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Original Research

Efficiency of immobilization technique for *Azotobacter chroococcum* and its effect on the growth and yield of wheat *Triticum aestivum* L.

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ABSTRACT:

Two local isolates of *Azotobacter chroococcum*, were used to study its nitrogen fixation one of which was isolated and identified in the Microbiology Laboratory of the Faculty of Agriculture, Al-Muthanna University. It was selected among the ten local isolates, isolated from different locations of Al-Muthanna province and its ability to nitrogen fixation in the liquid media and other isolates were originated from Agriculture college, University of Sulaymaniyah. The isolation was activated by Sucrose mineral-salts. A field experiment was conducted by Randomized Complete Block Design (RCBD) by three replicates to study Immobilization, inoculant effect and bentonite as a bacterium for the concentration of nitrogen increasing and growth of wheat plant using two factors. The first factor is the addition of bio-fertilizer at three levels *viz*., A_0 = without the addition of the bacterium, A_1 = *Azotobacter chroococcum* isolated from Al-Muthanna province soils, A_2 = *Azotobacter chroococcum* isolation sourced from the University of Sulaymaniyah. The second factor was the utilization of a bio-fertilizer carrier by two levels B_0 = bio-fertilizer and the addition of bentonite carrier as Conventional inoculant B_1 = immobilized inoculant. The study aims to investigate the immobilized inoculant technique and its effect, that favours bacteria capsulation by in a polymeric compound such as sodium alginate and bentonite as a carrier of bio-fertilizer in the growth and yield of wheat. The results showed that immobilized inoculant was superior to the bentonite carrier method in most characters studied with the isolation locally, The plant weight was 49.7 cm, with a significant increase of bentonite carrier. The dry weight of the plant was significantly increased in the same regard by immobilized inoculant (0.68 g). On the other side, the same treatment registered the weight of 500 grain and the total yield was registered as 4.69 g and 4.174 mega grams ha^{-1} sequentially.

Keywords:
Immobilized inoculant, Bentonite, Bio-fertilizer.

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INTRODUCTION

Bio-fertilizers are cheap sources for plants nutrient and alternative mineral fertilizers. They also reduce soil and water pollution, also benefits by reducing the of chemical fertilizers quantities (Al-Sefat, 2012). Agricultural development sustainability is requires optimal use of microorganisms effectiveness and their biological activity in agricultural soils, which is an environmentally safe alternative to the availability of plant nutrients compared to chemical fertilizers (Zaied *et al*., 2009). Nitrogen fixation is a process of reducing atmospheric nitrogen to ammonia**,** catalyzed by the nitrogenase enzyme complex (Rahim *et al*., 2014). The first studies of bio-fertilization were carried out at the beginning of the 20th century when the Russians used bacteria in bio-fertilization such as phosphate analyzer bacteria (Dommergues and Mangenot, 1970). Biofertilization can increase nutrients availability in the soil (El-Ghandour, 1992). Bio-fertilization also has some problems such as microorganisms at low number and it competition with the original microorganisms in the soil so researchers have recently sought to solve this problem by trying to prolong microorganisms life by in a polymer compound such as sodium alginate by using polymeric materials that can preserve bacteria for a long possible time when added to the soil. Sodium alginate, polyacrylamide, agar and Polyvinyl Alcohol (PVA) are synthetic polymers and materials that have been extensively applied in the cell immobilization (Dong *et al*., 2017). Immobilized cells had several advantages such as improved growth, catalytic activity, low production cost, reduced production time, extensive application prospects and high yield (Syiem, 2005). Schoebitz *et al*. (2013) explained that immobilized inoculant technique using with phosphate solubilizing microorganisms as a carrier has increased bio-fertilizer efficiency and uptake of phosphorus by 64%. Stozky (1968) confirmed that the presence of clay minerals, especially bentonite, greatly affects the efficiency of microorganisms in the soil because the effective clay retains water and is a condition of viscosity which leads to the lack of spread of pathogens. Bentonite protects the bacteria and other microorganisms from extremist environmental conditions such as humidity low, drought and high temperatures (Hamid and Saleh, 2002). The aim of this study is to investigate immobilized inoculant technique role with the nitrogen-fixation bacterium compared with the Conventional method by using bentonite and to study its effect in the growth and yield of wheat plant.

MATERIALS AND METHODS

Ten soil rhizosphere samples were collected

Table 1. Number of soil samples, location and field materials collected for the study

Sample number	Geographical location	Crop
1	Samawah / Eastern	Alfalfa
2	Rumaitha / Al-Fazaia	Wheat
3	Alkhder-Aljazera	Sun flower
4	Warka / Khudair	Wheat
5	Almajd	Barley
6	Samawah /Research Station 1	Alfalfa
7	Al-sweer	Sun flower
8	Samawah /Jarbouiyat	Barley
9	AL-Salman	Tomato
10	AL-najmi / extension station	Wheat

Table 2. Chemical, physical and biological characteristics of soil before planting

pH	EC ($ds.m^{-1}$)	OM (%)	Available N (%)	Available P (%)	Available K (%)	Sand $g.\ kg^{-1}$	Silt ($g.kg^{-1}$)	Clay ($g.kg^{-1}$)	Total bacterial count
7.75	4.25	0.75	46.0	12.5	275	230	390	380	$2.8x10^7$
						Silty clay		Soil texture	

from the fields planted with alfalfa, sun flower, wheat, barley and tomato from the geographical local of Al-Muthanna province as shown in Table 1. The sample were collected from the field and mixed them together to reduce the error rate and homogeneity the samples to form a representative sample of the field (Hsia *et al*., 2008). All samples were placed in alcohol-sterilized plastic bags and kept in the refrigerator until use. Soil samples were made by adding 10 g of soil to 90 mL of distilled and sterilized water in the 250 mL flask and well-mixed for sequential dilution (10^{-1}-10^{-7}) by transferring 1 mL of soil suspension to test tubes containing 9 mL of distilled and sterile water for each sample of the soil samples. Sucrose mineral-salts broth media was used for soil inoculation by *Azotobacter chroococcum* isolates. Soil dilution prepared were taken in 1 mL to the test tubes for inoculation by the above medium, and sterilized by autoclave for the 20 min at 121°C and 15 lb. Two replicates were maintained for each dilution. Tubes were incubated at 30°C for seven days and were tested for a thin white membranous observation on the surface, which is an indicator of *Azotobacter* sp. 0.1 mL of the culture was taken from the tubes and spread on a petri dish containing the sucrose mineral-salts agar medium. Dishes were incubated at 28°C for 2-3 days and were re-streak to obtain pure colonies of bacteria. White colonies were then extracted, by giving a brownish dye to the medium, brown colour over time was isolated in the pure environmental conditions (Zoghbi *et al*., 2007; Khan *et al*., 2007).

Field experiment

A field experiment was designed to study the effect of two *Azotobacter chroococcum* isolates using immobilized inoculate and bentonite carrier method. A Randomized Complete Block Design (RCBD) was designed using two factors, as shown below

The first factor

Bio-fertilizer by three levels **:-**

A_0 = Without bio-fertilizer

A_1 = Isolation of *Azotobacter chroococcum* from the soil of Muthanna province

A_2 = Isolation of *Azotobacter chroococcum* from the Faculty of Agriculture, University of Sulaymaniyah.

The second factor

Bio-fertilizer carrier method by two levels **:-**

B_0 = Bentonite carrier (conventional inoculant)

B_1 = Immobilized inoculant

experiment was carried out by three replicates

Table 3. Effect of bio-fertilizer type and carrier method on plant height (cm)

S. No	Bio-fertilizer type	Bio-fertilizer carried method		Average A
		B_0	B_1	
1	A_0	40.8	41.0	40.9
2	A_1	43.6	49.7	46.65
3	A_2	42.2	45.5	43.85
4	Average B	42.2	45.4	-
5	$L.S.D_{0.05}$	A= 2.82	B= 1.41	AB= 1.90

Table 4. Effect of bio-fertilizer type and carrier method on dry weight of the plant (g. plant^{-1})

S. No	Bio-fertilizer type	Bio-fertilizer carried method		Average A
		B_0	B_1	
1	A_0	0.51	0.54	0.52
2	A_1	0.60	0.68	0.64
3	A_2	0.57	0.62	0.59
4	Average B	0.56	0.61	-
5	L.S.D	A = 0.02	B = 0.01	AB = 0.04

Table 5. Effect of bio-fertilizer type and carrier method on plant branches (branch per plant)

S. No	Bio-fertilization type	Bio-fertilization carried method		Average A
		B_0	B_1	
1	A_0	3.66	3.33	3.49
2	A_1	5.83	7.98	6.90
3	A_2	4.33	5.99	5.16
4	Average B	4.60	5.76	-
5	L.S.D	A = 0.85	B = 0.65	AB = 1.50

Experimental units number = 3 × 2 × 3 = 18 units

Immobilized inoculant preparation

Immobilized inoculate was prepared according to the method (Schoebit *et al*., 2012) weighing three grams of sodium alginate and solvent in 100 mL distilled water. It was baked for 30 min to obtain a homogeneous solution and 47 g of potato-starch potato was added to the sodium alginate (matrix solution). The mixture was mixed for 30 min for homogenization. 30 mL of bacterial culture was taken and centrifuged for 10 min at 5,000 rpm. The pellet was taken and dissolved in 3 mL of 1% peptone and then mixed with 30 mL of matrix solution. 50 mL syrup was used as a for distillation matrix on sterile calcium chloride solution (15 g. mL^{-1}). It was left for 30 min to complete the production of bio-fertilizer balls and then the wet balls were collected and kept at the temperature of 4°C until use. It was centrifuged for 10 min at 5000 rpm per min. Pellet was taken and dissolved in 3 mL of 1% peptone and mixed with 30 mL matrix solution. A 50 mL syringe was used for forming matrix solution on the sterile sodium chloride solution (15 g.mL^{-1}). Leave it for 30 min to

Table 6. Effect of bio-fertilizer type and carrier method in the weight of 500 grains (g)

S. No	Bio-fertilization type	Bio-fertilization carried method		Average A
		B_0	B_1	
1	A_0	1.45	3.06	2.25
2	A_1	2.85	4.69	3.77
3	A_2	2.69	2.96	2.82
4	Average B	2.33	3.57	-
5	L.S.D	A = 1.90	B = 1.41	AB = 2.82

complete the pollen (Schoebitz *et al*., 2013).

Soil preparation

The experiment was conducted on the extension farm at Samawah for the agricultural season 2016-2017. Soil was plowed in a perpendicular manner and the leveling and softening process was carried out. The land was then divided into experimental units of 6 m^2 (3 m × 2 m). The wheat seeds were cultivated at Aba 99 variety in 15.11.2016 on straight lines between the line and the last 10 cm. Nitrogen fertilizer was added in the form of urea (46% N) at 20 $kg.ha^{-1}$, a stimulant for the action of the bacteria. The superphosphate P_2O_5 fertilizer (48%) weighing 60 $kg.ha^{-1}$ was added 120 $kg.ha^{-1}$ potassium was added in the form of potassium sulfate (K_2SO_4) (43% K). The panels were irrigated after reaching 80% of the field capacity and the bush was manually controlled. After the arrival of the crop to maturity at the end of the winter season of 2017. The average number of branches were measured and the wheat crop was harvested by cutting the plants from the area near the surface of the soil and recorded the following measurements as per El-Ghandour (1992): plant height, branches number, plant dry weight, 500 grain weight and grain yield. The trail date was statistically analyzed by GenStat method. The means were compared according to LSD test after level of 0.05 (Payne *et al*., 2011).

RESULTS AND DISCUSSION

Table 3 results showed the effect of biofertilizer in the plant height. Immobilized inoculum of *A. chroococcum* was locally isolated treatment for A1/B1 was found to be superior to the bentonite carrier and the same bacteria with a mean increase of 49.7 cm. With an increase of 21.81% compared to the control, immobilized inoculum promoted plant growth reflecting to the presence of rhizobacteria (PGPR) is an ideal reason to supply the soil with a high density of beneficial microorganisms. However, maintaining viable microorganisms is a major problem during seed treatment and storage (Gregorio *et al*., 2017). This increase in the height of plant may be due to the *A. chroococcum* characteristics by secretion. El-Gawad *et al*. (2009) and Zaied *et al*. (2009) refer that these secretions conduce an important role in the elongation of plant cells to the increased division of plant cells as well as their activity in the nitrogen fixation which provided some of the nitrogen at different stages of growth. The microorganisms in the rhizosphere lead to an important role in the increase of mineral circulation and the degradation of organic matter and its metal as the process is faster on the surface of the roots due to the increased activity and density of bacteria, and fungi (Davey and Rovira, 1974). The same treatment was also superior for bentonite carrier with immobilized inoculum of *A. chroococcum*, isolated from the Sulaymaniyah province soil (A_2B_1), which recorded 45.5 cm. This may be due to the effect of environmental factors in the isolates of bacteria from cold environmental zones which did not adapt to the new environment (Bashan *et al*., 2004).

The results shown in Table 4 refer to biofertilizer effect and carrier type in the dry weight of the plant. Immobilized inoculum treatment of the locally isolated *A. chroococcum* A_1B_1 was superior to the bentonite carrier with same bacteria A_1B_0 which recorded 0.68 g $plant^{-1}$ and 33.33% percentage increase maybe due to the effect of immobilized inoculum, which has the ability to release nitrogen-fixing bacteria. Through growing it nitrogen-fixing bacteria increases the efficiency of dissolved organic and inorganic phosphate (Rahim *et al*., 2014). Immobilized inoculum technique was possibly used in the nutrient-poor soils (Syiem, 2005). This technique was used with cyanobacteria nitrogen fixation, who spores tolerate extreme environmental conditions and can remain active in the soil for approximately three years (Syiem, 2005). The results refer that immobilized inoculum for local isolation (A_1B_1) was superior to the isolated bacteria from Sulaymaniyah soil (A_0B_1). It may be referred to the effect of

the environmental conditions of the isolated area, which has been adapted to the local isolation.

The results shown in Table 5, bio-fertilizer type and the carrier method effect in plant branches number and immobilized inoculum treatment of the isolated locally *A. chroococcum* (A_1B_1) were superior to the rest of the other treatments as it recorded 7.98 (branches plant^{-1}). It may be due to the immobilized inoculum effect, which has the ability to protect bacteria against tolerate extreme environmental conditions as it refer by (Schoebitz *et al*., 2013). The results also indicated the locally isolated A_1 which superior to the A_2, with regardless of carrier method, as it recorded 6.90 (branches plant^{-1}). This may be referred to the effect of environmental conditions on the isolation A_2, which was isolated from the low temperature and that have not adapted to the environmental conditions of Al-Muthanna province.

The results shown in Table 6 is referred to the bio-fertilizer type and the effect of carrier method in the weight of 500 grain as the results indicated that the superiority of the treatment A_1 (local isolation) on the rest of the other treatments and regardless of the carrier method as it is recorded with 3.77 g. It also has an increase of 67.55% control relative that may be referred to the increase in nutrients availability, especially nitrogen and phosphorus (Illmer and Schinner, 1995). Since *A. chroococcum* has the potential to dissolve mineral phosphorus from unavailable sources such as apatite rock and convert it to the available formula (Dobbelaere *et al*., 2003) which is also referred to during the isolation of *A. chroococcum*, and are effective in phosphorus dissolving by space formation in the colony center (Dhamangaonkar and Misra, 2009). Also ability of bacteria to dissolve and extract phosphorus from inorganic sources such as tricalcium phosphate and phosphate rock was found to be good (Illmer and Schinner, 1995). The results showed that the B_1 Immobilization inoculant was superior to B_0. This is referred to the bio-fertilizer efficiency increased which is conductive to the nutrient availability increasing readiness and bacteria providing around the root zone in the growing season as a result of the release of the bacteria from the immobilized inoculate (Schoebitz *et al*., 2013). The results showed that the interaction between locally isolated and immobilized inoculate (A_1B_1) was superior to the rest of the treatments, which recorded 4.69 g. This was referred to the immobilized inoculate efficiency by the nitrogen-fixation bacteria supply throughout the growth season as a result of their release from the sodium alginate; this bacteria protects it from the extreme environmental conditions (Syiem, 2005).

The results of the statistical analysis were shown in Table 7, which referred to the effect of bio-fertilizer type and carrier method in the grain yield, that showed the vigour of locally isolated bacteria which were superior to the other treatments regardless of the carrier method recorded (3.761 Mg.ha^{-1}), This is re-

Table 7. Effect of bio-fertilizer type and carrier method in grain yield (Mg.ha^{-1})

S. No	Bio-fertilization type	Bio-fertilization carried method		Average A
		B_0	B_1	
1	A_0	2.821	2.942	2.882
2	A_1	3.547	4.174	3.860
3	A_2	3.123	3.625	3.278
4	Average B	3.164	3.580	-
5	L.S.D	A = 0.239	B = 0.195	AB = 0.338

ferred to the bacterial ability to produce a growth stimulated such as Indole Acetic Acid (IAA); this is confirmed by Samurai and Rahi (2006) as well as its siderophores secretions, which is a sticking to the micro elements such as iron and zinc. The availability increases its as well as its enzymatic activity and nitrogen fixation (El-Gawad *et al*., 2009). This can supply sufficient N_2 and assist the hydrolysis of a wide range of phosphorous compounds leading to increased crop production (Garg *et al*., 2001). The results showed that Immobilization inoculant B_1 was superior to bentonite carrier method B_0 which recording 3.449 $Mg.ha^{-1}$. This referred to the increase in to bio-fertilization efficiency, which increased the availability of nutrients and of bacteria provided around rhizosphere through the growth season which referred to the release of bacteria from the Immobilization inoculant (Schoebitz *et al*., 2013). The results showed an interaction between the local isolation and immobilization inoculant A1B1 that was superior to the rest of the treatments which recorded 4.174 $Mg.ha^{-1}$.

This is due to the immobilized inoculum and efficiency to fix nitrogen throughout the growth season as a result of its release from the sodium alginate compound similar to the urea-coated fertilizer and in bacteria protecting it from the adverse environmental conditions as indicated by (Syiem, 2005). Numerous advantages related to the immobilized inoculum of rhizobacteria are found, for instance, the controlled release of bacteria into the soil, protection of microorganisms in the soil against biotic and abiotic stresses and contamination reduction during storage and transport (Mauricio *et al*., 2013).

CONCLUSION

The use of the immobilization inoculant has improved many growth characteristics compared to conventional inoculant. The use of isolates in the laboratories of the Faculty of Agriculture, Al-Muthanna University, was better than the isolation that originates from the cold-climatic province of Sulaymaniyah. This is due to the environmental adaptation of local bacteria, as well as, the use of bio fertilizers reduces the environmental pollution resulting from increased use of mineral fertilizers.

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