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New Strains of Plant Growth-Promoting Rhizobacteria in Combinations with Humic Acid to Enhance Squash Growth under Saline Stress

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THE STUDY aims at assessing the potentials of some new salt-tolerant isolatesas plant growth-promoting rhizobacteria (PGPR) under saline condition. Three of the 165 isolates that grew on the presence of 2-20% NaCl were high salt-tolerant and had many features of PGPR. They were identified by using 16S rRNA gene sequencing.The nearest species to our isolates were *Paenibacillus polymyxa* (GQ375783.1), *Ochrobactrum intermedium* (MG309678.1) and *Enterobacter cloacae* (MG309676.1) with nucleotide similarity 99, 97 and 99%, respectively. In 2017, a greenhouse trial was carried out to assess the efficiency of these novice isolates combined with humic acid and doses of inorganic fertilizerson squash (*Cucurbita pepo* L.) growth and productivity.Data showed that fertilizing the soil with a full dose of inorganic fertilizers only lead to decrease the values of dehydrogenase, alkaline phosphatase and nitrogenase activity at all determination periods. While, soil inoculation with PGPR strains combined with NPK 50% and humic acid spraying gave the higher records of all enzyme activities. Moreover, data showed that the highest values of peroxidase and polyphenol oxidase activity were observed in squash that sprayed with humic acid and inoculated with salt-tolerant PGPR strains combined with half dose ofinorganic-NPK. Generally, inoculating squash with salt-tolerant PGPR strains hasa positive effect on nutrients uptake,growth characteristics and yield and yield componentsas well as fruits quality. So, it could be recommended as biofertilizers to promote plant growth, increase crop production under salinity condition, decrease production costs and reduce pollution.

Keywords: PGPR, Microbial enzymes, Growth characteristics, Squash

Introduction

Salinity is one of the most severe biotic stress that limits plant growth and productivity. Stress negatively affects agricultural crop yields throughout the worldwide, affecting production, whether for subsistence or economic gain. At present, about 20 % of the world's cultivated land and approximately half of all irrigated land and 2.1 % of the dry agriculture land are affected by salinity (FAO, 2018).Salinization is spreading more rapidly in irrigated lands because of inappropriate management of irrigation, water quality and drainage (Hessini et al, 2015). It is an ever-increasing problem in the arid and semi-arid regions (Mwai, 2001). Salinity can negatively impact plants through three major components; osmotic, nutritious, and toxic stresses. When exposed to salinity, growth, development, and yield of most cultivated crops tend to decline with consequent reduction in their economic value (Rafie and El-Boraie, 2017).

Squash (*Cucurbita pepo* L.) is one of the most popular vegetable crops for humanoid nutrition and the most important cash crops, especially, in

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newly reclaimed areas of Egypt (Abou El Seoud and Abd El Hamid, 2020). Its fruits are very low in calories (19 kcal/100 g), moisture (94.8 g), edible portion (94%) and have large amounts of fiber (0.8 g) (El-Shoura, 2020). Squash is moderately tolerant to salinity. High salinity causes a reduction in both yield and quality of it (Rouphaelet al. 2006). Total fruit yield per plant significantly decreased with increasing salinity (Navarro et al, 2010).

Plant growth-promoting rhizobacteria (PGPR) can enhance crop growth in saline soil. It is suggested that root-colonizing bacteria that produce phytohormones may stimulate plant growth and help in nutrient recycling in the rhizosphere and thus PGPR can alleviate salinity effects. In addition, PGPR might also increase nutrient uptake by plants from soil and thereby reduce inorganic fertilizer requirements. As well as, PGPR suppress the pathogens by producing antibiotics and siderophores or bacterial and fungal antagonistic substances and/or by producing biologically active substances such as hydrogen cyanide (HCN) and ammonia (Nishma et al. 2014; El-Sayed andHagab, 2020; Yaseen et al. 2020; Riaz et al, 2021 and Abdel Latef et al. 2021).

The diversity of PGPR in the rhizosphere along with their colonization ability and mechanism of action would facilitate their wider application in the management of sustainable agricultural crop production. Further, PGPR have been used as a connecting link between plants and microbes that could express antagonistic and synergistic interactions with microorganisms and the soil (Shukla, 2019).

Recently, biostimulators as biological methods to prevent the application of chemical products and overcome injurious impact of salinity in agriculture have received considerable attention. Among the several categories of bio-stimulators are chitosan and humic acid (Ashour et al. 2021). Humic substances are well known as stimulators of plant growth by some mechanisms such as enhancing uptake and transport of nutrients, reducing uptake of toxic elements, increasing membrane permeability, respiration, photosynthesis and phosphate uptake and acting as growth hormones (Aydin et al, 2012 and De Hita, 2020). Furthermore, the effect of HA on amelioration salinity stress is related to its role on osmotic adjust by maintaining water uptake and cell turgor, inducing antioxidant enzymes that scavenging reactive oxygen species (ROS), enhancing levels of endogenous proline and decreasing membrane leakage that consider indicators of better adaptation to saline (Van Oostenet al, 2017 and Ashour et al, 2021).

The objective of this research is to evaluate the efficiency of salt-tolerant PGPR strains combined with either chemical fertilization or foliar application with humic acid on growth performance and yield of squash.

Materials and Methods

Soil sampling

Samples of salt-affected soil were collected from three locations: El-Behira (31°00'17.7"N 29°59'21.8"E), Kafr El-Sheikh (31°24'29.5"N 30°47'56.4"E) and Alexandria (31°18'49.6"N

TABLE 1. Particle size distribution andchemicalanalysesof soil samples

Soil sample	Organic Matter g.kg^{-1}	Mechanical Composition %			Textural class	pH	EC dS/m	Soluble anions and cations in soil mmolc kg^{-1}							
		Clay	Silt	Sand				Na^+	K^+	Ca^{++}	Mg^{++}	$CO_3^=$	HCO_3^-	Cl^-	SO_4^-
Soil (1)	12.4	23	27	50	Clay loam	8.41	8.95	28.02	23.15	18.63	19.7	0	27.93	32.55	29.02
Soil (2)	17.4	32	15	53	Sandy clay loam	7.26	8.62	25.86	21.75	21.8	16.82	0	28.7	26.03	31.5
Soil (3)	6.3	20	55	25	Silty clay loam	8.58	11.3	38.61	18.03	24.74	31.62	0	28.2	52.84	31.96

Soil (1): El-Behira; Soil (2): Kafr El-Sheikh; Soil (3): Alexandria

30°02'11.7"E) Governorates, Egypt, for isolating salt-tolerant PGPR. Mechanical and chemical analyses of soil (Table 1).

Isolation of salt-tolerant PGPR isolates

The isolation process was carried out using pouring and streaking plates method on different specific microbiological media named Ashby's medium (Abdel- Malek and Ishac,1986), King'smedium (King etal, 1954), modified Bunt & Roviraagar medium (Abdel-Hafez, 1966). Isolates were sub-cultured several times on their specific media for purification and then maintained as a stock culture at 4-5°C for the succeeding studies.

Screening for prospective PGPR characteristics

Primary screening of rhizobacterial isolates were conducted under saline stress in presence of different sodium chloride concentrations using nutrient broth to give final concentrations of 2, 4,6,8,10,12,15,18 and 20%. After inoculation, cultures were incubation at 37°C for 7 days in a rotary shaker (150rpm).

Biological activities of PGPR isolates

The secondary screening was considered under salinity condition (4%NaCl) for production of indole acetic acid (IAA) that was determined according to Gilickmann and Dessaux (1995); Gibberellic acid (GA) (Holbrook et al, 1961); siderophores (Alexander and Zuberer, 1991); catechol-type siderophores (Carson et al, 1992); HCN and ammonia (Lorck, 1948; and Cappuccino and Sherman, 1992); Nitrogenase activity (Dilworth, 1966). Moreover, colonization capability, phosphate-solubilization (Nguyen et al, 1992); phosphate solubilization (Nautiyal, 1999); and both qualitative and quantitative K solubilization (Manib et al, 1986).

Genetic analyses using16SrRNA sequences

The most potent isolate was completely identified using16SrRNA sequence technique as the following. The isolate was grown in nutrient broth onarotary shaker (120rpm)at 28°C for 24 hours. Bacterial Gene Jetgenomic DNApurificationKit(ThermoK0721)was usedtoextractDNAaccordingtoSIGMA company instructions. The obtained sequence for the 16SrRNA gene was analyzed by Vec Screen tool for vector contamination (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Also, NEbcuterV2.0 was used to create a restriction map and to identify the GC content of the obtained sequence (Vincze etal, 2003, http://nc2.neb.com/NEBcutter2/). ORFfinder software was used to obtain possible ORFs of the obtained sequence. Also, Jalview software was used to show SNPs and consensus resulted from the alignment of our bacteriali ate obtained sequence and the nearest bacterial strainin NCBI database (http://www.jalview.org/). The sequence was registered in NCBI database under accession number MG309677.1(http://www.ncbi.nlm.nih.gov/nuccore/MG309677.1). Construction of the phylogenetic tree was done by using Clustal Omega and MEGA6 software.

Greenhouse study

In 2017, a greenhouse experiment was conducted at the Faculty of Agriculture Experiment Station. Soil, from El-Behira Location, was put into 40-cm plastic pots. Each pot was filled withtwenty kilograms of soil that was mixed with a 100-g of 1:1 herbal plant residue to cattle manure. This whole mixture has: pH 7.6, EC 3.1 dSm-1, total N 1.21%, total P 0.91%, and porosity 62.67%. Prior to seeding, squash seeds, cv. Yara, were surface sterilized with 1% Ca (OCl) 2 for three min, rinsed thoroughly in running sterilized water and dried aseptically. The seeds in inoculated treatments were soaked for 30 min in the mixture of 10 % Arabic gum solution (as a sticker agent)cell suspension of either P. polymyxa (4 x 10^8 CFU/ml), O. intermedium (3.5 x108 CFU/ml) and E. cloaca (5 x10^8 CFU/ml) before sowing. To boost inoculation, mixture of PGPR inoculum was added three times throughout the growing season, each at a rate of 50 $mlpot^{-1}$with water irrigation. Following planting (two seed per pot), pots were directly irrigated with tap water to provide suitable moisture for inocula. In three equal doses, nitrogen, phosphorus and potassium were added at a rate of 1.2, 0.6 and 0.9 $g.pot^{-1}$ as ammonium sulphate (20.5% N), calcium superphosphate (15.5 P_2O_5) and potassium sulphate (48% K_2O), respectively. In addition, a foliar application of humic acid -HA- (83%) was applied at a rate of 0.02 g. pot^{-1} 15-, 30-, and 45-days after sowing (DAS). The experiment was laid out in three randomized complete blocks, each block contains 8 treatments: 100% NPK, PGPR+ 75% NPK, PGPR+ 50% NPK, PGPR+ 25% NPK, 100% NPK+ HA, PGPR+ 75% NPK+ HA, PGPR+ 50% NPK+ HA, PGPR+ 25% NPK+ HA. Treatment means were tested using Duncan's Multiple Range Test.

Inoculum preparation

PGPR inocula contain three salt-tolerant PGPR strains: *Paenibacillus polymyxa* (GQ375783.1), *Ochrobactrum intermedium* (MG309678.1), and Enterobacter cloacae (MG309676.1). All were 3-d old, and later on all were incubated at 30 0C for 3 d following each prepared in a specific broth medium. A modified nutrient-broth (Atlas,1995) was inoculated by P. polymyxa, Aleksandrov broth (Hu et al, 2006) by O. intermedium, and Pikovskaya broth (Pikovskaya, 1948) by E. cloacae.

Microbiological activities

Dehydrogenase, phosphatase and nitrogenase activities were estimated 15, 30 and 45 DAP. Dehydrogenase was assayed in the soil as previously mentioned according to Hardy et al, (1973). The alkaline phosphatase activity was measured according to Tabatabai (1982). Nitrogenase activity was assayed based on the reduction of acetylene to ethylene as quantities by gas chromatography. Acetylene reduction was performed by a modified protocol (Silvester, 1983).

Soil chemical analyses

Available nitrogen was determined according to Bremner and Keeny (1965). Available phosphorus was determined according to Watanabe and Oleson (1965). Soluble-potassium was determined according to Jackson (1973).

Peroxidase and polyphenol oxidase assessment

A 0.5 g-sample of fresh leaves was ground with 0.2 M tris HCl buffer (pH 7.8) containing 14 mM β-mercaptoethanol at a rate 1/3 w/v. The extracts were centrifuged at 10.000 rpm for 20 minutes at 4°C (Tuzunet al, 1989). The supernatants were used to determine peroxidase and polyphenol oxidase activity. Peroxidase and polyphenol oxidase activity were determined at 30 DAP according to Allam and Hollis, (1972)and Matta and Dimond (1963), respectively.

Squash growth, macro element content, and yield and fruit quality

A random 3-plant sample was selected to determine squash vegetative growth characters:plant height, total plant fresh weight, shoot and root dry weights, shoot and root lengths, leaves plant-1, flowers plant-1.At flowering, shoots were dried at 70°C and used for determination of total nitrogen, phosphorus and potassium (Chapman and Pratt, 1978; Page et al, 1982; and A.O.A.C., 2005). Fruits were harvested at proper maturity stage, then counted, weighed to estimate: fruit plant-1, plant yield. Total soluble solids (T.S.S.) were determined as fruit quality in the filtrate by Carl Zeiss refractometer. Total nitrogen content was estimated in fruits according to (A.O.A.C., 2005), total crude protein percentage was calculated by multiplying N-values by 5.7.

Statistical analysis

Statistical analysis was carried out according to Snedecor and Cochran (1989). The differences between the means value of various treatments were compared by Duncan's multiple range test (Duncan's, 1955).

Results and Discussion

Screening of salt-tolerant PGPR isolates

One hundred and sixty-five bacterial isolates were obtained and consequently used for primary and secondary screening. All examined rhizobacterial isolates showed salt tolerance up to 6% sodium chloride. While, 70.9%, 50.9% and 31.5% of the examined isolates showed salt tolerance at sodium chloride concentrations of 10%, 12% and 15%, respectively. Only 8.5% of the examined isolates showed salt tolerance at concentration of 20% sodium chloride. After secondary screening three isolates (STB6, STB121 and STB165) were chosen according to their superiority for NaCl tolerance, indole acetic acid (IAA), Gibberellins, siderophores, hydrogen cyanide (HCN) and ammonia production. Moreover, nitrogen fixation, phosphate and silicate solubilization (Table 2).

Identification of most potent PGPR isolates using 16S rRNA sequences

The most potent isolates were chosen and identified by 16S rRNA gene sequence analysis to ascertain their taxonomic positions (Table 3 and Fig. 1-3). Sequencing results were registered in NCBI database and analysis of the obtained sequence via the Vecscreen database showed no contamination with vector sequence.The FASTA homology showed that the16S rRNA gene sequences of the selected isolates had 99, 97 and 99% nucleotide similarity with that of Paenibacilluspolymyxa, Ochrobactrum intermedium and Enterobacter cloacae strains, respectively. These results were confirmed by the phylogenetic position of the obtained isolates. Also, the restriction Maps of the obtained 16S rRNA partial sequence were done. Calculating the pairwise alignment analysis, exhibited 4, 18 and 2 SNPs between the sequence of the obtained isolates and the nearest registered bacterial strain in NCBI database, *Paenibacillus polymyxa, Ochrobactrum intermedium* and *Enterobacter cloacae* strains, respectively for 16S rRNA gene.

Interaction effect of salt-tolerant PGPR strains, inorganic fertilizers and/or humic acid on some microbial enzymes activity in squash rhizosphere

Dehydrogenase (DH) is a guide of respiration rate and total microbial activity in soil. Whereas, alkaline phosphatase and nitrogenase activities are guides of mineralization processes of organic phosphorus substrates and as an indication of N2-fixers activity, respectively. Table 4 shows that DHA, PA and NA in saline soil that inoculated with salt-tolerant PGPR strains increased relative to the Control.PGPR strains can produce certain enzymes such as dehydrogenase, nitrogenase, lipases, phosphatases and proteases (Gupta et al. 2015). Through the activity of these enzymes, PGPR play a very significant role in plant growth promotion.

TABLE 2. Over-all activities by the selected and more potent salt-tolerant PGPR isolates in media amended with 4% NaCl

Beneficial activities	Description	Isolate code		
		STB6	STB121	STB165
Maximum NaCl concentration	%	20	10	10
IAA	(µg /ml)	8.02	11.74	21.46
Gibberellin	(µg /ml)	10.68	32.05	23.89
Siderophores	Qualitative	++	++	++
Catecholate	Qualitative	+	+	+
HCN	Qualitative	+++	++	+++
Ammonia	Qualitative	+	++	+
P. solubilization	Solubilization efficiency (%)	290	300	320
	Amounts of dissolved P (ppm)	11.92	17.90	15.00
Silicate. solubilization	Growth efficiency	++	++	++
	Amounts of dissolved K (ppm)	60	63	58.2
Nitrogenase activity	N moles C_2H_4/day/100 ml	7.68	24	7.92

+: low ++: moderate +++: high

TABLE 3. Molecular identification of the selected isolates (STB6, STB121and STB165)

Isolatescode	Closest relatives in NCBI	Accession number	Similarity %
STB6	*Ochrobactrum intermedium* strain (ACC.no.DQ 833764.1)	(ACC. no.MG309678.1)	97
STB121	*Paenibacillus polymyxa* strain (ACC. no. GQ375783.1).	(ACC. no. MG309677.1)	99
STB165	*Enterobacter cloacae* strain (ACC. no.FJ608249.1)	(ACC. no.MG309676.1)	99

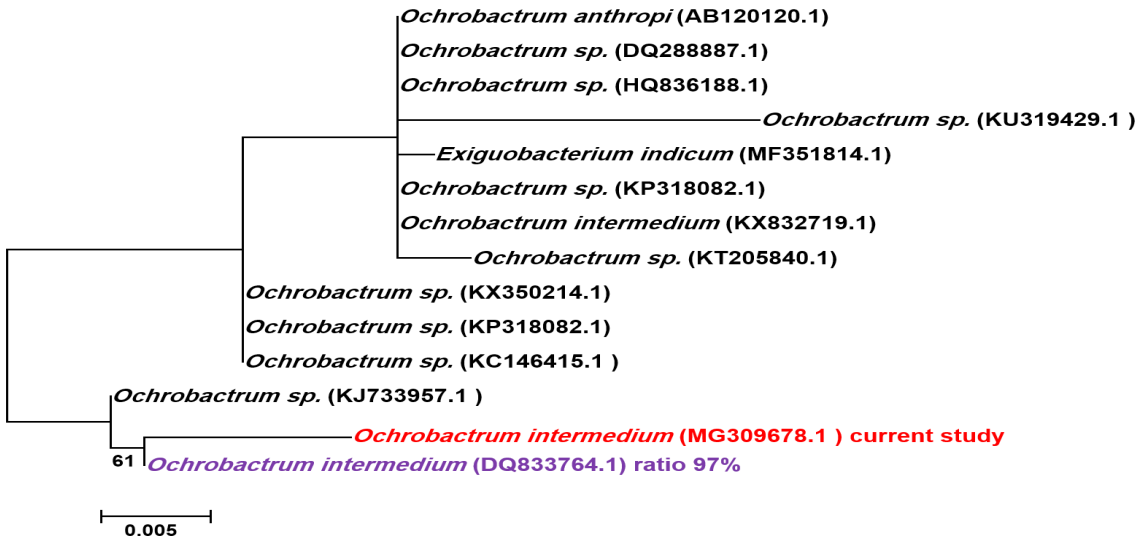

Fig. 1a. Phylogenetic trees recovered from maximum likelihood and neighbor-joining analyses of the 16S rRNA gene partial sequences

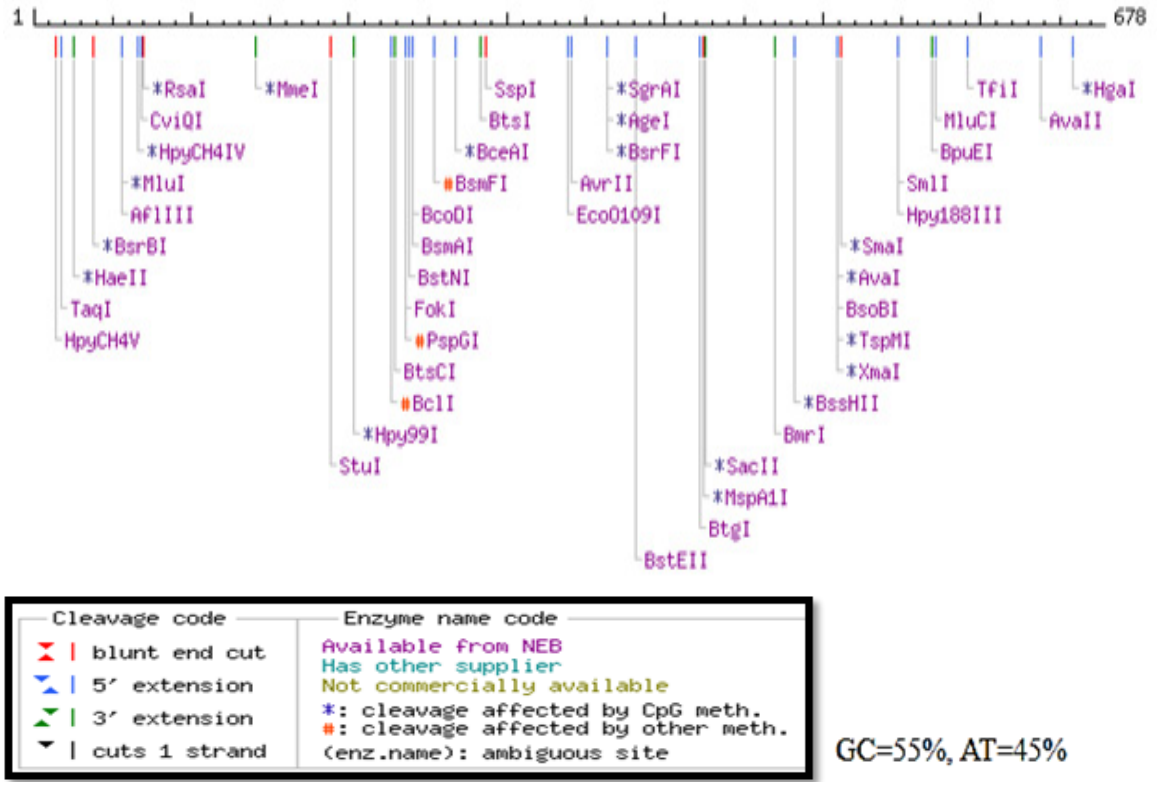

Fig. 1b. Restriction Map of the obtained 16S rRNA partial sequence

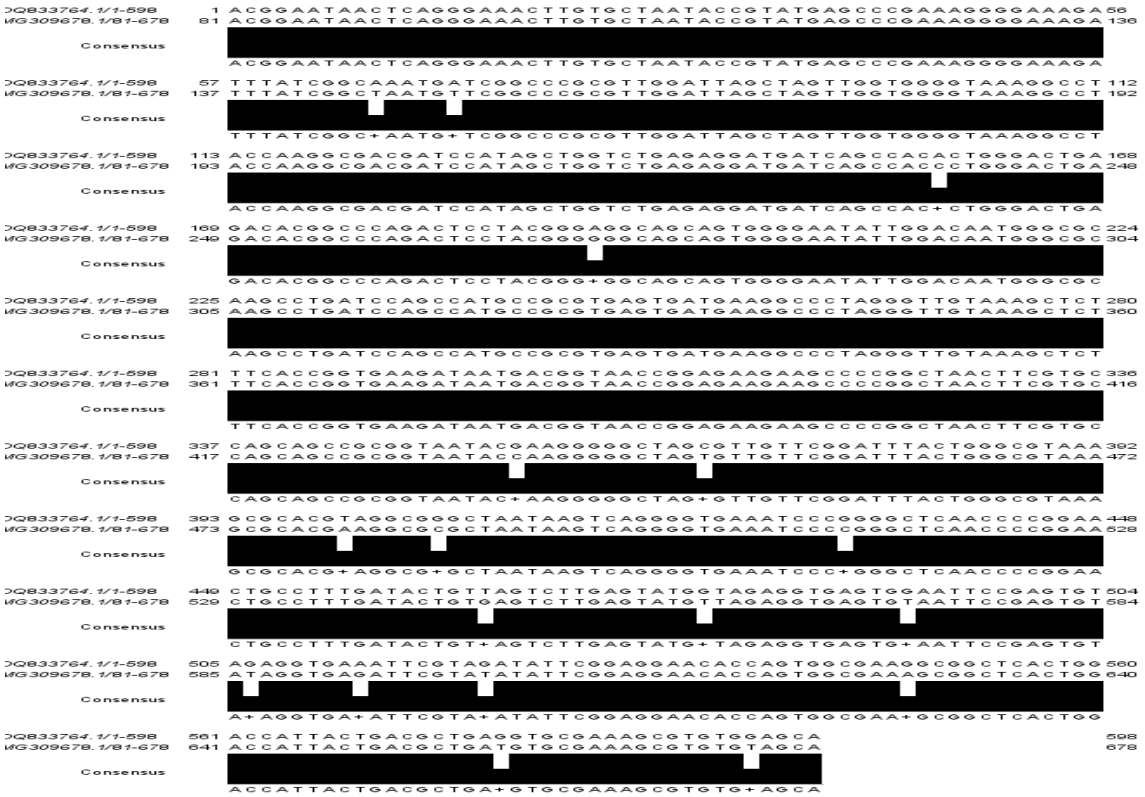

Fig. 1c. Single nucleotide polymorphism (SNPs) showed 18 SNPs between the obtained isolate (*Ochrobactrum intermedium* MG309678.1) and the nearest one on NCBI database based on a pairwise alignment analysis method

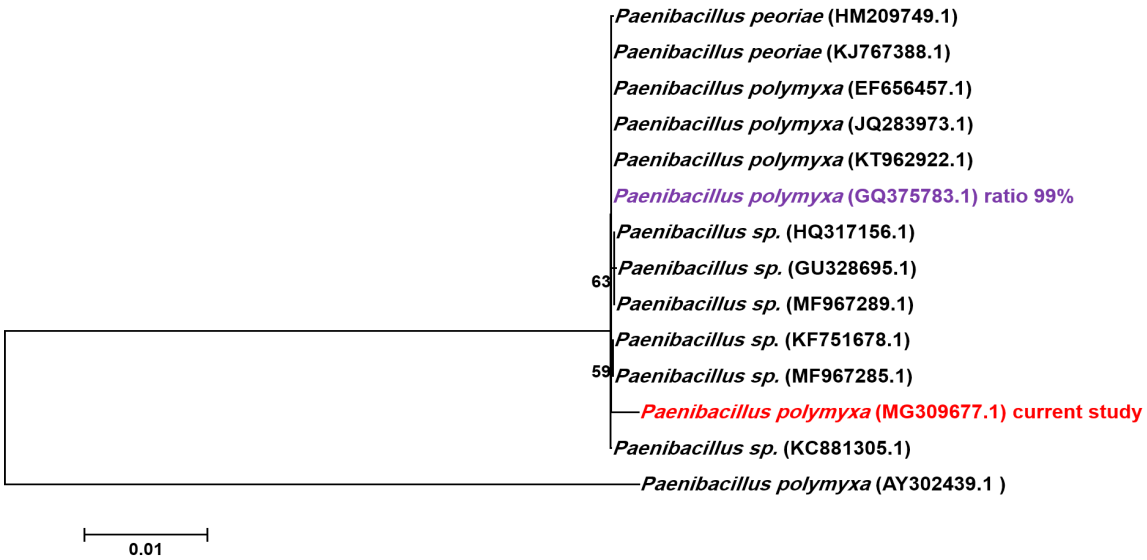

Fig. 2a. Phylogenetic trees recovered from maximum likelihood and neighbor-joining analyses of the 16S rRNA gene partial sequences

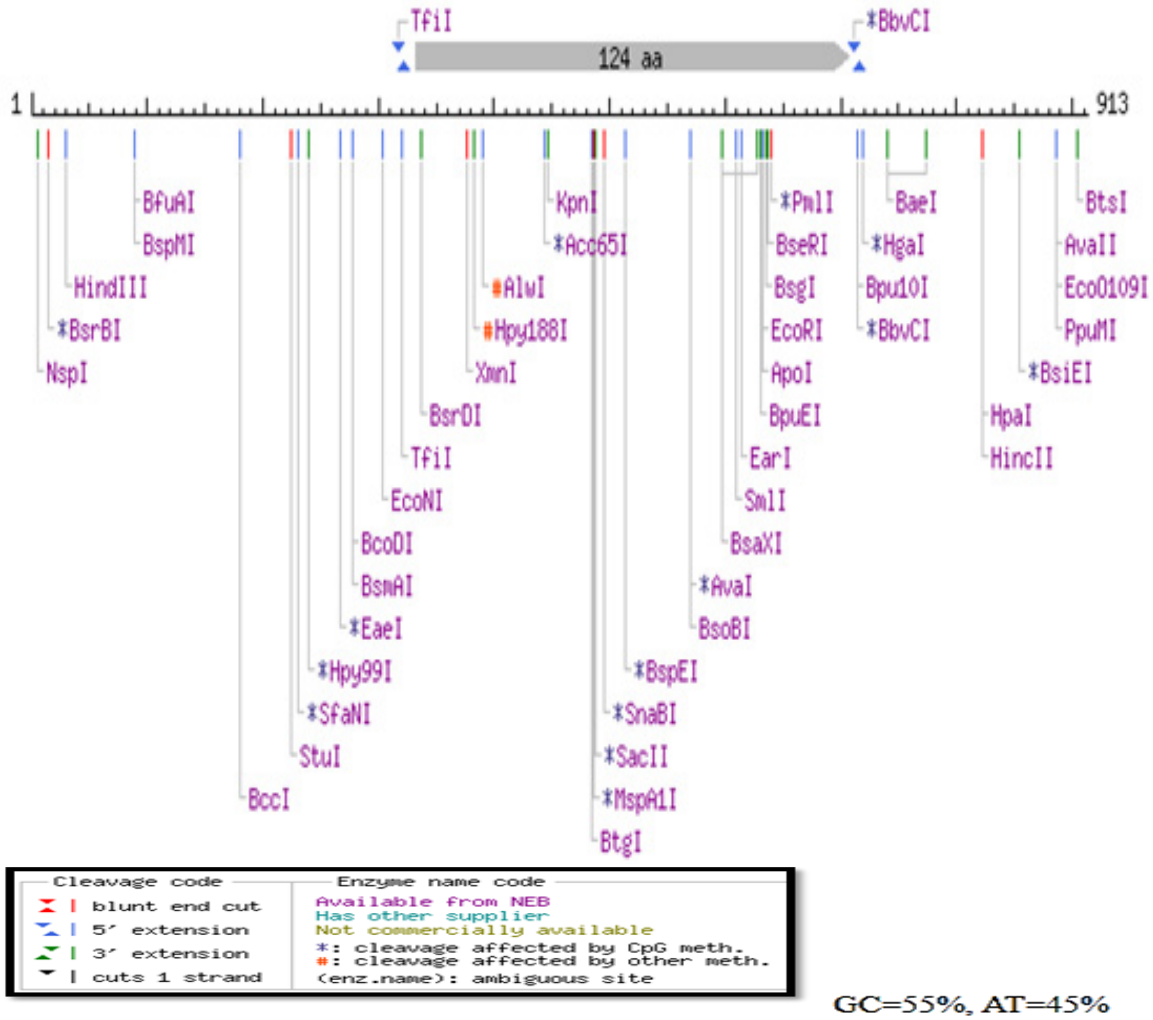

Fig. 2b. Restriction Map of the obtained 16S rRNA partial sequence

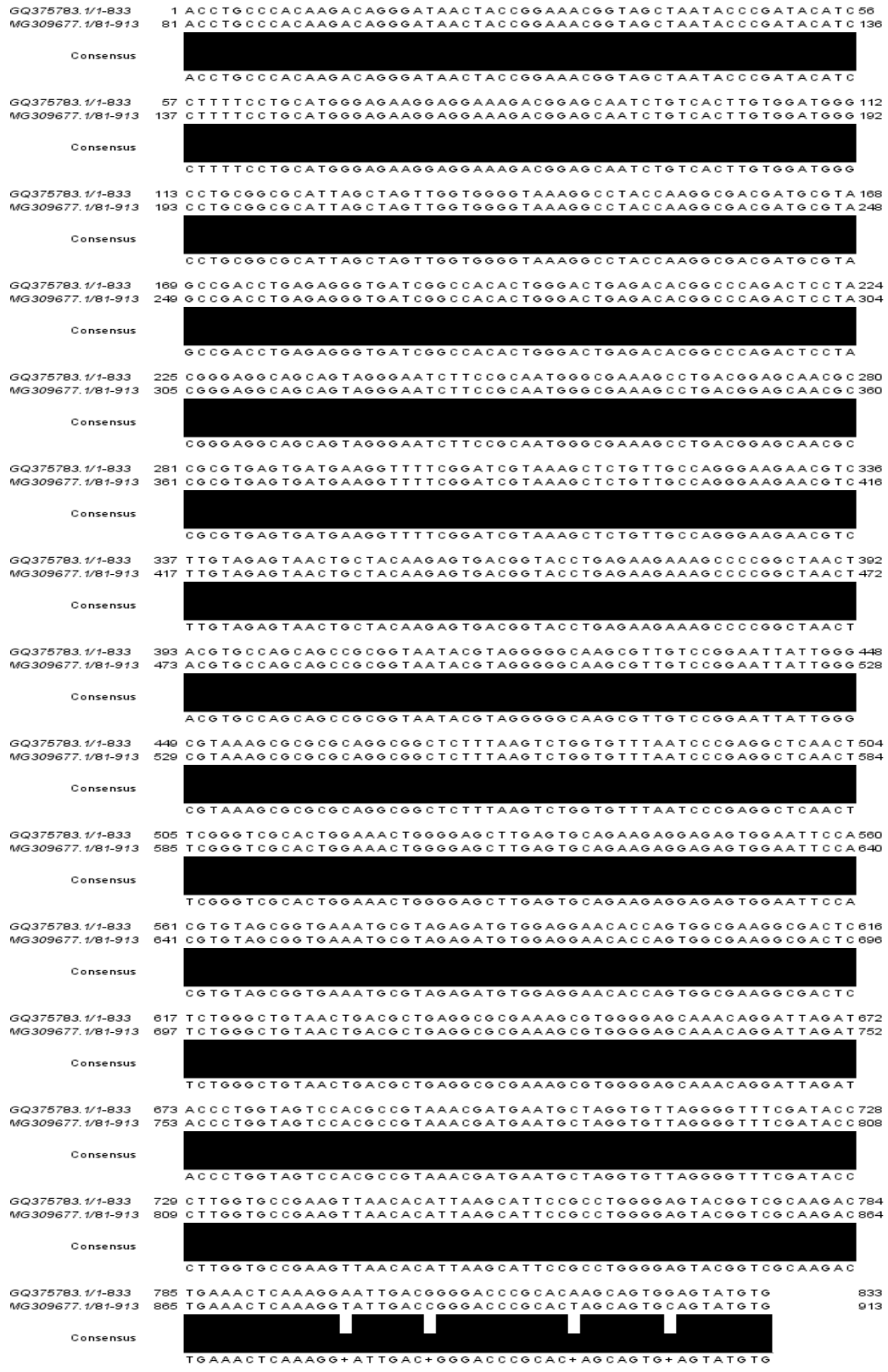

Fig. 2c. Single nucleotide polymorphism (SNPs) showed 4 SNPs between the obtained isolate *(Paenibacillus polymyxa* MG309677.1) and the nearest one on NCBI database based on a pairwise alignment analysis method

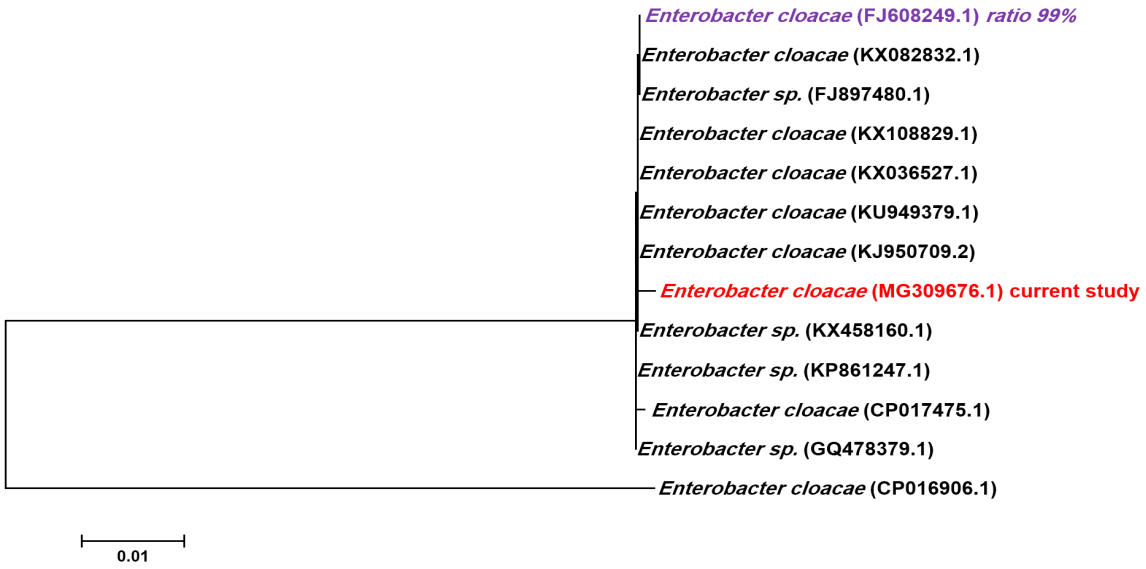

Fig. 3a. Phylogenetic trees recovered from maximum likelihood and neighbor-joining analyses of the 16S rRNA gene partial sequences

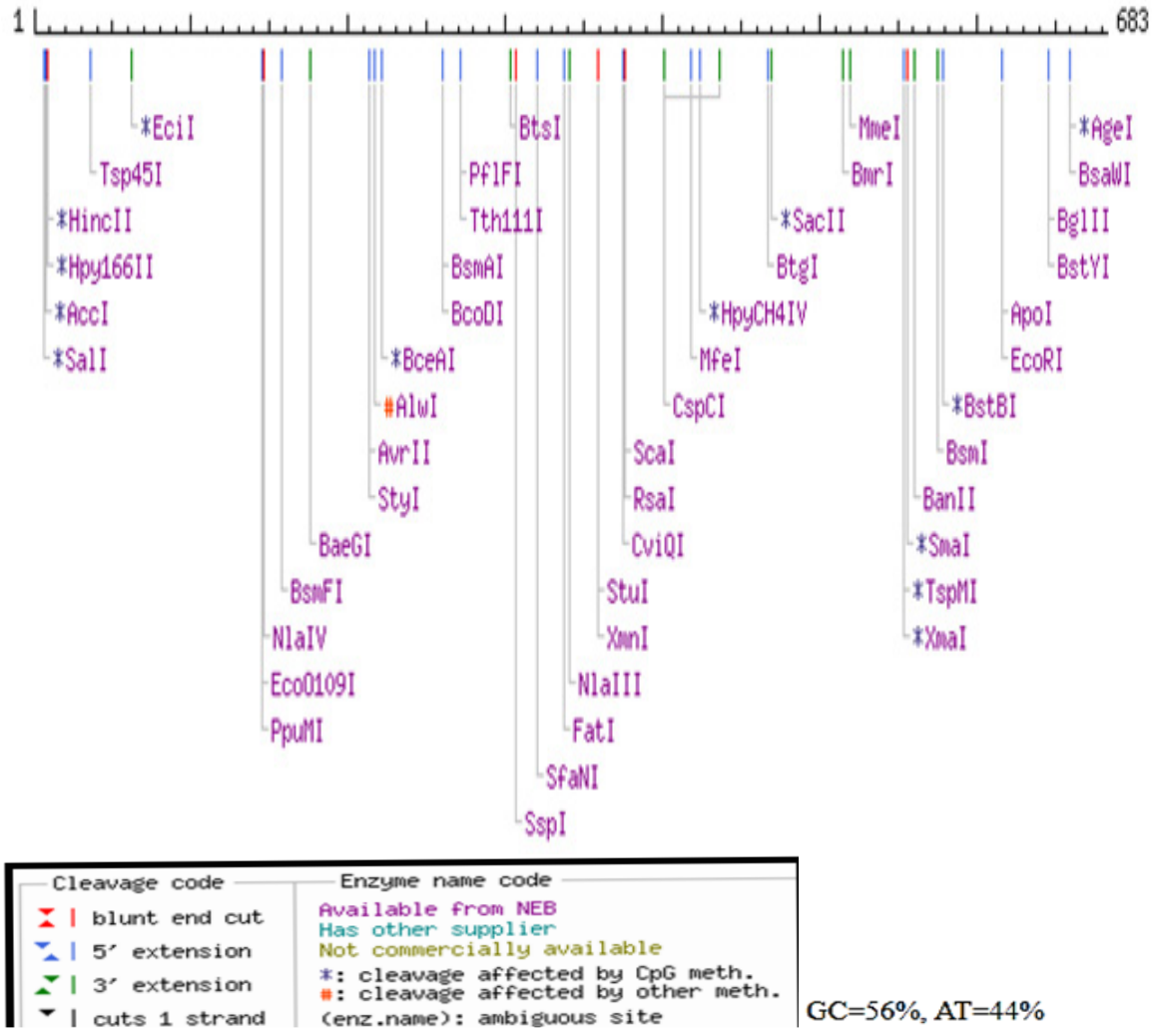

Fig. 3b. Restriction Map of the obtained 16S rRNA partial sequence

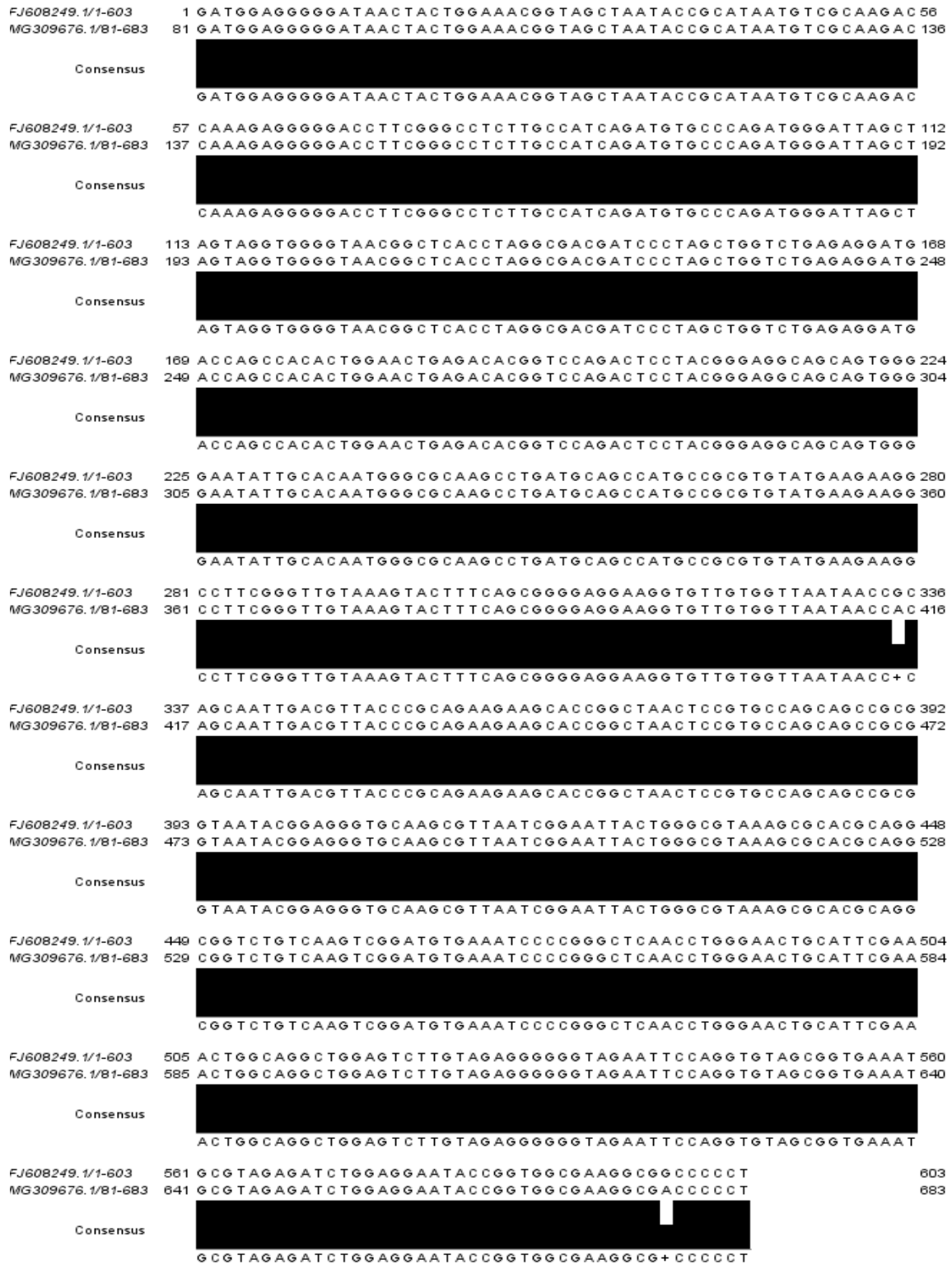

Fig. 3c. Single nucleotide polymorphism (SNPs) showed 2 SNPs between the obtained isolate (*Enterobacter cloacae* MG309676.1) and the nearest one on NCBI database based on a pairwise alignment analysis method

Additionally, all treatments contained humic acid increased DHA and PA compared to non-sprayed ones (Table 4). Humic acid may enhance growth of squash plants causing an increase in root exudates that is positively related to microbial activities. Bama et al. (2008) reported that the application of humic acid causes an increase of microbial enzymatic activities.

Furthermore, the soil inoculated with PGPR strains combined with NPK 50% gave relatively high concentrations of DHA,PA and NA.Concerning theNA (Table 4) shows that the increase of inorganic fertilizers led to decrease nitrogenase activity. This result may be due to the negative effect of nitrogenous inorganic fertilizer on NA.These results are in harmony with those obtained by Ayuni et al. (2015) who found that nitrogenase activity was reduced with the increase of urea -N application. In general, the high application of inorganic nitrogen fertilizers negatively affected nitrogenase activity.

Generally, DHA, PA and NA were increased with the increase of growth periods to reach their maximum valuesat 30 DAS (flowering stage)and decreased thereafter at 45 DAS.Root exudates which increase during flowering stage of cultivated plants increase the multiplication rate for different soil microorganisms and their enzymes.

Availability and uptake of N, P and K

PGPR-inoculated soil showed an increase in available and uptake of N, P and K compared to the uninoculate one (Tables 5 and 6). Co-inoculation of biofertilizer with inorganic fertilizer increased the fertility of the soil (Zahir et al, 2012 and Shedeed et al, 2014.). In addition, PGPR strains combined with 25% NPK gave lower available N, P and K than that amended with either 50% or 75%. Squash foliar application with humic acid gave significant higher available of N, P and K in comparison with the Control. Similar trend of results were observed in all investigated treatments. Vanitha & Mohandass (2014), and Masciandaro et al. (2002) emphasized that humic acid application improves plant physiological processes by enhancing the availability of macro and micronutrients. On the contrary, the lowest values of available and uptake N, P and K were observed in soil inoculated with PGPR strains combined with 25%NPK and without humic acid; however, high means were obtained when PGPR were applied combined with both 50% fertilizer and humic acid.

TABLE 4. Interaction effect of salt-tolerant PGPR strains, inorganic fertilizers with/without humic acid on some microbial enzymes activity in squash rhizosphere

	DHA (TPF/g dry soil/h.)			PA (µg P -nitrophenol /g drysoil/h.)			NA (nmole C2H4/dry soil/h.)		
	15	30	45 DAS	15	30	45 DAS	15	30	45DAS
Inorganic-NPK (full dose)	14.74^{h}	24.13^{h}	18.22^{h}	12.36^{h}	15.96^{e}	14.02^{h}	3.8^{e}	4.9^{h}	3.6^{g}
PGPR + NPK (75%)	34.56^{d}	64.12^{d}	42.72^{d}	43.66^{d}	80.2^{d}	76.66^{e}	12.3^{d}	22.6^{f}	17.9^{f}
PGPR + NPK (50%)	34.92^{b}	66.72^{c}	46.12^{b}	44.24^{b}	83.2^{b}	80.21^{c}	32.5^{b}	55.6^{b}	52.66^{c}
PGPR + NPK (25%)	16.35^{f}	27.57^{f}	20.34^{f}	36.25^{f}	80.0^{d}	78.4^{d}	28.3^{c}	43.3^{d}	38.46^{d}
NPK (full dose)+humic	15.92^{g}	25.43^{g}	19.98^{g}	12.82^{g}	16.3^{e}	14.8^{g}	4.0^{e}	4.6^{g}	3.6^{g}
PGPR + NPK (75%)+ humic	34.84^{c}	66.80^{b}	44.94^{c}	43.74^{c}	82.2^{c}	80.5^{b}	13.4^{bc}	22.6^{e}	20.4^{e}
PGPR + NPK (50%)+ humic	35.32^{a}	68.94^{a}	49.80^{a}	45.45^{a}	85.3^{a}	82.6^{a}	20.5^{b}	58.64^{a}	54.09^{b}
PGPR + NPK (25%)+ humic	17.42^{e}	29.34^{e}	21.91^{e}	38.82^{e}	80.2^{d}	76.02^{f}	35.4^{a}	46.3^{c}	42.66^{a}

a,b, c Means with a different superscript in the same column are significantly different at (P<0.05).
PGPR strains: *Ochrobactrum intermedium* (MG309678.1), *Paenibacillus polymyxa* (GQ375783.1) and *Enterobacter cloacae* (MG309676.1).

TABLE 5. Interactioneffect of salt-tolerant PGPR, inorganic fertilizers and/or humic acid on availableN, P and K

	Available – N		Available - P		Available – K	
	Without humic acid	With humic acid	Without humic acid	With humic acid	Without humic acid	With humic acid
Inorganic NPK (full dose)	116^{e}	121^{d}	102^{g}	105^{f}	56.02^{f}	56.90^{d}
PGPR + NPK (75%)	126^{c}	130^{b}	116^{d}	122^{a}	66.60^{g}	69.80^{c}
PGPR + NPK (50%)	130^{b}	133^{a}	120^{b}	118^{c}	68.04^{b}	72.44^{a}
PGPR + NPK (25%)	108^{g}	110^{f}	100^{h}	111^{e}	54.30^{h}	55.32^{e}

PGPR strains: as mentioned before in Table (4).

TABLE 6. Interaction effect of salt-tolerant PGPR, inorganic fertilizers and/or humic acid on N, P and K uptake

	N mg/plant		P mg/plant		K mg/plant	
	Without humic acid	With humic acid	Without humic acid	With humic acid	Without humic acid	With humic acid
Inorganic NPK (full dose)	101.48^{h}	103.34^{f}	29.44^{h}	31.80^{g}	52.81^{g}	53.62^{f}
PGPR + NPK (75%)	106.88^{e}	112.96^{b}	32.88^{e}	35.42^{b}	63.66^{e}	66.80^{b}
PGPR + NPK (50%)	110.44^{d}	114.42^{a}	33.46^{d}	35.58^{a}	66.48^{c}	68.42^{a}
PGPR + NPK (25%)	103.33^{g}	106.54^{c}	30.68^{f}	32.32^{c}	53.70^{e}	54.86^{d}

PGPR strains: as mentioned before in Table (4).

Peroxidase and polyphenol oxidase activities

Peroxidase and polyphenol oxidase activities were determined as a guide for the correlation between plant stress tolerance and oxidative enzymes activity.All treatments, which included PGPR, showed relatively higher peroxidase (Fig. 4) and polyphenol oxidase (Fig. 5) than the Control in squash leaves.regard to spraying the plants with humic acid, data in Fig. 1 and 2 showed that a significant increase of peroxidase and polyphenol oxidase activities was observed in plants sprayed with humic acid than that non-sprayed ones. A similar trend of results was observed in all investigated treatments. Zhang et al. (2008) reported that plants foliar application with humic acid gave significantly higher values of oxidative enzymes compared to non-sprayed ones.

In addition, the highest values of peroxidase and polyphenol oxidase activity were observed in squash inoculated with salt-tolerant PGPR strains combined with 50%NPK. Generally, oxidative enzymes increase at the beginning of plant life and protect plants against reactive oxygen species (ROS) result from abiotic stresses which formed under this condition. (Celik and Atak, 2012).

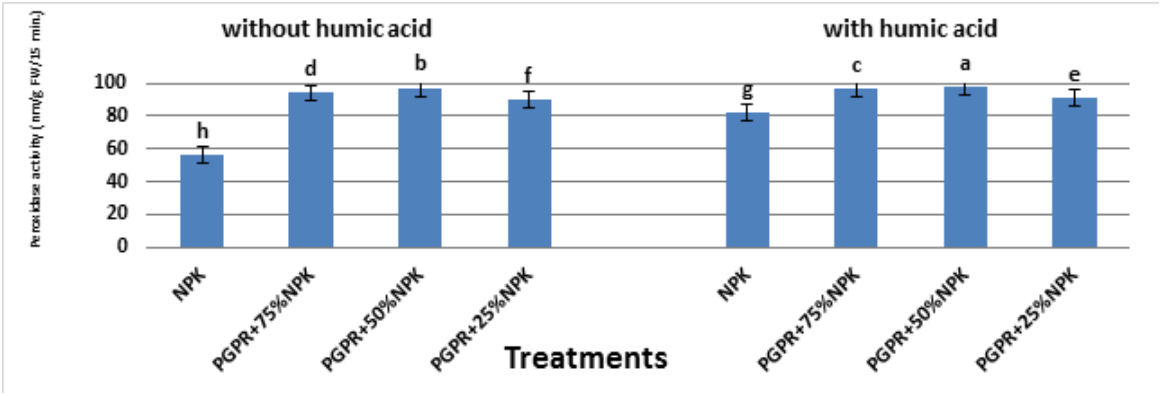

Fig. 4. Peroxidase activity in squash leaves

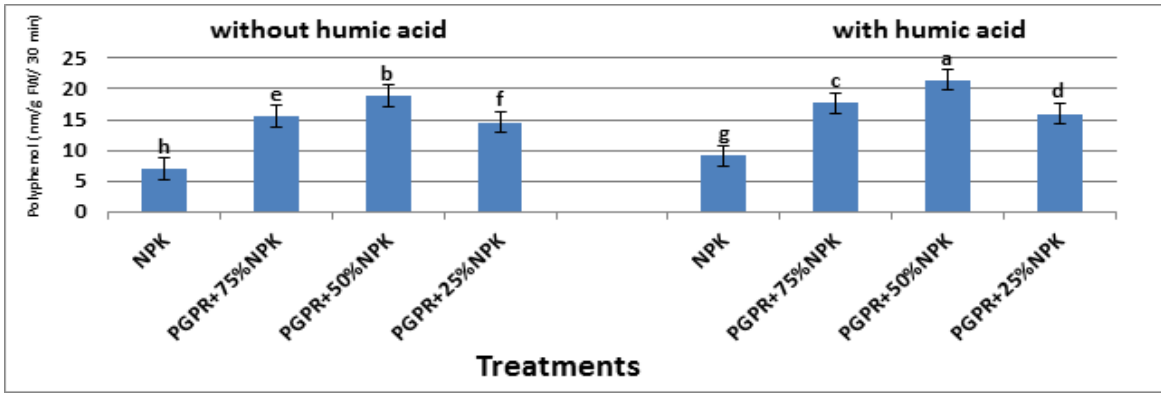

Fig. 5. Polyphenol oxidase activity in squash leaves

Interaction effect of salt-tolerant PGPR, inorganic fertilizers and/or humic acid ongrowth characteristics of squash

Data in Table 7 showed that squash inoculation with salt-tolerant PGPR strains has a positive effect on growth characteristics, i.e. plant height, plant fresh weight, root and shoot dry weight, leaves and flowers, root and shoot length (cm) compared to uninoculated squash. Also, data showed that squash inoculation with salt-tolerant PGPR strains in combination with 50% NPK gave higher records of growth characteristics than that fertilized with 25% NPK followed by 75% NPK. While squash growth characteristics were significantly decreased when fertilized with a full dose of NPK alone. These results are in agreement with (AbdAlla et al, 2015) who found that the lowest values of green onion yield were obtained from using 100% inorganic fertilizers under salinity stress. The lowest values of growth characteristics were observed in squash inoculated with salt-tolerant PGPR strains combined with 25% of inorganic fertilizers without foliar spraying by humic acid. Additionally, squash inoculation with salt-tolerant PGPR strains combined with 50% NPK and sprayed with humic acid gave the highest records of above-mentioned parameters.

The beneficial effect of the tested PGPR on growth characteristics may be attributed to their ability to produce IAA, gibberellins, solubilization of phosphate and potassium as well as their ability to fix atmospheric nitrogen under saline conditions. These results are in harmony with those obtained by Elsharkawy et al. (2014) who found that cucumber inoculated with plant growth-promoting rhizobacteria (PGPR) strains can increase plant height, fresh and dry weight. The ameliorative effects of PGPR application may be due to the increase of plant cell water potential and decrease the electrolytic leakage and also it reduces plant sodium ion concentration and increase salicylic acid and gibberellins synthesis (Kang et al, 2014).Also, salt-tolerant PGPR strains could promote plant growth under stress conditions by phytohormones production that promoted cell division and cell enlargement (Hrynkiewicz and Baum, 2011). Moreover, the use of beneficial PGPR might enhance the plant's tolerance to adverse saline stress (Zahir et al, 2012).

Squash yield

Figure 6 showed that squash inoculation with salt-tolerant PGPR strains significantly increased squash yield/plant in comparison with those un-inoculated ones.This resultis in agreement with Bloemberg and lugtenberg (2001) who stated that P. polymyxa strains can be used as biofertilizer or biostimulant in agriculture as efficient plant growth-promoting rhizobacteria (PGPR). PGPR competitively colonize plant roots and enhance plant growth by several mechanisms, including phosphate solubilization, nitrogen fixation and degradation of environmental pollutants and phytohormones production.Moreover, squash inoculation plants with salt-tolerant PGPR strains in combination with 50% NPK gave higher values of an above-mentioned criterion than plants inoculated and fertilized with 75% NPK followed by 25% NPK. This may be due to the nitrogen level increase leads to a decrease in the N2- fixers activity. This result agreed with Young et al. (2006) who found that using a half dose of iorganic fertilizers together with biofertilizers had a higher positive effect on microbial activity. Also, results indicated that squash sprayed with humic acid recorded a significant increase in yield and yield components compared to non-sprayed ones. This result may be due to that humic acid might increase the uptake of some nutritional elements. Therefore, it could be concluded that the application of humic substances could improve plant growth under salinity conditions (Masciandaro et al., 2002).

TABLE 7. Interaction effect of salt-tolerant PGPR, inorganic fertilizers and/or humic acid on growth characteristics of squash.

	Plant height (cm)	Plant fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)	No. of leaves/ plant	No. of Flowers/ plant	Root Length (cm)	ShootLength(cm)
Inorganic NPK (full dose)	20^{c}	58.26^{f}	1.36^{e}	9.44^{e}	14.0^{de}	10.0^{d}	6.0^{cd}	14^{ab}
PGPR + NPK (75%)	20^{c}	61.20^{c}	1.82^{d}	9.35^{e}	17.0^{c}	13.0^{bc}	7.0^{bc}	13^{bc}
PGPR + NPK (50%)	22^{b}	60.74^{d}	2.86^{b}	11.42^{c}	18.0^{bc}	13.0^{bc}	8.0^{ab}	14^{ab}
PGPR + NPK (25%)	15^{e}	52.77^{h}	0.69^{g}	6.44^{g}	9.0^{f}	6.0^{f}	3.0^{e}	12^{c}
NPK (full dose)+humic	21^{bc}	59.67^{e}	1.78^{d}	11.3^{d}	15.0^{d}	12.0^{c}	6.0^{cd}	15^{a}
PGPR + NPK (75%)+ humic	22^{b}	64.8^{b}	2.01^{c}	12.2^{b}	19.0^{ab}	14.0^{ab}	9.0^{a}	13^{bc}
PGPR + NPK (50%)+ humic	24^{a}	66.36^{a}	3.2^{a}	13.8^{a}	20.0^{a}	15.0^{a}	9.0^{a}	15^{a}
PGPR + NPK (25%)+ humic	18^{d}	56.12^{g}	0.8^{f}	7.33^{f}	13.0^{e}	8.0^{e}	5.0^{d}	13^{bc}

PGPR strains: as mentioned before in Table (4).

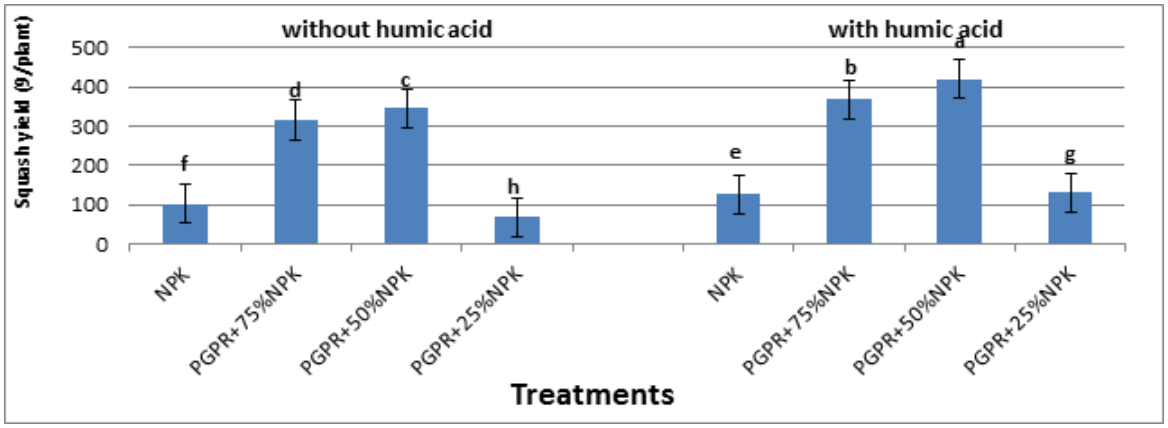

Fig. 6. Squash yield of fruits g / plant under various investigated treatments

Humic substances are well known as stimulators of plant growth by some mechanisms such as enhancing uptake and transport of nutrients, reducing uptake of toxic elements, increasing membrane permeability, respiration, photosynthesis and phosphate uptake and acting as growth hormones (Aydin et al., 2012). Recent progress in our understanding enhances the diversity of PGPR in the rhizosphere along with their colonization ability and mechanism of action that would facilitate their wider application in the management of sustainable agricultural crop production. Further, PGPR is being functioned as a connecting link between plants and microbes that could express antagonistic and synergistic interactions with microorganisms and the soil (Shukla, 2019).

Total protein and total soluble solids of squash fruits

Concerning the total protein and total soluble solids (T.S.S.) of squash fruits, data in Fig. 7 showed that the lowest records of total protein and total soluble solids were obtained when squash inoculated with PGPR strains in combination with 25% NPK.Moreover, squash inoculation plants with salt-tolerant PGPR strains in combination with 50% NPK gave higher values of the above-mentioned criteria than plants inoculated and fertilized with 75% NPK followed by 25% NPK. This may be due to the nitrogen level increase leads to a decrease in the N2- fixers activity.

Inoculation of squash with salt-tolerant PGPR strains combined with 50% NPK and sprayed with humic acid gave the highest records of total protein and T.S.S. being1.7% and0.87%, respectively. This explains that the beneficial role of PGPR which colonize plant rhizosphere and promote plant growth through different direct and indirect mechanisms (Nia et al, 2012 and Ramados et al., 2013).

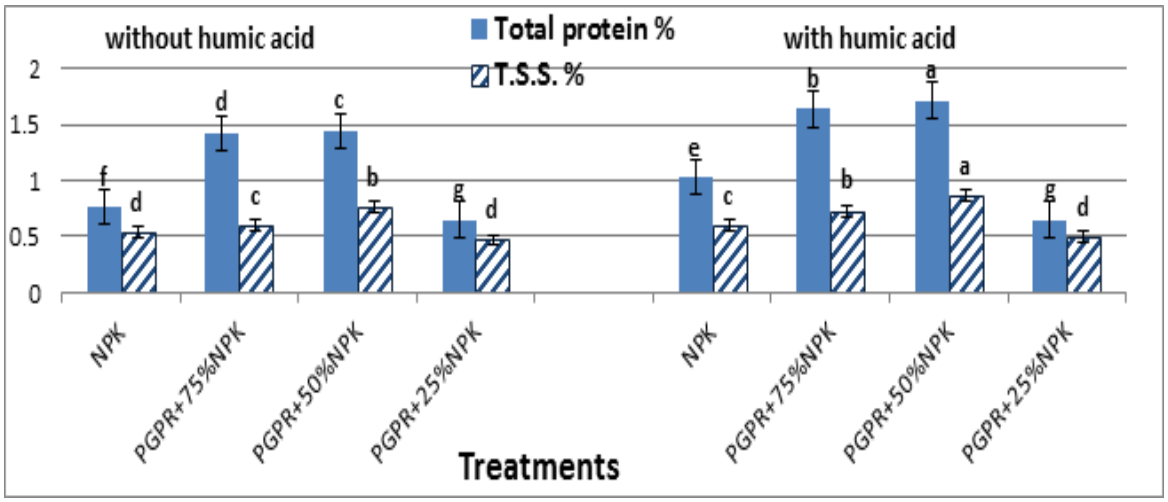

Fig. 7. Total protein and total soluble solids of squash fruits

Conclusion

As a consequence of soil salinity is one of the main factors that limit the spread of plants in their natural habitats,especially in the arid and semi-arid region. In addition, PGPR might alleviate salinity effects, increase nutrient uptake by plants from soil and thereby reduce inorganic fertilizer requirements So, this study for obtaining new salt-tolerant PGPR strains from salt-affected soil. The obtained isolate, *Paenibacillus polymyxa* (GQ375783.1), *Ochrobactrum intermedium* (MG309678.1) and *Enterobacter cloacae* (MG309676.1) could be exploited as plant growth-promoting rhizobacteria (PGPR) for squash and various vegetable crops since it exhibits reasonable potential characteristics. As well, the application of PGPR inocula can save half of the fertilizer requirements and minimize the environmental pollution resulted from the excessive use of chemical fertilizers especially nitrogenous fertilizers.Moreover, it could be recommended as a new effective rhizobacteria to promote plant growth, increase crop production under salinity condition, decrease production costs and reduce environmental pollution.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors declare their consent for publication.

Contribution of authors

This study was designed and implemented by all the authors, where all contributed to writing the manuscript, interpreting information presented and have read and agreed to the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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تعزيز نمو الكوسة تحت الاجهاد الملحي بسلالات جديدة من الريزوبكتريا المشجعة لنمو النبات مع حمض الهيوميك

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تهدف الدراسة إلى تقييم كفاءة بعض العزلات الجديدة المتحملة للملوحة من الريزوبكتريا المشجعة لنمو النبات (RPGP) تحت ظروف ملحية. ثلاثة من أصل ٥٦١ عزلة نمت في وجود ٢-٢٠٪ كلوريد الصوديوم كانت هي الأعلى في تحمل الملوحة ولديها العديد من خصائص الريزوبكتريا المشجعة لنمو النبات تم التعرف علي الثلاثة عزلات باستخدام16 S rRNA . فكانت الاجناس الأقرب للعزلات هي*Paenibacillus polymyxa* (GQ375783.1) و*chrobactrum intermedium*O (MG309678.1) و*Enterobacter cloacae* (MG309676.1) مع تشابه نيوكليوتيدي بنسبة ٩٩ و ٧٩ و ٩٩٪ على التوالي. في عام ٢٠١٧ ، تم إجراء تجربة صوبة لتقييم كفاءة هذه العزلات الواعدة جنبًا إلى جنب مع حمض الهيوميك وجرعات من الأسمدة الغير عضوية على نمو وإنتاجية الكوسة (*Cucurbita pepo* L.). وقد أظهرت النتائج أن تسميد التربة بجرعة كاملة من الأسمدة الغير عضوية بمفردها أدي إلى تقليل قيم نشاط إنزيمات الهيدروجينيز والفوسفاتيز القلوي والنيتروجينيز في جميع فترات التقدير. بينما أعطى تلقيح التربة بسلالات الريزوبكتريا المشجعة لنمو النبات RPGP مجتمعة مع٥٠٪ من الأسمدة الغير عضوية مع الرش بحمض الهيوميك أعلى المعدلات لنشاط الإنزيمات الثلاثة. علاوة على ذلك ، أظهرت النتائج أن أعلى قيم نشاط إنزيمات البيروكسيديز والبولي فينول أوكسيديز كانت مع نباتات الكوسة التي تم رشها بحمض الهيوميك وتلقيحها بسلالات الريزوبكتريا المشجعة لنمو النبات المتحملة للملوحة مع نصف جرعة التسميد الغير عضوي. بشكل عام ، كان لتلقيح الكوسة بسلالاتالريزوبكتريا المشجعة لنمو النبات المتحمله للملوحة تأثير إيجابي على امتصاص العناصر الغذائية وخصائص النمو ومكونات المحصول والمحصول بالإضافة إلى جودة الثمار. لذلك ، يمكن ان نوصي باستخدامها كأسمدة حيوية لزيادة نمو النبات وزيادة إنتاجية المحاصيل تحت ظروف الملوحة ولخفض تكاليف الإنتاج وتقليل التلوث.