrytis cinerea* (columns marked with different letters differed significantly according to Duncan's test at $p = 0.05$)

still similarly to Topsin M, they significantly controlled conidia germination already at the concentration of 1 ppm.

The preparations caused changes in biotic relations between *B. cinerea* and *T. viride* (Fig. 4). All preparations, unlike the fungicide, positively affected the relations between *B. cinerea* and *T. viride*. Preparations supplied to the medium favoured the development of the antagonistic fungus, which inhibited *B. cinerea* growth. The relation between *B. cinerea* and *T. viride* was most favourably affected by Biosept 33 SL, which even in 1 ppm concentration caused an increase of *T. viride* antagonism. At the concentration of 100 ppm this preparation caused poor development of *B. cinerea* colony and strong suppression by *T. viride*. A considerable growth of IBE was registered also after applying Antifung 20 SL at 100 ppm.

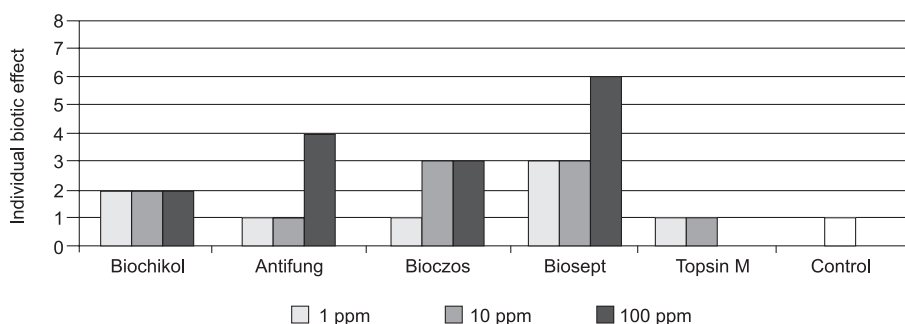


Fig. 4. Effect of preparations on biotic relations between *Botrytis cinerea* and *Trichoderma viride* (control – without preparation)

Discussion

Application of extracts based on composts may be one of the methods of plant protection against diseases (Wilk et al. 1996). The data presented in the paper by Orlikowski and Skrzypczak (1997) show that Antifung markedly hampered the development of *Phytophthora cinnamomi* on Lawson cypress and on pelargonium. Apart from numerous microorganisms inhabiting biohumus and their metabolites, this preparation brings to the environment also the compounds which may control the growth and sporulation of *P. cinnamomi*. Inhibitory effect of the compost extracts has been explained by their direct activity on spore germination, the growth of germ hyphae and induction of plant resistance (Vasyukova et al. 2001, Wojdyła 2001).

Most reports concerning the mechanism of chitosan effect on pathogenic fungi point to the fact that this compound does not restrain the growth of mycelium or spore germination *in vitro*, but identify this preparation as an inducer of systemic plant resistance to some pathogens (Orlikowski et al. 2002). Chitin, chitosan, and their derivatives are biologically active molecules regulating plant–phytopathogen interactions. Chitoooligosaccharides at high concentrations display fungal toxicity; inhibit fungal sporulation and growth of mycelium (Vasyukova et al. 2001). In the research conducted by Wojdyła (2001) it was observed that chitosan poorly inhibited or even stimulated the growth of *B. cinerea*, but caused a decrease in symptoms on roses infected with *Sphaerotheca pannosa* var. *rosae*. Microscopic observations revealed that this compound caused rapid dehydration and shrinkage of *S. pannosa* mycelium hyphae and spores. On the other hand, in *B. cinerea* chitosan causes changes and losses of amino acids and even proteins. Studies on the ultrastructure of fungi treated with chitosan also revealed changes in cell walls visible as their loosening, vacuolization and protoplasm disintegration at the final stage. These changes may be the result of chitin synthesis inhibition and appearance of larger amounts of chitosan in the cell membrane. It may be possible that chitosan applied to fungi from the outside stimulates deacetylation of the fungus chitin into chitosan and upsets the proportions between these components in the cell mem-

brane leading to its loosening (El Ghaouth et al. 1992, Pastucha 2001, Wojdyła 2001 a, 2003).

On the basis of research conducted so far it is difficult to determine which compounds contained in the grapefruit extract reveal antifungal effect (Orlikowski et al. 2002). Aliphatic aldehydes, monoterpenes, sesquiterpenes and nutkaton are dominant among many components of this extract. These diversified compounds may reveal synergism suppressing the development of some determined disease agent (Caccioni 1998). The effect of grapefruit extract on *B. cinerea* involves direct inhibition of its mycelium growth, sporulation and spore germination, as well as causing disorders in cytoplasm (Orlikowski et al. 2001, Orlikowski and Skrzypczak 2003, Pięta et al. 2004). If *B. cinerea* spores treated with grapefruit extract survive, they form very short spore hyphae, which may make the mycelium unable to infect the plant.

Extracts or homogenates of garlic reveal antifungal properties in relation to many pathogens. However, Wojdyła (2001 b) noted that garlic extract stimulated development of necroses on rose petals caused by *B. cinerea*. On the other hand, Piotrowski et al. (1995) observed that a macerate prepared of common garlic bulbs totally inhibited germination of *B. cinerea*, *Fusarium culmorum* and *Alternaria alternata* spores but it also caused a deformation of *Alternaria fabae* germ hyphae. Fungistatic properties are ascribed mainly to alliin in garlic, which is later metabolised to allicine and other sulphur derivatives, such as diallil sulphide, ajoene etc. It has been also pointed that ajoene reveals a stronger antifungal effect than allicine (Wojdyła 2001 b, Bianchi et al. 1997). Investigations on the mechanism of garlic extract activity revealed that it causes cytomorphological changes involving accumulation of fatty corpuscles in cells, a decrease in cell wall thickness and folding of cell membrane. These changes are similar to those which occur in fungi cells under the influence of synthetic fungicides (Bianchi et al. 1997).

Biological plant control of *B. cinerea* uses also among other *Trichoderma* fungi (Zimand et al. 1996, De Meyer et al. 1998, Elad 2000, Freeman et al. 2004). However, these fungi remain under the influence of biotic and abiotic environmental factors, which may affect their antagonism (Dłużniewska 2003, Kredics et al. 2003). Therefore it is recommended that various preparations used for plant protection should not lead to a decreased activity of biopreparations based on *Trichoderma* spp. So, the observation that the analyzed preparations favoured the development of antagonistic *T. viride* fungus, which inhibited *B. cinerea* pathogen growth, presented in this paper is favourable.

Presented investigation results show that application of natural compounds for plant protection against diseases may provide a good supplement of synthetic fungicides.

Streszczenie

AKTYWNOŚĆ *IN VITRO* PREPARATÓW ZAWIERAJĄCYCH SUBSTANCJE NATURALNE W STOSUNKU DO *BOTRYTIS CINEREA*

Celem pracy było określenie wpływu preparatów takich, jak: Biochikol 020 PC, Antifung 20 SL, Bioczoz BR oraz Biosept 33 SL, na wzrost grzybni, kiełkowanie zarodników *Botrytis cinerea* oraz oddziaływanie biotyczne tego grzyba wobec *Trichoderma viride*. Wyciągi z biohumusu w stężeniu 100 ppm (Antifung 20 SL) i grejpfruta (Biosept 33 SL) mocno hamowały wzrost *B. cinerea*. Wszystkie preparaty hamowały kiełkowanie zarodników *B. cinerea* i, w przeciwieństwie do fungicydu, działały korzystnie na relacje między *B. cinerea* i *T. viride*. Preparaty te dodane do podłoża hodowlanego sprzyjały rozwojowi grzyba antagonistycznego.

Literature

- Bianchi A., Zambonelli A., Zechini D'Aulerio A., Bezlesia F., 1997: Ultrastructural studies of the effects of *Allium sativum* on phytopathogenic fungi in vitro. *Plant Dis.* 81: 1241–1246.
- Borecki Z., 1984: Fungicydy stosowane w ochronie roślin. PWN, Warszawa.
- Borecki Z., 2001: Nauka o chorobach roślin. PWRiL, Warszawa.
- Botrytis: biology, pathology and control. 2004. Eds. Y. Elad, B. Williamson, P. Tudzynski, N. Delen. Kluwer, Dordrecht.
- Burgiel Z., 1984: Wpływ niektórych herbicydów na występowanie i rozwój patogenów powodujących choroby podszuszkowe pszenicy ozimej. Cz. II. Rozwój patogenów. *Acta Agr. Silv. Ser. Agr.* 23: 187–196.
- Caccioni D.R.L., Guizzardi M., Biondi D.M., Renda A., Ruberto G., 1998: Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. *Int. J. Food Microbiol.* 43, 1–2: 73–79.
- Chapeland F., Fritz R., Lanen C., Gredt M., Leroux P., 1999: Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). *Pestic. Biochem. Physiol.* 64, 2: 85–100.
- De Meyer G., Bigirimana J., Elad Y., Höfte M., 1998: Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *Eur. J. Plant Pathol.* 104, 3: 279–286.
- Dłużniewska J., 2003: Reaction of fungi of *Trichoderma* genus to selected abiotic factors. *Electr. J. Pol. Agric. Univ. Ser. Agron.* 6, 2, #4. [<http://www.ejpau.media.pl/volume6/issue2/agronomy/art-04.html>].
- El Ghaouth A., Arul J., Asselin A., 1992: Potential use of chitosan in postharvest preservation of fruits and vegetables. In: *Advances in chitin and chitosan*. Eds. Ch.J. Brine, P.A. Sandford, J.P. Zikakis. Elsevier, London: 440–451.
- Elad Y., 2000: Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes action. *Crop Prot.* 19, 8: 709–714.
- Freeman S., Minz O., Kolesnik I., Barbul O., Zveibil A., Maymon M., Nitzani Y., Kirshner B., Rav-David D., Bilu A., Dag A., Shafir S., Elad Y., 2004: *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *Eur. J. Plant Pathol.* 110, 4: 361–370.
- Kredics L., Antal Z., Manczinger L., Szekeres A., Kevei F., Nagy E., 2003: Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. *Food Technol. Biotechnol.* 41, 1: 37–42.
- Leroux P., Fritz R., Debieu D., Albertini C., Lanen C., Bach J., Gredt M., Chapeland F., 2002: Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Manage. Sci.* 58, 9: 876–888.

- Mańka K., 1974: Zbiorowiska grzybów jako kryterium oceny wpływu środowiska na choroby roślin. Zesz. Probl. Post. Nauk Roln. 160: 9–23.
- Myresiotis C.K., Karaoglanidis G.S., Tzavella-Klonari K., 2007: Resistance of *Botrytis cinerea* isolates from vegetable crops to anilino-pyrimidine, phenylpyrrole, hydroxylanilide, benzimidazole, and dicarboximide fungicides. Plant Dis. 91: 407–413.
- Orlikowski L.B., Skrzypczak Cz., 1997: Wapń, Antifung i wyciąg keratyno-koro-mocznikowy w ochronie niektórych roślin ozdobnych przed fuzariozą naczyniową i fytoftorozą. Prog. Plant Prot. / Post. Ochr. Rośl. 37, 1: 151–156.
- Orlikowski L.B., Skrzypczak Cz., 2003: Grapefruit extract as biocontrol agent of soil-borne and leaf pathogens. Bull. Pol. Acad. Sci. Biol. Sci. 51, 2: 79–85.
- Orlikowski L.B., Skrzypczak Cz., Jaworska-Marosz A., 2001: Influence of grapefruit extract on the growth and development of *Botrytis* spp. and grey mould development on lily and peony. Bull. Pol. Acad. Sci. Biol. Sci. 49, 4: 375–379.
- Orlikowski L.B., Skrzypczak Cz., Wojdyła A., Jaworska-Marosz A., 2002: Wyciągi roślinne i mikroorganizmy w ochronie roślin przed chorobami. Zesz. Nauk. AR Krak. 387, Ses. Nauk. 82: 19–32.
- Pastucha A., 2001: Oddziaływanie chitozanu na grzyby chorobotwórcze dla soi. Ann. Univ. Mariae Curie-Skłodowska Sect. EEE 9: 56–64.
- Pięta D., Patkowska E., Pastucha A., 2004: Oddziaływanie biopreparatów na wzrost i rozwój niektórych grzybów chorobotwórczych dla roślin motylkowatych. Acta Sci. Pol. Hortor. Cult. 3, 2: 171–177.
- Piotrowski W., Sas-Piotrowska B., Wroblekiewicz K., Czajkowski P., 1995: Wpływ wyciągów roślinnych na kiełkowanie zarodników niektórych gatunków grzybów patogennych dla roślin. Zesz. Nauk. AT-R Bydg. 190, Roln. 36: 139–145.
- Vasyukova N.I., Zinovyeva S.V., Ilinskaya L.I., Perekhod E.A., Chanelko G.I., Gerasimova N.G., Ilina A.V., Varlamov V.P., Ozeretskoykaya O.L., 2001: Modulation of plant resistance to diseases by water-soluble chitosan. Appl. Biochem. Microbiol. 37, 1: 103–109.
- Wilk S., Orlikowski L.B., Wojdyła A.T., 1996: Effectiveness of Antifung obtained from vermicompost against some soil-borne and leaf pathogens. In: Effectiveness of some microorganisms and plant extracts in the control of plant diseases. Eds. L.B. Orlikowski, Cz. Skrzypczak. Section for Biological Control of Plant Diseases of The Polish Phytopathological Society, Research Institute of Pomology and Floriculture, Skierniewice: 183–187.
- Williamson B., Tudzynski B., Tudzynski P., Van Kan J.A.L., 2007: *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8, 5: 561–580.
- Wojdyła A.T., 2001 a: Chitosan in the control of rose diseases – 6-year-trials. Bull. Pol. Acad. Sci. Biol. Sci. 49, 4: 243–252.
- Wojdyła A.T., 2001: Garlic juice in the control of some rose diseases. Bull. Pol. Acad. Sci. Biol. Sci. 49, 4: 253–263.
- Wojdyła A.T., 2003: Chitosan as the biocontrol agent of fungal pathogens; activity and mode of action. Bull. Pol. Acad. Sci. Biol. Sci. 51, 2: 159–165.
- Yourman L.F., Jeffers S.N., Dean R.A., 2001: Phenotype instability in *Botrytis cinerea* in the absence of benzimidazole and dicarboximide fungicides. Phytopathology 91: 307–315.
- Zimand G., Elad Y., Chet I., 1996: Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. Phytopathology 86: 1255–1260.

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