Antagonistic activity of *Trichoderma asperellum* and *Trichoderma harzianum* against genetically diverse *Botrytis cinerea* isolates

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ABSTRACT

Trichoderma species have been identified as potential biocontrol agents of many plant pathogenic fungi, including *Botrytis cinerea* Pers., one of the major pathogens in tomato (*Solanum lycopersicum* L.) production in the Republic of Macedonia. The aim of this study was to evaluate the *in vitro* antagonistic activity of *Trichoderma asperellum* and *T. harzianum* against 18 genetically diverse *B. cinerea* isolates. The results showed considerable antagonistic abilities of both *Trichoderma* species against all tested *B. cinerea* isolates. Both antagonists significantly (p < 0.01) inhibited the mycelial growth (*T. asperellum* from 74.246% to 96.915% and *T. harzianum* from 71.072% to 95.889%) and conidial germination (*T. asperellum* from 76.932% to 95.107% and *T. harzianum* from 76.933% to 93.658%) of *B. cinerea* isolates. The antagonistic abilities were not related to the genetic group, but apparent association with the region of origin of the pathogen isolates was observed. *Trichoderma asperellum* and *T. harzianum* are promising biocontrol agents for control of gray mold disease in tomato.

Key words: Gray mold, mycoparasitism, PICG, PIMG, *Solanum lycopersicum*, tomato.

INTRODUCTION

Botrytis cinerea Pers., the causal agent of the grey mold disease is a ubiquitous phytopathogenic fungus, which attacks more than 230 plant species. It is one of the most important diseases in commercial greenhouse production of vegetables all over the world, especially in tomato and cucumber (Borges et al., 2014; Soliman et al., 2015). In the Republic of Macedonia, grey mold of tomato is the leading disease in greenhouse production of tomato, with yield losses that can surpass 70% (Kuzmanovska et al., 2012). Despite the great phenotypic variability (Valiuskaite et al., 2010; Kuzmanovska et al., 2012), many authors confirmed that this fungus also possesses great genetic diversity, which is mainly due to the presence or absence of two transposable elements, *boty* (Diolez et al., 1995) and *flipper* (Levis et al., 1997). Based on the presence/absence of these two transposable elements, many authors confirmed that *B. cinerea* is a complex species, composed of at least two genetic groups, *transposa* and *vacuma*. *Transposa* group contains both transposable elements, whereas *vacuma* group has neither of these two elements (Vaczy et al., 2008; Tanovic et al., 2009). Furthermore, isolates that contain only *boty* (Tanovic et al., 2009) or only *flipper* (Vaczy et al., 2008; Kuzmanovska et al., 2012) are also detected. Control of grey mold disease in tomato is mainly based on common applications of fungicides, but the key problem is the ability of the fungus to become resistant to frequently applied fungicides (Zhao et al., 2010; Esterio et al., 2011; Walker et al., 2013; Hahn, 2014). Resistance to fungicides combined with the difficulty of registering new

fungicides and environmental reasons, has opened the way for other means of control of this pathogen, such as biological control (Barakat et al., 2014; Soliman et al., 2015; Haidar et al., 2016).

Trichoderma species have been identified as potential biocontrol agents of many plant pathogenic fungi (Herrera-Parra et al., 2017). *Trichoderma harzianum* is one of the most studied members within this genus as biological control agent against an array of plant pathogenic fungi, including *B. cinerea* (Cheng et al., 2012; Bendahmane et al., 2012). Recent studies has shown that *T. asperellum* is effective and promising biocontrol agent in the control of several soil-borne fungi, such as: *Phytophthora drechsleri* Tucker 1931 and *Phytophthora cryptogea* Pethybr. & Laff. 1919 (Moayedi and Mostowfizadea-Ghalamfarsa, 2010), *Phytophthora capsici* Leonian 1922 (Osorio-Hernández et al., 2011) and *Fusarium oxysporum* Schltdl. (Ommati and Zaker, 2012). However, there is lack of studies concerning the potential of *T. asperellum* as a biological control agent of *B. cinerea*, as one of the major pathogen in tomato production. As a result, the main objectives of this study were: (1) to evaluate the biological control potential of *T. asperellum* and *T. harzianum* against genetically diverse isolates of *B. cinerea in vitro*, (2) to investigate the mechanisms involved in biocontrol under microscopic observations that could be further used for effective *B. cinerea* control in tomato, and (3) to assess possible relationship between the antagonistic ability of both *Trichoderma* species and genetic group and the region of origin of the pathogen isolates.

MATERIALS AND METHODS

Pathogen isolates
A total of 123 *Botrytis cinerea* isolates were obtained from naturally infected tomato (*Solanum lycopersicum* L.) organs (stems, leaves, and fruits) from seven different regions in Republic of Macedonia, where tomato is grown under high tunnels and greenhouses. In order to define the genetic group of the isolates, according to the presence/absence of transposable elements (*boty* and *flipper*), molecular characterization was performed (Kuzmanovska et al., 2012). From a collection of 123 isolates, 18 isolates from all seven regions which belonged to different genetic groups (*transposa*, *vacuma*, and *flipper*) were selected for further *in vitro* analyses (Table 1).

Antagonists and *in vitro* evaluation of antagonistic activity
Two representative monoconidial isolates were used in this study: Ta (*Trichoderma asperellum* T1) from the commercial biological control product Trifender (NCAIM 68/2006, Biovéd 2005 Kft, Pinkamindszent und Kemestaródfa, Hungary) and Th (*T. harzianum* T22) from the commercial biological control product Trianum P (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands). Isolates were cultured in the dark at 25 °C on potato dextrose agar (PDA, Difco TM dehydrated culture media, Becton, Dickinson and Company, Pont de Claix, France) supplemented with 250 mg chloramphenicol in 1 L sterile water.

To determine the antagonistic potential of *T. asperellum* and *T. harzianum* against *B. cinerea* isolates, dual culture method (Rahman et al., 2009; Zivkovic et al., 2010) and method of inhibition of conidial germination (Imtiaj and Lee, 2008; Zivkovic et al., 2010) were used in this study.

Dual culture method
Mycelial disc (6 mm diameter) taken from the margin of the 5 d old culture of each pathogen isolate was placed 1.5 cm away from the periphery of the Petri plate (90 mm) and a disc with the same size of each tested *Trichoderma* antagonist

Table 1. Region of origin, *Botrytis cinerea* isolates and their genetic group.

Region	Isolate	Genetic group
Bogdanci (41°11'43'' N; 22°35'07'' E)	Bst1, Bst12	*flipper, transposa*
Dojran (41°14'27'' N; 22°41'56'' E)	Dl2, Dst10, Dst5	*flipper, transposa, vacuma*
Vinica (41°53'53'' N; 22°29'10'' E)	Vst3	*vacuma*
Gradsko (41°34'40'' N; 21°57'14'' E)	Grp15, Grp4, Grp6	*flipper, transposa, vacuma*
Strumica (41°24'55'' N; 22°40'10'' E)	Strp12, Strst1, Strl5	*vacuma, transposa, flipper*
Stip (41°48'26'' N; 22°13'27'' E)	Sst6, Sl4, Sp16	*flipper, transposa, vacuma*
Kocani (41°53'43'' N; 22°23'04'' E)	Kp7, Kst9, Kp8	*flipper, transposa, vacuma*

was placed in the same manner, but on the opposite end of pathogen sample. Paired cultures were incubated at 25 °C in the dark. Petri plates which contained only pathogens mycelial plugs, plated in the same manner, served as controls. The experiment was set in triplicate, and repeated twice.

Antagonistic activity was assessed after 4 d incubation by measuring the radius (mm) of the pathogen colony (from the point of inoculation) in the direction of the antagonist colony (R_2) and the radius of the pathogen colony in the control plate (R_1). Percentage of inhibition of mycelial growth (PIMG) was calculated according to the Skidmore and Dickinson (1976) by using the following formula:

$$PIMG\ (\%) = R_1\text{-}R_2/R_1 \times 100 \qquad [1]$$

where R_1 is the radius of the pathogen colony in the control Petri plate, and R_2 is the radius of the pathogen colony in paired culture.

After 7 d incubation, *T. asperellum* and *T. harzianum* were tested for mycoparasitism and antibiosis against *B. cinerea* isolates. For this purpose, the inhibition zone (confrontation zone) was cut using the sharp blade and transferred onto clean slides. Cover slips with a drop of lactophenol-cotton-blue (LCB) stain were mounted on the mycelia. Interactions between the antagonist and pathogen were observed under a light microscope (Leica-DMLB, Meyer Instruments, Houston, Texas, USA).

Inhibition of conidial germination method
The pure cultures of pathogen and antagonists were grown on PDA plates for 7 d at 25 °C. After the incubation period, plates were flooded with 10 mL sterile distilled water and Tween 20 (v/v 0.01%) and harvested by scraping with a rubber spatula. The suspension was then filtered through double layer of cheesecloth in order to remove mycelial fragments. The spore suspensions were adjusted to concentrations of 10^7 spores mL^{-1} by use of hemocytometer. After that, 50 μL of standardized suspensions of pathogen and antagonists were mixed in the sterilized watch glass and transferred to sterile microscope slides. Slides with the suspension of pathogen conidia in sterile distilled water, served as control. The slides were kept at 25 °C in the moisture chamber for 24 h. The experiment was set in triplicate, and repeated twice.

After the incubation period, a drop of LCB was placed over the spore suspensions to capture the germination process and observed under the light microscope (400X) in order to record the percentage of germinated and ungerminated conidia. In this manner, conidia were defined as germinated only if the germ tube had developed to longer than half of the cell length (Zivkovic et al., 2010). The percentage of germination was determined under a light microscope by counting 100 conidia from each pathogen isolate and the proportion that has germinated. Percentage of inhibition of conidial germination (PICG) was calculated according to the Skidmore and Dickinson (1976) by using the following formula:

$$PICG\ (\%) = A_1\text{-}A_2/A_1 \times 100 \qquad [2]$$

where A_1 is the number of germinated conidia of the pathogen in control slides; A_2 is the number of germinated conidia of the pathogen in mixed (paired) slides.

Statistical analysis
Obtained values for PIMG and PICG for each pathogen isolate and each antagonist were submitted for ANOVA. Mean values were compared with LSD test at level of 0.01. Principal component analysis was performed in order to assess possible relationship between the genetic group of the *B. cinerea* isolates and percentage of inhibition of mycelial growth and percentage of conidial germination (PIMG and PICG, respectively). For all analyses, R 3.3.1 statistical package (R foundation for Statistical Computing, Vienna, Austria) was used.

RESULTS AND DISCUSSION

Percentage of inhibition of mycelial growth
Obtained results from the dual culture method, regarding the percentage of inhibition of mycelial growth of 18 *B. cinerea* isolates as a result of two *Trichoderma* antagonists, are shown in Table 2. *Trichoderma asperellum* and *T. harzianum* showed strong antagonistic properties against all *B. cinerea* isolates. The percentage inhibition of mycelial growth of pathogen isolates in paired cultures with *T. asperellum* ranged from 74.246% to 96.915% and from 71.072% to 95.889% in paired cultures with *T. harzianum*.

Table 2. Percentage of pathogen mycelial inhibition and inhibition of conidial germination by two antagonists.

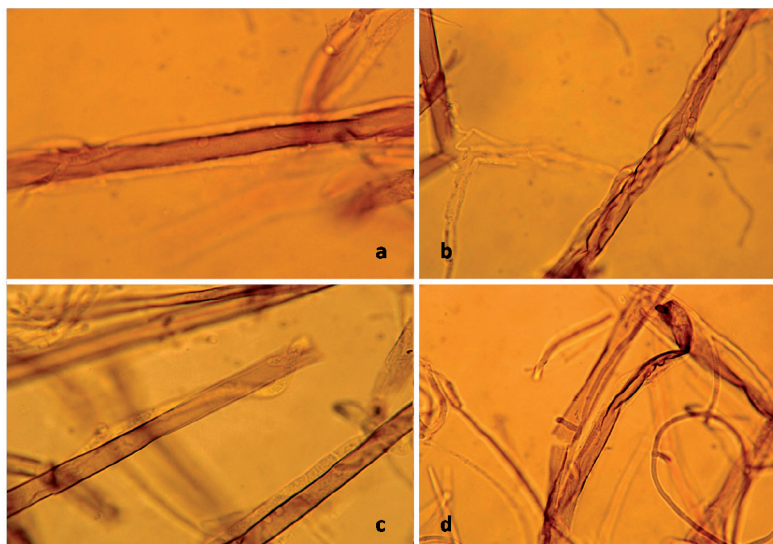| Pathogen isolate	Percentage of pathogen mycelial inhibition			Percentage of inhibition of conidial germination		
	Ta	Th	Average for both treatments	Ta	Th	Average for both treatments
	——— % ———			——— % ———		
Bst1	76.810	72.249	74.529	79.500	76.933	78.217
Dl2	85.569	88.435	87.002	81.121	85.519	83.320
Vst3	85.734	78.065	81.900	85.726	88.324	87.025
Grp15	79.104	73.134	76.119	86.969	79.744	83.356
Strp12	84.779	82.751	83.765	91.214	82.848	87.031
Sst6	90.216	88.149	89.183	93.861	89.245	91.553
Kp7	96.915	95.889	96.402	95.107	91.826	93.466
Bst12	77.393	75.758	76.575	79.490	79.496	79.493
Grp4	74.246	71.072	72.659	77.218	78.762	77.990
Grp6	75.509	72.874	74.192	76.932	78.500	77.716
Strst1	80.098	78.687	79.392	81.343	82.442	81.893
Strl5	83.333	80.308	81.820	82.177	84.727	83.452
Dst10	86.276	85.075	85.675	84.518	87.155	85.836
Dst5	89.400	87.824	88.612	88.418	89.868	89.143
Sl4	87.832	84.877	86.354	85.735	85.761	85.748
Sp16	84.861	82.090	83.476	84.714	85.927	85.320
Kst9	92.488	90.862	91.675	90.934	91.051	90.992
Kp8	95.485	93.939	94.712	93.556	93.658	93.607
Average	84.781	82.335	83.558	85.474	85.099	85.287
$LSD_{0.01}$ isolates		0.00008			0.611	
$LSD_{0.01}$ antagonists		0.061			0.049	
$LSD_{0.01}$ isolate × antagonist		0.061			0.049	

Ta: *Trichoderma asperellum*, Th: *Trichoderma harzianum*.

Significant difference (P = 0.01) in average antagonistic abilities between the two *Trichoderma isolates* (Ta and Th) against the array of *B. cinerea* isolates was detected (84.781% and 82.335%, respectively). There were also significant differences between the average PIMG values for all *Botrytis* isolates co-cultivated with both *Trichoderma* antagonists. The highest PIMG value was observed in isolate Kp7 (96.402%), followed by isolates Kp8 (94.712%) and Kst9 (91.675%). It is interesting that all 3 isolates with the highest PIMG value belonged to three different genetic groups (Kp7-*flipper*, Kp8-*vacuma*, and Kst9-*transposa*), but all originated from the same region (Kocani). Similar results regarding the genetic group and region of origin of pathogen isolates were detected for isolates from region Gradsko (Grp4, Grp6, and Grp15) and Bogdanci (Bst1 and Bst12). These isolates have shown the lowest PIMG value, regardless of the genetic group. *Trichoderma asperellum* (Ta) has shown greater antagonistic activity (PIMG) against all 18 *B. cinerea* isolates, except for the isolate Dl2, in which case *T. harzianum* (Th) achieved higher percentage of inhibition of mycelial growth. For each pathogen isolate, a significant difference was observed between the obtained values in paired cultures with two different antagonists (Ta and Th). The highest PIMG values were noticed in isolate Kp7 (96.915% for Ta and 95.889% for Th), significantly higher compared to all other pathogen isolates. *Trichoderma asperellum* (Ta) achieved lowest PIMG values in isolates Grp4 (74.246%) and Grp6 (75.509%), while *T. harzianum* (Th) has shown the lowest PIMG values for isolates Grp4 (71.072%) and Bst1 (72.249).

Microscopic examination of the inhibition zone displayed different interactions between the pathogen and antagonists, due to the mycoparasitism. Interactions such as parallel growth of the antagonist alongside the pathogen hypha (Figure 1a), coiling of the antagonists around the pathogen (Figure 1b), formation of appressorium-like structures (Figure 1c) and hyphal collapsing (disintegration) (Figure 1d) were observed in all paired cultures.

Similar observations regarding the hyphal coiling and lysis of different *Phytophthora* species as a result of direct mycoparasitism of *Trichoderma* spp. were previously reported by Barnett and Binder (1973), Elad et al. (1983), and Singh and Islam (2010). Zivkovic et al. (2010) have also reported direct mycoparasitism (coiling, penetration and parallel growth) of *T. harzianum* against *Colletotrichum acutatum* and *C. gloeosporioides*. Comparable results regarding the direct mycoparasitic activity (hyphal coiling, parallel growth and lysis) of *Trichoderma* spp. was observed against *Sclerotium rolfsii* (Jegathambigai et al., 2010) and *Sclerotinia sclerotiorum* (de Figueiredo et al., 2010).

Figure 1. Parallel growth of *Trichoderma* along the pathogen hypha (a), hyphal coiling (b), formation of appressorium-like structure (c), and disintegration (collapse) of pathogen hyphae (d).
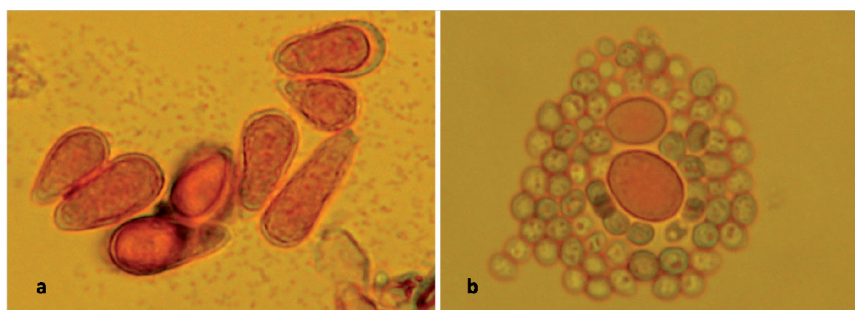

Percentage of inhibition of conidial germination

Germination of pathogen conidia was strongly inhibited in all co-cultivations with both antagonists. After 24 h co-cultivation, *T. asperellum* and *T. harzianum* showed significant inhibition of *B. cinerea* conidia germination. The percentage inhibition of pathogen conidial germination in co-cultivations with *T. asperellum* and *T. harzianum* ranged from 76.932% to 95.107% and 76.933% to 93.658%, respectively.

Opposite to the percentage of inhibition of mycelial growth of all pathogen isolates, there was nonsignificant difference between the average antagonistic abilities between the two *Trichoderma* isolates regarding the PICG values (Table 2). However, significant differences between the average PICG values for the analyzed *Botrytis* isolates co-cultivated with both *Trichoderma* antagonists were observed. Similarly as in the PIMG values, the highest PICG value were detected in the isolates from Kocani region, 93.607% and 93.466% for Kp8 and Kp7, respectively. The lowest values for PICG were also observed in isolates from Gradsko region, Grp4 (77.990%) and Grp6 (77.716%). Both antagonists, *T. asperellum* (Ta) and *T. harzianum* (Th) had the highest percentage of inhibition of conidial germination for the isolates Kp7, Sst6, and Kp8. When co-cultivated with *T. asperellum*, the lowest PICG values were observed for isolates Grp6 (76.932%) and Grp4 (77.218%), while for the isolates co-cultivated with *T. harzianum*, the lowest values were detected in Bst1 (76.933%), Grp6 (78.500%) and Grp4 (78.762%).

Figure 2. Germinated conidia of *Botrytis cinerea* in control (a) and non-germinated conidia of *B. cinerea* in the presence of *Trichoderma* spores (b).

During the microscopic examinations, very interesting phenomenon was observed. Namely, pathogen conidia were commonly surrounded by spores of antagonist (Figure 2). This phenomenon was detected in all antagonist-pathogen combinations. It was interesting that in such interactions between pathogen and antagonist (encirclement) swelling of conidia and germination (development of germ-tube) was strongly inhibited. Moreover, the conidia that did not germinate after 24 h, did not germinate at all. The inhibition of conidia germination is mainly due to the mechanism of competition for nutrients and space. According to Blakeman (1993), competition is effective when exogenous nutrients are needed for germination and germ-tube elongation of pathogen conidia. Elad (2000) has already demonstrated that *B. cinerea* control could be related to competition for resources that are in short supply for the pathogen.

Similar results regarding the PICG values were previously reported by Zivkovic et al. (2010). In this study, the screening of antagonistic activity of *T. harzianum* against *C. gleosporoides* and *C. acutatum*, revealed high percentage of inhibition of conidial germination (86% and 89% respectively), due to the antagonistic activity of *T. harzianum* spores. The phenomenon of surrounded pathogen conidia with spores of antagonist was also observed in this study.
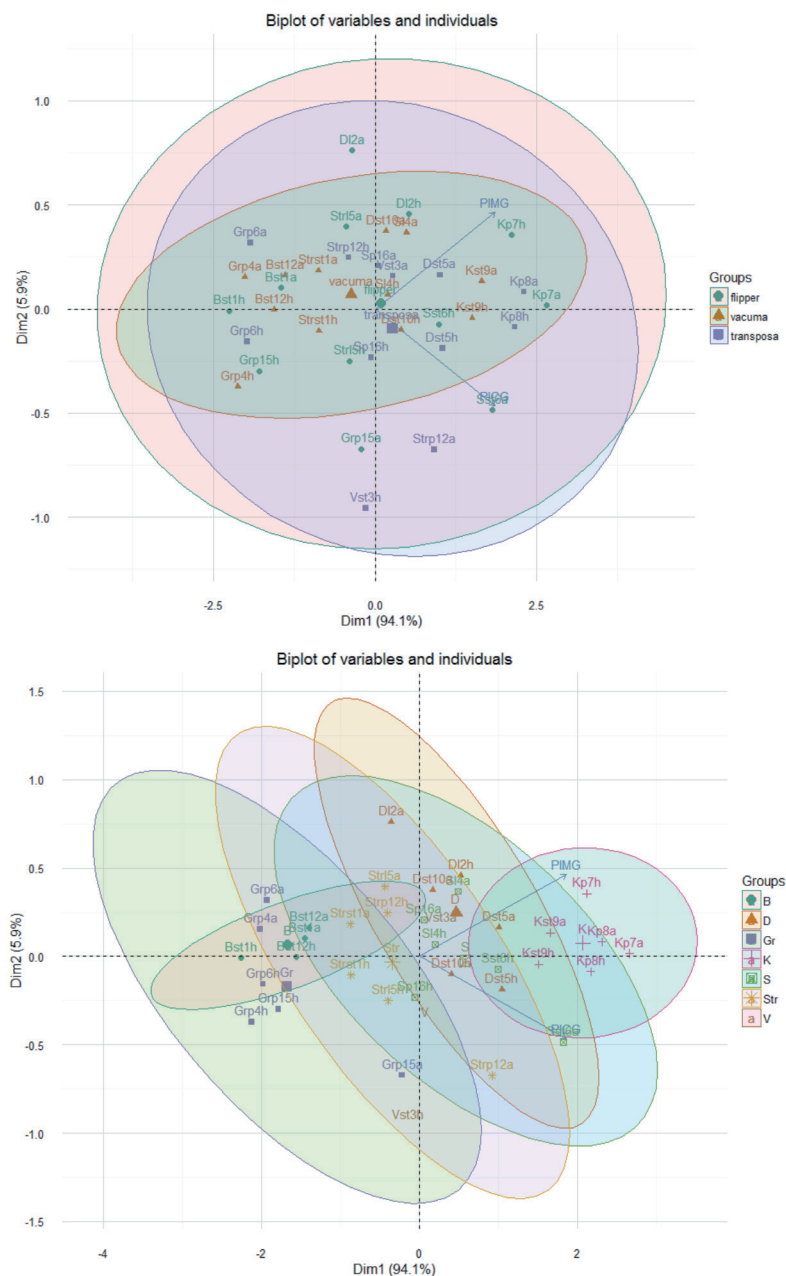
Principal component analysis
The principal component analysis was performed to evaluate the variability of analyzed *B. cinerea* isolates and to assess the relationship between the genetic group and the region of origin of the isolates with the obtained PIMG and PICG values.

The distribution of the isolates according to the determined values for PIMG and PICG can be easily distinguished on Figure 3. The isolates were marked based on the genetic group to which they belonged, and can be clearly seen that both the percentage of inhibition of mycelial growth and the percentage of inhibition of conidial germination was not dependent on the genetic group of the isolates. However, when the isolates are marked based on the region of origin, the previous notion that the PIMG and PICG values are associated with the region of origin, can be confirmed. All isolates from Kocani are characterized with the highest values for both PIMG and PICG, independent of the antagonist and most of the isolates from Gradsko and Bogdanci had low values. The PC analysis was useful for identification of the isolates which had the highest contribution to the variation of PIMG and PICG. It also facilitated the detection of possible association between the genetic group and region of origin of the isolates with their performance when co-cultivated with *Trichoderma* antagonists.

Generally, the results from this study did not confirm any relationships between the genetic group of the pathogen isolates and antagonistic abilities of two tested *Trichoderma* species. Considering this, we cannot select certain *Trichoderma* species for control of *Botrytis* isolates from specific genetic group. Moreover, the obtained results have shown that antagonistic abilities of *Trichoderma* species are more related to the region of origin of pathogen isolates, which additionally challenges the selection of certain *Trichoderma* species for specific *Botrytis* isolates.

If the relationship between the genetic group and region of origin of the pathogen isolates with *Trichoderma* species used for biological control could be established, it would be much easier to apply suitable strategies for control of grey mold disease. Since the results from this study did not confirm such relationships, we cannot predict in advance the antagonistic ability of certain *Trichoderma* species against *B. cinerea*. However, it is encouraging that both tested *Trichoderma* species has shown similar values for inhibition of mycelial growth and inhibition of conidial germination, meaning that using either *T. asperellum* or *T. harzianum*, adequate level of biological control of grey mold disease in tomato can be achieved. Moreover, several previous studies regarding the chemical control of *B. cinerea*, reported that *vacuma* isolates are resistant to fungicide fenhexamid (Fillinger et al., 2008; Esterio et al., 2011). Considering this, the obtained results from this study are even more significant for *B. cinerea* control, because they confirmed no relationships between the genetic group and antagonistic abilities of used *Trichoderma* species. On contrary, vacuma isolates were strongly inhibited by the both tested antagonists. At the end, it should be emphasized that the obtained results from *in vitro* evaluation do not necessarily reflect the antagonistic abilities of *Trichoderma* species in *in vivo* or field conditions.

Figure 3. Factor loadings of *Botrytis cinerea* isolates - marked according to the genetic group (left) and according to the region (right).

CONCLUSIONS

This research demonstrated that *Trichoderma asperellum* and *T. harzianum* inhibited the mycelial growth and conidial germination in *Botrytis cinerea*. The antagonistic abilities were not related to the genetic group, but obvious relationship with the region of origin of the pathogen isolates was detected. Both antagonists can be considered as promising biological control agents for control of grey mold disease in tomato. The obtained results from *in vitro* evaluation should be confirmed in field conditions.

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