

INDOLE ACETIC ACID (IAA) PRODUCTION POTENTIAL OF PGPR BACTERIAL ISOLATES AND THEIR EFFECT ON SEED GERMINATION IN *ZEA MAYS* L.

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Abstract

The study was conducted to determine the presence of indole-3-acetic acid (IAA) in PGPR isolates using PCR reactions and biochemical tests. Positive isolates were tested under in-vitro conditions for germination efficiency on maize (*Zea mays* L.) seeds. Salkowski method was used for IAA tests on 30 bacterial isolates with PGPR properties. The KH14.3 and KH24.1.2 isolates were biochemically active in terms of IAA test. The absorbance readings were made at 595 nm using microplate reader and resultant values were compared to an IAA standard. IAA production of isolates varied between 0.42-6.37 µg/ml. For germination experiments, 20 maize seeds were coated with IAA- positive isolates (1.3x10⁷ cfu/ml) in petri dishes in triplicates according to the randomized plot design. The germination test was monitored for 7 days in an incubator at 25°C. Experimental data were subjected to analysis of variance (one-way ANOVA). The difference between the bacterial coating and control group was significant at the level of P≤0.05. Germination efficiency was determined as 99.9% for KH14.3 and 2.7% for KH24.1.2. Present findings revealed that KH14.3 was ACC deaminase, phosphatase, and siderophore-positive and KH24.1.2 was positive for ACC deaminase and phosphatase. These two isolates were also biochemically active in terms of IAA and could increase germination success by coating on seeds.

Keywords: *Bacillus* sp., Indole Acetic Acid, Maize seeds, PGPR.

1. INTRODUCTION

The homeland of the corn plant is Mexico-Guatemala in Central America and has been grown as the main crop of the region for thousands of years. Archaeological studies revealed that corn plant existed prior to human habitation. In 15th century, it was transferred to Asia via North Africa and then to Far East countries such as India and China. It has entered to Türkiye through Egypt and has become a plant that provides the highest yield under suitable growing conditions. It was spread over a wide area from the Equator to the Baltic Sea (from around 60° north latitude and 42° south latitude up to 4000 m above sea level) around the world (Gençkan, 1983). During last three decades it was spread over large areas for silage feed production. It has become one of the most important silage crops in the world due to its high nutritional value and flavor (Sarıcan and Çete, 1998).

Corn is the most produced cereal in the world, with 1 billion tons per year. The average yield per decare is 580 kg (TOB, 2020). This amount is twice that of wheat and barley. According to the data of the International Grain Council (IGC); In 2020/2021 marketing season, global corn production was 1,146 billion tons and corn consumption was 1,169 billion tons. The qualification rate in the 2018/2019 marketing year is around 70%. In 2020-2021 production season, 6.5 million tons of corn was produced (TOB, 2020).

There are many ecological factors that limit quality and yield in agricultural production systems. The first step in eliminating these limiting factors is to provide a good seed bed and germination conditions (Yıldız et al., 2007). In providing these conditions, most sensitive periods of plants to environmental factors are germination, emergence and early seedling periods. Various researches are carried out to accelerate the germination of seeds as well as ensuring their homogeneous emergence (Duman and Eşiyok, 1998). For this purpose, priming applications (folding, soaking in water, acid etching, application of growth-regulating bacteria and treatment with hormones, etc.) are performed (Ercişli et al., 1999; Yıldız et al., 2017).

In this context, in PGPB isolates, nitrogen fixation, conversion of insoluble plant nutrients into forms that can be taken by the plant (mineralization), production of siderophores and ammonia (NH₃), competition in terms of carbon source and plant hormones (IAA, auxin, cytokinin, ethylene, gibberrellic acid, etc.) mechanisms are used (Weller, 1988).

Researchers have stated that IAA (Khalid et al., 2004) and ACC deaminase production abilities may be required for endophytic and rhizospheric bacteria to contribute plant growth and health. These characteristics can be used as markers (Etesami et al., 2015). It has been determined that isolates with these characteristics have high chance for their colonization in plants (Etesami et al., 2015). Researchers have reported that IAA production can enable bacteria to interact with plants (Etesami et al., 2015) and help them to enter into endophyte life form in plants (Verma et al., 2001). Gül et al. (2013) achieved an increase in yield in cucumbers with the application of PGPR in soilless farming and reported that there was a significant relationship between the in-vitro level of IAA produced by these bacteria and the increase in yield (Akköprü and Özaktan 2018).

In many studies, it was estimated that 80% of plant associated bacteria produce IAA (Patten and Glick, 2002; Spaepen et al., 2007). Calvo et al. (2010) determined that 81% of the isolates (Ashrafuzzaman et al. (2009) obtained from the potato rhizosphere, and 60% of the bacteria isolated from the rice rhizosphere produced IAA. However, the level of IAA produced by the isolates can vary and the amount of production is important in promoting growth. Poonguzhali et al. (2008) determined that PGPR isolates obtained from Brassica campestris rhizosphere produced IAA in the range of 6.02-27.75 ppm. Majeed et al. (2015), studied with the bacteria isolated by Babier et al. (2020) and found that 53.3% of the produced IAA was in the range of 1-25.03 ppm.

Phytohormones that provide hormonal control of vegetative development are auxin, cytokinin, strigolactones, gibberellins, and brassinosteroids. Among them indole-3-acetic acid (IAA) is considered the most abundant natural auxin. IAA is produced in two ways, from tryptophan (Trp) by several semi-independent pathways, and a Trp-independent pathway. Environmental and developmental control of genes regulating auxin biosynthesis, binding and degradation is performed by auxin balance (Mano and Nemoto, 2010). Most previous studies have shown that IAA was produced by Gram (-) organisms (Lindow et al., 1998; Datta and Basu, 2000). However, only a few strains of Gram (+) species in the genus Bacillus is known to produce IAA (Wahyudi et al., 2011). Bacterial isolates are more efficient auxin producers compared to the soil rhizosphere (Sarwar and Kremer, 1992). The amount of IAA production in bacterial strains are guessed by using the

Salkowski reagent (Ehmann, 1977). This reagent does not interact with IAA and L-tryptophan and is used as a marker (Vaghasiat et al., 2011). IAA synthesis is considered an effective tool for screening the beneficial microorganisms (Vaghasiat et al., 2011).

In present study, bacterial isolates known to have PGPR effect (Khaled, 2014) were searched in terms of IAA production efficiencies and their growth promoting effect were proved in vitro on germination of maize seeds.

2. MATERIALS AND METHODS

Bacterial isolates used in the study

Bacterial isolates were kindly supplied by Dr. Semih YILMAZ and activated from stock cultures for further studies. Isolates were incubated firstly in LB agar medium for overnight at 35 °C and 200 rpm and then LB medium at the same conditions (Tablo 1).

Table 1. Isolates used in the study

Bacterial isolates					
KH 3.3	KH 6.3	KH18.1	KH 6.4	KH 27.1	KH 2.1
KH 5.1	KH 9.1	KH 22.1	KH 6.1	KH 12.1	KH 28.2
KH 12.3.1	KH 18.2	KH 23.2	KH5-3	KH 8.1	KH 20.1
KH 10.1	KH 13.1	KH 14.3	KH 21.4	KH 15	KH 20
KH 24.1	KH 9.2	KH 24.1.2	KH 2.3	KH 12.2	KH 24.1.3

DNA isolation

Isolates were grown in LB medium at 35 °C and 200 rpm for overnight, and then a loop of cells was transferred into a microfuge tube containing 300-400 µl sterile ddH₂O. After mixing well, they were kept at -80 °C for half an hour and immediately immersed into boiling water for 10 minutes to fracture cells and release their content into medium. Subsequently the solution was centrifuged at 10.000 xg for 5 minutes and the supernatant was transferred to a new tube. The supernatant was used as DNA templates (Bravo 1998).

Screening IAA gene regions in isolates

PCR reactions were performed using the DNA templates and the primer pairs F-5'-TTCGAGCTGACCAAGGCGATGAT-3'/R-5'-CCAGGGTGATGAACTGGAAGCTTGT-3' (Rajkumar 2014) with product length of 200bp, and F-5'-CCAACATCATCAAGCTGCCGAACA-3'/R-5'-AGACCTTCATCATCGTGGCCTTCA-3' with product length of 250-300bp. For PCR reactions 1.4 µl of 25mM MgCl₂, 0.5 µl of dNTPmix (10mM each), 1.5 µl of 10XTaq Buffer, 0.1 µl of 0.5U taq polymerase, 2.5 µl of DNA (5 ng/µL), and 0.5 µl of 100 nmol primer pairs each. The final volume was completed to 25 µl with ddH₂O. The tubes were then placed in thermocycler (AB Veriti) and the reaction was adjusted to initial denaturation at 95°C for 2.5 minutes, followed by 30 cycles at 95°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 5 minutes. Then, 15 µL of PCR product were electrophoresed on a 1X Tris acetic acid EDTA (TAE with safe gel red) buffer in 1% agarose gel. The gel was prepared by dissolving and homogenizing 1g of agarose in 100 ml of Tris acetate EDTA (TAE) buffer. 15µl of PCR product

was mixed with 3 μ l of loading dye and loaded into wells and run at 100V for 2 h. The bands were visualized using BioRad ChemiDoc MP and analysed.

Determining the IAA capacity of bacterial isolates

For determining the amounts of IAA production of bacteria, they were incubated for 7 days at 28 ± 2 °C in nutrient broth (NB) medium containing 0.1% tryptophan, at a shaking speed of 150 rpm. Then the cultures were centrifuged at 10,000 rpm for 10 minutes and 2 mL of the supernatant was taken. It was mixed by adding 2 drops of orthophosphoric acid and 4 mL of Salkowski's reagent (Gordon and Weber 1951) and left in a dark environment for color change for 30 minutes. Clear color change was taken as positive result. The amount of IAA produced by candidate bacterial isolates in NB broth four days after inoculation was determined at ppm level by comparing with standard IAA absorbance values. IAA standard was adjusted to 10, 20, 30, 40, 50 ppm and treated like a sample. The absorbance of IAA was determined at 595 nm in a microplate reader (BioRad, iMark) (Ahmad et al. 2005).

Seed germination tests

Inoculation of seeds with isolates

Corn seeds were surface sterilized by keeping in 5% sodium hypochlorite for 8 minutes and washing thoroughly with sterile water. After drying the seeds for a while, they were inoculated with bacterial isolates at a concentration of 1.3×10^7 cfu/ml. 20 seeds for corn were inoculated with bacterial isolates separately and incubated for 30-50 min for continuous shaking at 50 rpm so that the bacterial cells can be attached to the surface of the seeds. Then seeds (20 seeds/per petri dish) were monitored till 7th and 14th days after placing them into incubator at 25°C. Percent germination rates were calculated using the formula: (Number of germinated seeds/number of seeds placed in germination medium) $\times 100$ (Akıncı and Çalışkan, 2010). Experimental data were subjected to analysis of variance (one-way ANOVA) using SPSS (ver. 10) for windows.

3. RESULTS AND DISCUSSIONS

Studies with PCR reactions on the DNAs of KH14.3 and KH24.1.2 isolates revealed specific bands around 200-300 bp (Figure 1). Biochemical test were also performed for verifying IAA production potentials of the isolates.

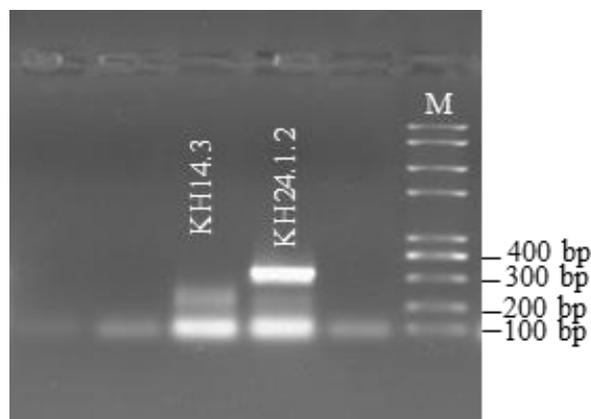


Figure 1. Band view of genes responsible for IAA production in KH14.3 and KH24.1.2 isolates. (M: Gene craft. GC-015-004).

Germination test

Among the 30 bacterial isolates used, KH14.3 and KH24.1.2 gave positive results in terms of IAA gene and biochemical activity. Germination rates (%) were calculated using the formula: (Number of germinated seeds/number of seeds placed in germination medium) x 100. Results revealed that the germination rate was perfect in seeds treated with KH14.3. Also, KH24.1.2 revealed considerable result with increase compared with control group (Table 2, Fig. 2). Our findings regarding the germination rates were in accordance with the results of Crevecoeur and Ledent (1985), Cutforth (1986), and Kınacı and Kün (1999).

Table 2. Statistical analysis of germination rates

Isolates	Germination rates (%)
Control	1,167±0,441 ^b
KH24-1-2	2,433±0,219 ^b
KH14-3	99,567±0,285 ^a

^{abc}different letters in the same column are statistically important (p≤0.05)



Figure 2. Germinating effect of isolates KH14.3 and KH24.1.2, and Control group

Determination of the capacity of isolates to produce IAA

In present study, the amount of IAA produced by KH14.3 isolate in the medium with and without L-tryptophan was searched. It was determined that while the amount in the medium without L-tryptophan was 0.76 µg ml⁻¹, it was 6.37 µg ml⁻¹ in the presence of L-tryptophan (Fig. 3-4, Table 3) as consistent with the results of Akbari et al., (2007) and Akköprü (2012). The absorbance rates at 595 nm was searched for isolates and the highest values were observed for KH14.3 and KH24.1.2 (Fig. 4). Ali et al (2009) reported higher values of IAA productions between 12–48 µg ml⁻¹ in their *Bacillus* isolates.

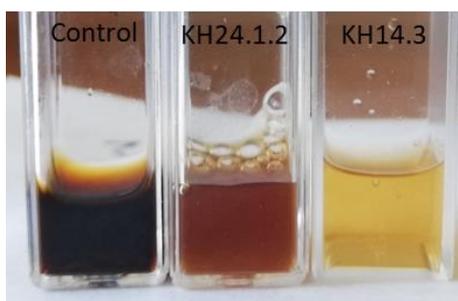


Figure 3. IAA production potentials of isolates in growth medium including L-tryptophane

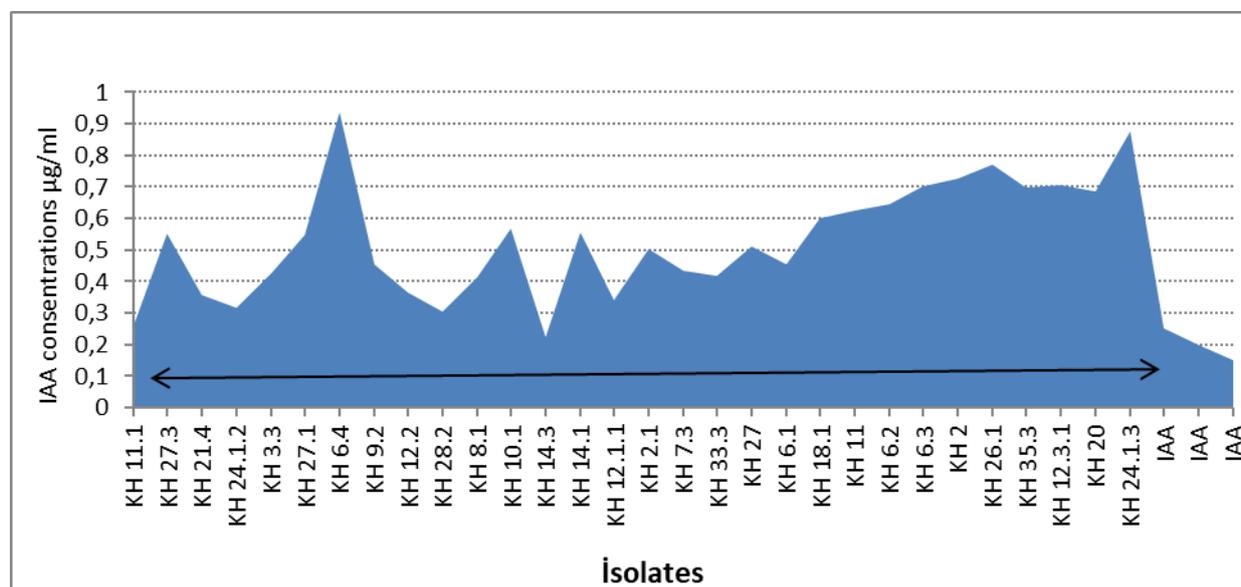


Figure 4. Absorbance values of IAA at 595nm

Table 3. IAA Statistical analysis for IAA production

Isolates and standart IAA	IAA (µg/ml)
KH24.1.2	0,92 ^a
KH14.3	6,37 ^b
IAA	0,76 ^c

Different letters next to the mean values in the same column indicate significant difference at P<0.05.

When the percentage values of germination rates in corn seeds in different PGPR applications were compared, it was seen that the effect of isolate KH14.3 was significantly higher than that of control and other isolates proving the higher amount of IAA in spectrophotometric measures (Table 4, Fig. 5). Our findings regarding the germination rates were in agreement with the findings of Crevecoeur and Ledent (1985), Cutforth (1986), and Kınacı and Kün (1999).

Table 4. Statistical analysis of germination percentage

Isolates	Germination percentage
Control	1,17±0,44 ^b
KH24.1.2	2,43±0,22 ^b
KH14.3	99,57±0,29 ^a

Different letters next to the mean values in the same column indicate significant differences at P<0.05.

Searching the interactions between plants and bacteria for finding active ones is an important process and requires a lot of time and effort. For this reason, determination of certain characters related with growth promoting activity under *in-vitro* conditions and selection of the most successful isolates or using those with desired characters in plant experiments is a method that is frequently used. Characters chosen as markers in general include; production of plant hormones

such as IAA, production of ACC deaminase that can reduce ethylene stress, production of siderophores that play a role in iron and phosphorus uptake, and the ability to dissolve phosphate.

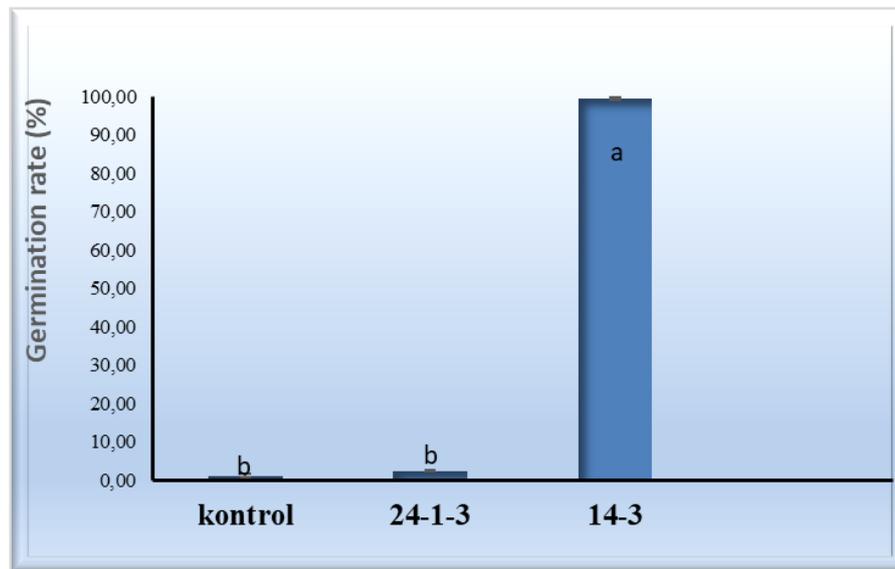


Figure 5. Seed germination rates after PGPR application

Researchers have stated that IAA (Khalid et al., 2004) production capabilities might be required for endophytic and rhizospheric bacteria to contribute to plant growth and health and that they could be used as markers (Etesami et al., 2015). It has been determined that isolates with these characteristics have a higher chance of colonization on plant roots (Etesami et al., 2015). It was reported that IAA production can enable bacteria to interact with plants and help them entering into endophyte life form in plants (Verma et al., 2001; Etesami et al., 2015).

There are studies demonstrating the effectiveness of PGPR bacteria, which promotes the production of IAA, in plant growth and development in many plant species. (Teale et al., 2006; Woodward and Bartel, 2005). Bacterial strains in genus *Bacillus* can produce IAA, solubilize P and produce siderophores for chelating iron, magnesium and some other minerals to make them available for plants (Raddadi *et al.*, 2008; Trivedi and Pandey, 2008). Not only bacteria but also fungi can synthesize IAA (Arshad and Frankenberger, 1992; Dobbelaere et al., 2003). Tryptophan as the precursor of IAA biosynthesis is demonstrated to have accelerating effect for efficient production. (Ahmad and Ahmad, 2008; Spaepen et al., 2007). In this study, IAA production was controlled though culturing the bacteria in mediums containing tryptophan. It was detected that all the strains grown in mediums with tryptophan produced IAA in varying amounts. Tryptophan-independent biosynthesis pathways of IAA has also previously reported by some researchers (Baca ve Elmerich, 2007; Spaepen et al., 2007).

PGPR is known to affect plant growth through various direct and/or indirect mechanisms. Researchers reported that these kinds of bacterial strains revealed to improve seed germination, root development and enhancement, mineral and water use (Dobbelaere et al., 2003). Therefore, the use of PGPR bacteria as seed inoculant or coating material is a practice used in many agricultural regions. It has been predicted that these kinds of bacterial strains can also be used to reduce potential worldwide pollutant chemical fertilizers and pesticide applications (Okon et al., 1998).

Some researchers have tested and reported the promoting efficacy of high endophytic diazotrophic bacterial strains associated with maize plants (Chelius and Triplett, 2001; Roesch et al., 2008).

4. CONCLUSIONS

In this study, it was seen that the strains KH14.3 and KH24.1.2 can be used as promising source of IAA-production in the rhizosphere. We concluded that when the bacterial growth medium is supplemented with tryptophan, the amount of IAA significantly increased. In addition, the presence of gene regions in the strains encoding IAA was revealed by PCR method. The bacterial isolates with this feature could be a good alternative and can be used to increase the germination rate of corn seeds as well as inducing root growth. Therefore, both environmental pollution and the use of hormones harmful to human health can be reduced or prevented by using these superior bacterial strains in seed germination and growth promotion. They can safely be used in cultivated areas to avoid excessive industrial applications.

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