Isolation and characterization of endophytic *Kosakonia radicincitans* to stimulate wheat growth in saline soil

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ABSTRACT

Among abiotic stresses, salinity is a significant limiting factor affecting agricultural productivity, survival, and production, resulting in significant economic losses. The goal of this study was to isolate and characterize the endophytic *Kosakonia radicincitans* bacteria and their plant growth-promoting (PGP) activities to determine the effect of its inoculation on wheat plants under salinity stress conditions. One endophytic bacterial isolate was obtained from root nodules of faba bean (*Vicia faba*) plants grown in the salt-affected clay soil of Egypt. The isolate was identified and characterized using 16S rRNA gene sequencing. This isolate Was phylogenetically related to *Kosakonia radicincitans* strain DSM 16656 with accession number OM980222.1. The isolate was evaluated for its ability to promote plant growth *in vitro*. Results revealed that the bacterial isolate of *Kosakonia radicincitans* produced indole-3-acetic acid (40.44 $\mu g ml^{-1}$) and exopolysaccharides (14.2 g/L), fixed nitrogen and could solubilize phosphate and potassium (273 mg/L and 42.8 µg/ml) respectively. In a field trial, this bacterial isolate reduced proline, and showed the best results for chlorophyll, grain yield, and 100-grain weight of wheat plants grown in the salt-affected soil as compared to the control plants. Wheat plants may benefit from the use of *Kosakonia radicincitans* as biofertilizers in sustainable farming techniques. Additional research in this area can support our findings.

***Keywords**: Endophytic bacteria, Kosakonia radicincitans, Wheat, 16S rRNA*

1. INTRODUCTION

Globally, rising salt levels are a serious concern and a significant barrier to food production [1]. Due to the rising annual need for food by a population that is expanding worldwide, the issue of soil salinity is critical. Yang et al.[2] confirmed that agriculture causes environmental damages, owing to large fertilizer inputs and land use practices. Living microbe cells called "biofertilizers" can replace artificial fertilizers because they give plants nutrition, prevent illnesses from spreading through the soil, and enhance the health and quality of the soil [3].

Endophytic N_2-fixing bacteria are of particular interest because they spend at least part of their lives inside plants and cause no visible harm to the host [4]. Despite the high specificity between Rhizobia and legumes, non-rhizobia have been discovered inside the root nodules, including *Pseudomonas fluorescence*, *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp., and *Paenibacillus* sp. [5, 6, 7]. Many studies confirmed the diversity of bacteria other than rhizobia that are associated with root nodules [8, 9]. De Meyer et al.[10] proved that *Enterobacter* and *Klebsiella*, members of the family Enterobacteriaceae, are known to live endophytically in a variety of crops' root nodules.
To comprehend the ecological significance of the relationship between endophytes and their host plants, studies on the interactions between them are crucial [11, 12]. These microorganisms could serve as biocontrol agents, release phytohormones like indole-3-acetic acid, cytokinins, and gibberellins, fix nitrogen so that it becomes available to the plant via cellular degeneration or active nitrogenous compound release [13].

Kosakonia radicincitans (DSM 16656) is a gram-negative, rod-shaped bacterium that stimulates plant growth and has been identified in the newly recognized genus *Kosakonia* of the Enterobacteriaceae [14]. Previously classified as Enterobacter, the genus *Kosakonia* was recently separated as a new genus [15]. The 16S rRNA gene is present in almost all bacteria and is usually used to their identification [16]. Many *Kosakonia* species have been isolated from different plants and have demonstrated the ability to promote plant growth [17]. The isolate of *K. radicincitans* (DSMT 16656) from wheat has plant growth promotion (PGP) [17] and nitrogen-fixing abilities [18]. Brock et al. [19] also demonstrated that *K. radicincitans* is capable of biologically fixing atmospheric nitrogen, producing hormones, and solubilizing rock phosphates. *K. radicincitans* DSM 16656 has shown distinct enhancement in growth, yield, and product quality in both glasshouse and field trials [20, 17], highlighting the strain's potential for various cultivation management systems.

Since wheat makes up most of the Egyptian diet, it is regarded as the country's first strategic food crop. Wheat straw is also a significant source of fodder [21]. This crop has been affected by water stress leading to decline in grain yield and quality. Different strategies to diminish the negative effects of salt stress have been developed to improve seed quality and production of wheat [22].

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The present work intend to isolate the endophytic bacterial strain *Kosakonia radicincitans* from the nodules of faba bean, characterize it through 16S rRNA based molecular technique and study of its plant growth-promoting activities as well as to determine the effect of its inoculation on wheat plants under salinity stress conditions.

2. MATERIAL AND METHODS

2.1 Isolation of endophytic bacteria

Nodules were chosen at random from roots of three faba bean plants grown in the salt-affected clay soil (Sahl El-Hussinia, El-Sharkia Governorate, Egypt), washed with sterile distilled water to remove soil particles, and surface sterilized with 95% alcohol for 30 s and 0.1 % (w/v) $HgCl_2$ for 2 min before being rinsed 6-8 times with sterile distilled water to completely remove $HgCl_2$. For the isolation of endophytic bacteria, the surface sterilized nodules were crushed and streaked on yeast-extract-mannitol agar (YEM) as indicated by [23, 24] and defined growth medium (DM) as indicated by [25]. Single colonies were purified further by streaking on the same medium repeatedly for 3 days at 28°C. The isolates were kept at -20°C in glycerol (20% v/v).

2.2 DNA isolation and 16S rRNA amplification

Isolation and purification of DNA was carried out according to the method described in [26]. The total genomic DNA was extracted from bacterial species and quality was evaluated on 1% agarose gel. Isolated DNA was amplified with 16S rRNA gene, the PCR amplification was carried out in 20 µl reaction solution containing 27F/1492R universal primers [27] as shown in Table 1. The amplification was performed: initial denaturation at 95ºC for 5 min followed by 35 cycles comprised of denaturation at 94ºC for 45 sec, 55º C for 60 sec for annealing, extension at 72º C for 60 sec and the final extension at 72ºC for 30 min. The PCR included a positive control (*E. coli* genomic DNA) and a negative control. The PCR products resolved by electrophoresis in 1.5 % agarose gel.

2.3 DNA Sequencing and Phylogenetic analysis

The PCR amplicon purification was carried out by column-based method using Montage PCR Clean-up kit (Macro gen). The purified PCR products were sequenced by using two primers, as described in Table 1. Sequencing was performed using Big Dye terminator cycle sequencing kit and the products were resolved on an Applied Bio systems model 3730XL automated DNA sequencing system (Applied Bio Systems, USA). Identification was performed by (GenBank) using the Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was done using the MEGA X program [28].

Table 1. The primer sequences used for 16S rRNA gene amplification.

Primers	Sequences
16S rRNA	27F 3' AGA GTT TGA TCM TGG CTC AG 5'
	1492R 3' TAC GGY TAC CTT GTT ACG ACT T 5'
Sequencing	518F 3' CCA GCA GCC GCG GTA ATA CG 5'
	800R 3' TAC CAG GGT ATC TAA TCC 5'

2.4 Plant growth promoting attributes

Indole acetic acid (IAA) production

According to the modified **Khamna et al.**[29] technique, isolate was used for IAA production. The test isolate was inoculated into Luria-Bertani (LB) broth that had been supplemented with tryptophan. In a shaker incubator, a flask was incubated at 35°C for three days at 150 rpm. The well-developed isolate culture was centrifuged at 8000 rpm for 20 min. Then, 2 mL of Salkowski reagent and three drops of orthophosphoric acid were added to 1 mL of the supernatant. For one to two hours, the mixture was incubated in darkness. At 530 nm, the isolate's absorbance was observed. The IAA concentration in cell-free supernatant was determined colorimetrically using a standard curve prepared from authentic IAA (Sigma Chemical, USA).

Exopolysaccharides (EPS) production

The bacterial isolate was inoculated into a conical flask containing 100 ml of yeast extract mannitol (YEM) broth to estimate EPS production. The inoculated flasks were incubated for 72 hours at 30°C and 200 rpm on a rotary shaker. The culture broth was centrifuged at 3500 *xg* after incubation, and the supernatant was mixed with two volumes of acetone. Centrifugation at 3500*xg* for 30 min was used to collect the crude polysaccharides that had developed. After being washed with distilled water and acetone alternately, the EPS was transferred to filter paper and weighed after being dried overnight at 105^{o}C. [30].

Nitrogen fixing ability

Nitrogen fixing ability was examined using Jensen's medium and Bromothymol Blue (BTB) as a color indicator according to [31]. Nitrogen fixing activity of the endophytic bacterial isolate was estimated by colour alteration of media to dark blue after 3-8 days. Here is the composition of Jensen's medium: 20 g sucrose, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 0.1 g Fe_2S0_4, 0.005 g $Na_2MoO_4 . 2H_2O$, 2 g $CaCO_3$, and 15g Bacto agar.

Estimation of phosphate solubilization

Qualitative determination of phosphate solubilization was performed on Pikovskaya's agar plate (PVK) medium [32]. Isolate was spot inoculated and incubated at 28 ± 2°C for 3-7 days. The halo zone formed surrounding the colony revealed phosphate solubilization and was expressed as solubilizing efficiency (SE%) [33]**.**

$$\text{Phosphate solubilization efficiency (\%)} = \frac{\text{Solubilization diameter (S) x100}}{\text{growth diameter}}$$

Estimation of phosphate solubilization was done quantitatively using ascorbic acid molybdate method [34]**.** Phosphorus content was expressed as mg/l and pH of the medium was recorded at the same time.

Estimation of potassium solubilization

The spotting method was used to investigate potassium solubilization by bacterial isolate on modified Alexandrov medium containing (5.0 g Glucose, 0.5 g $MgSO_4.7H_2O$, 0.1g $CaCO_3$, 0.006 g $FeCl_3$, 2.0 g Ca_3PO_4, 3.0 g insoluble mica powder as a potassium source and 20.0 g agar) in litre of deionized water [35]. Plates were incubated for 5 days at 28°C±2. The ability of bacterial isolate to form solubilization zones was used to detect potassium solubilization. Quantitative estimation of potassium release was performed by growing bacterial isolate in Aleksandrov broth medium and incubated for 5 days at 28°C±2. Following the incubation, the broth culture was filtered through Whatman No. 1 filter paper and centrifuged for 20 minutes at 12,000 rpm. The soluble K content in the supernatant was measured using flame photometer [36].

2.5 Field Experiment

A field experiment was conducted at the farm El-Rowad village in Sahl El-Hussinia, El-Sharkia Governorate, Egypt during the winter growing season of 2018. Wheat grains (*Triticum aestivum* L. cv. Sakha 93) were obtained from Field Crops Research Institute, Agricultural Research Centre, Giza, and were inoculated with vermiculite-based inoculants that had been gamma irradiated. Three treatments were tested and a randomized completed design with three replicates was used to determine the role of isolated bacteria in alleviating salt stress in wheat plants grown in a saline soil as compared to the control and *Azotobacter chroococcum* (a ref PGPB and nitrogen fixer for non legumes) that was obtained from Biofertilizers Production Unit, Agricultural Microbiology Research Department at Soils, Water and Environment Research Institute, Agricultural Research Centre, Giza. The treatments were as follow:

1. Control (Recommended dose of NPK)
2. *Azotobacter chroococcum* + half dose of NPK
3. *Kosakonia radicincitans* + half dose of NPK

Proline determination

Extraction and determination of proline in shoot of wheat plants (60 days from sowing) were performed according to the method of [37]. Proline content was measured by UV/Vis Spectrophotometer (Thermo Spectronic, Waltham, MA, USA) at 520 nm and calculated as µ mole proline / g of fresh weight material using a standard curve prepared from proline.

Estimation of total chlorophyll content

The chlorophyll content was determined at 60 days after planting using portable chlorophyll meter (SPAD-502) [38].

Determination of N, P, K contents and protein

Total contents of nitrogen, phosphorus and potassium of the harvested wheat grains were determined according to [39], and the protein was calculated by multiplying total nitrogen content by 5.75 [40].

Soil analysis

The main soil properties of the experimental soil were determined as shown in Table (2) according to the methods described by [41]. Soil pH and total soluble salt (EC) were measured in soil paste according to [42]. Available N was measured according to the modified Kjeldahel method [41]. Available P was determined colorimetrically according to Olsen’s method [42]. Available K was determined using the Flame-Photometer and Available micronutrients were determined as described by [43].

Table (2): Physical and chemical properties of the soil sample before planting

<table>
<tr><th>Course sand (%)</th><th>Fin sand (%)</th><th>Silt (%)</th><th colspan="2">Clay (%)</th><th>Texture</th><th colspan="2">O.M (%)</th><th>$CaCO_3$ (%)</th></tr>
<tr><td>4.2</td><td>15.2</td><td>35.5</td><td colspan="2">45.3</td><td>Clay</td><td colspan="2">0.47</td><td>11.5</td></tr>
<tr><td rowspan="2">pH (1:2.5)</td><td rowspan="2">EC (dS/m)</td><td colspan="4">Cations (meq^{-1})</td><td colspan="3">Anions (meq^{-1})</td></tr>
<tr><td>Ca^{++}</td><td>Mg^{++}</td><td>Na^{+}</td><td>K^{+}</td><td>HCO^{-}_{3}</td><td>Cl^{-}</td><td>SO^{--}_{4}</td></tr>
<tr><td>8.12</td><td>12.45</td><td>22.5</td><td>35.5</td><td>68.5</td><td>1.48</td><td>4.5</td><td>95.5</td><td>27.98</td></tr>
<tr><td>N</td><td>P</td><td>K</td><td>Zn</td><td>Mn</td><td>Fe</td><td colspan="3">Cu</td></tr>
<tr><td colspan="9">PPM</td></tr>
<tr><td>107.1</td><td>2.95</td><td>171.16</td><td>0.868</td><td>2.16</td><td>1.85</td><td colspan="3">0.61</td></tr>
</table>

2.6 Statistical analysis

The significance of variation in the yield, nutrients uptake and protein contents such as N, P, K, protein, Proline and chlorophyll contents in relation to the different treatments were assessed using one-way analysis of variance technique (ANOVA). The differences were tested using Tukey's test. The applications of these techniques were according to SPSS program ver. 20. [44] **spass**.

3. RESULTS AND DISCUSSION

3.1 Appearance of bacterial isolate

From root nodules of faba bean plants, gram-negative, rod-shaped bacterium was isolated. When cultivated on YEMA containing Congo red, the isolate did not absorb the color red.

3.2 Molecular characterization of the isolates by 16S rRNA gene

Molecular characterization of the isolate *Kosakonia radicincitans* strain DSM 16656 was achieved using the 16S rRNA gene. The amplified size designed for the 16S rRNA gene, about 1500 bp, was used (Table1). The PCR products were separated in gel, and the DNA bands conforming to the expected size of the gene were purified from the gel for sequencing. With the use of the forward and reverse primers, the product was sequenced, resulting in a 940 bp long sequence. This isolate's partial sequence was aligned with the partial sequences of the neighbor-joining sequences in GenBank and then deposited under accession number MO980222.1 and described by the phylogenetic tree(AM3) in Figure 1.

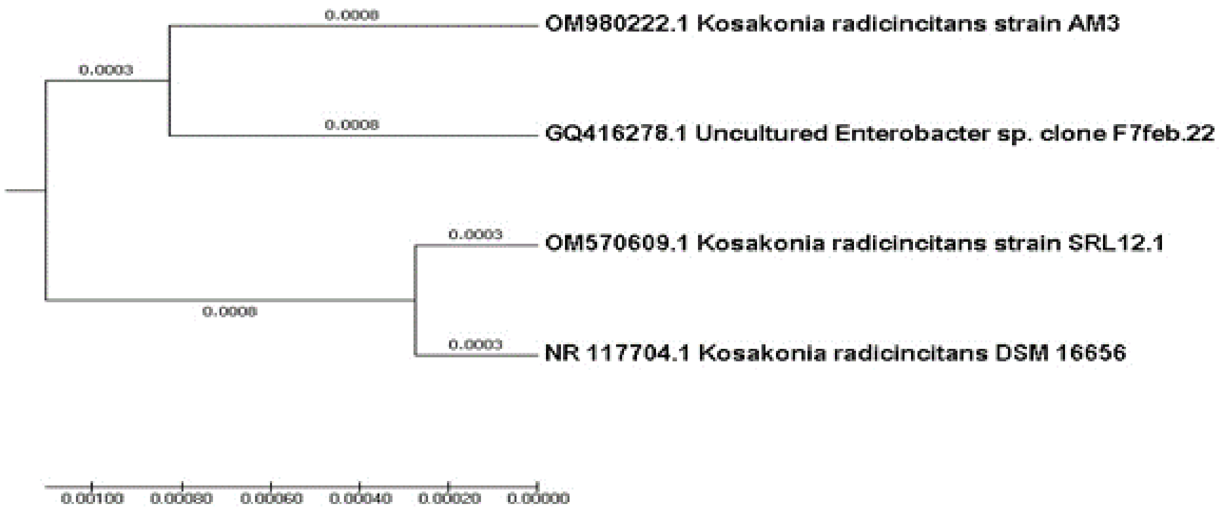

Figure 1. Phylogenetic tree based on 16S rDNA sequence analysis indicates the phylogenetic relationship of the bacterial isolate.

3.3 In Vitro Assessment of plant-growth-promoting activities

Bacterial isolate of *Kosakonia radicincitans* was examined for its plant growth promoting attributes in terms of indole-3-acetic acid (IAA) production, exopolysaccharides production, nitrogen fixation, phosphate (P) and potassium (K) solubilization (Table 3 and Fig. 2). Results indicated that the isolate was able to produce indole acetic acid (40.44 µg/ml) in the cultural filtrate. The results was in agreement with [45] who proved that *K. radicincitans* produced a significant IAA and the capacity to produce IAA suggested that the isolate could be used to regulate growth.

Concerning the releasing amounts of EPS, it revealed that the bacterial isolate could produce EPS (14.2 g/L). These results are in agreement with [46] who used EPS producing strain of *Kosakonia cowanii* for biosynthesis of EPS by several agro-industrial residues.

Jensen's medium, which is nitrogen-free, was created to find and grow bacteria that fix nitrogen. Several nitrogen fixing endophytic bacteria is acid producing and changes the color of media from greenish blue to yellow. The *in vitro* test on Jensen's nitrogen free media showed that the new bacterial isolate could grow on Jensen's medium and was acid producer. This agrees with [47] who suggested that *Kosakonia* sp. was an associative nitrogen-fixing bacteria or endophytic nitrogen-fixing bacteria.

It is known that microorganisms can solubilize phosphorous, making it easily accessible to plants and lowering the demand for phosphate fertilizers [48, 49]. In the current study, the new isolate of *K. radicincitans* showed halo zone on the Pikovskaya's agar medium that recorded solubilization efficiency up to 100%, and P liberated in the medium was 273 mg/L indicating its ability to solubilize tricalcium phosphate (Table 3 and Fig. 2). This result is in agreement with [25] who proved that osmotic stress on *K. radicincitans* cells increased their capacity to solubilize phosphate to 357.7 mg L^{-1} from 290.3 mg L^{-1} in non-stressed cells.

The ability to solubilize potassium by the bacterial isolate was tested. The new isolate of *K. radicincitans* was able to solubilize potassium effectively and recorded higher solubilization efficiency up to 266% as shown in (Table 3 and Fig. 2). Quantitative estimation of K solubilization was performed and the amount of K liberated in the broth medium was 42.8µg/ml. This may happened due to the production of organic and inorganic acids during growth of bacterial isolate which is a major mechanism for mineral solubilization as indicated by [50], where there was a decrease in final pH, reaching a value 5.3. This agrees with [51] who proved that pH decrease resulting from the release of organic acids could be related to P & K solubilization.These PGP activities may be due to sharing the bulk of genes that involved in plant growth-promotion as indicated with [47] who found that there were many genes related to promoting plant growth in Enterobacter FY-07.

Table (3). Production of IAA, EPS, solubilization of phosphate and potassium of the isolate

Isolate	IAA (µg ml^{-1})	EPS (g/l)	Nitrogen fixation	Final pH	Phosphate solubilization		Potassium solubilization	
					Solubilization efficiency (SE)%	P-liberated (mg/l)	Solubilization efficiency (SE)%	K-liberated (µg/ml)
AM3	40.44	14.2	+	5.3	100	273	266	42.8

a **d** **b** **e** **c**

Figure (2). (a) Isolate grown on Congo red YEMA medium (b) IAA production, (c) Exopolysaccharides production (d) Phosphate solubilization (e) Potassium solubilization by bacterial isolate.

3.4 Proline content in wheat

Under salt stress, most of plant species show a striking rise in their proline content [52]. It is obvious from the present results that application of new isolate onto wheat plants lowered the proline accumulation in these plants as compared to control and inoculant of *Azotobacter chroococcum* a ref bacterial strain (Fig 3). These results are consistent with [53]**Nadeem et al. (2007)** who confirmed that proline content in maize increased under saline stress however it was decreased by inoculation with plant growth promoting bacteria. These findings may be due to the synthesis indole acetic acid or exopolysaccharides by the bacterial isolate as reported by [54, 55] who showed that foliar applications of IAA and inoculation with EPS producing bacteria as *A. chroococcum* reduced proline content in the leaves of maize plants under salt stress.

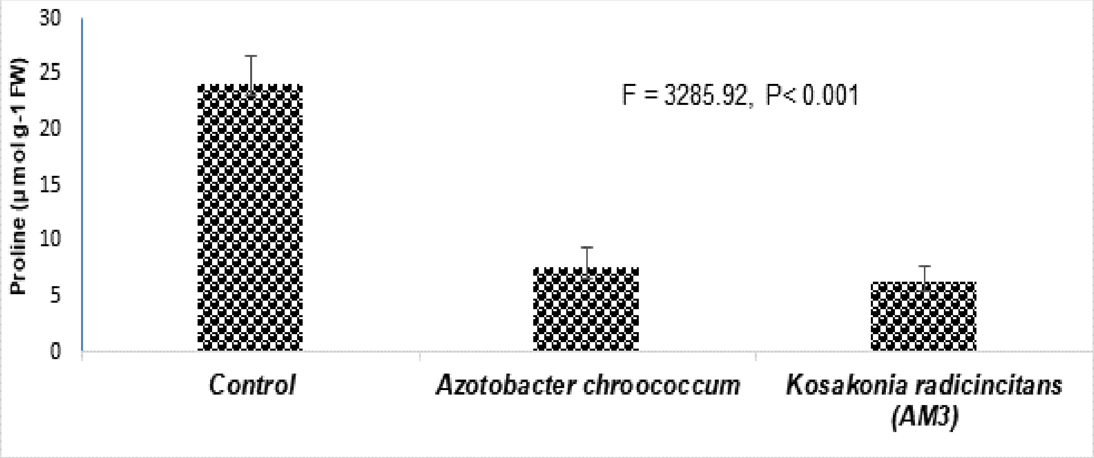

Figure 3. Proline content as affected by bacterial treatments

3.5 Total chlorophyll content

Results in Fig. 4 show that maximum content of chlorophyll in wheat leaves was obtained using bacterial isolate of *Kosakonia radicincitans* as compared to control or *Azotobacter* sp. In this respect Mehrvarz et al.[56] showed that inoculation of phosphate solubilizing bacteria increased the chlorophyll content and photosynthesis rates of barely plants

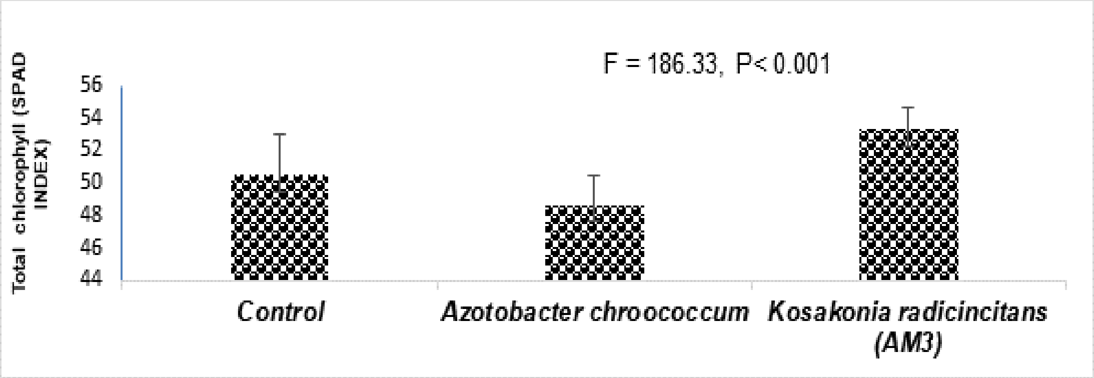

Figure 4. Total chlorophyll in wheat plant as affected by treatments

3.6 Yield, nutrients uptake and protein contents in wheat

There was a significant increase over *A. chroococcum* and the control in grain yield of wheat by the application of the new isolate of *K. radicincitans* (Table 4) that gave the highest value (17.6 Ardab/fed). Moreover, 100-grains weight of wheat was significantly influenced by the application of new bacterial isolate followed by *A. chroococcum* as compared to the control but there was non-significant increase in straw yield of wheat in case of using the new isolate. These outcomes might be attributable to the isolate's capacity to create IAA, as suggested by [57] who found that the administration of IAA boosted wheat grain production.

In terms of the concentration of the nutrients in wheat plants as a result of treatments, data in (Table 4) revealed that the contents of N, P, K, and protein are not significantly different by application of new bacterial isolate + half dose of NPK as compared to *A. chroococcum*+ half dose of NPK and the control treatment (Recommended dose of NPK). This could be due to the ability of the bacterial isolate to fix nitrogen and solubilize both phosphate, and potassium. Upon the inoculation of diverse plant species. Berger et al.[58] demonstrated that numerous Kosakonia bacterial strains could enhance plant development and increase yields where they could fix atmospheric nitrogen using nitrogenase that was present inside the bacteria [59]. Brock et al. [19] confirmed the capacity of *K. radicincitans* to synthesize hormones, physiologically fix atmospheric nitrogen, and dissolve rock phosphates. In this context, According to Nunes et al.[60]**,** *K. radicincitans* MUSA4 possessed a number of traits that aided in the promotion of plant growth, such as the production of indole acetic acid, the solubilization of phosphate, and the fixation of nitrogen. Additionally, the bacterial genome contained the nif and anf gene clusters, which, respectively, encode the Fe-Mo and Fe-Fe nitrogenase systems. This was supported by [61] who found that *K. radicincitans* had multiple copies of complex gene clusters that enabled it to colonize and have growth-promoting effects on a variety of plants.

Table 4. Yield components, N, P, K and protein content in wheat at harvest.

Treatments	**Grain Ardab / fed**	**Straw Ton/fed**	**Wt. 100 grains**	**N%**	**P%**	**K%**	**Protein**
Control (full dose NPK)	**10.7^{c}**	**1.7^{a}**	**3.33^{b}**	**1.08^{a}**	**0.560^{a}**	**0.3^{a}**	**6.21^{a}**
A. chroococcum+ half dose of NPK	**15.6^{b}**	**1.7^{a}**	**3.73^{ab}**	**1.09^{a}**	**0.590^{a}**	**0.6^{a}**	**6.26^{a}**
***K. radicincitans* (AM3) +** half dose of NPK	**17.6^{a}**	**1.8^{a}**	**4.02^{a}**	**1.56^{a}**	**0.585^{a}**	**0.5^{a}**	**8.97^{a}**
F-Value	**391.14*****	**0.23^{ns}**	**12.00****	**3.98^{ns}**	**0.01^{ns}**	**1.75^{ns}**	**3.99^{ns}**

**: P<0.01, ***: P<0.001, ns: non-significant. Means in the same column followed by the same letters are not significantly different (P<0.05) according to Tukey test.

4. CONCLUSION

The *Kosakonia radicincitans* PGPB isolate produced EPS, fixed nitrogen, indole-3-acetic acid, and was able to solubilize phosphate and potassium *in vitro*. In comparison to control plants, the PGPB isolate of *K. radicincitans* produced wheat plants with the highest levels of chlorophyll, grain production, and 100-grain weight. It can be concluded that using *K. radicincitans* as biofertilizers in sustainable farming methods may be advantageous for wheat plants. Our findings may be confirmed by further study in this area.

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