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Impact of formulated *Azospirillum lipoferum, Bacillus polymyxa* and *Nostoc muscorum* on Wheat productivity

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Abstract: Introduction of beneficial microbes into soil relies on the survival of microorganisms in a heterogeneous soil environment. A field experiment was conducted at Giza Research Experimental station, Agricultural Research Center (ARC), Giza, Egypt during winter season of 2013/2014 to evaluate formulations of some plant growth promoting microorganisms (PGPM) on their survival, biological efficiency and interaction with wheat plant under 75% of recommended nitrogen fertilizer dose. A*zospirillum lipoferum*, *Bacillus polymyxa* and *Nostoc muscorum* were formulated with three different carriers as follows: encapsulated with sodium alginate as beads, formulated with mixture of carboxymethyl-cellulose and talcum powder and carried on vermiculite. The inoculants were used individually and in combined mixtures. Results showed that application of the tested inoculants formulated with sodium alginate, either alone or in combination, recorded superiority over the other formulations to attain the highest values in most recorded data. Application of mixture of encapsulated *A. lipoferum*, *B. polymyxa* and *N. muscorum* at a ratio of 1:1:1 (T_{11}) gave a significant increase in rhizosphere microbial activities, which expressed as dehydrogenase (46.51 and 95.00 µg TPF g dry soil^{-1} day^{-1}), nitrogenase (5.09 and 12.17 µ mole C_2H_4 g dry soil^{-1} hr^{-1}) and total phosphatase enzyme activities (1.88 and 2.84 mg g dry soil^{-1}) after 45 and 75 days, respectively. While, the highest population of *A. lipoferum*, *B. polymyxa* and *N. muscorum* were attained due to the application of individual inoculants formulated with sodium alginate, comparing with the other formulation forms. Moreover, (T_{11}) exhibited the highest growth and yield components: 1000 grains wt. (52.99 g), grains and straw yield (2.48 and 4.51Ton/fed, respectively) comparing with the other treatments. Obviously, inoculation with mixture of organisms particularly when they entrapped with sodium alginate capsules achieved better growth and yield parameters. The highest increase percent of biological yield was achieved with T_{11}, which recorded 10.2 and 8.2 % in grain and straw yield, respectively, over the control treatment. Furthermore, the economic evaluation revealed that the increase in net return per fed of wheat was about 632 L.E., and the return of L.E. of wheat/fed has reached to about 1.36 L.E. /fed. due to the application of T_{11}.

Keywords: Biofertilization, formulated microorganisms, carriers, N_2-fixers, plant growth promoting microorganisms, *Azospirillum lipoferum*, *Bacillus polymyxa* and *Nostoc muscorum*.

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Introduction

Current trend in agriculture is focused on reducing the use of inorganic fertilizers on one hand and accelerating the search for alternative ways to sustainable agriculture on the other[1]. The use of PGPR inoculants as biofertilizers provides a promising alternative to chemical fertilizers and pesticides. The biofertilizers have the ability to convert nutritionally important elements from unavailable to available form through biological processes[2].

Different plant-growth promoting rhizosphere bacteria including associative bacteria such as *Azospirillum, Bacillus, Pseudomonas* and *Enterobacter* have been used for their beneficial effects on plant growth. The mechanisms of plant growth stimulation by associative bacteria are mobilization of nutrients. Survival of the plant growth-promoting bacteria at different temperatures and soils may be important for successful root inoculation[3].Cyanobacteria also may be the most important nitrogen-fixing agents in many agricultural soils[4]. Cyanobacteria are known by their ability to excrete growth-promoting substances such as hormones (Auxin, Gibberellins), vitamins and amino acids. They also increase the water- holding capacity through their jelly structure, increase in soil biomass after their death and decomposition, preventing weeds growth[5].

Carriers of microbial inoculants need to supply sufficiently large populations of viable, beneficial microorganisms in order to positively affect plant growth. Variety of materials used as carriers has been shown to improve the survival and biological effectiveness of inoculants by protecting bacteria from biotic and abiotic stresses[6]. Suitable carrier should be cheap, easily used, mixable, packageable and available. Also, the carrier must permit gas exchange, particularly oxygen, and has high organic matter content and high water holding capacity[7].

Among carriers that can sustain high levels of microbial load, the peat is considered the most widely and common commercial used carrier[8]. A variety of inoculants carriers (e.g., liquid growth medium, vermiculite and humus) and adhesive agents (e.g., Arabic gum, methyl cellulose and oil) have been used for microbial inoculation of seeds.

Other formulations useful to the application of beneficial microorganisms to seeds or plants make use of cross-linking organic polymers like alginate, carrageenan, polyacrylamide and talcum powder. These materials have been used extensively to experimentally immobilize plant, animal or microbial cells and even isolated enzymes[9]**.** Alginate is dry, synthetic, simple to use, uniform, biodegradable by soil microorganisms, and non-toxic in nature. It contains a large uniform bacterial population and provides slow release of the bacteria for long periods[10]. It causes no ecological pollution and can be produced on large scale by the proper industry. The beads can be stored for long periods in a relatively small volume without any apparent effect on the size of the bacterial population[11]. Formation of pelletized gels by mixing alginate with the microbial culture and then adding the mixture drop-wise into a solution of $CaCl_2$, which yields small beads of uniform size containing a high concentration of cells. The method has been used successfully to encapsulate a lot of beneficial microorganisms particularly the genera of *Azospirillum* and *Bacillus*[12] into small beads. The beads may be applied together with the seeds at sowing which keeping the same cell titer per gram of seeds. Encapsulated seeds showed above 90% germination rates that were similar to the non- capsulated controls. Microbial immobilization gives prolonged metabolic activity when microbial cells are reused. Organisms could be immobilized separately or co-immobilized together[13]**.**

The present study was designed to evaluate *Azospirillum lipoferum*, *Bacillus polymyxa* and *Nostoc muscorum* formulations on their survival, biological efficiency and interaction with wheat plant under field trial.

Materials and Methods

Sources and preparation of microbial inoculants

1. **Bacterial inoculants**

a) *Azospirillum lipoferum*

Azospirillum lipoferum was kindly provided from Department of Agric. Microbiology, Soils, Water and Environ. Res. Inst. (SWERI), Agric. Res. Center (ARC), Giza, Egypt. *A. lipoferum* was activated in nitrogen deficient malate medium[14]at 28°C for 48 hours, cell densities was adjusted to be $30x10^7$cfu /ml.

b) *Bacillus polymyxa*

An active strain of *Bacillus polymyxa* was obtained from Department of Agric. Microbiology, SWERI, ARC, Giza, Egypt. *B. polymyxa* was grown and maintained in nitrogen deficient medium of [15], then incubated for 48hr at 28°C (log phase), then the pure culture enriched on nutrient broth medium[16]for 48 hours at 28°C to reach the maximum growth (10^7cfu/ ml).

2. Algal inoculant

Nostoc muscorum was used through the current study, which kindly supplied by Department of Agric. Microbiology, SWERI, ARC, Giza, Egypt. An axenic culture of cyanbacterial strain (*N. muscorum*) was grown and propagated autotrophically on free nitrogen BG_{11}medium[17]. The culture was incubated in growth chamber under their optimum conditions and continuous illumination (2000 lux) up to obtain mass culture of 1g dry weight per liter at log phase stage.

The bacterial and algal cultures were collected and centrifuged at 3000 rpm for 10 minutes to separate the microbial cells from its supernatants. The supernatants were discarded and the cells were resuspended in 400 ml phosphate buffer pH 7 to reach its final volume and stored in refrigerator till use.

Inocula formulation

1. Vermiculite

Single or mixed cells of bacterial and algal strains were prepared and mixed with sterilized vermiculite (20% moisture) then adhesion using sticker such as Arabic gum (20%) to give homogenized inoculum.

2. Sodium Alginate

Macro-encapsulation process

Sodium alginate polymer (ALGOGGL 3001, SG 30- 60, Degussa, France) was sterilized as a dry powder in autoclave at 121°Cfor 20 min. before dispersion in distilled water where it dissolved in water for 30 min. Each inoculum was added into 30 ml of encapsulating matrix solution and mixed homogeneously, then introduced in a syringe and placed on the encapsulation device and extruded drop by drop through the needle (1.55mm) by acting the syringe pump at the rate of 120 ml / hr. The whole technique was done under aseptic conditions in a laminar air flow hood. The drops fell directly into 1.5% $CaCl_2$ solution for reticulation. After 30 min.; the minimum time required for total reticulation the macrocapsules (about 5-6 mm diameter); the capsules were washed three times with sterile tap water then stored in 0.85 % NaCl till used[13]**.**

3. Talcum powder

Mixture of 10 g carboxymethyl cellulose (CMC) and 1 kg of talcum powder were used to prepare the powder formulation; calcium carbonate was added to the mixture to adjust the pH to 7. The mixture was autoclaved at 121°C for 20 min. as described by[18]**.** Both bacterial and algal homogenized biomass were added to the carrier (1kg talc powder for 100 ml of each used strain) and mixed well under aseptic conditions to form pasta. The pasta was air dried under laminar flow hood for 24 hr. The dried product was powdered using a blender sieved and packed in sterilized polyethylene bags .One gram of each microbial type was taken to count the colony forming unit (10^7cfu/ml) by using dilution plate technique on specific media for both bacterial and algal strains.

To test the viability of encapsulated inoculants 10 beads were suspended in 10 ml solution of Na-tricitrate (10%) under gentle shaking for 30 min. Serial dilutions were carried out on specific medium of each used inoculum according to the method described by[13]**.**

The field work

A field experiment was designed and conducted at Giza Research Experimental Station, ARC, Giza Govern., Egypt in winter season (2013/2014). The experiment included thirteen treatments with three replicate, the plot area was 6 m^2. The experimental treatments were laid out in a complete randomized plots design as follow:

T1. Control (full dose of NPK)
T2. *A. lipoferum* en-capsulated with sodium alginate.
T3. *A. lipoferum* formulated with talcum powder.
T4. *A. lipoferum* carried on vermiculite.
T5. *N. muscorum* en-capsulated with sodium alginate.
T6. *N. muscorum* formulated with talcum powder.
T7. *N. muscorum* carried on vermiculite.
T8. *B. polymyxa* en-capsulated with sodium alginate.
T9. *B. polymyxa* formulated with talcum powder.
T10. *B. polymyxa* carried on vermiculite.
T11. Mix. of the microorganisms en-capsulated with sodium alginate.
T12. Mix. of the microorganisms formulated with talcum powder.
T13. Mix. of the microorganisms carried on vermiculite.

Soil

Soil samples representing the experimental location were analyzed before wheat cultivation process for some soil characteristics [19] and reported as following:

Particle size distribution (%): Clay 33.4, silt 35.6, fine sand 19.6, coarse sand11.2 and texture class was clay loam.
Chemical analysis: pH (1:10) 7.3, EC ds/m (1:10) 2.6.

Soluble cations and anions (meq L^{-1}):
Cations: K^{+}1.52, Na^{+}8.16, Mg^{++} 6.67 and Ca^{++} 9.20.
Anions: SO_4^{--}13.07, Cl^{-}11.13, HCO_3^{-} 1.35, CO_3^{--} 0.00.

Wheat seeds

Giza 168 cultivar of wheat (*Triticum aestivum*) seeds was used, which kindly obtained from field Crops Research Inst., (ARC), Giza, Egypt.

Field application of beneficial microorganisms

Wheat seeds were surface sterilized by soaking in chlorox solution (0.05%) for 2 min and then the seeds were rinsed five times with sterilized distilled water .The seeds were then coated with different formulated microorganisms according to the treatments. Directly after coating, the seeds were sown.

Fertilization

Nitrogen was applied as ammonium nitrate at a rate of 120 kg fed^{-1} in three equal doses 15, 30 and 60 days from sowing. Phosphorus was added as superphosphate (15.5% P_2O_5) at a rate of 200 kg fed^{-1} once during soil preparation. Potassium was added as potassium sulphate (48% K_2O) at a rate of 50 kg fed^{-1}once before flowering stage (60 days from sowing). All treatments received 75% of the recommended nitrogen dose except the control, which received the full dose of nitrogen. The agricultural practices were carried out as recommended by the Ministry of Agriculture and Land Reclamation, Egypt.

Measured parameters

1) Morphological parameters:

Plant height (cm), plant dry weight (g) and the number of reproductive tillers/plant were measured after 45 and 75 days of sowing respectively.

2) Biological parameters

The population dynamics of *A. lipoferum* and *B. polymyxa* were evaluated either in rhizosphere and rhizoplane areas, while, population density of cyanobacteria and *N. muscorum* were evaluated in rhizosphere area only. The population density of the tested microorganisms was followed after 45 and 75 days from wheat

cultivation on their specific media of each by using a plate count technique. The activities of nitrogenase (µmole C_2H_4/g rhizosphere), dehydrogenase (µg TPF/g dry soil) and alkaline and acidic phosphatases (mg/g dry soil) were determined according to the methods of [20, 21, 22] during 45 and 75 days, respectively.

Yield parameters

Plant samples from each treatment were collected by using 1 m^2 wooden frame to determine wheat yield and its components. Samples of straw and grains were oven dried at 70°C up to a constant dry weight, grounded and prepared for digestion method as described by[23]**.**The digests were then subjected for measurement of NPK. Nitrogen content was determined by Kjeldahel technique and potassium content was determined by Flame photometer as described by[24]**.** Phosphorus content was determined by inductively coupled plasma spectrometry (ICPS) (Ultima 2 JY Plasma).

Economic evaluation

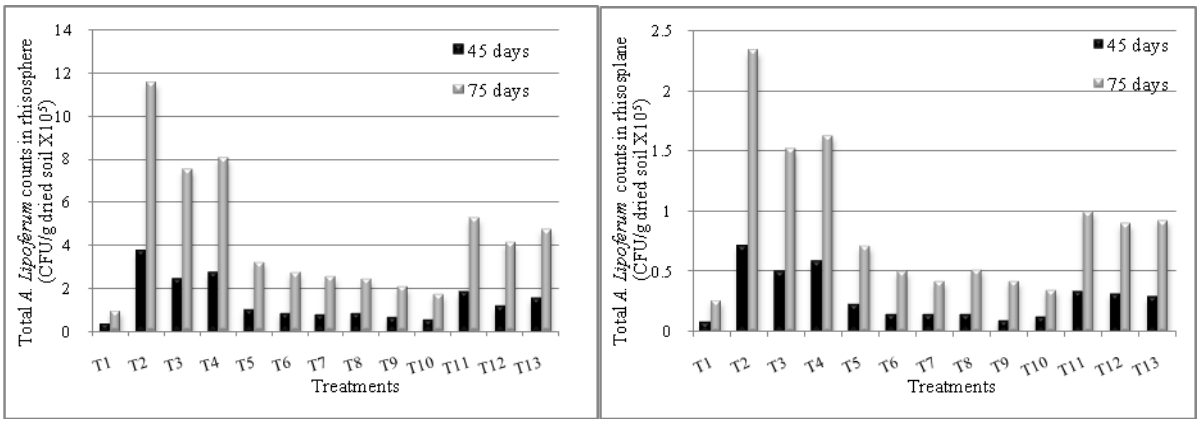
Cost of biofertilizers, Net return of wheat/fed. and the return of L.E. were evaluated for the most promising biofertilizer treatments and compared to the chemical fertilizer used under the experimental conditions.

Statistical analysis

Obtained results were subjected to statistical analysis through comparing means of the treatments by using least significant difference (L.S.D.) at 0.05 level of probability as described by[25]**.**

Results

The population density of the tested microorganisms were followed from the point of view of the action of different carrier forms and developmental plant stage on these bacteria under deficient of N fertilizer in wheat plants inoculated and non-inoculated with them. Results of the studies concerning this question are presented in Figures 1-4.



$T_{1:}$ Control, T_2: *A. lipoferum* capsulated with sod. alg., T_3: *A. lipoferum* formulated with T.P., T_4: *A. lipoferum* carried on vermiculite, T_5: *N. muscorum* capsulated with sod. alg., T_6: *N. muscorum* with T.P., T_7: *N. muscorum* carried on vermiculite, T_8: *B. polymyxa* capsulated with sod. alg., T_9: *B. polymyxa* formulated with T.P., T_{10}: *B. polymyxa* carried on vermiculite, $T_{11:}$ Mix. capsulated with sod. alg., T_{12}: Mix. formulated with T.P. and T_{13}: Mix. carried on vermiculite.

Fig. (1): Population of *Azospirillum lipoferum* in rhizosphere and rhizoplane regions of wheat plant inoculated with microorganisms in different formulated carriers.

The inoculation of wheat (*Triticum aestivum*) with *Azospirillum lipoferum* led to the colonization of these bacteria in rhizosphere and rhizoplane regions at different growth intervals. As shown in Fig (1) the total count of *A. lipoferum* was higher in the rhizosphere region than in the rhizoplane area. *Azospirillum* populations markedly increased at 75 days with all treatments more than at 45 days. *A. lipoferum* encapsulated with sodium alginate observed the highest colonization in both rhizosphere and rhizoplane regions compared to *A. lipoferum*

formulated with talcum powder and the ones carried on vermiculite. *Azospirillum* encapsulated with sodium alginate (T_2) recorded the maximum populations during the two intervals, where at 45 and 75 days it obtained 3.73 and 11.52x10^5cfu/g soil at rhizosphere area, respectively whereas it recorded 0.71 and 2.34x10^5cfu/g soil at rhizoplane, respectively. Additionally, the populations of *Azospirillum* formulated with talcum powder were slightly less than those carried on vermiculite. The individual treatments recorded more *Azospirillum* populations than the mixture ones.

Results in Fig (2) show the establishment of *Bacillus polymyxa* in wheat rhizosphere more than in rhizoplane in both growth intervals (45 and 75 days). *B. polymyxa* encapsulated with sodium alginate (T_8) recorded the maximum populations during the two intervals, where at 45 and 75 days it obtained 6.22 and 24.12x10^5cfu/g soil at rhizosphere area, respectively whereas it recorded 0.88 and 3.09x10^5cfu/g soil at rhizoplane, respectively. Data also showed that the increase of *Bacillus* populations with (T_8) was more than all other treatments including the control and mixture ones.

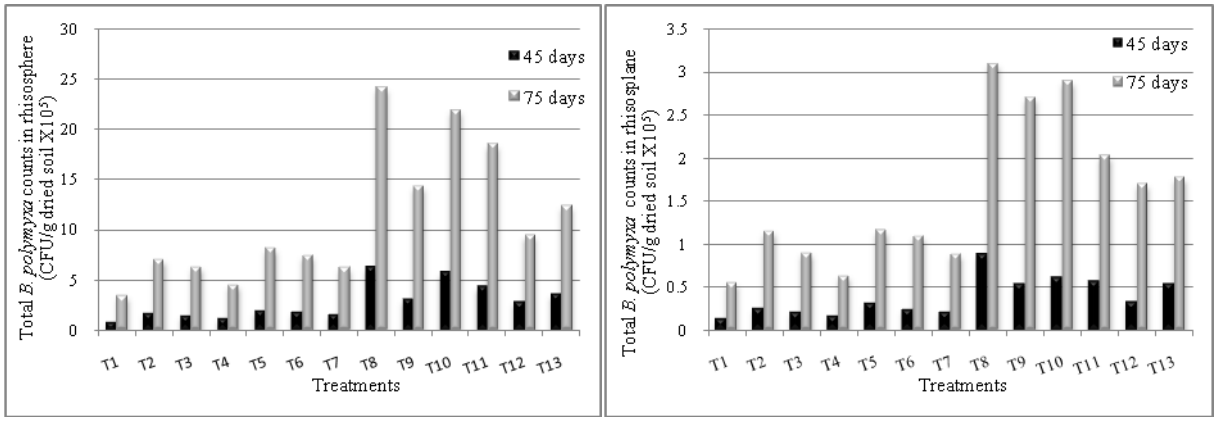


Fig. (2): Population of *Bacillus polymyxa* in rhizosphere and rhizoplane regions of wheat plant inoculated with microorganisms in different formulated carriers.

Inoculation of wheat with *Nostoc muscorum*; a nitrogen fixer cyanobacteria; individually or mixed with *A .lipoferum* and *B. polymyxa* increased the numbers of both total cyanobacteria and *N. muscorum* in wheat rhizosohere area as shown in Fig. (3). Treatment received capsulated *Nostoc* (T_5) exhibited the highest count comparing with other treatments and control. It recorded 7.66 and 29.13×10^3cfu/g soil as total cyanobacterial counts and 4.82 and 18.71 ×10^2cfu/g soil as *Nostoc muscorum* counts at 45 and 75 days, respectively.

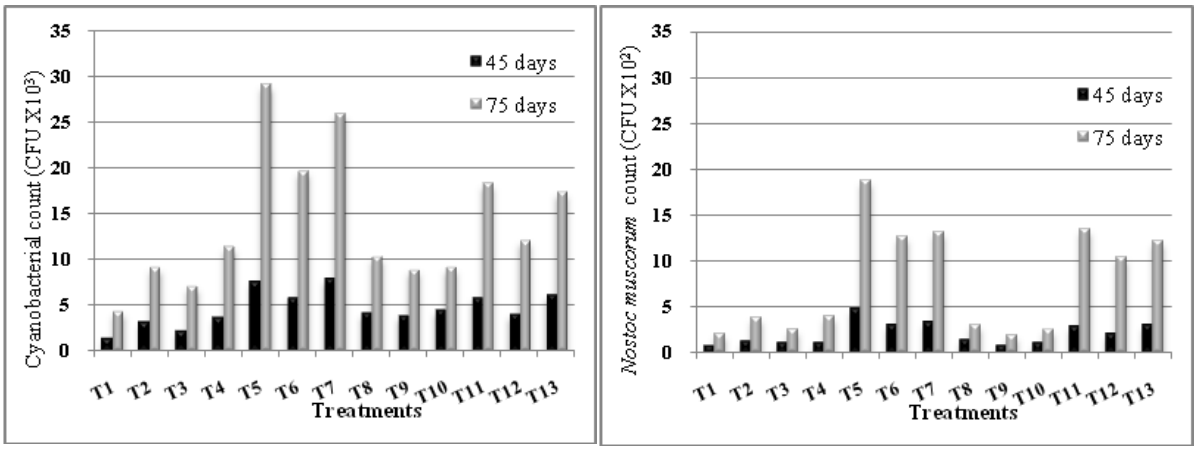


Fig. (3): Total cyanobacteria and *Nostoc muscorum* population in rhizosphere region of wheat plant inoculated with microorganisms in different formulated carriers.

As follows from our studies, inoculation of wheat plants with *A. lipoferum*, *B. polymyxa* and *N. muscorum* as plant growth promoters and N- fixers contributed to soil enrichment in these bacteria. The number of total bacterial was nearly always higher in plants inoculated with these microorganisms in rhizosphere region (Fig. 4). The highest bacterial population was attained on applying the capsulated form of the combined mixture

of the tested microorganisms (T_{11}), which recorded 68 x 10^6 and 116 x 10^6cfu/g soil along with 45 and 75 days of sowing.

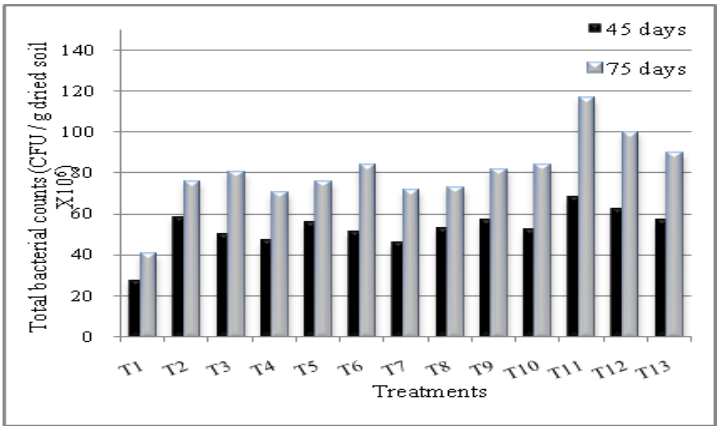


Fig. (4): Total bacterial counts in rhizosphere region of wheat plant inoculated with microorganisms in different formulated carriers.

The enzymes activity in rhizospheric area of wheat plants is governed by the activity of beneficial microorganisms that colonize the plant roots. According to the data presented in Table (1), there was a marked increase of dehydrogenase, nitrogenase and phosphatase (acid and alkaline) activities at 75 days more than at 45 days. Significant increases in the enzymes activities were attained with the plants inoculated with the microorganisms including; *A. lipofrrum*, *B. polymyxa* and *N. muscorum* in the form of capsulation more than the other carriers; talcum powder and vermiculite. The domination of the diverse beneficial microorganisms in the rhizosphere of wheat strongly correlated with the activity of the three main enzymes. Mixture of encapsulated microorganisms (T_{11}) exhibited the highest activity of dehydrogenase, nitrogenase and total phosphatase at the two time intervals, where it recorded 46.51 and 95.0 (µg TPF g dry soil^{-1} day^{-1}) with dehydrogenase enzyme, whereas it obtained 5.09 and 12.17 (µ mole C_2H_4 g dry soil^{-1} hr^{-1}) with nitrogenase at 45 and 75 days, respectively. Regarding to the total phosphatase (acid and alkaline), T_{11} still the unique one as it could establish the highest activity of this enzyme it recorded 1.88 and 2.84 mg/ g dry soil with both time intervals respectively. These results confirm the diversity of the microorganism in the rhizosphere and the role of each one without any antagonistic action or any inhibition.

Table (1): Some enzymes activities in rhizosphere area of wheat plants inoculated with microorganisms in different formulated carriers.

Treatments	Dehydrogenase activity (µg TPF g dry soil^{-1} day^{-1})		Nitrogenase activity (µ mole C_2H_4 g dry soil^{-1})		Total Phosphatase (mg g dry soil^{-1})	
	45 d	75 d	45 d	75 d	45 d	75 d
T_1	12.91^{j}	25.82^{k}	0.59^{l}	0.97^{g}	0.70^{f}	1.94^{gh}
T_2	27.57^{g}	56.15^{h}	3.06^{g}	7.66^{d}	1.40^{d}	2.31^{d}
T_3	20.51^{i}	41.02^{j}	2.22^{i}	5.55^{e}	1.10^{e}	2.06^{fg}
T_4	22.39^{h}	45.00^{i}	2.51^{h}	6.28^{e}	1.33^{d}	1.92^{h}
T_5	33.51^{e}	68.00^{f}	4.77^{b}	10.73^{b}	1.73^{b}	2.33^{d}
T_6	27.40^{g}	55.50^{h}	3.39^{f}	8.75^{c}	1.12^{e}	2.10^{ef}
T_7	30.70^{f}	61.40^{g}	3.94^{d}	9.11^{c}	1.20^{e}	2.20^{de}
T_8	38.21^{d}	78.00^{d}	1.76^{j}	4.25^{f}	1.60^{c}	2.60^{b}
T_9	35.00^{e}	67.20^{f}	1.43^{k}	3.54^{f}	1.41^{d}	2.46^{c}
T_{10}	33.60^{e}	72.00^{e}	1.52^{k}	3.71^{f}	1.44^{d}	2.21^{de}
T_{11}	46.51^{a}	95.00^{a}	5.09^{a}	12.17^{a}	1.88^{a}	2.84^{a}
T_{12}	40.10^{c}	80.20^{c}	3.77^{e}	9.33^{c}	1.43^{d}	2.60^{b}
T_{13}	43.40^{b}	87.00^{b}	4.23^{c}	10.50^{b}	1.62^{bc}	2.50^{bc}
LSD 0.05	1.43	1.68	0.10	1.04	0.11	0.13

Microbial inoculation affected the early plant growth stage of wheat, where the application of either capsulated or non- capsulated forms of *A. lipoferum, B. polymyxa* and *N. muscorum* significantly increased the shoot dry weight at 45 and 75 days compared with control treatment (T_1). As shown in Table (2), mixture of capsulated microorganisms (T_{11}) and mixture of microorganisms carried on vermiculate (T_{13}) recorded significant values of shoot dry weight in both treatments intervals compared to T_1. Where, T_{11} was unique one as it obtained the highest shoot dry weight (4.43 and 6.83 g) and number of tillers/ plant (4.3 and 4.7) at 45 and 75 days, respectively.

Table (2): Some morphological characters of wheat plants inoculated with microorganisms in different formulated carriers.

Treatments	Plant height (cm)		Number of tillers/plant		Shoot dry weight (gm)	
	45 d	75 d	45 d	75 d	45 d	75 d
T_1	65.5^{e}	100.3^{b}	3.0^{e}	3.4^{g}	3.55^{e}	6.04^{de}
T_2	64.0^{fg}	99.2^{b}	4.0^{b}	4.3^{c}	3.92^{c}	5.93^{e}
T_3	62.0^{h}	100.0^{b}	2.3^{f}	3.3^{g}	3.18^{g}	5.70f
T_4	67.3^{d}	103.2^{a}	3.0^{e}	3.2^{g}	3.49^{e}	5.74^{f}
T_5	65.1^{ef}	95.0^{c}	4.3^{a}	4.6^{ab}	3.67^{d}	6.13^{cd}
T_6	65.5^{e}	95.0^{c}	3.7^{c}	3.9^{ef}	3.17^{g}	6.01^{de}
T_7	60.3^{i}	105.3^{a}	3.3^{d}	4.0^{de}	3.30^{f}	6.25^{c}
T_8	63.0^{gh}	100.0^{b}	3.3^{d}	3.3^{g}	3.22^{fg}	5.93^{e}
T_9	70.2^{b}	95.0^{c}	3.7^{c}	3.7^{f}	2.80^{h}	5.45^{g}
T_{10}	60.5^{i}	98.5^{b}	3.0^{e}	3.3^{g}	3.16^{g}	5.74^{f}
T_{11}	68.6^{c}	92.0^{d}	4.3^{a}	4.7^{a}	4.43^{a}	6.83^{a}
T_{12}	72.3^{a}	98.2^{b}	4.0^{b}	4.2^{cd}	3.92^{c}	6.11^{cd}
T_{13}	65.3^{e}	105.4^{a}	4.1^{ab}	4.4^{bc}	4.25^{b}	6.54^{b}
LSD 0.05	1.22	2.58	0.23	0.21	0.09	0.16

In concern to yield components (Table 3), generally the mixed microbial inoculation resulted in significant increases in 1000 grain wt. and biological yield as compared to control and single inoculation treatments; except the single inoculation with capsulated *N. muscorum* (T_5). Weight of 1000 grain was improved in treatment with inoculants of combined mixture (T_{11}, T_{12} and T_{13}), which recorded 52.99, 52.00 and 52.26 gm. The highest increases of biological yield were achieved with T_{11}, followed by T_{13} and T_5, which recorded 10.2, 7.6 and 4.3 % in grain yield and 8.2, 1.9 and 1.9 % in straw yield, over the control treatment, respectively. Obviously, inoculation with mixture of combined organisms particularly when they entrapped with sodium alginate capsules achieved better growth and yield parameters comparing with the ones formulated with talcum powder or carried on vermiculite.

Table (3): Yield components of wheat plants inoculated with microorganisms in different formulated carriers.

Treatments	1000 grain wt. (g)	Seed index (%)	Grain yield (Ton/fed)	Relative Increase (%)	Straw yield (Ton/fed)	Relative Increase (%)	Harvest index (%)
T_1	47.44^{fg}	10.47^{b}	2.25^{cd}	------	4.17^{ab}	-----	35.05^{cde}
T_2	50.35^{bcd}	4.98^{ef}	2.21^{de}	- 1.7	4.05^{bc}	- 2.9	35.30^{bcd}
T_3	49.07^{def}	7.40^{d}	2.07^{f}	- 8.0	3.86^{bc}	- 7.4	34.91^{cde}
T_4	49.32^{de}	6.93^{d}	2.18^{def}	- 3.1	3.74^{c}	- 10.3	36.82^{a}
T_5	51.33^{abc}	3.13^{g}	2.35^{bc}	+ 4.4	4.25^{ab}	+ 1.9	35.60^{abcd}
T_6	50.13^{cd}	5.40^{e}	2.11^{ef}	- 6.2	3.85^{bc}	- 7.7	35.40^{bcd}
T_7	50.56^{bcd}	4.59^{f}	2.25^{cd}	-----	3.90^{bc}	- 6.5	36.59^{ab}
T_8	48.31^{efg}	8.83^{c}	2.14^{def}	- 4.9	4.17^{ab}	0.0	33.91^{e}
T_9	47.00^{g}	11.30^{a}	2.09^{f}	- 7.11	3.97^{bc}	- 4.8	34.49^{de}
T_{10}	47.65^{efg}	10.08^{b}	2.17^{def}	- 3.6	4.09^{bc}	- 1.9	34.66^{de}
T_{11}	52.99^{a}	0.00^{i}	2.48^{a}	+ 10.2	4.51^{a}	+ 8.2	35.48^{abcd}
T_{12}	52.00^{ab}	1.87^{h}	2.23^{d}	- 0.9	4.21^{ab}	+ 1.0	34.63^{de}
T_{13}	52.26^{a}	1.38^{h}	2.42^{ab}	+ 7.6	4.25^{ab}	+ 1.9	36.28^{abc}
LSD 0.05	1.58	0.53	0.10	----	0.37	----	1.22

With respect to the impact of different formulated carries of microorganisms on NPK contents of wheat grains and straw(Table, 4), it is arresting that, the mixture of capsulated bacterial strains and *N. muscorum* (T_{11}) attained optimum values of such elements. It recorded 2.30 and 0.56 % N, 1.05 and 0.34 % P and 0.5 and 1.96 % K in both wheat seeds and straw, respectively.

Table (4): Macronutrients content of wheat grains and straw inoculated with microorganisms in different formulated carriers.

Treatments	Grains			Straw		
	(%)					
	N	P	K	N	P	K
T_1	1.79^{de}	0.61^{h}	0.37^{bc}	0.67^{a}	0.31^{ab}	1.41^{g}
T_2	1.68^{ef}	0.71^{fgh}	0.42^{ab}	0.63^{ab}	0.22^{cd}	2.27^{a}
T_3	1.96^{c}	0.65^{gh}	0.43^{ab}	0.35^{f}	0.18^{de}	1.64^{ef}
T_4	1.58^{f}	$0.68f^{gh}$	0.41^{ab}	0.46^{de}	0.21^{cd}	1.86^{cd}
T_5	1.68^{ef}	0.78^{f}	0.31^{c}	0.46^{de}	0.14^{e}	1.74^{de}
T_6	2.17^{b}	0.73^{fg}	0.41^{ab}	0.39^{ef}	0.26^{bc}	1.67^{de}
T_7	1.96^{c}	0.69^{fgh}	0.44^{ab}	0.35^{f}	0.21^{cd}	1.62^{ef}
T_8	1.61^{f}	1.17^{bc}	0.47^{a}	0.49^{cd}	0.13^{e}	1.81cde
T_9	2.21^{b}	1.34^{a}	0.47^{a}	0.35^{f}	0.21^{cd}	1.77^{cde}
T_{10}	1.90^{cd}	1.24^{b}	0.44^{ab}	0.39^{ef}	0.31^{ab}	2.11^{ab}
T_{11}	2.30^{ab}	1.05^{d}	0.50^{a}	0.56^{bc}	0.34^{a}	1.96^{bc}
T_{12}	2.35^{a}	0.93^{e}	0.48^{a}	0.59^{ab}	0.29^{ab}	2.10^{ab}
T_{13}	2.18^{b}	1.12^{cd}	0.44^{ab}	0.49^{cd}	0.33^{a}	1.47^{fg}
LSD 0.05	0.12	0.09	0.08	0.08	0.063	0.18

Economic evaluation of the biofertilizers application

The most promising treatments recorded (T_{11}, T_{13} and T_5), due to the highest biological yield obtained, were evaluated economically according to our experimental conditions.

1. Cost of biofertilizers

The study shows that (Table 5) the production costs of wheat /fed increased as a result of biofertilization used to about 420 L.E. when compared with control treatment.

2. Net return of wheat/fed.

Furthermore, the study showed that the net return of wheat/fed. has increased as a result of biofertilizers application, moreover net return reached its maximum with treatment (T_{11}) which was about 7128 L.E./fed., followed by T_{13} (6648 L.E./fed.), and reached its minimum with T_5 (6452 L.E./fed.). Therefore, the best treatments were T_{11} and T_{13}, which was about 109.73%, 102.34% out of comparative control treatment.

3. The return of L.E.

The study showed that return of L.E. of wheat/fed has increased as a result of biofertilizers application, moreover, the return of L.E. reached about 1.36 L.E. /fed. with T_{11}. So, the best treatment was T_{11} which recorded about 100.74%, out with comparative to control treatment.

Assuming that wheat crop on the country level about 3.4 million fed. in 2013, and the increase in net return per fed. of wheat, about 632 L.E., so the national return are heading the use of bio-fertilizers to a 2.150 billion LE., which maximizes the yield of wheat adapter, as the increase in productivity estimated at 0.23 tons check increase at the national level achieved 782 thousand tons, which plays an important role in increasing the self-sufficiency and reduce dependence boil abroad and the provision of foreign currency rates.

Table (5): The economic evaluation of biofertilizers as a partial alternative source for mineral nitrogen fertilizer.

Cost items		Chemical fertilizer cost	Bio-fertilizer cost	Change
		(L.E.)		
Agricultural operations	Land Preparation	244	244	
	Seeding & Planting	333	333	
	Irrigation	356	356	
	Fertilization	662	554	
	Biofertilizers	0	528	
	Weeding	110	110	
	Pest Control	209	209	
	Harvesting	698	698	
	Transportation	165	165	
	Other Expenses	278	278	
Sub Total Without Rent		3055	3475	420
Rent		1753	1753	
Total Cost (L.E.)		4808	5228	420
Revenue of grain and straw yield (Ton/ fed.)				
Grains yield (control)		2.25	2.25	
Grain yield of T_5			2.35	0.10
Grain yield of T_{11}			2.48	0.23
Grain yield of T_{13}			2.42	0.17
Revenue of control (grains)		6300		
Revenue of T_5 (grains)			6580	280
Revenue of T_{11}(grains)			6944	644
Revenue of T_{13}(grains)			6776	476
Straw yield (control)			4.17	
Straw yield of T_5			4.25	0.08
Straw yield of T_{11}			4.51	0.34
Straw yield of T_{13}			4.25	0.08
Revenue of control (straw)		5004		
Revenue of T_5 (straw)			5100	96
Revenue of T_{11}(straw)			5412	408
Revenue of T_{13}(straw)			5100	96
Total Revenue (grain and straw) of control		11304		
Total Revenue of T_5			11680	376
Total Revenue ofT_{11}			12356	1052
Total Revenue ofT_{13}			11876	572
Net Returns (L.E.)				
Net returns of control		6496	0	
Net returns of T_5			6452	-44
Net returns of T_{11}			7128	632
Net returns of T_{13}			6648	152
Return of L.E.				
Return of control		1.35	0	
Return of T_5			1.23	-0.12
Return of T_{11}			1.36	0.01
Return of T_{13}			1.27	-0.08

Discussion

Several strains of a symbiotic nitrogen fixers (*Azospirillum* spp.) besides, phosphate solubilizers (*Bacillus polymyxa*) have been also proven to be efficient in plant growth promotion[26] the successful plant growth promotion by the free cell inoculum usually are restricted to gentobiotic, growth characteristics or green house studies but, in several instances this fails to yield under field conditions[27]. This is mainly due to constraints in maintaining a threshold level (10^6-10^7cfu/ml) of the initial bacterial inoculum under the heterogeneous soil conditions as to fix atmospheric nitrogen and to promote plant growth efficiently[12]**.**

The higher population densities in rhizosphere more than those in rhizoplane were attributed to the potential for biological nitrogen fixation to increase greatly by the fact that there is a close relationship between plants and nitrogen fixers' prokaryotes. Nitrogen fixers prokaryotes are able to make completely useful associations with plants from loose associations to intercellular symbiosis in which nitrogen fixing prokaryotes (e.g. *Azospirillum lipoferum, Azospirillum brasilense, Azotobacter chrococcoum and Bacillus polymyxa*) have been found in high population in rhizosphere of different plants such as sugarcane, maize, wheat, rice grasses and others[28].

The decrease of microbial population densities (*Azospirillum lipoferum, Nostoc muscorum* and *Bacillus polymyxa*) in both talcum powder and vermiculite form less than encapsulated ones as discussed in most cases the high populations densities initially established on roots then decline over time and distance from the inoculation source, and the introduced strain comprises a progressively smaller proportion of the total rhizosphere microflora[29]. Threshold population densities can be maintained by applying larger initial dose of inoculant (the covenantal methods) which is not economically feasible[12]**.** However, encapsulation enables slow and controlled release from the immobilization matrix of the alginate gel bead upon inoculation into soil and facilitates in establishing PGPRS nitrogen fixers population and the possibilities of decline over time can be minimized[29]**.**

Dehydrogenase indicates the microbial activity in soil and root surfaces. The increase of dehydrogenase activity as shown in table (1) with T_{11} (mixture of capsulated *A. lipoferum*, *B. polymyxa* and *N. muscorum*) more than other forms relied on the viability of these microorganisms and the existence in high population that could colonized the rhizosphere, which led to increase in CO_2 evolution and carbonic acids formation that decreased soil pH and consequently increases mineral absorption and enhances plant growth[30]**.**Inoculation with cyanobacteria had positively affected the soil fertility through enhancing rhizosphere soil biological activity in terms of total count bacteria, carbon dioxide evolution, dehydrogenase activity and nitrogenase activity as reported by[31]**.** The increase of the capsulated microorganisms more than formulated with talcum powder and those carried on vermiculite as the capsules protect the microbial cells and make them viable because this enzyme is an oxidoreductase, which only present in viable cells. This enzyme also has been considered as a sensitive indicator of soil quality and it has been proposed as a valid biomarker to indicate changes in total microbial activity[32]**.**

Nitrogenase enzyme catalyze the reduction of N_2 into NH_3 an evolution of H_2. *Azosprillum* bacteria could move from the capsules and successfully colonized the roots through the vast mobility ability with its flagella to penetrate the root of gramineous plant species and to grow intercellular to a degree, as well as growing in the rhizosphere[33] the increase of nitrogenase enzyme activity in the mixture of encapsulated microorganism (each group in each own capsule) was attributed to members of the nostocales could be related to the differentiation of more heterocyst that protect nitrogenase from inactivation by oxygen and thus increase their N-fixing capacity[34].[35] have also attributed the positive effect of *Nostoc muscorum* inoculation on growth of faba bean through the stimulation of other microflora by excretes a great number of substances that improve plant growth and productivity. Such *Nostoc* and cyanobacterial species can be promising candidatures for developing plant growth promoting associations for wheat plant. *A. lipoferum*, *B. polymyxa* and *N. muscorum* could produce auxins and vital enzymes involving nitrogenase, where the efficiency of this enzyme increased with increasing the efficiency of N_2- fixing bacteria[36].

The inoculation of wheat with encapsulated biofertilizers (*Azospirillum lipoferum*, *Nostoc muscorum and Bacillus polymyxa*) with 3/4 dose of mineral nitrogen fertilizer succeeded to improve the plant development and the improvement depended on the beneficial role of the used microorganisms where the increase of phosphatase (acid and alkaline) as recorded with $T_{11}$was due to the act of *Bacillus polymyxa* bacteria

in particular to produce organic acids which are considered as a solubizing agents of phosphorus compounds in soil leading to an increase of phosphorus rates in soil[37]**.**

Plant growth represented in shoot dry weight, wt. of 1000 grain, grain and straw yield enhanced by the beneficial viable microorganisms that could improve mineral nutrition mainly N, P and K[38]**,** protection against pathogens[39]. Among the free-living microorganisms plant growth promoting rhizobacteria (PGPR) included the used strains which could be exert a beneficial effect and increase of N, P and K percentages in both seeds and straw especially with T_{11} besides growth promoting sign of synergistic effects co-pored with single inoculated plants. The seed index assured the importance of inoculation with beneficial plant growth microorganisms in a mixture form, which positively reflected on the harvest index.

Conclusion

The overall conclusion has drawn from our data lead to say that the beneficial influence of inoculation with combined mixture of *Azosprillum lipoferum*, *Bacillus polymyxa* and *Nostoc muscorum* in capsule form on growth and yield of wheat under field trial. Moreover, our study carried out on the application of immobilized, mainly entrapped and capsulated, cells of microorganisms undoubtedly shows their advantages over traditional used of free cell inoculation[40]. The potential modification in formulation described here will be useful in agro-industry as there is a worldwide demand for biofertilizers to reduce input of chemical fertilizers to achieve environmental sustainability. It should be taken in consideration that our biofertilizers were made and prepared under laboratory scale, so their costs should be reduced when produced under large scale.

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