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IMPROVING RHIZOBIUM INOCULATION IN-EFFICIENCY BY CO INFECTION, GROWTH AND N, P, K UPTAKE OF COWPEA (*Vigna unguiculata* L. WALP.)

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ABSTRACT

The research aimed to study effect of mixedinoculation in promoting growth, efficiency of *Rhizobium* bacteria for purpose of infecting plants, enhancing growth, yield of cowpea plant and uptake of nitrogen, phosphorus and potassium. The idea of research was based on principle ofbenefiting from positive role of microbial interaction in the soil. The study included use of a *Rhizobium* isolate isolated from root nodules of cowpea plants (isolate R_6).

The mixed inoculation treatment of *Rhizobium*, *Pseudomonas*, and Mycorrhizae I_7 were exceeded of single and double inoculation treatments with microorganisms in increasing dry weight, number of root nodules and concentration of nitrogen and potassium in cowpea plant.

2- The necessity of inoculating cowpea with *Rhizobium* bacteria alone or in combination with *Pseudomonas* and Mycorrhizal or mixed inoculate in order to promote plant growth and the formation of root nodules.

3- The mixed inoculation treatment ($I_7 + N_3 + O_1$) did not differ significantly with treatment ($I_7 + N_2 + O_1$) in some of the studied parameters, such as dry weight, number of root nodules, nitrogen and potassium which reduced the recommendation of nitrogen fertilizer by 25 %, saving 10 kg N ha^{-1} and leading to reduce the cost production and reduced environmental pollution resulting lesse addition of fertilizer.

Keywords: Rhizobium; mycorrizae; *Pseudomonas*; co-inoculation.

INTRODUCTION

Agricultural areas around the world are decreasing by 250,000 hectares annually due to the expansion of cities and industrial areas, water scarcity and unexpected climate change, and prediction of a temperature increase of 2-4°C over the next hundred years will add a new complexity to drought and management of leguminous crops, especially in arid and semi-arid regions. Global food security as a result of the ever-increasing population depended on integrated nutrient management inputs but neglects the role of microorganisms [1,2]. As microorganisms that inhabit root growth can be of great importance because they are able to manage nutrient forms, obtain elements and nutrients and use them, and thus sustainability of crops, soil and efficiency of use of nutrients are enhanced by increase in number of microorganisms in rhizosphere through ofbetter for agricultural activities, such as plowing , nutrient management, use of organic fertilizers and bio-fertilizers [3,4,5,6].

The symbiosis of Rhizobium and legumes is a major source of bio-fixated nitrogen (ammonia) in biosphere, which leads to increased agricultural productivity while reducing dependence on nitrogenous fertilizers, and utilization of biotechnologies in agricultural fields [7,8] found that it is not possible to completely depended on local Rhizobium bacteria (native) to cause infection to roots of leguminous plants, as most soils contain a less number of local Rhizobium bacteria with high efficiency in causing infection, it is common that 1- 25% of local Rhizobium bacteria in soil are characterized by a low ability to cause infection, 50% of them are characterized by a medium capacity, and 25% of them are characterized by high efficiency in causing infection . Therefore, association of mycorrhizae and legumes is one of the beneficial relationships that can enhance plant resistance to biotic and abiotic stresses. This relationship is through which exchange of two different compounds is controlled: nitrogen (N) and carbon (C), as plant supplies microorganism with carbohydrates, which are used as a source of carbon and energy, and in return, the microorganisms stimulates N_2 fixation process by root nodules of legumes [9].

Mycorrhizae fungi were characterized by their high ability to exploit simple organic nitrogen sources, which contributes to enhancing plant growth as inoculation with mycorrhizae provides 50% of zinc, 60% of copper, 20-25% of water consumption and improves structural composition of the soil through secretion of polysaccharides, which helps adhesion of soil particles to each other, increases its ability to hold water and helps plantsف toresist drought [10] There are many types of bacteria that contribute to infecting root with mycorrhizal fungus, such as bacteria. *Pseudomonas spp.* which works to increase the speed of plant growth. These are known as Mycorrhization Helper Bacteria [11]. Types of *Pseudomonas* bacteria are distinguished by their ability to search for inorganic phosphates present within tissues or in agricultural media important in metabolic processes of this microbe. The growth of *Pseudomonas aeruginosa* was directly proportional to increase in phosphate concentration in culture medium. Many studies have shown that the results of double inoculation with biological fertilizers that dissolve phosphorous and fixed nitrogen have a positive effect on stimulating plant growth and improving nutritional status as well as increasing yield in quantity and quality, and for purpose of increasing number of Rhizobium bacteria and increasing infection rate of host plant, [12] indicated that Inoculation common legumes with Rhizobium and mycorrhiza fungi encourage formation of root nodules as well as it helps in resisting drought stress.

Dubney SK [13] found that co-inoculation with Bradyrhizobium, phosphate-dissolving bacteria, and ArbescularMycorrhizae fungi increased soybean production from 0.95 to 1.21 tons h^{-1}. [14] found that inoculating field pistachios with the BradyrhizobiumTtq strain alone or in combination with Pseudomonas (P52) at a level of 100% and 75% of the fertilizer recommendation for nitrogen and phosphorus gave the highest productivity of field pistachios of 1351 $kg.h^{-1}$ and an increase of 34.9% at level of 75% of the fertilizer recommendation for nitrogen and phosphorous, and that combined inoculation provided 25% of nitrogen and phosphorous and was superior to Rhizobium inoculation alone. This study aims to estimate effect of adding common Rhizobium inoculation with Mycorrhizal fungi and Pseudomonas bacteria and best level of nitrogen and organic fertilizer on growth and efficiency of Rhizobium bacteria in infecting host plant, formation of root nodules, growth and N, P, K uptake by cowpea plant.

MATERIALS AND METHODS

Loam clay soil was taken from Kataban area of Basra Governorate, with electrical conductivity up to 3.99 ds m^{-1}. which had not been previously cultivated with cowpea crops, The soil sample was dried, then milled and sieved to 2 mm diameter . The chemical, physical and biological properties of soilwere determined (Table 1). The trial parameters were 64 treatments resulting from the use of 8 inoculants coded I_0(Control), I_1 (RhizobumR_6), I_2 (Psedomonas), I_3 (Mycorrhizae), I_4 (Psedomonas+Mycorhizae), I_5 (Rhizobium R_6 +Mycorrhizae) I_6 (Rhizobium R_6+Psedomonas) and I_7 (Rhizobium R_6+Psedomonas + Mycorrhizae) and four levels of nitrogen and two levels of Organicsubstance with three replicates .The sterilized pots were filled topically with 5 kg of soil . Mycorrhizae treatments are inoculated by placing 100 grams of inoculant under surface of potted soil, 5 cm deep, and mixing another 100 grams of Inoculant with surface layer of soil and leaving some pots without Inoculation as a comparison .The homogeneous and superficially sterilized cowpea seeds were inoculated with *Rhizobium* (isolate R_6) and *pseudomonas*alone or with mixed [15] at a density of 0.85 * 10^8cfu.ml^{-1}. Uniquely, by soaking them in the Inoculant suspension for half an hour with the addition of Arabic gum to increase adhesion of bacteria to seeds , after which inoculated seeds were left to dry for an hour and a half away from direct sunlight [16]. nitrogen fertilizer were added in a ratio of 0, 50, 75 and 100% of fertilizer recommendation of 40 kg N h^{-1} in form of urea fertilizer as activated dose [17] organic fertilizer were added at levels 0 and 100% from fertilizer recommendation of 5 ton.h^{-1} Also, phosphate fertilizer was added in form of concentrated superphosphate (100 kg P_2O_5.h^{-1}) and potassium fertilizer in form of potassium sulfate (100 kg K_2O.h^{-1}) According to fertilizer recommendation of cowpea plant inoculated seeds were sown at 6 seed pot^{-1}, and some sterile seeds were left without inoculation as control treatment.

The pots were irrigated with RO water until field capacity and the lost moisture were compensated on basis of weight, and after germination the inoculation of pots was promoted again by adding 1 ml of bacterial inoculant with same numerical density around plant, and after 60 days of plant growth , plants were cut and dried at 70°C For 48 hours, their dry weight was recorded, plants were milled and digested, then their content of N was determined by a Kjeldal method according to [18]. Phosphorous was estimated using a spectrophotometer according to a wavelength of 700 nm, and potassium was estimated using a flam photometer [19]. Plant roots system were obtained for all treatments from soil and estimated the number of root nodules formed on root system, as well as number of active nodules of pink color, then nodules were carefully separated from roots and dried and taken their dry weight, samples of rhizosphere were taken from each treatment and the number of Rhizobium was estimated according to [20].

Table 1. Chemical, physical and microbial properties of used soil

value	subject	
7.2		pH
3.99		($ds.m^{-1}$) E.C.
20.3		($Cmol.kg^{-1}$) CEC
0.16	%	OM
22.5		$CaCo_3$
0.044		Total N
0.053		P
12.74	$Cmol.kg^{-1}$	Ca++
12.63		Mg^{++}
30.52		K^+
14.40		Na^+
29.15		$So_4^=$
6.07		Hco^{-3}
12.83		Cl^-
64.9	$g.kg^{-1}$	Sand
272.4		Silt
663.5		Clay
Clay loam		Texture
0.05 * 10^6	$cfu.gm^{-1}$	Bacteria
0.74 * 10^3		Fungi
0.20 * 10		Rhizobium

RESULTS AND DISCUSSION

The results of Table (2) indicate that single, double and mixed effects of Inoculates had a significant effect on increasing dry weight of cowpea, where highest rate was (26.96 g.pot^{-1}) due to effect of mixed Inoculate (I_7) and lowest rate (16.66)g.pot^{-1} by control without inoculation. The inoculation with Rhizobium, *Pseudomonas* and Mycorrhizae fungi aloneled to an increase in dry weight of cowpea plant as rate 25.33 , 24.51 and 23.51 g. pot^{-1} respectively compared to non-inoculated comparison treatment. The increase in plant growth parameters can also be attributed to positive interaction between Rhizosphere bacteria and Mycorrhizae fungi [21,11]. The addition of nitrogen fertilizer and organic fertilizer levels led to a significant increase in dry matter weight, as highest rate was (31.12)g . pot^{-1} when fertilizing with nitrogen level N_3 and the level of organic fertilizer O_1, while lowest rate was (18.43)g. pot^{-1} in comparison treatment without nitrogen and organic fertilizers.

The triple interaction between inoculation treatments, mineral nitrogen levels and added organic fertilizer significantly affected dry weight gain, as highest rate was (37.66)g . pot^{-1} due to effect of treatment (I_7 + N_3 + O_1), while lowest rate was (9.33) g . pot^{-1}for comparison treatment (without inoculation and without fertilization). It indicates positive effect of inoculation with micro-organisms in presence of organic matter and activated dose of mineral nitrogen (14,1). Also, the addition of organic fertilizers increases significant activity of different enzymes and thus improves the bio-enzymatic energy in the soil [20]. The results also indicate that dry weight of plant when fertilizing with levels N_0, N_1, N_2, N_3 and O_0, O_1 levels (9.33, 18.66, 20.33, 19.00, 13.66, 17.33, 17.00, 18.00 g .pot^{-1}) while that dry weight reached(20.00, 21.00, 21.33, 22.00, 25.66, 31.66, 36.33, 37.66 g. pot^{-1}) when fertilizing with previous levels and inoculating with isolate I_7 respectively, meaning that the increase in dry matter weight caused by inoculation with isolate I_7 i (10.67, 2.34, 1, 3, 12, 14.33, 19.33, 19.66 g. pot^{-1}) This means that dry weight due to inoculation with isolate I_7 is equivalent (10.29 g .pot^{-1}) without using fertilizer . So , that the inoculation with treatment (I_7+N_2+O_1) 31.66 g.pot^{-1} were not difference significantly with treatment (I_7+N_3+O_1) 37.66 gm.pot^{-1} this means that the inoculation with I_7 inoculant reduced the recommendation N fertilizer by 10kg N h^{-1} , which reduced the of production and ecosystem pollution.

Table 2. The effect of adding biological inoculation with levels of nitrogenous and organic fertilizers on the dry weight of cowpea (g.pot^{-1})

Average N*O	Biofertilizer								Organic fertilize / Nitrogen fertilizer	
	I_7	I_6	I_5	I_4	I_3	I_2	I_1	I_0		
18.43	20.00	18.66	13.00	19.33	19.00	26.00	22.12	9.33	O_0	N_0
19.08	21.00	16.33	18.00	20.66	22.33	22.66	13.00	18.66	O_1	
20.54	21.33	21.00	19.00	20	24.33	23.00	15.33	20.33	O_0	N_1
20.76	22.00	20.66	17.33	20.16	22.00	23.33	21.66	19.00	O_1	
20.16	25.66	20.33	18.00	19.66	22.33	21.66	20.00	13.66	O_0	N_2
23.99	31.66	20.33	20.00	26.33	20.00	26.33	30.00	17.33	O_1	
28.16	36.33	26.66	29.66	25.33	24.66	28.33	37.33	17.00	O_0	N_3
31.12	37.66	32.66	34.00	31.33	27.33	31.33	36.66	18.00	O_1	
	26.96	22.08	21.12	22.88	22.75	25.33	22.92	18.26	AverageI	
			I=2.262 , NO=2.262 , INO= 6.397						LSD	

Table 3. The effect of adding biological inoculation with levels of nitrogenous and organic fertilizers on the total number of nodules.pot^{-1}

Average N*O	Biofertilizer								Organic fertilizer / Nitrogen fertilizer	
	I_7	I_6	I_5	I_4	I_3	I_2	I_1	I_0		
6.24	28.66	.66	13.66	10.33	.00	.00	10.33	.00	O_0	N_0
12.03	26.66	2.66	28.33	13.00	4.66	7.66	12.66	.66	O_1	
9.62	34.00	.00	27.33	.00	.66	.00	15.00	.00	O_0	N_1
15.85	37.33	4.00	30.33	18.83	5.33	1.33	28.33	1.33	O_1	
14.03	38.00	.00	32.66	25.33	.66	.00	15.66	.00	O_0	N_2
22.95	58.00	7.00	42.66	37.00	6.00	8.00	24.00	1.00	O_1	
18.70	49.33	.00	26.33	26.66	.00	.00	23.66	.00	O_0	N_3
24.12	64.00	13.00	34.33	35.33	9.00	8.33	25.00	3.00	O_1	
	42.00	3.42	29.46	23.17	3.29	3.17	19.33	0.75	Average I	
			INO=10.732I=3.794 , NO=3.704 ,						LSD	

The results of Table (3) indicate that the single or double or mixed inoculation affected significantly on number of root nodules, as highest rate (42.00 $nodule.pot^{-1}$) when inoculated with mixed treatment (I_7) and lowest rate (0.75 $nodule.pot^{-1}$) when the non- inoculated comparison treatment (I_0) this indicates that the native Rhizobium were not able to infect the root of cowpea while using the inoculation with Rhizobium R_6 (I_1) alone or double or mixed were rise the number of nodules in other treatment this was agree with [8,12].

The addition of nitrogen fertilizer and organic fertilizer levels led to a significant increase in number of nodules, as highest rate was (24.12$nodule.pot^{-1}$) when fertilizing with nitrogen level N_3 and level of organic fertilizer O_1, while lowest rate was (6.24$nodule.pot^{-1}$) in non-fertilized comparison treatment.

The interaction between inoculation treatments and nitrogen and organic fertilizer added levels were significantly affected by increase in number of nodules. The highest rate was (64.00 nodule $.pot^{-1}$) due to effect of treatment ($I_7 + N_3 + O_1$), which did not differ significantly with treatment ($I_7 + N_2 + O_1$), while lowest rate was (0.00 nodule $.pot^{-1}$) the comparison treatment (without inoculation and without fertilization) this indicate that inoculate with I_7 treatment saved 10 kg $N.h^{-1}$.

The results of Table (4) indicate that inoculation with isolates single or double or mixed affected significantlyon concentration of nitrogen in plant, highest rate was (2.73 %) when inoculated with isolate I_7 (mixedinoculant) and lowest rate (1.733%) when compared to I0 (without inoculation).

The addition of nitrogen fertilizer and organic fertilizer levels led to a significant increase in nitrogen concentration in plant, as it reached highest rate (2.946%) when fertilizing with nitrogen level N_3 and level of organic fertilizer O_1, while lowest rate was (1.520 %) in comparison treatment without nitrogen and organic fertilizers.

The interaction between inoculation with treatments and levels of nitrogen and organic fertilizer significantly affected nitrogen concentration in plants, highest rate was (3.936 %) with effect of ($I_7 + N_3 + O_1$) treatment, while lowest rate was (1.100 %)comparison treatment (without inoculation and without fertilization), and their was not significant difference between ($I_7 + N_3 + O_1$) and($I_7+N_2+O_1$) treatment, this means that inoculation reduced the recommended nitrogen fertilizer by 25%, this result were agreement with [14]. The results also indicate that nitrogen concentration in plant when fertilizing with levels N_0, N_1, N_2, N_3 and O_0, O_1 levels is (1.100, 1.500, 1.466, 2.000, 1.433, 1.933, 2.066, 2.366%), while that concentration reached (1.966, 2.200, 2.200, 2.300, 2.466, 3.466, 3.300, 3.936) when fertilization with previous levels and inoculation with treatment I_7 respectively, meaning that increase in nitrogen concentration caused by inoculation with treatment I_7 (7.97%), and this means that nitrogen concentration due to inoculation with isolate I_7 Equivalent to adding (0.99% N pot) without using fertilizer.The result in Table (5) indicate thatthe interaction between all treatment were not significantly effect on phosphorus concentration in the plant.

The results of Table (6) indicate that inoculation with isolates single or double, or mixtures had a significant effect on potassium concentration in plant, as it reached highest rate (1,181) when inoculated with mixture treatment (I_7) and lowest rate (0.891) due to effect of treatment of single *Pseudomonas*inoculate (I_2). addition of nitrogen fertilizer and organic fertilizer levels led to a significant increase in potassium concentration in plant, as highest rate was (2.286) when fertilizing with nitrogen level N3 and level of organic fertilizer O_1, while lowest rate was (0.567) in the non-fertilized comparison treatment.

The interaction between inoculation with the isolates and different nitrogen fertilizer levels (N_0, N_1, N_2, (N_3) and organic fertilizer (O_0, O_1) added affected significantly in potassium concentration in plant, as highest rate was (2.83) due to effect of treatment ($I_1 + N_3 + O_1$), while lowest rate was (.503) for comparison treatment (without inoculation and without fertilization). The results also showed that there were no significant difference between the treatment ($I_1+N_3+O_1$) and treatment ($I_7+N_3+O_1$) this was indicated that inoculated with Rhizobium alone or mixed with other isolates were very importance to increase potassium concentration in cowpea plant.

Table 4. The effect of adding biological inoculation, levels of nitrogen fertilizer and organic fertilizer on nitrogen concentration in cowpea plant (%)

Average N*O	Biofertilizer								Organic fertilizer	Nitrogen fertilizer
	I_7	I_6	I_5	I_4	I_3	I_2	I_1	I_0		
1.520	1.966	1.633	1.633	1.500	1.366	1.300	1.666	1.100	O_0	N_0
1.713	2.200	1.566	2.333	1.613	1.466	1.600	1.433	1.500	O_1	
1.710	2.200	1.800	2.266	1.22	1.600	1.666	1.466	1.466	O_0	N_1
2.074	2.300	1.766	2.500	1.833	1.900	2.033	2.266	2.000	O_1	
2.083	2.466	1.933	2.200	2.033	2.200	2.000	2.400	1.433	O_0	N_2
2.407	3.466	2.103	2.600	2.066	2.333	1.900	2.860	1.933	O_1	
2.594	3.300	2.466	2.966	2.266	2.633	2.033	3.026	2.066	O_0	N_3
2.946	3.936	2.633	3.500	2.866	2.800	2.370	3.100	2.366	O_1	
	2.730	1.988	2.500	2.002	2.038	1.863	2.277	1.733	Average I	
	I= 0.1845 , NO= 0.1845 , INO=0.5217								LSD	

Table 5. The effect of adding biological inoculate with levels of nitrogenous and organic fertilizers on phosphorus concentration in cowpea plants (%)

Average N*O	Biofertilizer								Organic fertilizer	Nitrogen fertilizer
	I_7	I_6	I_5	I_4	I_3	I_2	I_1	I_0		
0.261	.286	.246	.276	.270	.276	.300	.220	.216	O_0	N_0
0.271	.300	.260	.320	.293	.283	.253	.220	.240	O_1	
0.273	.340	.276	.313	0.219	.313	.273	.233	.223	O_0	N_1
0.300	.123	0.500	.340	.300	.316	.290	.276	.256	O_1	
0.325	.333	.300	.310	0.501	.303	.310	.280	.263	O_0	N_2
0.329	.403	.330	.333	.296	.300	.310	.250	.280	O_1	
0.331	.433	.376	.343	.316	.316	.316	.253	.300	O_0	N_3
0.335	.446	.373	.366	.343	.276	.336	.290	.256	O_1	
	0.333	0.332	0.325	0.317	0.298	0.299	0.253	0.255	Average I	
	I= N.S. , NO=N.S. , INO= N.S.								LSD	

Table 6. The effect of adding biological inoculations, levels of nitrogen and organic fertilizers on potassium concentration in cowpea plant (%).

Average N*O	Biofertilizer								Organic fertilizer	Nitrogen fertilizer
	I_7	I_6	I_5	I_4	I_3	I_2	I_1	I_0		
0.567	.573	.570	.570	.576	.643	.566	.540	.503	O_0	N_0
0.673	.853	.636	.643	.670	.650	.610	.563	.766	O_1	
0.680	.863	.713	.650	0.510	.716	.646	.613	.733	O_0	N_1
0.797	.920	.736	.756	.663	.860	.676	.670	1.10	O_1	
0.924	.956	.893	.753	.736	1.05	.683	.963	1.36	O_0	N_2
1.039	1.27	.936	.826	.773	1.31	.72	1.05	1.43	O_1	
1.427	1.39	1.26	1.18	1.26	1.50	1.17	1.86	1.80	O_0	N_3
2.286	2.63	1.96	2.13	2.29	2.29	2.06	2.83	2.10	O_1	
	1.183	0.964	0.940	0.955	1.128	0.894	1.138	1.225	Average I	
	I= 0.1021 , NO= 0.1021 , INO= 0.2887								LSD	

CONCLUSION

We concluded from this study that the necessity of inoculating cowpea plant with Rhizobium bacteria to improve the growth, productivity, infection and increase the nodules formation. The inoculation with mixed inoculate were led to reduces the nitrogen requirement by 25% of recommended nitrogenous fertilizer.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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