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MORPHOLOGICAL IDENTITY AND POPULATION STRUCTURE OF HEMIBIOTROPHIC FUNGUS *Colletotrichum coccodes* COLONIZING PEPPER PLANTS

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ABSTRACT

Colletotrichum coccodes (Wallr.) Hughes has been recognized as one of casual agents of anthracnose roots pepper in south-eastern Poland. During 2007–2012 the species was isolated from roots of sweet pepper (*Capsicum annuum* L.) cultivated in the field. The purpose of the study was morphological characterization and biotic activity of *C. coccodes* isolates. Five randomly chosen isolates from fungus population and one reference isolate obtained from Bank of Plant Pathogens and Investigation of their Biodiversity of IPP-NRI in Poznan in Poland were studied. The character of culture like growth rate, the colour of averse and reverse and the formation of morphological structures of the fungus such as acervuli, conidia, sclerotia were studied. Ultrastructural observations of morphological structures were made using light and scanning electron microscopy. Biotic activity of *C. coccodes* was conducted using the method of biotic series on PDA. Seven species of test fungi were used in the study: *Alternaria alternata*, *Aureobasidium pullulans*, *Gibberella avenacea*, *G. intricans*, *Fusarium oxysporum*, *Penicillium aurantiogriseum*, *Trichoderma harzianum.* The biotic activity test showed that *C. coccodes* is a weak competitor, and its development in the rhizosphere of sweet pepper may be limited by numerous antagonists.

Key words: *Capsicum annuum*, rhizosphere fungi, morphology, biotic activity

INTRODUCTION

Colletotrichum is one of the most important genera of plant pathogenic fungi worldwide, particularly in subtropical and tropical regions. Fungi from genus *Colletotrichum* (teleomorph: *Glomerella)* are important for cultivation of pepper. They occur on the aboveground and underground parts of plants, causing spots on the leaves, fruits and roots. The anthracnose of pepper is caused by *Colletotrichum capsici* (Syd. & P. Syd.) E.J. Butler & Bisby, *C. gloeosporioides* (Penz.) Penz. & Sacc., *C. dematium* (Pers.) Grove, *C. coccodes* (Wallr.) Hughes, *C. acutatum* Simmonds and *C. boninense* Moriwaki, Toy. Sato et Tsukib [Diao et al. 2013]. The anthracnose of pepper is defined as dry fruit rot and it is the major disease of pepper in the hot regions of Asia, China, Korea, Indie and Thailand [Pearson et al. 1984, Roy et al. 1997, Hong and Hwang 1998, Shin et al. 2000, Xia et al. 2011, Choi et al. 2011].

Colletotrichum coccodes (Wallr.) Hughes colonizes the aboveground parts (stems, fruits) as well as the underground parts (roots) of the host plant. The species was described for the first time in 1833 on

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potato in Germany as *Chaetomium coccodes* (Wallr.) Hughes [Hyde et al. 2009]. Its synonyms are *Colletotrichum atramentarium* (Berk. et Broome) Taubenh and *Colletotrichum phomoides* (Sacc.) Chester. It is widely spread in the world and it is isolated from different plants although it mainly infects vegetables from the family Solanaceae and Cucurbitaceae. Anthracnose of cultivated plants caused by this pathogen was observed in Europe, Asia, North America, Africa and Australia [Hong and Hwang 1998]. This pepper disease was for the first time diagnosed on pepper seedlings cultivated in the ground in Korea in 1988 [Oh et al. 1988, Hong and Hwang 1998]. In Poland *C. coccodes* occurs on potato, tomato and pepper [Cwalina-Ambroziak and Czajka 2000, Cwalina-Ambroziak et al. 2007, Jamiołkowska and Buczkowska 2009, Jamiołkowska 2009a, b, Cwalina-Ambroziak and Trojak 2012]. In a few regions in the world (Great Britain, South Africa, the United States), *C. coccodes* is an important economic factor. The fungus brings about large yield losses, especially in warm areas and those with high humidity [Stevenson et al. 2004]. *C. coccodes* causes disease symptoms typical of anthracnose which occur on the fruits of tomato, pepper and pumpkin. The fungus was isolated from tomato leaves, the stems, bulbs and roots of potato, pepper, zucchini and weeds [Yu et al. 1987, Park and Kim 1992]. The polyphagous character of the fungus is confirmed by the isolations from the roots of chrysanthemum, cress, cabbage and lettuce [Dillard 1992].

Studies conducted in Poland on pepper healthiness showed that *C. coccodes* colonizes pepper roots and is capable of causing the disease at the stage of seedlings [Jamiołkowska and Buczkowska 2009, Jamiołkowska 2009a, b]. The main mass of the fungal inoculum is found in the soil and this is why it infects the germinating seeds and seedlings, causing their dying out. *C. coccodes* might also cause the rot of older roots. The bark layer of the roots becomes loosened and covered with numerous sclerotia so it can be easily separated from the other part of the root. Three types of symptoms of the brown root rot were observed in the infected roots of tomatoes cultivated in the field, namely the rot of the lateral root bark, corky root rot and stem base rot [Dillard 1992]. Studies on the pathogenicity of *C. coccodes* conducted on the roots of pepper seedlings indicate that the plant can be infected already during the 1st or 2nd leaf stage. In the case of older plants no disease expression was observed. The fungus gets into contact with the underground parts of the plant and occurs in a complex with other soil pathogens. However, it is not the major factor of the root system diseases [Hong and Hwang 1998]. Various pathotypes of *C. coccodes* were distinguished which cause different disease symptoms [Stevenson et al. 2004] and different degrees of pathogenicity [Jamiołkowska 2013].

Because the literature lacks information on the characteristics of species *C. coccodes* occurring on pepper cultivated in the weather conditions of Poland, studies were undertaken to explain this problem. The purpose of the present paper is morphological characterization and assessment of the structure of *C. coccodes* populations on pepper roots as well as biotic interactions of the species with fungi occurring the same community.

MATERIAL AND METHODS

Isolation and identification *Colletotrichum coccodes*. The studied material included isolates of *C. coccodes* obtained from the roots of sweet pepper (Barbórka, Caryca F_1, Mercedes, Ożarowska, Podstolina, Roberta F_1, Robertina, Rumba F_1cvs.) cultivated on the experimental field in Zezulin, Lublin province (N51°20', E22°49') in the years 2007–2012. The experiment was set up in the soil with a pH of 6.5. Wheat was the forecrop. In the year preceding pepper cropping, organic fertilization was applied at the rate of 40 t ha^{-1}. Spring mineral nutrition was done according to the soil analysis ($kg \cdot ha^{-1}$): N-100 (nitrogen nitrate), P-60 (superphosphate), K-140 (potassium sulphate).

The seedlings were produced in the greenhouse and planted in the field in the third decade of May at spacing of 0.67 m × 0.35 m. Eight plants at stage BBCH 86 (full fructification of pepper) were taken randomly from the assumed experimental combination and mycological analysis of roots was carried

out. Chosen fragments of roots were surface sterilised by soaking in 10% blench (0.525 sodium hypochloride) solution for 1 minute and then rinsing three times with sterile distilled water. Section of tissue were aseptically excised and placed into 90 mm diameter Petri plates (10 pieces/plate) containing the mineral medium (0.7 g NH_4NO_3, 0.3 g KH_2PO_4, 0.3 g $MgSO_4 \times 7\ H_2O$, 0.01 g $FeCl_3 \times 6H_2O$, 0.01 g $ZnSO_4 \times 7\ H_2O$, 0.01 g $CuSO_4 \times 7\ H_2O$, 0.01 g $MnSO_4 \times 5\ H_2O$ + 38 g saccharose + 20 g agar + 1000 ml H_2O). Within 7 days of incubation in the darkness at 24°C, small parts of colonies growing around the inocula were transferred into PDA medium (Potato Dextrose Agar) slants. After 10 days, the obtained isolates were segregated and identified according to the description given by Sutton [1980]. Five isolates of *C. coccodes:* K37/2011, K39/2010, K43/2010, K44/2010, K51/2011 obtained from pepper roots in 2010–2011 as a result of own research and one reference isolate IOR 316 obtained from Bank of Plant Pathogens and Investigation of their Biodiversity – IPP-NRI in Poznan (Poland), were randomly chosen from the collection of single – spore cultures for further studies. Each isolate was cultured in a thermostat, at the temperatures 24°C for 14 days. The character of the cultures: growth rate, the color of the averse and the reverse, the formation of morphological structures of the fungus like acervuli, conidia and sclerotia were studied after 14 days. To determine the structures mentioned above, the measurements of 50 acervuli and 50 spores for each isolate were made. Photographic documentation was made using a light and scanning electron microscopy Vega, Tescan.

Biotic activity of *Colletotrichum coccodes*. In the literature lacks information on the biotic activity of *C. coccodes*. The studies were conducted using the method of biotic series [Jamiołkowska and Thanoon 2016, Zimowska et al. 2016] on PDA medium. Two discs of 3 mm in diameter from 14-day-old cultures, one isolate of *C. coccodes* K51/2011 and seven test fungi representing the studied community of pepper plants in (*Alternaria alternata, Aureobasidium pullulans, Gibberella avenacea, G. intricans, Fusarium oxysporum, Penicillium aurantiogriseum, Trichoderma harzianum*) were taken [Jamiołkowska 2013, 2014]. Test fungi were obtained as a result of mycological analyzes of sweet pepper roots in 2012. They were placed mycelium down, 2 cm apart in the central of the Petri dish, on the solidified medium. The dishes with single fungi species constituted the control. For each experimental combination, 4 dishes were considered which were treated as replications. They were kept in a thermostat at 24°C in the dark. The biotic effect was estimated on the basis of an 8-degree scale after 10 days of common growth. While evaluating the biotic effect, the overgrowth of the fungus colony by the accompanying fungus were taken into consideration. The growth of tested fungus (*C. coccodes*) of the accompanying fungi representing the studied communities was expressed as an Individual Biotic Effect (IBE) [Mańka and Mańka 1992]. Next, the General Biotic Effect (GBE) was estimated which was the product of the individual biotic effect and the multiplicity of the occurrence of particular fungi species. The algebraic sum of general biotic effects made it possible to determine the Summary Biotic Effect (SBE). A positive value of IBE indicates the growth inhibition of the pathogen, whereas a negative value of IBE points to the lack of growth inhibition of the pathogen's colony. "0" value means a neutral effect of both fungi on each other [Mańka and Mańka 1992].

RESULTS

Isolation and identification of *Colletotrichum coccodes*. In total 157 isolates of *C. coccodes* were obtained during the mycological analysis of roots in the years 2007–2012, which constitutes from 0.8% to 9.7% of fungi obtained from the analyzed pepper roots (tab. 1). The population of *C. coccodes* colonies in the studied communities varied and included from a few (4 colonies – 2007) to a few tens of isolates (51 colonies – 2009; 49 colonies – 2010) (tab. 1).

Table 1. Participation of *Colletotrichum coccodes* isolates in fungal communities obtained from sweet pepper roots in 2007–2012

Fungi	Number of isolates					
	2007	2008	2009	2010	2011	2012
Colletotrichum coccodes (Wallr.) S. Hughes	4 (0.8%)	24 (5.2%)	51 (7.9%)	49 (9.7%)	24 (5.6%)	5 (1.1%)
Other species fungi	526 (99.2%)	442 (94.8%)	591 (92.1%)	459 (90.3%)	402 (94.4%)	458 (98.9%)
Total	530	466	642	508	426	463

Table 2. Growth rate (cm) and size (μm) of morphological structures of *Colletotrichum coccodes* on PDA (mean for 5 replicates)

Author	Isolate	Growth rate (cm)	Color	Conidial size (μm)	Conidial shape	Acervuli size (μm)	Sclerotia shape and color
Own data	K37/2011	8.4	Colorless substrate mycelium; reverse colorless	15.8 × 2.7	Few, fusiform, one cell conidia	289.5 × 63.2	Sclerotia abundant, globose, black, compact in central part of colony; sclerotia loose, arranged radially towards the edges of the colony
	K39/2010	9.0	Colorless substrate mycelium; reverse colorless	16.9 × 2.7	Few, fusiform one cell conidia	315.8 × 9.3	Sclerotia abundant, globose, black, compact in central part of colony; sclerotia loose, arranged radially towards the edges of the colony
	K43/2010	8.4	Colorless substrate mycelium; reverse colorless	13.3 × 2.3	Abundant, fusiform, one cell conidia	276.3 × 3.2	Sclerotia abundant, globose, black, compact on the whole surface of the colony
	K44/2010	8.4	Colorless substrate mycelium; reverse colorless	16.7 × 3.2	Abundant, fusiform one cell conidia	250.0 × 6.8	Sclerotia abundant, globose, black, compact in central part of colony; sclerotia scattered on the shore
	K51/2011	9.0	Colorless substrate mycelium; reverse colorless	17.3 × 2.7	Few, fusiform one cell conidia	276.3 × 3.2	Sclerotia abundant, globose, black, compact in central part of colony; sclerotia loose, arranged radially towards the edges of the colony
	IOR 316	8.6	Colorless substrate mycelium; diffuse grey aerial mycelium; reverse colorless	Lack of conidia	–	250.0 × 3.7	Sclerotia abundant, globose, black, compact in central part of colony; sclerotia loose, arranged radially Towards the edges of the colony
Sutton [1980]		–	Diffuse white aerial mycelium	16.0–22.0 × 3.0–4.0	Fusiform, straight conidia	–	Sclerotia abundant, black, setose, globose, separate or confluent

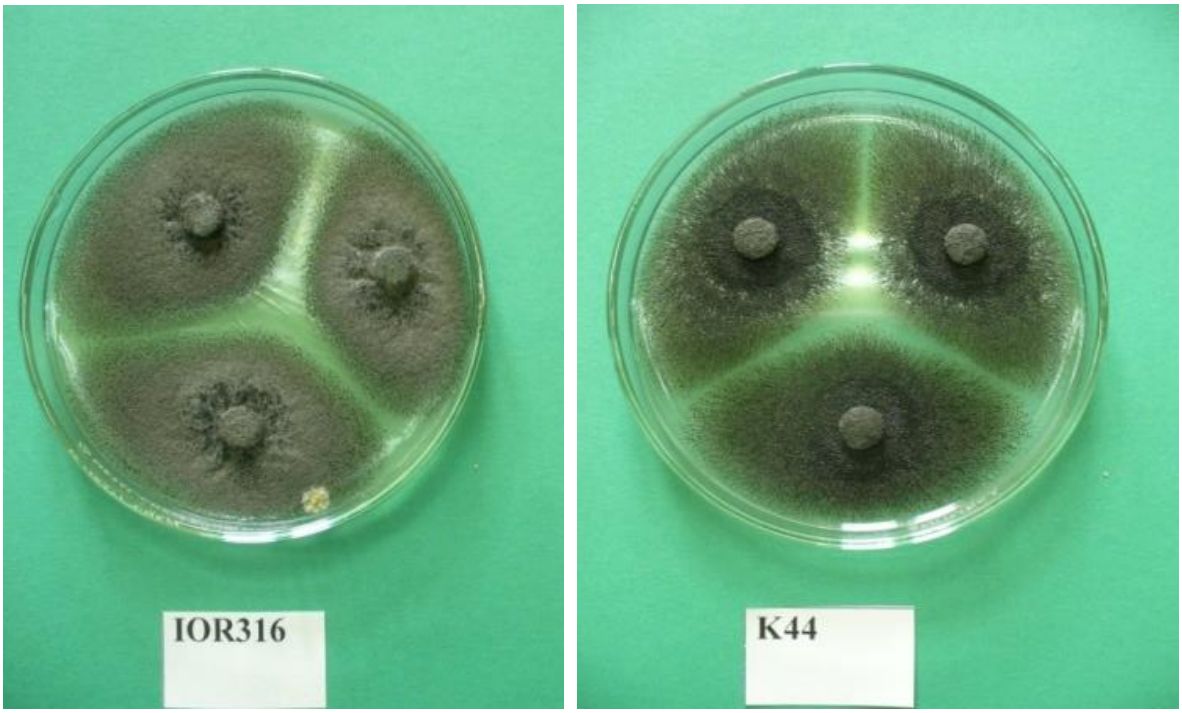

Fig. 1. 14-day-old colonies of *Colletotrichum coccodes* (isolates IOR 316, K44) on PDA (A. Jamiołkowska)

After 14 days of growth, colonies of fungus *C. coccodes* on PDA grew at a similar rate, reaching the size from 8.4 to 9.0 cm and overgrew almost all surface of the dish. The majority of *C. coccodes* isolates formed a structural, colourless mycelium with masses of microsclerotia on PDA. It was only isolate IOR 316 which formed aerial diffused, grey mycelium and substrate mycelium (fig. 1). All isolates formed very numerous, small, black, compact, globose and confluent microsclerotia, forming radially growth out to the edges of the colony (fig. 2).

The reverse of the colonies of the studied isolates was colourless. Numerous acervuli and conidia were observed on the whole surface of the colony (fig. 2–3). The acervuli were globose and lightly immersed in the medium. The diameter of the acervuli was 250.0–315.8 × 223.7–279.3 µm (tab. 2). Numerous setoses were visible on the surface of acervuli. One-celled, fusiform and straight spores were formed in the surface of acervuli. The size of the spores was 13.3–17.3 × 2.3–3.2 µm (tab. 2). The conidia were rounded on the base and slightly cut (fig. 2–3). It was only isolate IOR 316 which did not form spores (tab. 2).

Biotic activity of *Colletotrichum coccodes.* Among fungi achieved from roots: *Trichoderma harzianum Gibberella intricans*, *G. avenacea*, and *Alternaria alternata* reduced *C. coccodes* growth. Among the studied test fungi, the highest positive values of the individual biotic effect (IEB) was observed for *Trichoderma harzianum* (+8). This species overgrew the inoculum of *C. coccodes*, making the growth and sporulation of the pathogen impossible (tab. 3). The above-mentioned species did not only cause a growth decrease of the tested fungus but it also gave rise to the phenomenon of mycoparasitism (tab. 3, fig. 4).

Fungi limiting the growth of *C. coccodes* only to a small extent were *Gibberella intricans, G. avenacea, Fusarium oxysporum, Alternaria alternata, Penicillium*

aurantiogriseum (IEB +1) (tab. 3, fig. 4). The species that did not show any effect on the growth of *C. coccodes* was *A. pullulans* (IEB 0). The majority of the tested fungi showed a positive individual biotic effect (IEB) towards *C. coccodes*, which points to weak competitiveness of the tested fungus. Communities originating from roots could inhibit *C. coccodes* growth, because their SBE's were positive (tab. 3, fig. 4). The present study showed that SBE values of tested fungi were positive towards *C. coccodes*. This suggests that their growth can be inhibited by other fungi colonizing the rhizosphere of pepper plants.

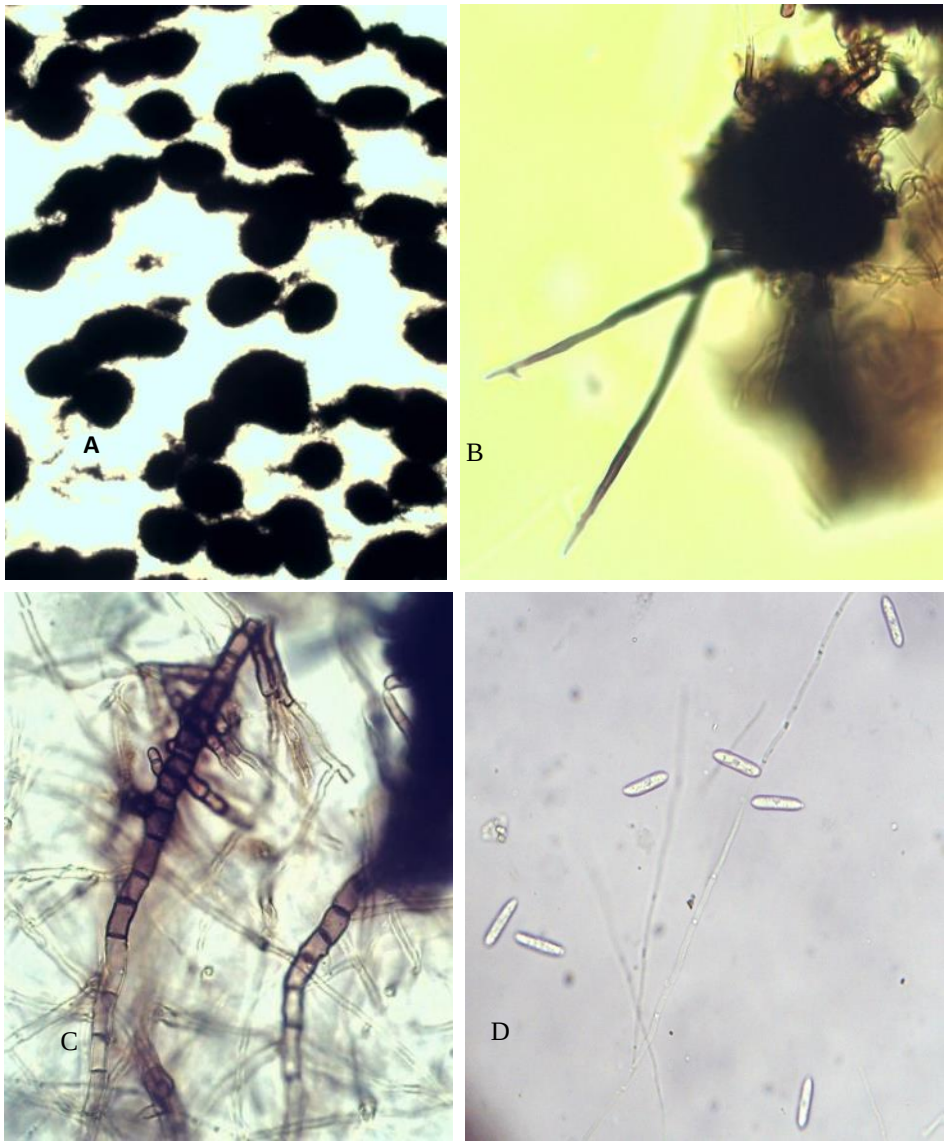

Fig. 2. *Colletotrichum coccodes* under light microscope: A) black, confluent microsclerotia ×10; B) acervulus with setoses ×40; C) thickening hyphae ×40; D) one-celled conidia ×40 (A. Jamiołkowska)

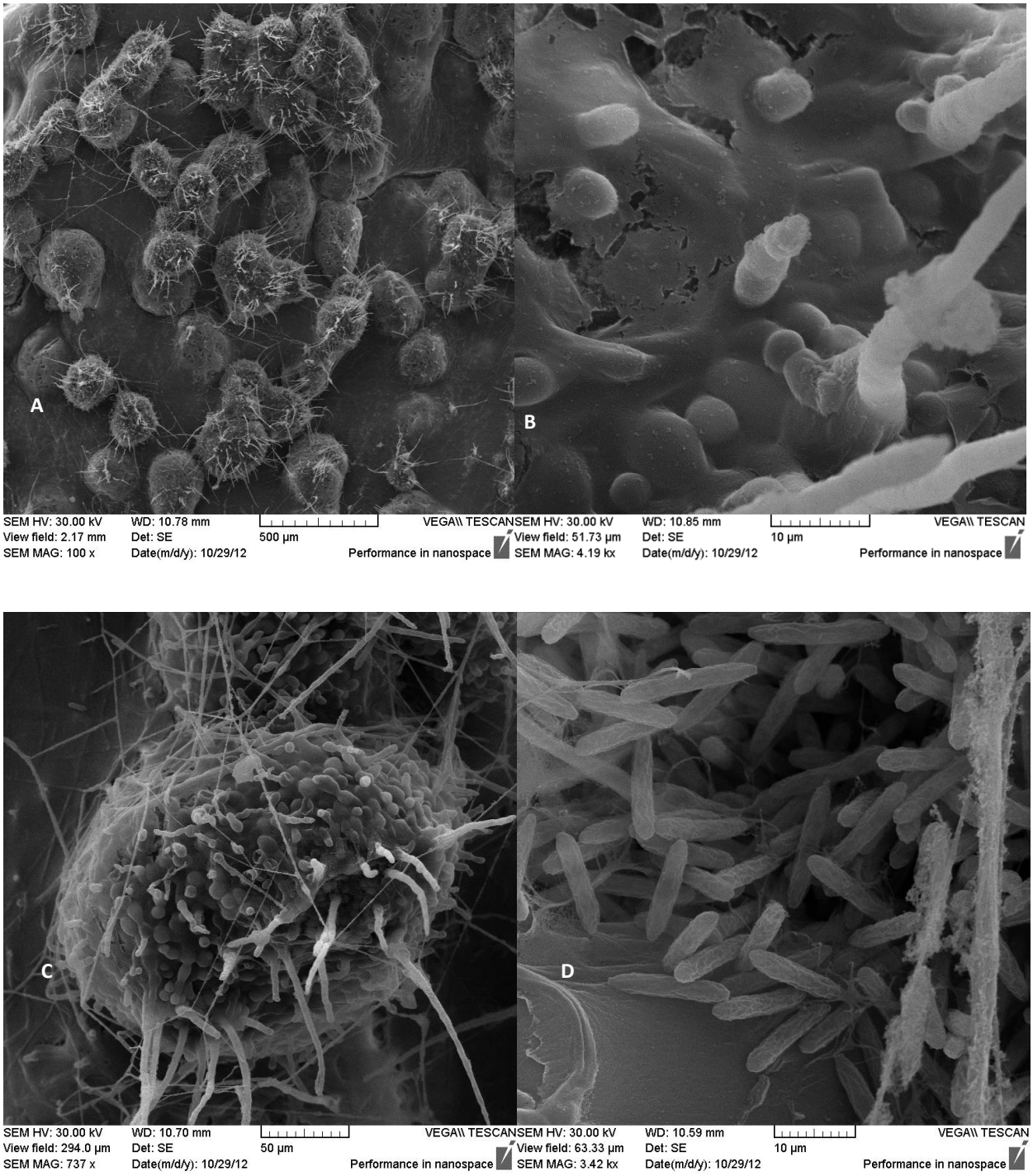

Fig 3. *Colletotrichum coccodes* in scanning electron microscopy: A) SEM micrographs showing globose acervuli; B) SEM micrographs showing setoses emerging from agar; C) SEM micrographs showing acervulus with setoses; D) SEM micrograph showing conidia (A. Wróbel)

Table 3. Biotic effect of fungi isolated from pepper roots on *Colletotrichum coccodes*, after 10 days of dual growth

Fungi	2007		2008		2009		2010		2011		2012		
	IBE*	frequency	GBE**	frequency	GBE**	frequency	GBE**	frequency	GBE**	frequency	GBE**	frequency	GBE**
Alternaria alternata (Fr.) Keissl.	+1	9	+9	4	+4	18	+18	30	+30	2	+2	0	0
Aureobasidium pullulans G. Arnaud	0	0	0	5	0	67	0	0	0	0	0	4	0
Fusarium oxysporum Schltdl.	+1	65	+65	81	+81	94	+94	34	+34	56	+56	128	+128
Gibberella intricans Wollenw.	+1	48	+48	36	+36	1	+1	1	+1	37	+37	32	+32
Gibberella avenacea R.J. Cook	+1	6	+6	52	+52	19	+19	27	+27	14	+14	28	+28
Penicillium aurantiogriseum Dierckx	+1	0	0	4	+4	10	+10	0	0	0	0	0	0
Trichoderma harzianum Rifai	+8	56	+448	82	+656	192	+1544	302	+2416	240	+1920	88	+704
Number of isolates		184		264		401		394		349		280	
SBE***			+576		+833		+1686		+2508		+2029		+892

* individual biotic effect, ** general biotic effect, *** summary biotic effect

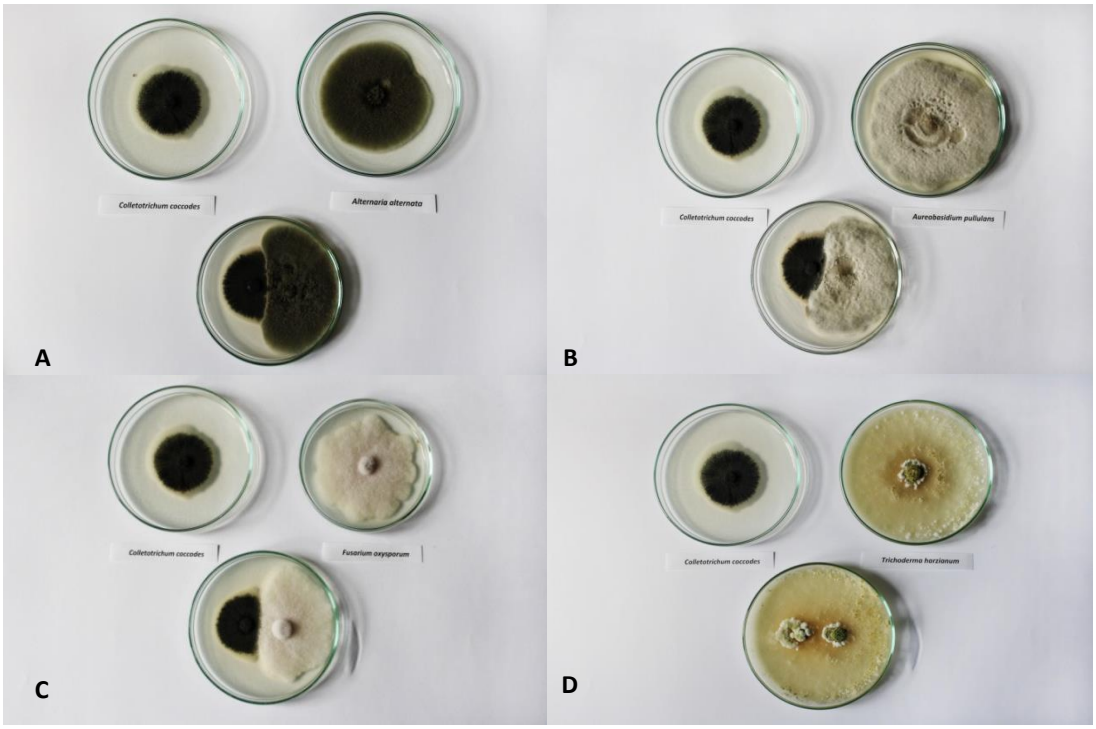

Fig. 4. Biotic activity of *Colletotrichum coccodes*: A) *C. coccodes* and *A. alternata*; B) *C. coccodes* and *A. pullulans*; C) *C. coccodes* and *F. oxysporum*; D) *C. coccodes* and *T. harzianum* (M. Pająk)

DISCUSSION

Mycological analyses conducted in the years 2007–2012 show that *Colletotrichum coccodes* is one of the more important fungi colonizing the roots of sweet pepper cultivated in the field in Poland [Jamiołkowska 2013, 2014]. The fungus colonized not only roots but also leaves, stems and fruits of pepper [Jamiołkowska and Buczkowska 2009, Jamiołkowska 2009a, b, 2011]. *C. coccodes* population varies in the years and depends on the weather conditions in a growing season. In warm and humid years fungus causes of pepper seedlings antracnose [Jamiołkowska 2009a, b, 2011]. Many authors write about pathogenic abilities of the fungus [Bailey et al. 1992, Jamiołkowska 2008, 2013]. Polyphagous and pathogenic character of *C. coccodes* were presented in Bailey and co-authors [1992] research. Similar studies were conducted by Jamiołkowska [2013], who showed the different harmfulness of *C. coccodes* isolated from *Capsicum annuum* roots. *C. coccodes* population included isolates non-pathogenic and highly pathogenic for seedlings peppers. Disease index of infected seedlings ranged from 24.9 to 66.6%. Pathogenicity of isolates was also dependent on their origin. Isolates from tomato and pepper roots were most pathogenic towards pepper seedlings than isolates provide from potato bulbs and pepper leaves.

The present morphological studies on *C. coccodes* will make it possible to get to know the species proper identification. The morphological study was carried out on PDA medium, which is recommended to identify genus *Colletotrichum* [Zimowska et al. 2016]. On PDA, all studied isolates of *C. coccodes* formed morphological structures characteristic of this species such as acervuli, spores and microsclerotia.

C. coccodes forms acervuli with setoses, globose, confluent microsclerotia and fusiform one-celled conidia. Contrary to other species of this genus, the fungus forms a very diffuse, delicate aerial mycelium of grey colour which disappeared quickly, and very numerous microsclerotia in black clusters radiating towards the edges of the colony. The studied morphological features of *C. coccodes* isolates are similar with the description by Sutton [1980].

The species occurs in a complex with other soil pathogens such as *Fusarium* spp., *Verticillium dahliae*, *Rhizoctonia solani* or *Ralstonia solanacearum* [Tsror Lahkim and Hazanovsky 2001, Jamiołkowska 2011, Fazli et al. 2012]. In mixed infections and in different combinations with vascular wilt agents such as *V. dahliae* and *F. oxysporum* and other root-infecting fungi such as *F. solani*, *R. solani*, *Macrophomina phaseolina*, this pathogen could be responsible for syndrome called "early dying of pepper" [Stoyanova et al. 2013]. A destructive effect of the fungus is caused by mechanical penetration of the plant through infection hyphae and the production of polygalacturonases (PGs) and pectin lyases (PL) causing enzymatic destruction of plant cells [Bailey et al. 1992, Redman and Rodriguez 2002, Ben-Daniel and Tsror Lahkim 2012].

Therefore, the assessment of microbiological activity of soil is important because microorganisms affect not only the healthiness of cultivated plants but also availability of nutrients in the soil [Martyniuk et al. 2007]. It is worth noticing that positive values of IBE were obtained in the case of *Penicillium aurantiogriseum* and *Trichoderma harzianum* which belong to fast growing fungi non-pathogenic towards a lot of vegetable plants [Jamiołkowska and Thanoon 2016]. The colonies of the studied *Trichoderma* sp. completely overgrew the inoculums of tested fungi and made their growth and sporulation impossible. This phenomenon have a positive and practical aspect in biological control against plant pathogens. Strong competitive abilities of *Trichoderma* spp. resulting from the production of endo- and exoenzymes, toxic metabolites and from overparasitism [Benitez et al. 2004, Suarez-Estrella et al. 2007, Jamiołkowska and Thanoon 2016]. The other species of tested fungi i.e. *Fusarium oxysporum*, *Gibberella avenacea* and *G. intricans*, *Alternaria alternata* cannot be regarded as positive antagonist since they belong to the species that are pathogenic towards pepper plants [Jamiołkowska 2008]. The study showed that *C. coccodes* is probably a weak competitor, and its development in the rhizosphere of sweet pepper may be limited by numerous antagonistic fungi. Communities that are rich in high number of antagonistic fungal species, are able to reduce the pathogen growth, therefore it is important – while performing chemical protection – to apply selective preparations that would not destroy antagonistic species. We can suppose that in the Polish weather conditions the community of fungi colonizing the pepper plant grown in the field, will inhibit the developing of *C. coccodes*. Despite the weak competitive abilities of the fungus, in a complex with other pathogens, it can provoke the symptoms of the wilting plants [Tsror Lahkim and Lazanovsky 2001].

CONCLUSIONS

Colletotrichum coccodes (Wallr.) Hughes is one of casual agents of anthracnose roots of sweet pepper cultivated in south-eastern Poland. *C. coccodes* is capable of causing the disease of roots already at the stage of seedlings. At the literature lacks information on the morphological characteristics and population structure of *C. coccodes* occurring on the roots of pepper cultivated in the weather conditions of Poland. Research on the morphology of species showed that the majority of *C. coccodes* isolates forms on PDA a structural, colorless mycelium with masses of microsclerotia. The reverse of the colonies of the studied isolates is colorless. Numerous acervuli and conidia are observed on the whole surface of the colony. The acervuli are globose and lightly immersed in the medium. Numerous setoses are visible on the surface of acervuli. One-celled, fusiform and straight spores are formed in the surface of acervuli. The size of the spores is 13.3–17.3 × 2.3–3.2 μm. The conidia are rounded on the base and slightly cut. The biotic activity test show that *C. coccodes* is a weak competitor, and its development on the roots of sweet pepper may be limited by numerous antagonistic fungi.

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