The use of bacterial indole-3-acetic acid (IAA) for reduce of chemical fertilizers doses

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Abstract

The standard technology of seed processing uses mainly chemical products. Recent researches showed that toxic materials from chemical fertilizers can be harmful to humans, animals and the environment. Currently the attention of researches is shifting away from chemical fertlizers and toward alternative that consumers perceive to be natural, Plant Growth Promoting bacteria (PGP). PGP bacteria could be a way to reduce chemical fertilizer doses. This was the reason to test the ability of *Bacillus megaterium*, *Azotobacter chroococcum* to produce hormone auxin (IAA). Bacterial strains were identified by PCR amplification and sequencing of the 16S rRNA gene. Indole-3-acetic acid (IAA) was detected and quantified by MRM experiment. This study conducted that maize seed inoculation with IAA from species mentioned above showed positive effects. They had statistically significantly higher root and steam height compared to control seedlings. Bacterial strains tested in this study may be recommended as PGP (Plant Growth Promoting) bacteria, due to their positive effects and eventually can be used to reduce chemical fertilizers doses.

Keywords: *Bacillus*, *Azotobacter*, auxin, IAA, maize seeds.

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Chemical fertilizers in agriculture can cause harm to humans, animals and the environment. Toxic materials found in fertilizers have been linked to a variety of adverse human health effects, including reproductive and developmental, as well as liver and blood toxicity issues [1]. Bouchard *et al*. [2] in their study showed that exposure to pesticides may be a contributing factor in Attention Deficit Hyperactivity Disorder (ADHD) among children. Excessive increase in the usage of chemical fertilizers destroys the soil properties (osmotic pressure, pH value, conductivity and water holding capacity) [3], damage water resources [4], it may also affect adversely on population of microorganisms. According to Naqvi *et al.* [5] increasing use of commercial fertilizers could contribute to global warming by decreasing oxygen and raising levels of nitrous oxide in coastal waters.

Currently the attention of researches is shifting away from chemical fertlizers and toward alternative that consumers perceive to be natural, Plant Growth Promoting bacteria (PGP). PGP are bacterial strains inhabiting rhizosphera and are benificial to plants. They promote plant growth by producing plant hormones.

Phytohormones are organic substances synthesized in plant organs that can be translocated to other sites, where they trigger specific reactions and are active in tissues where they are produced. There are two sources of phytohormones naturally available for the plants: endogenous production by the plant tissues and exogenous production by associated microorganisms [6]. Indole-3-acetic acid (IAA) is a simple metabolite that derives from tryptophan (Trp) by multiple enzymatic pathways and can also be synthesized by Trp-independent routes [7]. As biofertilizers, PGP is one of the most promising biotechnologies to improve primary agricultural production and an efficient approach to replace chemical fertilizers [8,9].

Maize is extremely important crop for agriculture. It has economic significance, because it participates in the diet of people, domestic animals and as raw materials in industrial production, and with different technological processes many products are produced from this plant.

Many studies showed that auxin-producing bacteria improve plant growth [10,11]. The main objective of this research was to determine if isolated strains from maize rizosphera in combination could affect growth parameters and seed germination of seedlings *in vitro.*

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EXPERIMENTAL

Identification of the bacterial isolates

Bacterial strains (*Bacillus megaterium, Azotobacter chroococcum*) were isolated from maize rizosphera. The isolates were tentatively identified following Bergey's Manual of determinative bacteriology. Identification was carried out by subjecting the isolates to cultural, morphological (colony morphology and pigmentation), microscopic (Gram staining and motility), biochemical (utilization of carbon sources and enzymatic activity) and physiological characteristics (temperature, pH value, salt and sugar tolerance).

PCR identification

Prior to DNA extraction, each isolate was subcultured on LB broth for 24 h at room temperature. PCR analysis using the universal primer pair was used to assess the identity of 2 isolates with colony morphology and biochemical test similar to *B. megaterium* and *A. chroococcum*. Reactions were conducted in a final volume of 30 µL, which contained EconoTaq® Plus 2× Master Mix, 0.5 mM of each primer and 10 ng of genomic DNA. The PCR amplification conditions in the thermocycler were set as follows: 5 min at 94 °C followed by 35 cycles at 94 °C for 30 s, 30 s at 55 °C, and 60 s at 72 °C with a final extension of 7 min at 72 °C. The amplicons were purified with the QIAquick PCR purification Kit/250 (Qiagen GmbH, Hilden, Germany). The amplicons were mixed with a Safe-Orange G dye and separated on a 1.5% agarose gel in 1 × Tris--acetate-EDTA (TAE) buffer at 80 V for 1.2 h. The gels were visualized under UV light with a UV transilluminator (UV-26, MRC LAB). After purification, the amplicons were sent for sequencing at Macrogen Sequencing Service (Seoul, Korea).

Assay for IAA production

The production of indoleacetic acid (IAA) was assayed by using Salkowski method [12]. The bacteria were inoculated in to the nutrient broth. After 48 h of growth at 120 rpm/28 ± 2 °C, the bacterial culture was centrifuged and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (2.0 mL 0,5 M $FeCl_3$ + H_2SO_4). The reaction mixture was incubated at room temperature for 30 min and then light absorbance was measured immediately at 535 nm. The amount of produced IAA was calculated using the standard curve prepared with known concentration of IAA (1, 5, 10, 25 and 50 µg/mL of filtrated IAA).

Avena straight growth test

Oat seed (*Avena sativa* L.), was used in bioassay Avena straight-growth [13,14]. Coleoptiles (5 mm length) of 3 days old seedlings were dipped in 2 mL of tested solutions and were incubated at 28 °C/24 h at 20 rpm. Length of section was measured before and after the experiment. A standard activity curve relating coleoptile growth to the dilutions of pure synthetic concentration of IAA was developed as a control. Final length for oat coleoptiles was measured after the tissues were floated in solutions containing different concentrations of each bacteria tested and their mixture. The concentration of the auxin was calculated from the Avena straight-growth test by interpolation on the IAA curve of the controls.

MRM detection of Indole-3-acetic acid

Indole-3-acetic acid (IAA) was detected and quantified in the ESI positive mode by monitoring the transition of ion 130 u. Capillary voltage was 3.5 kV, cone voltage 20 V, extractor 3 V, source temperature 400 °C, cone gas flow 100 l/h and desolvation gas flow 1100 l/h. MRM collision energy from 176→130 transition quantification was 10 V. The separation was accomplished with Acquity UPLC BEH C18 1.7 µm 2.1 mm×50 mm column heated to 30 °C with gradient of 0.02 formic acid: acetonitrile (0–8 min, 95 to 100 vol.%; 8–13 min, 100 vol.%; 13–14 min 100 to 95 vol.% and 14–17 min 95 vol.%). The compound was eluted at 0.35 mL/min over 13 min. Last 4 min (13–17 min) was column re-equilibration period.

Seed germination

Certified seeds (*Zea Mays* L.) of five different hybrids and FAO groups were obtained from Maize Research Institute, Zemun Polje, Serbia. ZP 366 and ZP 388 are FAO 300; ZP 560 is FAO 500, ZP 606 and ZP 666 are FAO 600. Seeds were disinfected with 2% NaClO, rinsed thoroughly in sterile distilled water. Inoculums were adjusted to OD 10^{-6} CFU/mL using the spectrophotometer and prior to inoculation they were mix in ratio 1:1. Seeds were submerged in the culture solutions of *Bacillus* and *Azotobacter* for 2 h and germinated 7 days at 25 °C (16/8 h), in germination chamber. Germination test was conducted in four replications of 100 seeds each by adopting between paper method as described by ISTA procedures [15] while 4×5 randomly selected normal seedlings were used to measure the root and shoot length. Seeds treated with comercial fungicide were used as control. The final count of germination and the measurement of the root and shoot lengths were estimated on the 7^{th} day.

Data analysis

The SPSS statistic program version 18.0 was performed for experiments involving calculations. Data were analyzed using Student t test $p \leq 0.05$.

RESULTS AND DISCUSSION

The DNA sequences from isolates were used to search for sequence similarity against the National Center for Biotechnology Information (NCBI) database [16] using the BLAST program. Amplicon of 1500 bp was amplified using universal primer set. Partial sequence of 16s rRNA (1500 bp) was compared with similar sequences in Gen-Bank NCBI and revealed a similarity to *Azotobacter chroococcum* (16s rRNA gene identity 99%) and amplification and sequencing of 16s rRNA gene of other species reveal similarity to *Bacillus megaterium* (16s rRNA gene identity 100%).

In this study, presence of auxin was first proved by colorimetric test and its biological activity was confirmed by Avena test. Colorimetric test exhibited higher values of IAA detected rather than Avena test. *Bacillus megaterium* exhibited slightly bigger values of produced hormone than *Azotobacter chroococcum* (Table 1).

Table 1. Results for IAA values (Auxin, µg/mL)obtained with Colorimetric test and Avena test; mean values of 10 replicates (mean ± SD)

Species	Colorimetric test	Avena test
Bacillus megaterium	8.86±2.18	3.89±1.93
Azotobacter chroococcum	7.55±2.01	3.68±1.76

IAA was reproducibly separated, detected and quantified using the HPLC–MS/MS with ESI mode. The limit of detection (*LOD*) of IAA in the extraction was 2 ng/mL and limit of quantification (*LOQ*) was 4 ng/mL. Linear range was from 10^{-6} to 0.1 mg/mL, while the correlation coefficient was r^2= 0.998. Final amount of IAA quantified by HPLC–MS/MS with ESI mode acid in inoculums were for *B. megaterium* 0.133 mg/L and for *A. chroococum* 0.127 mg/L (Figure 1).

Seed germination tests in present study revealed that in relation to all five tested hybrids, bacterial treatment had significantly more stimulating effect on shoot and root growth over controls (Figure 2).

The smallest increase in shoot or root length for all hybrids was exhibited by control seedlings (Table 2). IAA seed treatment showed no statistically significant positive effect on seed germination percentage.

Results of this study exibited plant growth effects of *B. megaterium* and *A. chroococcum.* Plant growth promoting effects of bacterial strains in relation to maize seeds were shown in many earlier tests [17,18]. Biological test and colorimetric test showed ability of these two bacterial strains to produce hormone auxin. It is one of the most important plant hormones. Auxin is regulator of early plant development and it has many different effects such as inducing cell elongation and cell

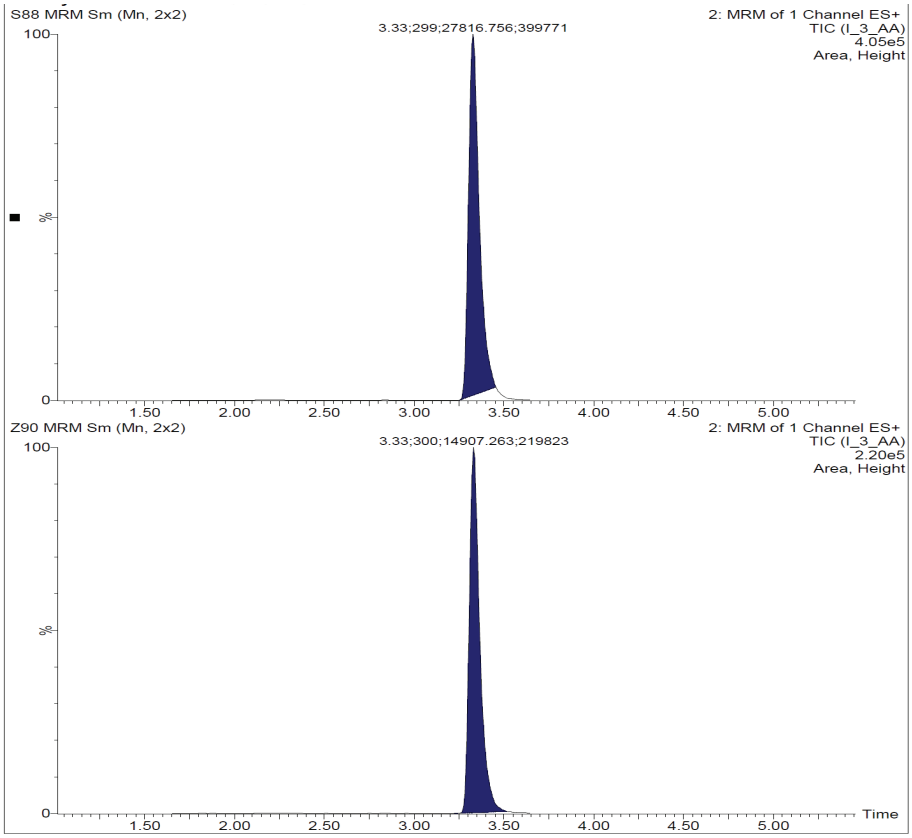

Figure 1. IAA detection and quantification by HPLC-MS/MS with ESI mode for B. megaterium (upper figure) and A. chroococcum (down figure).

Figure 2. On the left side are seedlings treated only with commercial fungicide, while at the right side are seedlings treated with mixture of bacterial inoculum (sample with one of the hybrids tested).

division; it influences elongation and branching of roots and shoots, development of vascular tissues, and response to light and gravity [19]. Seedlings of all maize genotypes tested treated with auxin had longer root and higher steam over control. Beneficial influence of IAA we have determined in previous work in which wheat seeds were examined [20]. By HPLC–MS/MS with ESI mode it was detected and quantified that these bacterial strains produce almost same amount of Indole-3-acetic acid as hormone of the auxin class. Szkop and Bielawski [21] in their study propose HPLC for determination of indolic compounds related to bacterial biosynthesis with a possibility of very low limits of detection. Improvement of root and shoot length observed in this study is due to detected IAA, which triggers cell division, elongation and differentiation [22]. The proposed mechanism for the auxin effect on cell elongation is triggered by the hormone induced opening of calcium channels in the plasmalema which, in turn, might cause changes on calcium homeostasis in the cytosol. It is very well know that increase in the concentration of tryptophan causes an increase in the amount of auxin produced, because precursor for the synthesis of IAA is tryptophan [23]. Analysis showed that bacteria tested in this study have ability for IAA production in the absence of L-tryptophan. Salkowski reagent was used for qualitative determination that assured the presence of the hormone in the supernatant of bacterial cultures. The amount of IAA produced by the bacteria was for *B. megaterium* 8,86 μg/mL and for *A.chroococcum* 7,55 μg/mL. Activity of species in producing IAA was also showed by Prashanth and Mathivanan [24]. In their work they detected that *Bacillus licheniformes* was able with addition of L-tryptophan to produce 23 μg/mL of IAA. Lim and Kim [25] similiar to our results reported that plant growth promotion was result of synergistic effect of produced substances by *B. subtilis* and *B. lichenoformis*. When the pots were simultaneously treated with a combination of isolated auxin from two tested species, the growth rates of plants were over 20% greater than observed with treatment with either auxin alone. Patil *et al.* [26] demonstrated also that combined inoculations of three beneficial organisms *Rhizobium*, *Azospirillum* and Phosphate Solubilizing Bacteria (PSB) were more superior over both single and dual inoculations. According to results of this study IAA had best stimulative effects in relation to maize genotypes of early growing season. Thereby early maize hybrids could gain an initial advantage for easier overcoming of unfavorable weather conditions. Seed quality plays a critical role in crop productivity and therefore agricultural production. Considering that standard technology of seed processing uses mainly chemical products, results of this study indicate that we would be able to reduce chemical fertilizer doses. Further investigations should be carried out in the fields.

CONCLUSION

The inoculation of maize seeds with indolacetic acid from *Bacillus* and *Azotobacter* strains mentioned above

Table 2. Mean values for shoot and root length and height (in cm) for 5 tested hybrids

Variant		ZP 366		ZP 388		ZP 515		ZP 606		ZP 666	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Control	Mean	20.06	10.01	13.89	5.45	14.00	5.25	17.49	5.24	17.95	12.31
	N	20	20	20	20	20	20	20	20	20	20
	Std. deviation	3.51	2.45	3.31	1.94	3.01	1.56	2.83	1.08	4.80	3.33
Auxin	Mean	22.61	14.42	20.47	11.61	16.07	10.93	20.78	6.90	23.09	14.32
	N	20	20	20	20	20	20	20	20	20	20
	Std. deviation	3.31	2.34	4.12	3.05	5.17	2.90	2.45	0.61	3.97	3.30
Total	Mean	21.33	12.22	17.18	8.53	15.03	8.09	19.13	6.07	20.52	13.32
	N	40	40	40	40	40	40	40	40	40	40
	Std. deviation	3.62	3.25	4.97	4.01	4.31	3.68	3.10	1.20	5.07	3.42

has direct positive effects on root and shoot growth. Seed germination tests in present study revealed that in relation to all five tested hybrids from all growing seasons, bacterial treatment had significantly more stimulating effect on shoot and root growth over controls. The smallest increase in shoot or root length for all hybrids was exhibited by control seedlings. IAA seed treatment showed no statistically significant positive effect on seed germination percentage. IAA treatment had best stimulative effects in relation to maize genotypes of early growing season thereby they could gain an initial advantage for easier overcoming of unfavorable weather conditions. These strains may be eventually recommended as PGP bacteria because of their multiple benefitial effects on maize seedlings. As biofertilizers, bacteria from this study present promising biotechnology to improve primary agricultural production and an efficient approach to replace chemical fertilizers.

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IZVOD

INDOL -3-SIRĆETNA KISELINA (IAA) IZ BAKTERIJSKIH KULTURA POSEDUJE MOGUĆNOST SMANJIVANJA UPOTREBE HEMIJSKIH ĐUBRIVA

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(Naučni rad)

Standardne tehnološke mere dorade semena podrazumevaju upotrebu hemijskih proizvoda. Međutim, u skorije vreme sve veći broj naučnih radova dovode u vezu toksične materije iz hemijskih đubriva kao izazivače mnogi obolenja ljudi, životinja i označavaju ih kao veoma štetne za životnu sredinu. Korišćenje Plant Growth Promoting Bacteria (ili skraćeno PGB) u savremenoj poljoprivredi predstavlja mogućnost da se smanje upotrebljene količine hemijskih đubriva. Ovo je bio razlog iz koga smo tetstirali sposobnosti *Bacillus megaterium, Azotobacter chroococcum* vrsta da produkuju hormon auxin (IAA), prirodni analog biljnom hormonu koji između ostalih efekata stimuliše rast biljaka. Bakterijski sojevi su identifikovani PCR umnožavanjem i sekvencioniranjem gena za 16s rRNA. Kvantitativno tandemno masenom spektormetrijom-MRM eksperimentom potvrđena je i kvantifikovana iz ovih bakterijskih kultura indol-3-sirćetna kiselina. Eksperimentalno je potvrđeno rezultatima rada da inokulacija semena kukuruza auksinima iz pomenutih bakterijskih vrsta pokazuje pozitivan efekat. Statističkom obradom relevantnih podataka dobijeni su rezultati koji ukazuju da tretirana semena imaju duže stablo i koren u odnosu na netretirana (kontrolna) semena. Bakterijski sojevi korišćeni u ovom radu mogu se prepručiti kao PGP bakterije zbog njihovog višestrukog pozitivnog dejstva na seme kukuruza i eventualno se mogu koristiti kako bi se smanjile upotrebne doze hemijskih đubriva.

Ključne reči: *Bacillus* • *Azotobacter* • auxin • IAA • Kukuruz • Seme