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Effect of Integrated Fertilization with Inorganic, Organic Fertilizers in Presence of *Enterobacter ludwigii* Local Strain on Growth, Yield and Fruit Quality of Anna Apple Trees

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THE investigation was conducted to search for novice strains of plant growth promoting rhizobacteria (PGPR) and apply them to apple trees with organic fertilizer to reduce the amount of chemical fertilizers. Twelve different bacterial isolates were isolated on Ashby's medium from rhizosphere of healthy fruit trees. Screening process was achieved based on *in vitro* plant-growth-promoting features. The more potent bacterial isolate (B3) was selected for the subsequent experiments. Morphological and cultural characteristics and 16S rRNA gene partial sequence were used for identification obtained isolate. The 16S rRNA gene sequencing results revealed that the nearest bacterial species to our isolate was *Enterobacter ludwigii*, KJ638989.1, with about 98% matching. During 2019 and 2020 field traits were carried out to evaluate the effective role of *E. ludwigii* strain combined with doses of inorganic and organic fertilizers on "Anna" apple trees growth, yield and fruit quality. Data revealed that inoculation by *E. ludwigii* combined with compost could compensate 50% of chemical fertilizer with significant increment in growth, yield and fruit quality. Since, data showed high yield and fruits quality by using 50% mineral-NPK, compost (14 kg/tree) and *E. ludwigii* inoculant followed by treatment of 75% NPK, compost (7 kg/tree) and *E. ludwigii* inoculant. Finally, the obtained strain, *E. ludwigii* could be capitalized on promoting plant growth for various fruit crops since it exhibits reasonable potential characteristics.

Keywords: Apple, PGPR, *Enterobacterludwigii*, Compost and Mineral-NPK.

Introduction

Apple (*Malu sdomestica* Borkh), family Rosaceae, is one of the most widely cultivated tree fruits. It is considered one of the major and the most important deciduous fruit crop in the world (Karlidaget al. 2007). "Anna" apple is considered one of the leading apple cultivars in Egypt, being of low chilling requirements. It needs chilling about 300-350 h below 7.2°C to break their bud dormancy (Soliman et al. 2018). The cultivated area of "Anna" apple cultivar is being increased rapidly especially during the last three decades to reach 66869.05 feddan in 2018 which produced 704727 ton according to FAO (2018).

An intensive farming practice that warrants high yield and quality requires extensive use of chemical fertilizers, which are costly, and have deleterious effects on soil, water and atmosphere pollution, and reflected on animal and human health. It had also adversely affected the soil fertility, water quality, yield and quality of the products (Aslantas et al. 2007 and Srivastava 2012). Subsequently, more recently there has been a reawaking of interest in environmental friendly, sustainable and organic agricultural practices (Esitken et al. 2005). The development and application of sustainable agricultural techniques and biofertilization would reduce the need for chemical fertilizers and alleviating environmental pollution (Esitken et al.

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2003; Vessey 2003; Aslantaset al. 2007 and Cen et al. 2020). Thus, PGPR as biofertilizing agents can be important components of integrated nutrients management. The organic and biofertilizers would play an important role in productivity and sustainability of soil, protect the environment as eco-friendly, and cost-effective inputs for the farmers. By using the organic and biofertilizers, a low input system can be carried out and it can be help achieving sustainability of farms.

The mechanisms by which PGPR promote plant growth directly include: atmospheric nitrogen fixation that is conducted to the plant, siderophores production that chelate iron and make it available to the plant root, bioavailability of minerals such as phosphorus, and synthesis of phytohormones. PGPR that synthesize Auxins and cytokinins or that interfere with plant ethylene synthesis have been identified (Khehra 2014; Babitaet al. 2015; Hadoleet al. 2015; Abel-Rahman et al. 2017 and Aloo et al. 2020). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported. PGPR strains may use one or more of these mechanisms in the rhizosphere. Environmental and economic benefits can include raised income from high yields, reduced fertilizer costs and reduced emission of the greenhouse gas and environmental pollution. Plant growth promoting bacteria are important in organizing plant growth because of their effects on soil conditions, nutrient availability, plant growth and yields. As stated earlier, the focus on biofertilizing PGPR may be reflective of the selection criteria that researchers use in looking for a biofertilizing PGPR strains.

The objectives of this study are mainly to look for novice strains of PGPR and apply them to apple trees with organic fertilizer to reduce the amount of chemical fertilizers. Besides, the study is evaluating tree growth, yield and fruit quality under experimental treatment.

Materials and Methods

Samples collection

Soil samples were collected from rhizosphere of healthy fruit trees at the Experimental Farm at Faculty of Agriculture, Benha University, Qalyoubeia Governorate, Egypt. (Latitude: 30.35 N Longitude: 31.22E). Samples of soil were transferred to sterile plasticbags and were delivered to the laboratory within half hour after their collection under aseptic conditions.

Isolation and selection

Serial dilution and spread plate method was used to isolate the desired bacteria as described byAneja(2003).Toget10^{-1}to10^{-6},0.1ml concentration range of each dilution,10 g sample of soil from fruit trees rhizosphere was diluted using phosphate buffering solution. Each sample was uniformly spread on Modified Ashby's medium agar poured in plates. Then, the plates were incubated at 35°C for 24–72 hr (AbdEl–Malek and Ishac, 1986).The single bacterial colonies were sub-cultured to etapure culture. The obtained bacterial isolates were kept on trypticsoyaagarslants at 4°C .

Lightmicroscopy examination

The more potent isolate was studied using a lightmicroscope (Nikon Ci-L clinical microscope, SEO ENTERPRISES, INC, USA) to check for the shape, Gram stain reaction, sporulation, motility by methods according to Harrigan and Mac-Cance (1976) and Bertrand etal. (2001). The cultural characteristics were recorded as colony morphology, i.e., color, shape, size and pigmentation.

Scanning electronmicroscopy (SEM)

Morphological changes of selected bacterial isolate and photomicrographs were observed and carried out with a JEOLJSM-5500LV scanning electron microscope (JEOL InstrumentsInc., Japan) at an accelerating voltage of 20kV at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt. The samples were fixed and covered with gold by method according to Abdel-Rahman et al. (2017).

Plant-growth-promoting features of isolates

Activity of nitrogenase was measured as a guide for nitrogen fixation using the acetylene reduction technique given by Okaforand MacRae (1973). To determine the ability of the isolates to solubilize phosphate, the Pikovskaya's (PVK) medium was used according to Nguyen et al. (1992). Phosphate solubilization was evaluated quantitatively on the National Botanical Research Institute's Phosphate (NBRIP) broth medium according to Naik etal. (2008). Moreover, Yeast Extract Manni to lbroth medium was used to determine qualitatively the isolate ability to produce indole aceticacid (IAA) and gibberellins (GA3) accordingt o Sarwart etal. (1992) and Pandya and Desai (2014) methods, respectively.

Bacterial genomic DNA extraction

The bacterial isolate was inoculated LB broth and incubated overnight with shaking. 5 ml from overnight culture was centrifuged. The pellet was washed in 1 ml TE buffer three times. The pellet was resuspended in 500 µl TE buffer. 10 µl Proteinase K (20mg/ml), 20 µl lysozyme (50mg/ml) and 100 µl 10%SDS were added to cell suspension. The bacterial cell suspension tube was incubated overnight to obtain the complete cell lysate. 100 µl 5M NaCl and 100 µl 10% CTAB were added and mixed well by invert the tubes. The mixture was incubated at 70°C for thirty minutes after that the tubes incubated in ice for 10 minutes. The tubes were centrifuged at high speed for 15 minutes. The upper phase transferred to 1.5 ml clean tube and equal volume from 25:24:1 (phenol: chloroform: isoamyl) added and mixed the mixture well by inverting the tubes. The tubes centrifuged at 15000 rpm for 15 minutes. The upper phase transferred to clean tubes and equal volume of 24:1 (chloroform: isoamyl) added and centrifuged at 15000 rpm for 15 minutes. The upper phase was transferred to clean tube and double volume of ethanol absolute added and incubated overnight at -20°C. The tubes were centrifuged at high speed for 20 minutes. The DNA pellet washed three times by 1 ml 70% ethanol after that the DNA pellet resuspended in 50 µl sterilized TE Buffer and DNA samples stored at -20°C for further studies.

PCR reaction, cloning and sequencing

The primers were used 27F and 1492R for 16S rRNA gene according to (Jiang et al. 2006). The PCR reaction was 50 µl in 0.2ml PCR tube. The PCR reaction components were as follow: 2.5 µl from each forward and reverse primer, 5 µl dNTPS mix, 5 µl 10x buffer, 0.5 µl TAKARA Taq (Cat. #:R001AM), 1 µl DNA template 10 ng/µl and the volume adjusted with free nuclease water or double distilled and sterilized water. The PCR program was as follow: initial step 3 min at 95°C, (95°C for 50 sec, 52°C for 1 min and 72°C for 1 min were repeated 35 cycles), and the extension step was 72°C for 10 min and the PCR tubes stored at 4°C for overnight. After the PCR program was complete then the PCR tubes was stored at -20°C for future analysis. Three µl from PCR product was run on 1.2 % agarose gel and visualized under UV Transilluminator. The DNA band eluted from agarose gel and purified according to manufactures of QIAquick Gel Extraction Kit (Cat. #: 28704). The purified PCR band was ligated in pGEM®-T Easy Vector Systems (Cat. #: A1360) according to Manufactures. The competent cells from top 10 strain ware prepared according to Inoue et al.(1990). The competent cells ware transformed according to Inoue et al. (1990). The white colonies picked up from LB/Amp/Xgal plates and inoculated on LB/Amp broth and incubated overnight at 33°C for stabilizing the plasmid inside the transformed cells with shaking. The plasmid was isolated according to alkaline method (Birnboim and Doly, 1979). The recombinant plasmids ware sent to Macrogen Company (South Korea) for sequencing.

DNA analysis

The 16S rRNA gene sequence was analyzed by FASTA screening to determine its similarity to known bacterial species in the DNA database. The sequence was registered at DNA database under accession number KP325139.1. Construction of the phylogenetic tree was done through two bioinformatic processes. In the first processes, the nucleotide sequence of the recovered 16S rRNA gene phylotype and its homologue sequences, from the DNA database, beside out-group sequences, were aligned using the online program ‘‘Clustal Omega’’, http://www.ebi.ac.uk/Tools/msa/clustalo/. In the second processes, the aligned sequences, including the sequence gaps, were submitted to the MEGA software, V. 6.06, http://www. megasoftware.net/, for drawing the phylogenetic tree.

Evaluation of E. ludwigii inoculum

The investigation was carried out during the two successive seasons of 2019 and 2020 respectively, in addition to preparation season during 2018 at the Experimental Farm at El-Kanater Horticultural Research Station, Qalyoubeia Governorate, Egypt (Latitude: 30. 19N Longitude: 31. 11 Elevation: 16.9 m), to study the effect of inorganic, organic and biofertilizers as well as their interactions onfruitful trees of “Anna” apple budded on MM106 rootstock. The selected trees were about twelve years old grown on clay loamy soil at planting distance 4 x 4 meters. Trees were carefully selected as being healthy and approximately uniform in their vigor, shape and size and received regularly the common horticultural practices in the region.The soil texture in this experiment was clay loamy textured. Moreover, particle size distribution and chemical analysis of the experimental soil from (0) to (60) cm depth was determined according to the methods described by Piper (1950) as shown in Table 1.

The commercial name of the organic fertilizer (compost) used in this experiment was "Biobinta", its physical and Chemical is shown inTable 2.

The experimental design was a randomized complete block with three replicates for each treatment and two trees for each replicate.The control fertilized with 100 % recommended N, P and K inorganic fertilizers i.e., 380 g N/tree (1145 g ammonium nitrate 33.5 % N/tree/year), 110 g P_2O_5 /fed. (760 g calcium superphosphate 15.5 % P_2O_5 /tree/year) and 381g K_2O/fed. (763 g potassium sulphate 48 % K_2O/tree/year). Three levels of inorganic N, P and K fertilizers (25, 50, and 75 % of full dose) were appliedin the soil integrated with organic manure "Compost biobinta " at (21, 14 and 7kg/tree), respectively. The addition rates of organic fertilizer were calculated on basis of N %.The apple trees that were fertilized with inorganic and organic fertilizers were divided into two groups, one of them inoculated with *E. ludwigii* inoculum and the anther one not.*E. ludwigii* inoculumwas prepared by pure culture of strain reserved in the Agricultural Microbiology Department, Faculty of Agriculture, Benha University, Egypt, by method described by Abdel-Rahman et al. (2017). The cell suspension of bacterial strain (9×10^{11} CFU ml^{-1}) is divided into two parts. The first one added with compost (50 ml/tree) and the second one(50 ml/tree) is drenched before irrigation directly in the first of growing season. The boost addition of bacterial strainsuspension (100 ml/tree) was added three times during growing season.

TABLE 1. Physical and chemical analysis of orchard soil at (0 - 60 cm) depth before starting the experiment in 2019 season

Physical analysis			Chemical analysis					
Silt (%)	Clay (%)	Soil	EC (dSm^{-1})	pH (1:25)	$CaCO_3$ (gKg^{-1})	T. N. ($mgKg^{-1}$)	O. M ($mg\ Kg^{-1}$)	
		Clay	1.05	7.96	28.7	384.33	10.2	
			Soluble Cation and Anions ($cmol_c$ kg soil)					
			Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Cl^{-}	CO_3^{--}	SO_4^{--}
			3.23	1.71	3.96	0.88	3.93	4.03	-	1.83

Abbreviations: E.C: electric conductivity, T.N.: total nitrogen, O.M.: organic matter

TABLE 2.Chemical and physical analysis of "Compost Biobinta"

Parameter	Value
Weight of/m^3 (kg)	577 kg
Humidity (%)	20%
pH (1:10)	6.53
EC (1:10 extract),dS m^{-1}	2.75
Total-N	1.29%
Organic matter	35%
Organic-C	17.57%
Total-P	0.59%
Total potassium	1. 5 %
C:N ratio	13.6:1
Fe (ppm)	1200
Mn (ppm)	100
Cu (ppm)	50
Zn (ppm	250

The corresponding amount of each (N and K) fertilization treatment was fractionated into was added at three unequal batches as 40 % in the first week of Feb., 30 % just after fruit setting (3rd week of April) and 30% at 21 days intervals after fruit setting. However, in late December of each season, four trenches (80 cm length x 30 width x 40 cm depth) were digged along four sides of the tree at one meter apart from tree trunk in the direction of irrigation furrows. Thereafter, the calculated amount of organic manure source (Compost biobinta) was equally divided into fourquarter and applied in the four trenches (quarter amount of organic manure/ trench) as well as the corresponding amount of phosphorus in the form of superphosphate (15.5 % P_2O_5) was added and covered with trench soil.

Soil and plant analyses

Random soil samples were taken from apple rhizosphere for assessing themicrobialenzymatic activitiesand available-potassium content at 30, 60,90 and 150 days after biofertilizer inoculation.The activities of dehydrogenase (DH) and alkaline phosphatase (Alp) were measure dusings pectrophotometer (SCO-Tech,SPUV-19, Germany)at 464 and 400 nm, respectively, as described by Schinneretal.(1996).However, nitrogenase(N_2-ase) activity was measured as previously mentioned with some modification by Oka for and MacRae(1973). Available-potassium was determined according to the method described by Jackson (1973).

Four main branches, in different directions of each replicate were labeled. All current shoots developed on those branches on Aug. were used for measuring shoot length (cm). Leaves were taken at random from the middle of branch during mid-August in both seasons to determine leaf number, leaf area (cm^2) using Li-core 3100 area meter and leaf chlorophyll content using a chlorophyll meter (Model SPAD 502; Minolta Corporation, N.J., USA. To determine leaf nutritional status, samples of twenty mature leaves were collected at random, ground and digested. Total nitrogen and phosphorus were determined colorimeterically, also potassium andcalcium contents were determined using Flam Photometer and the concentration of N, P, K and Ca were expressed as percent according to A.O.A.C. (1990).

Yield component and fruit properties

For determining yield and fruit quality, eight selected branches around tree were labeled for recording of different data during seasons. Fruit set % and fruit drop were calculated as follows:

Fruit set % = (No. of developing fruitlets)/ (Total No. of flowers)×100

Fruit drop % = (No.of fruitlets at setting – No.of fruits at picking time)/(No.of fruitlets)×100

At harvest time (first week of July), yield of each tree was recorded as number of fruits per tree and kg per tree or ton per feddan during the two seasons of study. Samples of twenty fruits from each replicate under treatment at harvest were randomly collected and the following characters were determined as follows:

Physical fruit properties

Fruit weight (g), fruit volume (cm^3), length (cm), diameter (cm), fruit shape index (fruit length/fruit diameter) and firmness. Firmness was determined by Magness and Taylor (1925), pressure tester using 7/18-inch plunger two reading were taken on the flesh of each fruit.

Chemical fruit properties

Total soluble solids (TSS %) and acidity (as malic acid) of fruit juice was determined according to A.O.A.C. (2016).Total sugars %, was determined in pulp fruit samples according to A.O.A.C. (2016). Vitamin C (Ascorbic acid) was assessed by the method of A.O.A.C. (2016) as mg/100g fruit.

Statistical analysis

Data were statistically analyzed according to the analysis of variance as described by Waller and Duncan (1969).

Results and Discussion

Isolation and screening of diazotrophs

Twelve different bacterial isolates were detected on Ashby's medium after 48–72 hr of incubation at 35°C. Bacterial isolates were screened based on *in vitro* plant-growth-promoting features (Table 3). Data reveled that bacterial isolate (B3) was the more potent bacterial isolate since it has the highest values of all growth promoting substances tests and are thus qualified as plant growth promoting rhizobacteria. So it was selected for the subsequent experiments.

Morphological characters of the more potent bacterial isolate

After 48 hr of incubation on nitrogen free Ashby's medium, at 35°C, the colonies of the more potent bacterial isolate ranged in size from 1.6 to 3 mm in diameter, smooth and mucoid. Regarding, microscopic morphology, the obtained bacterial isolate was straight rods Fig.1, rounded ends. It was Gram negative, single, non-sporeforming and motile with peritrichously-arranged flagella.

TABLE 3. Evaluation of the obtained bacterial isolates as plant growth promoting rhizo bacteria

		Alkaline	Phosphate	Phosphate	IAA	GA3	Siderophores production		HCN
B1	48.9	ND	90.2	21.25	ND	ND	+	-	++
B2	60.1	22.4	55.6	13.30	ND	ND	-	-	
B3	61.5	41.5	104.7	25.52	55.2	11.8	++	+++	+++
B4	55.6	39.6	101.2	24.86	32.2	ND	+	++	-
B5	57.6	ND	101.3	24.75	ND	9.4	-	-	+
B6	45.8	35.6	80.5	19.41	22.5	ND	-	-	-
B7	57.8	33.2	95.3	23.68	15.6	5.2	-	-	+
B8	55.4	ND	101.2	24.55	ND	4.9	+	++	+
B9	44.5	22.1	102.5	24.58	10.6	7.5	+	+++	-
B10	56.9	ND	55.6	13.63	25.6	ND	-	-	-
B11	38.6	ND	85.6	20.77	ND	7.4	-	-	-
B12	45.2	ND	74.8	18.48	14.6	8.9	-	-	+

ND= not detect

DNA and phylogenetic analysis

The PCR could amplify 1500 bp of the 16S rRNA gene. The FASTA homology showed that the16S rRNA gene of the current isolate had` 98 % nucleotide identity with that of *E. ludwigii*, strain recorded in Malaysia (acc. no. KJ638989.1). this result was confirmed by the phylogenetic position of the current isolate, forming polyphyletic clade with *E. ludwigii*, but with an obvious phylogenetic distance (Fig. 2). Overall distance 0.145 ± 0.757 SE obtained from Mega 6 software

The trees show the phylogenetic position of recovered *Enterobacter species* within the phylogenetic branches of the family Enterobacteriaceae. Average Bootstrap values, of compared algorithms, are indicated at the branch roots. The bar represents 0.1 changes per nucleotide. Accession numbers of database extracted sequences are in brackets.

E. ludwigii inoculum andapple production

In present study, the impact of *E. ludwigii* inoculum, inorganic and organic fertilizers on apple productivity have been studied. To display the role of *E. ludwigii* as plant growth promoting rhizobacteria, some microbial enzymes in soil were determined. Microbial enzymes activity are the direct expression of the soil environment to metabolic requirements and available nutrients. The enzymes are essential to the nutrients cycling and are thus critical to availability of nutrients to both microorganisms and plants. Frequently, enzymes are regulated and externalized as a response to exogenous soil conditions (Tarafdar and Claassen, 1988 and Shams et al. 2013).

Periodical changes of dehydrogenase activity

Dehydrogenase activity (DHA) was determined in the soil as a criterion for respiration rate and total microbial activity. Data presented in Table 4, showed widely varied of DHA values among the studied treatments. The values of DHA in soil amended with inorganic fertilizers were significantly lower than soil treated with compost with or without biofertilizing PGPR strain. This result was observed in almost experimental periods. This result was consistent with Krishnakumar et al. (2007) who reported that the recommended NPK fertilizer treatment have significantly lower values of DHA than organic manure and Garciá-Gil et al. (2000) who mentioned that DHA was higher in organic manure treatments than in the un-amended soil, indicating an increase in the microbial metabolism in soil as a result of mineralization of biodegradable carbon compounds.

Higher records of DHA were observed in manured soil and inoculated with *E. ludwigii* than soil amended with organic fertilizer only. Similar trend of results was obtained in both growing seasons. Higher records of DHA with biofertilization are likely be due to the effective role of inoculation for enhancing colonization of introduced biofertilizing PGPRstrain for plant roots. Moreover, the inoculation might lead to accumulation of available nutrients and stimulate the microorganisms in rhizosphere. This was true in all experimental periods and in the two growing seasons. Similar trend of results was observed by Zaghloul et al. (2010) and Shams et al. (2013).

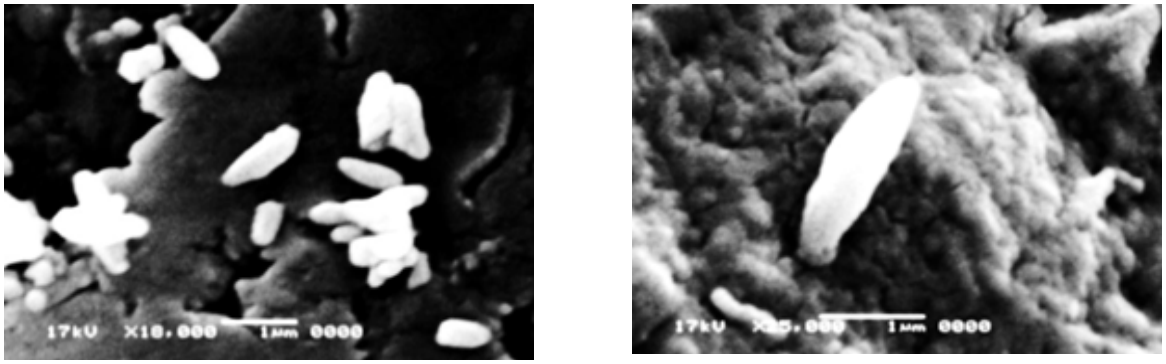

Fig. 1. Morphological structure images of the obtained isolatefrom current study by scanning electron microscopy (SEM)

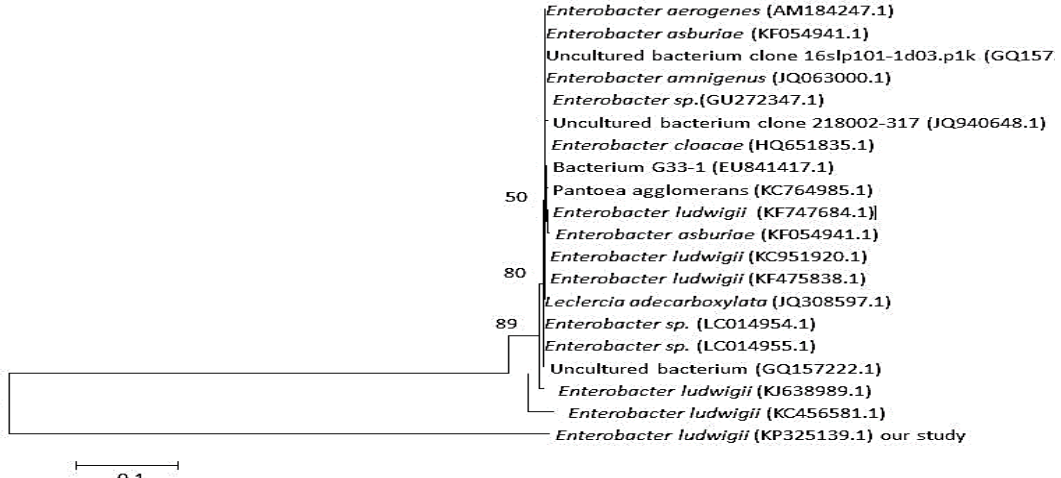

Fig. 2. Phylogenetic trees recovered from maximum likelihood analyses of the 16S rRNA gene Partial sequences

TABLE 4. Periodical changes of dehydrogenase activity in apple rhizosphere

Treatments	Dehydrogenase activity (µg TPF g^{-1}dw h^{-1})							
	30		60		90		150 days	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	13.15f *	14.52f	17.35f	17.46f	30.48f	31.44g	22.25d	25.39f
75% NPK+COMP(7 kg/tree)	15.12e	21.07e	20.92e	22.48e	40.21e	44.88f	22.92d	29.41e
50%NPK+COMP (14 kg/tree)	20.08d	22.82d	23.18d	35.06d	41.75e	48.30e	24.45c	32.23d
25% NPK+COMP (21 kg/tree)	22.91c	24.93c	36.14c	36.74c	57.09d	54.33d	24.62c	33.81c
75% NPK+COMP (7 kg/tree)+BIO	25.18b	25.89b	36.54c	39.11b	67.82c	64.79c	35.52b	37.78b
50%NPK+COMP (14 kg/tree)+BIO	27.90a	28.74a	45.58a	47.34a	78.88a	89.46a	54.76a	41.50a
25% NPK+COMP (21 kg/tree)+BIO	25.89b	28.30a	39.31b	40.00b	70.90b	76.44b	35.72b	41.47a

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.*
*Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

It is worthily to mention that DHA was increased in manured soil by increasing amounts of compost. Since, DHA in soil treated with high dose of compost (21 kg/tree) gave higher values followed by low doses. This result was observed in the two growing seasons. This result is in agreement with those obtained by Lizarazo et al. (2005) and Chen et al. (2002) who found that dehydrogenase activity was independent on the organic manuring dose, which means that even the lowest dose provided enough carbon to maintain microbial activity. Dehydrogenase activity of soil increased by increasing the amounts of organic matter.

Data revealed that soil treated with 50% mineral-NPK, compost (14 kg/tree) and inoculated with *E. ludwigii* gave the highest values of DHA. This result is logic and was expected and this may be not due only to the effect of inoculation on microbes' number in rhizosphere but also to the beneficial effect of compost on indigenous and introduced biofertilizing PGPR strain proliferation and activities.This result confirms the importance of organic manure that has direct contribution to soil organic matter levels thereby increasing the microbial population. In addition, the positive effect of inoculation with biofertilizing PGPR on microbial activities in the rhizosphere. This result is in accordance with Balakrishnan et al. (2007) who found that the application of compost in combination with phosphate solubilizing bacteria significantly increased soil microflora such as bacteria, fungi and actinomycetes and soil enzyme activities such as dehydrogenase and phosphatase.Also, data showed that DHA records were higher in the second season than the first one. This difference between the two growing seasons may be due to boost addition of organic and biofertilization form season to season.From the obtained data in Table 4, it's worthily to mention that the DHA activity increased gradually from growing season up to 90 days and decreased again.

Periodical changes of nitrogenase activity

Nitrogenase (N_2-ase) activity was periodically determined as an indication of a symbiotic N_2-fixers activity. Data in Table 5,showed that N_2-ase activity was decreased in soil amended with inorganic fertilizer only compared with manured soil with compost. This was true in the two growing seasons. Lower values of N_2-ase activity may be due to the inhibition of N_2-ase activity with the amendment of inorganic nitrogen fertilizer at a high rate. This result is in agreement with Anne-Sophie et al. (2002) and Shams et al. (2013) who found that the addition of NH_4NO_3 decreased the nitrogenase activity. Higher records of N_2-ase activity were observed in manured soil that inoculated with *E. Ludwigii* than that treated with compost only. This result externalized the importance of inoculation on proliferation and enhancement of N_2-fixers in rhizosphere. Enhancement of biological activities caused by organic manure might be due to the introducing of a large amount of living microorganisms and readily utilizable carbon source on which microorganisms live. Similar trend of results was observed at all determination periods.

By increasing the dose of compost and decreasing inorganic fertilizer, the N_2-ase activity showed significant increase either inoculated soil or non-inoculated one. Therefore, N_2-ase activity increased with the increasing of compost dose. Similar trend of results was observed in both growing seasons. These results are in harmony with Cheng and Zhiping (2007) and Shams et al. (2013) who found that when increase the amount of compost application, soil enzymes activities were increased. The highest values of N_2-ase activity were obtained with treatment that received 25% NPK-mineral and compost (21 kg/tree) combined with *E. ludwigii* inoculation.

As a result of continuous addition of biofertilizers during growth season, obtained data revealed that values of N_2-ase activity in inoculated soil at all periods were higher than un-inoculated one. This result explains the synergistic effect of inocula addition on survival and activities of beneficial microorganisms. From the obtained data in Table 5, it's worthily to mention that the N_2-ase activity values were higher at flowering stage (90 days) rather than anther growing stages. Higher records of N_2-ase activity at flowering stage could be attributed to the beneficial effect of root exudates which increase during this stage of cultivated plants on microbial activities. This result is in harmony with those obtained by Neweigy et al.(1997) Hanafy et al. (1998) and Abdel-Rahman (2009) who found that the densities of N_2-fixer bacteria in rhizosphere were higher at heading (flowering) stage of plants than other plant growth stages.

Periodical changes of Phosphatase activity

Alkaline phosphatase (AP) activity was determined for its importance in the mineralization process of organic phosphorus compounds into available phosphorus. Data tabulated in Table 6,

TABLE 5. Periodical changes of nitrogenase activity in apple rhizosphere

Treatments	Nitrogenase activity (µL C_2H_4 g^{-1}dw h^{-1})							
	30		60		90		150 days	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	23.34e*	16.89f	19.34g	10.61f	20.46g	9.67g	5.88e	3.18g
75% NPK+COMP (7 kg/tree)	24.99d	21.17e	23.84f	12.00f	29.47f	18.15f	5.88e	12.87f
50%NPK+COMP (14 kg/tree)	27.48c	26.89d	31.89e	21.21e	38.88e	38.67e	18.28d	21.20e
25% NPK+COMP (21 kg/tree)	33.99b	27.24d	47.03d	43.72d	49.50d	51.08d	18.83cd	23.46c
75% NPK+COMP (7 kg/tree)+BIO	27.78c	39.73c	58.26c	55.71c	60.94c	59.70c	18.96c	22.14d
50%NPK+COMP (14 kg/tree)+BIO	34.25b	42.32b	69.69b	68.52b	73.00b	72.22b	26.38a	27.47b
25% NPK+COMP (21 kg/tree)+BIO	49.82a	48.22a	77.87a	76.45a	78.18a	77.51a	23.59b	38.98a

NPK: nitrogenous, phosphate and potassium mineral fertilizers (control) COMP: compost BIO: inoculation with *E. ludwigii.* *Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

showed that AP activity in the soil amended with compost was significantly higher than inorganic fertilized one. This result is in agreement with Cheng and Zhiping (2007) who reported that size and activity of soil microbial enzymes are greatly stimulated by the addition of manure. Also, Krishnakumar et al. (2007) found that the application of recommended chemical fertilizer showed significant lower phosphatase activity than all organic manure treatments. Moreover, data emphasized that inoculation of soil with *E. ludwigii* led to a significant increase in AP activity. Similar trend of results was observed in the both seasons. This result externalized the beneficial effect of biofertilizer strains in phosphatase production. Moreover, the efficiency of biofertilizers on phosphatase production as well as the beneficial effect of compost as nutritional substances for stimulating of different soil microorganisms specially P-solubilizers. This result is in accordance with Balakrishnan et al. (2007), Abdel-Rahman (2009) and Takeda et al. (2009) who found that the application of compost in combination with phosphate solubilizing bacteria significantly increased soil microflora such as bacteria, fungi and actinobacteria and soil enzyme activity such as dehydrogenase and phosphatase.

Data in Table 6, showed increasing of phosphatase activity by increasing of dose of compost. These results are in harmony with Cheng and Zhiping (2007) who found that when increasing the amount of compost application, phosphatase activity was increased. It is doubtless that the combination of biofertilizers and compost showed the greatest effect on increasing phosphatase activity. The highest records of phosphatase activity were observed in soil treated with 25% NPK-mineral and high dose of compost (21 kg/tree) in combination with *E. ludwigii* inoculation. From the obtained data we can notice that phosphatase activity recorded maximum values at flowering stage. This result can be attributed to the positive effect of the root exudates of cultivated plants.

Periodical changes of soluble potassium

Soluble potassium was periodically determined as an indicator for silicate solubilizing bacteria activity. In this respect, data presented in Table 7,showed that concentration of soluble-K in apple rhizosphere was significantly increased by inoculation with *E. ludwigii*. Concerning the combination between inoculation with PGPR strain and compost, obtained data showed that dual treatments showed higher records of soluble-K than soil treated with compost singularly. Higher values of soluble-K in case of inoculated soil and fertilized with compost may be due to the beneficial effect of compost on multiplication rate of silicate dissolving bacteria. Moreover, it supplies all major nutrients (N, P, K, Ca, Mg and S) necessary for plant growth. Similar trend of

results was observed by Brandjeset al. (1996) and Ancheng and Xi (1994) who found that organic manure significantly increased K-solubilizing, organic P mineralization, and soil respiration rate and enzymes activity.

Moreover, soluble-K was increased with the increasing of compost rate. In this respect, results showed that highly significant increases in soluble-K values occurred during the two successive growing seasons in soil amended with high dose of compost (21 kg/tree) than with lowone (14 and 7 kg/tree). The highest values of soluble-K were observed in soil treated with 25% mineral-NPK combined with compost (21 kg/tree) and inoculated with *E. ludwigii*. Concerning the trend of soluble-K during the experimental periods, obtained data revealed that soluble-K values were higher at flowering stage. This result can be explicated by increasing the microbial activity in this stage where the beneficial root exudates are abundance. These results are in harmony with those obtained by Abdel-Rahman (2009). Generally, data recorded that the soluble-K fluctuated during the growth period. This was true under all investigated treatments. This fluctuation is likely being due to the temperature changes, soil drying & remoistening and plants uptake during the experimental period.

Apple vegetative growth

The vegetative growth parameters i.e., Shoot length, number of leaves, leaf area and chlorophyll were significantly affected by PGPR strain inoculation in combination with compost and mineral-NPK doses in both seasons. Data in Table 8,clearly showed that all vegetative growth characteristics in inoculated apple tree with *E.ludwigii* were increased significantly over the uninoculated ones. Moreover, they also improved by substituting the quarter of mineral-NPK

TABLE 6. Periodical changes of alkaline phosphatase activity in apple rhizosphere

Treatments	Alkaline phosphatase activity (µgρNP g^{-1} h^{-1})							
	30		60		90		150 days	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	11.04e	11.52f	13.83e	12.44d	13.12e	11.60f	9.03g	10.04f
75% NPK+COMP (7 kg/tree)	12.28d	13.76e	14.72d	14.94c	15.76d	16.56e	10.35f	11.60e
50%NPK+COMP (14 kg/tree)	13.83c	14.67d	15.08d	15.01c	18.08c	17.65d	12.04e	12.51d
25% NPK+COMP (21 kg/tree)	14.15c	14.77d	15.70c	15.76b	23.54b	24.65c	13.01d	12.84cd
75% NPK+COMP (7 kg/tree)+BIO	16.01b	16.08c	16.24bc	16.31b	23.62b	26.89b	13.89c	13.28c
50%NPK+COMP (14 kg/tree)+BIO	16.24ab	16.72b	16.78b	17.94a	24.40a	27.21b	17.78b	18.00b
25% NPK+COMP (21 kg/tree)+BIO	16.71a	17.81a	19.05a	18.25a	24.63a	30.16a	19.98a	20.89a

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.* *Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

TABLE 7. Periodical changes of soluble potassium in apple rhizosphere

Treatments	Soluble potassium (ppm)							
	30		60		90		150 days	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	1427c	1433c	1547bc	1556b	1467d	1525d	1387e	1377d
75% NPK+COMP (7 kg/tree)	1427c	1054e	1387e	1424d	1347e	1312f	1427de	1403d
50%NPK+COMP (14 kg/tree)	1467c	1096e	1467d	1471cd	1387e	1444e	1467cd	1471c
25% NPK+COMP (21 kg/tree)	1547b	1222d	1507cd	1411d	1467d	1517d	1501bc	1472c
75% NPK+COMP (7 kg/tree)+BIO	1547b	1476bc	1587b	1510bc	1547c	1630c	1507bc	1473c
50%NPK+COMP (14 kg/tree)+BIO	1787a	1517ab	1707a	1743a	1677b	1771b	1552b	1561b
25% NPK+COMP (21 kg/tree)+BIO	1787a	1560a	1707a	1751a	1743a	1897a	1627a	1646a

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.* *Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

by compost (7 kg/tree). The highest values of vegetative growth characteristics were recorded with using 50% mineral-NPK + organic-N (compost 14 kg/tree) + *E. ludwigii* inoculation for first and second seasons. While, the lowest values of vegetative growth characteristics were recorded with using the 25% mineral-NPK + 75% organic-N without inoculationin both seasons.

The increments in vegetative growth parameters in case of using compost and biofertilizing PGPR strain may be attributed to their role on increasing soil content of available nutrient elements which have main role on formation of protoplasmic material, cells division and elongations biochemical interaction which affect the rate of plant growth.Additionally, the role of PGPR in increment growth promotion by production of phytohormones (Aslanta et al.2007), improving availability of nutrients, non-symbiotic nitrogen fixation and stimulation of disease resistance mechanisms (Zdor and Anderson 1992), which all together may promote the vegetative growth. Obtained results are coincided with those mentioned by Abdel-Rahman et al. (2017); Doklega (2017); Hosseini et al. (2017); Lallawmkima et al. (2018); Verma et al. (2018) and Ramandeep et al. (2018).

In general, the obtained increment in vegetative growth that result fromthe integration of inorganic, organic and biological fertilization maybe due to high nutrient and mineral content and improve nutrient efficiency as use of different sources of nutrients. Application of organic manure and biofertilizers improved the soil cation exchange capacity (CEC) and porosity due to bulkiness in nature, which in turn helped the plant root development and enhanced the uptake of available nutrients resulting into faster cell division and cell elongation; and consequently increased the tree growth and size.

Leaf nutrient contents

Data in Table 9, show the effect of integrated fertilization with inorganic (NPK), organic (COMP) fertilizers in presence of *E. ludwigii* local strain on leaf chemical composition of Anna apple trees.all examined fertilization positively affect leaf N, P, K and Ca content of apple leaves in the 1st season and the 2nd season.The treatment (50% NPK+ COMP (14 kg/tree) +BIO) recorded maximum leaf N, P, K and Ca content of apple leaves followed by (75%NPK+COMP (7 kg/tree) +BIO) and (100% NPK) in both seasons. On the contrary,thetreatment (25% NPK+COMP (21 kg/tree) had the lowest values of these parameters in most cases in both seasons.Moreover, data obtained that by increasing dose of compost the P, K and Ca leaf content increase. The positively effect of compost and biofertilizing PGPR strain on leaf N, P, K and Ca content of apple leaves was agree with those obtained byDarwesh (2012) on persimmon trees,Rathi and Bist (2004) and Kabeel et al. (2008) on pear, Shddad et al. (2005) on apricot fruit trees..

Respect to inoculation with *E. ludwigii,* data revealed that macronutrients content in apple leaves significantly increased in inoculated apple trees compared with uninoculated ones. These results could be attributed to *E. ludwigii* local strain increasing leaf nitrogen, phosphorus and potassium as well as calcium content by creating certain microbial environment in the root rhizosphere zone for better uptake of N, P, K and Ca, smaller data was confirmed by Srivastava et al. (2002).

TABLE 8. Effect of studied treatments on vegetative growth of apple trees

Treatments	Shoot length (cm)		No. of leaves/ shoot		Leaf area (cm^2)		Chlorophyll (SPAD)	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	32.23d	34.51d	29.92c	36.10d	20.19c	20.89c	43.46c	42.42d
75% NPK+COMP (7 kg/tree)	33.02c	35.64c	31.78b	39.08c	20.43c	20.76bc	45.40b	44.00c
50%NPK+COMP (14 kg/tree)	28.27e	30.14f	25.37e	30.14f	18.27e	16.86e	40.87d	40.21e
25% NPK+COMP (21 kg/tree)	27.16f	30.27f	24.70e	29.87f	17.60f	15.28f	40.54d	39.23f
75% NPK+COMP (7 kg/tree)+BIO	37.10b	40.05b	34.17a	44.37b	23.57b	21.55b	45.99b	45.74b
50%NPK+COMP (14 kg/tree)+BIO	41.39a	44.49a	35.10a	50.25a	24.50a	22.63a	49.02a	47.87a
25% NPK+COMP (21 kg/tree)+BIO	28.19e	32.00e	27.15d	32.27e	19.38d	18.64d	42.67c	41.50d

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.*
*Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

Fruit set, fruit drop and yield

It appeared from data presented in Table 10, that, percentage of fruit set was significantly increased by integration between mineral, organic and biological fertilizer while fruit drop percentage decreased. The highest values of fruit set showed with apple tree that treated with 50% mineral-NPK and 14 kg compost combined with inoculation by *E. ludwigii*. However, the same treatment had the lowest values of fruit drop percentage.On the other hand, fertilized trees with 25% mineral- NPKand compost with rate 21 kg/ tree gave the lowest values of fruit set percentages and the highest values of fruit drop percentage in both seasons.

Generally, the organic and biological fertilizers with doses of mineral-NPK were positively affected fruit set and fruit drop of apple trees in both seasons. Where, they gave the best fruit set and also reduced fruit drop. This is because compost and bacterial strain not only add macro and micronutrients to soil, but also improves the physical and chemical properties of soil; that causes nutritional balance of the soil as well as the plant. Thus, the improved plant growth and development caused by nutritional balance increases fruit set % and reduces fruit drop. The similardata were obtained by Ennab (2016). Moreover, the inoculation with PGPR stain may have increased various endogenous hormonal levels in plant tissue which might be responsible for enhancing flowering, pollen germination and pollen table which might have ultimately increased fruit set (Gabr and Nour El-Din 2012).

Data in Table 10, showed that yield (kg/ tree or ton/fed) were significantly affected by different treatments. The integration between biofertilizers and organic manure treatments with mineral-NPK dose were found to be significantly superior for enhanced apple fruit yield compared to mineral-NPK only. Thus, the inoculated apple trees that treated with 50% mineral-NPK and 14 kg compost recorded the highest records of yield followed by that received 75% NPK and 7 kg compost. The beneficial effect of organic and biofertilizer with mineral-NPK dose on improving yield of apple maybe due to positive effect on nutrients uptake Table 9, which reflected on active vegetative growth parameters in Table 8. The role of biofertilization strain in production of phytohormones and/or improving the availability and acquisition of nutrients or by both Table 3, may explain the encouraged growth and yield of inoculated tree with biofertilizing PGPR strain. Also the positive response of yield as a result of biofertilizers treatments may be attributed to the high ability of biofertilizing PGPR in nitrogen fixation and the secretion of several compounds that increase soil fertility, and enhanced absorb elements by apple tree. Furthermore, the role of compost in increasing bacteria activity in tree rhizosphere, that reflected to tree's ability to grow and increase productivity. This conclusion agrees with the result obtained by Kumeret al.(2011), Hadoleet al. (2015) and Ennab (2016).

TABLE 9. Effect of studied treatments on leaves nutrients content of apple trees

Treatments	N (%)		P (%)		K (%)		Ca (%)	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	1.97c	1.86c	0.263b	0.276b	1.27c	1.31b	1.23c	1.21b
75% NPK+COMP (7 kg/tree)	1.87d	1.75d	0.236b	0.343a	1.26c	1.24c	1.13d	1.20b
50%NPK+COMP (14 kg/tree)	1.54f	1.54f	0.250b	0.276b	1.12d	1.13d	0.97e	0.95d
25% NPK+COMP (21 kg/tree)	1.51f	1.44g	0.336a	0.236b	0.95e	0.96e	0.96e	0.87e
75% NPK+COMP (7 kg/tree)+BIO	2.03b	2.03b	0.340a	0.323a	1.44b	1.51a	1.34b	1.25b
50%NPK+COMP (14 kg/tree)+BIO	2.19a	2.42a	0.346a	0.353a	1.63a	1.54a	1.43a	1.36a
25% NPK+COMP (21 kg/tree)+BIO	1.78e	1.64e	0.250b	0.230b	1.10d	1.15d	1.12d	1.11c

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.* *Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

Physical and chemical fruit properties

It is clear from the obtained data in Fig (3 and 4) that fertilizing apple trees with 50% NPK, compost (14kg/tree) and PGPR strain led to significant increase physical and chemical fruit properties compared to that fertilized with inorganic-NPK only. Moreover, the lowest values of observed with treatments that fertilized with 25% NPK+ compost (21 kg/tree). The same Tables declared that, during the two experimental seasons, fertilized apple with organic and inorganic fertilizers was associated with remarkable and significant decrease in juice total acidity, spatially when the apple trees inoculated with examined microorganism. These results were agreement with obtained by Sharaf et al. (2012) who displayed that organic manure (compost) coupled with bio-stimulants as soil fertilization gave the highest values of chemical and physical fruit parameters of persimmon. Moreover, the same trend were obtained by Fathi et al. (2002), on apple and peach trees, Kabeel et al. (2007) on apple trees and El-Naggar (2009) on apricot trees.

Generally, the fruit quality in all treatment which received dual application of organic and inorganic fertilizer improved by increasing dose of inorganic fertilizers.Except acidity, all physical and chemical fruit properties were remarkable high in inoculated apple trees than un-inoculated ones, during the two experiment seasons.The obtained results were in agreement with these reported by Neilsenet al. (2004) and El-Shenawi and Moursy (2010) who reported that organic amendment increases all considered fruit quality parameters but acidity was decreased.

Conclusion

Consequently from the abovementioned results, it was clear the main role of integrated nutrients management using compost and new biofertilizing PGPR strain with different doses of mineral-NPK on "Anna" apple trees growth and for supplying nutrients which indispensable for improvement of the nutritional status of trees and production of maximum yield with a good quality. Moreover,inoculation by *E. ludwigii*(KP325139.1) combined with compost (14 kg/tree) could compensate 50% of chemical fertilizer with significant increment in growth, yield and fruit quality of apple. As well, increasing soil content of most nutrients without harmful effects on the tree and environment.Finally, the obtained isolate, *E. ludwigii* (KP325139.1) could be exploited as plant growth promoting rhizobacteria for various fruit crops since it exhibits reasonable potential characteristics. Moreover, it could be recommended as a new effective rhizobacteria to increase crop production, decrease production costs and reduce environmental pollution. Considering these facts as well as other features of PGPR new strains, further investigations are ongoing in our laboratories.

TABLE 10. Effect of studied treatments on fruit set, fruit drop and yield of apple trees

Treatments	Fruit set (%)		Fruit drop (%)		Yield (kg/tree)		Yield (ton/fed.)	
	1st S	2nd S	1st S	2nd S	1st S	2nd S	1st S	2nd S
100% NPK	20.20c	18.61c	38.61d	35.03c	36.47b	31.54bc	9.48b	8.20bc
75% NPK+COMP (7 kg/tree)	17.45d	17.45d	39.73c	36.46b	31.55d	27.28de	8.20d	7.09de
50%NPK+COMP (14 kg/tree)	17.05d	16.89e	44.55b	40.54a	28.88e	26.71de	7.51e	6.95de
25% NPK+COMP (21 kg/tree)	16.65d	16.45e	49.50a	41.03a	27.59f	25.49e	7.17f	6.63e
75% NPK+COMP (7 kg/tree)+BIO	21.43b	20.12b	33.66f	28.88e	40.54a	35.17ab	10.54a	9.14ab
50%NPK+COMP (14 kg/tree)+BIO	24.20a	22.00a	28.22g	27.59f	41.04a	38.63a	10.67a	10.04a
25% NPK+COMP (21 kg/tree)+BIO	19.55c	18.37c	34.65e	31.55d	35.03c	30.63cd	9.11c	7.96cd

NPK: nitrogenous, phosphate and potassium mineral fertilizers COMP: compost BIO: inoculation with *E. ludwigii.* *Values followed by the same alphabetical letter(s) in common, within a particular group of means in each character, do not significantly differ, using revised L.S.D test at 0.05 level of probability.

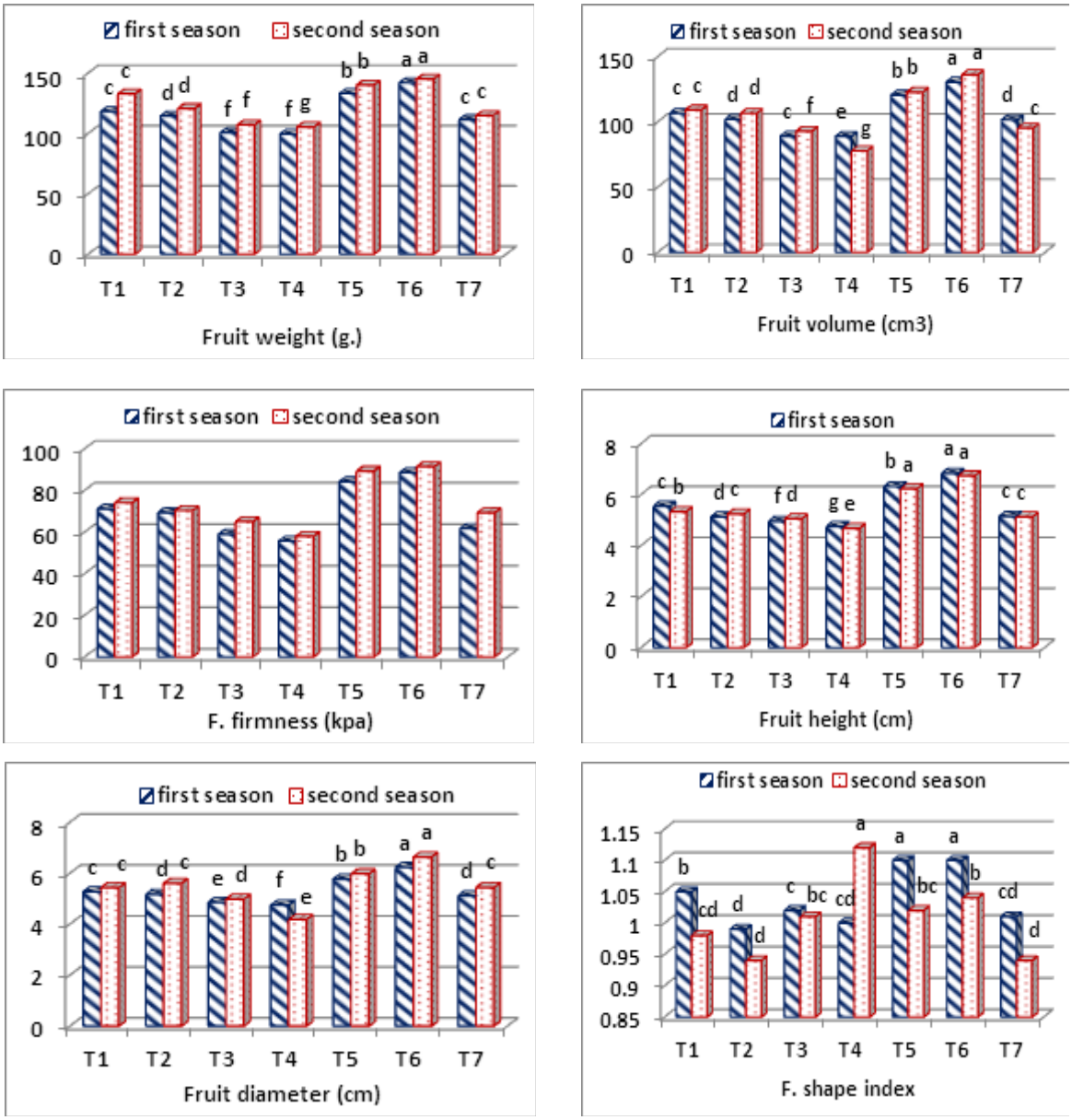

Fig. 3. Effect of studied treatments on physical fruit properties of apple trees.T1: 100% NPK, T2: 75% NPK+compost (7 kg/tree), T3: 50%NPK+ compost (14 kg/tree), T4: 25% NPK+ compost (21 kg/tree), T5:75% NPK+ compost (7 kg/tree)+ *E. ludwigii*, T6: 50%NPK+COMP (14 kg/tree)+ *E. ludwigii*and T7: 25% NPK+COMP (21 kg/tree)+ *E. ludwigii*

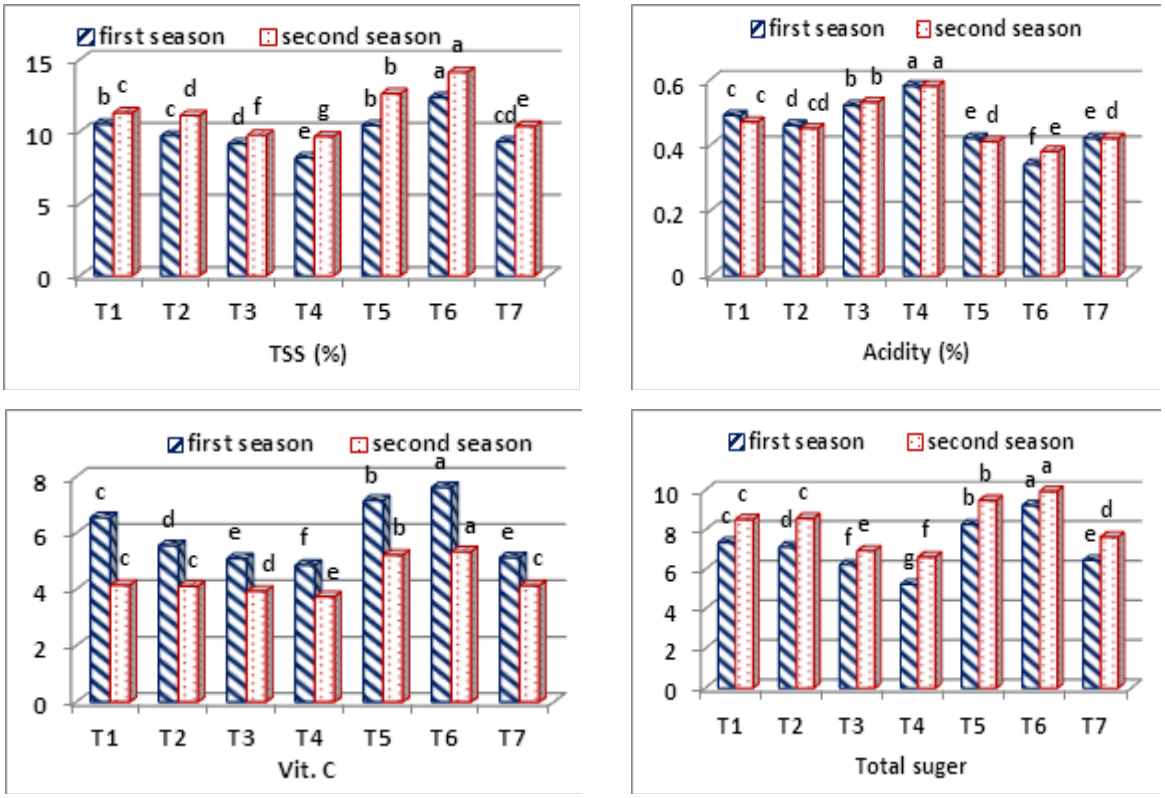

Fig. 4. Effect of studied treatments on chemical fruit properties of apple trees. T1: 100% NPK, T2: 75% NPK+compost (7 kg/tree), T3: 50%NPK+ compost (14 kg/tree), T4: 25% NPK+ compost (21 kg/tree), T5:75% NPK+ compost (7 kg/tree)+ E. ludwigii, T6: 50%NPK+COMP (14 kg/tree)+ E. ludwigii and T7: 25% NPK+COMP (21 kg/tree)+ E. ludwigii

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