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ANTIBACTERIAL ACTIVITIES OF SOME *BACILLUS* SPP. AND *TRICHODERMA HARZIANUM* AGAINST PHYTOPATHOGENIC BACTERIA

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SUMMARY

Biological control is an innovative, cost effective and eco-friendly approach for control of many plant diseases. *Bacillus* sp. and *Trichoderma* sp. were known for its mycoparasitic and antagonistic mechanism in the control of wide range of phytopathogenic diseases in many types of crops. This research is based on the antibacterial activities of some *Bacillus* spp. and *Trichoderma harzianum* in relation to the 10 selected phytopathogenic bacteria in *in vitro* study.
The results indicated that among 52 strains of *Bacillus* spp. presented against phytopathogenic bacteria, only 6 of them showed inhibition but only against *Xanthomonas* genera. Other *Bacillus* spp. strains weren't effective against tested phytopathogenic bacteria. Results of testing of *T. harzianum* efficacy against the growth of selected phytopathogenic bacteria showed that this fungi could be effective to strains from genera *Pseudomonas* and *Xanthomonas* but for other tested genera was ineffective. In further work, all of this trial need to be supported by evaluation of antimicrobial activity in *in vivo*.

Key words: biocontrol, *Bacillus* spp., *Trichoderma harzianum*, *in vitro*, effects, phytopathogenic bacteria

INTRODUCTION

Many plant diseases are caused by phytopathogenic bacteria which greatly determine the quality of plant production (Agrios, 1998). The biological control of plant pathogenic bacteria is an alternative method to the application of chemicals, which may be accomplished through the destruction of existing inoculums, exclusion from the host, or the suppression or displacement of the pathogen after infection (Campbell, 1989). It offers an environmentally friendly approach to the management of plant disease and can be incorporated with cultural and physical control and limited chemical usage for an effective and integrated disease-management system (Monte, 2001; Mérillon and Ramawat, 2012). Biological control includes the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause. Most of antagonists are mass-produced, commercialized, and perhaps patented (Cook and Baker, 1983). Common organisms among them include members of the bacterial genera *Pseudomonas* and *Bacillus*, and the fungal genus *Trichoderma*. They have different mechanisms of disease suppression, fungal antagonists depend mainly on physical contacts with their pathogen while, bacteria mainly use antibiotics as weapon for killing of the pathogens (Howell, 2003).

Bacteria of the genus *Bacillus* has showed antimicrobial activity against plant pathogenic microorganisms (Handelsman and Stabb, 1996; Compant et al., 2005; Živković et al., 2010; Popović et al., 2012a, 2012b; Ivanović et al., 2013). *Bacillus* spp. are natural inhabitants of the phyllosphere (Arias et al., 1999) and rhizosphere (Sessitsch et al., 2003; Berg et al., 2005). These bacteria are involved in the control of plant diseases through a variety of mechanisms of action, such as competition, systemic resistance induction and antibiotic production. The mechanism

of antibiosis has been shown to be one of the most important (Tomashow and Weller, 1996). *Bacillus* spp. have the advantage of being already adapted to the environment where they can be applied as biological control. They have the characteristics of having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. It is considered safe biological agents and their potential as biocontrol agents is considered to be high (Kim et al., 2003).

The fungus *Trichoderma harzianum* is an efficient biocontrol agent against a wide range of soilborne pathogens and has plant growth promote capacity (Chet, 1990; Rabeendran et al., 2000; Yedidia et al, 2001; Howell, 2003; Harman et al., 2004; Bal and Altintas, 2006). For a long time, *Trichoderma* species have been known as biological agents for control of plant diseases (Howell, 2003; Ranasingh et al., 2006). They interact with root, soil and leaf surroundings, and produce many components, which induce local or systemic plant resistance to abiotic stress. The main factor for ecological success of this genus is a combination of very active mycoparasitism mechanisms and an effective defensive strategy, induced in the plants (Rosado et al., 2007).

This study was performed to examine the antimicrobal activity of *Bacillus* spp. and *T. harzianum* against different plant pathogenic bacteria in *in vitro*.

MATERIALS AND METHODS

Phytopathogenic bacterial strains

From the several bacterial diseases that received high attention from research scientists because of their serious effects on many national economical crops, we have selected the following phytopathogenic bacteria showed in Table 1.

In vitro screening of *Bacillus* spp. for biocontrol activity

We used 52 native strains of *Bacillus* spp. isolated from phyllosphere (codes B1–B52) (Ivanović, unpublished data). The strains of *Bacillus* sp. were screened for inhibitory activity against 10 selected bacterial pathogens (Table 1) by following the modified well-diffusion assay (Harris et al., 1989). Petri dishes with appropriate solid medium were poured with 7 ml of soft LB medium, previously inoculated with 70 µl of the culture of indicator strain (10^6 CFU/ml). For agar well-diffusion assay, a well was made in the medium (ø 5 mm) using of sterile bottom parts of the 200 µl pipette tips. Well-diffusion assay was completed by adding tested substances into the well in the final volume of 50 µl. The sterile distilled water was served as a control. The plates were incubated overnight at optimal temperature

Table 1. Plant pathogenic bacteria tested for selection of antagonistic *Bacillus* spp. strains and *T. harzianum*.
Tabela 1. Fitopatogene bakterije korišćene za selekciju antagonističkih *Bacillus* spp. sojeva i *T. harzianum*.

Species	Collection	Code	Originated from	References
Pseudomonas syringae* pv. *syringae	Institute for Plant Protection and Environment, Serbia	IZB-26	Pear	(Ivanović, 2009)
Pseudomonas savastanoi* pv. *phaseolicola	Institute for Plant Protection and Environment, Serbia	TP11	Bean	(Popović, 2008)
Ralstonia solanacearum	La Collection Française de Bactéries Phytopathogènes, France	CFBP 3857	Potato	http://www-intranet.angers.inra.fr/cfbp/doc_pdf/catalogue.pdf
Xanthomonas axonopodis* pv. *phaseoli	Institute for Plant Protection and Environment, Serbia	TX11	Bean	(Popović, 2008)
Xanthomonas campestris* pv. *campestris	Institute for Plant Protection and Environment, Serbia	TKU1	Cabbage	(Popović et al., 2013)
Xanthomonas campestris* pv. *vesicatoria	Institute for Plant Protection and Environment, Serbia	TXv5	Tomato	(Popović et al., 2012)
Xanthomonas arboricola* pv. *juglandis	Institute for Plant Protection and Environment, Serbia	IZB-320	Walnut	(Ivanović et al., 2012)
Erwinia amylovora	Institute for Plant Protection and Environment, Serbia	TEad1	Quince	(Popović et al., 2012)
Brenneria nigrifluens	Institute for Plant Protection and Environment, Serbia	TOr1	Walnut	(Popović et al., 2013)
Clavibacter michiganensis* subsp. *sepedonicus	La Collection Française de Bactéries Phytopathogènes, France	CFBP 3561	Potato	http://www-intranet.angers.inra.fr/cfbp/doc_pdf/catalogue.pdf

for indicator strains, at 27°C. The result was obtained by measuring the zone of inhibition from the edge of the well and expressed in mm.

In vitro screening of *Trichoderma harzianum* for biocontrol activity

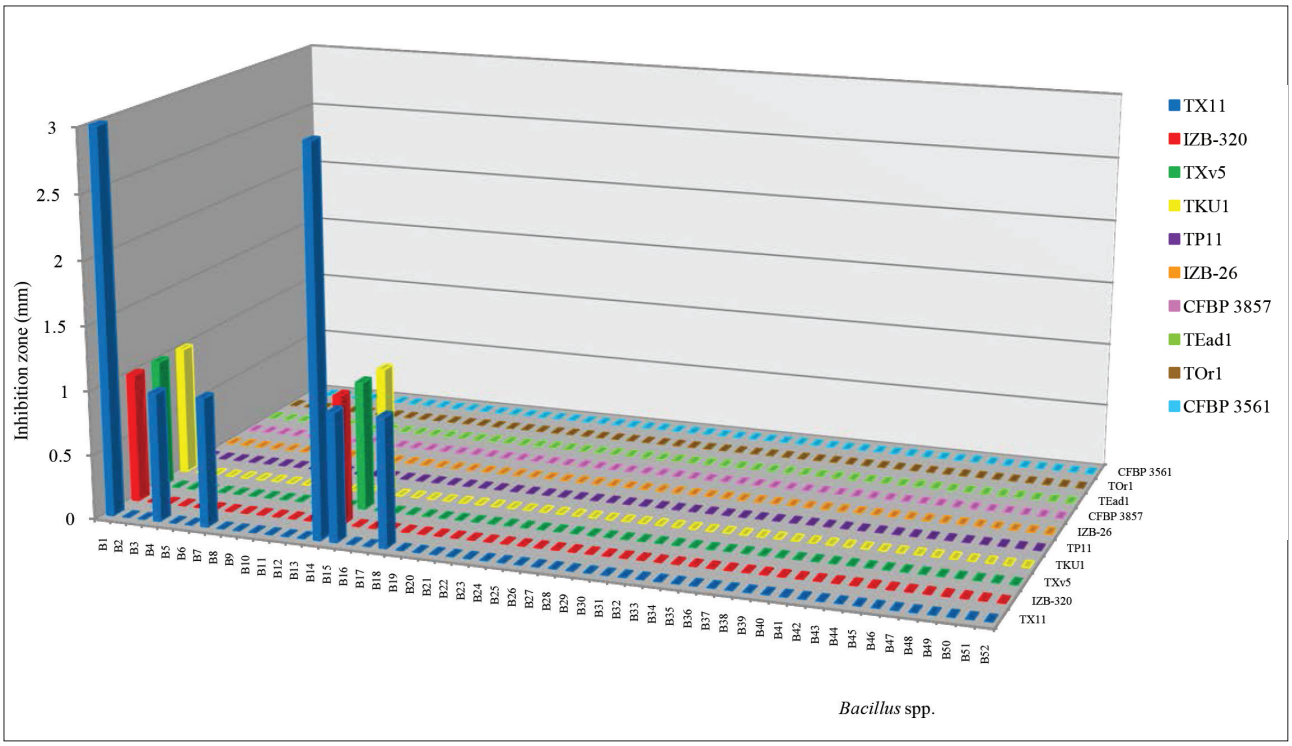
T. harzianum was evaluated for efficacy against the growth of 10 different bacterial pathogens (Table 1) by inhibition zone assay method (Raju, 2010). The antagonistic microorganism *T. harzianum* (DSM 63059), was obtained from the German Collection of Microorganisms and Cell Cultures.

A heavy suspensions (3 day old) of used bacterial strains multiplied in nutrient broth (per 20 ml for each strain) were mixed with Nutrient agar (1000 ml) contained in Erleyenmayer's flask. Fifteen to twenty ml of seeded medium was poured into the sterilized Petri dishes and allowed to solidify. Mycelial discs (ø 5 mm) taken from actively growing *T. harzianum* culture from Potato Dextrose Agar (PDA) were placed in the centre of Petri dishes containing the seeded medium. The inoculated Petri dishes were incubated at 27°C for 72 hours. Observations were recorded for the zone of inhibition produced by antagonistic microorganism around the growth of the pathogen.

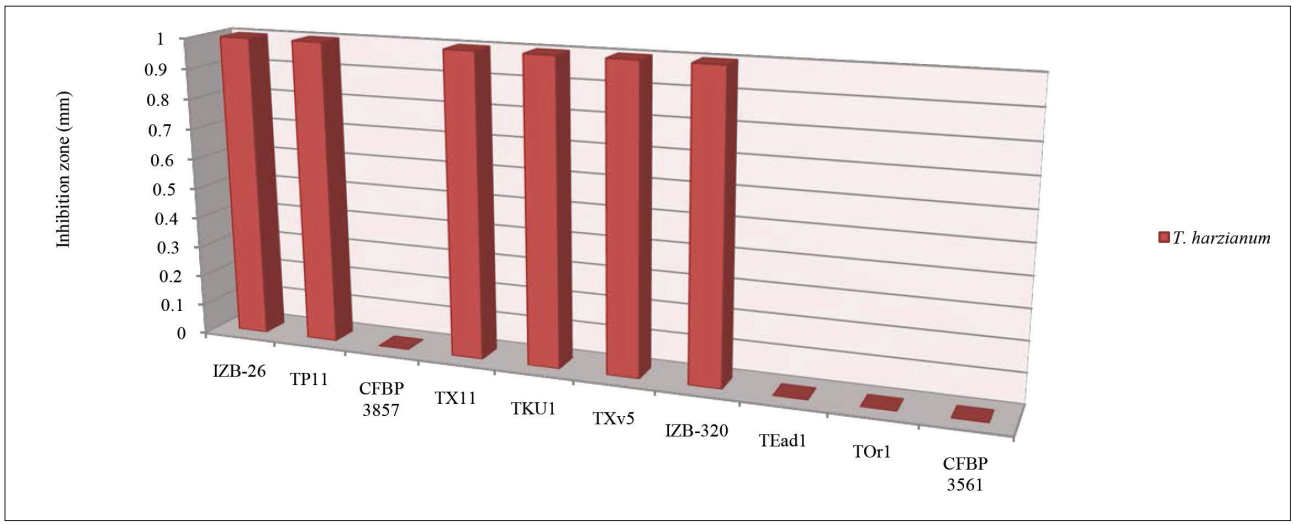
RESULTS

Study conducted on effect of *Bacillus* spp. on growth of 10 different phytopathogenic bacteria indicated that antagonism is showed only to *Xanthomonas* strains (Graph 1). Among tested *Bacillus* spp. strains only two of them (B1 and B14) were found significantly superior in inhibiting the growth of *Xanthomonas* sp. strains (ø inhibition zone 3 mm), while four of them (B4, B7, B15, B18) were less effective (ø inhibition zone 1 mm). Other *Bacillus* spp. strains were not showed antibacterial activities against tested phytopathogenic bacteria.

The results of *T. harzianum* effect against phytopathogenic bacteria showed that the growth of *Pseudomonas* and *Xanthomonas* genera was reduced (Graph 2). After three days, inhibition zones of 1 mm were recorded. *Trichoderma harzianum* was found ineffective to other phytopathogenic bacteria as it failed to inhibit the growth of tested strains.



Graph 1. Antibacterial effect of *Bacillus* spp. against tested phytopathogenic bacteria.
Grafikon 1. Baktericidno delovanje *Bacillus* spp. na testirane fitopatogene bakterije.



Graph 2. Antibacterial effect of *T. harzianum* against tested phytopathogenic bacteria.
Grafikon 2. Baktericidno delovanje *T. harzianum* na testirane fitopatogene bakterije.

DISCUSSION

In recent years, there has been substantial interest in the biological control of plant pathogens especially plant bacteria over the world (Cooksey and Moore, 1980; Volksch and May, 2001; Wilson et al., 2002; Krause et al., 2003). Under biological control, various antagonistic organisms have been identified, which fight against the pathogens by different mechanisms such as competition, lysis, antibiosis, siderophore production and hyper parasitism (Pal i McSpadden Gardener, 2006). Among the different antagonists tried as biocontrol agents, *Bacillus* spp. have been commonly used. Assis et al. (1996) tested the antagonism of 32 epiphytic *Bacillus* spp. isolated from cabbage, kale and radish and found among these isolates, 13 who reduced 100% the incidence of black rot in kale under greenhouse conditions. In field experiments, those 13 isolates reduced incidence in cabbage, ranging from 48–78% (Assis et al., 1997). Popović et al. (2012a) also studied antagonism activity of *Bacillus* soil isolates collected from the rhizosphere of different cultivated plants against *Xanthomonas campestris* pv. *campestris* from cabbage in *in vitro*. Growth inhibition given by Q18 and Q3 of *Bacillus* isolates showed significant value comparing to others. Tested *Bacillus* soil isolates were also used for their antagonistic activities against *Pseudomonas syringae* pv. *syringae* and among them two *Bacillus* (Q7 and Q13) formed inhibition zones (Popović et al., 2012b).

Berić et al. (2013) made screening of 203 natural isolates of *Bacillus* sp. for their antimicrobial activity against *Burkholderia glumae, B. cepacia, B. plantarii, Erwinia carotovora, Pseudomonas fuscovaginae, P. aeruginosa, Agrobacterium tumefaciens, Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, as well as *Bacillus subtilis* 168 and *Escherichia coli*. Authors observed that 127 tested strains inhibit at least one sensitive strain, which illustrates their potential use as biocontrol agents.

Many species of *Bacillus* are known to suppress growth of several bacterial pathogens such as *Agrobacterium tumefaciens* (Ivanović et al., 2013), *Xanthomonas vesicatoria* (Al-Arabi, 2002; Filho et al, 2010), *Xanthomonas axonopodis* pv. *punicae* (Raju, 2010), *Xanthomonas campestris* pv. *campestris* (Monteiro et al., 2005), *Ralstonia solanacearum* (Seleim et al., 2011), *Pseudomonas savastanoi* pv. *savastanoi* (Krid et al., 2010), *Clavibacter michiganensis* subsp. *michiganensis* (Al-Arabi, 2002), *Xanthomonas arboricola* (Dimkić et al., 2013), *Pectobacterium carotovorum* (Dimkić et al., 2013).

Our preliminary screening performed with 10 strains of phytopathogenic bacteria used as indicator strains, showed that two *Bacillus* strain (B1 and B14) from the collection exhibited antagonistic activity against isolates from genera *Xanthomonas*. Therefore, these strains could be evaluated *in vivo* for further biological control potential. Strains from other genera were not showed sensitivity to tested *Bacillus* strains.

This research work also describes use of *T. harzianum* in control of plant pathogenic bacteria *in vitro*. It was found to be possible effective to strains from genera *Pseudomonas* and *Xanthomonas* but for other tested genera was ineffective. Trials should be supported by *in vivo* testing because of slow fungi growing comparing to fast growing of

bacteria. Literature sources which are concerning on antimicrobal activity of *T. harzianum* on phytopathogenic bacteria are poor. Raju (2010) evaluated *T. harzianum* efficacy against the growth of *Xanthomonas axonopodis.* pv. *punicae* by inhibition zone assay method. It was found ineffective to inhibit the growth of *X. a.* pv. *punicae.*

T. harzianum is described as a biocontrol agent in control of soil-borne plant pathogens *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Fusarium oxysporum* (Mishra et al., 2011; Elad et al., 1980), *Pythium* sp. (Hadar et al., 1984), *Macrophomina phaseolina*, *Sclerotinia sclerotiorum* (Mishra et al., 2011), *Alternaria alternata* (Rocco and Perez, 2001; Monte, 2001), *Bipolaris oryzae* (Abdel-Fattah et al., 2007).

Evaluations of antimicrobial properties of selected *Bacillus* spp. and *T. harzianum* by *in vivo* studies are currently in progress.

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BAKTERICIDNO DELOVANJE NEKIH *BACILLUS* SPP. I *TRICHODERMA HARZIANUM* NA FITOPATOGENE BAKTERIJE

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REZIME

Biološka kontrola predstavlja inovativni, isplativ, ekološki pristup za suzbijanje mnogih biljnih bolesti. Vrste roda *Bacillus* i *Trichoderma* su poznati po svom mikoparazitskom i antagonističkom mehanizmu suzbijanja širokog spektra fitopatogenih prouzrokovača bolesti kod brojnih poljoprivrednih kultura. U ovom radu proučavano je baktericidno delovanje nekih vrsta roda *Bacillus*, kao i gljive *Trichoderma harzianum* na 10 odabranih fitopatogenih bakterija u *in vitro* uslovima.

Prema dobijenim rezultatima, od ukupno 52 testirana izolata iz roda *Bacillus*, samo 6 je inhibiralo porast fitopatogenih bakterija i to samo iz roda *Xanthomonas*. Rezultati ispitivanja efikasnosti gljive *T. harzianum* su ukazali na mogućnost delovanja ove gljive na bakterije iz roda *Pseudomonas* i *Xanthomonas*, ali ne i na druge rodove fitopatogenih bakterija testirane u ovom radu. Dalja istraživanja treba bazirati na proučavanjima antimikrobne aktivnosti testiranih agenasa prema fitopatogenim bakterijama u *in vivo* ogledima.

Ključne reči: biološka kontrola, *Bacillus* spp., *Trichoderma harzianum*, *in vitro*, efekat, fitopatogene bakterije

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