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Isolation and Characterization of Plant Growth-Promoting Rhizobacteria from *Trigonella foenum- graecum L* Rhizosphere and Evaluation of their Potential Substances Produced.

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ABSTRACT

The aim of this study was to isolate rhizobacterial strains (PGPR) that have the ability to produce phytohormones [Indol acetic acid, (IAA)], Hydrogen cyanide (HCN), Siderophore, Ammonia, Phosphate solubilization, chitinase enzyme activity and also have broad spectrum against phytpathogenic fungi. A total of thirty bacterial strains isolated from the rhizosphere of *Trigonella foenum- graecum L*. They are characterized according to their morphological characteristics. Twenty three bacterial isolates were found to have broad spectrum antifungal activities against pathogenic fungi, and found to be able to produce hydrogen cyanide ranging from dark brown to faint brown. Only one strain identified as *Bacillus cereus* KU058893 was able to produce dark brown, which indicated that it produced high amount of HCN and *Brevundimonus diminuta* KT277492 gave brown colour which indicate moderate production of HCN. Also, twenty bacterial isolates out of thirty with percentage 66.67% were found to be siderphore producers. Ten isolates with a percentage 33.3% were able to produce IAA in the absence of tryptophan while 66.7% were unable to produce IAA in the absence of tryptophan, *B. diminuta* KT277492 was the most active producer strain of IAA (24.912±0.045µg/ml).While *B. cereus* produced 15.034±0.0707µg/ml. While in the presence of tryptophan at 0.1% concentration, *B. diminuta* KT277492 was also the most active producer of IAA (31.723±0.0059µg/ml). While *B. cereus* KU058893 produced 22.2±0.046µg/ml. while 80% of the bacterial isolates were able to produce ammonia, 6 isolates represented 20% didn't produce ammonia.We found that about eleven isolates were able to hydrolyze Phosphate which represents about 36.667% of the total isolates, nineteen isolates were not able to hydrolysis phosphate with percentage 63.333%. *B. diminuta* KT277492 showed the highest chitinase enzyme activity 95 IUL^{-1} on the (NB+ chitin) medium.

Keywords: Chitinase enzyme, *Bacillus cereus* KU058893, *Brevundimonus diminuta* KT277492, Plant growth-promoting bacteria, *Trigonella foenum,* .

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INTRODUCTION

Plant pathogenic fungi cause reduces in crop yield. To overcome the fungal plant infection and increase the crop yield was by using chemical fertilizers and pesticides. On the other hand use of chemical fertilizers and pesticidesincreases environmental pollution, health hazards and sometimes induces Phytotoxicity (Shehata *et al* 2012).We can say they have bad effect to the entire ecosystem. So scientists had to search for new tools to overcome this problem. In the recent year, scientists gave great attention to the beneficial microbes that habitat the rhizosphere. The rhizosphere is the narrow zone of soil which is rich in nutrients due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005, Beneduzi *et al.,* 2012 and Hamed *et al* 2015). Plant-associated bacteria can be classified into beneficial, deleterious and neutral groups on the basis of theireffects on plant growth (Dobbelaere *et al.*, 2003). Beneficialfree-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria (Kloepper *et al.*, 1989). Lwin *et al.,* (2012) stated that plant growth promoting rhizobacteria (PGPR) are bacteria that have the ability to colonize plants root hairs and increase plant growth and yield (Davis, 1995). Datta *et al.,* (2011) stated that, Plant growth promoting rhizobacteria (PGPR) constitute approximately 2-5% of the total rhizomicrobial population (Kloepper *et al.* 1980). Beneduzi *et al.* (2012) showed that, PGPR works indirectly or directly. The direct promotion of plant growth by PGPR is either by providing the plant with a compound that the bacterium synthesize, for example phytohormones or by facilitating the uptake of certain nutrients from the soil (Glick, 1995) and fixation of atmospheric nitrogen, solublization of minerals such as phosphorus, production of siderophores, and synthesis of plant growth hormones i.e. Indole-3- acetic acid (IAA), gibberellic acid, cytokinins, and ethylene (Nelson. 2004 andKumar, *et al.,* 2012).The indirect promotion of plant growth occurs when PGPR decrease or prevent the deleterious effects of one or more phytopathogenic organisms(Anjum *et al.,* 2007 and Lwin *et al.,* 2012). This can happen through the production ofantibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophore or through competition for nutrients and space canimprove significantly plant health and promote growth, as evidencedby increases in seedling emergence, vigor, and yield (Khan, 2006). Chitinase may be applied as insecticides and fungicides to control pests and fungal pathogens of plants respectively (Wang, et al 2006). Various species of bacteria have been recorded as plant growth promoting rhizobacteria that promote and enhance plant growth such as *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Entrobacter, Alcaligenes, Arthrobacter, Bacillus* and *Serratia* (Lwin, *et al.* 2012).

Ashrafuzzaman *et al.,* (2009) reported that, bio-fertilizers such as microbial inoculantspromote plant growth, productivity and increase the nutrient status of the host plant have internationally been accepted as an alternativesource of chemical fertilizers (Vessey, 2003). Significant increases in crop yieldshave been reported by applying PGPR microbial inoculants (Salamone, 2000). So, keeping all this in view, the present study was carried out to isolatethe mostactive plant growth promoting rhizobacteria trains from the rhizospheric soils of *Trigonella foenum- graecum-L*. The objectives of this research were to screen potential PGPR from *Trigonella foenum* rhizosphere which produce phytohormones, Hydrogen cyanide (HCN), Siderophore, Ammonia, Phosphate solubilization and extracellular chitinase.

MATERIALS AND METHODS

Source of samples

The rhizospheres samples and plant roots were collected from the green house of Ministry of Agriculture, Giza, Egypt. The collected samples were brought to the laboratory in clean plastic bags.

Isolation of PGPR from rhizosphere

Five grams of rhizosphere soil were taken into a 100 mL of conical flask, and 45mL of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One milliliter of suspension was added to 5mL vial and shaken for 1min. An aliquot (0.1mL) of this suspension was spread on the plates of Luria Bartany (LB) agar medium. Plates were incubated for 3 days at 28°C to observe the colonies of bacteria. Bacterial colonies were streaked to other LB agar plates and the plates were incubated at 28°C for 3 days. Well isolated single colony was picked up and re-streaked to fresh LB agar plate and incubated under the same condition. (Ashrafuzzaman *et al.,* 2009).

Isolation from the root sample

The root sample (1 g) was taken and surface sterilized with 0.2% $HgCl_2$ for 3-5 minutes and then washed thoroughly (10 times) with sterilize distilled water to make it free from $HgCl_2$. The samples were then used for isolation of rhizobacteria on LB agar by using modified replica plating technique. (Abou-Zeid *et al.*, 2004, Abou-Zeid *et al.,* 2008 and Kaushal *et al.,* 2011).

Characterization of bacterial isolates

Morphological characteristics (shape, color and odor) of the colony of each isolate were examined on nutrient agar plates. (Ashrafuzzaman *et al*., 2009).

Morphological characteristics

The suspected organisms were subjected to Gram's staining **(Vincent, 1970).** The bacteria which retained the primary stain called gram $^{+ve}$, while those that lost the crystal violet and counter stained by safranin were referred as gram $^{-ve.}$

Antagonistic effects of isolated bacteria towards soil borne plant pathogen

Isolated bacteria were tested toward soil borne plant pathogens; *Fusarium solani, F. verticillioides* and *Rhizoctonia solani* which were isolated previously in National Research Center. The test was carried out according to the method of (Varese *et al.,* 1996). The pathogens were grown on Petri dishes containing Potato Dextrose Agar (Difco) Media. Colony of bacteria was placed on one side of the Petri dish and the pathogen disk placed on the other side. The Petri dishes were incubated at 25 °C for 7 days. The growth of mycelium was measured and compared to the control treatment (Budi and Nunang, 2012). The evaluation of bacterial growth is performed by measuring the diameter of the colony of the pathogen and the bacterial antagonist. The evaluation of inhibition by the tested bacterial strains was estimated by calculating the percentage of mycelia growth inhibition by using the following formula

$$\%= (1-Cn/Co)\times 100.$$

Cn is the average of pathogen colonies diameter in the presence of antagonist and Co is the average diameter of control. (Bouziane *et al.,* 2011).

Screening of PGPR for multiple plant growth promoting activities

Detection of IAA

Culture growth conditions

According to Kumar *et al.* (2012) Fifty milliliter of Nutrient broth (NB) containing (00.0 % and 0.1%)DLtryptophan was inoculated with 500 Nl of 24 h old bacterial cultures and incubated in refrigerated incubator Shaker at 30±0.1 °C and 180rpm for 48 h in dark. The bacterial cultures were centrifuged at 10,000 rpm for 10min at 4°C. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using colorimetric assay (Loper*, et al.* 1986; Ruci, *et al.,* 2012). Appearance of pink color in test tubes indicated IAA production described by (Gordon and Weber, 1951).

Production of Ammonia

All the bacterial isolates were tested for the production of ammonia as described by (Cappuccino and Sherman, 1992). Overnight grown bacterial cultures were inoculated in 10ml peptone broth and incubated at 30±0.1°C for 48h in Incubator shaker. After incubation 0.5ml of Nessler's reagent was added. The development of faint yellow (+), deep yellow (++) to dark brown color (+++) indicated the production of ammonia.

HCN production

According to Geetha, *et al*. (2014) Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on King's B agar medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed air-tight with Para-film and incubated at 30°C for 48h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as an indication of HCN production (Bakker and Schipperes, 1987).

Siderophore production

According to Sujatha and Ammani, (2013) Siderophore production was detected by adding 0.5ml of culture filtrate to 0.5ml of 2% aqueous $FeCl_3$ for the appearance of orange or reddish brown colour which was positive indication of siderphore production.

Identification of selected PGPR strains by sequencing of 16S rRNA gene

The most two active strains were aerobically grown in Nutrient Broth (NB) for 24 h at 28C, and genomic DNA was extracted according to the method of (Higgins *et al.,*2007). The 16S rRNA gene of the selected bacteria was PCR amplified by using forward and reverse primers, fD1 (5'AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'- ACGGCTACCTTGTTACGACTT -3'), respectively (Weisburg *et al.,* 1991). The nucleotide sequence of purified PCR products was analyzed at the Macrogen Service Center (Seoul, Korea). The derived DNA sequences were aligned with DNA sequences present in GenBank (http://www.ncbi.nlm.nih.gov/Blast).

Effect of media component on chitinase enzyme production

Five different broth media were screened for chitinase production by *B. diminut*a KT277492 and *B. cereus* KU058893 isolates. The first was Lauri bertaini broth (LB) (gl^{-1}): tryptone, 10; yeast extract, 5.0; NaCl, 5.0. King broth (KB) (gl^{-1}): peptone, 20.0; dipotassium hydrogen phosphate, 1.5; magnesium sulphate heptahydrate 1.5. Nutrient broth (NB) (gl^{-1}): yeast extract, 1.5; NaCl 5.0; beef extract 1.5. Wheat bran media (WB) (gl^{-1}): wheat bran, 60 and Nutrient broth medium supplemented with 1% chitin as substrate (Kuddus and Ahmad 2013).

Assay of chitinase activity

According to Sudhakar and Nagarajan (2011) Chitinase activity was determined by a dinitrosalicylic acid (DNS) method (Miller,1959) with some modification. This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymatic action (Ulhoa and peberdy 1991). The 2ml reaction mixture contained 0.5 ml of 0.5% colloidal chitin in acetate buffer (pH 5.5), 0.5 ml crude enzyme extract and 1ml distilled water. The well vortexed mixture was incubated in a water bath shaker at 40˚C for 2 h. The reaction was arrested by the addition of 3ml DNS reagent followed by heating at 100˚C for 10 min with 40% Rochelle's salt solution. The colored solution was centrifuged at 10,000 rotations per minute for 5 min and the absorption of the appropriately diluted test sample was measured at 530 nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of (Roberts and Selitrenkoff, 1988). One unit (U) of the chitinase activity is defined as the amount of enzyme that is required to release 1µmol of *N*-acetyl-d-glucosamine per minute from 0.5% of dry colloidal chitin solution under assay conditions.

RESULTS AND DISCUSSION

Thirty bacterial isolates were isolated, 17 bacterial isolates were isolated from the rhizosphere (Prhizo) with a percentage 56.67% and 13 bacterial isolates from the root (Proot) of the *Trigonella foenum-graecum-L.* with percentage 43.33%. Colonies showing different morphological characteristics on the plates were selected for further characterization. A total 30 strains were isolated with different morphological characteristics and their PGPR characteristics were studied.

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Data in Table (1) show that, the isolated bacterial strains varied between smooth shiny, crinkled shiny surface and colours ranging between light yellow, off white, milky and creamy, one strain was mustard and one isolate was pink. All the isolates were odourless except 3 isolates had rotten egg odour. On contrast UmaMaheswari *et al.,* (2013) studies proved thatMajority of the isolates appeared colourless, circular in form and with entire margin. Nine isolates were coloured such as yellow, orange and pink.Our resultsrevealed that, the isolated bacteria were both Gram positive with percentage 90% and Gram negative 10%, and 80% were long rod and 20% cocci shape.Similar results were reviewed by UmaMaheswari *et al.,* (2013) who isolated twenty five isolates, eighteen isolates were rod shaped with percentage 72% and other 7 with percentage 28% were coccalshaped .Gardner *et al.,* (1982), Zinniel *et al*., (2002) studies showed that (41%) of the bacterial isolates were gram-positive while the percentages of gram-negative was (42%).

Table (1): Morphological characteristics of the isolates on nutrient agar

Isolate	shape	Gram test	colour	odour
Prhizo1	short rod	-ve	Light yellow	odourless
Prhizo2	long rod sporulated	+ve	off white	odourless
Prhizo3	cocci	+ve	off white	odourless
Prhizo4	long rod	+ve	Light yellow	odourless
Prhizo5	long rod	+ve	off white	odourless
Prhizo6	cocci	+ve	off white	odourless
Prhizo7	cocci	+ve	Light yellow	odourless
Prhizo8	cocci	+ve	off white	odourless
Prhizo9	cocci	+ve	Pink	odourless
Prhizo10	long rod	+ve	Light yellow	Rotten egg
Prhizo11	long rod	+ve	off white	odourless
Prhizo12	long rod	+ve	off white	odourless
Prhizo13	long rod	+ve	mustard	odourless
Prhizo14	long rod	+ve	off white	odourless
Prhizo15	long rod	+ve	off white	odourless
Prhizo16	cocci	+ve	Light yellow	odourless
Prhizo17	cocci	-ve	off white	odourless
Proot 18	cocci	+ve	off white	odourless
Proot 19	long rod	+ve	Milky	odourless
Proot 20	short rod	+ve	off white	odourless
Proot 21	long rod	+ve	off white	odourless
Proot 22	long rod	+ve	Light yellow	odourless
Proot 23	long rod	+ve	off white	odourless
Proot 24	cocci	-ve	off white	odourless
Proot 25	long rod	+ve	yellow	Rotten egg
Proot 26	long rod	+ve	off white	odourless
Proot 27	cocci	+ve	off white	odourless
Proot 28	long rod	+ve	Light yellow	Rotten egg
Proot 29	long rod	+ve	off white	odourless
Proot 30	cocci	+ve	off white	odourless

Bacterial isolates from rhizosphere (Prhizo), bacterial isolates from the root (Proot)

Antagonistic effects of isolated bacteria towards soil borne plant pathogen

Thirty bacterial isolates were tested against 3 different pathogenic fungi; *Fusarium solani*, *Rhizoctonia solani* and *F. verticillioides.* Eleven isolates with a percentage 36.67% have been found to have broad spectrum antifungal activities against these pathogenic fungi. The antagonistic effect varied between the bacterial isolates as shown in Table (2) and Fig. (1). on the other hands Prhizo1which was identified as *B. diminuta* KT277492 showed high antagonistic activities against the three pathogenic fungi and burn the pathogen on the pathogenic inoculated disk with percentage 99%, 90% and 90%.Sri and Nunang (2012) work revealed that the bacterial isolates *Bacillus subtilis, Pseudomonas diminuta* , *Proteus penneri* and *Enterobacter hormaechei* have theability to inhibit the growth of three tested phytopathogens *Sclerotium* sp., *Rhizoctonia* sp. and *Ganoderma* sp. The percentages of inhibition produced by *Bacillus subtilis Pseudomonas diminuta* was 79-80%.

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Furthermore Prhizo'2' which is also identified as *Bacillus cereus* showed a very clear zone with *F. solani, R. solani* and *F. verticillioides* with inhibition zone percentage 50%, 45% and 55% respectively.

Sri and Nunang (2013) work proved that *Bacillus cereus* inhibited the growth of *Sclerotium* sp, *Rhizoctonia* sp. and *Ganoderma* sp. with percentage 59-90%. While bacterial isolate Prhizo14 which isolated from the rhizosphere and Proot 20and Proot 18 which isolated from the root showed antagonistic effect against *Rhizoctonia solani* and *F. verticillioides* while they showed no activities against *Fusarium solani.* Moreover Prhizo17 which isolated from the rhizosphere and Proot 28 which isolated from the root showed antagonistic effect against *Fusarium solani* and *F. verticillioides* while it showed no antagonism against *Rhizoctonia solani* Furthermore Proot 23 and Proot 29 which isolated from the root showed antagonistic effect against *Fusarium solani* and *Rhizoctonia solani* while there were no activities against *F. verticillioides.*

Kabir *et al.* (2012) isolated 125 bacterial strains from rhizosphoric soil, only seven isolate were capable of inhibiting the phytopathogenic fungi *Colletotrichum acutatum in vitro* dual culture screening tecnique. They added that all the seven bacterial isolates produced inhibition zone more than 50% of *C. acutatum*on dual culture plates and The greatest inhibition ability of mycelia growth of the fungi was produced by isolate AB15(69.22%); moreover, AB05, AB11, AB12, and AB17 also produced more than 60% inhibition of the radial growth of *C. acutatum*.

Table (2): Percentage of antagonistic activity between the isolated bacterial strains against three pathogenic fungi

	Pathogenic fungi		
Bacterial isolate no.	***Fusarium solani***	***Rhizoctonia solani***	***Fusarium verticillioides***
Rhizosphere			
Prhizo 1*	99%	90%	90%
Prhizo2**	50%	45%	55%
Prhizo3**	30%	27%	33%
Prhizo4	9%	0	0
Prhizo5	0	0	0
Prhizo6	5%	0	9%
Prhizo7	0	0	0
Prhizo8	15%	18%	9%
Prhizo9	0	0	0
Prhizo10	0	0	7%
Prhizo11**	33%	25%	12%
Prhizo12**	45%	34%	0
Prhizo13	0	0	0
Prhizo14	0	15%	15%
Prhizo15	5%	5%	10%
Prhizo16	0	4%	0
Prhizo17	5%	0	5%
Root			
Proot 18	0	5%	5%
Proot 19	10%	10%	5%
Proot 20	0	10%	10%
Proot 21	15%	5%	5%
Proot 22	0	0	0
Proot 23	15%	10%	0
Proot 24	5%	5%	15%
Proot 25	10%	5%	9%
Proot 26	0	0	0
Proot 27	6%	15%	10%
Proot 28	15%	0	10%
Proot 29	5%	5%	0
Proot 30	5%	0	5%

*It suppress the pathogen growth and burn it on the desk turned its color to dark brown ** show a very clear zone

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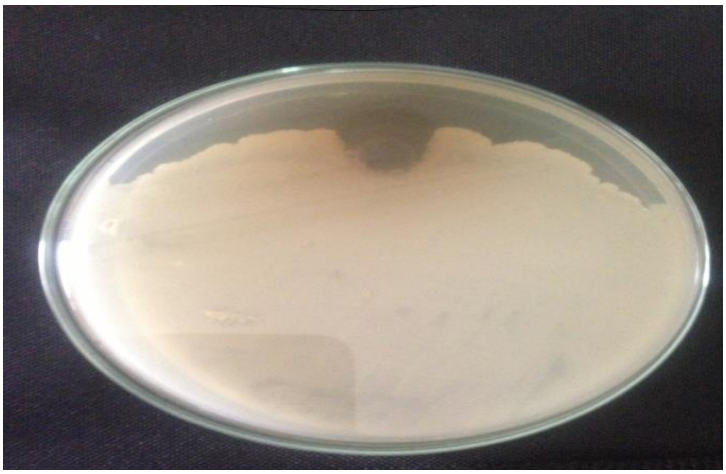
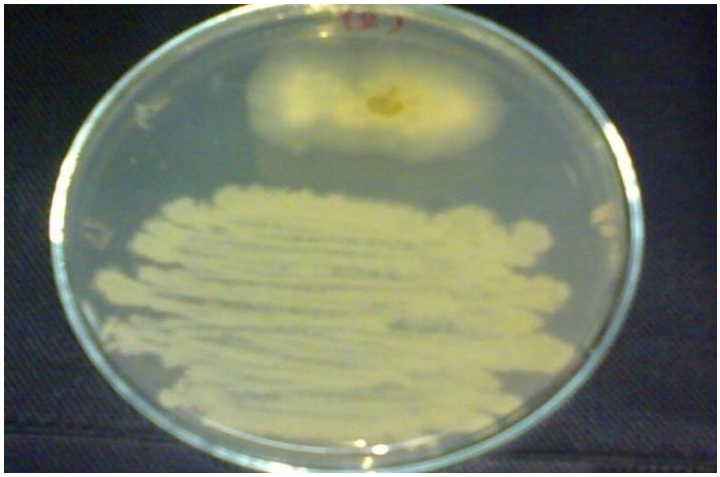


(A)



(B)

Fig. (1): Illustrated the antagonistic effect, (A) *Brevundimonus diminuta* KT277492 against *F. solani.* (B) *Bacillus cereus* KU058893 against *R. solani.*

Screening of bacterial isolates for indole acetic acid (IAA) production

All the isolates were screened for their ability to produce IAA. Data recorded in Table (3) revealed that only ten isolates with a percentage 33.3% of the isolates were able to produce IAA in the absence of tryptophan while 66.7% were unable to produce IAA in the absence of tryptophan. While only 13 isolate with percentage 43.33% were only able to produce IAA in the presence of tryptophan. In the absence of tryptophan, *B. diminuta* was the most active producer strain of IAA and it produced 24.912±0.045 µg/ml. while *B. cereus*produced 15.034± 0.0707 µg/ml and the minimum production was 0.94 ±0.064 µg/ml by Proot29. While in the presence of tryptophan at 0.1% concentration, *B. diminuta* was also the most active producer of IAA and it produced 31.723±0.0059 µg/ml. while *B. cereus* produced 22.2±0.046 µg/ml. and the minimum IAA production was by Prhizo13and it was 0.032 ±0.043 µg/ml as in Table (3). The amount of IAA produced by *B. diminuta* is less than that have been reported by (Edi, 2003) which was 33.82 µg/ml. while it was higher than that reported by De Freitas *et al.* (1997) which was 9.43 µg/ml. on the other hand these results was similar to that produced by (UmaMaheswari *et al.,* 2013) isolated twenty five bacterial isolates and found thatall the bacterial isolates produced higher amount of IAA in the presence of tryptophan. The IAA production ranged from 1.06µg $ml^{-1}$to 6.46µg ml^{-1} (with tryptophan presence) and in the absence of tryptophan 0.03µg ml^{-1} to 0.12µg ml^{-1}. They added In the presence of tryptophan, the tow bacterial isolates RS10 and BS25 produced the maximum amount of IAA production was (6.46 µg ml^{-1}) while in the absence of tryptophan the isolate BS22 produced the maximum amount of IAA which was (0.12µgml^{-1}).

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Table (3): production of Indole Acetic Acid (IAA) by the bacterial strains in the absence and presence of tryptophan

Bacterial isolate no.	Tryptophan 0% IAA (µg/ml)	Tryptophan 0.1% IAA (µg/ml)
Prhizo1	24.912 ±0.045	31.723 ±0.0059
Prhizo2	15.034± 0.0707	22.2±0.046
Prhizo3	ND	10.45±0.0096
Prhizo4	ND	ND
Prhizo5	ND	ND
Prhizo6	9.753±0.0093	11.65±0.016
Prhizo7	ND	ND
Prhizo8	ND	ND
Prhizo9	ND	0.85 ±0.0266
Prhizo10	ND	ND
Prhizo11	12.26±0.043	9.89 ±0.0246
Prhizo12	ND	ND
Prhizo13	ND	0.032 ±0.043
Prhizo14	ND	ND
Prhizo15	14.659±0.012	15.821 ±0.126
Prhizo16	ND	ND
Prhizo17	ND	ND
Proot 18	3.312±0.0061	4.756 ±0.237
Proot 19	ND	ND
Proot 20	0.568±0.022	0.845 ±0.0655
Proot 21	10.11±0.032	10.39± 0.453
Proot 22	ND	ND
Proot 23	18.756±0.017	20.991±0.022
Proot 24	ND	ND
Proot 25	ND	ND
Proot 26	ND	ND
Proot 27	ND	0.15
Proot 28	ND	ND
Proot 29	0.94 ±0.064	1.71 ±0.002
Proot 30	ND	ND

ND: the IAA production is not detected

Production of Ammonia

The data showed in Table (4) revealed that twenty four bacterial isolate with percentage 80% of the bacterial isolates were able to produce ammonia, while 6 isolates represented 20% didn't produce ammonia. Almost 5 isolates which is equal to16.6% of the isolate produced dark brown colour (+++) as shown in fig, while 12 bacterial isolates with percentage 40% showed deep yellow (++), also 7 isolates with percentage 23% produced faint yellow (+) as shown in table (3). While as illustrated in table (4) *B. diminuta* and *B. cereus* produced dark brown color (+++) which indicate high yield production of ammonia. Sajani and Muthukkaruppan (2011) studied eight bacterial species; *Bacillus* spp., *Pseudomonas* spp, *Azotobacter* spp, *Azospirillium* spp, *Phosphobacteria* spp., *Glucanoacetobacter* spp. and *peanobacillus* spp., 100% of them were able to produce ammonia.

Production of HCN and siderphore

The data recorded in Table (4) showed that about twenty bacterial isolates with percentage 90% of the isolates produced HCN ranging from dark brown (++++) to faint brown (+). Only one isolate *B. cereus* were able to produce dark brown (++++) which indicated that it produced high amount of HCN and *B. diminuta* gave brown colour which indicate moderate production of HCN. while 8 isolates represent 26.67% were able to produce moderate amount of HCN and 7 isolates with 23.33% produce sufficient amount of HCN and 11 isolates represent 36.67% produced small amount of HCN. Fig. (2) Showed production of HCN by bacterial isolate *B. diminuta* and *B. cereus* compared with control. Ruchi *et al.,* (2012) studied proved that almost all the eight isolates of *pseudomonas* sp produce HCN with (++++) while the other nineteen isolates showed production of HCN ranging between (++) to (+++). The data recorded in Table (4) revealed that Twenty isolates

out of thirty with percentage 66.67% were found to be siderphore producers on the other hand 33.33% lack the ability to produce siderphore. While Ruchi *et al.,* (2012) reported that twenty bacterial isolates represent 66.667% the bacterial isolates were found to be siderphore producers while 10 isolates with percentage 33.333% didn't produce siderphore. Moreover Ladwal *et al.,* (2012) studied five isolates, only two strains DKC2 and DKM5 were found to produce siderophore while the other three isolates were not able to produce siderophore.

Phosphate solubilizing bacteria

The data recorded in Table (4) showed that about eleven isolates were able to hydrolyze Phosphate which represents about 36.667% of the total isolates. While nineteen isolates were not able to hydrolysis phosphate with percentage 63.333%. *B. diminuta* were able to hydrolysis phosphate with range (++++) and produce a large inhibition zone after one week but after thirty days it hydrolysis all the phosphate. While *Bacillus cereus* produced lesser inhibition zone than that is produced by *B. diminuta*. Fig (3) showed Phosphate solubilization by different bacterial isolates. Tilak *et al*. (2005) results confirm our results and stated that *Pseudomonas* and *Bacillus* are the main phosphate solubilizer bacteria.

Table (4): Screening of PGPR for multiple plant growth promoting activities: Ammonia NH_4, HCN, siderphore production and phosphate solubilization

Bacterial isolate no.	Ammonia production	HCN production	Siderphore production	Phosphate solubilization
Prhizo1	+++	+++	+	++++
Prhizo2	+++	++++	+	+++
Prhizo3	++	+++	+	ND
Prhizo4	+	++	ND	ND
Prhizo5	++	ND	ND	ND
Prhizo6	ND	++	+	+++
Prhizo7	++	+	ND	ND
Prhizo8	++	++	+	ND
Prhizo9	ND	+	ND	ND
Prhizo10	ND	+	+	+
Prhizo11	++	+++	+	ND
Prhizo12	+	ND	+	+
Prhizo13	++	+	+	ND
Prhizo14	ND	+	ND	ND
Prhizo15	+++	+++	+	+
Prhizo16	++	+	+	ND
Prhizo17	ND	+	+	ND
Proot 18	+++	+++	ND	+
Proot 19	+	++	+	ND
Proot 20	++	++	ND	ND
Proot 21	+	+++	ND	+
Proot 22	ND	ND	+	ND
Proot 23	++	+++	+	ND
Proot 24	+	+	+	+
Proot 25	+	+	+	ND
Proot 26	++	+	+	ND
Proot 27	++	++	+	+
Proot 28	++	++	ND	ND
Proot 29	+++	+++	+	+
Proot 30	+	+	ND	ND

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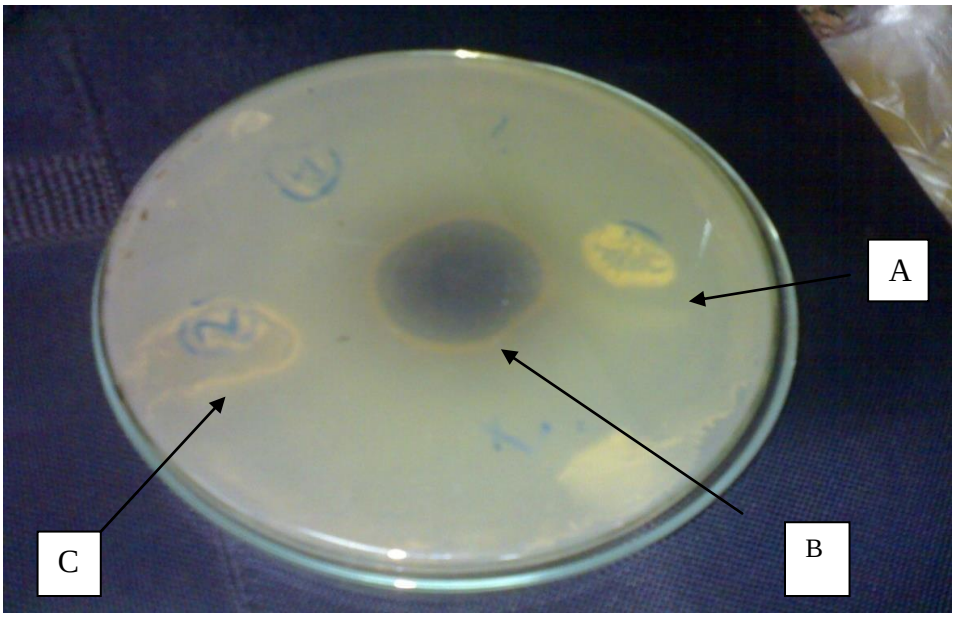
Fig (2) Production of HCN by bacterial isolate *B. diminuta* (Prhizo 1) with strength (+++) and *B. cereus* (Prhizo 2) with strength (++++)



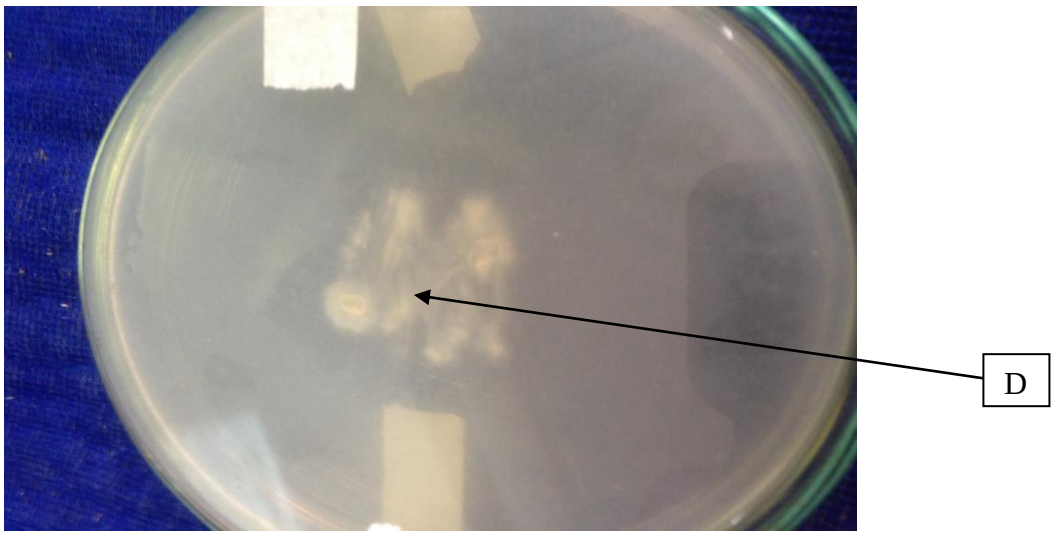


Fig (3) Phosphate solubilization by different bacterial isolates, A-Prhizo17 , B- *B. cereus* , C- Prhizo15, D- *B. diminuta*.

Chitinase enzyme activity

From all previous data, *B. diminuta* KT277492 and *B. cereus* KU058893 were most active plant growth promoting rhizobactria. They were screened for chitinase enzyme production using different media. The result in Table (5) showed that the bacterial isolate *B. diminuta* KT277492 showed the highest chitinase enzyme

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activity 95 IUL^{-1} on the (NB+ chitin) medium that will be further studied for the optimization of medium components in the coming work . Chitinase production was reported in different species of *Bacillus* such as *B. amyloliquefaciens*, *B. cereus*, *B. subtilis* and *B. megaterium* (Wang *et al.,* 2002 and Wang *et al.,* 2006).

Table (5): Screening of different media for chitinase enzyme production by *B. diminuta* KT277492 and *B. cereus* KU058893.

Media	Chitinase activity IUL^{-1}	
	B. diminuta KT277492	*B. cereus* KU058893
LB	70	0.0
KB	20	32
NB	79	39
WB	65	0.0
(NB+chitin)	95	54

Lauri bertaini broth (LB), King broth (KB), Nutrient broth (NB), yeast extract (WB)

Molecular Identification of the most dominant bacterial isolates (Prhizo1 &Prhizo 2).

The molecular identification using partial sequencing of 16S rDNA with available NCBI GeneBank database was tested for the selected isolates Prhizo 1 and Prhizo**2**. The nucleotide sequence of bacterial isolates were submitted to the GenBank and deposited in the GeneBank under the accession numbers (KT277492 and KU058893, respectively). Results obtained from alignments showed that *Brevundimonas diminut*a KT277492 (Prhizo**1**) revealed high identity (98%) to *Brevundimonas diminuta* strain NBRC 12697 (GeneBank accession number NR113602) and revealed high close similarity (98%) with species *Brevundimonas diminuta* JCM2788 (GeneBank accession number NR113238).

Also the alignment results showed that *Bacillus cereus*KU058893 (Prhizo**2**) revealed high identity (95%) to *Bacillus cereus* strain MVSV4 (GeneBank accession number JN089708) and show high similarity 94% with species *Bacillus cereus*B64 with (GeneBank accession number HM588150).

The phylogenetic trees of the bacterial isolates were displayed using the TREEVIEW Program that showed the sequences of close relatives obtained from GenBank to resolve the phylogenetic relations with ancestor. Fig. (4 and 5)

Mahwish *et al.,* (2013) reported that *Bacillus* and *Pseudomonas* have been found to be as themostdominantgenera isolates in various plant research **(Hallmann and Berg, 2006**).

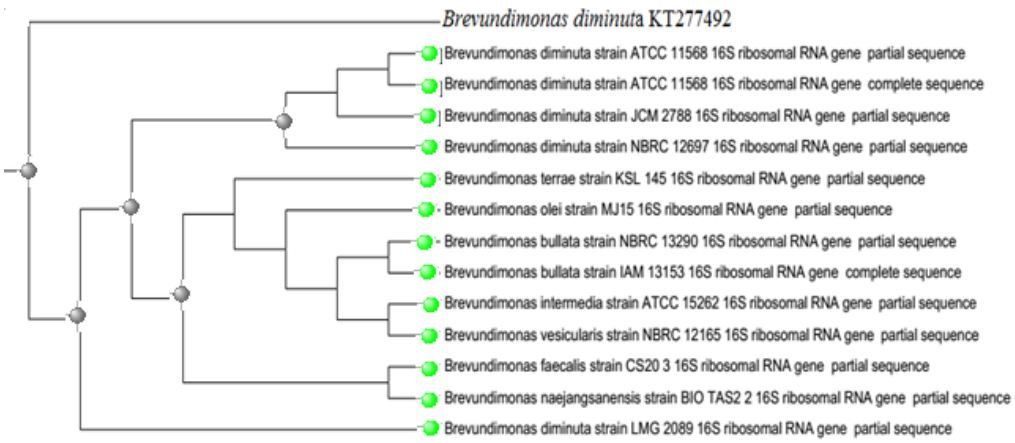

Fig. (4):The phylogenetic tree based on partial sequencing of 16S rDNA gene showing relationship neighbor-joining between the bacterial isolate *Brevundimonas diminut*a KT277492 and other closely related sequences on NCBI GeneBank reference taxa.

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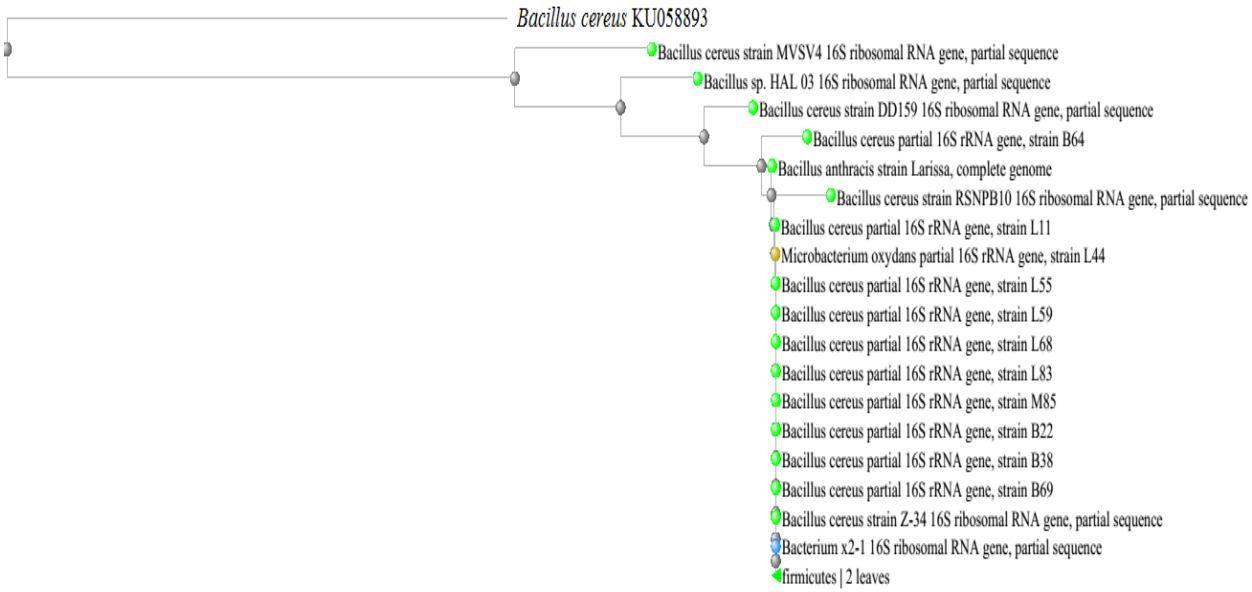

Fig. (5): The phylogenetic tree based on partial sequencing of 16S rDNA gene showing relationship neighbor-joining between the bacterial isolate *Bacillus cereus* KU058893 and other closely related sequences on NCBI GeneBank reference taxa

CONCLUSION

The ability of rhizobacteria to produce phytohormones differs from bacterial strain to another and may be essential in suppression of pathogenic fungi. Our study conclude that the bacterial isolates Prhizo 1 and Prhizo 2 genetically identified as *Brevundimonus diminuta* KT277492 and and *Bacillus cereus* KU058893. They are the most active plant growth promoting rhizobactria which have the ability to produce different types of phytohormones as well as good chitinase enzyme activity and suppress various pathogenic fungi with variable degree.

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