INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 8, No. 4, p. 43-54, 2016

RESEARCH PAPER OPEN ACCESS

Identification and molecular characterization of endophytic bacteria isolated from wheat roots with biotechnological potential in agriculture

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Key words: Wheat, Endophytic bacteria, Plant growth promotion, RAPD-PCR, 16S rRNA sequencing.

http://dx.doi.org/10.12692/ijb/8.4.43-54 Article published on April 23, 2016

Abstract

Bacterial root endophytes reside in a vast number plant species as a part of their root microbiome. Some of them have been shown to positively influence in plant growth. Here, six bacterial isolates namely W1, W3, W7, W11, W12, and W15 were obtained from surface sterilized healthy roots of wheat. This isolates showing positive ability for IAA production, nitrogen fixation and phosphate solubilization activity. As an indication of favorable bacterial action, isolateW11 showed the highest functional potentialities in relation to plant growth promoting activities among the other isolates and increased the total dry weight of root and shoot by 200% and 180%, respectively. Moreover, the bio-inoculation of wheat root with W11 resulted in significant increase in the N, P and K concentrations of the shoot after 30 days of inoculation by 82%, 37.5%, and 59%, respectively. Consequently, the three more efficient isolates namely W11, P31 and P35 were identified as *Arthrobacter arilaitensis, Bacillus anthracis* and *Achromobacter spanius*, respectively, by sequencing of the 16S rRNA gene. Furthermore, molecular biodiversity of these three isolates were done using eight random primer of RAPD-PCR and the fragments size ranged from 100-5920 bp. From These results showed that W11 strain proposed as potential microbial inoculants or biofertilizers for sustainable wheat production in reclamation soil in Egypt because of its benefit and biosafety.

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Introduction

Bacterial endophytes are living inside plants, colonizing inner tissues of hosts without causing signs of plant diseases. Endophytes can enter plants from soil through cracks due to emerging lateral root; transfer to roots, then spread in leaves, flowers and fruits via vascular plant system (Rashid *et al.*, 2012; Vacheron *et al.*, 2013**).**Moreover, use of chemicals in agriculture and livestock production has created potential health hazards, not only to livestock and wildlife, but also for living organisms (Nair *et al.*, 2011; Ali *et al.*, 2012). In this work, some isolates were chosen for plant bio-inoculation experiments in greenhouse conditions. Recently, the importance of these bacteria due to their ability to promote plant growth rhizobcteria is being used as a biofertilizer and a bioenhancer for different plants as an alternative source of chemical fertilizer (Compant *et al.*, 2010; Beneduzi *et al.*, 2013**).** The bacteria inside the plant take advantage of a major availability of nutrients, and plants receive from bacteria both protection against pathogens and enhanced growth. Thus, these types of bacteria have concerned attention because of the need to reduce the use of chemicals, especially in view of the context of sustainable agriculture and environmental protection (Compant *et al.*, 2011; Etesami *et al.*, 2015**).** Bacteria promoting plant growth can act directly, through one or more mechanisms, including nitrogen fixation(Rashid *et al.*, 2012), phosphate solubilization (Mandal *et al.*, 2007; Etesami *et al.*,2015**),** production of hormones such as auxins, gibberellins and zeatin (Cassan *et al.*,2009; El-Awady *et al.*, 2015**),** ammonium ion production (Compant *et al.*, 2011;Pandya *et al.*,2015**),** or act indirectly by means of biological control of pathogens (Szilagyi-Zecchin *et al.*, 2014). Moreover, several studies have indicated that endophytic bacteria can promote plant growth by altering plant physiology including osmotic pressure regulation, changes in stomata responses, adjustment in root size morphology, modification of nitrogen accumulation and metabolism, increasing uptake of certain minerals (Pérez-Garcìa *et al.*,2011; Paul and Sinha, 2013**).** Studying plant bacterial endophytes is important for understanding ecological interactions and for developing biotechnological applications (Ryan *et al.*, 2008**).** Recent studies have shown the positive effects of endophytic bacteria inoculation with different plants, e.g. potato inoculated with endophytic bacteria increased the total dry weight of roots and shoots (Dawwam *et al.*, 2013), sugarcane with endophytic bacteria leading to increase contribution of biological nitrogen fixation, to promotion of root development, increasing biomass and productivity (Oliveira *et al.*, 2003), tomato (*Lycopersicum esculentum* L.) with endophytic bacteria increased plant height, leaf area, leaf number, together with fresh and dry plant weight (Barretti *et al.*, 2008**).** Moreover, studies of agricultural and native plants showed that endophytic bacteria have the capacity to control plant pathogens, and positively contribute to plant nutrient levels and promote plant growth (Compant *et al.*, 2010; Reinhold-Hurek and Hurek 2011; Dawwam *et al.*, 2013; Szilagyi-Zecchin *et al.*,2014).

The main objective of this study was to evaluate and characterize the properties of endophytic bacteria that isolated from wheat roots. Also, molecular identification of the three new endophytes (W11, P31, and P35) isolated from a number of geographically different rhizospheric plants. Screen them for their plant growth promoting capacities, and finally to use them as environmentally friendly adjuncts to agricultural practice, with the potential to have biotechnological interest for their use in sustainable agriculture.

Materials and methods

Biological materials

Freshly collected roots of wheat were carefully washed with tap water for removing adhering soil. The roots were surface sterilized using 70% ethanol for 30 sec and 2% sodium hypochlorite for 5 min, and then washed twice with sterilized distilled water (Elbeltagy *et al.*, 2000). The sterilized roots were aseptically cut into 1-2 cm sections, macerated with 0.8% saline solution and quartz sand, and then decimally diluted in 0.8% saline solution. The last dilutions were used to spread on different specific

cultural media, namely yeast extract manitol agar medium(YEM), The National botanical research institute's phosphate growth medium (NBRIP) and Pikovskaya's (PVK) agar medium were used for isolation of indole acetic acid (IAA) and phosphate solubilizing bacteria, respectively. The isolates were sub cultured on their specific media for purification and maintained as a stock culture at 4-5°C for further studies.

Screening of bacterial isolates for their IAA production

The ability of endophytic bacterial isolates to produce IAA was determined qualitatively on Yeast Extract Mannitol broth medium (YEM) amended with tryptophan (0.1g/L) as described with (Mandal *et al.*, 2007). Bacterial cultures were grown in malate medium supplemented with tryptophan (100 mg/L) as the precursor of IAA and compared to those grown without the addition of tryptophan precursor. IAA production was determined using colorimetric methods (El-Awady *et al.*, 2015). The experiment was repeated twice with three replicates each and mean was calculated.

Evaluation of phosphate solubilizing ability

Bacterial isolates were screened for their phosphate solubilizing ability on NBRIP and PVK media respectively according to El-Awady *et al.* (2015). Quantitative assessment of phosphate solubilization in liquid culture was determined according described by (El-Awady *et al.*, 2015).

Effects of rhizobacterial inoculum on wheat plants

One of the strategies is to exploit the benefits that several endophytic bacteria may give to plants when added as inoculants (Lucy *et al.*, 2004). Therefore, for determining the effectiveness of the isolated bacterial isolates as bio-inoculants; plastic pots (12 cm width) were filled with mix of sterilized soil/sand in 1:1 ratio, 100 g of sterilized vermiculite and 4g of rock phosphate was added to sterilized mixture soil (3 kg). Wheat seeds were coated with the selected isolates grown in liquid culture medium for 15 minute using 10% Arabic gum, and left to dry for 15 minute. Wheat seeds were sown in pots under soil surface and irrigated weakly. Pot experiments were carried out at glass house of Shibin El-Kom farm, Egypt. After 30 days of planting, wheat plants were collected and measured for different growth parameters; shoot and root length, fresh and dry weight for shoot and root. Nitrogen, phosphorus and potassium contents were determined according to the methods described by (AOAC, 2005).

RAPD-PCR technique

Total bacterial genomic DNA was extracted using Mini-Prep Kit (Axygen cat. No. V110440-05) according to the manufacturer's instructions and RAPD analysis was performed according to (Moschetti *et al.*, 2005) using eleven primers OPA-01, OPA-03, OPA-04, OPA-05, OPA-09 OPA-10 OPB-02 and OPD-05. A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram using NTSYS-PC package (Rohlf, 2000).

The 16S rRNA amplification and sequencing

The DNA of each isolate was amplified with the primers F1-5'AGAGTTTGCATCCTGGCTCAG-3' R1-5' ACGGACTACCTTGTTACGACTT-3'.

The amplificationswere carried out in thermo cycler as following. Amplified 16S-rRNA gene using Primers and conditions described by (Hassan and Ismail, 2014). About 1200 bp 16S rRNA fragments were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and sequenced using specific primers as mentioned previously on an Applied Biosystems model 373A DNA sequencer (Sigma Co., Hamburg City, Germany).

The sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, WI). BLAST searches were done using the NCBI server at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The 16S rRNA sequences for the three endophytic bacterial strains were deposited in Gene Bank under the

accession numbers KF707490, KF707491, and KF707492 for isolates W11, P35 and P31, respectively.

Statistical analysis

The results were presented as mean±SD of three replicates. The obtained data were subjected to one-way ANOVA and differences between means were tested at the 5% probability level using Duncan test. All the statistical analyses were done using SPSS program version 15 (SPSS, Richmond, VA, USA) as described by Dytham (1999).

Results and disscusion

Isolation and characterization of endophytic bacteria

In this study, six endophytic bacterial isolates, namely W1, W3, W7, W11, W12, and W15 were isolated from surface sterilized roots of healthy wheat plants from Moshtohor village, Al-Qalyubia Government, Egypt, to evaluate their plant growth promoting activities. Preliminary characterization of these bacteria showed that isolates W3, W11 and W15 were Gram positive, while, isolates W1, W7 and W12 were Gram negative bacteria (Table 1).

Table 1. The biochemical characteristics ofthe six endophytic bacteria.

Bacterial isolate	Gram reaction	Sporulation	Catalase production	Rock phosphate solubilization	IAA production
W1	-	-	+	+	+
W3	+	+	+	+	+
W7	-	-	+	+	+
W11	+	-	+	+	+
W12	-	-	+	+	+
W15	+	-	+	+	-
P31*	+	+	+	+	+
P35*	-	-	+	+	+

* Used as a references (Dawwam *et al.*, 2013).

Regarding catalase test and spore formation, all bacterial isolates showed catalase activity. The W3 isolate formed spores, while all other isolates were non-spore forming bacteria. All the six isolated bacteria were screened for their potential in rock phosphate solubilization and IAA production. Recently, endophytic bacteria are commonly used successfully as inoculum in Egypt and worldwidecultivated land, covering over million hectares (Compant *et al.*, 2011; Rashid *et al.*, 2012; Dawwam *et al.*, 2013).

Table 2. Quantitative amount of phosphate solubilization efficiency by the bacterial isolates.

Bacterial isolate	Solubilization efficiency of phosphate %**	Amounts of dissolved Phosphate (µg/ml) ***
W1	150.3	199.6
W3	101.2	129.2
W7	155.7	231.6
W11	195.7	255.7
W12	107.3	147.3
W15	103.6	207.9
P31*	350.0	354.3
P35*	108.3	240.6

* Used as a references (Dawwam *et al.*, 2013),** Amount on solid medium and *** Amount on liquid medium

Enzymatic and physiological characterization of the six isolates Phosphate solubilization

All isolates performed phosphate solubilization in the range of pH 6-9; however, optimum pH for better solubilization was 7 for all isolates. On the other hand, all isolates were able to grow at temperature ranging from 25 to 40°C. But, the optimum temperature for better solubilization was 30°C for all isolates. Thus, the bacterial isolates were screened for its ability to solubilize phosphate in liquid and solid medium (Table 2, Fig. 1). The direct measurement of phosphate solubilization in NBRIP broth assay always resulted in reliable results. It is apparent clearly that the ability to dissolve phosphate was remarkably differed among the tested bacterial isolates and reflected by the variation of clearing zone around colonies in case of solid medium (Fig. 1) and variation in the intensity of blue color in case of liquid medium for each tested isolate. Interestingly, the isolate W11 gave the highest solubilization efficiency on PVK agar medium (195.7%) and the highest amount of dissolved phosphorous on NBRIP broth media (255.7 µg/ml) (Table 2). The isolate W7 was the second most efficient isolate which produced 155.7 % and 231.6 µg/ml on solid and liquid media, respectively, whereas, isolate W3 showed the lowest solubilization efficiency.

Table 3. Quantification amount of IAA production by Bacterial isolates using Salkowski assay**.**

Bacterial isolate	IAA production µg/ml
W1	6.36
W3	0.44
W7	6.08
W11	3.48
W12	5.21
W15	ND
P31*	4.91
P35*	8.38

* Used as a references (Dawwam *et al.*, 2013).

Table 4. Different vegetative growth parameters of wheat as affected by inoculation of the six bacteria isolates after 30 days of inoculation.

Bacterial isolates	Root length (cm/plant)	Root dry wt (g/10plants)	Shoot length (cm/plant)	Shoot dry wt (g/10plants)
control	7.4^{c}	0.107^{d}	31.6^{d}	0.31^{d}
W1	7.7^{fgh}	0.011^{jk}	31.8^{no}	0.043^{l}
W3	7.5^{hi}	0.013^{j}	31.7^{op}	0.045^{k}
W7	7.9^{def}	0.021^{h}	32.3^{k}	0.047^{j}
W11	8.1^{b}	0.33^{c}	33.3^{c}	0.87^{c}
W12	8^{cde}	0.032^{ef}	33.2^{gh}	0.062^{jh}
W15	8^{cde}	0.031^{f}	33.9^{e}	0.051^{i}
P31	8.5^{a}	0.39^{b}	34.9^{b}	0.90^{b}
P35	8.6^{a}	0.44^{a}	35.1^{a}	0.98^{a}

Values within the same vertical area with the same letter are not significantly different at 5% probability level by Duncan's Multiple Range test.

Moreover, the efficiency phosphate solubilization either from the source of rock phosphate or tricalcium phosphate was tested by W11, P31, and P35 isolates (Fig. 2). The results indicate that rock phosphate was best source for solubilization by endophytic bacteria. The W11 isolate showed 200 % of solubilization efficiency with rock phosphate comparing to 125 % of solubilization efficiency from tricalcium phosphate.

On the other hand, the P31 isolate produced 350 % and the P35 isolate produced 101 % of solubilization efficiency with rock phosphate (Fig. 2).All the phosphate solubilizing bacteria strains isolated from North Egypt rhizosphere soils showed efficient solubilization of insoluble phosphate as reported earlier (Rashid *et al.*, 2012; El-Awady *et al.*, 2015). Phosphate solubilizing microorganisms not only provide phosphorus to the plant but also at the same time provide growth promoting substances like hormones, vitamins, and amino acids (Paul and Sinha, 2013; El-Awady *et al.*, 2015). The phosphate solubilizing bacteria effect on wheat plants is gaining scientific scrutiny (Mamta *et al.*, 2010; Pandya *et al.*, 2015).

Table 5. Effect of inoculation with bacteria isolates on NPK percentage in wheat shoot after 30 days of inoculation.

Bacterial isolate	Total N (%)	Total P (%)	Total K (%)
control	1.54^{k}	0.16^{m}	4.26^{n}
W1	1.88^{j}	0.17^{m}	4.69^{j}
W3	1.99^{i}	0.2^{l}	4.31^{m}
W7	2.21^{g}	0.3^{ef}	5.22^{g}
W11	2.81^{a}	0.22^{k}	6.78^{e}
W12	2.52^{d}	0.25^{j}	7.77^{c}
W15	2.62^{c}	0.33^{d}	7.01^{d}
P31	2.24^{g}	0.41^{b}	4.58^{k}
P35	2.7^{b}	0.39^{c}	8.91^{a}

Values within the same vertical area with the same letter are not significantly different at 5% probability level by Duncan's Multiple Range test.

Table 6. Polymorphic bands of each genetic primers and percentage of polymorphism in three strains of endophytic bacteria.

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
OPA-01	12	1	11	08.3	91.6
OPA-03	14	3	11	21.4	78.6
OPA-04	13	2	11	15.4	84.6
OPA-05	7	0	7	00.0	100
OPA-09	11	1	10	09.1	90.9
OPA-10	12	2	10	16.7	83.3
OPB-02	11	0	11	00.0	100
OPD- 05	14	0	14	00.0	100
Total	94	9	85		

IAA production

Results presented in Table 3 indicate to the ability of bacterial isolates to produce IAA as a representative of auxins using Salkowski assay (El-Awady *et al.*, 2015). It is apparent from these results that their ability to secrete IAA compound was greatly varied among isolates. The highest production of IAA obtained by the W1 isolate (6.36 µg/ml), while the W11 isolate produced 3.48 µg/ml of IAA. The W3 Isolate showed the lowest production of IAA (0.44 µg/ml). IAA production from endophytic bacteria increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from soil (Compant *et al.*, 2011; Etesami *et al.*, 2015). The presence of IAA and related compounds could be demonstrated for many

diazotrophs, for example, *Arthrobacter arilaitensis, Bacillus anthracis*, and *Achromobacter spanius* (Compant *et al*., 2010; Ali *et al*., 2012; El-Awady *et al*., 2015; Jhala *et al*., 2015).

Effect of inoculation with bacteria isolates on wheat growth parameters

The efficiency for plant growth promoting activities by the isolated endophytic bacteria was tested in pot experiments (Table 4 and Fig. 3). In this regard, the six isolates were tested as bio-inoculants for wheat plants. The different growth parameters (root length, root dry weight, shoot length and shoot dry weight) were estimated. Most of the isolated bacteria showed significant increase in all of the tested parameters over the control. The W11 isolate showed highest increase in root length (8.1 cm/plant) and root dry weight (0.33 g/10 plants) as compared with control plants.

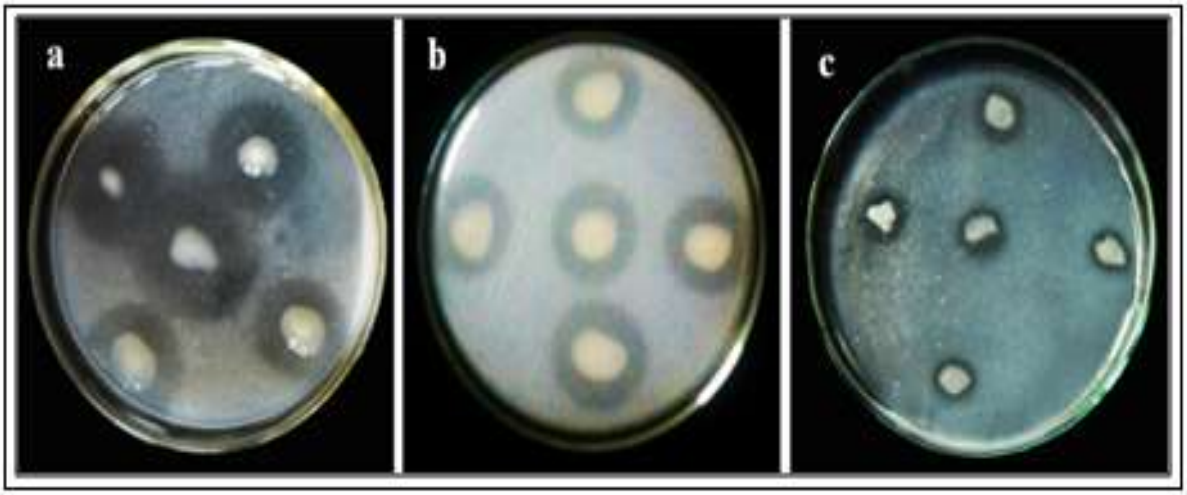

Fig. 1. Phosphate solubilization assay of some bacterial isolates (*in vitro)*.

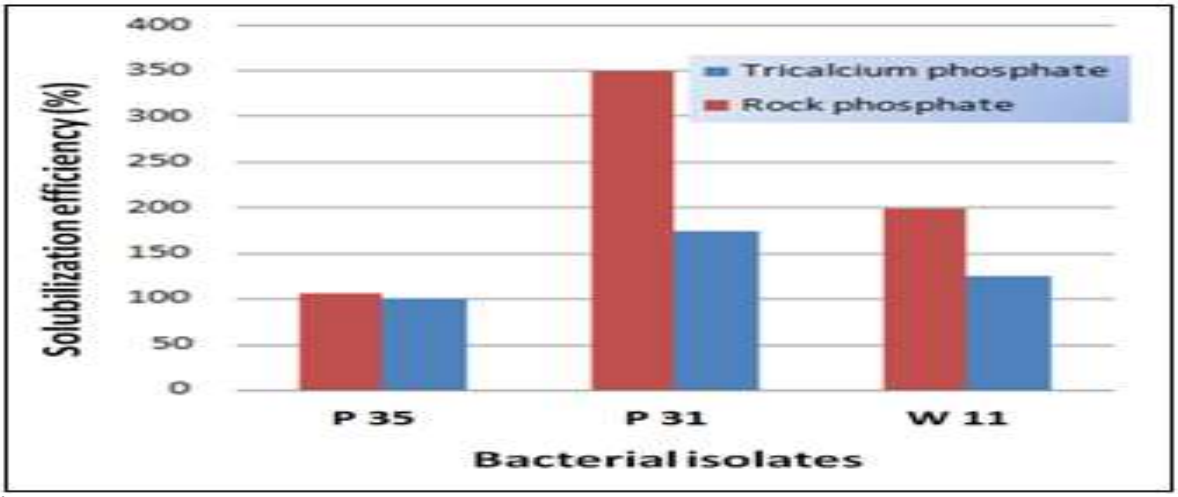

Fig. 2. Solubilization efficiency of rock phosphate and tricalcium phosphate by endophytic wheat bacteria.

From the morphological study, it is being reaffirmed that the phosphate solubilization by different endophytic bacteria involved may be with the production of organic acids (Reinhold-Hurek and Hurek, 2011; Dawwam *et al*., 2013). Moreover, they have been reported that bacteria mobilize insoluble phosphate very efficiently, via producing gluconic acid during the extracellular oxidation of glucose catalyzed by quino-protein glucose dehydrogenase. In this sense, the obtained results are similar to (Mamta *et al*., 2010). The inoculation of phosphate solubilizing bacteria significantly increases the plant

growth (shoot length, root length, leaf dry weight, stem dry weight, and biomass), available phosphate content in soil as well as its uptake (Das *et al.*, 2008; Mamta *et al.*, 2010).

Effect of endophytic bacteria inoculation on NPK content of wheat

The bio-inoculation of wheat plant with the six endophytic bacteria showed significantly their influenced on the N-P-K content in shoot of wheat (Table 5). The W11 isolate and reference P35 isolate showed highest nitrogen uptake (2.81 and 2.7%, respectively). Moreover, the significant phosphorus uptake was recorded by the W15 isolate (0.39%) and potassium uptake was recorded by the W12 isolate (8.91%) respectively when compared with un-inoculated plants. The solubilization of phosphate in the rhizosphere is the most common mode of action implicated in endophytic bacteria that increases availability to the host plant (Rashid *et al.*, 2012;Ali *et al.*, 2012).

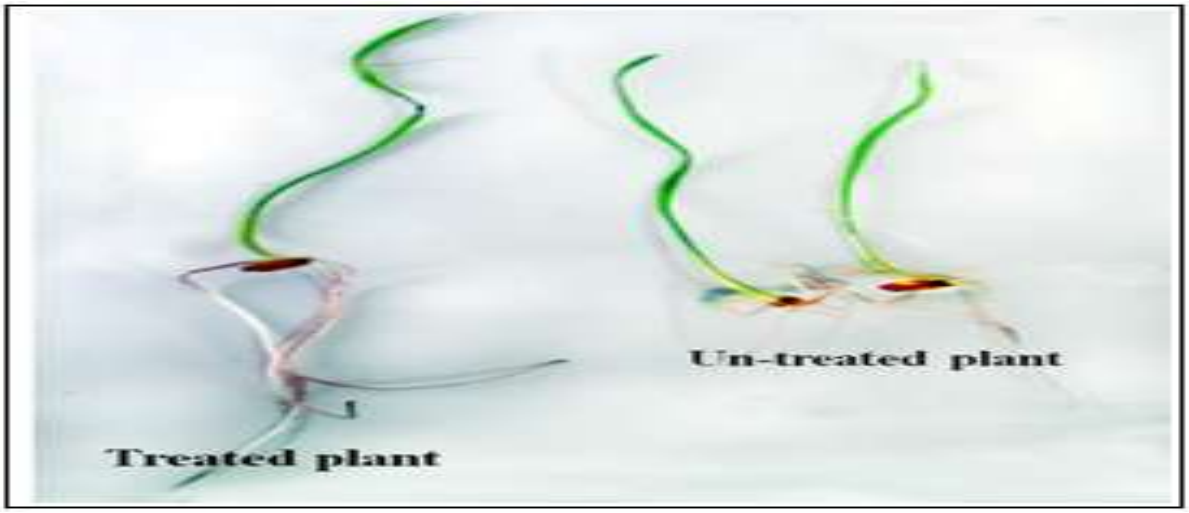

Fig. 3. Root colonization of wheat plants after 14 days of inoculation with endophyticbacteria isolateW11.

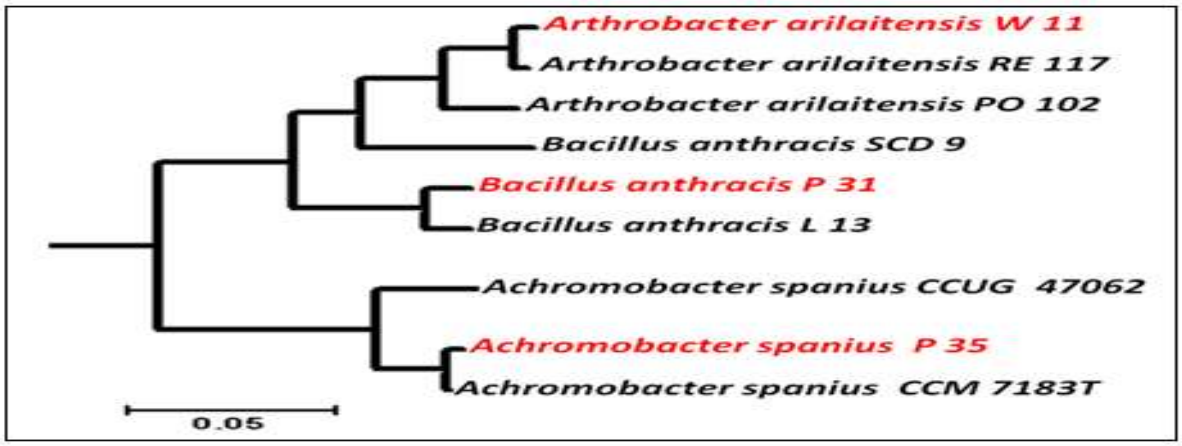

Fig. 4. Phylogenetic tree based on the 16S rRNA gene sequence of the isolates in this study and reference strains obtained from the GenBank database using the neighbor joining method in Mega.5.2.1.

Molecular phylogeny of the bacterial isolates

After aligning the sequences of the gene 16S rRNA, a region of 1200 bp was used for phylogenetic analysis. The 16S rRNA sequences of the following strains representing the three identified *Arthrobacter arilaitensis, Bacillus anthracis*, and *Achromobacter spanius* genotypes were determined with isolates code W11, P31, and P35, respectively (Fig. 4). These sequences were aligned and compared with the 16S rRNA sequences of other members of their families

available in the GeneBank database. These strains are deposited in GeneBank under the accession numbers KF707490, KF707491 and KF707492 for W11, P35, and P31isolates, respectively. The phylogenetic tree based on 16S rRNA sequences showed the relationship among these isolates. Nucleotide sequences of 16S rRNA genes obtained in this research and from the GenBank database were aligned, sequence similarities were calculated and a phylogenetic tree generated, bootstrapped with 1000 subsamples and visualized as described.

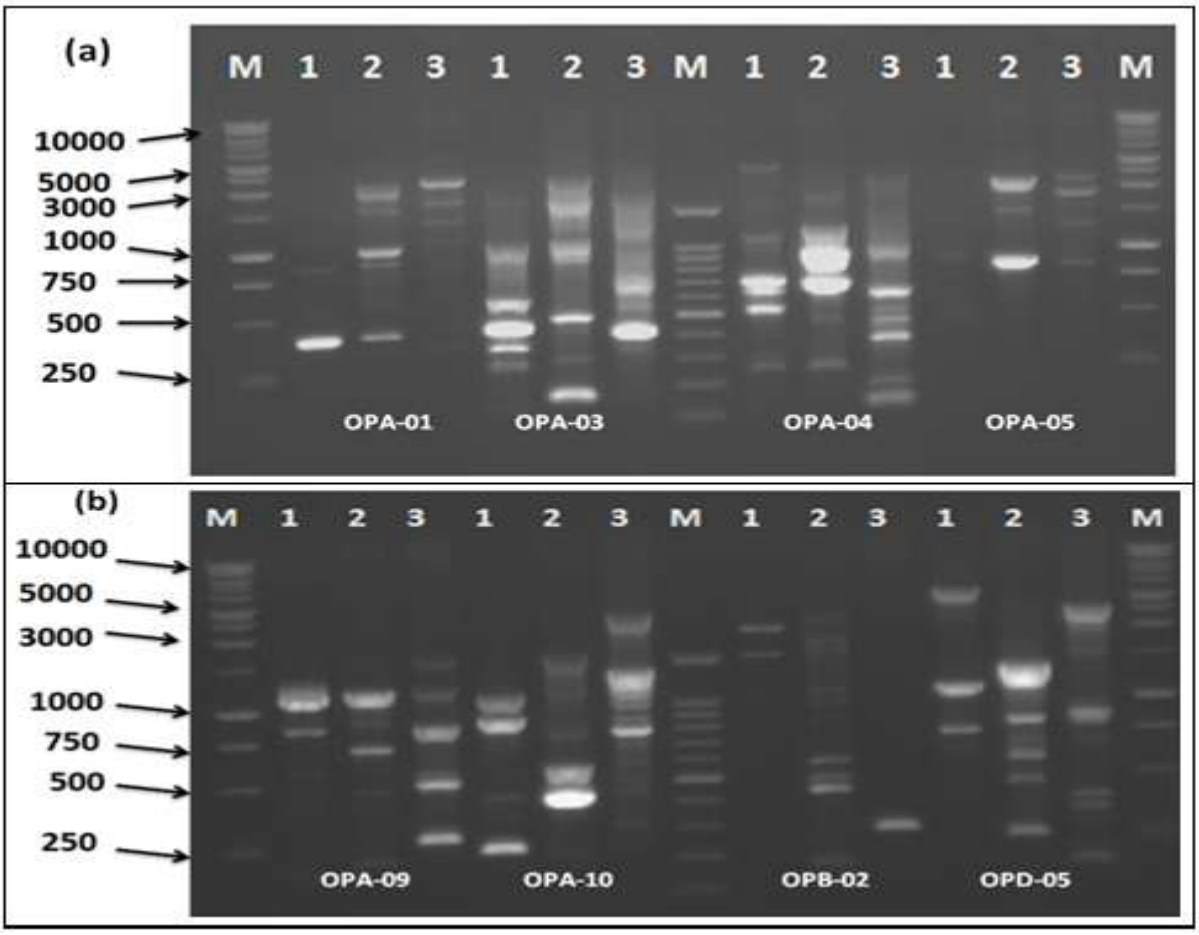

Fig. 5. RAPD profile of endophytic bacterial isolates generated with different primers; M = marker (1 kb. DNA ladder); 1 = W11, 2 = P31 and 3 = P35.

RAPD-PCR analysis

The genetic diversity among W11, P31, and P35 isolates was investigated using RAPD-PCR technique (Table 6 and Fig. 5). Out of eleven primers, eight were amplified successfully and given about 94 different bands that scored to determine genetic similarities among these strains. The fragments size range was 100-5920 bp (Fig. 4). Some primers were very successful in generating polymorphism with about 91.6 % polymorphic bands and were scored for analysis by NTSYS-PC Statistical Package (version 2.1) software program, using Gower general similarity coefficient. The three endophytic bacteria separated into two clusters. The W11 and P31 isolates belonged to the same cluster, while the P35 isolate belonged to the other cluster (Data not show). The W11 and P31 isolates was related to a similarity value of 64 %. On the other hand, the P35 strains were closely related to each other, which were isolated from potato plant; also, that quite different with a low similarity of about 62 %. Molecular-based approaches (e.g. rRNA cloning, sequencing, terminal restriction fragment length polymorphism and Pyrosequencing) to the study of microbial ecology generally reveal a broader microbial diversity than can be obtained by traditional cultivation methods (Hassan and Ismail

2014; Jhala *et al*., 2015). RAPD analysis is commonly used to evaluate differences among total bacterial aquatic communities (Hassan and Ismail 2014; Hassan *et al*., 2014). Oligonucleotide primers in RAPD characterization showed genetic variation among the Bacterial strains. In this study, amplified fragments showed 90 % unique polymorphic bands, the rest was monomorphic bands. We also, sequenced 16S rRNA from the W11, P31, and P35isolates. The analysis of the RAPD results and 16S rRNA showed that W11, P31 and P35 belongs to the *Arthrobacter arilaitensis, Bacillus anthracis*, and *Achromobacter spanius*. The extent of relatedness between bacterial isolates can be scrutinized by the construction of phylogenetic trees or dendrograms. The phylogenetic tree ascertains the genus to which the strain belongs and its closest neighbors, i.e., those sharing clade showing >97% 16S rRNA gene sequence similarity. These results agree with those of (Dawwam *et al*., 2013; Hassan and Ismail, 2014) who demonstrated that *Pseudomonas, Azotobacter* and *Azomonas* were close branches of the same phylogenetic tree.

Conclusion

Our study shows thatW11, isolates from the 42 tested Egyptian endophytic bacteria, show high proportion of phosphate solubilizing, growth promoting substances and increased shoot biomass. Therefore, these strains (biological fertilizer) are very promising for use as inoculants in crops. Future studies are needed to test the biotechnological potential of these strains under field conditions as a bioenhancer for different plants as an alternative source of chemical fertilizer.

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