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Research Article

Capability of Plant Growth-Promoting Rhizobacteria (PGPR) for producing indole acetic acid (IAA) under extreme conditions

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ABSTRACT

Plant Growth-Promoting Rhizobacteria (PGPR) inhabiting the area around the plant roots or in plant tissues and stimulate plant growth directly or indirectly. Synthesis of the phytohormone auxin indole-3-acetic acid (IAA) is one of the direct effects of PGPR on plant growth. This study aimed to isolate and screen IAA producing bacteria from soil and study the impacts of the alkalinity and salinity on IAA production and total antioxidant activity of the highly IAA producing strain. From the fifteen isolates tested, six were selected as efficient IAA producer, from which one isolate was highly IAA producer. The highly producing isolate was identified based on molecular characteristics using 16S rRNA. The sequence analysis showed 99% similarity with *Bacillus subtilis* from GenBank data base. The strain yielded IAA in a wide range of pH (5-9), giving its maximum IAA production at pH 8. High IAA concentration was also observed in the presence of 0.5% and 1% NaCl in comparison with control (with no NaCl). Furthermore, the results indicated that, total antioxidant was increased in acidic (pH 5 and pH 6) and alkaline (pH 8) media, as well as in salinity up to 2%. This study could be stated as the prospective of IAA producing bacterial isolate in the field, as a result, using it as alternative valuable biofertilizer.

Keywords: *Bacillus subtilis*; Indole acetic acid; Soil; Salinity; Antioxidant; Sequencing.

1. INTRODUCTION

Microorganisms especially bacteria produce promoting compounds which promote and improve nutrient uptake, plant growth and plant tolerance to abiotic and biotic stress. Rhizosphere bacteria (rhizobacteria) and soil-borned bacteria enhance plant growth by many mechanisms referred to as Plant Growth Promoting Rhizobacteria (PGPR) [1]. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production [2]. As well as they can stimulate plant growth through mobilizing soils nutrients, producing numerous plant growth hormones, protecting plants from phyto-pathogens, improving soil structure and bioreme-diating of toxic heavy metal species and degrading xenobiotic compounds [1, 3, 4]. Indole-3-acetic acid (IAA), a plant hormone compound, is a natural auxin produced by rhizobacteria is one of phytohor-mones [1]. Consequently, IAA plays an important role in rhizobacteria-plant interactions [5]. More-over, down-regulation of IAA as signaling is associated with the plant defense mechanisms against a number of phytopathogenic bacteria as

evidenced in enhanced susceptibility of plants to the bacterial pathogen [5]. Several IAA producing bacterial species have been isolated previously, such as *Streptomyces* sp., *Bacillus subtilis* spp. [6], *Pseudomonas syringae* [7], *Pseudomonas fluorescens* [8], *Agrobacterium tumefaciens*, *Alcaligenes faecalis* and *Azotobacter tumefaciens* [9] and *Bacillus megaterium* [10].

Recent studies focused on the response of antioxidant system of bacteria, which are important in biotechnology, such as *Streptomyces* growth in various oxidative stress conditions [11, 12]. Reactive oxygen species (ROS) arise by the transfer to O_2 of one, two or three electrons to form superoxide (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH^-), respectively [13]. Excess of ROS is highly cytotoxic, due to their reactivity with various cellular components. High ROS concentrations cause a major disturbance to intracellular ionic homeostasis, the oxidation of unsaturated fatty acids in lipids (affecting cell membrane integrity), amino acid residues in proteins (inhibiting enzyme activities) and DNA [13]. The collective effect can lead to cell death [14]. Salinity stress cause oxidative stress through the increase formation of ROS [15]. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to the living organisms [16]. Antioxidant compounds that are capable of protecting the cells from free radical mediate oxidative stress [12]. The antioxidant activities of bioactive compounds are mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals [17]. Living organisms have an abundance of antioxidant compounds that have been shown to be effective at removing ROS [18].

The objective of this study was to isolate and screen IAA producing bacteria from different soil samples, and optimize conditions for maximum IAA production. The effect of different pH values and NaCl concentrations (salt stress) on total antioxidant activity of bacterial isolate was also assessed.

2. MATERIALS AND METHODS

2.1. Isolation of IAA producing bacteria from soil

Rhizosphere samples were collected from soil cultivated with faba bean (*Vicia faba*), wheat (*Triticum aesativum*) and Helba (*Trigonella foenum-graecum*) and uncultivated soil (one sample). Three samples from each studied soil were collected from Botanical Garden of Botany and Microbiology Department at Assiut University in Assiut Governorate, Egypt. The chemical and biological properties of all soil samples were determined and presented in table 1. Five Gram from each soil sample were added to 45 ml sterilized distilled water. The mixture was shaken vigorously at 150 rpm for 1 hour and the soil suspensions were serially diluted to 10^{-6}. Two hundreds µl were spread on modified Pikovskaya agar medium plates [19] with composition (g/l): glucose, 10.0; dipotassium hydrogen phosphate, 10.0; $MgCl_2.6H_2O$, 5.0; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.2; $(NH_4)_2SO_4$, 0.1; NaCl, 10 and agar 15. The seeded plates were incubated at 30°C for 3 days. Pure colonies were picked up and transferred to 5 ml tryptone broth medium (g/l): tryptone 5, yeast extract 5, NaCl 3, tryptophan 0.2) and incubated at 30°C with shaking for 4 days. The bacterial suspension was centrifuged at 4000 rpm for 5 min. The reagent Salkowski was added to the bacterial supernatant (1:2) to determine IAA producing capability.

2.2. Determination of IAA produced by bacterial isolates

Six out of 15 bacterial isolates were identified as IAA producers. The amount of IAA produced by each bacterial strain was determined [20]. Each bacterial strain was grown in 100 ml Erlenmeyer flask containing 25 ml tryptone broth medium. All flasks were incubated under shaking at 100 rpm at 30°C, for 3 days. Then, the bacterial culture was harvested by centrifugation at 4000 rpm for 5 minutes. Bacterial supernatant was used to determine the concentration of IAA production by using Salkowski reagent (1.2% $FeCl_3$ in 37% H_2SO_4) and measured at 530 nm wavelength using spectrophotometer (MniCam-UV-vis Spectrophotometry, Helios Gamma, Germany). Concentration of IAA was calculated using a standard curve of known concentration of synthetic IAA (1-100 µg per ml) [21].

2.3. Assessment of total antioxidant activity

Total antioxidant activity was assessed by mixing Three ml of bacterial supernatant with 3 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate). The mixture was incubated at 95°C for 90 min in water bath, the developed blue color was measured by spectrophotometer at 695 nm. The total anti-oxidant activity was expressed as the number of equivalent of ascorbic acid [22].

2.4. Determination of sodium and potassium

Assessment of sodium and potassium in soil samples (1:10) contents was determined using Flame photometer technique (Flame photometer M7D, Germany) [23].

2.5. Molecular identification of IAA producing bacterial isolate

The identification of IAA producing isolate (CW-2) was done on the basis of 16S rRNA gene sequencing as described previously by Yousef [24]. PCR product was sequenced in both direction in Solgent company (South Korea) using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGCTACCTTGTTACGACTT). The sequences obtained were then aligned with known 16S rDNA sequences in GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). After obtaining the DNA sequences a phylogenetic tree was constructed with MEGA version 4.0. The sequence of the strain has been deposited in the GenBank nucleotide sequence databases (NCBI).

2.6. Effect of different NaCl concentrations on IAA production by bacterial isolate CW-2

To evaluate the effect of different concentrations of NaCl on IAA production by the highest producing isolate CW-2, different NaCl concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5% and 3% NaCl) was added to 100 ml Erlenmeyer flasks containing 29 ml tryptone broth medium, then 1 ml bacterial suspension was added to obtain $3x10^4$ CFUs/ml as the initial bacterial density. Three replicates were prepared and the flasks were incubated on a shaker incubator at 100 rpm. Concentration of IAA was measured after 3 days of bacterial inoculation.

2.7. Effect of different pH values on IAA production by bacterial isolate CW-2

The effect of different pH values of tryptone broth medium (5,6,7,8 and 9) on IAA production by strain CW-2 was assessed in medium with initial bacterial counts $3x10^4$ CFUs/ml. Five treatments were established including five different pH values of liquid medium 5, 6, 7, 8 and 9. The flasks (in replicates for each pH) were incubated on a shaker at 100 rpm. Concentration of IAA was measured at 3 days after inoculation.

2.8. Effect of IAA producing *Bacillus subtilis* strain on wheat and faba bean growth

To study the effect of IAA producing bacteria on plant growth, an experiment was conducted in sandy soil. Hoagland's nutrient solution containing 0.3% sodium chloride was used. This solution was sterilized at 121°C for 20 minutes [25].

Wheat grains and faba bean seeds were surface sterilized with 0.5% sodium hypochlorite solution for 1 minute and 70° alcohol for 1 minute, following washed consecutively with sterilized distilled water for four times and then dried. For coating the seeds with bacteria, the surface sterilized seeds were immersed in bacterial suspension (10^8 cfu/ ml) for 30 min with shaking [10]. The coated seeds were incubated on 1% agar plates in the dark and at room temperature for 5 days. After germinating, the seedlings were transferred to small pots containing washed sandy soil containing Hoagland's solution. The experiment was carried out in 4 treatments, four replicates for each treatment at room temperature. At the 10th day after transplanting, root and shoot plant fresh weight were determined and then dried in oven at 105°C to determine the dry weight. The treatments were compared with the control without bacterial coating.

2.9. Statistical analysis

The data were analyzed by SPSS, version 10 for windows (SPSS Inc; Chicago, IL, USA), Basic statistical parameters (mean and standard deviation) were estimated.

3. RESULTS AND DISCUSSION

3.1. Isolation of IAA producing bacteria

The analysis of soil samples indicated that the pH of all samples were moderately alkaline and total soluble salts and sodium ions were high in uncultivated soil (Table 1). A total 15 of bacterial isolates were isolated on Pikovskaya agar medium. Six of these isolates exhibited pink color when reacted with Salkowski's reagent indicating IAA production.

3.2. IAA and antioxidant production potential of the isolates

Six out of 15 bacterial isolates had the ability to produce high concentration of IAA as well as antioxidant production in tryptone broth medium containing 0.3% NaCl (Figure 1). These bacterial isolates (CW-1, CW-2, CW-3, CF-1, CF-2, and CH-1) produced IAA with variable degrees ranged between 13.0-25.5 mg/ l. Among these strains, the maximum IAA concentration in broth medium was observed by isolate CW-2 (25.5 mg/l) followed by CF-1 (21.7 mg/l) which gave the highest optical density (good growth) and CW-3 (19.4 mg/l). Moreover, these six isolates were antioxidant producers as shown in Figure 1. In previous studies 12 of 20 isolates, showed higher concentration of IAA, 100 µg ml^{-1} [26]. However, lower amount of IAA produced by *Bacillus* isolates ranging from 0.75 to 21.3 µg ml^{-1} [27]. According to Mirza et al. [28], IAA production by microorganisms may vary between different species and strains of the same species. The culture conditions and substrate stage growth conditions may cause variations in IAA production.

Results in Figure 1 showed also that, the all 6 bacterial isolates tested exhibited antioxidant activities. In the range of 1.75-2.72 µg/ml with little variation between isolates and this may be due to that they were isolated from the same habitats. However, Selim et al. [18] found that the bacterial isolates from marine habitats gave higher antioxidant activity.

Table 1. Chemical and biological properties of the studied soil samples.

Soil samples	pH (1:10)	EC/mS (1:10)	Na (mg/g soil)	K (mg/g soil)	Total soluble salt (%)	Bacterial counts/g soil
Cultivated Wheat (CW)	8.1	25.9	37.8	3.99	0.2901	35 x 10^6
Cultivated Faba (CF)	8.0	39.3	54.2	9.04	0.4402	19 x 10^7
Cultivated Helba (CH)	7.8	32.3	51.90	9.88	0.3618	11 x 10^7
Uncultivated (UN)	8.2	611	97.8	1.53	0.6843	25 x 10^4

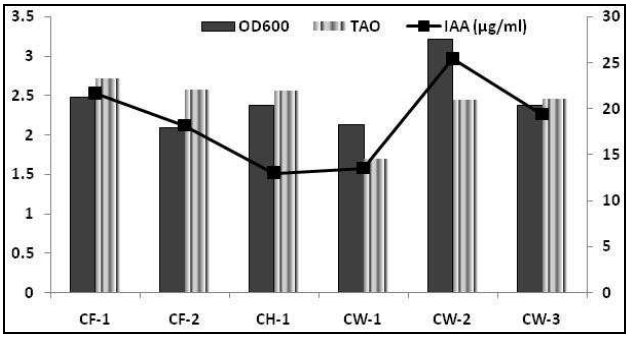

Figure 1. Average production of indole acetic acid (IAA), Total antioxidant (TAO) and optical density (OD_{600}) of selected 6 bacterial isolates.

Presences of antioxidant in normal conditions due to small amounts of reactive oxygen species (ROS) are by-products of normal cell metabolism, formed in vital processes such as respiration [13]. Inconsistently, under non-stressful conditions, ROS at low concentrations play an important role as signaling molecules involved in plant growth, development, and many normal physiological processes [29, 15]. Such low-level ROS functions include triggering of antioxidant defense mechanisms for adapting to abiotic stress [14].

3.3. 16S rRNA Gene Sequencing of bacterial isolate CW-2

The BLAST results of the 16S rRNA gene sequences allowed to assign the isolate to family Bacillaceae. The sequence was deposited in the GenBank under accession number MG642784. Phylogenetic tree was constructed using closely related *Bacillus* species from Genbank data base (Figure 2). The evaluated strain was aligned against sequences available from GenBank data base; the isolated strain, CW-2 matched to *Bacillus subtilis* with 99% similarity through GenBank data base (Figure 2). The identified bacterium in this study have been associated with plant rhizosphere and its IAA production and phosphate solubilizing activity has also been reported earlier [30, 31].

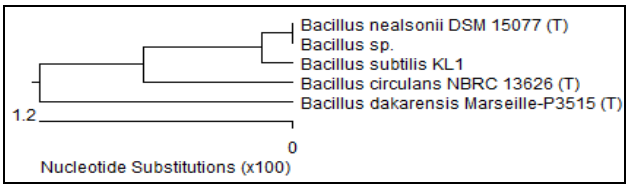

Figure 2. The phylogenetic homology tree based on multiple sequence alignments of the 16S rRNA of the *Bacillus* spp. reference to international isolates.

3.4. Effect of NaCl concentration on the IAA and total antioxidant production by *Bacillus subtilis*

Salt tolerance of the selected bacterial strain, CW-2 (the highest IAA producing strain) was determined tryptone broth medium supplemented with different NaCl concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%). The results showed that, this strain was able to grow and produce IAA product in medium supplemented with 0-3% (w/v) NaCl (Figure 3). The IAA production by the tested isolate was significantly decreased with increasing NaCl concentrations. The maximum IAA production varied between 28.20 and 37.65 mg l^{-1}, significantly higher than that of the control treatment (24.92 mg l^{-1}). However, at the concentrations of 1.5 and 2% NaCl, IAA productions were higher than that produced in 2.5 and 3% NaCl. The highest production level of IAA (28.20-37.65 mg l^{-1}) were found at 0.5 and 1% NaCl and this strain can tolerant the environmental salt stress to 3% NaCl. Egamberdiyeva [32] reported that IAA-producing bacteria significantly increased plant growth under salt stress. In addition, Nakbanpote et al. [33] also reported that IAA producing *Pseudomonas* sp. isolated from Zn/Cd contaminated soil was classified as salt-tolerant bacteria. Similar results were obtained by Nghia et al. [10] who found that *Bacillus* sp. tolerant salt stress to 3% NaCl. It is worthy to mention that, isolate CW-2 could be used as plant growth promoting halotolerant bacteria in saline soil. Nadeem et al. [34] found that *B. megaterium* was less affected by high salinity and can alleviate the negative impacts of salinity on cucumber growth.

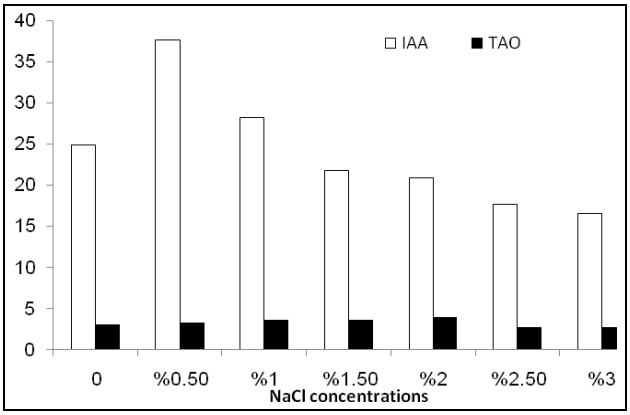

Figure 3. Effect of different NaCl concentrations on IAA and TAO production by bacterial strain CW-2.

In order to evaluate the relationship between salt stress and the antioxidant response, the activity of total antioxidant was determined. The As compared to control, total antioxidant activity (TAO) increased in lower NaCl concentrations but it showed decreasing trend with increasing salt concentrations. In this respect increased antioxidant response has been shown to be positively associated with decreased oxidative damage and improved salinity tolerance [35].

The result of the current experiment also revealed that, the total antioxidant activity increased in media supplemented with lower concentrations NaCl, the higher values was recorded with increasing salinity up to 1%. The highest value of total antioxidant was recorded at 0.5% and 1% (w/v) NaCl, but decreased thereafter. Antioxidant compounds are capable of protecting the cells from free

radical mediate oxidative stress due to their redox properties, which play an important role in absorbing and neutralizing free radicals [18]. These results are in agreement with those of Hu et al. [36] who reported that the antioxidants play an important role as free radical scavengers for the prevention of oxidative damage in living organisms.

Salinity represents the common abiotic stress increases ROS production resulting in a dramatic promotion of oxidative damage [15]. High ROS concentrations cause a major disturbance of cellular components [13]. To help avoid excessive ROS accumulation during stress, plants activate enzymatic and nonenzymatic antioxidant systems and antioxidant response increased to be positively associated with decreased oxidative damage and improved salinity tolerance [35]. PGPR impart drought tolerance by producing exopolysaccharides and phytohormones inducing accumulation of antioxidants as well as causing alteration in root morphology in acquisition of stress [37].

3.5. Effect of pH of culture medium on IAA and total antioxidant production by *Bacillus subtilis*

The IAA production by the strain CW-2 in different pH values (5, 6, 7, 8, 9) is presented in Figure 4. IAA production was significantly different among different pH values, the maximum amount 41.19 mg/l of IAA was produced at pH 8, followed by pH 7 (33.93), pH 6 (32.56) and pH 5 (16.72). These results are in agreement with those of Nghia et al. [10]. Acidic pH medium (below 6) as well as alkaline (over 8) was found to be unfavorable for IAA production by the tested isolate. Mandal et al. [38] have reported that the *Rhizobium* strain, VMA 301 elaborated high IAA concentration in pH 7.2 medium, and Khamna et al. [39] recorded that pH 7.0 was suitable for maximum IAA production by *Streptomyces* sp. IAA production by *Bacillus* spp. MQH.19 showed highest value at pH 6.0, but decreased by 62% at pH 5.0. For *Paenibacillus* spp. SPT.03, IAA production was highest at pH 5.0 and decreased by 42% at pH 7.0 [40]. The property of IAA is considered as effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effect on plant growth [41]. Furthermore, the current experiment showed that, total antioxidant increased in acidic (pH 5-6) and alkaline (pH 8) media. The antioxidant activities of bioactive compounds are mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals [18].

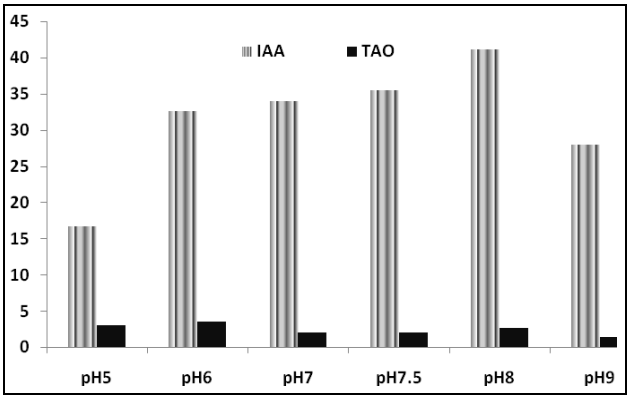

Figure 4. Effect of different pHs on IAA and TAO production by bacterial strain CW-2.

3.6. Effect of IAA producing bacterial strain *Bacillus subtilis* on wheat and faba bean growth

To study the effect of IAA producing strain on plant growth, wheat and faba bean cultivars were chosen for this assay due to their important as the most common cultivated crops. The results showed that, the plants treated with *B. subtilis* stimulated the germination and seedlings and exhibited improvement in plant growth through the fresh and dry weights compared with untreated plants (Table 2). The current results are in agreement with previous results of Mena-Violante and Olalde-Portugal [42], who found that isolates of *Bacillus subtilis* promoted size, mass and texture fruit of tomato (*Solanum lycopersicum*) and also the yield per plant, and they attributed these results to a possible production of hormones by *Bacillus*. Fatima et al. [43] concluded that, the plants inoculated with IAA producing bacteria induced the proliferation of lateral roots and root hairs. Reetha et al. [31] found that *B. subtilis* caused increase in fresh and dry weight of onion roots and shoots. It has been also found that the strain *Bacillus subtilis* produce phytohormones during its development, which also provided encouragement in developing soybean root [27]. Ghosh et al. [44] found that, *Bacillus* spp. promote and facilitate seeding of *Canola* and *Brassica*. Yousef and Hussein [45] found that, the fresh and dry

weight of faba bean increased when inoculated with *Rhizobium* sp.

Table 2. Effect of IAA producing bacteria on growth of faba bean and wheat plants.

Treatments	Germination %	Fresh weight/g		Dry weight/g	
		Shoot	Root	Shoot	Root
Control-faba bean	90	1.76 ± 0.12	1.26 ±0.17	0.151 ± 0.023	0.133 ± 0.014
Treated-faba bean	94	1.954±0.21	1.66 ±0.15	0.185 ±0.21	0.153 ±0.017
Control-wheat	92	0.104±0.023	0.120±0.02	0.0138±0.003	0.048 ± 0.011
Treated-wheat	94	0.117±0.031	0.253 ±0.13	0.180 ±0.24	0.105 ±0.019

4. CONCLUSION

Cultivated soils harbor IAA producing bacteria. Fifteen IAA producing strains were isolated from soil samples, among which, 6 were highly IAA and total antioxidant producers. From the six isolates, the isolate CW-2 showed the maximum IAA production, identified and characterized as halotolerant bacteria and considered as plant growth promoting bacteria. Furthermore, the IAA production was performed in wide range of pH (5-9). The significance of this study is the potential of IAA producing bacteria in salinity condition, which can stimulate the plant growth in the field and prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields. Plant growth promoting bacteria (PGPB) are good alternative biofertilizer and they can be used as safe and ecofriendly fertilizers. They can be used as bio-inoculants to promote plant growth and development under various stresses.

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CONFLICTS OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this article.

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