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THE EFFECT OF IAA PRODUCING *BACILLUS* SP. Q3 STRAIN ON MARSHMALLOW SEED GERMINATION

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Abstract

STAROVIC, M., D. JOSIC, S. PAVLOVIC, S. DRAZIC, D. POSTIC, T. POPOVIC and S. STOJANOVIC, 2013. The effect of IAA producing *Bacillus* sp. Q3 strain on marshmallow seed germination. *Bulg. J. Agric. Sci.,* 19: 572-577

Marshmallow (*Althea officinalis*) is one of the important medicinal plants in Serbia. One of the bigest problem in growing of marshmallow is poor seeds germination. Rhyzospheric bacteria able to produced plant growth stimulating hormones can inpruve seed germinations and decreace seed infections with patogens. IAA production by *Bacillus* sp. Q3 strain estimate in this investigation ranged from 3.76-10.62 $\mu g ml^{-1}$. The application of soil bacteria as the antagonists to the growth of pathogenic fungi, indicated that IAA producing *Bacillus* sp. strain Q3 demonstrated not only a high level of antagonism towards the seed mycoflora, but significantly increased the germination rate of the marshmallow seeds. Soaking marshmarrow seeds for 24 h in the 10^5 CFU ml^{-1} of investigated strain *Bacillus* sp. strain Q3 increased the 55.1% germination and decreased the percentage of the seed infection by the fungus *Alternaria alternata*, compared to the control (32%). Strong increasing percent germination of marshmallow's seeds from 26.9-55.1% and decreasing seed infection with phytopathogen *Alternaria alternate*, as predominant marshmallow seeds pathogen, can recommend this strain for seed protection and as PGPR.

Key words: *Bacillus* sp., marshmallow, seed germination

Introduction

Medicinal plants are among the most economically significant plants in Serbia. Among them marshmallow (*Althea officinalis*) is one of the important medicinal plant in Serbia. Marshmallow is cultivated because of medical properties of its root (*Althaeae radix*), leaves (*Althaeae folium*) and flowers (*Althaeae flos*). That was the reason why the Institute for Medicinal Plant Research "Dr Josif Pancic" started the cultivation of marshmallow in cooperation with other producers, as well as on its own land.

It is already established that there is a problem with poor germination of marshmallow seeds, averaging only 29% (Lekić et al., 2009). This is a serious issue in the cultivation of marshmallow on a large scale.

The application of the germination stimulans such as giberellin acid did not yield positive results, due to a very hard seed coat. Our preliminary investigation on the application of soil bacteria as the antagonists to the growth of pathogenic fungi, indicated that *Bacillus* sp. strain Q3 demonstrated not only a high level of antagonism towards the seed mycoflora, but significantly increased the germination rate of the marshmallow seeds.

Marshmallow seeds are rich in protein and carbohydrates and therefore an excellent substrate for the growth of microorganisms, especially fungi. A large number of pathogenic fungi are identifying on the seed, leaf, stem and root of marshmallow (Pavlović and Stojanović, 2001; Pavlović et al., 2002; Pavlović et al., 2006; Pavlović et al., 2007). Fungi from the genus *Alternaria* and *Fusarium* are dominant populations on the seed, *Puccinia malvacearum* on the leaves, *Fusarium* species and *Sclerotinia sclerotiorum* in the necrotic tissue of the root and stalk of the marshmallow (Pavlović et al., 2007). *Alternaria alternata* is permanently present on the seed (Pavlović et al., 2006), causing serious problems in seed germination and plant growth.

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Suppression of diseases caused by plant pathogens and promotion of plant growth using beneficial plant growth promoting rhizobacteria (PGPR) allowed us to avoid the use of fungicides in medicinal plants cultivation. The principal mechanisms of growth promotion include production of growth stimulating phytohormones, siderophores, antibiotics (Steenhoudt and Vanderleyden, 2000), decreasing ethylene levels in root cells (Li et al., 2000), limitation of plant nutrients (nitrogen, phosphorus, B-vitamins, amino acids) in the rhizosphere (Nautiyal et al., 2000), colonizing-roots competition with pathogenic microorganisms (Dekkers et al., 1998) and induction of plant systemic resistance to pathogens (Gutierrez-Manero et al., 2001; Richardson et al., 2009).

Genus *Bacillus* is the member of aerobic endospore forming bacteria (AEFB) able to survive under adverse environmental conditions for an extended period. Many *Bacillus* strains are able to promote plant growth using single or combined PGP mechanisms (Whipps, 2001; Idris et al., 2007, Richardson et al., 2009). One of them is the production of plant hormones, which stimulate plant cell elongation, division, and differentiation, and play an important role in the response to biotic and abiotic stresses. One of the phytohormones - auxin (IAA) is involved in the initial processes of lateral and adventitious root formation (Gaspar et al., 1996) and root elongation (Yang et al., 1993). A majority of rhizosphere colonizing bacteria, which are capable of producing more than one type of plant hormone, are Gram-negative. Some Gram-positive soil bacteria are also producers of substances with IAA or (IAA)-like bioactivity (Loper and Schroth, 1986; Idris et al., 2004; Vandeputte et al., 2005). Other indolic compounds, such as indole-pyruvic, indole-acetamide and indole-carboxylic-acid, can be involved in root formation (Nelson, 2004).

Previous studies carried out in our laboratories pointed out the indigenous *Bacillus* Q3 strain cause hyphal deformation, inhibition of hyphal elongation and growth inhibition of marshmallow pathogenic fungi - *Myrothecium verrucaria* isolated from collar root, *Alternaria alternata* and *Sclerotinia sclerotiorum* isolated from seed (Josic et al., 2011). The aim of our investigation was to estimate IAA production of *Bacillus* Q3 strains detected as promising biocontrol agent and effect of this strain on seed germination.

Materials and Methods

Qultivation of bacteria. Bacteria were grown aerobically on nutrient agar (NA) or on a rotating shaker (150 rpm) in nutrient broth (NB) for 24 h at 28ºC. The density of culture was measured spectrophotometrically at 600 nm, diluted in sterile medium to a final concentration of 5 x 10^8 CFU $ml^{-1,}$ and the resulting suspensions was used for PCR amplification and IAA quantification.

PCR identification. Bacterial culture was grown 24h on NB and supernatant with DNA was collected after incubation at 95^0C for 10 min and 5 min at 4^0C.Primers fD1 and rD1 (Weisburg et al., 1991) were used for amplification of the conserved regions of 16S rRNA genes. PCR amplification with 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 57°C) and extension (2 min at 72°C) was used. Initial denaturation at 95°C for 3 min and final extension at 72°C for 6 min were applied. Product of amplification was subjected to horizontal electrophoresis through 1.2% agarose in 0.5x TBE buffer, and visualized after ethidium bromide staining. Amplicon of ~ 1500bp was purified using PCR purification kit (Fermentas, Lithuania). The nucleotide sequence of *Bacillus* Q3 strain determined in this study using facility of Macrogen (Korea) has been deposited in the GenBank database under accession number JX143762.

Quantification of IAA production of *Bacillus* Q3 strain. *Bacillus* Q3 were tested for indole-3-acetic acid (IAA) production, using the Salkowski method (Glickman and Dessaux, 1995). Bacterial strain in concentration of 1x 10^9 CFU ml^{-1} was added to nutrient broth and NB supplemented with of 2,5 and 5 mM L-tryptophan. Incubation with rotary shaking at 28°C for 24 and 48h was done, the density of the culture was measured spectrophotometrically at 600 nm for optimisation to value 1 and the bacterial cells were removed from the culture medium by centrifugation. Salkowski's reagent (50mL 35% $HClO_4$ + 1mL $FeCl_3$) was used for color development and the absorbance at 530 nm (Shimadzu Spectrophotometer UV-160) was measured. The concentration of IAA was determined by comparison with a standard curve (1 - 50 μg ml^{-1}). The IAA produced was measured in triplicate.

Marshmallow seed treatment with *Bacillus* Q3 strain. Marshmallow seeds were collected randomly from the entire population to get an adequate representation of genetic diversity in locality Pancevo. Marshmallow seeds were surface sterilized with the 70% ethanol for 5 min, and rinsed five times with distilled water. The sterilized seeds were submerged in the culture solutions of *Bacillus* Q3 (concentration 10^4, 10^5 and 10^6 CFU ml^{-1}) for 2, 12 and 24 hours. Four replications (with 100 seeds per replication) were placed in filter paper on Petri dishes. The seeds immersed in Giberellic acid (GA_3 concentration 10^6 μg ml^{-1}) and distilled water was used as a negative control. The seeds were incubated at 25°C and the percentages of infected seeds were recorded. Percentage of seed germination was recorded after 20 days. The rate of germination was estimated by using a modified Timson's index of germination velocity = ΣG/t, where G is percentage of seed germination at 2-days intervals, and t is total germi-

nation period (Khan and Ungar, 1998). The obtained results were analysed by Duncan test.

Results and Discussion

PGPR application in medicinal plants cultivation is efficient method for environmentally friendly growth stimulation and plant diseases suppression. Identification of key antimicrobials produced by indigenous rhizobacteria, well adapted to local environmental condition, can be exploited for plant growth stimulation and antifungal activity against medicinal phytopathogens. As IAA is among the most important native auxin and its production by PGPR can vary among different species and strains, we have estimated the IAA produced by *Bacillus* Q3.

PCR identification. Amplicon of 1500 bp from *Bacillus* Q3 strain was amplified using fD1/rD1 primer set. Partial sequence of 16S rDNA (944 bp) under accession number JX143762 was compared with similar sequences in GenBank NCBI and revealed a similarity to *Bacillus altitudinis* (HQ336306.1, GU001891.1) and *B. pumilus* (EU379270.1, GU323367.1), *Bacillus* sp. BCHMAC41 (GU188893.1). Additional analyses for better characterization of *Bacillus* Q3 strain are needed.

IAA production of *Bacillus* Q3 strain. *Bacillus* Q3 strain was able to produce indole-3-acetic acid without the addition of tryptophan as a precursor of IAA (Figure 1). Tryptophan has been identified as a main precursor molecule for biosynthesis of IAA in bacteria. A higher level of IAA was detected after 24 and 48h of *Bacillus* Q3 strain cultivation in a medium supplemented with 5 mM L-tryptophan. During the investigation of rooting and root growth in kiwifruit, Erturk et al. (2010) reported similar value of IAA production in non-supplemented medium for PGPR strain *Bacillus* RC23 (4.3 µg/ml). *Paenibacillus polymyxa* RC05, *B. subtilis* OSU 142, *Bacillus* RC03, *B. megaterium* RC01 and *B. simplex* RC19 produce more IAA (5.6-7.2 µg/ml) growing in the same condition. Appreciable IAA level from 20.4 μgml^{-1} (*Bacillus* RC23) to 33.6 μgml^{-1} (*Bacillus simplex* RC19) are quantified in the presence of 25 µg/ml of tryptophan, which is higher than the value obtained for *Bacillus* Q3 IAA production. The indigenous auxins-producing *Bacillus subtilis* AH18 and *Bacillus licheniforims* K11 strains are able to promote plant growth of red-pepper and tomato synergistically (Lim and Kim, 2009). In addition to auxins, these strains produce antifungal β-glucannase, siderophores, and antibiotic iturin (*B. licheniformis* K11) and were capable of solubilizing insoluble phosphates.

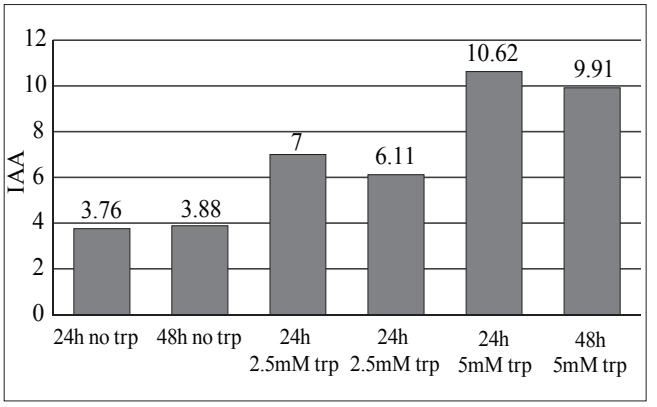

Fig. 1. *Bacillus* Q3 production of IAA in the absence and presence of tryptophan (trp)

Results of effect on seed germination due to treatment by *Bacillus* Q3 strain are given in Table, 1, Figures 2 and 4. These results suggest a general increase in seed germination. Soaking marshmarrow seeds for 24 h in the solution of soil bacteria, *Bacillus* sp. strain Q3 significantly increased the percentage germination and decreased the percentage of the seed infection by the fungus *Alternaria alternata*, compared to the control (Figure 3). When the seeds were soaked for 2 hours only, in any concentration of the *Bacilus* sp. strain Q3, the effect on the germination and contamination was not significant. The best results were obtained after the soaking of the seeds for 24 h in the bacterial suspension as the percent germination increased and the percent of infection decreased (Table 1). Bacterial suspension in concentrations of 10^4 and 10^5 cfu/ml affected the percent germination most, while the concentration increase didn't change the level of seed contamination significantly. The total rate of germination increased between 26.9 – 55.1 % depending on the concentration of the suspension.

The average marshmallow seed infection with the fungus *Alternaria althernata* in the negative control was 34%, while in the variant treated with *Bacillus* Q3 strain concentration 10^4 CFU ml^{-1} for 24 hours, percentage of infection was re-

Table 1
A percentage of germination and infection of marshmallow seeds immersed in culture solutions of Bacilus sp. strain Q3 for 24 h

Concentration of antagonistic *Bacillus* Q3 strain	Percentage of germination [a]	Percentage of infected seeds [a]
10^4	58.25 a	2.2 b
10^5	55.75 a	5.5 b
10^6	47.75 b	3.8 b
GA_3	45.50 b	37.50 a
Control	37.75 c	34.0 a

a - values in the columns marked wiht the same letter are not statistically significant according to the Duncan's test (P=0.05)

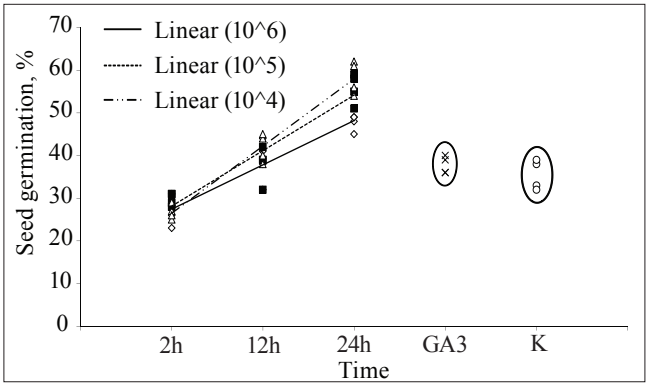

Fig. 2. Influence of length of exposure and different concentrations of bacterial isolates in culture solutions isolate Q3 to percent of the germination of marshmallow's seeds

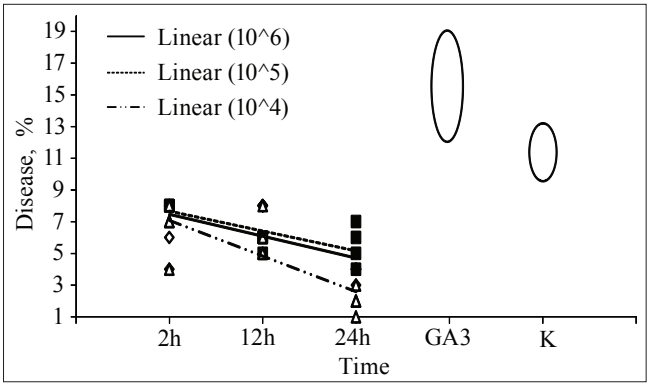

Fig. 3. Influence of length of exposure and different concentrations of bacterial isolates in culture solutions isolate Q3 to the infection marshmallow's seeds with *A.alternata*

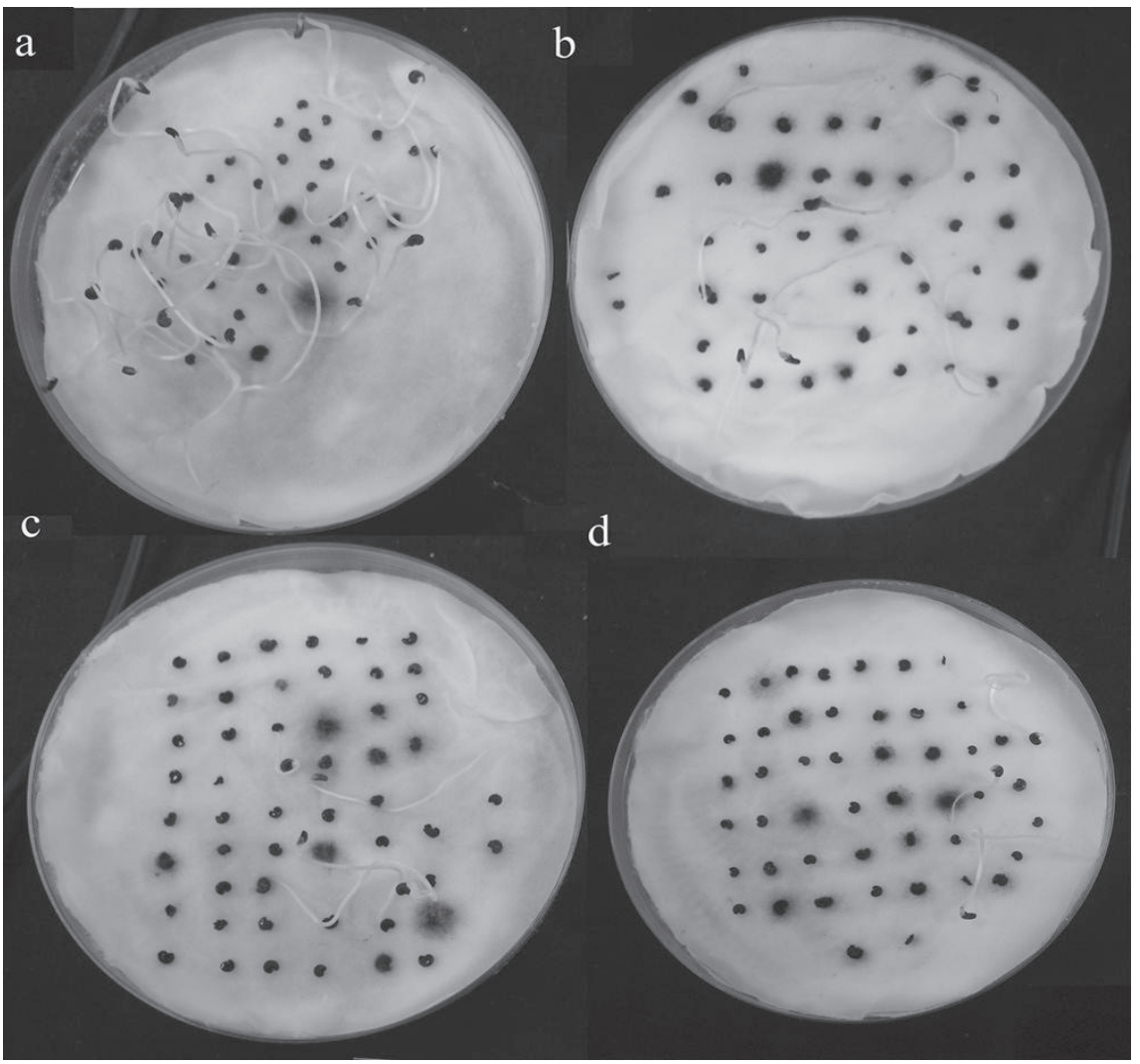

Fig. 4 . Seed germination: a) *Bacillus* Q3 24^h Conc. 10^4, b) *Bacillus* Q3 12^h Conc. 10^6, c) GA3, d) Control

duced by 32 %. The lowest decrease in the percentage of infection was 26% in the variants treated with concentrations of 10^5 CFU ml^{-1} for 2 hours and concentration 10^6 CFU ml^{-1} for 12 hours (Figure 3).

This results from marshmallow seed are corroborated with Kaur et al. (2007). They found that some bacterial isolate from rhizosphere, as *Pseudomonas* spp. could inhibit *Aspergilus* and *Fusarium* growth. Umechuruba (2004) investigate antagonistic properties of *Bacillus subtilis* against *Alternaria* spp. isolated from seed and found the inhibitory effect ranging between 26-58%, while Mishra et al. (2011) showed that their isolate *B.subtilis* can completly inhibit *A.alternata*. Sharma et al. (2007) compared different dilutions of *Pseudomonas* and *Bacillus* strains on seed germination and obtained the best results with 10^{-4} dilution.

IAA producing strains *B. subtilis* AH18 and *B. licheniformis* K11 stimulate seed germination of red pepper, tomato, green onion, spinach, and radish plants (Lim and Kim, 2009). In this investigation, the germination rate of seeds is about 13% higher than the control seeds. Germination of soybean seeds decreased in the presence of *Bacillus thuringiensis* from 18 to 55% as described Reyez-Ramirez et al. (2004).

Conclusion

Considering that indigenous strains are well adapted to the environmental conditions, we confirmed that the indigenous *Bacillus* strain Q3 is able to produce IAA. Strong increasing percent germination of marshmallow's seeds from 26.9-55.1% and decreasing seed infection with phytopathogen *Alternaria alternate*, as predominant marshmallow seeds pathogen, recommended this strain as PGPR.

Further investigations will include a full-scale laboratory and field testing to find out the optimum conditions, which would make *Bacillus* Q3 completely safe as a biological agent, and compatible with sustainable, environmentally friendly agriculture.

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