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MICROBIOLOGY

Endophytic Colonization of tomato plants by *Gluconacetobacter diazotrophicus* and its effect on crops improvement and yield promotion

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KEY WORDS

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G. diazotrophicus,
Colonization,
crop improvement,
N-fix®,
Ailsa Craig tomato,
Micro Tom tomato,
PGPB

ABSTRACT

Gluconacetobacter diazotrophicus (G.d) is a nitrogen-fixing, endophytic and non-nodulating bacterium isolated from sugarcane. The aim of this study was to investigate the effect of different G.d concentrations on tomato plants grown at very low nitrogen levels to examine its ability to colonize plant tissues and improve crop yield. Tomato seeds were inoculated with different doses of G.d (10^9, 10^{10}, and 10^{11} CFU/ml) and grown in the glasshouse under nitrogen deficiency conditions. Estimation of endophytic bacterial population was performed by bio-PCRs analysis using specific primers for G.d. Three months after sowing, significant variations in the phenotypes and biomass production were observed: the inoculated plants with 10^{10} and 10^{11} of G.d-Nfix-3%sucrose showed increased plant height, root length, nitrogen content, and flower number. They also exhibited a healthier phenotype and higher fruit production in comparison to 10^9 of G.d-Nfix-3%sucrose and non-inoculated control plants. These results showed that coating tomato seeds with specific concentrations of G.d-Nfix-3%sucrose (10^{10} and 10^{11} CFU/ml) was an effective enhancer for crop production at low nitrogen levels and proved the ability of G.d to enhance production of tomato fruits. In conclusion, this study aimed to develop a natural nitrogen fixing seed coating technology to provide a sustainable solution to fertilizer overuse and nitrogen pollution. This technology is environmentally friendly because it based on a food-grade bacterium (G.d) derived from sugarcane. It aims to address the need of international market for sustainable agricultural fertilizers. Moreover, it provides a cost benefit to the farmers via reduced fertilizer costs and improved crops yields.

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1. Introduction

Chemical fertilizers are the most considerable factor of environmental contamination which reach rivers, lakes, resources of groundwater, and consequently cause destructive effects on human health (Kummu *et al*., 2012; Savci, 2012). Moreover, chemical fertilizers are highly costive and should be replaced by a safe and inexpensive source. In recent agriculture, application of nitrogen chemical fertilizers is not economically viable because only 50% of applied nitrogen fertilizers is consumed by plants and the other 50% is being wasted via leaching in the soil. However, the biological nitrogen fixation is very economic due to its direct combination with crops proteins and consequently very low accumulation of nitrogen in the soil (Kale, 2013). Plant growth-promoting bacteria (PGPB) are the most promising alternative source for chemical fertilizers which has a significant impact on crops improvement through biochemical, physiological and molecular mechanisms (Palacios *et al*., 2014). PGPB could help the host plants through nitrogen fixation process, production of phytohormones and vitamins, solubilization and uptake of phosphorus, zinc and potassium (Hardoim *et al*., 2008; Burgess *et al*., 2009; Compant *et al*., 2010).

Gluconacetobacter diazotrophicus is gram negative (-ve), catalase (+ve), oxidase (-ve), sucrose-loving, nitrogen fixing and endosymbiont bacterium (Kale, 2013). Also, *G. diazotrophicus* can fix up to 300 kg / ha of atmospheric nitrogen and solubilize the inorganic phosphates in liquid cultures as a result of acid production (Bhowmik, 1995; Sarita and Bhattacharya, 2000). Moreover, *G. diazotrophicus* has the potential to produce different types of plant growth hormones in the cultures such as gibberellins and Indole Acetic Acid (IAA) (Fuentes-Remirez *et al*., 1993; Bastian *et al*., 1998). Therefore, *G. diazotrophicus* could be used as a cheap and economic biofertilizer and its production technology is very simple and applicable. Muñoz-Rojas and Caballaro-Mellado (2003) demonstrated that the response of different varieties of sugarcane to *G. diazotrophicus* is dependent on the type and age of sugarcane variety, the bacterial genotype, and the level of applied N2 fertilizers. Also, the environmental factors which could have influence on *G. diazotrophicus* colonization and consequently on nitrogen fixation were studied and addressed by Chapman *et al*., (1993).

Inoculation of different crops with *G. diazotrophicus* has been performed in the previous studies to determine the ability of this bacterial strain to colonize and improve growth of plants rather than sugarcane. Inoculation of maize with *G. diazotrophicus* showed an internal colonization of maize with *G. diazotrophicus* (Caballero-Mellado *et al*., 1998). However, Adriano-Anaya *et al*., (2006) reported that inoculation of sorghum with *G. diazotrophicus* increased its dry matter, while inoculation of maize with *G. diazotrophicus* had no effect.

In the current study, inoculation of different tomato varieties with *G. diazotrophicus* has been performed to examine if *G. diazotrophicus* could colonize and improve growth of different varieties of tomato plants rather than sugarcane and therefore could increase tomato fruit yields.

2. Materials and Methods

2.1. Bacterial strain, media and Growth Conditions

Gluconacetobacter diazotrophicus strain used in this study was kindly provided by Azotic Technologies Ltd., BioCity, Nottingham, United Kingdom, where a strain proprietary to this company and grown at 28 ˚C in ATGUS (Cocking *et al*., 2006) and LGIP (Stephan *et al*., 1991) growth media. The bacterial growth was monitored by reading the absorbance at 600 nm as a wavelength (OD_{600} or optical density) using a TECAN Infinite F200 automated, multifunctional luminometer spectrometer.

2.2. Plant Materials and Growth Conditions

The wild type tomato (*Solanum lycopersicum* L) (Ailsa Craig and Micro Tom

varieties) were kindly provided by Prof. Rupert Fray's and Graham Seymour's labs (Division of Plant and Crop Sciences, University of Nottingham, UK). Unless specific conditions are stated, the tomato plants were grown in Levington M3 compost consisting of 20 mg/L Intercept (Scotts, Ipswich, UK) insecticide.

The greenhouse for growing tomato was adjusted to be 16 h: 8 h photoperiod and 20: 26°C, light: dark.

2.3. Greenhouse experiment

A. Preparation of bacterial inoculum

Gluconacetobacter diazotrophicus (WT) (1 mL) was grown overnight in 1 L flasks containing 100 ml of ATGUS medium on a rotatory shaker at 200 rpm and at 28 ˚C. Then, culture was centrifuged at 4000 rpm and 4 ˚C for 10 minutes and resuspended in some of supernatant and this thick bacterial suspension was used as inocula. For greenhouse experiment, the bacterial suspension was used to prepare three different doses by serial dilution through mixing with Nfix-3% sucrose solution and it was adjusted to be approximately 10^{11} CFU/ml (O.D~2.15), 10^{10} CFU/ml (O.D~1.325) and 10^{9} CFU/ml (O.D~0.995). The number of CFU was determined by serial dilution, plating on LGI-P medium (Reis *et al*., 1994) and counting bacterial colonies after 5 days of incubation at 28˚C.

B. Inoculation of tomato seeds with *G. diazotrophicus* bacterial suspension

The experiment was performed between October and January (2016-2017) (4 months) at the glasshouses of Nottingham University, Sutton Bonington Campus, Loughborough, United Kingdom.

Tomato seeds "Ailsa Craig and Micro Tom varieties" (*Solanum lycopersicum*) (0.36 gm/treatment) were soaked in 1 mL of *G. diazotrophicus*-Nfix-3% sucrose at 10^{11}, 10^{10}, 10^{9} CFU/ml at room temperature for 30 minutes with gentle shaking. The inoculated seeds and controls (untreated seeds) were placed in sterile petri-plates and let them to dry in the hood for about 3 hours. Next day, the seeds were sown and grown in seedling trays (24 cells per tray and each cell with dimensions 5cmx5cmx5cm, W/L/H) which filled with Levington M3 compost consisting of 20 mg/L Intercept (Scotts, Ipswich, UK) insecticide. Seeds were germinated and grown under a light/dark cycle of 16 and 8 h, respectively, at 20 ˚C during night and 26 ˚C during day.

The experiment comprised 5 treatments: A) untreated seeds or non-inoculated (control), B) inoculated with 10^{11} CFU/ml of *G. diazotrophicus*(wt), C) inoculated with 10^{10} CFU/ml of *G. diazotrophicus*(wt), D) inoculated with 10^{9} CFU/ml of *G. diazotrophicus*(wt).The experimental design was a randomized complete block design as follows; tomato seeds were sown as one seed per each cell, so the total number was 24 seeds per treatment. Plants were not fertilized. All treatments received water daily by overhead irrigation.

Four-week-old seedlings were transferred from the germination trays into larger individual pots (7L) with dimensions 5cmx5cmx5cm, W/L/H) (25cmX25cmX21cm, W/L/H) in case of Ailsa Craig variety and (5L) (16cmX16cmX15cm, W/L/H) in case of Micro Tom variety which filled with low nitrogen compost (%50: %50 silver sand: klasman low N seed compost). Prior to transfer, seedling roots were thoroughly rinsed to ensure the removal of any adhering soil from the germination trays. Five plants per treatment were replanted and all treatments received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. During transferring, some plants were photographed to examine the differences in the structure of shoot and root systems of treatments.

Crop yield was determined by measuring total tomato production throughout the season. Some parameters were recorded once a week such as length of shoot, seedling vigour index, and number of flowers and fruits. Moreover, the Soil Plant Analysis Development (SPAD) leaf chlorophyll meter (KONICA MINOLTA) was used to estimate the status of leaf nitrogen. The evaluation was performed by triplicate at vegetative and yield stages. Determination of color was carried out on the fully expanded leaves of plants from each treatment and the results are expressed as SPAD values.

Moreover, Bio-PCRs were performed on the leaves of crops to detect *G. diazotrophicus*

at different growth stages. Tomato yield production is expressed as means of the total measurements per replicate during four months of evaluation.

C. Growth observations

1- Germination percentage

The two varieties of tomato seeds started to germinate at the fourth day after sowing (DAS). The percentages of germination were calculated by using the following formula.

Germination Percentage (%) =

$$\frac{\text{Number of germinated seeds}}{\text{Number of sown seeds}} X\ 100$$

2- Seedling vigour index (SVI)

It was calculated 30 days after sowing (DAS) by measuring the shoot height and root length of seedlings, then the following equation was used to calculate the vigour index.

SIV = [Shoot Length + Root Length] x % germination

3- Height of the plant

It was recorded at seedling, vegetative, and fruiting stages by measuring the length of plant from the base of the stem to the apical bud of the plant.

4- Length of root

It was recorded at the seedling stage by measuring the distance between the base of stem and the root tip.

5- Number of flowers

It was recorded during flowering stage by counting and recording the blossomed flowers.

6- Number of fruits

It was recorded at the yield stage by counting the harvested fruits from each treatment after 4-months from sowing.

7- SPAD measurement

The hand-held Chlorophyll Meter SPAD-502 device (Konica-Minolta, Japan) (kindly provided by Azotic Technologies Ltd, BioCity Nottingham, UK) was used for fast measurement of chlorophyll content of tomato plant leaves without damaging them. SPAD values for each treatment were the average of 3 replicates and using the same leaves from the same plants every time.

D. Bio-PCR analysis

Specific sets of primers for *G. diazotrophicus* strain (Gd11F&R and Gd12F&R) (Table 1) were used for this analysis.

The samples of tomato leaf tissues were collected from different and random positions by cutting off 3 cm^2 of leaf after 6 weeks from sowing. The leaf samples of tomato plants were surface sterilised with 5% bleach and rinsed several times with sterilised distilled water (SDW), placed in eppendorf tubes and macerated using autoclaved sterile plastic pestles for each sample at room temperature (RT) in the tissueLyser II machine. A 440 µL of LGIP were added to each macerated sample to resuspend the macerated tissues. Then, 20 µL of extract were added to 180 µL of LGIP media (Neat) and 1:10 serial dilutions were prepared. The plates were incubated at 28˚C for 6 days to be used for bio-PCR analysis as a DNA template (Figure 1). A 3 µL of DNA template (different serial dilutions were examined) was mixed with 10 µL of 2x Taq-red master mix, 0.6 µL of Gd11 (F), 0.6 µL of Gd11 (R), 0.4 µL of Gd12 (F), 0.4 µL of Gd12 (R) and complete up to 20 µL of sterile distilled water. The PCR reaction conditions were as follows; initial denaturation at 95˚C for 1 minute, followed by 34 cycles of 95˚C for 20 s, 63˚C annealing for 30 S and 72˚C extension for 20 s. A final extension step was performed for 2 min at 72 °C. The samples were then held at 12 °C. All PCR reactions were set up on ice. Genomic DNA of G.d(WT) was used as a positive control and distilled water was used as negative control.

Primer name	Nucleotide Sequence (5´- 3´)
G.d11-F	TCAGGGCAATCACTAGCCGG
G.d11-R	TCGAGCAGCCGTTTCATCCA
G.d12-F	TGATGCGCTTGTTCGTGACG
G.d12-R	CGTTCGCCCTTGTCGTCATG

Table 1. Details of the primers used for Bio-PCR analysis

in the control plants (uninoculated) and the germination percentage increased by 16.95%

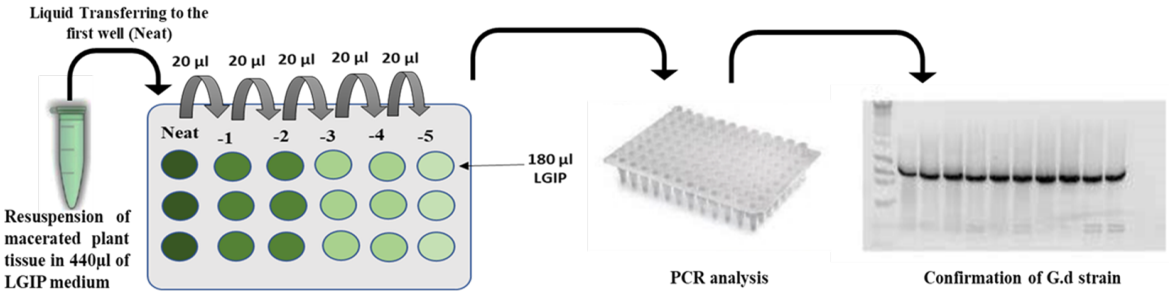

Fig.1. Schematic diagram of the bio-PCR analysis for the inoculated and non-inoculated tomato plant leaves.

E. Statistical Analysis

The results of this study were statistically analysed using one-way of variance (ANOVA) test to determine the significance degree for the obtained variations by using treatments at probability level $p \leq 0.05$. The statistical methods applied in our study were according to Bishop (1983). Results are presented as the mean ± standard deviation (SD) from three to eight replicates. COSTAT statistical software program was used for determination of L.S.D, F-value and significance of the obtained results.

3. Results

3.1. Seedling Stage

3.1.1. Germination percentage

The germination percentage of the plants ranged between 81.94% to 95.83% and 88.88% to 100% in Ailsa Craig and Micro Tom varieties respectively, reflecting variation responses towards *G. diazotrophicus* concentrations as shown in Table 2. The germination percentages of all samples treated with *G. diazotrophicus* bacteria were higher than that of corresponding control.

The results show that successive increase of *G. diazotrophicus* concentration resulted in a successive increase in percentages of germination reached 95.83% and 100% at 10^{11} CFU/ml *G. diazotrophicus*-Nfix-3%sucrose compared with the control. The lowest germination percentages were recorded and 12.5% at 10^{11} CFU/ml *G. diazotrophicus*-Nfix-3%sucrose treatments of Ailsa Craig and Micro Tom seedlings respectively, compared with the control seedlings (untreated).

Table 2. Effect of different concentrations of *G. diazotrophicus*-Nfix-3%sucrose on the germination percentage (%) of Tomato seeds "Ailsa Craig and Micro Tom varieties" (Solanum lycopersicum) grown in Levington M3 compost and received normal tap water.

Germination percentage (%) at 7 Days After Sowing (DAS)		
Treatments	**Ailsa Craig variety**	**Micro Tom variety**
Un treated	**81.94±2.41^{a}**	**88.89±4.81^{a}**
G.d (wt) 10^{9}	**83.33±0.00^{a}**	**97.22±2.41^{b}**
G.d (wt) 10^{10}	**88.89±2.41^{b}**	**98.61±2.41^{b}**
G.d (wt) 10^{11}	**95.83±1.70^{c}**	**100.0±0.00^{b}**
L.S.D	**0.76**	**1.33**
F-value	**41.33**	**8.61**
Significance	**0.00001*** **	**0.0069** **

Values are the mean of three replicates ± SD, Values with different letters (a, b, and c) are significant at $p \leq 0.05$ and Values within the same column for each factor designed by the same letter are insignificant at $p \leq 0.05$.

Four-weeks-old seedlings were transferred from the germination trays into larger individual pots. Five plants per treatment were replanted and all treatments received zero nitrogen feed (ZeroN2:36P:36K g/L) instead of water to study the effect of *G. diazotrophicus* on plant growth at very low levels of nitrogen and determine which concentration of bacteria

is enough to supply the plant with the required amount of nitrogen and help the plant to grow normally. At this stage, the structure of root and shoot systems shown some variations between all treatments as represented in Figure 2. The given pictures show that the length of root, root hairs, height of stem, and number of leaves were increased gradually by increasing the concentration of *G. diazotrophicus* used for inoculation of both varieties of tomato plants.

3.1.2. Shoot height and root length

The given results in Figure 3 show that gradual increase in concentrations of *G. diazotrophicus* caused a gradual increase of shoot and root of Ailsa Craig and Micro Tom seedlings. The percentage of increase of shoot height of Ailsa Craig seedlings reached 20.59%, 21.57% and 42.16% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared with the uninoculated seedlings (control). Also, the percentage of increase of root length of the same variety reached 28.57%, 40.48% and 76.19% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared with the uninoculated seedlings (control). Where the length of shoot of Micro Tom seedlings increased by 28%, 36%, 40% and the length of root of the same variety increased by 6.84-, 9.47- and 11.57-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* respectively compared with the uninoculated seedlings (control).

As shown, a remarkable increase in the shoot height and root length of Ailsa Craig and Micro Tom seedlings was recorded at the highest concentration of *G. diazotrophicus*-Nfix-3%sucrose (10^{11} CFU/ml).

3.1.3. Seedling vigour index

The given results in Figure 4 indicate that the gradual increase in concentrations of *G. diazotrophicus* inoculum resulted in a gradual increase of the vigour index of seedlings. The percentages of increase in the seedling vigour index of Ailsa Craig seedlings reached 24.02%, 35.81% and 73.05% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared with the uninoculated seedlings (control). On the other hand, there was a highly significant increase in all inoculated treatments of Micro Tom variety and the percentages of increase reached 50%, 66.41% and 76.79% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared with the uninoculated seedlings (control).

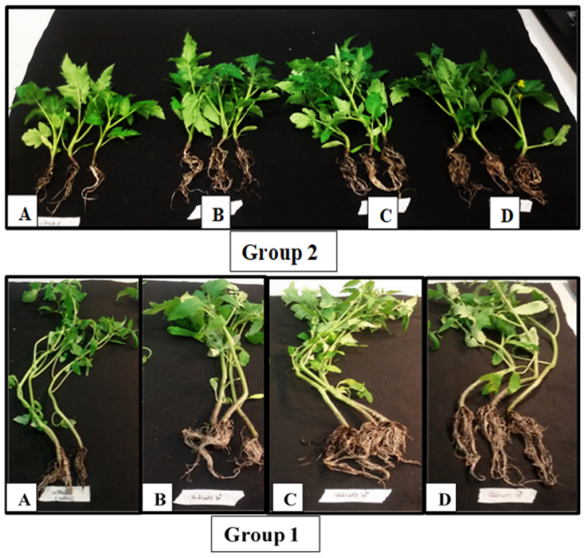

Fig.2. Root and shoot structure of one-month-old plants of Ailsa Craig (Group 1) and Micro Tom (Group 2). A. Untreated seedlings, B. Treated with G.d_{wt} 10^9 (CFU/ml), C. Treated with G.d_{wt} 10^{10} (CFU/ml) and D. Treated with G.d_{wt} 10^{11} (CFU/ml).

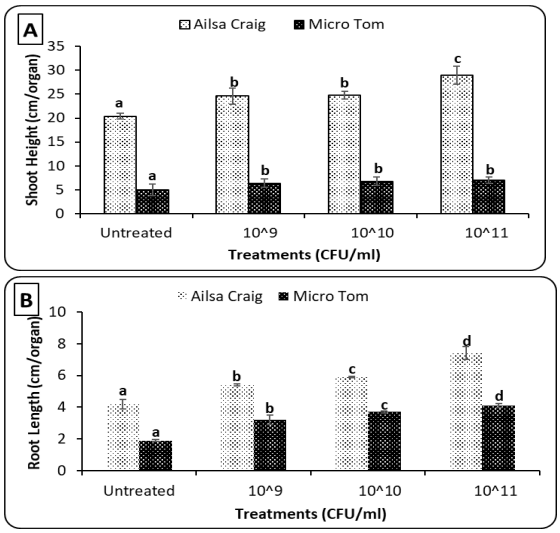

Fig.3.Effect of different concentrations of *G. diazotrophicus*-Nfix-3%sucrose on height of shoot (A) and length of root (B) of 30-day-old tomato seedlings "Ailsa Craig and Micro Tom varieties" (Solanum lycopersicum) grown in Levington M3 compost and received normal tap water. Each column represents the mean value of five replicates. Error bars represent

the standard deviations. Different letters (a, b, c, and d) indicate the differences in significance at $p \leq 0.05$.

3.2. Vegetative Stage

3.2.1. Shoot height

Figure 5 indicates that the high doses of *G. diazotrophicus* caused a significant increase in shoot height of both tomato varieties and the plants looked more greener and healthier. At 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose treatments, the bacteria showed a strong and significant phenotype on the size and number of leaves. The shoot lengths of Ailsa Craig plants were increased by 11.27%, 52% and 85.82% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared to the uninoculated seedlings (control). Moreover, the increase percentages in shoot lengths of Micro Tom variety were 11.54%, 17.31% and 25% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared to the uninoculated seedlings (control) as well.

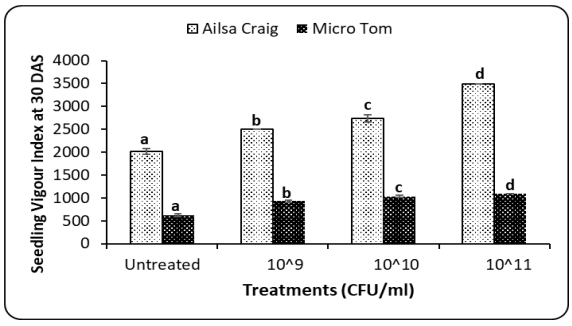

Fig.4. Effect of different concentrations of *G. diazotrophicus* on the seedling vigour index of different tomato varieties "Ailsa Craig and Micro Tom varieties" (Solanum lycopersicum) grown in Levington M3 compost and received normal tap water. Each column represents the mean value of five replicates.

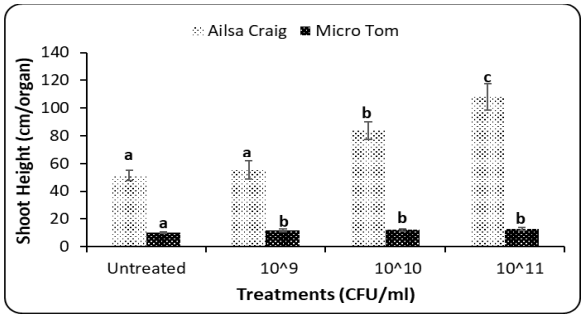

Fig.5. Effect of *G. diazotrophicus* on height of shoot of 5-weeks-old tomato (Solanum lycopersicum) (Ailsa Craig and Micro Tom varieties) plant grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. Each column represents the mean value of five replicates. Error bars represent the standard deviations. Different letters (a, b, and c) indicate the differences in significance at $p \leq 0.05$.

3.2.2. SPAD measurements

The provided results in Figure 6 show that the SPAD values were significantly higher than those noticed in the uninoculated plants (control). Gradual increase in *G. diazotrophicus* concentrations caused a gradual increase in SPAD values. In Ailsa Craig tomato plants, the percentages of increase were 2.78-, 1.83- and 8.78-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared with the control plants. On the other hand, the increase percentages in the other variety (Micro Tom) were 1.50-, 3.19- and 4.78-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared to the control.

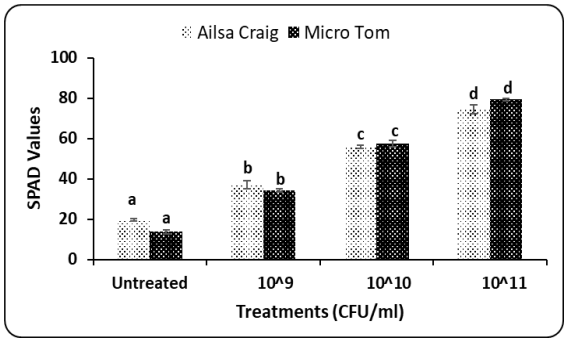

Fig.6. Effect of *G. diazotrophicus* on greenness index of 5-weeks-old tomato (Solanum lycopersicum) (Ailsa Craig and Micro Tom varieties) plant grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. Each column represents the mean value of five replicates. Error bars represent the standard deviations. Different letters (a, b, c, and d) indicate the differences in significance at $p \leq 0.05$.

3.3. Flowering Stage

3.3.1. Number of flowers

The flowers started to appear when the plants reached 6-weeks and 8-weeks-old after sowing in Ailsa Craig and Micro Tom varieties, respectively. The fruits started to grow after

one week from flowering in both varieties. The flowering stage here included the period between appearance of flowers and growing of first fruit.

The provided figure No. 7 shows that the flowers number in the inoculated plants was significantly higher than those observed in the untreated ones. In Ailsa Craig tomato plants, the average number of flowers ranged between 13.4 and 38.6 flower per plant and the increase percentages reached 0.87-, 1.51- and 1.88-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared to the control. On the other hand, the increase percentages in the other variety (Micro Tom) were 28.45%, 32.76% and 81.89% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared to the control.

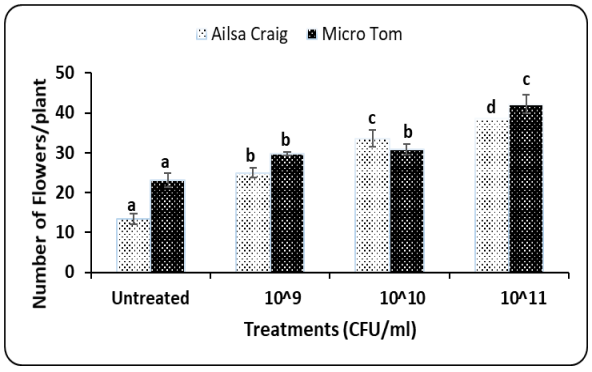

Fig.7. Effect of *G. diazotrophicus* on number of flowers of two varieties of tomato (Solanum lycopersicum) (Ailsa Craig and Micro Tom) plants grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. Each column represents the mean value of five replicates. Error bars represent the standard deviations. Different letters (a, b, c, and d) indicate the differences in significance at $p \leq 0.05$.

3.4. Fruiting or Yield Stage

3.4.1. Shoot height

At this stage of growth, the shoot height of Ailsa Craig showed a significant increasing till the end of the experiment, while Micro Tom plants stop to grow more in their lengths after 10-weeks from sowing.

The provided Figure No. 8 shows that the high doses of *G. diazotrophicus* caused a significant increase in shoot height of both tomato varieties. In case of Ailsa Craig variety, the percentages of increase reached 0.1-, 13.60- and 13.72-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared with the uninoculated plants (control). The shoot length of Micro Tom plants was increased by 3.39%, 15.25% and 35.59% at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared with the control plants.

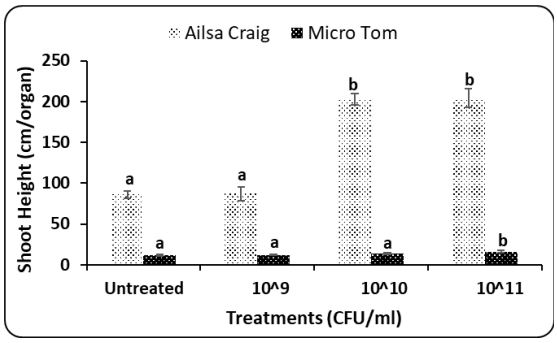

Fig.8. Effect of *G. diazotrophicus* on height of shoot of 4-months -old tomato (*Solanum lycopersicum*) (Ailsa Craig and Micro Tom varieties) plant grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. Each column represents the mean value of five replicates. Error bars represent the standard deviations. Different letters (a and b) indicate the differences in significance at $p \leq 0.05$.

3.4.2. SPAD measurements

In the inoculated treatments of both tomato varieties, the SPAD values were significantly higher than those observed in the uninoculated plants (control). As represented in Figure No. 9, a gradual increase in *G. diazotrophicus* concentrations resulted in a gradual increase in SPAD values. In Ailsa Craig tomato plants, the percentages of increase were 0.93-, 2.11- and 3.15-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, respectively compared with the uninoculated plants (control).

On the other hand, the increase percentages in the other variety (Micro Tom) were 1.70-, 3.80- and 5.69-fold at 10^9, 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* N-fix-3% sucrose, respectively compared to the control.

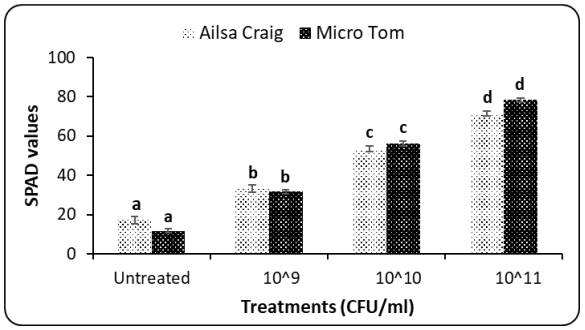

Fig.9. Effect of *G. diazotrophicus* on greenness index of 4-months -old tomato (Solanum lycopersicum) (Ailsa Craig and Micro Tom varieties) plant grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water. Each column represents the mean value of five replicates. Error bars represent the standard deviations. Different letters (a, b, c, and d) indicate the differences in significance at $p \leq 0.05$.

3.4.3. Number of fruits

Inoculation of tomato seeds with *G. diazotrophicus* affected crop yields under nitrogen limitation conditions. Yield per plant of tomato varieties showed statistically significant variation due to differences in *G. diazotrophicus* concentrations.

Table 3 indicates that the number of harvested fruits of inoculated Ailsa Craig plants significantly increased at 10^{10} and 10^{11} CFU/ml of *G. diazotrophicus* Nfix-3% sucrose, while the fruits number at 10^{9} CFU/ml of *G. diazotrophicus* was approximately similar to the uninoculated plants (control). However, the harvested fruits number in Micro Tom plants was increased in all inoculated plants at all doses of *G. diazotrophicus* Nfix-3% sucrose (10^{9}, 10^{10} and 10^{11} CFU/ml) when compared to the untreated plants.

In the inoculated plants, the maximum number of fruits per plant (16 and 38.8) was recorded from the plants which inoculated with high doses of bacteria (10^{11} CFU/ml) in Ailsa Craig and Micro Tom varieties, respectively. The minimum number of fruits (3 and 16.8) was recorded from the plants which received low doses of bacteria (10^{9} CFU/ml) in Ailsa Craig and Micro Tom varieties, respectively.

Results showed that the highest dose of *G. diazotrophicus* (10^{11} CFU/ml) caused a highly significant increase in tomato crop yield of both varieties, where the percentages of increase in Ailsa Craig and Micro Tom varieties were 7- and 6.19-fold, respectively compared to control.

Table 3. Effect of *G. diazotrophicus* on fruit numbers of 4-months -old tomato (Solanum lycopersicum) (Ailsa Craig and Micro Tom varieties) plant grown in %50: %50 silver sand: klasman low N seed compost and received zero nitrogen feed (Zero N2:36P:36K g/L) instead of water.

	Fruit Number/plant	
Treatments	**Ailsa Craig plants**	**Micro Tom plants**
Un treated	**2.0±1.22^{a}**	**5.40±1.14^{a}**
G.d (wt) 10^{9}	**3.0±0.71^{a}**	**16.8±2.28^{b}**
G.d (wt) 10^{10}	**9.6±2.07^{b}**	**20.0±3.74^{b}**
G.d (wt) 10^{11}	**16±1.87^{c}**	**38.8±4.27^{c}**
L.S.D	**2.09**	**4.17**
F-value	**86.44**	**99.32**
Significance	**0.0000*****	**0.0000*****

3.1. Bio-PCR analysis for Ailsa Craig and Micro Tom tomato leaves

The leaves of Ailsa Craig and Micro Tom varieties were sampled at 6-week of growth and tested for presence of *G. diazotrophicus*. The extracts of tomato leaves were used as templates of DNA in the PCR reactions and amplified using the forward primers G.d11 and G.d12 in combination with the revers primers G.d11 and G.d12 (Table 1). The two sets of primers were used in the same reaction and the expected band sizes were (1118 bp and 478 bp) at (G.d11F&R and G.d12F&R), respectively. The entire 20 µl PCR volume was electrophoresed on a 1% agarose gel with respective positive and negative controls (Figure 10).

The gels of bio-PCR analysis using G.d11 and G.d12 primers show that G.d(wt) bacteria were present at (-2 dilution) in Ailsa Craig (Figure 10, Ae) and Micro Tom (Figure 10, K) plant leaves which treated with 10^{9} CFU/ml with the expected band sizes (1118 and 478 bp), while the other dilutions (-3, -4, -5 dilutions) gave negative results (Figure 10, Af, A, B) (Figure 10, L, M & N) of Ailsa Craig and Micro Tom varieties, respectively. The plants which inoculated with 10^{10} CFU/ml of G.d(wt) showed positive results with the expected band sizes at (-2 & -3 dilutions) for Ailsa Craig and Micro Tom varieties (Figure 10, C&D) and (Figure 10, O&P), respectively. However, the lower dilutions (-4, -5) of 10^{10} CFU/ml of G.d(wt) plants of both varieties did not give

positive results (Figure 10, E & F) (Q & R) for Ailsa Craig and Micro Tom varieties, respectively. On the other hand, the plants which inoculated with 10^{11} CFU/ml of G.d(wt) showed positive results at (-2, -3 & -4 dilutions) for Ailsa Craig and Micro Tom varieties (Figure 10, G, H&I) and (Figure 10, S, T&U), respectively. The lower dilution (-5) of 10^{11} CFU/ml G.d(wt) inoculated plants did not give positive results as shown in figure 10 (J&V) of Ailsa Craig and Micro Tom varieties, respectively. The untreated plants (control) of both tomato varieties showed negative results with the G.d primers at all dilutions (Figure 10, Aa, Ab, Ac, Ad & W, X, Y, Z).

4. Discussion

To achieve a sustainable management and alleviate the environmental problems by minimizing the application of fertilizers, an efficient inoculant must be detected (Alves *et al*., 2004; Adesemoye *et al*., 2009; Hungria *et al*., 2010, 2013). Production of biologically treated seeds with one of Plant Growth Promoting Bacteria (PGPB) will enable farmers to purchase pre-inoculated seeds and use them for direct sowing in similar way they have obtained seeds treated with pesticide (Catroux *et al*., 2001).

Our present study shows that application of *G. diazotrophicus* using seed coating process resulted in an efficient colonization of tomato plants and consequently crop improvement. Our results are in accordance with numerous studies that reported the efficiency of seed coating process on germination of seeds, growth of seedlings, root length, shoot height, leaf area, dry plant biomass and significant yield (Zelonka *et al*., 2005; Gevrek *et al*., 2012; Tavares *et al*., 2013). In addition, Widnyana (2018) reported that the best method for application of bacterial inoculant on tomato seeds is soaking them in the bacterial suspension and it is better than watering plants or drenching the roots. Moreover, this advanced technology enhances plant growth, reduces insecticide leaching from treated seeds and minimizes production of dust from seeds (Avelar *et al*., 2012).

In the present study, a mixture of Gum Arabic, tween 80 and sucrose is called N-fix solution was used as a sticker agent to fix the bacterial inoculum on the surface of all tested plants. A good adhesive is necessary to bind the bacterial inoculum to plant seeds and thereby improve colonisation efficiency. Our study reveals that seed coating with N-fix solution is effective in colonisation of plants with *G. diazotrophicus* strain. Moreover, N-fix solution in our study was provided with 3% of sucrose, because it is well-known that this sugar is a very necessary carbon source required for *G. diazotrophicus* growth (Cavalcante and Dobereiner, 1988) and the natural plant hosts of this bacterial strain have high concentrations of sucrose (Muthukumarasamy *et al*., 2002). Moreover, sucrose plays a critical role during nitrogen fixation process because it supplies the bacterial cells with enough amount of energy molecules required for this biological process (Galar and Boiardi, 1995).

Although high availability of nitrogen in soil can minimize the plant root quality, the deficiency of nitrogen can result in reduced biomass production and yield (Werker *et al*., 1999). Two different varieties of tomato seeds were used in this experiment to examine how colonization would occur in different types of tomato genotypes. The biochemistry and physiology may be different in various genotypes and can affect the bacterial distribution in the plant tissues; this has been reported in previous studies on *G. diazotrophicus* (Tian *et al*., 2009) and other types of bacteria such as Paenibacillus (Silva *et al*., 2003).

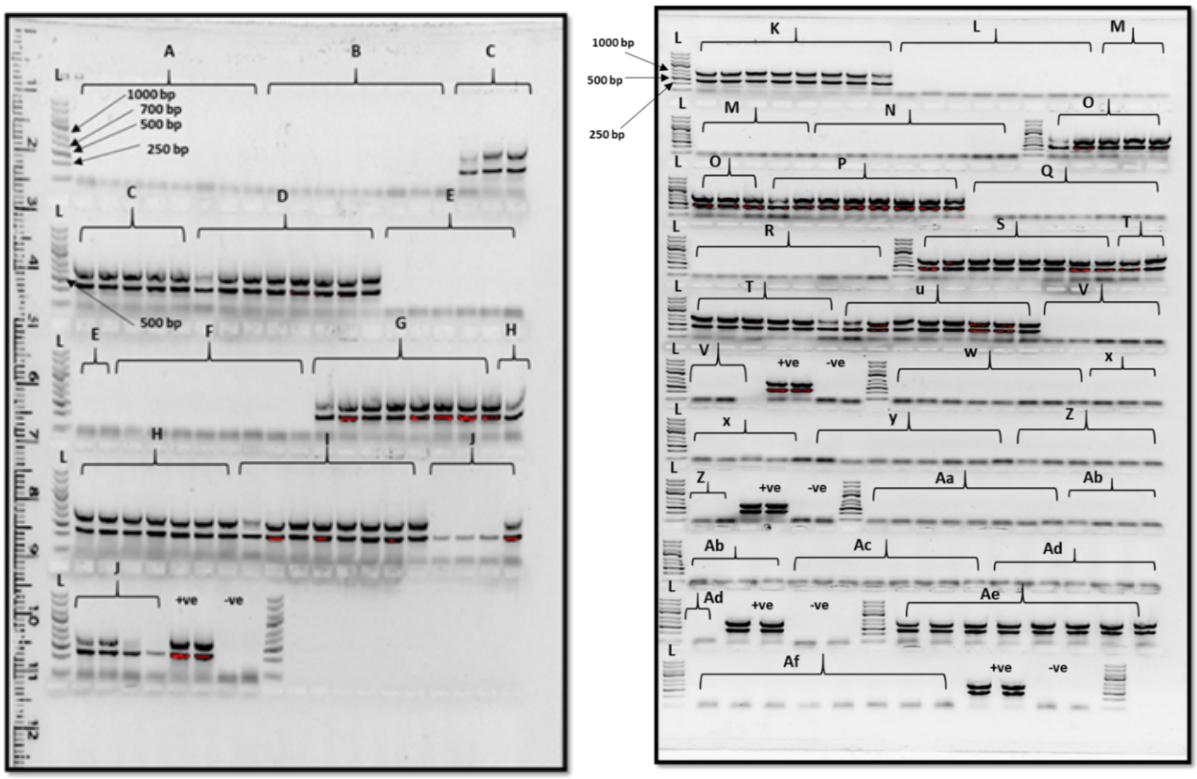

Fig. 10. Bio-PCR for Ailsa Criag and Micro Tom tomato leaves using Gd11&Gd12 primers (appendix I). Leaf extracts of different concentrations of G.d(wt) (10^9, 10^{10}, 10^{11} CFU/ml) at different serial dilutions. A- Ailsa Criag (10^9 at -4 dilution), B- Ailsa Criag (10^9 at -5 dilution), C- Ailsa Criag (10^{10} at -2 dilution), D- Ailsa Criag (10^{10} at -3 dilution), E- Ailsa Criag (10^{10} at -4 dilution), F- Ailsa Criag (10^{10} at -5 dilution), G- Ailsa Criag (10^{11} at -2 dilution), H- Ailsa Criag (10^{11} at -3 dilution), I- Ailsa Criag (10^{11} at -4 dilution), J- Ailsa Criag (10^{11} at -5 dilution), K- Micro Tom (10^9 at -2 dilution), L- Micro Tom (10^9 at -3 dilution), M- Micro Tom (10^9 at -4 dilution), N- Micro Tom (10^9 at -5 dilution), O- Micro Tom (10^{10} at -2 dilution), P- Micro Tom (10^{10} at -3 dilution), Q- Micro Tom (10^{10} at -4 dilution), R- Micro Tom (10^{10} at -5 dilution), S- Micro Tom (10^{11} at -2 dilution), T- Micro Tom (10^{11} at -3 dilution), U- Micro Tom (10^{11} at -4 dilution), V- Micro Tom (10^{11} at -5 dilution), W- Micro Tom (Untreated at -2dilution), X- Micro Tom (Untreated at -3 dilution), Y- Micro Tom (Untreated at -4 dilution), Z- Micro Tom (Untreated at -5 dilution), Aa- Ailsa Criag (Untreated at -2 dilution), Ab- Ailsa Criag (Untreated at -3 dilution), Ac- Ailsa Criag (Untreated at -4 dilution), Ad- Ailsa Criag (Untreated at -5 dilution), Ae- Ailsa Criag (10^9 at -2 dilution) and Af- Ailsa Criag (10^9 at -3dilution.

The current study shows that the most effective *G. diazotrophicus* concentration was exhibited using 10^{11} CFU/ml of this bacterial strain. On the other hand, 10^{10} CFU/ml of *G. diazotrophicus* ranked the second order showing high colonization efficiency, while 10^9 CFU/ml of inoculated bacterium has the lowest efficiency in which led to similar effect of untreated control plants and that may be refer to low levels of the bacterium inoculum.

The present study revealed that the growth criteria including root length, shoot height, leaf chlorophyll content, number of flowers and fruits at seedling, vegetative and fruiting stages were significantly increased by inoculation of tomato seeds with 10^{11} CFU/ml of *G. diazotrophicus*-Nfix-3%sucrose due to its beneficial effects on plant and improve the growth. This ameliorative impact on the inoculated tomato plants may be referred to production of plant growth promotion substances, solubilization of insoluble mineral phosphorus and the activity of nitrogenase enzyme (caballero-Mellado *et al.*, 2007; Luna

et al., 2012). The significant increase in tomato production is due to colonization of their roots and shoots with *G. diazotrophicus* (Luna *et al*., 2012). Our results are consistent with those reported by Bernabeu *et al*. (2015) on tomato plants where they reported that inoculation of seedlings with diazotrophic Burkholderia species resulted in an effective root colonization followed by travelling of the bacterial population to the aerial tissues and improvement of tomato yield production in two various seasons. A similar trend was reported by Mattos *et al*. (2008) who demonstrated that Burkholderia kururiensis as a diazotrophic bacterium has the ability to endophytically colonize rice crop and improve both whole plant growth and grain yield of rice.

The present study indicated that the germination percentage increased by 16.95% and 12.5% at 10^{11} CFU/ml *G. diazotrophicus*-Nfix-3%sucrose treatments of Ailsa Craig and Micro Tom seedlings respectively, compared with the seedlings of control (untreated). Our results are in accordance with the findings of Kale (2013) who studied the effect of different treatments of *G. diazotrophicus* on growth of pearl millet and showed that the germination percentage of inoculated plants ranged from 92.14% to 96.46% indicating different responses of *G. diazotrophicus* treatments. In addition, Bhowmik (1995) and Jambukar (2003) reported that the germination percentage of beet root was significantly increased due to inoculation with *G. diazotrophicus* strain alone. A similar pattern of results was obtained by Widnyana (2018) who demonstrated that fast germination and high germination percentage can be obtained by soaking seeds of tomato in a mixture of Bacillus sp and Pseudomonas spp. The significant effect of *G. diazotrophicus* on germination percentage of plants may be due to production of some plant growth promoting substances such as Gibberllins and IAA (Fuentes-Ramirez *et al*., 1993 and Bastian *et al*., 1998).

Our results showed that 10^{11} CFU/ml treatment of *G. diazotrophicus* recorded maximum seedling vigour index comparing with uninoculated plants. A similar trend was reported by Reis *et al*. (1990) who demonstrated that plant root length and shoot height were improved due to inoculation with Acetobacter strains in sugarcane. In the present study, the maximum root length and plant height of tomato at different stages of crop growth was obtained by inoculation of tomato seeds with *G. diazotrophicus* and it was increased gradually by increasing *G. diazotrophicus* concentration. The significant increase of plant height may be due to the increased nitrogen uptake or production of some phytohormones such as auxins, gibberellins and cytokinins which are responsible for stimulation of plant cell division and stem elongation. Similar observation was reported in sugarcane (Reis *et al*., 1990) and sweet sorghum (Bhowmik, 1995) and maize (Pandey, 2004).

The SPAD meter has been widely applied in the agricultural and research sectors as an alternative method for the measurement of leaf chlorophyll content. Accordingly, in the present study, the chlorophyll content of tomato crops was measured at vegetative and fruiting stages using SPAD device and it was found to be statistically significant increased in the *G. diazotrophicus* treated plants when compared with untreated plants. Our results are in concordance with the previous findings by Rangel *et al*. (2015) who reported that photosynthetic capability and water use efficiency were improved due to colonization of *Arabidopsis thaliana* with *G. diazotrophicus*. Growth of leaf has been affected by nitrogen because this element has a role on the leaf area of plants and affects photosynthesis process and consequently chlorophyll content (Bojović and Marković, 2009). Moreover, they confirmed the relation between chlorophyll content and level of nitrogen. In addition, Evans (1983) demonstrated that the content of chlorophyll is proportional to nitrogen content of plant leaf. These reports may explain the significant increase of leaf chlorophyll content of *G. diazotrophicus* inoculated plants when compared with the untreated plants. Nitrogen fixation may be attributed to the activity of nitrogenase enzyme produced by *G. diazotrophicus* and accordingly increased the nitrogen and chlorophyll contents in tomato leaves. Our results are in harmony with those obtained by Meenakshisundaram and Santhaguru (2011) who reported that the chlorophyll content and leaf nitrogen were

increased in sorghum due to *G. diazotrophicus* effect.

Moreover, the highest increase in length of roots was recorded at 10^{11} CFU/ml of *G. diazotrophicus* when measured at seedling stage. These results are consistent with those observed by Abdallah *et al.* (2018) on tomato plant (*Solanum lycopersicum* L) "Rio Grande variety" where they demonstrated that a significant increase in the height of stem and root length can be obtained by soaking the tomato seedings in 10^8 cells/ml of the bacterial suspensions of different types of PGPB.

This study show that 10^{11} CFU/ml of *G. diazotrophicus* treatment improved the flowering stage and increased the productivity of yielded tomato fruits of both tested tomato varieties due to the role of this growth promoting bacterium in improving the physiological processes involving nitrogen fixation through the activity of nitrogenase enzyme, production of some phytohormones and increasing the biomass of roots and therefor increasing uptake of some essential nutrients from soil (Kuklinsky-Sobral *et al*., 2004).

Bio-PCR analysis on the leaves of both tomato varieties indicated that *G. diazotrophicus* strain can not only establish and live in the root tissues of tomato plant, but also can travel to other plant tissues and aerial parts after colonization, showing the bacterial cells adapted to the new environment and conditions (Tian *et al*., 2009). In addition, the root exudates provided enough nutrients to sustain the growth of bacterial population (Luna *et al*., 2012). A similar finding was reached by Bernabeu *et al*. (2015) who reported that Burkholderia tropica as a plant-growth promoting bacterium was isolated from the stem of tomato plants suggesting that this endophytic bacterium can travel throughout the root tissues till reach aerial organs. These findings are an addition to the previous reports in which the study of colonization was performed only in the plant root tissues (Sevilla and Kennedy, 2000; Cocking *et al*., 2006). Several studies reported that *G. diazotrophicus* may move through the xylem and spread into different plant tissues from the point of primary infection (Olivares *et al*., 1997; James 2000; Tian *et al*., 2009).

In conclusion, soaking tomato seeds in specific concentrations of *G. diazotrophicus*-Nfix-3%sucrose for 30 minutes seem to be efficient method to colonize and improve different varieties of tomato plants and this significant effect may be due to the activity of nitrogenase enzyme and therefor nitrogen fixation, or to production of phytohormones, or solubilization of in organic phosphate in the soil, or to a combination between all those factors (Bernabeu *et al*., 2015). In our study, improvement of plant growth at low levels of nitrogen and presence of *G. diazotrophicus* in the glasshouse experiment, suggesting the role of *G. diazotrophicus* in nitrogen fixation from surrounded air and soil.

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