

Assessment of *Azotobacter chroococcum*'s Ability to Produce Hydrolytic Enzymes and Enhance Growth of *Vicia faba*

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Received 10/4/2018, Accepted 19/12/2018, Published 11/3/2019



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Abstract:

A. chroococcum isolate was examined for its ability to produce the hydrolytic enzymes chitinase, pectinase, protease, and lipase, in an effort to enhance the growth of fava bean (*Vicia faba*). Biological experiment was conducted in pots with complete random design (CRD). The experiment includes three treatments: control (plant without treatment) (P), NPK fertilizer (plant + fertilizer) (PF), and *A. chroococcum* inoculum (plant + *A. chroococcum*) (PA). These treatments were performed with sterile and non-sterile soil, which were planted with fava beans. At the end of experiment (seven weeks from planting), length and weight of plant shoot and plant root were calculated. The results show that the isolate was able to produce chitinase, pectinase, protease, and lipase enzymes. Plant growth increased with fertilizer and *A. chroococcum* inoculum addition. Fertilizer treatments (PF) were the best growth indicators, with the averages of shoot length and weight, and root length and weight of 19.52 cm, 15.55 gm, 19.93 cm, and 10.52 gm respectively. The averages of these indicators in *A. chroococcum* treatment (PA) were 16.4 cm, 8.85 gm, 17.52 cm, and 6.88 gm respectively. The percentages of increase caused by *A. chroococcum* inoculum addition (PA) in contrast with control treatment (P) were 12.83% and 34.4% in plant shoot length and weight respectively, and 60.98% and 38.99% in plant root length and weight. In general, growth indicators were higher in non-sterile than sterile soil, but these increases were not significant.

Key words: *Azotobacter chroococcum*, Biofertilizer, Fava bean, Hydrolytic enzyme, Plant growth promoting.

Introduction:

Chemical fertilizers are used to achieve maximum crop production in agricultural fields. The frequent usage of this fertilizers is associated with health hazards, environmental pollution, and loss of soil fertility. Therefore biofertilizers, substances which contain living microorganisms, are gaining importance as they are non-hazardous, ecofriendly and enhance soil fertility. Several bacteria have been used as biofertilizers such as *Azotobacter*, *Azospirillum*, *Pseudomonas* and *Rhizobium* (1). *Azotobacter* are aerobic, free-living soil microbes which play an important role in the nitrogen fixation. The isolates of *Azotobacter* show positive nitrogen fixing capacity ranging from 3.16 to 12.66 mg N per gram of sucrose consumed (2). *Azotobacter* have a full range of enzymes needed to perform the nitrogen fixation such as ferredoxin, hydrogenase and an important enzyme nitrogenase (3). Being free living N₂-fixer, *Azotobacteria* synthesize growth materials such as auxins, cytokinins, and gibberellin, which are the primary

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substances regulating the enhanced growth (4). *A. chroococcum* has been shown to promote the growth of different crops under different soil types and climates (5). Enzymes are major components of biological soil processes, such as the degradation of organic compounds, mineralization and the liberation or recycling of nutrients (6). Hydrolytic enzymes catalyze the hydrolysis of a chemical bond. There are several types of hydrolytic enzymes such as protease, glucanase, cellulase, pectinase, lipase, and chitinase. Large numbers of soil microorganisms produce lytic enzymes such as *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Acetobacter*, and fungi (7). The present study was evaluated *A. chroococcum* as a plant growth promoting bacteria by investigate its ability to produce hydrolytic enzymes and its effect on length and weight of fava beans plant shoot and root.

Materials and Methods:

Determination *Azotobacter* ability to produce hydrolytic enzymes

Chitin, pectin and milk agar-containing plates were inoculated with 24 h aged bacterial

cultures (the optical density = 0.84 at 600 nm) and incubated at 30°C. Formation of a clear zone after incubation time (3-7 days for Chitin, and Pectin, and 24h for milk agar) indicated the positive result for production of chitinase, pectinase and protease enzymes respectively (8, 9).

Lipase production was investigated on mineral salt olive oil agar, which was inoculated with 24 h age of bacterial culture and incubated for 3-7 days at 30°C. Bacterial growth, appearance and reduction of medium oil reflected a positive result (10).

Azotobacter inoculum

The *A. chroococcum* isolate used in this study was provided and identified by The Agriculture Research Center of Abu Ghraib (ARC). This bacterium was isolated from the rhizosphere of wheat. Morphologically, it was oval to spherical shape, gram-negative, motile and produced smooth white colonies on nutrient agar. Biochemically, it was positive for oxidase, catalase, urease and indole tests (11). *A. chroococcum* is reactivated in Burks medium (10 gm/ L Glucose, 0.64 gm/ L K₂HPO₄, 0.16 gm/ L KH₂PO₄, 0.2 gm/ L NaCl, 0.2 gm/ L MgSO₄.7H₂O, 0.05 gm/ L CaSO₄.2H₂O, 0.01 gm/ L NaMoO₄.2H₂O, 0.003 gm/ L FeSO₄, 1000 ml distilled water, pH 7) and incubated at 30 °C for six days (12). Inoculum preparation was achieved by growing of *A. chroococcum* isolate in 100 ml of the broth culture for six days at 30°C, where the optical density of bacterial culture was 0.84 at 600 nm.

Plant seeds preparation

Fava bean (*Vicia faba*) seeds, which were obtained from ARC, were prepared by surface sterilization using 2% HgCl₂ and 95% Ethanol for 2 min, and then washed well with distilled water (13).

Soil preparation

Soil was obtained from Baghdad University fields from the depth of (10-15) cm, and Table (1) showed some of soil properties. Soil was divided into two groups: sterile and non-sterile soil, where the sterile soil was autoclaved three times at 121°C for 1 hour each time (14).

Table 1. The properties of study soil.

Properties	Soil content
pH	7.3
Electric conductivity (EC)(ds.m ⁻¹)	1.8
Cation exchange capacity(CEC) (cmol.Kg ⁻¹)	18.6
Organic matter (gm.Kg ⁻¹)	1.4
Nitrogen (mg.Kg ⁻¹)	2.21
Phosphor (mg.Kg ⁻¹)	14.53
Sand (gm.Kg ⁻¹)	233
Silt (gm.Kg ⁻¹)	273
Clay (gm.Kg ⁻¹)	494
Texture	Sandy loam

Biological experiment

Sterile and non-sterile soil was distributed in 18 sterile, 5 Kg pots, 9 for each set of trials. Seeds, treated with inoculum, were soaked with 100ml of *A. chroococcum* fresh culture (the optical density = 0.84 at 600 nm) and mixed with sterilized Arabic gum. NPK fertilizer supplied by ARC.

Biological experiment performed (CRD) with three replicates and two factors; sterile and non-sterile soil and three treatments: control, include plant without treatment (P), NPK fertilizer (100 Kg. hec.⁻¹) (PF), and addition of *A. chroococcum* inoculum (PA). Thus there were 18 experimental units (2*3*3=18). Seeds were cultured, pots were arranged randomly inside a plastic house and irrigated with tap water.

Plant growth promoting effect estimation

Seven weeks after germination, an assessment of length and weight of each plant shoot and plant root were conducted.

Statistical Analysis

The experiment was statistically analyzed using ANOVA, and the averages was compared with least significant difference (LSD) values at the level of 0.05 (15).

Results and Discussion:

***A. chroococcum* ability to produce hydrolytic enzymes**

A. chroococcum isolate was able to form hydrolytic zones on chitin, pectin and milk agar (Table 2), so it was able to produce chitinase, pectinase and protease enzymes (8 and 9). The isolate also was able to grow and reduced the oil in mineral salt olive oil agar, verifying the ability to produce a functional lipase enzyme (16). Production of hydrolytic enzymes including cellulose, pectinase, protease, chitinase and lipase by some rhizospheric bacterial community indicate its benefit and plant growth promoting activity (17). Rodrigues and his team (18) isolated 136 sugarcane associated bacteria, of them 83 were presenting some plant growth mechanism: 47 % phosphate solubilizers, 26 % nitrogen fixers and 57 % producing IAA, 0.7 % HCN and chitinase, 45 % ammonia, 30 % cellulose and 8 % pectinase. They found that best isolates, which belong to the **Enterobacteriaceae** family and the **Klebsiella**, **Enterobacter** and **Pantoea** genera, promoted maize plant growth in greenhouse experiments.

Table 2. Hydrolytic enzymes produce by *A. chroococcum* isolate.

Enzymes	Production ability (Diameter of clear zone (mm))
chitinase	+ (3.7)
pectinase	+ (4.5)
protease	+
lipase	+

***Azotobacter chroococcum* inoculum effect on plant shoot**

-Shoot length

The average shoot length of fava bean in non-sterile soil treatments (17.02 cm) was more than in sterile soil treatments (16.61 cm), but this difference is not significant. *Azotobacter* inoculum increased shoot length in sterile and non-sterile soil (Figs. 1 and 2).

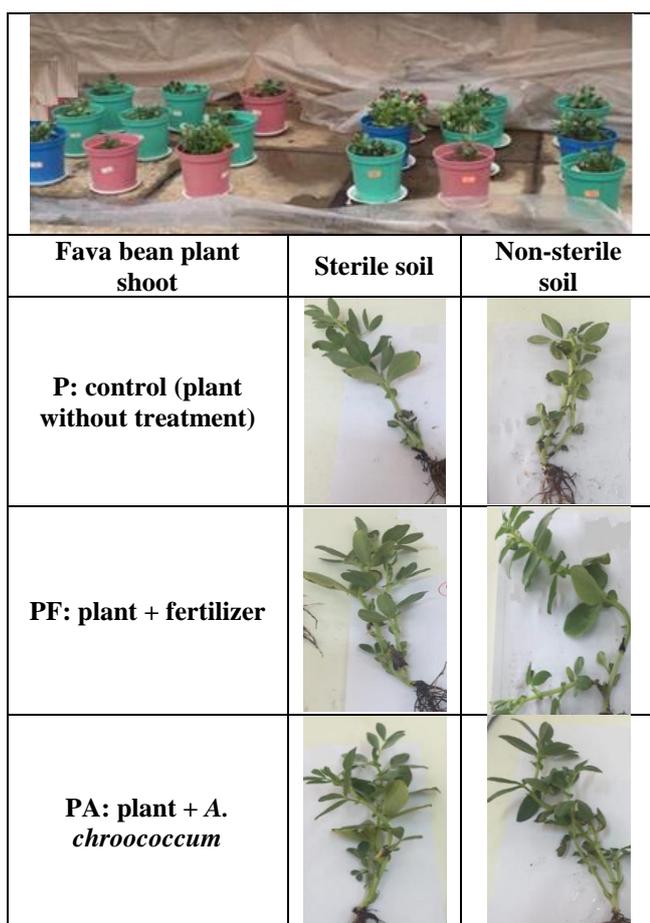


Figure 1. Fava bean shoot in the treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*.

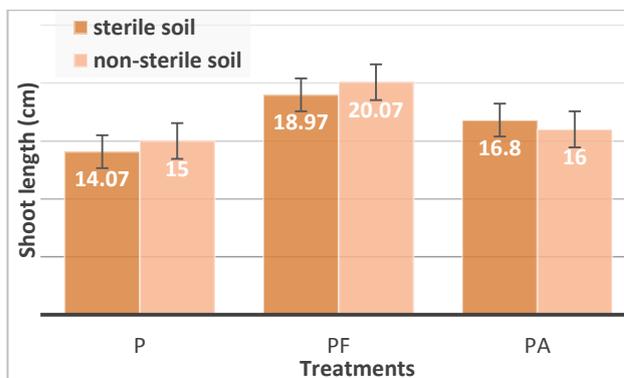


Figure 2. Fava bean shoot length (cm). The treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*. (LSD_{0.05}=3.64) (SE=1.180).

The percentage of increase caused by *Azotobacter* inoculum addition was 12.83 %. Fertilizer treatments show significant increase in shoot length from control treatments. There were no significant differences between fertilizer treatment (18.97 cm) and *Azotobacter* treatment (16.8 cm) in sterile soil. While these two treatments (20.07 and 16 cm respectively) exhibited significant differences in non-sterile soil, this may have related to the computation of indigenous microorganisms. *Azotobacter* inoculum increased the growth by supplying the crop with nitrogen through asymbiotically fixation and phytohormone synthesis. Kalay and his team (19) mention that applying *A. chroococcum* on choy sum (*Brassica rapa* L.) did not increase the yield. Felipe and his team (5) observed a beneficial effect on greenhouse planted cotton inoculated with *A. chroococcum*, and suggested that co-inoculation allow to reduce nitrogen fertilization doses up to 50%.

Shoot weight

There were no significant differences between the averages of plants shoot weight in sterile soil treatments (10.09 gm) and non-sterile soil treatments (10.56 gm). Fertilizer treatments (15.03 and 16.07 gm respectively) showed significant increase in shoot weight from the control (6.92 and 6.25 gm respectively) and *Azotobacter* treatments (8.33 and 9.37 gm respectively) (Fig. 3). Shoot weight gained increase by 34.4% in *Azotobacter* treatment compared with control treatment, which may have been related to production of indol-3-acetic acid and siderophores, which can increase nutrient availability and improve plant growth (20). Felipe and his team (5) evidenced an increase ranging between 8 and 53% in all parameters of plant growth, suggesting that ***A. chroococcum*** inoculation has a higher potential to promote cotton plant growth.

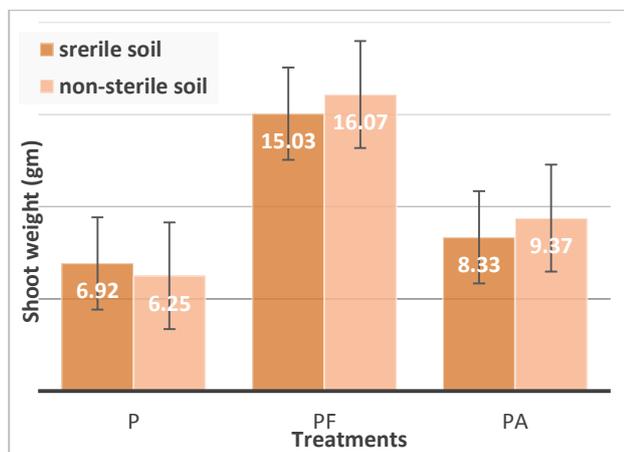


Figure 3. Fava bean shoot weight (gm). The treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*. (LSD_{0.05}=4.41) (SE=1.431).

***Azotobacter chroococcum* inoculum effect on plant root
-Root length**

The results in figs. (4 and 5) show that fertilizer treatments exhibited highest length of bean plant root in non-sterile and sterile soil (19.6 and 20.26 cm respectively) followed by *Azotobacter* treatments (16.6 and 18.43 cm respectively). *Azotobacter* inoculum increased the length of plant roots in contrast with control treatments, with an increase of 60.98%. Root lengths in non-sterile soil (16.85 cm) are improved better than in sterile soil (15.37 cm). Wani Sartaj and his team (21) mentioned that *Azotobacteria* affect the growth of the closely associated higher plants. They can benefit plant development through multiple mechanisms of action, directly through the production of substances which promote growth and increase nutrient availability in soil and their uptake, or indirectly through the suppression of plant pathogens (20).

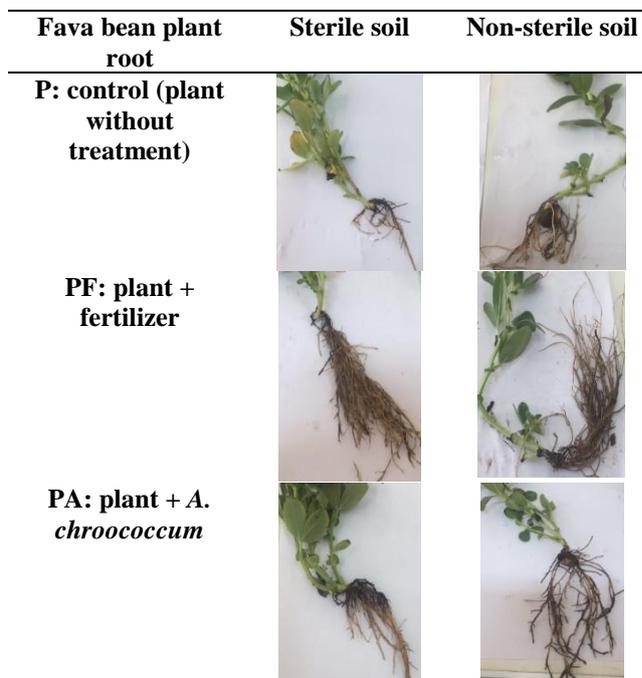


Figure 4. Fava bean root in the treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*.

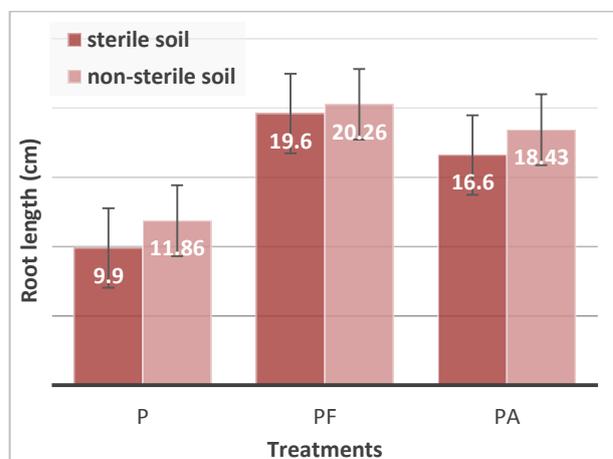


Figure 5. Fava bean root length (cm). The treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*. (LSD_{0.05}=9.4) (SE=3.049).

Root weight

The treatments of non-sterile soil presented no significant increase in the average of fava bean plants root weight (8.05 gm) from sterile soil treatments (6.84 gm). Fertilizer treatments obtained maximum root weight in sterile soil and non-sterile soil (10 and 11.03 gm respectively), which differ significantly other treatments (fig. 6). *Azotobacter* inoculum enhanced the roots weight of bean plant in sterile soil and non-sterile soil by 6.13 and 7.63 gm respectively. The percentage of increase in inoculum treatments from control treatments was 38.99%. This increase may have been related to synthesized hormonal substances

which originate from the rhizosphere or root surface (21). Tiwari and his team (1) reported that *Azotobacter* sp. inoculum enhanced plant shoot length, root length, leaves number, shoot and root fresh and dry biomass, but mixed inoculations of *Rhizobium* sp. and *Azotobacter* sp. revealed further enhancement.

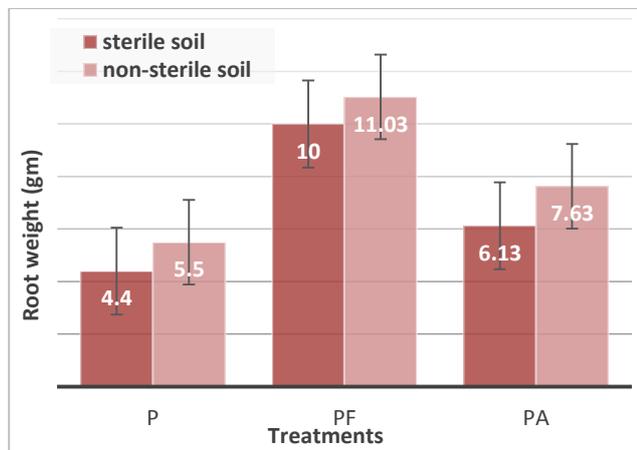


Figure 6. Fava bean root weight (gm). The treatments: P: control (plant without treatment), PF: plant + fertilizer, and PA: plant + *A. chroococcum*. (LSD_{0.05}=2.58) (SE=0.856).

Conclusion:

The concept of this study is to use natural alternatives that are effective and not harmful to the environment. Therefore, *A. chroococcum* isolate, which is known for its capability to fix atmospheric nitrogen, was used. Moreover, *Azotobacter* strains have been well known for their siderophores, indol-3-acetic acid and exopolysaccharides (EPS) production, which may benefit plant growth/nutrient availability and soil water retention properties. This isolate was also able to produce chitinase, pectinase, protease and lipase enzymes. *A. chroococcum* isolate improved length and weight of fava bean plants shoot and root. Therefore, such addition should be recommended to replace chemical fertilizers, albeit partially, in order to reduce the risk of chemical toxicity and preserve soil fertility and health.

Conflicts of interest: None

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تقييم قدرة بكتريا *Azotobacter chroococcum* في إنتاج الانزيمات الحالة وتحسين نمو الباقلاء (*Vicia faba*)

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الخلاصة:

اختبرت قدرة عزلة الأزوتوباكتر *Azotobacter chroococcum* في إنتاج الانزيمات الحالة (الكابتينيز والبروتينيز واللايبينز) وتعزيز نمو نبات الباقلاء (*Vicia faba*). اجريت تجربة بايولوجية باستعمال السنادين وبالتصميم العشوائي التام (CRD). شملت التجربة ثلاث معاملات هي: معاملة السيطرة (نبات بدون معاملة) (P)، ومعاملة سماد الـ NPK (نبات + سماد) (PF)، ومعاملة لقاح بكتريا *Azotobacter chroococcum* (نبات + *A. chroococcum*) (PA)، ولقد نفذت هذه المعاملات في التربة المعقمة و التربة غير المعقمة وزرعت ببذور نبات الباقلاء (*Vicia faba*). وفي نهاية التجربة تم حساب الاطوال والاوزان لسيقان وجذور النبات. أظهرت النتائج أن العزلة كانت قادرة على إنتاج انزيمات الكابتينيز والبروتينيز واللايبينز. ازداد نمو النبات مع إضافة السماد ولقاح بكتريا *A. chroococcum*. حصلت معاملة السماد (PF) على أفضل مؤشرات نمو، إذ بلغ متوسط طول ووزن الساق وطول ووزن الجذر 19.52 سم و 15.55 غم و 19.93 سم و 10.52 غم على التوالي. وكانت متوسطات هذه المؤشرات في معاملة لقاح بكتريا *A. chroococcum* (PA) 16.4 سم و 8.85 غم و 17.52 سم و 6.88 غم على التوالي. ان النسب المئوية للزيادة الناجمة عن إضافة لقاح بكتريا *A. chroococcum* (PA) مقارنة بمعاملة السيطرة (P) كانت 12.83% و 34.4% في طول ووزن الجزء الخضري للنبات على التوالي، و 60.98% و 38.99% في طول و وزن جذور النبات على التوالي. ان مؤشرات النمو في التربة غير المعقمة كانت أعلى من التربة المعقمة عموماً، ولكن هذه الزيادات لم تكن معنوية.

الكلمات المفتاحية: *Azotobacter chroococcum*، المخصب الحيوي، الباقلاء، الانزيمات الحالة، تعزيز نمو النبات.