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APPLICATION OF ALGINATE BEAD ENCAPSULATED N_2-FIXING BACTERIA IS IMPROVING WHEAT YIELD UNDER DROUGHT STRESS

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Abstract

Water scarcity is one of the most pressing and threating environmental issues facing the human population. Plant growth promoting bacteria (PGPB) are presented as safe and ecological complementary solution to the food security problem along with the traditional crop breeding and genetic engineering. Plant-associated microbial communities, such as, nitrogen-fixing bacteria, improve crop productivity and provide stress tolerances for different biotic and abiotic factors. In this study, we isolated and characterized fourteen *Azotobacter* spp from rhizosphere of wheat plants, among these isolates; we identified the most effective plant growth promoting isolate as *Azotobacter chroococcum* using Bio-log identification system. The encapsulated of the *A. chroococcum* with sodium alginate shows beds integrity intact bacterial cells using the scanning electron microscopy. Encapsulated *A. chroococcum* was tested for growth promotion of wheat under different water regime at field experiments, the results indicated that encapsulated *A. chroococcum* was effective in lowering the harmful effect of water deficit on several wheat agronomical criteria. Wheat treated with encapsulated or liquid culture significantly diminish the reductions in relative water content recorded 66.37% and 59.23% , respectively under water defiant 80% from actual evapotranspiration plus 100% and 75% mineral nitrogen . Moreover, wheat inoculated with *A. chroococcum* in two forms showed reduction in proline accumulations in shoots, as well as lowest antioxidant enzymes contents. The inoculation with *A. chroococcum* either encapsulated or liquid culture significantly enhanced wheat grain yield and yield components, as well as nitrogen (N), phosphorus (P), and potassium (K) contents in grains of wheat under water defiant 80% from actual evapotranspiration.

Key words : Encapsulation, Alginate, Scanning electron microscopy, wheat, water relations

Introduction

Water availability is the most limiting factor for rising production of agricultural and an important factor for wheat production in Egypt as well as arid and semi-arid regions as they face shortage in water demands of agriculture. Efficient utilization of available water resources is crucial for a country facing severe water scarcity in Egypt, where water consumption in agriculture constitutes more than 85% of the total annual water resources. Sustainability of agricultural production depends on the conservation and appropriate management of scarce water resources especially in arid and semi-arid areas, where irrigation is required for the production of food and cash crops. Drought controls crops productivity worldwide in the majority of agricultural fields and recent global climate change has made this situation more adverse (Al-Ghamdi, 2009), besides, it affect the morphological, physiological, biochemical and molecular processes in plants resulting in growth inhibition. The extent of these changes is dependent on the time, stage and severity of environmental stress (Cao *et al*., 2011). Many biological micro, macro-molecules are affected with drought, such as nucleic acids (DNA, RNA, micro RNA), proteins, carbohydrates, lipids, hormones, ions, free radicals and mineral elements (Ingram and Bartles, 1996). As a result of drought stress, an increased production of reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, hydroxyl radicals and hydrogen

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peroxide (Agarwal *et al*., 2005). Generation of ROS causes rapid cell damage (Imlay, 2003). A variety of strategies has been used to improve the drought tolerance of crops, including traditional selection methods and genetic engineering (Fleury, 2010).

Azotobacter is an aerobic, free living, non-symbiotic nitrogen fixing diazotroph (Wani, 1990). It can beneficial to plant by secrete the vitamins , amino acids , produce siderophores and auxins which are among the direct mechanisms of increasing root development and plant growth (Akbari *et al*., 2007). Moreover, they produce thiamin, riboflavin, indole acetic acid and gibberellins (Kader, 2002). Beneficial effects of *Azotobacter* on growth various plants was reported and consider as Plant Growth Promoting Rhizobacteria (PGPR) (Nasaruddin, 2014).

Encapsulation has been studied as carriers of plant beneficial microorganisms to increase the efficacy and quality of bio inoculants and reduced the costs and environmental impact (Bashan and Gonzalez 1999; Amalraj *et al*., 2013). These encapsulated of bacteria, protecting them from adverse environmental conditions and allowing their gradual release when these polymers are degraded. In addition, encapsulated bacteria can be dry stored at room temperature for long periods of time (Bashan *et al*., 2002). Moreover, these formula with encapsulated bacteria have been suggested for seed treatment, it can be improved the environmental persistence of bead-immobilized microorganisms (John *et al*., 2011).However, the use of encapsulated bacteria has two main disadvantages; it requires additional seed treatment during sowing. This may be objected because of inadequate agricultural education or the conservative cultural traditions of some small-scale growers wary of new technologies. The use of micro-alginate beads could resolve these difficulties by employing seeds coated with "bead powder" at the handling facility, which are sold to the grower as "improved seeds" (Bashan *et al*., 2014). However, seed coating with microencapsulated bacteria requires additional adhesive substances such as lecithin and Resitol (Bashan *et al*., 2002) and, being no easy task, it has, until now, only been conducted on an experimental scale (Bashan *et al*., 2014).

Wheat (*Triticum aestivum* L.) is considered the thread cereal crops, it is the dominant crop in temperate countries being used for human food and animals feed. Limiting crop yields already today in more than 70% of arable lands, and the drought limitations further gain in importance in the near future as agricultural activities expand to less fertile areas to satisfy growing demands for food (Flexas, 2013). The urgent need to increase global wheat production requires greater progress in improving wheat tolerance to biotic and a biotic stresses, whose production reached more than 730 million in 2017/2018 (FAO, 2017). Wheat is one of the most important crops in Egypt. However, national production remains low and does not meet the needs of the growing population Shrief and Abd El-Mohsen (2015). The most limiting factor for wheat productivity is water deficit, which affects yield depending on its intensity and wheat phenological stage (Okuyama *et al*., 2004; Araus *et al*., 2008). Thus, the aims of this study to provide a multi-function approaches using N_2 fixing bacteria as plant growth promoting bacteria to help plant growth under drought stress. We selected the most efficient free nitrogen fixing bacteria isolated from the rhizosphere and apply these bacteria as encapsulated inoculum to the wheat growing under drought.

Materials and Methods

Samples collection, isolation, and identification of *Azotobacter*

Soil samples were collected from the soil rhizosphere for different wheat plants collected from Agricultural Research Center (ARC) (30°01'13.6"N 31°12'30.4"E: 30°01'11.3"N 31°12'20.5"E, Giza Govern., Egypt. Fourteen isolates of *Azotobacter* spp isolated by plating method (Brown *et al*., 1962). These bacteria were grown on selective free nitrogen Ashby's medium (Abd El-Malak and Ishac. 1968) and incubated at 28±2°C for 7 days. Isolates of *Azotobacter* morphological characteristic e.g. flat, slimy, paste-like colonies with a diameter of 5–10 mm were purified by subsequent streaking on new plate. Morphological characteristics of all isolates such as cell shape, color, consistency and biochemical reaction of isolates were recorded as described in Bergey's Manual of Systemic Bacteriology (Krieg *et al.*, 1994). Motility of isolates was screened according to Rhodes (1958). Gram staining were determined after 2-5 days of incubation at 28 ± 2°C according to (Hegazi and Neimela, 1976). Catalase test was estimated according to (Schaad, 1992). HCN production was examined as described by (Bakker and Schippers (1987). Acetylene reduction assay was determined according to (Cappuccino and Sherman, 2002). The extracellular exopolysaccharide production (EPS) was determined according to (Damery and Alexander 1969). The identification of the selected isolate was done according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) then we conformed this identified through (Biolog GN2 Microplate Biolog, 2000) in the VACSERA Cairo, Egypt using gram negative and

positive systems in Microlog database.

Preparation and verification of bacterial encapsulation

Bacteria was grown in nutrient broth medium Difco Manual (1985) for 48 hours at 28°C to reach the maximum growth (10^7cfu/ ml), encapsulation was done using sterile solution of sodium alginate (ALGOGGL, Degussa, France) according to Ivanova *et al*., (2005), the air cooled sodium alginate were mixed with bacterial inoculum to prepare the beads of alginate, and kept it in saline solution. The Survival of encapsulated beads inoculants was tested as described by (Abo-Koura and Maie 2016). Scanning electron microscopy was used to study the beads formation and *Azotobacter* morphology using the (SEM, QUANTA FEG 250) at National research center, Cairo, Egypt according to the manufacture protocols.

Preparation bacterial inoculums

Bacteria was grown in nutrient broth medium Difco Manual (1985) for 48 hours at 28°C to reach the maximum growth (10^7cfu/ ml). Two types of *Azotobacter* inoculums were used, either encapsulated or liquid form, seeds were mixed with encapsulated bacteria as described by (Bashan *et al*., 2002), while other forum of bacteria culture were carried on (1:1) vermiculite: beat moss using Arabic gum as adhesive agent to form slurry. The slurry was then mixed with the seed until it was evenly coated. The coated seeds were lifted to dry in the shed for 60 minutes and planted in soil.

Experimental design, field practices and data collection

Field experiment was conducted at the Giza Agricultural Research Station experimental farm (latitude of 29°26'N and longitude of 31°13'E) during winter season (2016/2017). Wheat (*Triticum aestivum* L.) grains Giza 168 cultivar were obtained from field Crops Research Inst., Agricultural Research Center, Giza, Egypt. Bulk density, physical and chemical properties of the soil at the experimental site were determined according to Klute (1986) and Page *et al*., (1982), while particle size according to (Piper, 1950) and chemical properties according to Ryan *et al*., 1996). The experimental design was split plot with four replicates, the plot area was 6 × 7 m each. The treatments were, main plots (irrigation regimes based on crop evapotranspiration): 100%, 80% and 60% ET crop. Sub-plots (N-fertilizer rates): 100%, 75%, 75+ encapsulated beads of *Azotbacter* and 75+ liquid of *Azotobacter*. From each treatment, six plants were used to estimate growth criteria. Nitrogen (N), phosphors (P), potassium (K) mineral fertilization were applied as recommended dose for Egyptian Ministry of Agriculture.g The control plots received 100% from recommended dose from NPK, while the other treatment received 75% mineral nitrogen and full dose from PK.

Biochemical assays

Proline content was estimated in shoots of wheat after 35 days as described by (Bates *et al*., 1973). For Antioxidant enzymes assays, Catalase (CAT) enzyme was determined according to the method of Aebi (1983). Ascorbate Peroxidase (APX) was determined according to Nakano and Asada (1981) and Super Oxide Dismutase (SOD) was determined according to Donahue *et al*., (1997).

Relative water content (R.W.C%)

For the determination of RWC % after 35 days after sowing, fresh mass (FM) was estimated for shoots wheat and dry mass (DM). Turgid weight (TM) was estimated after holding shoots in 100% humidity conditions in the dark at 4^0C for 4 h. Then, the samples were dried at 70^0C to estimate the dry mass (DM). The RWC % of shoots was determined using the equation as described by Sharp *et al*., (1990) as follows:

RWC = (FM-DM / TM-DM) X 100

Cell membrane stability index (C.M.S %)

Leaf membrane stability was determined after 35 days, according to protocol described by Sairam (1994). 0.2g was taken from leaf and put it in test tubes containing 10 ml of double distilled water in two sets. Test tubes in one set were kept at 40°C water bath for 30 min then we measured (C_1) using a conductivity bridge. Test tubes in the other site incubated at 100°C in the boiling water bath for 15 min, then we measured above (C_2). MSI was calculated using the formulae

MSI = (1-C1/C2) × 100: Where:- C1= reading at 40^0C and C2=reading at 100^0C

Yield component

Plants were collected from a Random chosen $1m^2$ using wooden frame. Samples of straw and grains were dried at 70°C up to steady dry weight, and then grounded and digested according to the method recorded by (Page *et al.,* 1982).The digests were used for measurement of NPK. Nitrogen was determined using micro Kjeldahl, while phosphorous and Potassium was determined to the procedure outlined by (Allen, *et al.,* 1974).

Harvest index was calculated are described by the following Equation as described by (Kozak and M¹dry, 2006) as follow: HI= grain yield / biomass yield

Water relations:

CROPWAT model was used to calculate reference evapotranspiration with Penman Monteith.

1. Crop evapotranspiration (ETc) :- (Allen 1998)

$ETc = ET0 \times Kc$

Where:-

ETc = Crop evapotranspiration.

ET0 = Reference evapotranspiration.

Kc = Crop coefficient (from FAO 56)

2. Applied irrigation water (AIW)

A furrow surface irrigation method was used to conduct this treatment. Applied irrigation water was measured by a flow meter installed in the main pumping unit of irrigation water. The depth of applied irrigation water (AIW) to the experimental plots was calculated according to the following equation:

$AIW = ETc / Ea$

Where:

ETc = water consumptive use (CU, mm/d), or actual evapotranspiration (ETc).

Ea = application efficiency (fraction) = 0.6 for surface system at the site.

A submerged flow orifice with fixed dimensions was used to measure the amount of water to be applied to the experimental plots. The discharge of the orifice is calculated according to the following equation (Michael, 1978).

$$Q = CA\sqrt{2gh}$$

where:

Q = discharge through orifice, (cm^3/sec)

C = coefficient of discharge (0.6 up to 0.8).

A = cross-sectional area of the orifice (cm^2)

g = acceleration of gravity (981 cm/sec).

h = head of water causing discharge through the orifice (cm).

3. Water utilization efficiency (WUtE)

Water utilization efficiency (WUtE) values were calculated according to **Jensen (1983)** as follow:

WUtE (kg m^{-3}) = Grain yield (kg fed^{-1})/Applied irrigation water (m^3 fed^{-1}).

Applied irrigation water was recorded by a flow meter installed in the main unit of irrigation water.

Statistical analyses

The study design was split plot. Least significant difference test was used to compare means using the statistical analysis software; CoStat (CoHort Software, U.S.A) version 6.4. The values of probability $p<0.05$ were considered statistically significant. Based on the least significant difference test.

Results

Physical and chemical properties of experimental soil

The experimental site were characterize with clay loam soil with 34% clay and 36% silt and 28% sand The total orange mater with approximately 1% with normal, salinity of soil was 2.0 dSm^{-1} with neutral/ slit alkane pH, which is average of this region (Table 1).

Water capacity of the exponential filed

In order to recorded the water constants of field sites different measurement was done in different soil depth as inducted in the (Table 2).

Characterization of *Azotobacter* isolates

Fourteen isolates of *Azotobacter* bacterial were isolated from rhizosphere of wheat plants.

Morphological characters of *Azotobacter* isolates obtained are presented in Table 3. *Azotobacter* isolates have variation in cell shapes on plates after 7 days from growth of incubation. Majority of *Azot* 1, 6, 5, 10, 13, and *Azot* 14 have large rods of shape, while *Azot* 2, 7, 8, 11 and *Azot* 12 have coccid shapes. *Azot* 3 has medium rod shape while *Azot* 9 has small rod shape. Most of isolates are producing insoluble pigment creamy and slime changes to brown like *Azot* 1,2,7,8,9,10,13 and *Azot* 14 while *Azot* 4, 5, 6 and *Azot* 11 are not capable to produce the pigments in the plates after 7 days from growth. Consistency also differed between the isolates of *Azotobacter spp*, ranged from mucoid, viscid and milky in plates. All isolates are negative to gram reaction stain, motility and positive to catalase test. Also all of isolates have the ability to synthesis the hydrogen cyanide on Kings

Table 1: Physical and chemical properties of the soil at the experimental site.

Seasons	* Particle size distribution			Textural class	**Chemical properties					
	Clay	Silt %	Sand		O.M.(%)	EC dS/m	Available (ppm)			pH
							N	P	K	
2016/17	34.9	36.8	28.3	Clay loam	1.34	1.95	31.6	16.3	215.8	7.9

Table 2: Water constants and bulk density values of the soil at the experimental site.

Depth (cm)	Field capacity (%, w/w)	Wilting point (%, w/w)	Available water (%, w/w)	Bulk density (g cm^{-3})
00 - 15	42.9	18.3	24.6	1.26
15 - 30	37.9	16.8	21.1	1.30
30 - 45	32.2	15.9	16.3	1.35
45 - 60	26.8	16.8	10.0	1.44
Mean	34.95	16.95	18.0	1.34

B agar medium amended with glycine.

Nitrgenase activities and exopolysaccharides production from *Azotobacter* isolates

All of *Azotobacter* isolates screened for their N_2-fixing ability, producing, exopolysaccharide production (EPS) (Fig.1). *Azot* 7 recorded highest amounts of nitrogenase activity compared to other isolates followed by *Azot* 4 and *Azot* 2 being 47.23, 45.17 and 41.03 (µ mole C2H4 / ml/h) respectively. While *Azot* 12 recorded lowest nitrogenase activity. Fourteen isolates distrusted to produce exopolysaccharide. Dry weight EPS weighing results indicate that the *Azotobacter* isolate 7 resulted in a higher dry weight compared to other isolates of azotobacter. *Azot* 7 recorded higher producing EPS (7.9 g/L) followed by *Azot* 2, *Azot* 4 recorded (7.6 and 7.5 g/L). EPS weighing results ranged between (2.8 to 7.9 g/L). *Azot* 11 recorded lower producing EPS compared to other isolates. So the active selective isolate for nitrogenase activity and producing exopolysaccharides has been identified to *Azotobacter chroococcum*. Therefore, it was used to encapsulated and later as inoculants for wheat plant.

Evaluation of encapsulated *Azotobacter chroococcum* by Scanning Electron Microscope (SEM)

Using the SEM to visualize the encapsulated bacteria, (Fig.2), single and double coccid of *Azotobacter* of sodium alginate was obtained through SEM image has a size of range about 236.0 nm to 680.3 and no sign of contamination of other bacteria were observed..

Antioxidant Enzymes

The results showed in Fig. 3, significantly increased antioxidant enzymes in leaves of wheat plants under water deficit, while inoculation with *A. chroococcum* led to reduce the activity of antioxidant enzymes in shoots of wheat. CAT enzyme recorded 9.0 and 9.0 **(**µmol H_2O_2 mg^{-1} protein min^{-1}), respectively with inoculation either encapsulated or liquid culture under 80% from ETC, while recorded 10.3 and 10.4 **(**µmol H_2O_2 mg^{-1} protein min^{-1}), respectively under 60 % from ETC. Also APX and SOD enzymes recorded lowest activity in shoots of wheat treated with encapsulated or liquid *A. chroococcum* under 80% from ETC compared with wheat untreated with bacteria, recorded 3.5 and 3.8 (µmol ASA mg^{-1} protein min^{-1}) for APX, respectively, while 9.2 and 9.9 (µ /100g FW), respectively for SOD. On the contrary, the highest enzyme activity obtained when wheat plants were treated with 100 % N nitrogen only under water deficit. It is interesting to observation that antioxidant enzymes decreased on the following order, 75%N+*A. chrococcum* encapsulated and 75% N+ *A. chrococcum* liquid under 80 % and 60% from ETC as indicated in Fig. 3.

Table 3: Morphological characteristics of *Azotobacter spp* isolates.

No of isolates	Cell shape	Color	Consistency	Gram staining	Motility	Catalase Test	HCN
Azot.1	Large rod	Brown	Mucoid,viscid	-	+	+	+
Azot.2	Coccid	Creamy white	Viscous	-	+	+	+
Azot. 3	rod	Dull white	Weak slimy	-	+	+	+
Azot. 4	rod	Clear white	Mucoid	-	+	+	+
Azot. 5	rod	white	Mucoid,viscid	-	+	+	+
Azot. 6	Large rod	Dull Brown	Dry	-	+	+	+
Azot. 7	Coccid	Dull Brown	Mucoid	-	+	+	+
Azot. 8	Coccid	Brownish black	Milky	-	+	+	+
Azot. 9	Small rod	Dull Brown	Slimy	-	+	+	+
Azot. 10	Large rod	Brownish black	Mucoid	-	+	+	+
Azot. 11	Cocci single	White	Gummy	-	+	+	+
Azot. 12	Cocci single	Clear translucent	Viscous	-	+	+	+
Azot. 13	Large rod	Brownish black	Viscous	-	+	+	+
Azot. 14	Large rod	Dull Brown	Milky	-	+	+	+

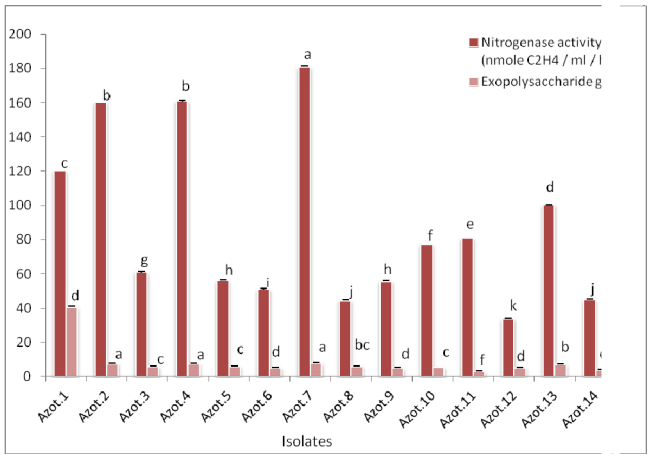
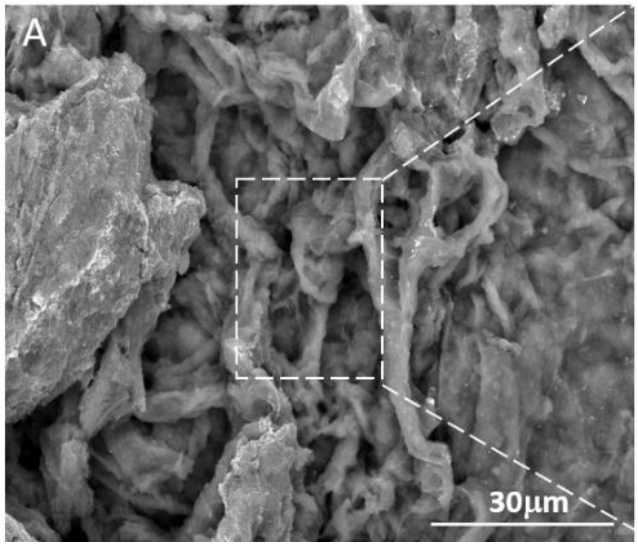
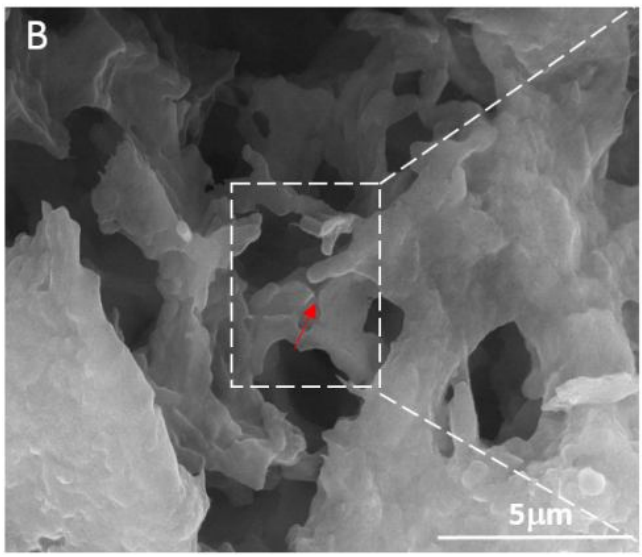


Fig. 1: Nitrogenase activity and exopolysaccharides production of *Azotobacter spp* isolates.



(A)



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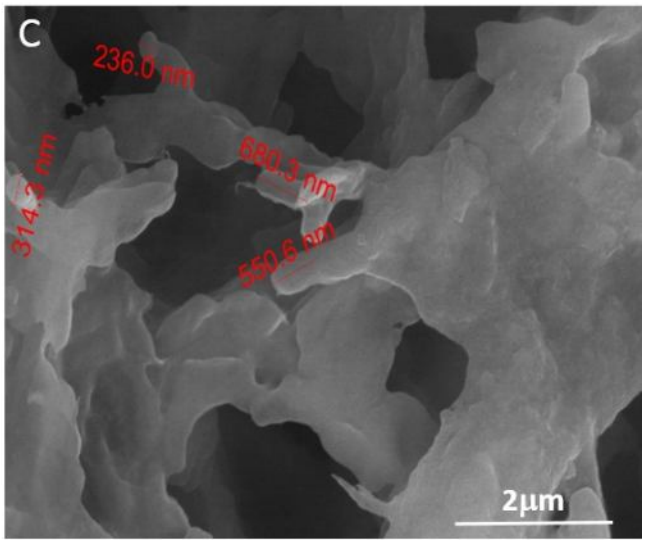
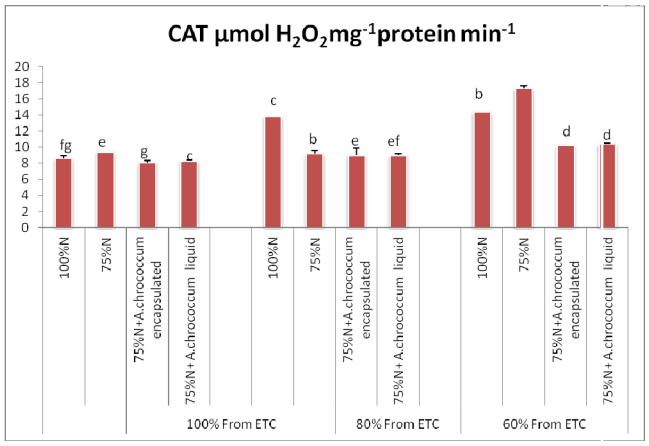


(C)

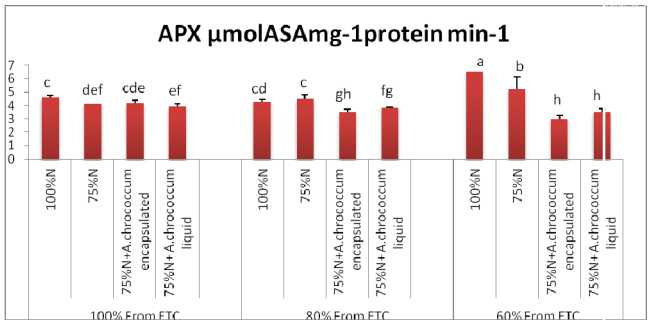
Fig. 2: SEM image of *Azotobacter* encapsulated beads with sodium alginate: A (encapsulated media with sodium alginate), B and C (encapsulated beads of alginate covering *A. chroococcum*).

Physiological characteristics

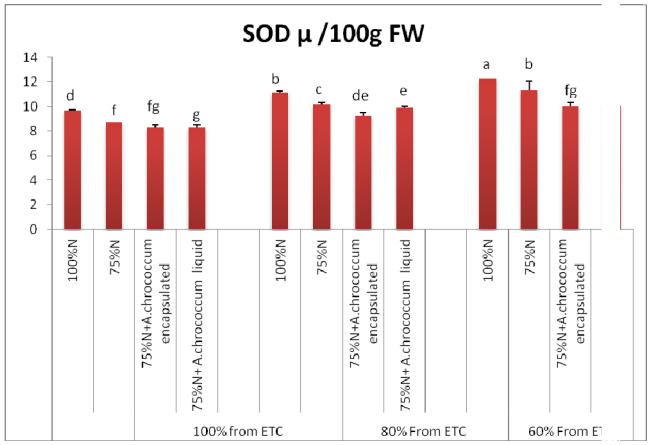
Physiological characteristics including R.W.C%, C.M.S% and proline content are presented in Table 4. Water defiant stress induced a massive decrease in R.W.C (%) and C.M.S (%) in shoots of wheat plants, In R.W.C recorded 55.93% and 55.87%, respectively with 100 %N and 75 N % under 80% from ETC, while the inoculation with *A. chrococcum* either in two forms significantly diminished these reductions being 66.37% and 59.23% , respectively in wheat treated with 75% N + *A. chrococcum* encapsulated and 75% N+ *A. chrococcum* liquid under 80% from ETC, the increased in R.W.C were 18.6% with 75% N + *A. chrococcum* encapsulated and 6.0 % with 75% N+ *A. chrococcum* liquid under 80% from ETC compared to the treatment supplemented with 100 %N under 80% from ETC. On the other hand the R.W.C% under water defiant 60% from ETC were 49.23% and 44.00 %, respectively with 100 %N and 75 %N whereas the inoculation increased the R.W.C % with two forms of bacteria under 60% from ETC. Regarding to C.M.S % there are a significant variation for cell membrane stability, water stress induced reduction in C.M.S % of the wheat cultivar while under irrigation C.M.S% were higher than water defiant condition. Under 80% from ETC wheat treated with encapsulated bacteria recorded C.M.S 43.9% ,while with liquid culture were 42.9% compared to wheat treated with 100% and 75 % N only. On the other hand, 60 % from ETC the C.M.C% recorded 49.9% and 48.7%, respectively with wheat treated either encapsulated form or liquid culture.



(A)



(B)



(C)

Fig. 3: Antioxidant Enzymes Assays, (A,B and C) affected by either encapsulated bead of *A.chrococcum* or liquid under three levels of water.

Regarding to Proline, it was significantly increased in shoots of wheat under 80 and 60 % from ETC compared to wheat plants under 100% from ETC, while inoculation wheat with encapsulation beads of *A. chroococcum* and liquid culture induced reduction in proline accumulation under 80 and 60 % from ETC, recorded 5.07 and 4.81 (mg /g d. w), respectively under 80 from ETC, while recorded 6.90 and 6.05 (mg /g d. w), respectively under 60 % from ETC compared to un- inoculated wheat, recorded 11.0 and 10.20 (mg /g d. w), respectively under 80 and 60 % from ETC plus 75 %N.

Yield components

In concern to yield components (Table 5) data obtained showed that *A. chroococcum* inoculant either encapsulated or liquid could markedly improve grains, straw yield and harvest index of wheat under water defiant. Maximum grain yield was obtained with encapsulated of *A. chroococcum* inoculant plus 75% N under 80 % from actual evapotranspiration recorded 3.60 Ton /fed followed by treatment obtained with liquid of *A. chroococcum* recorded 3.36 Ton/ fed. While 60 % from actual evapotranspiration maximum grain yield recorded 3.25 Ton /fed with encapsulated beads of *A. chroococcum* followed by treatment obtained with liquid of *A. chroococcum* recorded 3.15 Ton/ fed compared to treatment containing 100% N and 75% N only. The increased in grain yield was 28.57% with inoculation 75%N+ encapsulated of *A. chroococcum* ,while the increased was 20.0% with 75%N+*A. chrococcum* liquid compared to wheat treated with 75%N only under 80% from ETC, while the increased were 41.30% and 36.95%, respectively with 75%N+ encapsulated of *A. chroococcum* and 75%N+*A. chrococcum* liquid compared with wheat treated by 75%N only under 60% from ETC. Similar pattern was also observed for straw yield and harvest index. Maximum straw yield obtained with encapsulated beads of *A. chroococcum* under 80% from ETC ,recorded 4.14 Ton /fed compared to treatment supplemented with mineral nitrogen only either 100% N or 75% N were recorded 3.86 and 3.46 Ton/fed, respectively. On the other hand the maximum straw yield with 60% from ETC recorded 3.99 and 3.86 Ton/fed, respectively with encapsulated beads and liquid culture. The increased in straw yield was 19.65 % with encapsulated beads+ 75%N, while the increased was 18.20% with 75%N+*A. chrococcum* liquid compared to wheat treated with 75%N only under 80% from ETC. Whereas under 60% from ETC the increased were 33.00% and 28.66%, respectively with wheat treated with encapsulated of *A. chroococcum* + 75%N and *A. chroococcum* liquid + 75%N compared with wheat treated by 75%N only. The highest harvest index recorded with wheat treated with encapsulated *A. chroococcum* + 75%N followed by wheat treated with *A. chroococcum* liquid + 75%N under 100% from ETC.

Macronutrients of wheat grains

All the treatments under 100% from ETC irrigation significantly enhanced nitrogen, phosphors, potassium content in grains of wheat plants (Table 6). On the

Table 4: Physiological characteristics affected by either encapsulated bead of *A.chrococcum* or liquid under three levels of water.

IrrigationA	TreatmentsB	R.W.C (%)	C. M.S (%)	Proline (mg /gd. w)
100% from ETC	100%N	69.47	55.2	5.02
	75%N	65.77	58.0	4.79
	75%N+*A.chrococcum* encapsulated	69.70	49.3	5.05
	75%N+ *A.chrococcum* liquid	63.50	48.4	5.02
80% From ETC	100%N	55.93	43.2	10.05
	75%N	55.87	39.9	11.00
	75%N+*A.chrococcum* encapsulated	66.37	43.9	5.07
	75%N+ *A.chrococcum* liquid	59.53	42.9	4.81
60% from ETC	100%N	49.23	48.5	10.78
	75%N	44.00	45.5	10.20
	75%N+*A.chrococcum* encapsulated	50.23	49.9	6.90
	75%N+ *A.chrococcum* liquid	56.57	48.7	6.05
L.S.D at 0.05				
A		2.704	73.45	0.913
B		4.471	57.09	1.810
A×B		7.745	98.89	3.135

Table 5: Grain, straw and harvest index of wheat affected by either encapsulated bead of *A.chrococcum* or liquid under three levels of water.

IrrigationA	TreatmentsB	Grain yield Ton/fed.	Straw yield Ton/fed.	Harvest Index %
100% from ETC	100% N	3.87	4.45	46.51
	75%N	3.44	4.13	45.44
	75%N+*A.chrococcum encapsulated*	3.85	4.31	47.18
	75%N+*A.chrococcum* liquid	3.53	4.00	46.87
80% from ETC	100%N	3.03	3.86	43.97
	75%N	2.80	3.46	44.72
	75%N+*A.chrococcum* encapsulated	3.60	4.14	46.51
	75%N+*A.chrococcum* liquid	3.36	4.09	45.10
60% from ETC	100%N	2.70	3.26	45.30
	75%N	2.30	3.00	43.39
	75%N+*A.chrococcum* encapsulated	3.25	3.99	44.89
	75%N+*A.chrococcum* liquid	3.15	3.86	44.93
L.S.D at 0.05				
A		5.531	7.2	——
B		6.2	7.2	——
A×B		7.2	1.24	——

contrary, wheat treated with minerals content only, recorded decreased in NPK % in grain under 80% and 60% from ETC. Maximum N in grains of wheat recorded with wheat treated encapsulated of *A. chroococcum* with 75% N recorded 1.39 N% in grains, while wheat treated with liquid *A. chroococcum*, recorded 1.35 N% under 80% from ETC. Under 60% from ETC the nitrogen in grain recorded 0.98% in wheat treated with 75%N+*A. chrococcum* encapsulated while, it record 0.88% with wheat treated with 75% N+*A. chrococcum* liquid. Regarding to P%, inoculation significantly improvement the phosphors in grains, recorded 0.88% in grains of wheat treated with 75%N+*A. chrococcum* encapsulated under 80% from ETC and giving 0.82% with 75%N+*A. chrococcum* liquid compared to wheat untreated with bacteria, also under 60% from ETC phosphors in grains recorded 0.59% and 0.50%, respectively with 75%N +*A.chrococcum* encapsulated and 75% N+*A. chrococcum* liquid culture compared to wheat untreated with bacteria, recorded 0.48% and 0.53% with 100%N and 75%N, respectively. Likewise inoculation with of *A. chroococcum* either encapsulated or liquid culture increased the K% in grains of wheat under drought stress compared to wheat treated with 75% N only. Highest K% recorded with wheat treated with 75% N +*A.chrococcum* encapsulated under 100% from ETC compared with other treatments. Generally, application of bacteria either encapsulated or liquid culture enhancement the mineral concentration in wheat grains.

Soil water relations

Applied irrigation water (AIW)

Amount of applied irrigation water throughout the growing season for different treatments were presented in Table 7. Results showed that the amounts of applied irrigation water were recorded under 100% ETo as compared with 80 and 60% ETo treatments through the growing season. The total amount of applied irrigation water for wheat were 2391, 1924 and 1836 m3 fed^{-1} for the 100, 80 and 60% ETo, respectively For the relation between of applied irrigation water (100, 80%

Table 6: Macronutrients of wheat affected by either encapsulated bead of *A.chrococcum* or liquid under three levels of water.

Irrigation	Treatments	N%	P%	K%
100% from ET_0	100%N	1.43	0.89	0.52
	75%N	1.33	0.83	0.51
	75%N+*A.chrococcum* encapsulated	1.54	0.99	0.55
	75%N+*A.chrococcu*m liquid	1.52	0.87	0.54
80% From ET_0	100%N	1.02	0.65	0.43
	75%N	0.90	0.61	0.41
	75%N+*A.chrococcum* encapsulated	1.39	0.88	0.54
	75%N+*A.chrococcum* liquid	1.35	0.98	0.51
60% from ET_0	100%N	0.80	0.48	0.33
	75%N	0.76	0.53	0.31
	75%N+*A.chrococcum* encapsulated	0.98	0.59	0.42
	75%N+*A.chrococcum* liquid	0.88	0.50	0.40
L.S.D at 0.05				
A		0.4241	0.0358	0.0035
B		0.5269	0.2712	0.0313
A×B		0.9126	0.4698	0.0542

Table 7: Applied water and water utilization efficiency of wheat affected by either encapsulated bead of *A.chrococcum* or liquid under three levels of water.

IrrigationA	TreatmentsB	AIW	Yield kg/fed.	WUtE
100% from ETC	100%N	2400	3.87	1.61
	75%N	2395	3.44	1.44
	75%N+*A.chrococcum* encapsulated	2375	3.85	1.62
	75%N+*A.chrococcu*m liquid	2392	3.53	1.48
	Mean	2391	3.67	1.53
80% from ETC	100%N	1935	3.03	1.56
	75%N	1931	2.80	1.45
	75%N+*A.chrococcum* encapsulated	1914	3.60	1.88
	75%N+*A.chrococcu*m liquid	1915	3.36	1.75
	Mean	1924	3.19	1.65
60% from ETC	100%N	1814	2.70	1.48
	75%N	1812	2.30	1.26
	75%N+*A.chrococcum* encapsulated	1809	3.25	1.79
	75%N+*A.chrococcu*m liquid	1910	3.15	1.65
	Mean	1836	2.85	1.72

and 60% ETC) and four fertilizer treatments (100% N, 75%N, + encapsulated beads of *A.chrococcum* + 75%N and 75%N+*A.chrococcum* liquid) results revealed that fertilizer of 100% N treatment resulted in average higher amount of AIW were (2400) of 100% ETC (1935 m3/ fed.) and (1814 m3/fed.) comparing with 80 and 60% ETC treatments through the growing season. While the lowest ones amount of IWA were recorded by fertilizer treatment 75% N + encapsulated beads of *A.chrococcum* recorded (2375, 1914 and 1809 m^3/fed) respectively.

Water utilization efficiency (WUtE, kg m^{-3})

Efficiency of water utilization is an important limiting factor to crop production. Water utilization efficiency (WUtE) values of wheat yield affected by the tested variables during 2016 /2017 growing season are presented in (Table 7). Results show that the average values of water utilization efficiency (WUtE) were affected by irrigation and fertilizer treatments. Results indicated that the average water utilization efficiency (WUtE) as affected by irrigation treatments, was 1.53, 1.65 and 1.72 kg m^{-3} under (I_1), (I_2), and (I_3) irrigation treatments, respectively. The interaction shows that highest values of WUtE were 1.88, 1.79 and 1.65 kg grain m^{-3} water applied respectively; obtained from (I_2, I_3 and I_1) with 75%N+ *A.chrococcum* encapsulated treatment. Whereas, the lower values of WUtE recorded 1.44kg m^{-3} water applied, was obtained by (I_1) with 75%N

Discussion

Drought is the major reasons for damages and losses in Agriculture, different effort are conducted to reduce or minimize the effect of drought in agriculture, one of the initiatives is using the nitrogen fixing bacteria to decrease the water using by the plant as well as to decrease the negative environmental impact from using chemical fertilizers. In this study a total of fourteen *Azotobacter* isolates were isolated from the rhizosphere of wheat plants. All isolates were characterization as *Azotobacter* spp. and studied to the morphologically & biochemically tested according to the Burges manual. All of *Azotobacter* isolates were motility, catalase positive and synthesis the hydrogen cyanide, these results are harmony with Abdel-Hamid *et al.*, (2010) Mazinani *et al* (2012). Aquilanti *et al.*, (2004) found that the difference in cell shape, colony size may be because of various factors like presence of confused, shapeless masses-symbolisms, may also be indorsed to

the structure of the medium. *Azotobacter* is a free–living N_2– fixer diazotroph, the fixation of N_2 depends on the activation of nitrogenase activity, which may differ from strain to strain (Sandeep *et al* 2015); Stella and Suhaimi (2010) also found that three species of *Azotobacter*, *A. chroococcum*, *A.vinelandii* and *A. beijerinckii*, showed high growth, nitrogen fixation and in vitro production of phyto-hormone.

Different bacterial metabolites and extracellular polymeric substance (EPS) have been found to have an effect impact in the plant health (Seifan *et al*., 2016). Higher EPS producing help the plants to assume a higher volume of water and enhanced the nutrients from rhizosphere soil, causing in a better growth of plants, and also, this was useful to stabilize the damaging effects of drought stress (Sandhya *et al.*, 2011). EPS producing the cells of *Azotobacter*, which leads to an increase in aggregates stability size destruction as indirect additional effect, which improves the plant growth under drought stress (Alami *et al*., 2000). Husam and Shatha (2013) studied the exopolysaccharides and found variation in exo-polysaccharide polymer due to differed between molecular weight between the isolates. Encapsulated technique with sodium alginate positively immobilized with the above-mentioned polymer Damasceno *et al.*, (2014); Krishnamoorthy *et al.*, (2016) generally sodium alginate as safe which has a high oxygen block when dry without disturbing bacterial bioactivity. Sodium alginate had no effect the survival of bacteria even for several days from immobilized as well as is an ecological hydrophilic. Results obtained from this study showed that immobilization of bacteria in sodium alginate beads a favorable style for improve the cell protection and salvage. This ticking for immobilization provides a new protocol in future application of sustainable bio self-healing material. Also the encapsulation of *Azotobacter* in sodium alginate beads is a hopeful method to improve the cell protection and recovery, and supply a new technique to address the limitations in future application of ecological bio self-healing material. Under drought stress condition nitrogen fixing bacteria inoculation, caused a decreased in antioxidant enzymes 'activities there are a significant interaction between antioxidant enzyme activity and drought stress, wheat inoculation with PGPRs led to reduce the activity of enzymes (Han and Lee 2005). As a result of drought, SOD,CAT and APX activates significantly increased in leaves of wheat, as a result of ROS formation and due to closed of the stomata and following decrease in CO_2 concentration in the leaf mesophylls tissue, then NADPH is accumulation; and the oxygen doings an alternative acceptor of electrons, then the superoxide radicals are formed (Cadenas, 1989) Also, Lee *et al*., (2009) found that there are a positive a correlation between all of antioxidants enzymes in the case of water deficit stress conditions. *Azotobacter* inoculation led to changes in enzymes activity also be a result of change of synthesis and accumulation of less activity of the enzymes (Chaparzadeh *et al*., 2004).

A reduction in relative water content is a typical plant reply to osmotic stress (Fahad *et al.*, 2015). RWC increased for *Azotobacter* inoculated treatments under stress condition. This is a signal of enhanced water uptake due to the bacterial inoculation under drought stress conditions. Under drought stress inoculation of PGPR could increase plant water status and thus increased biomass accumulation (Nadeem *et al.*, 2007). Drought stress made a loss in cell membrane stability due to a major reason for reduce the growth of various plant species, this reduction might be to the enhanced production of damaging ROS molecules. *Azotbacter* inoculation significantly reduced the amount of leakage from plant tissue (Samira *et al.*, 2014). Many authors found that in crop varieties of wheat under drought stress proline accumulation were significantly increased in wheat plant free proline might be involved in membrane stabilization during water stress (Kocheva and Georgiev, 2003), or it might be a reserve of readily mobilize N_2 available upon relief of stress. PGPRs inoculation reduced proline content in the leaves of wheat plants under drought stress. May be *Azotobacter* inoculation could compensate drought effects plus to enhanced water status in wheat plant (Samira *et al*., 2014). Here also 60% irrigation from actual evapotranspiration without *Azotobacter* inoculation has lowest grain yield these result are harmony with Ehsan *et al.*, (2017) cleared that under drought stress and PGPR plus nitrogen application gave maximum grain and straw yield on the other hand 60% irrigation without PGPR treatment gave lowest grain yield. Further, Moser *et al*., (2006), found that adverse effect of drought stress on the grain yield. Water deficit induced reducing in the gaining of nutrients by the root and their transport to shoots, as well as induced reduction in the inorganic nutrients as a result from interfering in nutrient uptake and the unloading mechanism, P and K was disadvantaged (Garg, 2003; McWilliams, 2003). Under water stress PGPRs have been reported to improvement to root hair propagation and increase root branching, and uptake of minerals and water (Spaepen *et al.*, 2007). On the other hand (Yang and Zhang, 2006) cleared that applied of N fertilizer application under drought stress conditions improved remobilization and enhanced grain-filling rate. Generally, encapsulated, cells of microorganisms absolutely shows their leads over usually used free cell inoculation like N_2 fixation (Bashan *et al.*, 2002; Fenice *et al.*, 2000). Soil water content at field capacity (FC) and wilting point (WP) are important for irrigation planning, assessing plant water requirement and assessing soil rightness for diverse land uses (Mbah, 2012).

The increase in irrigation water applied under 100% ETo may be attributed to the increase in direct evaporation. Therefore, the seasonal irrigation water applied is higher under 100% ETC followed by 80% and 60% ETC for wheat during the growth season. The present results are in harmony with those obtained by the results of (Morsy *et al.*, 2018). Regarding to results in irrigation frequency of irrigation and interval of irrigation are closely related and are often interchangeable (Majumdar, 2002 and Yavuz *et al.*, 2010). Results indicated that applied irrigation water and water utilization efficiency were in harmony with those obtained by Abd El-Hady and Ebtisam, (2016) and Ewis *et al*., (2016). Morsy and Abd El- Latif (2012) found that increasing water applied for onion yield gave lower water productivity for all varieties. Under the conditions of the present experiment and to conserve the limited irrigation water resources, as an important national issue, it is advisable to 80% from ETC with fertilizer 75%N+*A.chrococcum* encapsulated in order to obtain reasonable water productivity.

Concerning to the results *Azotobacter* is able to alleviate drought stress on growth of wheat plant through colonization in the rhizosphere of plant, this is probably may be to producing EPS and this EPS might be deliver a microenvironment that grips water and dry out more slowly, and protecting the bacteria from drying (Sandhya *et al*., 2009). This will open the horizons for further illustration of the genetic and metabolic capacity on those strains for that a genome sequence will be conducted as a the next step. As well as the capsulation will be applied to different bacterial strains as safe effecting way for inoculated bacteria.

Conclusions

The present study prospers to isolate nitrogen fixing bacteria and studied the morphological and biochemical characterization. The best active isolate for nitrogenase activity and exopolysaccharides production was selected to identify by Bio log system techniques and encapsulated it, these capsule beads proved a promising technique for protect the bacteria from drought stress. This technique improved the growth, Physiological characteristics under drought stress. As well as enhancement the oxidative enzymes besides that encapsulated beads of bacteria succeeded to increase the yield components with 80 and 60 % from actual evapotranspiration and enhancement the applied irrigation water and water utilization efficiency under drought stress. Encapsulate in alginate is presented as great carried for of inoculated plant growth promoting bacteria on the filed application.

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