Plant Archives Volume 20 No. 1, 2020 pp. 2851-2859 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

AZOTOBACTER CHROOCOCCUM AND *RHIZOBIUM LEGUMINOSARUM* INOCULUMS SURVIVAL IN SOIL AND EFFICIENCY IN ENHANCING PLANT GROWTH

Hutaf A.A. Alsalim

Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

The efficacy of *Azotobacter chroococcum* and *Rhizobium leguminosarum* inoculums in nitrogen fixation (nitrogenase enzyme activity), inorganic phosphate solubilization, siderophores and Indole acetic acid (IAA) production were estimated. The inoculums viability in soil and ability to enhance the growth of faba bean (*Vicia faba*) then tested, using pot experiment. Pot experiment contains two factors; sterile and non-sterile soil, three replicates and five treatments; (C) control or non-inoculated seeds, (F) non-inoculated seeds with NPK fertilizer (100 Kg.hec.$^{-1}$), (A) seeds inoculated with *A. chroococcum*, (R) seeds inoculated with *R. leguminosarum*, and (A+R) seeds inoculated with both *A. chroococcum* and *R. leguminosarum*. Soil samples were taken weekly, from inoculated pots (A, R and A+R), to estimate the number of *A. chroococcum* and *R. leguminosarum* bacteria. After seven weeks from seeds germination, length and weight of each vegetative part and root were estimated. The results showed that *R. leguminosarum* isolate was more efficient in nitrogen fixation (622.86n.mol.h^{-1}.ml^{-1}), solubilizing inorganic phosphate (29.90µg.ml^{-1}), producing siderophores (+++) and IAA (13.08 µg.ml^{-1}) than *A. chroococcum* isolate (317.40n.mol.h^{-1}.ml^{-1}, 27.80 µg.ml^{-1}, ++ and 10.85 µg.ml^{-1} respectively). *A. chroococcum* and *R. leguminosarum* were survived in the soil until the end of the pot experiment (7 weeks). *A. chroococcum* log numbers (CFU.g^{-1}) were very strongly and positively correlated with time in sterile soil, while the correlation was less in non-sterile soil. *R. leguminosarum* numbers were very strongly correlated with time in sterile soil and non-sterile soil, in most treatments. *A. chroococcum* inoculum treatment (A) showed increase from control in plant vegetative part length (19.49%, 6.67%) and weight (20.38%, 49.92%) and root length (39.7%, 55.27%) and weight (39.32%, 38.73%) in sterile and non-sterile soil, respectively. *R. leguminosarum* inoculum treatment (R) showed more increase from control, than *A. chroococcum* inoculum, in the vegetative part length (25.6%, 12.4%) and weight (43.35%, 60.48%) and root length (67.68 %, 58.64%) and weight (46.14%, 82.36%) in sterile and non-sterile soil, respectively. Combination of *A. chroococcum* and *R. leguminosarum* inoculums treatment (A+R) exhibited the highest percentages of increase in vegetative part length (32.0%, 20.2%) and weight (105.2%, 107.2%) and root length (77.07%, 67.9%) and weight (76.59%, 78.18%) in sterile and non-sterile soil, respectively.

Key words: *Azotobacter chroococcum*, *Rhizobium leguminosarum*, Bio-fertilizers, siderophores, IAA, nitrogenease.

Introduction

The presence and number of bacteria in the soil are related to its fertility, and considered an indicators of healthy soil. Healthy soil can withstand impacts, such as agriculture, without loss of structure, fertility and biological activity. Hence the idea of replacing the chemical fertilizers with bio- fertilizers arose, even partially to diminish environmental burden of chemical fertilizer. Bio-fertilizers are substances that contain living microorganisms that have no hazard effect on human or environment and enhance soil fertility by fixing nitrogen, solubilizing phosphate and producing many substances, such as siderophore, indole acetic acid, that increase nutrients availability and then enhance plant growth. Numerous bacteria have been used as bio-fertilizers such as *Rhizobium*, *Azotobacter*, *Pseudomonas* and *Azospirillum* (Tiwari *et al.*, 2017). *Rhizobium* can lives in the soil or in legumes root-nodules that provides biologically fixed ammonia fertilizer (Robledo *et al.*, 2008). Their ability to solubilize phosphate, produce siderophore (Gopalakrishnan *et al.*, 2015), and IAA have been reported (Hassan Etesami *et al.*, 2009). *Azotobacter*, which are free-living soil microbes, can fixing nitrogen asymbiotically. Some researchers indicate the efficacy of Azotobacter spp. to solubilize phosphate

***Author for correspondence:** E-mail: hutafalsalim67@scbaghdad.edu.iq

(Rahim *et al.*, 2014), produce siderophores, IAA and HCN (Rajaee e*t al.*, 2007; Bjelic Dragana *et al.*, 2015; Viscardi *et al.*, 2016). *Azotobacter* synthesize auxins, cytokinins, and gibberellin, which are growth materials (Satai *et al.*, 2016), and ferredoxin, hydrogenase and nitrogenase, which are enzymes needed to achieve the nitrogen fixation (Amutha *et al.*, 2014). *A. chroococcum* and *R. leguminosarum* are involved in hydrolytic enzymes production, such as pectinase, chitinase, lipase and protease, which leads to enhance plant growth (Alsalim 2019; Alsalim *et al.*, 2018). Increasing the biomass of plant and seed production by *Rhizobium* and *Azotobacter* inoculums have been reported by several researchers (Tiwari *et al.*, 2017; Gano-Cohen *et al.*, 2016; Muhammad and Umar 2012; Argaw and Abere 2017). The inoculums must be able to withstand, avoid or compete, antagonism or parasitism from other soil micro-organisms, and they also must be able to tolerate fluctuations of soil moisture and temperature. The present study was assessed the efficacy of *A. chroococcum* and *R. leguminosarum* inoculums in nitrogen fixation (nitrogenase enzyme activity), inorganic phosphate solubilization, siderophores and IAA production, their effect (alone and together) on faba beans vegetative part and root length and weight, and evaluate the survival of this inoculums during the experiment time.

Materials and Methods

Bacterial isolates

Azotobacter chroococcum and *Rhizobium leguminosarum* isolates used in this study were provided and identified by The Agriculture Research Center of Abu Ghraib (ARC). *A. chroococcum*, which isolated from the wheat rhizosphere, was reactivated in Burks medium (10 gm/L Glucose, 0.64 gm/L K_2HPO_4, 0.16 gm/L KH_2PO_4, 0.2 gm/L NaCl, 0.2 gm/L $MgSO_4.7H_2O$, 0.05 gm/L $CaSO_4.2H_2O$, 0.01 gm/L $NaMoO_4.2H_2O$, 0.003 gm/L $FeSO_4$, 1000 ml distilled water, pH 7) and incubated at 30^0C for six days (Pozo *et al.*, 2002). *A. chroococcum* was gram-negative, oval to spherical shape, motile, and its growth on Burks medium was smooth, whitish and flat with mucoid texture. It was showed positive results for oxidase, catalase, urease tests and sugar fermentation (glucose, mannitol) (Williams *et al.*, 1984). *R. leguminosarum* was isolated from the rhizosphere of fava bean. It was reactivated in yeast extract mannitol agar (15 gm/L agar, 10gm/L mannitol, 0.5 gm/L K_2HPO_4, 0.2 gm/L $MgSO_4.7H_2O$, 0.1 gm/L NaCl, and 1 gm/L yeast extract, in 1000 ml distilled water at pH 6.8-7.0) and incubated at 30^0C for 3 days (Wang *et al.*, 1998). Morphologically, *R. leguminosarum* was gram-negative, rod shape, motile in hanging drop preparations from 24 h old yeast extract mannitol broth cultures, and produced moist, smooth, whitish and gummy colonies on yeast mannitol agar. Biochemically, it was positive for oxidase, catalase, and urease tests. Moreover it was able to utilize different carbon sources (lactose, Mannitol, Pectin, Sucrose, and Galactose) (Niste *et al.*, 2015).

Detection of inoculums traits that promote plant growth

A. chroococcum and *R. leguminosarum* inoculums ability for nitrogen fixation (nitrogenase enzyme activity), solubilize inorganic phosphate, produce siderophores and IAA were detected. These tests were carried out with control treatment and in three replicates. Acetylene reduction method was used to measure nitrogenase activity. Autoclaved vials containing media were inoculated with 1 ml of bacterial inoculums, OD =0.85, and incubated at 28^0C for 48h. Then locked tightly and injected with acetylene gas and incubated at 28^0C for 2h. Ethylene gas formed was estimated by Gas chromatography device (Turner and Gibson, 1980). Phosphate solubilizing was determined by inoculating 100 ml of NBRIP (National Botanical Research Institute Phosphorus) broth media (glucose 10.0 g, µg$SO_4.H_2O$ 0.25 g , KCl 0.2 g, $Ca_3(PO_4)_2$ 5.0 g, µg$Cl_2.6H_2O$ 5.0 g and $(NH_4)_2SO_4$ 0.1g /1L DW at pH 7) with 24 h old bacterial culture and then incubated in incubator shaker for 14 days (180 rpm) at 28°C, DW used in control treatment, and then centrifuged at 10,000 rpm for 10 min. Released phosphate was determine in the separated supernatant by spectrophotometer, at 880 nm (Behera *et al.*, 2014; Murphy and Riley 1962).

The production of siderophores was performed streaking the isolates in nutrient agar (99ml) with 2, 2 dipyridyl (0.2mg in 1ml DW), then incubated at 28°C for 48h. The appearance of bacterial growth indicates their ability to produce siderophores (Payne, 1980).

Isolates ability to produce IAA was determined by incubating inoculated Luria Bertani (LB) broth medium (peptone 10.0g, yeast extract 5.0g, NaCl 5.0g, L-tryptophan 1.2g, agar 15g and DW 1 L, pH7) for 3 days at 30°C. Then 4 ml of Salkowski reagent (70% perchloric acid 49 ml, 0.5 M $FeCl_3$ 2 ml and DW 49 ml) were added and incubated for 30 min at 25°C in the dark. Developing of a pink to red color indicates a positive result (Bric *et al.*, 1991).

Preparation of inoculums

Preparation of *A. chroococcum* inoculum was achieved by growing the isolate in 100 ml of Burks broth culture for six days at 30^0C. The optical density at 600

nm of bacterial culture was 0.88. *R. leguminosarum* inoculum preparation was done by growing the isolate in 100 ml of yeast extract mannitol broth culture for three days at 30^0C. The optical density of bacterial culture was 0.90 at 600 nm.

Pots experiment

Fava bean (*Vicia faba*) seeds, provided by ARC, were surface sterilized with 2% $HgCl_2$ and 95% Ethanol for 2 min, and then washed with distilled water (Vincent, 1970). Soil, which was obtained from Baghdad University (Aljadria) fields, was air dried and sieved through 2mm, some of the soil properties are illustrated in Table 1. Sterilization of soil was carried out by autoclave at 121^0C for 1 hour three times (Bashan *et al.*, 1995). Sterile and non-sterile soil was distributed in 30 sterile, 5Kg, pots. Seeds, treated with inoculum, were soaked with 100ml of bacterial fresh culture (the optical densities at 600 nm were 0.88 and 0.90 for *A. chroococcum* and *R. leguminosarum* respectively) and mixed with sterilized Arabic gum. Pot experiment, which achieved with complete random design (CRD), includes two factors; sterile and non-sterile soil, three replicates and five treatments; (C) control or non-inoculated seeds, (F) non-inoculated seeds with NPK fertilizer, supplied by ARC, (100 Kg. $hec.^{-1}$), (A) seeds inoculated with *A. chroococcum*, (R) seeds inoculated with *R. leguminosarum*, and (A+R) seeds inoculated with both *A. chroococcum* and *R. leguminosarum*. Thus there were 30 experimental units (2*3*5=30). Seeds were cultured in pots soil, then pots were arranged randomly inside a plastic house and irrigated with tap water. Pots experiment are showed in Fig. 1.

Survival of inoculum in soil

Samples of soil were taken from inoculated pots (A, R and A+R) weekly, after mixing the pots top soil, and kept in sterile plastic bags. Serial of dilutions were prepared from 1 gm of soil, using sterile distilled water. The dilutions were cultured on petri dishes containing Burks medium, for *A. chroococcum* count, and yeast extract mannitol agar, for *R. leguminosarum* count. Burks medium plates were incubated at 30°C for 5-6 days, while yeast extract mannitol agar plates were incubated at 28°C for 2-3 days. Plates were used for counting the colony forming unit (CFU) to evaluate the bacterial inoculum viability in soil during the seven weeks of plant growth.

Assessment of plant growth

Plant growth was assessed by estimating length and weight of each plant vegetative part and plant root, after seven weeks from germination.

Statistical analysis

The coefficient of determination (r^2), which is the proportion of the variance in the dependent variable (x) that is expectable from the independent variable (y), was calculated between inoculums log numbers (y) and time in weeks (x). Statistical analysis for pot experiment was performed using ANOVA, and the averages were compared with least significant difference (LSD) values at the level of 0.05 (SAS, 2012).

Results and Discussion

Inoculums traits that promote plant growth

The results showed that *A. chroococcum* and *R. leguminosarum* inoculums were able to fix nitrogen, solubilize inorganic phosphate, and produce siderophores and IAA. Table 2 show that *R. leguminosarum* was more efficient in nitrogen fixation ($622.86 n.mol.h^{-1}.ml^{-1}$), solubilizing inorganic phosphate ($29.90 \mu g.ml^{-1}$), producing siderophores (+++) and IAA ($13.08 \mu g.ml^{-1}$) than *A. chroococcum* ($317.40 n.mol.h^{-1}.ml^{-1}$, $27.80 \mu g.ml^{-1}$, ++ and $10.85 \mu g.ml^{-1}$ respectively). Researchers recorded *Azotobacter* spp. efficacy to fix nitrogen (158.6 and 326.4nmol $C_2H_4.h^{-1}vial^{-1}$) and solubilize phosphate (Rahim *et al.*, 2014). They also reported *A. chroococcum* ability to produce siderophores, IAA and HCN (Rajaee *et al.*, 2007; Bjelic Dragana *et al.*, 2015; Viscardi *et al.*, 2016), with the present of all these properties, *Azotobacter* spp. considered one of the most important biofertilizers. Fixing nitrogen symbiotically by Rhizobium spp. have bene known long time ago, hence, researchers started to detect additional activities, which can be other reasons for their supporting to plant growth. (Gopalakrishnan *et al.*, 2015) mentioned that *R. leguminosarum* can solubilize phosphate and produce siderophore. Many *Rhizobium* strains capable to produce IAA, which is an important way for this bacteria to affect plant development (Hassan *et al.*, 2009).

The survival of bacterial inoculum in soil

The results in Fig. 2 showed that *A. chroococcum* survived in sterile soil and non-sterile soil until the end of the pot experiment (7 weeks). R treatment (*R. leguminosarum* inoculum) in non-sterile soil exhibited lower numbers of *A. chroococcum*, while in sterile soil no *Azotobacter* found. Combination addition of *A. chroococcum* and *R. leguminosarum* inoculums (A+R treatment) revealed the highest number of *A. chroococcum* in sterile soil and non-sterile soil. This result indicates that there was no antagonisms between the two inoculums.

Linear equations and regression squared values (r^2)

between *A. chroococcum* inoculum log numbers (CFU) (y) and time in weeks (x) were showed in Table 3. The results showed that the bacterial numbers were very strongly correlated with time in sterile soil, while the correlations were less in non-sterile soil. This equation depends on the experiment conditions, so it is possible that the reason for this association is due to the sterilization condition that provides reducing competition for these bacteria.

The results in Fig. 3 showed that *R. leguminosarum* survived in sterile soil and non-sterile soil until the end of the pot experiment (7 weeks). Different soil treatments exhibited different numbers of *R. leguminosarum*. Combination addition of *R. leguminosarum* and *A. chroococcum* inoculums (A+R) treatment exhibited an increase of *Rhizobium* number in sterile and non-sterile soil, which indicates that the addition of *A. chroococcum* inoculum did not contradict with *R. leguminosarum* inoculum.

Linear equations and regression squared values (r^2) between *R. leguminosarum* inoculum log numbers (CFU) (y) and time in weeks (x) were showed in Table 4. The numbers of bacteria were very strongly correlated with time in sterile soil and non-sterile soil, in general.

Rhizospheric soil is a unique niche that offers nutrition and habitation to plant growth promoting microorganisms (Gopalakrishnan *et al.*, 2015). The survival rate and effectiveness of bacterial inoculum depend on organism physiological state, climatic situation and soil properties, such as texture, pH, water content and the existence of protozoan populations (Cunliffe *et al.*, 2006). Soil structural microsites, which influenced by the presence and type of plant grown in the soil, are vary in their suitability's to protect and support the growth of bacteria. *Azotobacter* spp. numbers varied widely in different soils, according to their climate and properties, from several to almost 10000 cfu.g^{-1} (Martyniuk and Martyniuk, 2003). Chaudhary and his team (2013) found that *Azotobacter* strains survived for 90 days after sowing, but the population number decreased compared to count observed at 60 days after sowing. Mendes and Bottomley (1998) found that *R. leguminosarum* population was distributed heterogeneously across the different size of soil aggregates, and their numbers were influenced by the treatment of the crop and sampling time.

Table 1: The properties of soil used in the pot experiment.

The properties	Soil content
pH (1:1)	7.3
Electric conductivity (EC) (1:1) ($ds.m^{-1}$)	1.8
Cation exchange capacity (CEC) ($cmol.Kg^{-1}$)	18.6
Nitrogen ($mg.Kg^{-1}$)	2.21
Phosphor ($mg.Kg^{-1}$)	14.53
Organic matter ($gm.Kg^{-1}$)	1.4
Calcium carbonate ($gm.Kg^{-1}$)	235
Sand ($gm.Kg^{-1}$)	233
Silt ($gm.Kg^{-1}$)	273
Clay ($gm.Kg^{-1}$)	494
texture	Sandy loam

Inoculums effect on plant

The length and dry weight of vegetative part

The average length of fava bean vegetative part in sterile soil treatments (17.21 cm) showed no significant increase than in non-sterile soil treatments (17. 19 cm). Fertilizer (F) treatments showed significant increase in vegetative plant part length compared with control treatments, in sterile and non-sterile soil Fig. 4. *A. chroococcum* (A treatment) and *R. leguminosarum* (R treatment) inoculums increased vegetative plant part length in sterile soil (19.49% and 25.6% respectively) and non-sterile soil (6.67% and 12.4% respectively), with superiority to *R. leguminosarum* inoculum. Combination addition of *A. chroococcum* and *R. leguminosarum* inoculum (A+R) exhibited an obvious increase in the length of vegetative plant part, which was significant in sterile soil compared with control treatment. The percentage of increase caused by A+R treatment was 32% in sterile soil and 20.2% in non-sterile soil. There were no significant differences between fertilizer treatments and combination addition of *A. chroococcum* and *R. leguminosarum* inoculum treatments in sterile soil (18.96 cm and 18.56 cm respectively) and non-sterile

Fig. 1: Pot experiment.

soil (20.06 and 18.03cm respectively).

Fig. 5 showed that there were no significant differences between the averages weight of plants vegetative part in sterile soil treatments (10.88 gm) and non-sterile soil treatments (10.73 gm). In sterile soil, fertilizer treatment (15.03 gm) increased the vegetative plant part weight significantly compared with control (6.92 gm) and *Azotobacter* treatment (8.33 gm), and non-significantly compared with *Rhizobium* treatments (9.92 gm). In non-sterile soil, fertilizer treatment (15.07 gm) showed significant increase from control, *Azotobacter* and *Rhizobium* treatments (6.25, 9.37 and 10.03 gm respectively). *A. chroococcum* inoculum increased the vegetative part weight in sterile and non-sterile soil by 20.38% and 49.92% respectively, while *R. leguminosarum* inoculum increased the vegetative part weight by 43.35% and 60.48%, in sterile and non-sterile soil respectively. Combination addition of *A. chroococcum* and *R. leguminosarum* inoculums (A+R) revealed a significant increase, which was 105.2% in sterile and 107.2% in non-sterile soil, compared with control treatment. There were no significant differences between combination addition (A+R) and fertilizer treatments in sterile soil (14.2 gm and 15.03 gm respectively) and non-sterile soil (12.95 and 15.07 gm respectively).

Table 2: Production of plant growth promoting compounds by *A. chroococcum* and *R. leguminosarum* isolated.

Inoculums traits	*A. chroococcum*	*R. leguminosarum*
Nitrogenase activity (n.mol.h^{-1}.ml^{-1})	317.40	622.86
Soluble P (µg.ml^{-1})	27.80	29.90
Siderophores	++	+++
Indole acetic acid (µg.ml^{-1})	10.85	13.08

Presented results are means of three replicates

Table 3: Linear equation and regression squared value between *A. chroococcum* inoculum log numbers (CFU) (y) and time in weeks (x).

The treatments		R-squared value (r^2)	Linear equation
Sterile	A	0.928	Y=0.2579 X + 4.8194
soil	A+R	0.943	Y=0.3204 X + 4.8395
Non-	A	0.439	Y=0.2842 X + 4.6161
sterile	R	0.939	Y= 0.2127 X + 4.2203
soil	A+R	0.765	Y=0.2884 X + 5.3006

r = 0.4 - 0.59 regarded as moderate, r = 0.6 - 0.79 as strong, r = 0.8 - 0.99 as very strong.

The efficiency of *A. chroococcum* and *R. leguminosarum* inoculums to fix nitrogen, solubilize inorganic phosphate, and produce siderophores and IAA, which improve the root system and subsequently increase nutrients uptake by plant, Table 2 indicate their importance for the development of plants and the superiority of *R. leguminosarum* inoculum.

A. chroococcum inoculum increased plant vegetative part length and dry weight, i.e. able to promote plant growth, because, beside its efficiency to fix nitrogen asymbiotically (Din Misbahud *et al.*, 2019), it's able to synthesis phytohormone (Viscardi *et al.*, 2016) and produce hydrolytic enzymes (Romero-Perdomo *et al.*, 2017). Alsalim (2019) showed that *A. chroococcum* ability to produce pectinase, chitinase, lipase and protease was a reason for enhancing shoot length and weight by an increase of 12.83% and 34.4% respectively, compared with control. Viscardi and his team (2016) mentioned that *A. chroococcum* strains can benefit tomato plant development either directly, through the production of

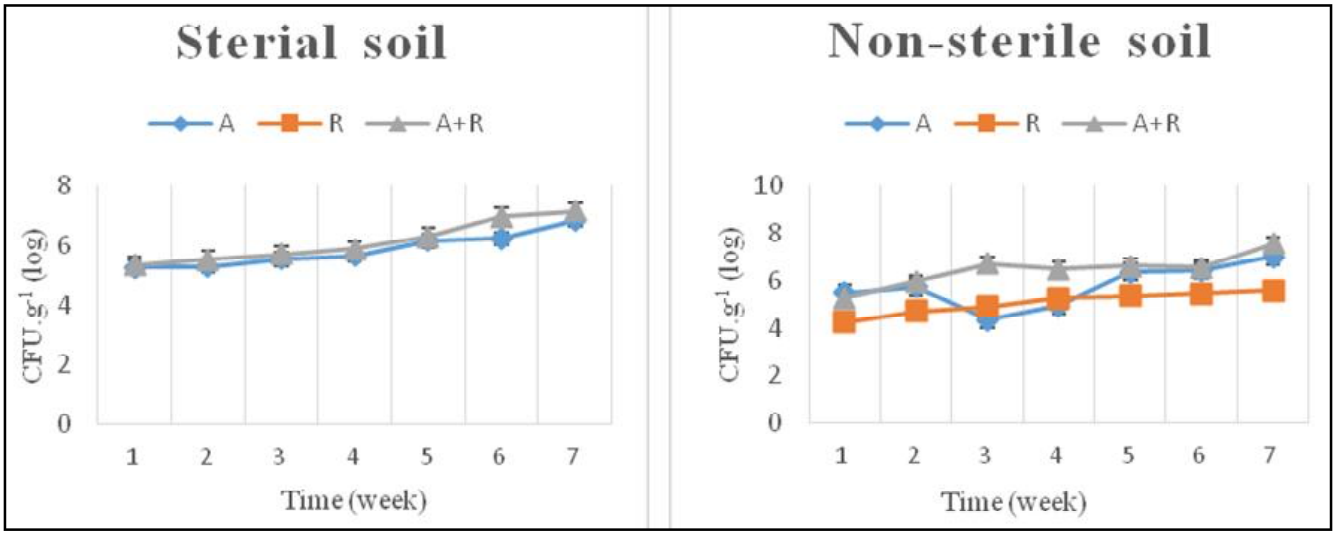

Fig. 2: The log numbers of *A. chroococcum* (CFU) in sterile and non-sterile soil during plant growth. The treatments: A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum and R. leguminosarum* inoculums).

Table 4: Linear equation and regression squared value between *R. leguminosarum* inoculum log numbers (CFU) (Y) and time in weeks (X).

The treatments		R-squared value (R^2)	Linear equation
Sterile soil	R	0.808	Y=0.1148 X + 8.2556
	A+R	0.951	Y=0.1764 X + 8.0929
Non-sterile soil	A	0.965	Y=0.4524 X + 2.2093
	R	0.740	Y= 0.417 X + 6.3161
	A+R	0.792	Y=0.3462 X + 7.0448

r = 0.4 - 0.59 regarded as moderate, r = 0.6 - 0.79 as strong, r = 0.8 - 0.99 as very strong.

siderophores and indole-3-acetic acid that promote growth and increase the availability of nutrients in soil and their uptake, or indirectly, through plant pathogens suppression.

R. leguminosarum inoculum caused extra increase in plant vegetative part length and dry weight through soil nutrient enrichment by nitrogen fixation, phosphate solubilization, siderophore production and phytohormones production (Gopalakrishnan *et al.*, 2015). Alsalim and her team (2018) attributed growth enhancement of fava bean plants to *R. leguminosarum* inoculum ability to produce hydrolytic enzymes, such as pectinase, chitinase, lipase and protease. Argaw and Abere (2017) mentioned that *Rhizobia* increased field pea growth over N-fertilizered plant. Plants inoculated with both *A. chroococcum* and *R. leguminosarum* inoculums have performance improved than those inoculated with each biofertilizer alone. This benefit effect may related to their previously mentioned capabilities and properties, or/and there was no antagonism between the two isolates.

The length and dry weight of root

The treatments of non-sterile soil revealed an increase in the average of plants root length (17.86 cm) from sterile soil treatments (15.49 cm), but this increase is not significant. The average length of plant root in F treatment exhibited highest value, followed by A+R, then R and A. Fig. 6 showed that the increase of root length in fertilizer treatments was significant in non-sterile (20.26 cm) and sterile soil (19.6 cm). *A. chroococcum* inoculum increased the length of plant roots, in sterile and non-sterile soil (13.83 and 18.43 cm respectively), in contrast with control treatments by 39.7% and 55.27% respectively. *R. leguminosarum* inoculum also increased the length of roots significantly in sterile (16.6 cm) and non-sterile soil (18.83 cm) with an increase of 67.68% and 58.64%, respectively, from control. Combination addition of *A. chroococcum* and *R. leguminosarum* inoculums showed a significant increase in root length, which was 77.07% in sterile and 67.9% in non-sterile soil, compared with control treatment. There were no significant differences between combination addition treatments (17.53 cm) and fertilizer treatment (19.6 cm) in sterile and non-sterile soil (19.93 and 20.27 cm respectively).

The results in Fig. 7 presented that non-sterile soil treatments presented an increase in the average of roots dry weight (8.798 gm) from sterile soil treatments (6.946 gm), but this increase is not significant. *A. chroococcum* (A) and *R. leguminosarum* (R) inoculums increased root dry weight in sterile soil (39.32% and 46.14% respectively) and non-sterile soil (38.73% and 82.36% respectively), of them *Rhizobium* inoculum showed significance in non-sterile soil in contrast with control. Combination addition of *A. chroococcum* and *R. leguminosarum* inoculums exhibited significant increase in non-sterile soil (9.8 gm), and non-significant increase in sterile soil (7.77 gm), compared with control treatment (5.5 and 4.4 gm

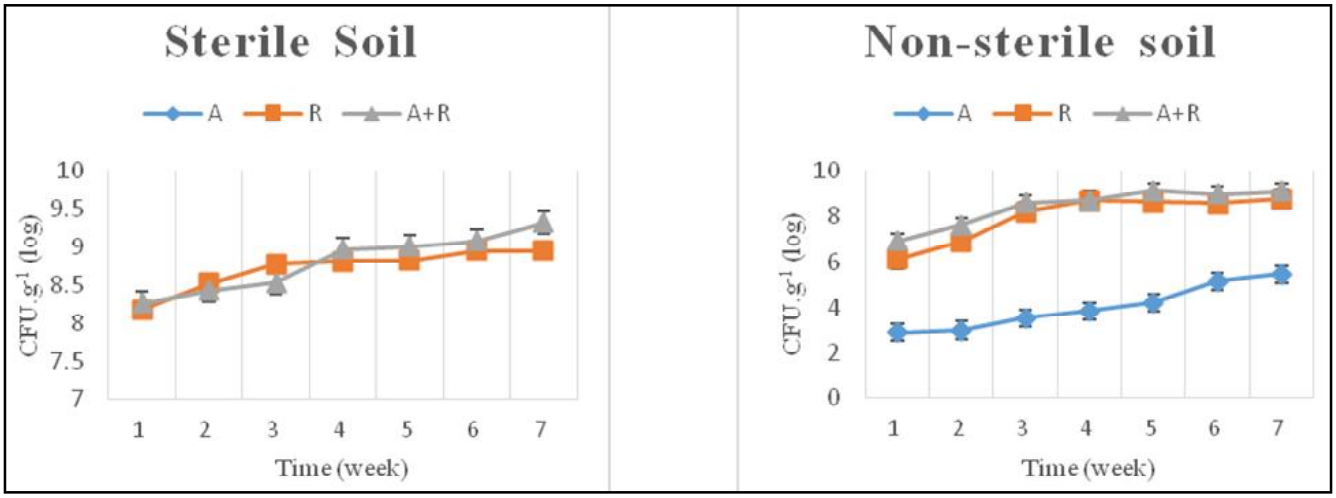

Fig. 3: The log numbers of *R. leguminosarum* (CFU) in sterile and non-sterile soil during plant growth. The treatments: A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum and R. leguminosarum* inoculums).

respectively). The percentage of increase in combination addition treatment was 76.59 % in sterile soil and 78.18% in non-sterile soil. Fertilizer treatments, which obtained higher root weight in sterile (10 gm) and non-sterile soil (11.03 gm), showed no significant differences from combination addition of *A. chroococcum* and *R. leguminosarum* inoculum treatment in sterile and non-sterile soil.

Both *A. chroococcum* inoculum alone and *R. leguminosarum* inoculum alone showed a positive effect

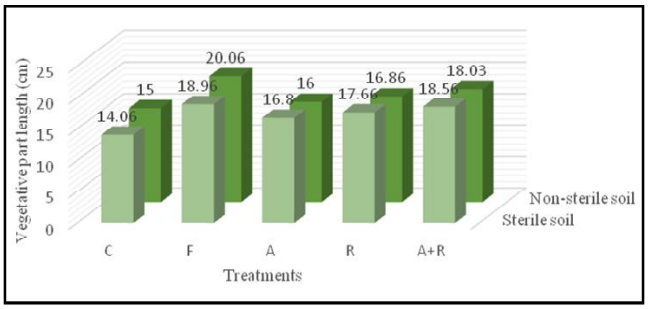

Fig. 4: The length of fava bean vegetative part (cm). The treatments: C (Control), F (Fertilizer), A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum* and *R. leguminosarum* inoculums) ($LSD_{0.05}$=3.67).

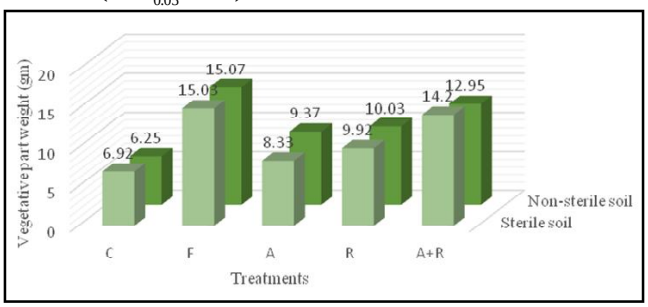

Fig. 5: The weight of fava bean vegetative part (gm). The treatments: C (Control), F (Fertilizer), A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum* and *R. leguminosarum* inoculums) ($LSD_{0.05}$=6.40).

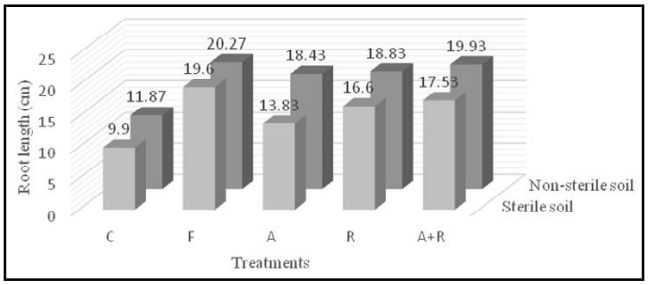

Fig. 6: The length of fava bean root (cm). The treatments: C (Control), F (Fertilizer), A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum* and *R. leguminosarum* inoculums) ($LSD_{0.05}$=6.87).

on roots length and dry weight, but combination inoculums addition had distinctive effect. Inoculums ability to produce IAA hormone Table 2 maybe the most effecting factor for their root growth development, as many researchers pointed (Viscardi *et al.*, 2016; Hassan *et al.*, 2009). Previous studies mentioned that *A. chroococcum* inoculum increased fava bean roots length (60.98%) and weight (38.99%) (Alsalim, 2019) and they suggested that this may associated with synthesized hormone and other growth promoter substances (Wani *et al.*, 2013). Alsalim and her team (2018) acquire 49.28% and 56.2% increase in faba bean roots length and weight by adding *R. leguminosarum* inoculum. Prior study found that the *R. leguminosarum* inoculum promote the growth of carrots and lettuce by increasing nitrogen and phosphor uptake, as well as shoot and root dry matter (Flores-Felix *et al.*, 2013). Tiwari and his team (2017) have reported that mixed inoculations of Azotobacter sp. and *Rhizobium* sp. were significantly enhanced shoot length, root length, shoot fresh and dry biomass, root fresh and dry biomass, leaves number, nodules numbers and chlorophyll content compared with single inoculum of them. They suggested that *Azotobacter* support plant growth in the early stage, by fixing atmospheric nitrogen asymbiotically, while *Rhizobium* need more time in nodules formation and then nitrogen fixation starts.

Conclusion

Biofertilizers are eco-friendly and safe alternatives to chemical fertilizers. They are important plant growth promoters as they producing many substances that increase nutrient availability and subsequently plant growth. *A. chroococcum* and *R. leguminosarum* strains, which used as biofertilizers in this study, were capable of fixing nitrogen, solubilize phosphate, producing siderophore and IAA. Therefor they can be used potentially to improve plant nutrition of nitrogen, phosphor, and micronutrients such as Fe. They encourage faba bean plant growth

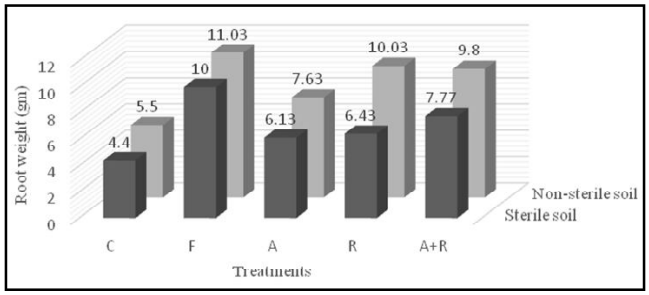

Fig. 7: The weight of fava bean root (gm). The treatments: C (Control), F (Fertilizer), A (*A. chroococcum* inoculum), R (*R. leguminosarum* inoculum) and A+R (combination addition of *A. chroococcum* and *R. leguminosarum* inoculums) ($LSD_{0.05}$=3.46).

through soil nutrient enrichment and promote the defenses of plant against diseases, through hydrolytic enzymes production. Combined addition of *Azotobacter chroococcum* and *Rhizobium leguminosarum* was found more effective than single one. *A. chroococcum* starts fixing nitrogen in the soil, asymbiotically, and help improved plant growth at the early period of seedling growth. *R. leguminosarum*, which have symbiotic relation with legume crop, requires time for nodule formation before nitrogen fixation start. Such situation make *A. chroococcum* as a substitute for chemical fertilizer dose supplementing the biofertilizer, *R. leguminosarum*. This friendly association may recommended for better consequences instead of single inoculum.

References

Alsalim Hutaf, A.A. (2019). Assessment of *Azotobacter chroococcum*'s ability to produce hydrolytic enzymes and enhance growth of *Vicia faba*. *Baghdad Science Journal,* **16(1):** 34-39.

Alsalim Hutaf, A.A., Azhar abood and Lamees M.R. Abbas (2018). Ability of *Rhizobium leguminosarum* inoculum to improve faba beans (*Vicia faba*) growth and produce some hydrolytic enzyme. *Iraqi Journal of Science*, **59(3A):** 1231-1236.

Amutha, R., S. Karunakaran, S. Dhanasekaran, K. Hemalatha, R. Monika, P. Shanmugapriya and T. Sornalatha (2014). Isolation and mass production of biofertilizer (*Azotobacter* and *Phosphobacter*). *International J. of Latest Res. in Sci. and Tech.,* **3:** 79-81.

Argaw Anteneh and Abere Mnalku (2017). Symbiotic effectiveness of *Rhizobium leguminosarum* bv. *vicieae* isolated from major highland pulses on field pea (*Pisum sativum* L.) in soil with abundant rhizobial population. *Annals of Agrarian Science,* **15:** 410-419.

Bashan, Y., M.E. Puente, M.N.R. Mendoza, G. Toledo, G. Holguin, R.F. Cerrato and S. Pedrin (1995). Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. *App. and Environmental Microb.,* **16(5):** 1938-1945.

Behera, B.C., S.K. Singdevsachan, R.R. Mishra, S.K. Dutta and H.N. Thatoi (2014). Diversity, mechanism and biotechnology of phosphate solubilizing microorganism in mangrove - A review. *Biocatalysis and Agricultural Biotechnology,* **3:** 97-110.

Bjelic Dragana, D., B.M. Jelena, B.T. Branislava, Lj.T. Sonja, M.N. Aleksandra and B.M. Nastasija (2015). Screening of *Azotobacter* isolates for PGP Properties and antifungal activity. *J. Nat. Sci.,* **129:** 65-72.

Bric, J.M., R.M. Bostock and S.E. Silverstone (1991). Rapid In-Situ assay for indole-acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied Environmental Microbiology,* **57:** 535-538.

Chaudhary Deepika, Neeru Narula, S.S. Sindhu and R.K. Behl (2013). Plant growth stimulation of wheat (*Triticum aestivum* L.) by inoculation of salinity tolerant *Azotobacter* strains. *Physiol. Mol. Biol. Plants,* **19(4):** 515–519.

Cunliffe Michael, Akitomo Kawasaki, Emma Fellows and Michael A. Kertesz (2006). Effect of inoculum pretreatment on survival, activity and catabolic gene expression of *Sphingobium yanoikuyae* B1 in an aged polycyclic aromatic hydrocarbon-contaminated soil. *Microbiology Ecology,* **58(3):** 364–372.

Din Misbahud, Rubina Nelofer, Muhammad Salman, Abdullah, Faisal Hayat Khan, Asad Khan, Munib Ahmad, Fazal Jalil, Jalal Ud Din and Mudassir Khan (2019). Production of nitrogen fixing *Azotobacter* (SR-4) and phosphorus solubilizing *Aspergillus niger* and their evaluation on *Lagenaria siceraria* and *Abelmoschus esculentus*. Biotechnology Reports. V, 22.

Flores-Felix Jose, Esther Menéndez, Lina P. Rivera, Marta Marcos-García, Pilar MartínezHidalgo, Pedro F. Mateos, Eustoquio Martínez-Molina, Ma de la Encarnación Velázquez, Paula García-Fraile, and Raúl Rivas (2013). Use of *Rhizobium leguminosarum* as a potential biofertilizer for *Lactuca sativa* and *Daucus carotacrops*. *Journal of Plant Nutrition and soil.* **176(6):** 876-882.

Gano-Cohen, K.A., P.J. Stokes, M.A. Blanton, C.E. Wendlandt, A.C. Hollowell, J.U. Regus, D. Kim, S. Patel, V.J. Pahua and J.L. Sachs (2016). *Nonnodulating bradyrhizobium* spp. modulate the benefits of legume-Rhizobium mutualism. *Appl. Environ. Microbiol.,* **82(17):** 5259-5268.

Gopalakrishnan, Subramaniam, Arumugam Sathya, Rajendran Vijayabharathi, Rajeev Kumar Varshney, C.L. Laxmipathi Gowda and Lakshmanan Krishnamurthy (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech, Springer Link.,* **5(4):** 355–377.

Hassan Etesami, Hossein Ali Alikhani, Mahdieh Jadidi and Abolfazl Aliakbari (2009) Effect of superior IAA producing Rhizobia on N, P, K uptake by wheat grown under greenhouse condition. *World Applied Sciences Journal,* **6(12):** 1629-1633.

Martyniuk S. and M. Martyniuk (2003). Occurrence of Azotobacter spp. in some Polish soils. *Polish Journal of Environmental Studies,* **12(3):** 371-374.

Mendes Ieda C. and Peter J. Bottomley (1998). Distribution of a population of *Rhizobium leguminosarum* bv. trifolii among different size classes of soil aggregates. *Applied and Environmental Microbiology,* **643:** 970-975.

Muhammad Javed and Umar Bacha (2012). Role of *Rhizobium* bacteria in bio-fertilizers production. LAP LAMBERT Academic Publishing. Germany.

Murphy, J. and J.P. Riley (1962). A modified single solution method for determination of phosphate in natural waters. *Analytica Chimica Acta.,* **27:** 31-36.

Niste Monica, Roxana Vidican, Carmen Puia, Ioan Rotar and Rodica Pop (2015). Isolation and Biochemical

Characterization of *Rhizobium leguminosarum* bv. *Trifolii* and *Sinorhizobium meliloti* using API 20 NE and API 20 E. *Bulletin UASVM Agriculture,* **72(1):** 173- 178.

Payne, S.M. (1980). Synthesis and utilization of siderophores by *Shigella flexneri. Journal of Bacteriol.,* **143(3):** 1420–1424.

Pozo, C., M.V. Martinez-Toledo, B. Rodelas and J. Gonzalez Lopez (2002). Effect of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpechin (wastewater from olive oil mills) as primary carbon sources. *J. Biotech.,* **97(2):** 125- 131.

Rahim Nosrati, Parviz Owlia, Horieh Saderi, Iraj Rasooli and Mohammad Ali Malboobi (2014). Phosphate solubilization characteristics of efficient nitrogen fixing soil *Azotobacter* strains. *Iran J. Microbiol.,* **6(4):** 285–295.

Rajaee, S., H.A. Alikhani and F. Raiesi (2007). Effect of plant growth promoting potentials of *Azotobacter chroococcum* native strains on growth, yield and uptake of nutrients in wheat. *Journal of Science and Technology of Agriculture and Natural Resources.*

Robledo, M., J.I. Jimenez-Zurdo, E. Velazquez, M.E. Trujillo, J.L. Zurdo-Pineiro, M.H. Ramirez-Bahena, B. Ramos, J.M. Diaz-Minguez, F. Dazzo, E. Martinez-Molina and P.F. Mateos (2008). Rhizobium cellulase CelC2 is essential for primary symbiotic infection of legume host roots. *Microbiology,* **105(19):** 7064–7069.

Rodrigues, A.A., M.V. Forzani, R.S. Soares, S.T. Sibov and J.D.G. Vieira (2016). Isolation and selection of plant growth-promoting bacteria associated with sugarcane. *Pesquisa Agropecuaria Tropical,* **46(2):** 1983-4063.

Romero-Perdomo Felipe, Jorge Abril, Mauricio Camelo, Andrés Moreno-Galván, Iván Pastrana, Daniel Rojas-Tapias and Ruth Bonilla (2017). *Azotobacter chroococcum* as a potentially useful bacterial biofertilizer for cotton (*Gossypium hirsutum*): Effect in reducing N fertilization. *Revista Argentine Microbiologia,* **49(4):** 377-383.

SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS Inst. Inc. Cary N C. USA.

Satai, A. Wani, Subhash Chand, Muneeb A. Wani, M. Ramzan, and Khalid Rehman Hakeem (2016). *Azotobacter chroococcum* – A potential biofertilizer in agriculture: an overview. Soil Sci: Agricultural and Environmental Prospectives, 333-348.

Tiwari, S., R.K. Chauhan, R. Singh, R. Shukla and R. Gaur (2017). Integrated Effect of *Rhizobium* and *Azotobacter* Cultures on the Leguminous Crop Black Gram (*Vigna mungo*). *Adv in Crop Sci. and Tech.*, **5(3):** 1-9.

Turner, G.L. and A.H. Gibson (1980). Measurement of nitrogen fixation by indirect means F.J. Bergersen (Ed.), Methods for evaluating biological nitrogen fixation, John Wiley & Sons. New York. 111-138.

Vincent, J.M. (1970). A manual for the practical study of root-nodules bacteria. *International Biological Program. Handbook,* **15:** Black well Scientific Publication. Oxford, England. 125 – 126.

Viscardi, S., V. Ventorino, P. Duran, A. Maggio, S. De Pascale, M.L. Mora and O. Pepe (2016). Assessment of plant growth promoting activities and abiotic stress tolerance of *Azotobacter chroococcum* strains for a potential use in sustainable agriculture. *J. Soil Sci. Plant Nutr.,* **16(3):** 848-863.

Wang, E.T., P. van Berkum, D. Beyene, X.H. Sui, O. Dorado, W.X. Chen and E. Martinez-Romero (1998). Rhizobium huautlense sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int. J. Syst. Bacteriol.,* **48:** 687–699.

Wani Sartaj, A., Subhash Chand and Tahir Ali (2013). Ptential use of *Azotobacter chroococcum* in crop production: An overview. *Curr. Agriculture Res. J.,* **1(1):** 35-38.

Williams, S.T., M.E. Sharpe and J.G. Holt (1984). Bergey's Manual of Systematic Bacteriology. Baltimore, USA: Williams and Wilkins.