نظام مبتكر لإنتاج البروتين من يرقات الذباب المنزلي عن تغذيتها على مخلفات الدواجن للاستهلاك الحيواني

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ملخص البحث. تم دراسة إمكانية إنتاج البروتين المركّز من يرقات الذباب المنزلي (*Musca demostica*) النامية على مخلفات الدواجن من أجل استخدامه كغذاء للحيوانات. ولتحقيق ذلك فقد تم تصميم نظام الإنتاج المكون وتقييمه من وحدة تعقيم مخلفات الدواجن، ووحدة إنتاج البيض، ووحدة إنتاج اليرقات. كما تم تطوير أنظمة حصد اليرقات واستخلاص البروتين. وأثبتت التجارب أن استخدام الأشعة فوق البنفسجية لتعقيم مخلفات الدواجن فعالة لمعدلات التغذية المنخفضة، بينما انخفضت فعالية نظام التعقيم بصورة جذرية عند زيادة معدل تغذية المخلفات في جهاز التعقيم. كما وُجد أن زمن الاستبقاء عامل مهم خلال عملية التعقيم. ولوحظ من التجارب أن الاختلاف في زمن فقس بيض الذباب المنزلي يؤدي إلى تداخل في مراحل الحياة المختلفة لهذه الحشرة، مما أدّى إلى ظهور يرقات في وحدة فقس البيض وعذاري في وحدة إنتاج اليرقات. كما لوحظ أن عزل اليرقات من المخلفات لم يكن سهلاً. ويقترح تصميم نظام جديد لحصد اليرقات لجمعها قبل تطورها لمرحلة الحشرة الكاملة. وأثبتت النتائج أن الذباب المنزلي ذو كفاءة عالية لتحويل المخلفات إلى بروتين، حيث تحتوي اليرقة على ٦٤٪ بروتين و ١٦٪ دهون وأحماض أمينية مهمة، ويمكن حصاد اليرقات وتجفيفها واستخدامها كمصدر للبروتين في العلائق الحيوانية.

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Impact of Inoculation with Nitrogen Fixing Bacteria on the Productivity of Legume and Non-legume Crops

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Abstract. The present work was performed to examine colonization patterns of certain diazotrophs associated with wheat and faba bean plants under aseptic conditions in a laboratory experiment, and to evaluate the beneficial effects of bio-fertilization with N_2-fixing bacteria at different rates of mineral nitrogen fertilizer on the economical leguminous faba bean and non-leguminous wheat crops in greenhouse experiments.

The combination of diazotrophic inoculation and N application resulted in highly significant plant heights of wheat. Inoculation with *Bacillus polymyxa* gave higher plants than inoculation with *Azospirillum* or *Azotobacter*. The presence of *Rhizobium leguminosarum* and co-inoculation with *Azotobacter*, *Azospirillum* or *Bacillus polymyxa* increased significantly the heights of faba plants.

Dry weights of shoots and roots of both crops increased significantly in the presence of both inoculation with symbiotic and associative N_2-fixing bacteria. Diazotrophs inoculation significantly increased the dehydrogenase and nitrogenase activity of wheat rhizosphere soils. Moreover, inoculation of faba bean with *Rhizobium* and co-inoculation with *Azotobacter*, *Azospirillum* or *Bacillus polymyxa* before sowing resulted in an increase in the activity of dehydrogenase and nitrogenase in rhizosphere soil. Grain and straw yields of wheat increased when inoculated with diazotrophs as compared with control, and inoculation by *Rhizobium* and co-inoculation significantly increased both seed and straw yields of faba bean. Extending the ability of these bacteria to fix N_2 in non-legumes such as cereals would be a useful technology for increased crop yields.

Introduction

In Saudi Arabia, as in most developing countries, the extension of desert reclamation is very important due to the increase in population and to the urgent need for more cultivated areas to meet their needs. Unfortunately, desert soils are very poor in organic matter and microflora. Therefore, biological activities are very low and cultivation of such areas need special agricultural practices.

Diazotrophs are bacteria that fix atmospheric nitrogen gas into a more usable form such as ammonia (Halbleib and Ludden, 2000). A diazotroph is an organism able to grow without external sources of fixed nitrogen. Examples of these organisms include rhizobia and Frankia (in symbiosis) and *Azospirillum*. Two of the most studied systems are those of *Klebsiella* and *Azotobacter*. These systems are used because of their genetic tractability and their fast growth (Berman-Frank *et al.*, 2003).

Considering the large amount of nitrogen added to soils by biological fixation and the important of nitrogen in plant growth, biological N_2-fixation can be considered as one of the most important processes in nature and it is as important as photosynthesis (Berman-Frank *et al.*, 2003). The beneficial effect of N_2-fixers namely *Azotobacter chroococcum*, *Azospirilhim* sp. and *Bacillus polymyxa*, in addition to Rhizobium, are related not only to their N-fixing proficiency, but also to their ability to improve soil quality and produce antibacterial and antifungal compounds as well as growth regulators (Hoflich *et al.*, 1995).

Rhizobium is a symbiotic bacterium, colonizes on roots of the specific legumes to form root nodules and fixes atmospheric nitrogen in the nodules of the host plant anaerobically from N_2 to NO_3. *Azotobacter* is a non-symbiotic or free living nitrogen bacteria which are aerobic and free living. They fix nitrogen in rhizosphere and provide it to

the plants, particularly cereals like wheat (Kavimandan, 1985). *Azospirillum* colonizes the root mass and fixes nitrogen in loose association with plants in an environment of low oxygen tension. The bacteria induce the plant roots to secrete a mucilage which creates low oxygen environment and helps to fix atmospheric nitrogen. These organisms are found in association with the roots of cereals. *Bacillus polymyxa* increased the yield of wheat, rice, chickpea, sugarcane and potato markedly by secreting organic acids that make phosphorus and other nutrients soluble and available to plant roots (Kavimandan, 1985; Hoflich, 1999; Matiru and Dakora, 2004).

Wheat (*Triticum astivum*) and faba bean (*Vicia faba*) are very important crops in most countries especially in the Arab zone, as a source of food for humans and animals, but their production are still limited and are depending on foreign countries to satisfy their needs. So increasing production is one of the major targets of the agricultural policy and can be achieved by both increasing the cultivated area and its productivity. In recent years, many attempts have been undertaken to use various nitrogen fixing bacteria for the purpose of increasing the productivity of non-leguminous. In an associative nitrogen fixing system, plants supply the diazotrophs with organic exudates, while microorganisms fix atmospheric nitrogen that is directly or indirectly transferred to the plant in a symbiotic relationship.

Research on the colonization of roots by introduced organisms has largely been reported to increase plant growth in general rhizosphere dynamics. In this respect, colonization and efficient association of *Azospirillum* and other bacterial species with non-leguminous crops could potentially provide an environment for useful biological nitrogen fixation (BNF), similar to *Rhizobium*/legume symbiosis (Wiehe and Hoflich, 1995).

The present work was performed to examine colonization patterns of certain diazotrophs associated with wheat and faba bean plants under aseptic conditions in a laboratory experiment, and to evaluate the beneficial effects of bio-fertilization with N_2-fixing bacteria at different rates of mineral nitrogen fertilizer on the economical leguminous faba bean and non-leguminous wheat crops in greenhouse experiments. In addition, the study included influence of inoculation and rate of N-fertilizer on total bacterial count, dehydrogenase and nitrogenase activities in the rhizosphere soil.

Materials and Methods

Preparation of inoculation

Inoculation with *Rhizobium leguminosarum*, *Azotobacter chroococcum, Bacillus polymyxa,* and *Azospirillum brasilense* was investigated for their ability to colonize and to stimulate the growth of wheat (*Triticum astivum*) and faba bean (*Vicia faba*) after seed inoculation with peat inoculum (10^8 cfu/g) in pot experiments using sandy soil. The activities of dehydrogenase and nitrogenase enzymes were also analyzed. Experiments were conducted in the Agricultural Experimental Station of the College of Agriculture and Veterinary Medicine, Qassim University, KSA, during winter seasons of 1428/1429 and 1429/1430 H.

Colonization was characterized as the ability of some bacterial cells to develop into a large population attached to the roots. The colonization patterns of diazotrophs associated with legume and non-legume crops were demonstrated in two major experiments, as follows:

a) The first experiment

Colonization patterns of N_2-fixing bacteria, in spermosphere model and glass tubes, on root surface under aseptic conditions in a laboratory experiment were investigated. The germinating seeds were held at the surface of the nutrient medium in germinating tubes which were closed and incubated in the dark (Amellal *et al*., 1998). Semi-solid agar (0.5%) mineral medium was prepared according to Watanabe *et al*. (1979). Strains of *Azotobacter chroococcum*, *Bacillus polymyxa*, and *Azospirillum brasilense* were used. *Azotobacter* was pre-cultured in modified Ashby's medium, and the other two strains were grown in nutrient broth liquid medium for 2 days. Cultures were then centrifuged and the sediments were dissolved in 0.8% KCl, shaken for 5 min and used as inoculums.

Seeds of wheat and faba bean were sterilized in saturated calcium hypochlorite for 2 hrs, then washed thoroughly in distilled water and immersed in 10% hydrogen peroxide for 20 min. Seeds were, then, germinated in sterilized culture dishes for 3 days at 30°C in dark conditions. One germinated seed was then sown in a semisolid agar, N-free and C-free, Watanabe medium in spermosphere tubes for wheat, and in glass test tubes for faba seeds. Root colonization was assayed by a technique that detects the color produced from the bacteria *in situ*. Using this method, bacteria reduce 2,3,5-triphenyl tetrazolium dichloride in 3-4 hrs to red colored formazan has been successfully traced (Patriquin and Döbereiner, 1978).

b) The second experiment

This experiment evaluated the beneficial effects of bio-fertilization (with N_2-fixing bacteria) at different rates of mineral nitrogen fertilizer on wheat and faba bean crops in greenhouse experiments. Pots of 30 cm diameter were filled with 9 kg of sandy soil. The main physical and chemical characteristics of the experimental soil are obtained in Table 1.

Table 1. Chemical and physical analyses of the soil

Chemical Properties		Physical Properties	
pH (2.5:1):	8.20	Fractions (%):	
ECe* (ds/m):	2.06		
Soluble cations (meq.L^{-1}):		Sand:	95.30
		Silt:	3.60
Na^+	11.00	Clay:	1.10
Ca^{2+}	4.35		
Mg^{2+}	2.50	Texture:	Sandy soil
Soluble anions (meq.L^{-1}):			
$CO_3^{2-} + HCO_3^-$	2.99		
SO_4^{2-}	11.70		
Cl^-	7.60		
$CaCO_3$	4.00%		
O.M.	**0.23%**		

* ECe = Electric conductivity of the extract.

In this part, the response of wheat to inoculation with the non-symbiotic diazotrophs, *A. chroococcum*, *B. polymyxa* and *A. brasilense* at 125, 225 and 300 kg N/ha, representing 50, 75 and 100% of recommended dose, was detected. As for faba bean, it is not usually fertilized with regular N_2 fertilization because it fixes atmospheric N_2 through root nodules, but low (50 kg N/ha) or high (100 kg N/ha) doses were applied as a starter in the presence of the same non-symbiotic and the symbiotic strain *Rhizobium leguminosarum*.

Inoculation was performed by mixing wheat and faba bean seeds with the centrifuged bacterial culture of each strain. Arabic gum (5%) was used as sticky agent, and seeds were then sown after air drying. Control treatments were treated as inoculated ones but without bacterial culture. Phosphorus, potassium and other nutrients were applied as recommended. Fifteen days after sowing, the plants were thinned to three seedlings per pot and the nitrogenous fertilization was applied as recommended by Mengel (2006).

Microbial analysis

Total microbial count, dehydrogenase and nitrogenase activities, in rhizosphere soil, were determined periodically at 30, 60 and 90 days after sowing (DAS).

The total viable bacteria in the rhizosphere (colony forming units, cfu) was counted according to the method described by Hoflich *et al.* (1995) using the nutrient agar media. Dehydrogenase activity in rhizosphere was determined using 2,3,5 triphenyl tetrazolium chloride and Nitrogenase activity in the rhizosphere was measured as described by Jagnow *et al.* (1991).

Plant growth characters

Plant height, dry weights of shoots and roots were measured at different stages of growth. Grain and straw yields were determined at harvest time.

Statistical analysis

Experiments were arranged in a completely randomized design and were analyzed by analysis of variance (ANOVA). All data were statistically analyzed according to Snedecor and Cochran (1980) with the aid of COSTAT computer program for statistics. Differences among treatments were tested with LSD at 5% level of significance.

Results and Discussion

Colonization

Within 1 or 2 hrs of immersing roots in 2,3,5 triphenyltetrazolium chloride (TTC), the roots of wheat and faba bean and teterazolium reduction were visible to the eye, and bacteria reducing tetrazolium could be observed in a red color with inoculated plants, while the un-inoculated roots were colorless. The inoculating bacteria were presumed to be attracted to roots through chemo and air tactics, and then colonized the plant roots, so that red color developed. It seems that colonization sites corresponded mainly to the areas where root mucigel was present. The area around the point of emergence of lateral roots usually showed maximum colonization (data not shown). In this respect, Hoflich (1999) found that colonization was mostly present on the root hair, and the colonization of the main root surface was also observed. The pattern of colonization was probably due to the distribution of receptor structures on the plant root surface, relating to the sites of greater root exudation. Pectinase and exopolysaccharide often play important roles in the association between the host plant and bacteria (Hoflich *et al.*, 1997).

Light microscopy showed that bacteria colonized on roots, *Azotobacter chroococcum*,

Azospirillum sp. and *Bacillus polymyxa*, were attached and adsorbed on plant roots and reduced TTC, while un-inoculated plants weekly reduced TTC. These results are in harmony with those obtained by Hoflich and Ktihn (1996). It was obvious that bacterial strains caused changes in morphology of roots, hair density, length of elongation zone, and total root surface area. In an early study, Han and New (1998) attributed these changes to the production of growth promoting substances; indole acetic acid and gibberellic acid (IAA, GA_3), and cytokinin like-substances.

Prerequisites for plant growth promotion include not only bacterial phyto-effective metabolites, but also the survival and establishment of the inoculated strains in the rhizosphere in competition with the native microbial flora. Since young plant roots are sparsely colonized by microorganisms, it is possible to establish a population of selected plant growth promoting microorganisms via inoculation. Therefore, the present work showed that after seed inoculation with inoculants, *Rhizobium* colonized the roots of gramineae (wheat) and leguminoseae (faba) when grown in pot experiments. These results are in harmony with those recorded by Hoflich *et al.* (1995). The strain was able to grow along the growing roots to the tip.

In field experiments, Wiehe and Hoflich (1995) showed that *Rhizobium* colonized the rhizosphere (3.0-6.0 log cfu/g root of legumes (pea) as well as of non-legumes (maize, wheat)). They also found that, *Rhizobium* also colonized non-inoculated spontaneous weed plants at a distance of 40 cm from the inoculated plants. The *Rhizobium* strain was able to re-establish in the rhizosphere of these plants, even after dry storage of the soil for a period up to 12 months. The highest cell numbers were detected in legumes. The microbial analysis of surface sterilized roots indicated the colonization of the tissues of the root tip and in the zone of emerging laterals of lupin, broad bean, wheat and maize (Hoflich *et al.*, 1995; Wiehe and Hoflich, 1995). With monospecific polyclonal antisera, the colonization of *Rhizobium* in the inner root tissue of non-legumes maize was detected occasionally. *Rhizobium* were detected in the cells of the root cortex as well as in intercellular spaces of central root cylinder cells (Schloter *et al.*, 1997).

Second experiment (greenhouse)

Growth parameters

The effect of inoculation with associative N_2-fixing bacteria at different rates of N-fertilization on plant heights and shoot and root dry weights of wheat and faba bean plants were presented in Tables 2 and 3. The data in Table 2 shows that variability in shoot lengths were attributed to the rate of N-fertilizer application and also to the diazotrophic inoculation. Plant heights increased linearly and significantly with increasing N application. The obtained data show some kind of integration between diazotrophic inoculation and N application since their combination resulted in highly significant plant heights as compared with the non-inoculated wheat plants. In their effects on plant height, some differences between bacterial inocula were recorded, in this respect data in Tables 2 and 3 show that *Bacillus polymyxa* gave higher plants than *Azospirillum* or *Azotobacter* inocula. Similar results were obtained by Egener *et al.* (2002) and Kataputiya *et al.* (1995) who declared that the inoculation of wheat seeds with *Azospirillum*, *Azotobacter* and *Bacillus polymyxa*, alone or in combination, increased the heights of wheat plants.

Recorded data indicate that the inoculation of faba bean plants with *Rhizobium leguminosarum* and co-inoculation with *Azotobacter chroococcum* or *Azospirillum* sp. or *Bacillus polymyxa* under half or full rate of chemical N-fertilizer increased significantly plant height compared to un-inoculated treatment. It is obvious that inoculation with *Rhizobium* alone increased plant height significantly, as compared to control. However, further increase was recorded with the co-inoculation treatments. The highest response to the co-inoculation was recorded at full rate of nitrogen fertilization. This enhancement could be attributed to the possible N_2-fixation and to the production of growth promoting substances by the nitrogen fixing bacteria.

Dry weights of shoots and roots (Tables 4 and 5) increased significantly with the presence of both inoculation and associative N_2-fixing bacteria, particularly at 90 DAS. The increased weights of shoots and roots over un-inoculated wheat plants were highly significant with all inoculate strains. However, the positive effect of *B. polymyxa* on dry weights was much more higher than that recorded for *Azospirillum* and *Azotobacter* treatments. It is clear that shoot and root dry weights responded positively to N application. The greatest effect of N was recorded with the highest rate of fertilization (300 kg N/ha). In this respect, an early study by Hoflich *et al.* (1997) showed that both the uptake of nitrogen, phosphorus, potassium in maize, and the yield of shoots and roots were significantly stimulated when plants were inculated with some strains of diazatrophs.

Table 2. Effect of N_2-fixing bacteria and N-fertilization on heights and shoot dry weights of wheat plants at different stages of growth

Bacterial inocula (A)	N rate kg/ha	Plant height (cm)(B)				Shoot dry weight (g)(B)			
		Days after sowing (DAS)							
		30	60	90	mean	30	60	90	mean
Control	150	12.5	31.4	55.8	33.2	0.52	2.67	8.66	3.95
	225	14.7	35.7	60.2	36.9	0.60	3.44	11.23	5.09
	300	18.6	40.8	62.6	40.7	0.65	3.82	12.30	5.59
	mean	15.3	35.9	59.5		0.59	3.31	10.7	
L.S.D. 5%		A = 3.7 B = 2.1 AB = 7.8				A = 1.4 B = 5.7 AB= 6.4			
Azotobacter	150	17.6	36.6	60.8	38.3	0.66	3.11	13.12	5.63
	225	19.2	40.2	65.5	41.6	0.72	3.76	15.22	6.61
	300	22.6	46.6	68.4	45.9	0.84	4.80	17.65	7.76
	mean	19.8	41.1	64.9		0.74	3.89	15.33	
L.S.D. 5%		A = 2.8 B = 12.6 AB = 18.7				A = 1.2 B = 8.8 AB = 11.6			
Bacillus	150	16.9	41.5	62.2	40.2	0.61	3.54	13.55	5.90
	225	18.8	43.3	66.7	42.9	0.78	4.45	17.88	7.70
	300	20.6	44.8	72.3	45.9	0.91	5.25	20.24	8.80
	mean	18.8	43.2	67.1		0.77	4.41	17.22	
L.S.D. 5%		A = 1.5 B = 28.9 AB = 31.2				A = 1.1 B = 4.2 AB = 4.7			
Azospirillum	150	15.6	39.4	60.2	38.4	0.63	3.32	12.76	5.57
	225	18.8	41.0	61.4	40.4	0.68	3.88	14.54	6.37
	300	21.2	42.2	66.5	43.3	0.71	4.56	16.82	7.36
	mean	18.5	40.9	62.7		0.67	3.92	14.71	
L.S.D. 5%		A = 2.1 B = 23.3 AB = 35.6				A = 1.1 B = 3.7 AB = 4.6			

A = means of treatment data, B = means of DAS data.

Table 3. Effect of N_2-fixing bacteria and N-fertilization on heights and shoot dry weights of faba bean plants at different stages of growth

Bacterial inocula(A)	N rate kg/ha	Plant height (cm)(B)				Shoot dry weight (g)(B)			
		Days after sowing (DAS)							
		30	60	90	Mean	30	60	90	Mean
Control	100	17.6	25.6	46.5	29.9	16.8	20.5	28.8	22
	150	22.2	34.7	55.6	37.5	18.3	23.2	32.5	25
	mean	19.9	30.15	51.05		17.6	21.9	30.7	
L.S.D. 5%		A = 6.6 B = 8.4 AB = 16.4				A = 1.5 B =3.5 AB = 9.5			
Rhizobium	100	30.5	55.2	80.3	55.3	20	26	38	28
	150	36.8	62.4	88.4	62.5	28	30	42	33
	Mean	33.7	58.8	84.4		24	28	40	
L.S.D. 5%		A = 2.4 B = 10.5 AB = 32.4				A = 2.2 B = 2.7 AB = 6.5			
Azotobacter + Rh	100	23.4	35.7	65.8	41.6	18	22	34	25
	150	29.2	42.2	70.5	47.3	22	26	39	29
	mean	26.3	38.9	68.2		20	24	36.5	
L.S.D. 5%		A = 4.2 B = 5.2 AB = 5.2				A = 2.5 B = 3.4 AB = 6.9			
Bacillus + Rh	100	28.8	44.6	75.5	49.6	19	26	36	27
	150	33.7	52.5	80.8	55.7	26	29	40	32
	mean	31.3	48.6	78.2		22.5	27.5	38	
L.S.D. 5%		A = 4.7 B = 8.8 AB = 11.7				A = 3.5 B = 4.2 AB = 9.7			
Azospirillum + Rh	100	30.2	40.2	70.5	50.7	16	24	36	25.3
	150	32.8	46.6	74.4	51.3	27	28	40	31.7
	mean	31.5	43.4	72.5		22	26	38	
L.S.D. 5%		A = 1.1 B = 5.3 AB = 7.6				A = 3.1 B = 3.5 AB = 7.8			

A = means of treatment data, B = means of DAS data.

Table 4. Effect of N_2-fixing bacteria and N-fertilization on root dry weights and microbial count of wheat plants at different stages of growth

Bacterial inocula[(A)]	N rate kg/ha	Root dry weight (gm)[(B)]				Total microbial count (log No cfu/g soil)[(B)]			
		Days after sowing (DAS)							
		30	60	90	mean	30	60	90	mean
Control	150	0.22	0.32	0.82	0.45	7.11	7.66	6.40	7.1
	225	0.28	0.42	1.10	0.60	7.20	7.75	7.00	7.3
	300	0.32	0.55	1.56	0.81	7.88	7.88	7.50	7.8
	mean	0.27	0.43	1.16		7.40	7.76	6.97	
L.S.D. 5%		A = 0.14 B = 0.23 AB = 0.53				A = 0.11 B = 0.54 AB = 0.75			
Azotobacter	150	0.28	0.45	1.45	0.73	7.90	8.10	7.50	7.83
	225	0.33	0.65	2.22	1.06	8.10	8.30	7.80	8.07
	300	0.40	0.75	2.34	1.16	8.75	9.00	8.00	8.58
	mean	0.34	0.62	2.00		8.25	8.47	7.76	
L.S.D. 5%		A = 0.24 B = 0.88 AB= 0.91				A = 0.21 B = 0.25 AB = 0.33			
Bacillus	150	0.33	0.60	2.65	1.19	8.20	8.40	7.60	8.1
	225	0.38	0.88	3.85	1.70	8.80	8.20	8.20	8.4
	300	0.47	1.22	4.15	1.94	9.40	9.80	9.00	9.4
	mean	0.39	0.90	3.55		8.80	8.80	8.27	
L.S.D. 5%		A = 0.31 B = 0.45 AB = 0.72				A = 0.11 B = 0.21 AB = 0.27			
Azospirillum	150	0.30	0.55	2.20	1.01	7.88	8.00	7.50	7.79
	225	0.37	0.74	2.85	1.32	8.35	8.50	8.00	8.28
	300	0.42	0.95	3.65	1.67	9.00	9.10	8.40	8.83
	mean	0.36	0.75	2.90		8.41	8.53	7.97	
L.S.D. 5%		A = 0.12 B = 0.22 AB = 0.31				A = 0.11 B = 0.13 AB = 0.21			

A = means of treatment data, B = means of DAS data.

Table 5. Effect of N_2-fixing bacteria and N-fertilization on root dry weights and microbial count of faba bean plants at different stages of growth

Bacterial inocula[(A)]	N rate kg/ha	Root dry weight (gm)[(B)]				Total microbial count (log No cfu/g soil)[(B)]			
		Days after sowing (DAS)							
		30	60	90	Mean	30	60	90	
Control	100	1.12	2.30	5.65	3.1	6.2	6.9	5.8	6.3
	150	1.48	3.50	7.22	4.1	7.3	8.2	7.2	7.6
	mean	1.3	2.9	6.4		6.8	7.6	6.5	
		A = 0.33 B = 1.1 AB = 2.5				A = 0.24 B = 0.31 AB = 0.34			
Rhizobium	100	1.76	3.55	6.45	3.9	7.2	8.8	6.8	7.6
	150	2.05	5.21	8.42	5.2	9.6	10.4	8.5	9.5
	Mean	1.9	4.4	7.4		8.4	9.6	7.7	
L.S.D. 5%		A = 1.11 B = 2.1 AB = 4.5				A = 1.2 B = 1.8 AB = 2.2			
Azotobacter + Rh	100	1.40	2.87	5.55	3.3	6.8	7.8	6.0	6.9
	150	1.65	4.12	7.22	4.3	8.2	9.4	8.0	8.5
	mean	1.5	3.5	6.4		7.5	8.6	7.0	
L.S.D. 5%		A = 0.81 B = 1.32 AB = 2.25				A = 1.1 B = 1.2 AB = 2.1			
Bacillus + Rh	100	1.6	3.42	5.87	3.6	7.6	8.2	7	7.6
	150	1.95	4.65	7.90	4.8	8.9	9.9	8	8.9
	mean	1.8	4.0	6.9		8.3	9.5	7.5	
L.S.D. 5%		A = 0.63 B = 2.22 AB = 3.65				A = 1.1 B = 1.2 AB = 1.8			
Azospirillum + Rh	100	1.55	3.26	5.33	3.4	6.9	7.4	6	6.8
	150	1.76	4.45	6.85	4.4	7.5	8.9	7	7.8
	mean	1.7	3.9	6.6		7.2	8.2	6.5	
L.S.D. 5%		A = 0.9 B = 1.4 AB = 1.9				A = 0.6 B = 0.9 AB = 1.2			

A = means of treatment data, B = means of DAS data.

Similarly, most significant increase in dry weights of faba bean shoots and roots was recorded under co-inoculation combined with N-fertilization treatments, at which dry weights of shoot and root were increased substantially over control. Similar results were obtained by Han and New (1998) who found that plants inoculated with both *Azospirllluin* and *Rhizobium* had a two-fold dry weight increases compared to those inoculated with *Rhizobium* only. They also found that a mixed inoculation of *Vicia faba* with four different *Rhizobium* + *Azospirillum* and *Rh.* + *Azotobacter* increased significantly the dry weights compared with plants inoculated with *Rhizobium* alone. They ascribed this increase in dry

weights to changes in total content and distribution of mineral macro- and micronutrients, K, P, Ca, Mg, Fe, B, Mn, Zn, and Cu. In addition, available nitrogen and growth promoting substances supplemented by the inoculating organisms may positively affect the plant growth.

Early studies by Hoflich *et al.* (1997) showed that some strains of diazatrophs stimulated the growth of legumes and non-legumes. In this respect, in greenhouse experiments, *R. leguminosarum*, promoted the shoot growth of wheat, maize, oil radish, rape and mustard about 19-33% in comparison with non-inoculated controls. In field experiments, inoculations significantly and consistently stimulated the growth of red clover, clover-grass-mixtures (7%), maize (10%), spring wheat (8%), spring barley (16%) and oil radish (21%). It is well known that *Rhizobium* strains stimulated the growth of legumes by about 10%. *Rhizobium* spp. are also able to colonize the rhizosphere of cereals (Webster *et al.*, 1997) stimulating the nitrogenase activity and/or phytohormone production of native rhizosphere microorganisms (Jagnow *et al.*, 1991) and promoting the growth of wheat (Kavimandan, 1985). Moreover, rhizobia produce chemical molecules that promote plant growth including phytohormones, riboflavin, H_2 molecules and node factors that collectively or individually affect biodiversity in the cropping system. Nod factors, for example, stimulate seed germination, promote plant growth and increase grain yields in legume and non-legume crops (Dakora and Phillips, 2002). H_2 gas, which is a byproduct of N_2 fixation by rhizobia in symbiotic legumes, is also known to stimulate biomass accumulation in soybean, wheat, barley, and canola, as well as increase tillering, head number and grain yields of field-grown wheat and barley. Additionally, rhizobia can suppress soil pathogen populations, and thus contribute to plant health in natural and agricultural ecosystems.

Microbiological aspects

Total microbial count

Data in Table 4 shows the total microbial count or colony forming units (cfu/g dry soil) in wheat rhizosphere soil increased by inoculation with diazotrophs compared with un-inoculated treatment. Both inoculation and N-fertilizer recorded the highest total microbial count (cfu) in rhizosphere area in comparison with those obtained from control or low nitrogen fertilizer. It is obvious that *B. polymyxa* caused highest increase in cfu/g soil compared to *Azospirillum brasilense* or *Azotobacter chroococcwn*. As for the rate of nitrogen fertilization, the recommended rate (300 kg/ha) gave highest increase in cfu/g soil compared with those obtained at lower doses. It is clear that maximum increase in cfu/g was recorded at 60th DAS then decreased as time passed.

Data in Table 5 show that total bacterial count in rhizosphere soil cultivated with faba bean increased significantly with *Rhizobium leguminosarum* alone or in combination with *Azotobacter chroococcum*, *Azospirillum* spp. or *Bacillus polymyxa*. However, co-inoculation with *Bacillus* gave the highest result followed by *Azospirillum* then by *Azotobacter*. The enhancement of total bacterial count may be due to increase in total microbial population which resulted from stimulation with inoculation and co-inoculation. These data are in harmony with those obtained by Halbleib and Ludden (2000) who stated that inoculation of *Phaseolus vulgaris* with *Azospirillum* increased total bacterial count in rhizosphere area.

Nodulation status

Results in Table 6 shows that *R. leguminosarum* either alone or with co-inoculation treatments and chemical N-fertilization increased significantly the number and the fresh weight of nodules, on faba roots, at 60 days after sowing, as compared to the un-inoculated treatment. Nodule numbers were almost constant with time. The greatest effect of inoculation was recorded with co-inoculation compared to *Rhizobium* alone. Co-inoculation gave a significant increase in number and the weight of nodules with respect to *Rhizobium* alone, the increases were even more when compared with the un-inoculated control plants. An important observation was that diastrophic inoculation together with half rate of N-fertilization stimulated nodulation. Further increase in N rate decreased nodulation processes.

The nodulation status was obvious at 60 DAS compared to 30 DAS. This enhancement in number and weight of nodules were ascribed to the production of plant growth promoting substances when plants were inoculated with N_2-fixing bacteria (Halbleib and Ludden, 2000) and to the presence of Nod factors that stimulate seed germination, promote plant growth and increase grain yields in legume and non-legume crops (Dakora and Phillips, 2002).

Enzymes activities

a) Dehydrogenase: Data in Table 7 shows that inoculation with diazotrophs significantly increased the dehydrogenase activity (DHA) of wheat rhizosphere soil. A gradual response in DHA of wheat rhizosphere with the increase in N-fertilizer rates was recorded. The greatest effect of N-fertilizer was recorded with the highest rate of N application. The integration of N_2-fixing bacteria with N-fertilizer

resulted in a significant increase in DHA. The highest value was obtained by *Bacillus polymyxa* compared to that resulted from *Azotobacter*, *Azospirillum* or control.

As for faba bean, data in Table 8 shows that inoculation with *Rhizobium* and co-inoculation with *Azotobacter*, *Azospirillum* or *Bacillus polymyxa* before sowing generally increased in dehydrogenase in rhizosphere soil cultivated by faba bean. These increases were in the presence of *Rhizobium* and other strains in the order of *Bacillus* > *Azospirillum* > *Azotobacter* > *Rhizobium* alone. The highest increase in DHA for wheat and faba bean plants was recorded at 60 DAS compared to 30 and 90 DAS. It is obvious that enzyme activity coincides with the number of nodules per plant.

Table 6. Effect of N_2-fixing bacteria and N-fertilization on number and fresh weight of nodules on faba bean at different stages of growth

Bacterial inocula[(A)]	N (kg/ha)	No of nodules/ plant[(B)]				F.wt of nodules g/plant[(B)]			
		Days after sowing (DAS)							
		30	60	90	mean	30	60	90	mean
Control	50	6.2	20.5	25.4	17.4	3.4	12.1	14.2	9.9
	100	5.5	17.4	19.8	14.2	3.0	10.4	11.6	8.3
	mean	5.9	19.0	22.6		3.2	11.3	12.9	
L.S.D 5%		A = 2.3 B = 3.7 AB = 7.5				A = 1.1 B = 2.7 AB = 3.8			
Rhizobium	50	10.8	45.5	50.2	35.5	4.6	28.2	30.2	21.0
	100	12.7	33.8	36.6	27.7	4.2	19.2	21.3	14.9
	mean	11.8	39.7	43.4		4.4	23.7	25.8	
L.S.D 5%		A = 4.8 B = 6.7 AB = 13.8				A = 5.5 B = 2.1 AB = 5.3			
Azotobacter + Rh.	50	13.5	30.6	33.7	25.9	4.1	18.6	21.6	14.8
	100	10.3	20.9	27.5	19.6	3.5	15.3	18.8	12.5
	mean	11.9	25.8	30.6		3.8	16.9	20.2	
L.S.D 5%		A = 2.4 B = 4.5 AB = 5.7				A = 1.2 B = 3.7 AB = 5.6			
Bacillus + Rh.	50	15.2	55.4	62.6	44.4	5.3	20.5	23.2	16.3
	100	13.2	37.6	41.4	30.7	4.5	16.6	19.9	13.7
	mean	14.2	46.5	52		4.9	18.6	21.6	
L.S.D 5%		A = 4.5 B = 14.7 AB = 28.8				A = 2.4 B = 4.3 AB = 8.3			
Azospirillum + Rh.	50	12.3	33.7	37.5	27.8	4.2	18.3	22.5	15
	100	10.8	22.5	30.5	21.3	3.8	14.2	16.8	11.6
	mean	11.6	28.1	34.0		4.0	16.3	19.7	
L.S.D 5%		A = 3.2 B = 5.6 AB = 11.4				A = 3.2 B = 4.6 AB = 9.4			

A = means of treatment data, B = means of DAS data.

Table 7. Effect of N_2-fixing bacteria and N-fertilization on nitrogenase and dehydogenase activities in wheat plants at different stages of growth

Bacterial inocula[(A)]	N rate kg/ha	Nitrogenase (mol C_2H_4/hr/g dwt)[(B)]				De hydrogenase (L H/g soil/24 hr)[(B)]			
		Days after sowing (DAS)							
		30	60	90	mean	30	60	90	
Control	150	5.75	14.45	7.48	9.22	116	206	125	149
	225	7.55	20.12	12.23	13.30	128	242	138	169
	300	4.20	15.10	6.77	8.69	156	285	140	193
	mean	5.83	16.55	8.83		133	244	134	
L.S.D 5%		A = 2.4 B = 3.5 AB = 4.2				A = 15 B = 35 AB = 46			
Azotobacter	150	28.70	37.66	18.76	28.37	325	446	258	343
	225	49.55	52.14	22.67	41.45	388	488	340	405
	300	30.33	38.50	18.43	29.09	408	534	400	447
	mean	36.19	42.77	19.95		374	489	333	
L.S.D 5%		A = 1.3 B = 4.3 AB = 6.2				A = 22 B = 34 AB = 45			
Bacillus	150	35.32	44.20	20.43	33.32	385	485	250	373
	225	59.45	75.63	45.27	60.12	412	550	350	437
	300	30.23	45.46	16.45	30.71	538	620	465	541
	mean	41.66	55.09	27.38		445	551	355	
L.S.D 5%		A = 19 B = 12 AB = 22				A = 45 B = 64 AB= 77			
Azospirillum	150	30.40	40.54	21.20	30.71	321	368	244	311
	225	51.22	62.14	32.16	48.51	395	442	280	372
	300	28.40	41.66	14.25	28.10	428	560	350	446
	mean	36.67	48.11	22.54		381	456	291	
L.S.D 5%		A = 12 B = 14 AB = 23				A = 32 B = 42 AB = 51			

A = means of treatment data, B = means of DAS data.

Table 8. Effect of N_2-fixing bacteria and N-fertilization on nitrogenase and dehydogenase activities in faba bean plants at different stages of growth

Bacterial inocula[(A)]	N rate kg/ha	Nitrogenase[(B)]				De hydrogenase[(B)]			
		Days after sowing (DAS)							
		30	60	90	mean	30	60	90	
Control	100	15	20	12	16	226	255	230	237
	150	27	32	16	25	245	345	260	283
	mean	21	26	14		236	300	245	
L.S.D 5%		A = 3	B = 4	AB = 6		A = 22	B = 28	AB = 34	
Rhizobium	100	48	64	38	50	514	623	477	538
	150	56	78	48	61	716	780	688	728
	mean	52	71	43		615	702	583	
L.S.D 5%		A = 6	B = 8	AB = 11		A = 80	B = 95	AB = 104	
Azotobacter + Rh	100	42	56	34	44	398	512	355	422
	150	50	66	45	54	587	674	460	574
	mean	46	61	40		493	593	408	
L.S.D 5%		A = 5	B = 6	AB = 8		A = 65	B = 72	AB = 86	
Bacillus + Rh	100	44	57	35	45	467	588	377	477
	150	66	70	55	64	688	712	545	648
	mean	55	64				650	461	
L.S.D 5%		A = 8	B = 9	AB = 13		A = 87	B = 94	AB = 112	
Azospirillum + Rh	100	47	59	36	47	443	549	335	442
	150	63	65	58	62	688	680	560	643
	mean	55	62	47		57	615	448	
L.S.D 5%		A = 9	B = 10	AB = 12		A = 111	B = 118	AB = 125	

A = means of treatment data, B = means of DAS data.

b) Nitrogenase: Data declared that nitrogenase (N_2-ase) activity increased in the rhizosphere soil for wheat plants inoculated with diazotrophs, *Bacillus polymyxa*, *Azospirillum brasileuse* and *Azotobacter chroococcwn* (Table 7). These results indicated that *Bacillus polymyxa* enhanced N_2-ase activity more than other varieties. A comparison between the N_2-fixers for their effect in increasing N_2-ase activity in rhizosphere of wheat plants showed the following descending order: *Bacillus polymyxa* > *Azospirillum brasilense* > *Azotobacter chroococcum*. The maximum increase in N_2-ase activity was recorded at 60 DAS then decreased with time. It is noteworthy to indicate that dehydrogenase activity is positively correlated with the total number of microorganisms in soil. The presence of appreciable amounts of combined nitrogen in soil stimulates the total microflora, including diazotrophs which became in no need to fix atmospheric nitrogen, thus N_2-ase activity is diminished. These results are in harmony with those obtained by Matiru and Dakora (2004) who found that the inoculation of wheat plants with *Bacillus polymyxa*, *Azospirillum* and *Azotobacter* increased total microbial count and enzyme activities.

As for faba bean, the obtained results showed the inoculation with *R. leguminosarum* individually or when mixed with *Azotobacter*, *Azospirillum* or *Bacillus* as co-inoculation increased N_2-ase activity of faba bean (Table 8). In addition, it is obvious that co-inoculation gave considerably higher N_2-ase activity compared with those obtained with *Rhizopium* alone. The highest N_2-ase activity levels were recorded at 60 DAS. When comparing different N_2-fixers for their effect in increasing N_2-ase activity in rhizosphere of faba bean plants, data revealed that *Rhizopium* + *Bacillus* was superior in enhancing N_2-ase activity as compared with other strains. Early investigation by Hoflich (1999) on the metabolism of the *Rhizobium* in pure culture showed no nitrogenase activity, but the activity of nitrate reductase and the production of phytohormones (cytokinin, auxin) was detected. The bacterium was also able to metabolize pectate and cellulose. It has been found that, in sterile hydroponic cultures (without N) and in pot experiments (with loamy sand), *Rhizobium* stimulated nitrogenase activity on clover but not on wheat (Table 8), and that in tubes without plants, *Rhizobium* co-inoculated with rhizosphere microorganisms significantly stimulated nitrogenase activity by about 175%, *Rhizobium* also increased the auxin content of the culture medium by about 131%.

The yield

Grain and straw yields of wheat increased by inoculation with diazotrophs when compared with control (Fig. 1). In this respect, *Bacillus polymyxa* gave higher increase compared to *Azotobacter chroococcum* or *Azospirillum brasilense*, particularly under the high level of N fertilizer. The positive effect of inoculation on growth parameters, which was ascribed to the improvement of nitrogen fixation activity in the rhizosphere zone as a result of

increasing numbers of diazotrophs, may be a reason for improving the yield. More amounts of fixed N in addition to the growth regulators (IAA and CK) produced by the bacteria, may cause an improve in plant productivity (Dakora and Phillips, 2002).

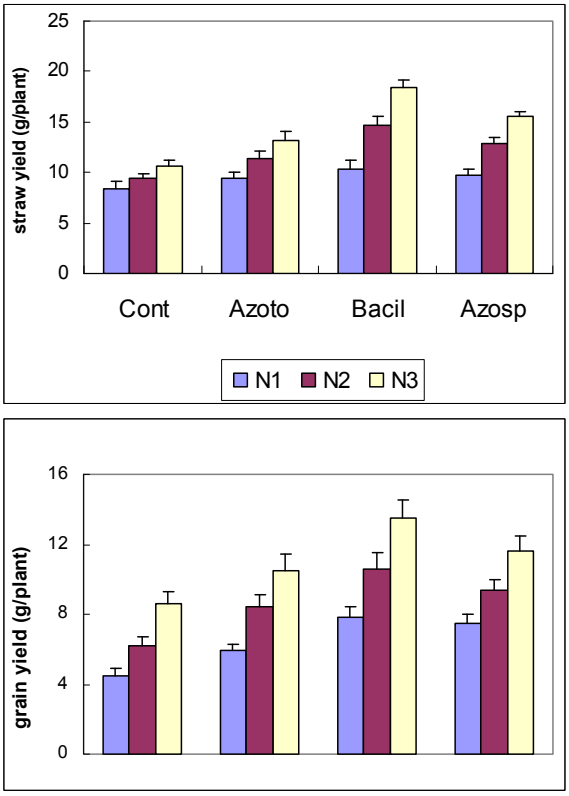

Fig. 1. Effect of diazotrophs and N fertilization on wheat yield.

Results in Fig. 2 indicated that inoculation by *Rhizobium* and co-inoculation significantly increased both seed and straw yields of faba bean compared with un-inoculated plants. The increase in either yield recorded higher values under co-inoculation treatment than that recorded under control or *Rhizobium* treatment. Moreover, there was a gradual increase in grain and straw yields of faba bean when increasing N level. This increase in yield with increasing N supply might be attributed to the increase in the dry weight of vegetative organs as a result of the expected increase in photosynthetic rates.

A comparison between effects of the different inocula on the grain and straw yields showed that *Rhizobium* + *Bacillus* was found to be the best treatment followed by *Rhizobium* + *Azospirillum* then *Rhizobium* alone. The available nitrogen, supplemented by the inoculation and the high N_2-fixation activity, assumed to be the key factor in increasing the yield of faba bean. The production of growth substances, under inoculation treatment, might also lead to this improvement in the yield. It has been reported that the increased yield under inoculation was due to increased photosynthetic rates in plants (Dakora and Phillips, 2002).

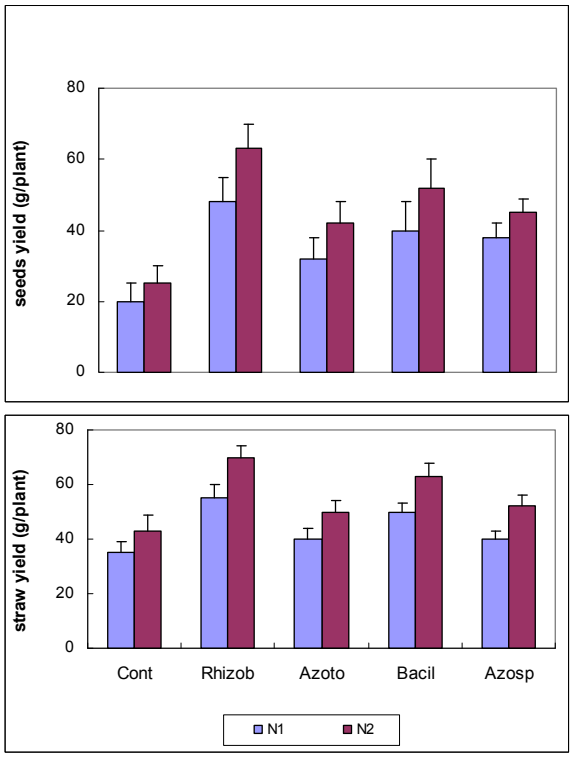

Fig. 2. Effect of diazotrophs and N fertilization on faba yield.

Conclusion

Rhizobium bacteria and co-inoculation with *Azotobacter*, *Azospirillum* or *Bacillus polymyxa* stimulated significantly the growth of legume as well as of non-legume crops. The *Rhizobium leguminosarum* colonized the rhizosphere of wheat and faba bean during plant growth. The colonization of active growing root parts [cortex ruptures of emerging laterals, root tip mucigel, root hairzone] and the production of effective metabolites is important for the root growth and the nutrient uptake of the plants. The importance of plant growth promotion by factors such as phytohormone production, N_2 fixation was obvious in the present study. Rhizobia form root nodules that fix nitrogen (N_2) in symbiotic legumes. Extending the ability of these bacteria to fix N_2 in non-legumes such as cereals would be a useful technology for increased crop yields among resource-poor farmers.

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أثر التلقيح بالبكتيريا المثبتة للنيتروجين على إنتاجية المحاصيل البقولية وغير البقولية

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كلمات مفتاحية: تثبيت النيتروجين، رايزوبيوم، بكتريا غير تكافلية، بقوليات، محاصيل حبوب، إنزيمات.

ملخص البحث. أجريت هذه الدراسة بمحطة التجارب والبحوث الزراعية التابعة لكلية الزراعة والطب البيطري بجامعة القصيم لفحص أنماط المستعمرات التي قد تتكون على جذور المحاصيل البقولية متمثلة في الفول والمحاصيل غير البقولية متمثلة في القمح وذلك عند تلقيحها ببكتريا تثبيت النيتروجين تكافلياً أو لا تكافلياً. كما تهدف الدراسة إلى التعرف على مدى التعاون الذي قد ينشأ بين البكتريا المثبتة للنيتروجين تكافلياً أو لا تكافلياً في تحسين نمو هذه المحاصيل وإنتاجيتها. وقد تمت التجارب على قسمين إحداها أجري في المعمل والآخر في البيت المحمي. وقد تم تلقيح التربة بالبكتريا المختلفة (رايزوبيوم، وباسيلس، وأزوسبيريللم، وأزوتوباكتر) تحت مستويات مختلفة من التسميد النيتروجيني المعدني في البيت المحمي. وقد أوضحت النتائج أن الرايزوبيوم كان له أثر جيد في تحسين النمو والمحصول وعندما أضيف مع البكتريا الأخرى غير التكافلية ازداد الأثر الموجب زيادة معنوية في كل من المحصولين (الفول والقمح). وقد أوضحت النتائج أيضاً أن وجود هذه الكائنات مع بعضها أدى إلى زيادة نشاط كل من إنزيمي الديهيدروجينيز والنيتروجينيز الهامين في عملية تثبيت النيتروجين في التربة وجذور النبات. وقد نتج عن التلقيح بهذه البكتريا زيادة معنوية في كل من محصول القش والحبوب في كل من المحصولين. ويتبين من هذه الدراسة أنه يمكن استخدام بعض أنواع البكتريا المثبتة للنيتروجين مع المحاصيل غير البقولية مثل محاصيل الحبوب لتحسين نموها وإنتاجيتها.