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Yield and Nitrogen Assimilation of Winter Wheat Inoculated with New Recombinant Inoculants of Rhizobacteria

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Abstract: The effects of bacterial inoculants on the growth and yield of winter wheat were studied using three strains of *Azospirillum*, four strains of *Azotobacter* and seven new recombinant transconjugants isolated from five intraspecfic hybrids as an inoculants tested. Two varieties of winter wheat were evaluated in their response to biofertilizer inoculants. Both varieties inoculated with each bacterial strain was inconsistent and varied from inoculant to another. Inoculants exhibited increases in chlorophyll synthesis in Sakha 69, also enhance the chlorophyll synthesis in Gemaza 90, whereas, Sakha 69 showed more response to most inoculants than Gemaza 90. Application of biofertilizer strains had directly and beneficial effect on chlorophyll formation, as compared with full N fertilized controls. Plants inoculated with *Herba spirillum* strain exhibited significantly increases in grain yield spike^{-1} in both of two varieties used. The variety Sakha 69 showed more response to *Azospirillum* transconjugants than Gemaza 90 in grain yield relative to the control amended with full N recommended dose. Whereas, transconjugant 6 of *Azotobacter* and *Herba spirillum* stimulated 100-grains weight in Gemaza 90. However, most inoculants enhanced both grain yield spike^{-1} and 100-grains weight of Sakha 69 in relative to N unfertilized (chemical or biological) control. Practical use of efficient rhizobacteria as inoculants for winter wheat may have limited value to overcome 50% deficiency of N below the recommended dose in this study. *Azospirillium* exhibited increases in dry matter production in Sakha 69 due to their effect on stimulating growth rate, whereas Gemaza 90 variety did not show any enhance increase in dry matter yield, as compared with Sakha 69, in relative to the control amended with N full dose. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed three major bands of activity corresponding to molecular weights of 12, 10 and 6 (KDa) in recombinant transconjugant isolates of *Azotobacter* and also two major bands of activity corresponding to molecular weights of 14 and 10 (KDa) in *Azotobacter* parental strains. Whereas, two major bands of activity corresponding to molecular weights of 10 and 7 in parental *Azospirillum* strains and also corresponding to molecular weights ranged between 6-13 in recombinant *Azospirillum* isolates. *Azospirillum* transconjugant isolates derived from the same conjugation were differed in the presence or absence from one or two bands. These differences reveal a possible biochemical differences in their efficiency to fix nitrogen free in the rhizosphere of wheat plants.

Key words: *Azotobacter, Azospirillum*, biofertilizer inoculants, molecular weight, protein pattern, transconjugant, winter wheat (*Triticum aestivum*)

Introduction

Biofertilizer are the preparations containing live or latent cells of efficient strains of nitrogen fixing used for application to seed or composting areas with the objective of increasing the numbers of such micro-organisms and accelerating those microbial processes which augment the availability of nutrients that can be easily assimilated by plants. Biofertilizers harness atmospheric nitrogen with the help of specialized micro-organisms, which may be free living in soil or symbiotic with plants. Microbial inoculants are carrier based preparations containing beneficial micro organisms in a viable state, intended for seed or soil application, designed to improve soil fertility and help plant growth by increasing the number of desired micro-organisms in plant rhizosphere. *Azotobacter* and *Azospirillum* are aerobic, nitrogen fixers and non-symbiotic bacteria, used for cereals , millets, and vegetables, in contrast with *Rhizobium* a symbiotic bacteria used as inoculants for legumes. Thus, soil microorganisms play an important role in improving crop yield and they could replace the application of chemical fertilizers (Margaret *et al*., 1964). These microorganisms are playing an important role in mobilizing P for plants by bringing about changes in rhizospheric soil pH and also by producing chelating substance, which lead to solubilization of phosphates (Kim *et al*., 1997).

P-solubilizing rhizobacteria have a capability to produce plant-growth-regulating substances of the auxin type leading to contribute their stimulating effects on the plant growth (Leinhos and Bergmann, 1995). The phytohormone indole-3 acetic acid affects root elongation and lateral-root formation (Pilet and Saugy, 1987). The effects of inoculating cereals and grasses with various bacteria are well documented (Kloepper *et al*., 1988). Plant growth and yields are usually affected as a result of such inoculations. For example, significant increases in plant dry matter and/or nitrogen accumulation by wheat inoculated with various *Azospirillum* spp. have been reported (Christiansen and Van Veen, 1991). Smith *et al*. (1977) reported that inoculation of field grown guinea grass (*Panicum maximum*) and pearl millet (*Pennisetum americanum* with *Azospirillum brasilense*) increased plant dry matter by 25%, however, no significant increase in the nitrogenase activity (N_2 fixation) was observed. In constant, Rennie *et al*. (1983) reported enhanced N_2 fixation in several spring wheat varieties inoculated with *A. brasilense*. Other rhizobacteria, including *Klebsiella pneumoniae, Enterobacter cloacae* and *Erwinia herbicola* have been isolated from winter wheat rhizospheres. Nitrogen fixation in the root segments, estimated by the acetylene reduction assay, was 2.5 g of N ha day^{-1} (Pedersen *et al*., 1978). *Azospirillum* is receiving much attention in view of its nitrogen fixing property and enhancing mineral uptake associated with a large number of grasses and grain crops (Rai, 1988a,b). The possible effects of inoculation with *Azospirillum* on plant growth can be explained by its effect on root development. Inoculation has been shown to increase the total root surface under laboratory conditions (Sumner, 1990), thereby increasing potential mineral and water uptake by the plants (Sarig *et al*., 1988). In addition, *Azospirillum* has been shown to increase nitrogen fixation in the rhizosphere, thus enhancing the overall environment conducive to plant growth. In many cases, *Azospirillum* inoculation promoted growth and crop yield of agronomically important forage, grain grasses and legumes (Sumner, 1990). The bacteria may also affect plant growth by increasing nitrogen uptake in low-nitrogen soils and more importantly by producing plant growth substances such as indole-3-acetic acid (IAA) (Fallik *et al*., 1988). In many field experiments, it has been demonstrated that *Azospirillum* inoculants do not cause either environmental hazard (Fages, 1992) or any health problems in the plants (Okon, 1985). The objective of this study was to induce new recombinant isolates of *Azotobacter* and *Azospirillum* through transconjugational process to investigate their stimulating effects on plant growth and yield of winter wheat and also to reduce N chemical fertilizers of wheat.

Materials and Methods

Plant material: Two varieties of winter wheat (*Triticum aestivum* L.) were used in this study, i.e. Sakha 69 and Gemaza 90, which kindly provided from Field Crop Research Institute, Agriculture Research Center, Giza, Egypt.

Bacterial inoculation: Bacterial strains and their transconjugants used in this study are presented in Tables 1 and 2. Wild type strains were kindly supplied from Water, Soil and Environmental Research Institute, Giza, Egypt.

All strains of *Azospirillum* and their transconjugants were grown in LGI medium contained K_2HPO_4, 0.065 g; $K_2H_2PO_4$, 0.095; $MgSO_4$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.2 g; malic acid, 5.0 g; yeast extract, 0.05 g; $NaMoO_4$, 0.002 g; $MnSO_4$, 0.001 g; H_3BO_3, 0.0014 g; $CuSO_4$, 0.004 g; $ZnSO_4$, 0.0021 g; Fe EDTA, 4.0 ml (1.64% w/v); $NaOH_3$, 3.0 g; bromothymol blue, 2 ml (0.05% alcoholic solution); distilled water, 1.0 and pH was maintained by 0.1 N HCl before autoclaving whereas, Fe EDTA was separately sterilized and added after sterilization of the medium (Rai, 1988b).

Antibiotic susceptibility assays: Antibiotic susceptibility was measured by plate diffusion method, according to Collins and Lyne (1985) with cultures grown to logarithmic growth phase in nutrient agar medium of each microbe. Bacterial suspension (1.0 ml) was mixed with 15 ml of

Table 1: Bacterial strains used in this study

Strains	Relevant characteristics	Designation
Azotobacter bijerenkii ATCC 130	Epamox^r Strep^s Ospan^s tetra^r	St_1
Azotobacter vinelandii SMR 230	Epamox^r Strep^s	St_2
Azotobacter chroococcum ARRL-B.14346	Epamox^s Strep^r Ospan^r tetra^s	St_3
Azotobacter chroococcum ARC RU 22	Neomycin^r tetra^s	St_4
Azospirillum brasilense B-14647	Epimox^r Neobiotic^s	St_8
Azospirillum lipoferum 265	Epimox^s Neobiotic^r Amoxyllin^s	St_9
Herba spirillum SMR 422	Epimox^r Neobiotic^s Amoxyllin^r	St_{10}

r - recessive s = successive

Table 2: Bacterial transconjucgation process and their transconjugants used in this study

Strains	Relevant characteristics of derived transconjugants	Designation
St_1 Epamox^r strep^s ospan^s tetra^r x St_3 Epamox^s strep^r ospan^r tetra^s	Epamox^r strep^r ospan^r tetra^r	Tr_5
St_1 Neomycin^s tetra^r x St_4 Neomycin^r tetra^s	Neomycin^r tetra^r	Tr_6
Str_2 Epamox^r streptomycin^s x Str_3 Epamox^s streptomycin^r	Epamox^r streptomycin^r	Tr_7
St_8 (Epimox^r Neobiotic^s) x	Epimox^r Neobiotic^r	Tr_{11}
St_9 (Epimox^s Neobiotic^r)		Tr_{12}
Str_9 (Epimox^s Neobiotic^r Amoxyllin^s) x St_{10} (Epimox^r Neobiotic^s Amoxyllin^r)	Epimox^r Neobiotic^r Amoxyllin^r	Tr_{13} Tr_{14}

nutrient agar medium in petri/dishes. Wells (8 mm diameter) were punched in the agar using stainless steel borer, and were filled with 0.1 ml of the antibiotic concentration. The plates were incubated overnight at 28°C and the diameter of resulting Zones of inhibition was measured (Toda *et al*., 1989). Different antibiotics were used with the concentration of 400 μg ml^{-1}, according to Roth and Sonti (1989), they were the product of Hoechst Orient S.A.E., Cairo, Egypt.

Conjugation procedure: Nutrient broth cultures, in the late-exponential growth phase were used. Quantitative spot mating of conjugal transfer was carried out, according to Lessel *et al*. (1993) by inoculating 10 μl samples of the donor cultures onto the surface of selective medium, previously seeded with 100 μl of the recipient culture. A single colony of transconjugants was picked up and transferred to slant nutrient agar medium.

IAA-detection with Salkowski colorimetric technique: *Azospirillum* and *Azotobacter* cells were grown overnight in nutrient broth at 30°C. Production of IAA in the culture supernatant was assayed by using the PC method, as described by Pilet and Chollet (1970). Salkowski-based colorimetric technique (Glickmann and Dessaux, 1995). For the reaction, 1 ml of reagent R_1, consisting of 12 g $FeCl_3$ l^{-1} in 7.9 M H_2SO_4 was added to 1 ml of the sample supernatant, mixed well and left in the dark for 30 min at room temperature. Absorbance was measured at 530 nm. IAA concentration was calculated from a standard curve using different concentrations ranged from 2 to 20 μg ml^{-1} of 3-indolylacetic acid as a standard.

Experimental design: The experiment was conducted in the Experimental and Agricultural Research Station, Faculty of Agriculture, Mansoura University, during the year winter of 2001/2002 in a randomized complete block design with three replications. Soil was amended with the equivalent of 17 kg P ha^{-1} as super phosphate and ammonium sulfate, equivalent to 35 kg N ha^{-1}. Sixteen treatments were imposed, two of them did not inoculate with biofertilzer strains as follows: (a) fertilized only with recommended dose of super phosphate (17 kg P ha^{-1}), (b) fertilized with recommended full dose of N as ammonium sulphate equivalent to 78.54 kg N ha^{-1} with super phosphate (17 kg P ha^{-1}); all other treatments inoculated with biofertilizer strains + super phosphate (17 kg P ha^{-1}) + 50% of N recommended dose (39.27 kg N ha^{-1}). This N rate was chosen based on 50% of fertilizer recommendations, except for control which amended with 78.54 kg N ha^{-1}, for growing stimulation water wheat in this soil. The other 50% of N recommended dose depending on the efficiency of biofertilizer strains to reduce the addition rate of chemical fertilizer to cereals. After addition of fertilizer, the soil was moistened with distilled water and maintained at 22% (v v^{-1}) moisture (-0.33 Mpa) through the experiment (De Freitas, 2000).

Bacterial inoculation and plant growth: *Azospirillum* strains and their transconjugants were grown in nutrient broth (LGI medium). *Azotobacter* strains and their transconjugants were grown in *Azotobacter* basal medium. Strains of *Azotobacter* were growing aerobically at 30°C in Burk's sucrose medium as described previously by Santos and Flores (1995).

Cultures (125 ml) were grown in 250 ml Erlenmeyer flasks on a rotatory shaker (120 rpm) for three days at 28°C. Winter wheat plants after one week of planted seeds were inoculated with bacterial suspension (5 ml pot^{-1}) for four times, one week intervals between one time and each other. As determined by plate counting techniques, the procedure yielded 10^7-10^8 colony-forming units ml^{-1}. Plants were thinned and considering plant uniformity to three plants per pot. Three replicates (each containing 3 plants) were harvested at 58 days (end of vernalization period-corresponding to stage 5 of Feeks scale (Large, 1954) and 170 days (maturity).

Harvest and N analysis: Plants were harvested from the field experiment and root fragments recovered by passing

the soil through a 5 mm sieve. Plant dry matter and photosynthetic activity (Mackinney, 1941) were determined at 45 days after sowing. Shoots (leaves plus stems) and seeds were dried at 65°C in a forced air oven for 72 h, ground and analyzed for total-N according to Bremner and Mulvaney (1982). The uninoculated plants were divided in two controls, the first inoculated with super phosphate alone and the second inoculated with super phosphate and the full dose of ammonium sulfate. N-content and grain yield were recorded after maturity. Nitrogen content in dried plant materials was determined by the wet digestion at dried and finely pulverized plant material using microkjeldahl method (Jackson, 1958). Samples of 0.20 g dry material were digested by sulphoric and perchloric acids. Distillation was carried out with 40% NaOH and ammonia was received in 4% boric acid solution. The distillates were then titrated with 0.041 N HCl using the mixed methyl red-bromocresol green indicator. Nitrogen concentration was determined according to Burris and Wilson (1957).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): This technique was carried out according to Bilal *et al.* (1990). Parental strains and their recombinant isolates were grown to stationary phase in 5 ml of nutrient broth at 30°C with shaking. Cultures were centrifuged at 1000g for 10 to 15 min. The cell pellet was processed for total soluble protein profile analysis by employing the method described by Shivikular *et al.* (1986). Samples were subjected to dimensional SDS-PAGE using 10% (w/v) resolving and 4.5% (w/v) stacking gels. A 10 μl sample containing 50 μg of protein was loaded per well on the gels. After electrophoresis, the gels were stained overnight in 25% (v/v) ethanol and 5% (v/v) glacial acetic acid containing 0.025% (w/v) Coomassie Brilliant Blue R-250, destained for 2 to 3 h in 40% (v/v) absolute ethanol and then 5% (v/v) glacial acetic acid. The gels were left in methanol-glycerol-water (55:2:43 by volume) for 12 to 24 h. Coomassie Brilliant Blue stained protein patterns in the gels were photographed using a Fortepan 200 black and white film.

Statistical analysis: Data were subjected statistically to analysis of variance according to Steel and Torrie (1960). Least significant difference (LSD) was calculated to compare between treatment means other than the control.

Results and Discussion

Chlorophyll formation: In this study, inoculation of winter-wheat with different strains of *Azotobacter, Azospirillum* and their transconjugants influenced chlorophyll concentration (Table 3). Different strains (St_3, St_9 and Tr_{11}) had a significant stimulatory effect of total chlorophyll in both varieties of winter wheat used in this study, in relative to chemical N fertilizer control. Some other strains enhanced chlorophyll formation in one variety than the other. However, plant responses to the various inoculants were not consistent from one variety to another. Thus are due to plant and/or bacterial genotype might be the controlling factor in an inoculation response. The results obtained here are in agreement with Sharma *et al.* (2001), who reported that vigorously growing nitrogen fertilized plants were able to absorb a large quantity of mineral nutrients through their well developed root system and also increased the synthesis of photosynthates. Application of biofertilizer strains and their transconjugants had directly beneficial effect on chlorophyll formation. The highest chlorophyll content in leaves was achieved in variety Gemaza 90 rather than Sakha 69. The possible reason may be that nitrogen is involved in chlorophyll synthesis. The better availability of nutrients through increased absorption surface may have brought this significant increase in most of yield contributing characters and finally it is being reflected in grain yield.

The results obtained in this study are in harmony with Sharma (2001), who reported that inoculation with *Bradyrhizobium* culture exhibited significant differences in photosynthetic efficiency at all growth stages of mungbean (*Vigna radiata*). The same author also reported that this inoculation might have increased the nitrogen concentration which ultimately led to improved growth and photosynthetic surface and finally increased the crop growth and relative growth rate. The use of efficient biofertilizer strains are very useful to use in Egyptian new soils with poor fertility to improve plant growth in these soils. Because winter wheat are very needed to nitrogen and phosphorus which are indispensable element for growth, development, metabolism uptake of nutrients, photosynthesis and ultimately yield of any crop. It is generally believed that winter wheat and other cereals can meet their nitrogen requirement by application of chemical fertilizer or free nitrogen fixing strains to get addition nitrogen requirements from *Azotobacter* and *Azospirillum* strains, which fixed nitrogen in loose association with plants in an environment of low oxygen tension.

Biological yield and total-N: Dry matter production showed significant differences between the treatments (Table 4). S_4 strain of *Azotobacter*, S_{10} strain of *Azospirillum* and also all different transconjugants of *Azospirillum* showed significantly influence to increase dry matter production in Sakha 69 variety over the plants

Table 3: Effect of biofertilizer on chlorophyll synthesis in winter wheat inoculated with rhizobacteria

	Sakha 69			Gemaza 90		
Treatments	Chl. A	Chl. B	Total Chl.	Chl. A	Chl. B	Total Chl.
A	1.13	3.54	4.67	0.80	4.18	4.98
B	0.83	4.09	4.92	1.34	4.19	5.54
St_1	1.14	4.58	5.72	1.27	5.27	6.54
St_2	1.09	5.72	6.81	1.11	5.10	6.22
St_3	1.08	5.63	6.71	1.58	6.64	8.22
St_4	1.13	3.54	4.67	0.51	6.59	6.83
Tr_5	1.20	5.32	6.52	1.41	5.69	7.10
Tr_6	1.59	4.58	6.17	0.62	7.95	8.27
Tr_7	1.22	5.45	6.67	1.33	4.73	6.06
St_8	1.16	4.58	5.74	1.03	5.06	6.09
St_9	2.76	4.14	6.89	1.90	8.62	10.52
St_{10}	1.12	4.75	5.86	1.06	4.82	5.88
Tr_{11}	2.64	4.41	7.06	0.59	6.99	7.58
Tr_{12}	2.81	2.88	5.69	2.02	3.80	5.82
Tr_{13}	2.34	4.42	6.76	0.73	6.16	6.90
Tr_{14}	2.72	4.00	6.72	1.60	4.74	6.51
L.S.D 0.05	2.15	3.09	1.51	0.72	2.21	1.62
0.01	2.88	4.15	2.03	0.96	2.97	2.20

Chl. = Chlorophyll

Table 4: Total nitrogen and dry matter production at 170 DAP (maturity) of winter wheat inoculated with rhizobacteria

	Sakha 69					Gemaza 90				
Treatments	Dry weight (g) 3 plants^{-1}	N%	Protein content %	Total N mg plant^{-1}	N_2 uptake	Dry weight (g) 3 plants^{-1}	N %	Protein content %	Total N mg plant^{-1}	N_2 uptake
A	6.2	0.41	2.84	2.52	25.22	6.17	0.46	2.86	2.83	28.28
B	8.73	0.52	3.27	4.57	45.69	14.33	0.51	3.20	7.44	73.35
St_1	11.08	0.58	3.59	6.33	63.26	16.83	0.56	3.47	9.36	63.57
St_2	13.77	0.48	3.00	6.31	63.09	13.47	0.42	2.61	5.62	56.22
St_3	11.93	0.53	3.47	4.22	42.23	13.00	0.42	2.61	5.43	54.01
St_4	14.80	0.49	3.09	7.32	40.88	9.83	0.59	3.69	5.81	58.06
Tr_5	13.17	0.60	3.73	7.86	78.62	10.80	0.41	2.54	4.40	43.52
Tr_6	7.53	0.43	2.83	3.42	34.15	17.80	0.58	3.64	10.39	103.87
Tr_7	9.63	0.46	2.90	4.47	44.75	10.87	0.55	3.43	5.96	59.58
St_8	9.77	0.54	3.37	5.27	52.70	9.27	0.52	3.24	4.82	48.18
St_9	10.63	0.52	3.26	5.54	55.44	9.83	0.49	3.09	4.86	63.61
St_{10}	19.63	0.63	2.91	12.29	92.90	9.13	0.44	2.75	4.02	40.19
Tr_{11}	21.37	0.49	3.03	10.37	103.67	9.20	0.46	2.85	3.06	30.60
Tr_{12}	16.60	0.57	3.59	9.54	95.39	10.50	0.50	3.10	5.22	52.18
Tr_{13}	14.20	0.45	2.81	6.40	63.97	11.37	0.62	3.84	6.99	69.93
r_{14}	16.27	0.55	3.46	9.01	90.07	12.33	0.53	3.10	2.28	22.82
F test	**	NS	NS	**	**	*	NS	NS	**	*
L.S.D. 0.05	5.41			3.59	53.75	4.60			2.74	36.51
0.01	7.29			4.95	74.18	6.21			3.79	50.40

*, ** P<0.05 and P<0.01 respectively NS = Non-significant,

fertilized with N full dose. This are due to their effects on stimulating growth rate led to improved plant growth. These results indicated that these isolates are superior than other strains and it was be recommended to use with Sakha 69 variety, as a highly sensitive host to these biofertilizer strains. The formation of dry matter production did not show any significant increase by any of biofertilizer strains used with Gemaza 90, in relative to the control applied with the full dose of recommended N. In fact, it has been reported that *Azotobacter, Azospirillum* and their interaspecific hybrids are able to affect wheat crop growth through mechanism such as provision of plant growth regulators (Tien *et al*., 1979). Plant growth hormones may be supplied by both bacteria and/or roots as a reaction to bacterial infection at the root interface (Kapulnik *et al*., 1985). In many cases, the shoot and root growth altering effects induced by these rhizobacteria were comparable to those produced by

additions of indole-3-acetic acid and gibberellic acid (Kucey, 1988). It is apparent from this study that inoculation with rhizobacteria may enhance the early vegetative growth of winter wheat. However, plant response to these bacteria was inconsistent, as shown here from the different responses of different varieties of wheat crop.

Bacterial enhancement plant growth is exhibited by only a few cultivars (De Freitas, 2000). Similarly, the application of efficient biofertilizer strains affect significantly on dry matter production caused an increase in plant growth surface, than others failed to significantly affect this trait. Thus, biofertilizers are taken a great attention in the last few decades because they are environment friendly and low cost or almost nonmonetary input, which can play a significant role in plant nutrition. Following they are used in plant production and they are available at almost all the agricultural colleges, universities, Agriculture Research Centers in a packets with specific quantity that is 250 g per packet.

Total N (mg plant^{-1}) in plant tissue was significantly higher in Sakha 96 variety as affected by some *Azospirillum* strains and their transconjugants (St_{10}, Tr_{11}, Tr_{12} and Tr_{14}) in relative to control plants fertilized with full N recommended dose from ammonium sulphate. However, significant increases in the total N contents (mg plant^{-1}) of the plants over unfertilized control plants were observed when the inoculant was *Azotobacter* strains (*A. bijerenkii* ATCC132, *A. vinelandii* SMR230 and *A. chrococcum* ARC Ru.22) and their transconjugants (Tr_5). Recombinant isolate of *Azospirillum* (Tr_{11}) significantly increases N_2 uptake over control plants fertilized with N full dose in Sakha 69 variety, whereas some other recombinant isolates of *Azospirillum* and also *Herba spirillum* SMR 422 strain increases N_2 uptake over unfertilized control plants.

In Gemmaza 90 variety of winter wheat, a recombinant isolate of *Azotobacter* (Tr_6) significantly greater total N (mg plant^{-1}) over control plants fertilized with N full dose. *Azotobacter* strains (St_1, St_2 and St_4), some of their recombinant isolates (Tr_7) and some of *Azospirillum* recombinants (Tr_{13}) affect to significantly greater total N (mg plant^{-1}) in plant tissue over unfertilized control plants. Recombinant isolates of *Azotobacter* (Tr_6) and *Azospirillum* (Tr_{13}) still affect to significantly increase N_2 uptake, in similar to their significantly affect on total N (mg plant^{-1}). This indicated that different genotypes of winter wheat showed different responses to inoculation with the same genotype of microbial inoculants in the same environmental conditions. However, inoculation treatments had no significant effect on the percentage of nitrogen and protein content among two genotypes of winter wheat. Thus, as suggested by Rennie *et al.* (1983), plant and/or bacterial genotype might be the controlling

Table 5: Grain yield at 170 DAP (maturity) of winter wheat inoculated with rhizobacteria

	Sakha 69		Gemaza 90	
Treatments	Grain yield/5 spikes	100-grain weight	Grain yield/5 spikes	100-grain weight
A	7.3	3.75	7.11	3.75
B	10.64	4.51	15.30	5.04
St_1	13.21	4.17	18.05	5.21
St_2	7.62	4.39	18.32	5.29
St_3	7.88	4.61	13.34	5.39
St_4	9.73	4.13	16.09	4.68
Tr_5	8.15	4.17	16.94	4.27
Tr_6	18.30	5.23	13.48	5.63
Tr_7	15.55	5.52	16.13	3.97
St_8	13.06	5.49	17.91	4.80
St_9	12.09	4.15	20.13	4.71
St_{10}	15.05	4.53	25.51	5.72
Tr_{11}	12.33	4.96	13.57	4.40
Tr_{12}	17.68	5.19	16.38	5.05
Tr_{13}	16.59	4.79	19.62	5.47
Tr_{14}	18.67	5.21	16.69	5.44
L.S.D. 0.05	1.30	0.52	9.17	0.44
0.01	1.73	0.69	12.37	0.61

factor in an inoculation response. The results obtained here are in agreement with Boddey *et al.* (1986), who reported that spring wheat inoculated with different *Azospirillum* species and grown under semi-tropical conditions, presented increases in N concentration during the reproductive stages of plant growth. This study suggested that rhizobacteria inoculants may be capable to enhance uptake of nutrients e.g., N-fertilizer and grain yield. In fact, it has been reported that *Azotobacter* and *Azospirillum* are able to affect crop growth through mechanisms such as provision of plant growth regulators (Tien *et al*., 1979). Plant growth hormones may be supplied by both bacteria and/or roots as a reaction to bacterial infection at the root interface (Kapulnik *et al*., 1985). In many cases, the shoot and root growth-altering effects induced by these rhizobacteria were comparable to those produced by additions of indole-3-acetic acid and gibberellic acid (Kucey, 1988). Use of these rhizobacteria for winter wheat may have limited value until such time as we better understand factors which influence rhizosphere competence of bacterial inoculants. The improved of total N contents in water wheat shoots through biological free nitrogen fixation in the rhizosphere will improve the content of protein leading to improve productivity of cattle and buffalo from meat and milk to avoid the poor quality of protein in the shoots of winter wheat.

Grain yield: Significant increases in grain yield (g 5 spikes^{-1}) of the plants were observed (Table 5) in Sakha 69 variety by some inoculant strains and some transconjugants of Azotobacter. A significantly greater of grain yield in two varieties of winter wheat was observed when the inoculant was *Herba spirillum* strain. Conversely, the plants inoculated with all *Azospirillum* strains and their transconjugants showed significant increase of grain yield in relative to the control amended with full recommended dose of N. Whereas, both transconjugant 6 of *Azotobacter* and *Herba spirillum* stimulated 100-grains weight of Gemaza 90 variety. In addition, transconjugants 6, 7 of *Azotobacter, Azospirillum brasilense* B-14647 and transconjugants `12, 14 of *Azospirillum* affect to significantly increase 100-grains weight in Sakha 69 variety, in relation to control amended with N full dose. This indicated that transconjugants 12 and 14 of *Azospirillum* indicating plasmids from *Azospirillum lipoferum* could stimulate grain yield/spike and 100-grains weight in Sakha 69 variety.

In this study, inoculation of winter wheat with fourteen different rhizobacteria influenced spike grain yields, tended to exhibit a higher yield response than plants fertilized with N recommended full dose. However, plant responses to the various inoculants were not consistent from one stage to another. Bacterial enhancement of plant growth is exhibited by only a few cultivars. For example Millet *et al*. (1984) reported that when 20 different spring wheat genotypes were inoculated with *A. brasilense*, only two cultivars (exhibiting yield increases of up to 8.0%) responded to inoculation. Similarly, Rennie and Thomas (1987) observed that of 10 different cultivars of spring wheat inoculated with *B. polymyxa* C-11-25 and *A. brasilense* ATCC 29729, only one cultivar (Cadet, inoculated with *B. polymyxa* C-11-25) showed consistent plant yield responses due to inoculation. Thus, as suggested by Rennie and Larson (1979), Baldani *et al.* (1983) and Rennie *et al*. (1983) plant and/or bacterial genotype might be the controlling factor in an inoculation response.

The obtained results indicated that grain yield response to these bacteria was inconsistent. This may reflect a problem with inoculant formation or delivery to the seed, or a failure of the strains to adequately colonize roots. However, most inoculation treatments significantly increases both grain yield/spike and 100-grains weight in relative to treatment A (unfertilized with N fertilizer such biological or chemical). In fact, it has been reported that rhizobacteria are able to enhance the yield of winter wheat even with the absence of N fertilizer. Some biofertilizer strains could overcome 50% deficiency in N below recommended dose, these isolates may be a more efficient inoculant than others. Vigorously growing nitrogen chemical or biofertilizer plants were able to absorb a large quantity of mineral nutrients through their well develop root system. Application of nitrogen such chemical or biological had directly beneficial effect on 100-grains weight and grain yield. These results are in accordance with the results of Sarker and Banik (1991).

Application of rhizobacteria had marked influence on vegetative traits of Gemaza 90 (Table 6). These traits affected markedly in Gemaza 90 including chlorophyll A, B, total chlorophyll and plant dry weight. Similar marked effect was shown in Sakha 69 concerning plant dry weight, shoot N content and grain yield/spike. Application of rhizobacteria increased accumulation of dry matter and shoot nitrogen content in both varieties of winter wheat. This indicated that rhizobacteria improved growth rate of both cultivars, also showed a significant influence on physiological parameters in Gemaza 90 other than Sakha 69, and also significantly influence on yield parameter of Sakha 69 other than Gemaza 90. Inoculation with rhizobacteria culture might have increased the nitrogen concentration, which ultimately led to improved growth and photosynthetic surface as shown in Gemaza 90 and also improved growth and finally increased grain yield/spike in Sakha 69. The positive effect of Rhizobacteria inoculation in increasing about mentioned growth and physiological traits is in accordance with the findings of Thakur and Panwar (1997). The results

Table 6: Mean squares of the analysis of variance of biological traits in winter wheat inoculated with rhizobacteria

Traits	Sakha 69	Gemaza 90
Chlorophyll. A	1.62NS	0.632**
Chlorophyll. B	1.84NS	5.76**
Total chlorophyll	1.49NS	5.39**
Plant dry weight	53.81**	26.94**
Shoot N content	14.30**	9.71**
Nitrogen (%)	0.0076NS	0.0086NS
Protein content of shoots	0.190NS	0.332NS
Nitrogen uptake	2272.88**	782.53*
Grain yield/spike	8.54**	1.51NS
100-grain weight	0.37NS	1.45NS

*, **P< 0.05 and P< 0.01 respectively
NS = Non-Significant

obtained herein are in agreement with Venkateswarlu and Ran (1983), who found that inoculation of pearl millet with *A. Brazilians* resulted significantly increase in growth and dry matter both under sterilized and unsterilized conditions and they also reported that nitrogenase activity of the roots was very low and did not support the increase in plant growth due to inoculation. Baldani *et al.* (1987) also found that inoculation of wheat plants with *Azospirillum* spp. resulted in significant increase in grain yield and total nitrogen yield. The results obtained in this study stress the importance of factors other than nitrogen fixation (other factors like the phytohormones secreted by *Azospirillum* contribute to the beneficial responses in wheat) for the observed inoculation responses seen plant growth of both varieties. The significant effect in the nitrogen yield of inoculated wheat found in this study without increasing the concentration of applied nitrogen over than 50% of recommended dose, suggested the importance of factors other than nitrogen fixation in the N-assimilation of *Azospirillum*-inoculated wheat (Baldani *et al.*, 1987).

It was concluded that nitrogen fixation, the growth stimulating effects of bacteria seen in dry matter production affected significantly by inoculation were also possibly responsible for the positive impacts of *Azotobacter* and *Azospirillum* inoculation of wheat. Application of biofertilizer as shown in this study may have led to improve physiological traits and yields of winter wheat, because the dry matter productivity and grains yields recorded significantly affected with the application of rhizobacteria. The increase in the yields of crops could be due to the enhanced moisture content by Rhizobacteria (Narender *et al.*, 2001.

Indole acetic acid (IAA) production: The quantitative estimation of IAA from culture filtrates of different *Azotobacter* and *Azospirillum* strains (Table 7) revealed the largest amounts of IAA using tryptophane precursor were from *Azotobacter* mid-parents of St_1 x St_3 in relation to all other mid-parents. From IAA produced using tryptophane precursor, *Azospirillum* strains caused the highest mid-parents rather than *Azotobacter* strains. In case of lactic acid and ethanol precursors, *Azotobacter* strains caused higher mid-parents in IAA production rather than *Azospirillum* strains. These observations confirm the results of Tien *et al.* (1979), who have detected 3 or 4 types of indoles including IAA and indole lactic acid besides gibberellins and cytokinins in the culture filtrates of *A. Brazilians* and demonstrated that these compounds cause similar effects on the plant growth as that of pure auxins and gibberellins. Since inoculation of roots with optimal concentrations of *Azospirillum* enhances root proliferation, consisting mainly of increased lateral root and root hair formation (Kapulnik *et al.*, 1985), the production of plant growth substances by *Azospirillum* has often been proposed as one of the key factors responsible for the observed plant growth promotion (Dobbelaere *et al.*, 1999). In this study, the mid-parents of *Azotobacter* strains produced largest amounts of IAA in relative to *Azospirillum* mid-parents showed a broad range of largest indoleacetic acid (IAA) biosynthetic intermediates using tryptophan (Trp), indoleacetic acid and indoleethanol. In contrast, mid-parents of *Azospirillum* strains produced largest amounts of indole tryptone in relative to mid-parents of *Azotobacter* strains. Together, these results confirm the important role of IAA produced by *Azotobacter* and *Azospirillum* strains and their recombinant transconjugants in altering growth morphology and illustrate the power of combining genetic tools and bioassays to elucidate the mechanism of a beneficial *Azospirillum*-plant interaction. Since three types of plant growth substances could be detected in the supernatant of *Azospirillum* cultures: auxins (Crozier *et al.*, 1988), cytokinins (Cacciari *et al.*, 1989) and gibberellins (Bottini *et al.*, 1989).

Although all *Azotobacter* transconjugants (Table 8) exhibited more indole ethanol biosynthesis than their mid-parents, as well as, the isolate of Tr_7 achieved significant production of indole lactic acid, in relative to other transconjugants of *Azotobacter* and other indolic compounds. All *Azospirillum* transconjugants has been shown to produce largest amounts of indole tryptone in relative to their mid-parents. Some transconjugants of *Azospirillum* revealed significant production of indole lactic acid and indole ethanol other than their mid-parents. The marked increase over the mid-parents in indole tryptone, indole lactic acid and indole ethanol biosynthesis can be obtained by the *Azospirillum* recombinant Tr_{11}. There is an evidence that phytohormones produced by bacteria in the rhizosphere can increase plant growth and improve the yields (Barea and Brown, 1974). In this study, strains which produced more indoles were found to be more effective in

Table 7: Mid-parents of IAA production (μg ml^{-1}) by bacterial strains

Transconjugants	Source or reference	Indolic compounds			
		Tryptophane	Tryptone	Lactic acid	Ethanol
Tr_5	St_1 x St_3	29.58	6.93	0.68	1.18
Tr_6	St_1 x St_4	19.13	7.22	0.44	0.94
Tr_7	St_2 x St_3	18.36	7.62	0.76	1.15
Tr_{11}	St_8 x St_9	19.77	40.91	0.36	0.69
Tr_{12}	St_8 x St_9	19.77	40.91	0.36	0.69
Tr_{13}	St_9 x St_{10}	11.36	43.01	0.28	0.50
Tr_{14}	St_9 x St_{10}	11.36	43.01	0.28	0.50

Table 8: Production of IAA by *Azotobacter, Azospirillum* strains and their recombinant transconjugants

Strains	Tryptophane	Tryptone	Lactic acid	Ethanol
St_1	30.20	6.39	0.570	0.89
St_2	7.77	7.77	0.733	0.83
St_3	28.95	7.46	0.780	1.47
St_4	8.05	8.05	0.317	0.99
Tr_5	17.73	8.36	0.630	1.71
Tr_6	3.02	7.74	0.337	2.58
Tr_7	6.53	7.79	1.033	2.19
St_8	22.05	37.86	0.450	0.54
St_9	17.49	43.95	0.264	0.84
St_{10}	5.22	42.07	0.299	0.16
Tr_{11}	24.07	70.90	0.589	1.18
Tr_{12}	19.88	46.26	1.160	0.73
Tr_{13}	4.26	54.38	0.125	1.13
Tr_{14}	11.93	45.65	0.357	0.95
F test	**	**	**	**
L.S.D. (0.05)	7.60	5.10	0.173	0.49
(0.01)	10.25	8.09	0.233	0.66

** = $P<0.01$

enhancing plant growth, chlorophyll content, grain yield/spike and 100-grains weight. These results are in agreement with Venkateswarlu and Rao (1983), who found that *Azospirillum* which lives on or in the roots can continuously release the phytohormones affecting the plant growth by inducing the proliferation of lateral roots. The marked increase shown in this study in biological biomass and grain yield particularly suggested the role of growth hormones produced in the rhizosphere by these rhizobacteria. Interestingly, striking differences among strains of *Azotobacter* and *Azospirillum* were noticed with regard to their effectiveness. The increased biological biomass affected by rhizobacteria might have resulted in a higher nutrient uptake and a better growth than N_2-fixation by the bacteria. Together, this evidence points towards an important role of indole biosynthesis in the observed plant growth promotion upon inoculation. In addition, IAA biosynthesis in the phytostimulatory effect observed upon recombinant transconjugants of *Azotobacter* and *Azospirillum* inoculation over their mid-parents, are in agreement with the results reported by Dobbelaere *et al.* (1999), who found that *Azospirillum brasilense* Sp245 (pFAJ055) strain, containing an extra copy of the *ipdc* gene was expected to exert an enhanced stimulatory effect on wheat root morphology compared to the wild type. The lack of such an enhancement can be explained in two ways. First of all, the increase in IAA production resulting from an extra copy of the *ipdc* gene is too low to produce any visible effect on root morphology. On the other hand, since *ipdc* expression is strictly regulated in *A. brasilense* (Vande Broek *et al.*, 1999) and since the introduced copy of the *ipdc* gene still contains its own promoter and upstream regulatory sequences, this strict regulation might overrule the effect of adding extra copies of *ipdc*. The same authors hypothesized that the *orf*, located immediately

Table 9: Detection in SDS-polyacrylamide gels of the proteins produced by rhizobacteria strains and their transconjugant recombinant isolates

		Number of bands											
		1	2	3	4	5	6	7	8	9	10	11	12
St_1	Relative flow (Front)	0.133	0.152	0.187	0.246	0.354	0.389	0.464	0.496	0.550	0.667	0.0	0.0
	Molecular weight (KDa)	251.0	206.0	141.0	99.0	59.0	44.0	29.0	24.0	18.0	9.0	0.0	0.0
	Band density (OD)	51.4	48.7	35.04	18.5	29.7	36.9	39.5	39.04	39.4	73.7	0.0	0.0
	Average of peak area (%)	0.83	0.78	0.96	0.58	1.08	1.15	1.61	1.10	2.01	4.92	0.0	0.0
St_2	Relative flow (Front)	0.130	0.151	0.202	0.242	0.328	0.360	0.447	0.495	0.563	0.598	0.663	0.0
	Molecular weight (KDa)	260.0	208.0	120.0	100.0	70.0	56.0	32.0	24.0	17.0	14.0	10.0	0.0
	Band density (OD)	26.9	26.1	19.5	9.1	15.8	15.8	18.8	17.4	13.4	22.1	36.1	0.0
	Average of peak area (%)	0.99	1.17	1.17	0.93	0.58	0.73	0.88	1.04	0.41	0.67	1.09	0.0
St_3	Relative flow (Front)	0.095	0.121	0.153	0.184	0.235	0.344	0.374	0.444	0.502	0.549	0.595	0.663
	Molecular weight (KDa)	378.0	288.0	203.0	147.0	103.0	65.0	50.0	32.0	24.0	18.0	14.0	10.0
	Band density (OD)	38.3	35.8	29.9	25.8	18.3	15.3	20.4	24.1	27.7	23.5	28.5	39.8
	Average of peak area (%)	1.14	0.87	1.38	1.58	0.66	0.47	0.61	0.61	0.84	0.59	0.68	0.99
St_4	Relative flow (Front)	0.162	0.190	0.246	0.339	0.355	0.445	0.483	0.559	0.587	0.668	0.0	0.0
	Molecular weight (KDa)	184.0	137.0	99.0	67.0	59.0	32.0	26.0	17.0	15.0	9.0	0.0	0.0
	Band density (OD)	59.1	48.0	27.1	32.5	33.1	39.2	43.3	43.5	52.0	75.9	0.0	0.0
	Average of peak area (%)	2.82	0.88	0.48	0.59	0.48	0.44	0.48	0.91	0.57	16.4	0.0	0.0
Tr_5	Relative flow (Front)	0.148	0.190	0.237	0.325	0.360	0.434	0.483	0.555	0.0	0.0	0.0	0.0
	Molecular weight (KDa)	214.0	137.0	103.0	71.0	56.0	34.0	26.0	18.0	0.0	0.0	0.0	0.0
	Band density (OD)	61.4	51.8	24.6	43.5	36.2	44.8	44.2	39.3	0.0	0.0	0.0	0.0
	Average of peak area (%)	0.64	1.14	0.51	2.05	0.40	0.81	0.65	0.58	0.0	0.0	0.0	0.0
Tr_6	Relative flow (Front)	0.151	0.239	0.188	0.327	0.364	0.452	0.485	0.571	0.0	0.0	0.0	0.0
	Molecular weight (KDa)	209.0	102.0	140.0	70.0	54.0	31.0	26.0	16.0	0.0	0.0	0.0	0.0
	Band density (OD)	57.0	26.9	52.5	36.8	31.6	38.0	40.8	40.4	0.0	0.0	0.0	0.0
	Average of peak area (%)	1.03	0.50	4.99	0.83	0.49	1.0	0.92	1.14	0.0	0.0	0.0	0.0
Tr_7	Relative flow (Front)	0.123	0.148	0.190	0.237	0.329	0.381	0.455	0.503	0.564	0.594	0.668	0.0
	Molecular weight (KDa)	281.0	214.0	137.0	103.0	69.0	47.0	31.0	23.0	17.0	14.0	9.0	0.0
	Band density (OD)	48.0	37.3	31.7	18.2	23.9	29.5	35.4	35.7	24.4	36.0	72.2	0.0
	Average of peak area (%)	1.5	0.67	0.73	0.53	0.52	0.68	0.97	0.82	0.42	0.81	1.96	0.0

O.D. = Optical density of band.

Table 9: Continued

		Number of bands											
		1	2	3	4	5	6	7	8	9	10	11	12
St_8	Relative flow (Front)	0.118	0.160	0.197	0.238	0.336	0.384	0.438	0.491	0.539	0.574	0.650	0.729
	Molecular weight (KDa)	297.0	190.0	128.0	102.0	67.0	45.0	34.0	25.0	19.0	16.0	10.0	7.0
	Band density (OD)	48.9	41.8	34.0	18.7	24.3	26.6	30.9	28.7	23.7	24.8	63.8	63.2
	Average of peak area (%)	0.93	1.53	3.57	0.77	0.69	0.71	0.97	0.95	0.45	0.55	7.14	2.09
St_9	Relative flow (Front)	0.120	0.162	0.199	0.234	0.329	0.373	0.44	0.495	0.569	0.667	0.0	0.0
	Molecular weight (KDa)	289.0	185.0	124.0	104.0	70.0	50.0	32.0	24.0	16.0	9.0	0.0	0.0
	Band density (OD)	43.08	40.0	34.6	20.3	20.2	22.2	32.2	31.8	31.3	66.0	0.0	0.0
	Average of peak area (%)	1.03	1.23	3.50	0.85	0.54	0.51	0.77	0.77	1.35	2.49	0.0	0.0
St_{10}	Relative flow (Front)	0.132	0.164	0.182	0.233	0.312	0.365	0.464	0.515	0.554	0.614	0.654	0.0
	Molecular weight (KDa)	257.0	181.0	149.0	104.0	75.0	54.0	29.0	22.0	18.0	13.0	10.0	0.0
	Band density (OD)	26.3	24.4	20.5	14.7	17.2	14.7	22.3	19.7	15.3	26.4	39.4	0.0
	Average of peak area (%)	0.88	2.21	0.71	1.10	0.70	0.51	1.38	0.95	0.43	0.73	1.96	0.0
Tr_{11}	Relative flow (Front)	0.097	0.139	0.199	0.250	0.354	0.394	0.449	0.500	0.546	0.623	0.650	0.736
	Molecular weight (KDa)	371.0	237.0	124.0	97.0	59.0	43.0	32.0	24.0	18.0	12.0	10.0	6.0
	Band density (OD)	17.6	20.7	15.6	8.2	8.5	12.1	13.8	11.8	10.8	17.3	21.5	24.5
	Average of peak area (%)	1.21	1.31	1.90	0.76	0.50	0.68	0.78	0.81	0.75	1.21	1.72	2.3
Tr_{12}	Relative flow (Front)	0.118	0.151	0.183	0.251	0.343	0.383	0.469	0.513	0.578	0.615	0.664	0.0
	Molecular weight (KDa)	296.0	209.0	147.0	97.0	65.0	46.0	28.0	22.0	15.0	13.0	10.0	0.0
	Band density (OD)	52.7	42.7	34.2	13.4	18.4	23.6	32.8	28.8	28.2	35.1	43.0	0.0
	Average of peak area (%)	0.85	1.10	1.00	0.40	0.40	0.62	1.23	0.93	0.87	1.62	1.83	0.0
Tr_{13}	Relative flow (Front)	0.123	0.164	0.190	0.262	0.343	0.377	0.468	0.495	0.532	0.567	0.609	0.674
	Molecular weight (KDa)	282.0	181.0	137.0	93.0	66.0	48.0	29.0	24.0	20.0	16.0	13.0	9.0
	Band density (OD)	58.4	44.7	41.2	19.5	28.8	31.0	41.7	43.3	37.4	30.1	41.6	59.4
	Average of peak area (%)	1.30	1.06	1.30	0.82	0.76	1.86	1.21	1.23	0.89	0.43	1.60	1.69
Tr_{14}	Relative flow (Front)	0.144	0.167	0.204	0.251	0.336	0.385	0.448	0.517	0.580	0.617	0.675	0.0
	Molecular weight (KDa)	225.0	175.0	118.0	15.3	67.0	45.0	32.0	22.0	15.0	12.0	9.0	0.0
	Band density (OD)	37.7	33.6	28.8	205.0	21.1	27.4	36.3	32.7	26.4	33.9	45.1	0.0
	Average of peak area (%)	2.78	2.98	4.03	0.77	0.80	0.99	2.66	1.63	0.63	1.57	1.36	0.0
Tr_{15}	Relative flow (Front)	0.153	0.206	0.252	0.343	0.387	0.468	0.500	0.558	0.609	0.678	0.0	0.0
	Molecular weight (KDa)	205.0	116.0	97.0	66.0	45.0	29.0	24.0	17.0	13.0	9.0	0.0	0.0
	Band density (OD)	49.6	40.1	14.3	20.6	26.10	32.8	27.2	21.7	31.2	49.5	0.0	0.0
	Average of peak area (%)	1.56	1.70	0.59	0.75	1.23	1.40	1.15	0.99	1.31	1.21	0.0	0.0

downstream of the *ipdc* gene, probably as an operon might also be involved in IAA biosynthesis via the IPYA pathway, but this *orf* gene plays no or only a minor role in IAA biosynthesis (Dobbelaere *et al*., 1999).

Differential detection of rhizobacteria proteins after SDS-polyacrylamide gel electrophoresis: Protein pattern technique of rhizobacteria strains and their tramnsconjugant recombinants after SDS polyacrylamide gel electrophoresis detected genetic differential between parental strains and their recombinants (Table 9, Fig. 1). Physiological genetic characterization of these strains and their recombinant demonstrated that all *Azotobacters strains* (St_1, St_2, St_3 and St_4) and their recombinants (Tr_5, Tr_6 and Tr_7) indicated common bands numbered from one to eight, whereas , they differed slightly from bands number 9, 10, 11 and 12. Bands numbered 9 and 10 were present in all *Azotobacter* strains, whereas number 11 was present in *Azotobacter vinelandii* SMR 230 and also number 11 and 12 were present in *Azotobacter chrococcum* NRRL strain. In addition, only recombinant *Azotobacter* isolate (Tr_7), indicate the bands number 9, 10 and 11 than other recombinants, resembled closely

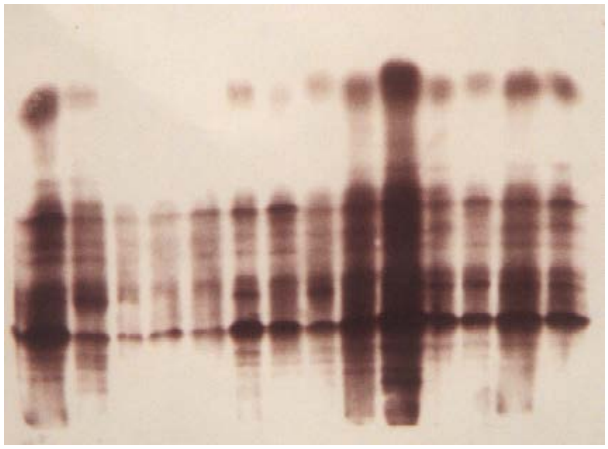

Fig. 1: SDS-PAGE pattern of total soluble proteins of different rhizobacteria strains and their recombinant transconjugants numberd one to 14 from right to left

related to their parental strains *A. vinelandii* and *A. chrococcum*. *Azotobacter* strains and their recombinants were differed slightly in their molecular weight proteins might reveal a possible biochemical differences. *Azotobacter chrococcum* NRRL B-14346 indicated higher molecular weight proteins and maximum number of bands than other strains and recombinants. In contrast, *Azospirillum* strains and their recombinants indicating common bands numbered from one to ten, but they were differed slightly in the bands numbered 11 and 12. Two recombinant isolates isolated from the same cross were also differed slightly in the presence or absence of the band number 11 and 12. *A. brasilense* B-14647 represent the maximum number of bands (12 band), whereas *A. lipoferum* 265 represent the common bands (10 band) only, although *Herba spirillum* SMR422 indicate 11 bands. The differences obtained between *Azospirillum* strains and their recombinants might reveal a possible biochemical differences in their efficiency of fixed nitrogen in loose association with wheat plants. Relative flow (front), band optical density (O.D.) and average of peak area (%) were differed from strain to another and from recombinant to another, even isolated from the same cross. In addition, this further suggest that the common bands present in *Azotobacter* and *Azospirillum* strains are 10 number of bands, whereas the common number of bands in recombinant isolates of *Azotobacter* and *Azospirillum* were 8 and 10, respectively. This indicated that recombinant isolates of *Azotobacter* and *Azospirillum* were differed in three and two number of bands, respectively. Whole cell protein pattern comparison by appropriate choice of separation method can discriminate microorganisms at the genus, species or strain levels required (Jackman, 1988). Sundaram *et al*. (1988) have characterized various *Azospirillum* isolates using a one-dimensional SDS-PAGE method. The obtained results were in agreement with those reported by Bilal *et al*. (1990), who found that comparison with *Azospirillum lipoferum, A. brasilense* and *A. amazonense* strains depending on SDS-PAGE for total soluble bacterial proteins showed that their strains including the genera *Azospirillum, Herbaspirillum, Acetobacter* and *Azotobacter* resembled closely *A. brasilense* strain, whereas these differed slightly from *A. brasilense* strain Sp-7 in their lower molecular weight proteins and from *A. amazonense* in several low and higher molecular weight proteins. SDS-polyacrylamide gel electrophoresis in small slab gels provides a rapid and high resolution method for analyzing a complex mixture of rhizobacterial proteins. The detection technique is based on the ability of rhizoproteins diffusing form bands in polyacrylamide gel. These manipulation allow the rapid assignment of molecular weight to each component of bacterial protein complex.

In conclusion, biofertilizers are environment friendly and low cost which can play a significant role in plant nutrition. It is apparent that inoculation with rhizobacteria may enhance the early vegetative growth of winter wheat.

However, plant response to these bacteria was inconsistent as shown in this study, resulting from different plant genotypes response to the same and different strains of *Azotobacter* and *Azospirilla* giving different patterns of colonization, which may have different consequences for the N_2-fixing ability of the association. The use of rhizobacteria inoculation appears to be much cheaper than fertilization based on a comparison of the cost of its application in agricultural crops such as winter wheat, corn, sorghum, and sunflower as compared with NP fertilization (Okon, 1985). Inoculation has been shown to have no hazardous effects on the environment or the plants (Fages, 1992), making it an ecological sound alternative to fertilization.

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