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ANTIFUNGAL ACTIVITY OF INDIGENOUS *BACILLUS* SP. ISOLATE Q3 AGAINST MARSHMALLOW MYCOBIOTA

ABSTRACT: Marshmallow is a host of a number of saprophytic and parasitic fungi in Serbia. The seeds of marshmallow are contaminated with fungi from different genera, especially *Alternaria* and *Fusarium*, which significantly reduced seed germination and caused seedling decay. In this study we investigate antagnonism of indigenous *Bacillus* sp. isolate Q3 against marshmallow mycopopulation. *Bacillus* sp. Q3 was isolated from maize rhizosphere, characterized by polyphasic approch and tested for plant growth promoting treats. *Bacillus* sp. Q3 produced antifungal metabolites with growth inhibition activity against numerous fungi in dual culture: 61.8% of *Alternaria alternata*, 74.8% of *Myrothecium verrucaria* and 33.6% of *Sclerotinia sclerotiorum*. That effect could be caused by different antifungal metabolites including siderophores, hydrolytic enzymes, organic acids and indole acetic acid (IAA). Suppression of natural marshmallow seed infection by Q3 isolate was observed. The seeds were immersed in different concentrations of bacterial suspension during 2h and their infections by phytopathogenic fungi were estimated. The results showed significant reduction of seed infection by *Alternaria* spp.

The presented results indicate possible application of this isolate as promising biological agent for control of marshmallow seed pathogenic fungi.

KEY WORDS: *marshmallow, Alternaria, Bacillus sp., antagonism, mycopopulation*

INTRODUCTION

Marshmallow as medicinal plant is among the most economically significant herb in Serbia. Its cultivation has been started in Serbia because of medical properties of root, leaves and flowers.

Marshmallow is the host of many fungal species (Pavlović and Stojanović, 2001, Pavlović et al., 2002, 2006). Fourteen species from 10 genera were identified on the seed, leaf, stem and root of marshmallow (Pavlović et al., 2007). Fungus from the genera *Alternaria* and *Fusarium*, were dominant on the seed. In smaller percentage, fungus from the genera *Phoma*,

Epicoccum, *Cladosporium*, *Penicillium*, *Aspergillus* and *Rhizopus* were present. *Alternaria alternata* was permanently present on the seed. A significant pathogen of the leaves was *Puccinia*. Very destructive pathogens on the stem and root were *Fusarium* species and *Sclerotinia sclerotiorum* causing tissue necroses and white rot of root and stem (P a v l o v i ć and et al., 2006).

Pathogenic fungi decrease the yield and quality of herbal raw material. The use of chemicals is not allowed in plantation of marshmallow. Therefore, alternatives in plant protection are very important. The biological control of soil-born pathogens with antagonistic bacteria has received special attention because of the dural role of these bacteria in plant-growth promotion (PGPR) and disease control (Z e h n d e r et al., 2001). PGPR enhance the adaptive potential of their hosts through a number of mechanisms, such as the mobilization of recalcitrant soil nutrients, the fixation of molecular nitrogen, the control of phytopathogens and the synthesis of phytohormones and vitamins. Direct promotion by PGPR occurs by providing the plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. Indirect promotion of plant growth occurs when PGPR lessens or prevents the deleterious effect of one or more phytopathogenic microorganisms.

Bacillus spp. are well known rhizosphere residents of many crops and usually show plant growth promoting activities that include biocontrol capacity against some phytopatogenic fungi. Recently, intensive agricultural production has been paying greater attention to crop protection from pathogens that lessen yields, as well as to the microbial quality of these crops as raw materials. From the initial implementation of sustainable agriculture, the availability of alternative protective strategies has been reassessed and consequently, the development of environment-friendly and food-hygienically-safe plant-protecting methods based on biological agents has been greatly emphasized (W a r r i o r, 2000).

In order to find effective biocontrol agents which act through the combination of several different mechanisms, the procedures that allowed selection of strain, positive for more than one antagonistic mechanism, were used. Isolate *Bacillus* Q3 seems to be more promising biocontrol agent then other *Bacillus* isolates from soil rhizosphere. The results of testing of cultural, biochemical and PGPR traits of Q3 isolate, as well as the estimations of its antifungal activity to *A. alternata* on seed are presented in this paper.

MATERIALS AND METHODS

Influence of different cultivation conditions on growth rate of indigenous *Bacillus* sp. – isolate Q3. Influence of different nutrient agar media on *Bacillus* Q3 growth was tested: Waksman agar (B e r g, 2002), Yeast Mannitol agar – YMA (V i n c e n t, 1970), Nutrient agar, King B, Potato Dextrose agar – PDA and Triple sugar Iron (Biomedics, Spain). O/F basal medium supplemented with different carbon sources was used for testing the utilization of carbohy-

drates as only carbon source. In order to test the effect of NaCl, temperature and pH on *Bacillus* Q3 growth, the isolate was grown in nutrient broth after inoculation with $5x10^6$ cells. Growth was measured at 600 nm using Shimadzu UV-160 spectrophotometer.

Enzymatic activities assay. Catalase, lysine decarboxylase, gelatinase and urease were detected as recommended by S m i b e r t and K r i e g (1994).

Enzymatic activities for protease, chitinase, pectinase, amylase, and cellulase were identified by clear zone formation around the cell (S m i b e r t and K r i e g, 1994) after incubation for 3 days at 28°C. Protease production was assayed using skim milk agar, chitinase by using Waksman agar supplemented with colloidal chitin, pectinase by using M9 medium supplemented with pectin, cellulase by using M9 with carboxymethyl cellulose (CMC), amylase using M9 with starch.

Estimation of main PGPR trait of *Bacillus* Q3. Phosphate solubilization ability of isolate Q3 was tested on Pikovskaya agar plates (P i k o v s k a y a, 1948) with 0.5% tricalcium phosphate [Ca_3 $(PO4)_2$] and identified by clear halo zones around the colonies.

The production of indole acetic acid (IAA) by Q3 strains and the effect of L-tryptophan on IAA production were assayed according to the Salkowski method (G l i c k m a n and D e s s a u x , 1995). The bacteria were inoculated into the nutrient broth containing L-tryptophan in the concentrations of 2 and 5 mM.

Production of siderophore was determined by Chromazurol Sulphonate (CAS) agar method (A l e x a n d e r and Z u b e r e r, 1991). After incubation at 28°C for 5 days, siderophore production was assayed by the change in medium color, turning from blue to orange.

Hydrogen cyanide production was assayed according to the method suggested by C a s t r i c (1977). Bacteria were streaked onto Nutrient agar plates supplemented with glycine. Petri plates were inverted and a piece of filter paper impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with parafilm and incubated at 28°C for 96 h. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide.

Sampling and fungal isolation. Marshmallow plant parts expressing pathological changes were collected during the period 2008-2010 at the localities of Pančevo, Ruma and Zrenjanin. Seed samples were collected from the plantation intended for marshmallow seed production in Pančevo.

Isolation of the collected samples of leaves, leaf stalks, stems and roots was conducted in in a common manner, by taking the fragments from the zones between healthy and diseased tissues. Before transferring to PDA, plant material was sterilized with 2% of NaOCl solution for 2 minutes, washed with distilled water and incubated for 7-10 days at 25°C. Isolation of fungi from the seeds was carried out according to the procedure supplied by ISTA (M a t h u r and K o n g s d a l, 2003), using the incubation methods that include filter paper and nutritive medium. After the incubation of seeds for 5-10 days, formed mycelia were transferred to PDA. Twelve isolates were obtained from marshmallow (Table 1).

Tab. 1 – Fungi isolated from marshmallow used in this study

Fungal	Reference of source
Alternaria alternata	Seed
Aspergillus niger	Seed
Aspergillus flavus	Seed
Myrothecium verricaria	Seed
Fusarum oxysporum	Seed, root
F. verticillioides	Seed, root
F. proliferatum	Seed, root
F. semitectum	Stalk
F. sporotrichoides	Seed
F. equiseti	Seed
F. solani	Root
Sclerotinia sclerotiorum	Collar root

Antifungal activities of Bacillus Q3 isolate against phytopathogenic fungi. Antifungal activities were tested with 15 phytopathogenic fungi (Table 1.) A dual plate method was used for *in vitro* screening of biocontrol PGPR. Percentage of growth inhibition was calculated using the formula proposed by V i n c e n t (1947): I = (c- T)/c x 100, where I is the percentage of growth inhibition; C the growth of fungi in control; T the growth of fungi in treatment.

Marshmallow's seed treatment with *Bacillus* sp. – isolate Q3. Marshmallow seeds were suface sterilized with 70% ethanol for 5 min, and rinsed five times with destilled water. The sterilized seeds were submerged into the culture solutions of *Bacillus* Q3 isolate (concentration $5x10^4$, $5x10^5$ and $5x10^6$ CFU ml^{-1}) for 2 hours. Four replications (with 100 seeds per replication) were placed in filter paper in Petri dishes. The seeds immersed in distilled water were applied as negative control. The seeds were incubated at 25°C and the percentages of infected seeds were recorded. The obtained results were analyzed by Duncan test.

RESULTS AND DISCUSSION

Recent developments have encouraged the research into PGPR commercialization. These developments include the need for alternatives to soil fumigants to control soil borne plant pathogens. Current fumigants are being banned or restricted in use or are too costly for annual crop producers. Medicinal plants are to be targeted for PGPR application as these producers may replace costly soil fumigants with as likely costly PGPR.

Influence of different cultivation conditions on growth rate of indigenous *Bacillus* sp. – isolate Q3. On the basis of cultural, morphological and biochemical characteristics, the isolate Q3 was presumptively identified as *Bacillus* strain. Growth on different carbon sources added in O/F basal medium, on different nutritive agar media and at different conditions is presented in Table 2.

Tab. 2 – Effect of different nutritive media, carbon sources, temperature, saline and pH conditions on *Bacillus* Q3 growth

Growth on nutritive agar media		Growth on carbon sources (in O/F basal medium)		Growth in Nutrient broth at pH 7		Growth in Nutrient broth at 37°C			
Waksman	+	glycerol	+	t° C		% NaCl		pH	
Yeast Mannitol	+	mannitol	+	4	-	1	+	5	-
Potato Dextrose	+	lactose	+	10	±	3	+	5.5	±
Triple Sugar Iron	+	sucrose	+	26	+	5	+	7	+
Nutrient	+	glucose	+	37	+	7	+	8	+
King B	-	maltose	+	41	±	8	±	8.5	±

Bacillus Q3 showed optimal growth at pH 7-8 in temperature range 26-37 °C. We observed tolerance to saline concentration up to 8%.

Enzymatic activities. Enzymatic activities of catalase and lysine decarboxylase were observed, while gelatinase and urease activities were not detected (Table 3). The absence of clear zone formation around the cells on media for chitinase, pectinase, amylase and celulase were identified suggesting negative results for enzyme activities. Protease activity was poor and zones on skim milk agar were observed, but with not clear appearance. L e o n et al. (2009) reported that several enzymatic activities (proteolytic, chitinolytic and cellulolytic) of *B. amyloliquefaciens* BNM340 were detected.

Estimation of PGPR traits of *Bacillus* Q3 isolate. Phosphate solubilizing microorganisms (PSM) play a significant role in making phosphorus available to plants by bringing about favorable changes in the soil microenvironment and leading to solubilization of inorganic phosphate sources. Solubilization ability of isolate *Bacillus* Q3 was detected as clear halo zones around the colonies on Pikovskaya agar plates supplemented with 0.5% tricalcium phosphate. The solubilization of inorganic phosphate has been attributed to the production and release of organic acids or some additional mechanisms as it is confirmed by weak or even lack of linear correlation between pH and the amount of P-solubilized (A l a m et al., 2002).

Tab. 3 – Enzymatic activities and main PGPR traits of *Bacillus* Q3 isolate

		PGPR traits			
Enzymatic activity				Solubilization of insoluble phosphates	±
katalase	+	Protease	±	Antifungal activity	+
lysine decarboxilase	+	Pectinase	-	Production of	
gelatinase	-	Cellulase	-	indole acetic acid	+
urease	-	Chitinase	-	siderophores	+
amylase	-			HCN	±

Another important trait of PGPR – production of low-molecular-weight compounds called siderophores was observed. Indigenous isolate *Bacillus* Q3 showed large amount of siderophores production detected by the color change intensity of the CAS medium from blue to orange. Siderophores may indirectly influence the plant growth and health. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a serious competition (L o p e r and H e n k e l s, 1997). Under iron-limiting conditions, PGPR produces siderophores in order to acquire ferric ion (W h i p p s, 2001). They bind to the available form of iron (Fe^{3+}) in the rhizosphere, thus making it unavailable for the phytopathogens and protecting the plant's health. Although various bacterial siderophores differ in their abilities to sequester iron, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (L o p e r and H e n k e l s, 1999).

Indigenous isolate *Bacillus* Q3 was inoculated into the nutrient broth containing L-tryptophan in the concentrations of 2 and 5 mM and production of indole acetic acid (IAA) was observed. IAA production by microorganisms promotes the root growth by directly stimulating plant cell elongation or cell division (I d r i s et al., 2004). *B. amyloliquefaciens* BNM340-inoculated soybean plants were protected from a high *P. ultimum* infestation, since only 30% of seedlings emerged in the control treatments (L e o n et al. 2009). This strain was able to produce auxins, as well as excreted surfactin and some iturin-like lipodepsipeptides, such as iturin A. These mechanisms have been previously correlated with antifungal activity (Z e h n d e r et al., 2001; I d r i s et al., 2004).

Bacillus Q3 showed very light discoloration of the filter paper from orange to brown, and it was considered to be a poor cyanide producer. Hydrogen cyanide is a general biocide forming stable compounds with divalent ions and inhibiting cytochrome oxidase of many organisms (V o i s a r d et al., 1994). A h m a d et al. (2008) published that three strains of *B. subtilis* were unique in their characteristics, being antagonistic to *C. falcatum,* deficient in HCN production and producers of surfactin lipopeptide only. Inability of strains to produce HCN will make them biocontrol agents of choice, since HCN imposes negative effects on plant growth (S c h i p p e r s et al., 1990).

Antifungal activities of Bacillus Q3 isolate against phytopathogenic fungi. Biocontrol activity of *Bacillus* strains against multiple plant pathogens have been widely reported and well documented (L e o n et al., 2009; K l o e p p e r et al., 2004). Indigenous isolate *Bacillus* Q3 showed hyphal deformation, inhibition of hyphal elongation and different percent of growth inhibition of tested marshmallow pathogenic fungi (Table 4). Maximum inhibitory zone and antifungal activity was observed against *Myrothecium verrucaria* (about 75%). Bacillus Q3 isolate caused high percent of inhibition (61.75) on *Alternaria alternata* growth. We assume that inhibitory effect may be caused by different antifungal metabolites including siderophores, organic acids, IAA and antifungal antibiotics. These results are in agreement with earlier report on *Bacillus* sp. producing antifungal metabolites with activity against a number of mycelial fungi (R a m i r e z et al., 2004; C a z o r l a et al., 2007).

Tab. 4 – The inhibitory effects of the bacterial isolates *Bacillus* Q3 on tested pathogenic fungi

Phytophatogenic fungi	Hyphal deformation	Inhibition of hyphal elongation	Percent of growth inhibition
Alternaria alternata	+	+	61.75 ± 2.70
Aspergillus niger	+	±	ns
Fusarium solani	+	±	ns
F. verticillioides	+	±	ns
F. semitectum	+	±	ns
F. sporotrichioides	+	±	ns
F. equiseti	+	+	ns
Sclerotinia sclerotiorum	+	+	33.63 ± 2.50
Myrothecium verrucaria	-	+*	74.80 ± 4.91
A. flavus	-	-	-
Fusarium oxysporum	-	-	-
F. proliferatum	-	-	-

(–) no inhibition; (±) inhibition during 5 days; (+) inhibition longer than 7 days; ns- not significant, less than 10%; *- hyphal elongation in opposite direction of bacteria;

VVV reported that *B. circulans* MTCC 8983 also showed antifungal activity against *Rhizoctonia solani, Fusarium oxysporum, Sclerotinia sclerotiorum.*

Efficacy of indigenous *Bacillus* sp. – Q3 isolate against marshmallow seeds mycoflora. The average percentage of seed infection with *Alternaria althernata* was 34% in control, but only 6.5% in the variant treated with *Bacillus* sp. isolate in the concentration of $5x10^6$ CFU ml^{-1} for 2 hours. The obtained results showed that isolate Q3 reduced infection of seeds by *A. althernata* for more than five times. The same results were obtained by using bacterial concentration of $5x10^5$ CFU ml^{-1} and $5x10^4$ CFU ml^{-1} for 2 hours.

Our results coincided with the results of K a u r et al., (2007) who found that some bacterial isolate from rhizosphere, such as *Pseudomonas* spp., could inhibit *Aspergilus* and *Fusarium*. U m e c h u r u b a (2004) studied antagonistic activity of *Bacillus subtilis* against *Alternaria* spp. isolated from seed and found the inhibitory effect of 26-58%.

CONCLUSION

Indigenous *Bacillus* sp. – isolate Q3 showed strong *in vitro* antagonistic activity against *Myrothecium verrucaria, Alternaria alternata* and *Sclerotinia sclerotiorum* that continued for eight days. It has now been confirmed that a single PGPR – indigenous *Bacillus* Q3 has several modes of action. So far, this has been the first report of a *Bacillus* sp. isolated from maize rhizosphere in Serbia with phosphate solubilizing ability simultaneously producing siderophore, IAA and antagonistic activity against marshmallow mycobiota. This study confirmed the potential of rhizoplane and rhizosphere to protect medicinal plants, in this case marshmallow, from some diseases.

Bacillus sp. isolate Q3, appeared to be very promising biocontrol agent against *Alternaria* spp., a predominant marshmallow seeds pathogen.

Identification of key antimicrobials produced by *Bacillus* Q3 can be exploited for the testing of antifungal activity against other medicinal plant pathogenic fungi. Also, *Bacillus* Q3 could be further exploited both as a biofertilizer and an effective biocontrol agent.

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АНТИФУНГАЛНА АКТИВНОСТ АУТОХТОНОГ ИЗОЛАТА *BACILLUS* SP. Q3 НА МИКОПОПУЛАЦИЈУ БЕЛОГ СЛЕЗА

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Резиме

Бели слез гајен у Србији је домаћин многим сапрофитним и паразитским гљивама. Семе је заражено гљивама из различитих родова, нарочито *Alternaria* и *Fusarium*, које значајно редукују клијавост семена и изазивају сушење. У овом раду испитиван је антагонизам аутохтоне бактерије *Bacillus* sp. изолата Q3 и микопопулације белог слеза. *Bacillus* sp. Q3 је изолован из ризосфере кукуруза, карактеризација је извршена полифазном методологијом и тестиране су особине одговорне за стимулацију раста биљака. *Bacillus* sp. Q3 продукује антифунгалне метаболите са израженом активношћу против фитопатогених гљива са различитим процентом инхибиције раста у двојној култури: 61.75% код *Alternaria alternata*, 74.80% код *Myrothecium verrucaria* и 33.63% код *Sclerotinia sclerotiorum*. Овај ефекат је последица продукције различитих антифунгалних метаболита, укључујући сидерофоре, хидролитичке ензиме, органске киселине и индолсирћетну киселину (IAA). Установљено је сузбијање природне инфекције семена белог слеза применом изолата *Bacillus* Q3. Семена су потапана у различите концентрације бактеријске суспензије током 2 h и праћен је степен инфекције фитопатогеним гљивама. Резултати су показали значајан степен редукције инфекције семена белог слеза гљивом *Alternaria* spp.

Ови резултати указују на могућу примену овог изолата као потентног биолошког агенса за контролу инфекције семена белог слеза фитопатогеним гљивама.