Comparison of the effectiveness of wheat roots colonization by *Azotobacter chroococcum* and *Pantoea agglomerans* using serological techniques

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ABSTRACT

Colonization of *Azotobacter chroococcum* Mac 27 strain on wheat roots was monitored using double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA) and immuno-fluorescence in terms of colonization sites, migration and survival of the bacteria. Furthermore, the effectivity of inoculation of *A. chroococcum* and *Pantoea agglomerans* D5/23 strain on wheat plant parameters under greenhouse conditions was investigated. Studies with DAS ELISA revealed that root tip had significant titre of inoculants as compared to the basal root parts. *A. chroococcum* colonized roots as well as soil and also migrated along with roots. An overall increase in plant growth was observed with inoculation of these bacteria. Both *A. chroococcum* and *P. agglomerans* were found to increase plant dry matter, nitrogen and phosphate uptake.

Keywords: *Azotobacter chroococcum*; *Pantoea agglomerans*; root colonization; serological testing; wheat; N and P uptake

Root colonization is one of the most important steps in the interaction of bacteria and host plants. In the past two decades, various groups of bacteria like diazotrophs, plant growth promoting rhizobacteria (PGPR) including biological control agents were reported to be beneficial to plants (Weller 1988, Verma et al. 2001). These bacteria must grow on, in or around the roots for the colonization of plant roots, which is of primary importance for an effective plant-microbe interaction (Kloepper and Beauchamp 1992). Nevertheless, effects of nutritional conditions of the plants on root colonization or bacterial effectivity are still not fully understood.

Reports on the correlation between root colonization density and bacterial effectivity are often inconsistent or contradictory. Jiang and Sato (1994) reported a positive correlation between the titre of phosphate solubilizing bacteria in the rhizosphere and the growth of wheat plants. Similarly, Bashan (1986) found an association between the inoculating concentration of *Azospirillum brasilense* and its effects on wheat. The best results were found with 10^5 to 10^8 cells/ml of inoculation suspension as observed by Baldani et al. (1997). Döbereiner (1995) found a highly significant correlation (r = 0.92) between population density of *A. brasilense* SP245 and total nitrogen incorporation in wheat.

A. chroococcum Mac 27 strain was used as biofertilizer for wheat, mustard, sunflower and vegetables and it showed an increase in the crop yield (Narula et al. 1991, 2000, Lakshminarayana et al. 1992, Lakshminarayana 1993, Yadav et al. 2003). However, its colonization or settlement behaviour has not been studied sufficiently to confirm its beneficial effects. Therefore, in the present study, an attempt has been made to study the colonization behaviour of *A. chroococcum* Mac 27 in wheat using DAS ELISA and immuno-fluorescence under hydroponic conditions. Greenhouse experiments were prepared to investigate the colonization behaviour as well as the effectivity of inoculated

A. chroococcum Mac 27 strain, and also to compare them with the behaviour of known colonizers like *P. agglomerans* D5/23 (Remus et al. 2000) on German wheat varieties at different levels of applied nutrients.

MATERIAL AND METHODS

Bacterial cultures. *A. chroococcum* Mac 27 (a methyl ammonium chloride resistant strain) was obtained from the Department of Microbiology, CCS Haryana Agricultural University, Hisar (India). *P. agglomerans* D5/23 was obtained from the Institut für Gemüse- und Zierpflanzenbau Grossbeeren/Erfurt (Germany).

Seeds. German wheat varieties (*Triticum aestivum* var. Munk and GREIF) were obtained from the Department of Plant Breeding, Martin Luther University, Halle and Muncheberg (Germany).

Hydroponic experiment. Seeds of wheat variety GREIF were surface sterilized with 1% Bromine water (Remus et al. 2000) and germinated on 1% agar. Germinated seeds were transferred in a semi-solid nutrient media in 100 ml wide mouth screw cap glass bottles and inoculated with bacterial strains (10^5 cells/ml hydroponic media). These were then transferred to phytotron chamber (12 h light, ca 30 000 lux, 15–17°C during the day and 8–10°C during the night) and kept there over a period of 28 days. This experiment was done for the quantification of *A. chroococcum* in the hydroponic media and on and within the wheat roots and shoots.

Immuno-fluorescence microscopy of inoculated plants from hydroponic experiment. Ten micro liters (10 µl) of cell suspension of *A. chroococcum* was placed onto a slide (cleaned with acetone and then dried with tissue paper), air-dried and passed over the flame to fix the bacteria. For detection of *A. chroococcum* in "squeeze preparation", cleaned slides were coated with a glycerol-protein mixture (1:1, glycerol:ovalbumin). Root fragments of hydroponically grown plants were placed on the slides and then squeezed with a second slide and covered with blocking-buffer for 2 h. Slides were washed with PBS-buffer and dried with tissue paper, incubated with the first antibody (IgG from rabbit against *A. chroococcum*) over night at 6°C. Next morning, the slides were washed with PBS-buffer and covered with blocking-buffer for 2 h again, rewashed with PBS-buffer and dried with tissue paper. Subsequently, the second antibody (IgG from goat against IgG from rabbit labeled

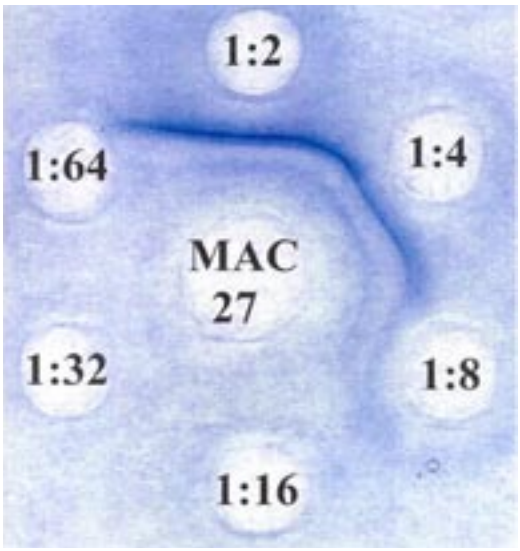

Figure 1a. Double Radial Immuno-diffusion of Azotobacter Mac 27 (center) with different serum (2S1B) dilutions (1:2 ... 1:64) after 72 h

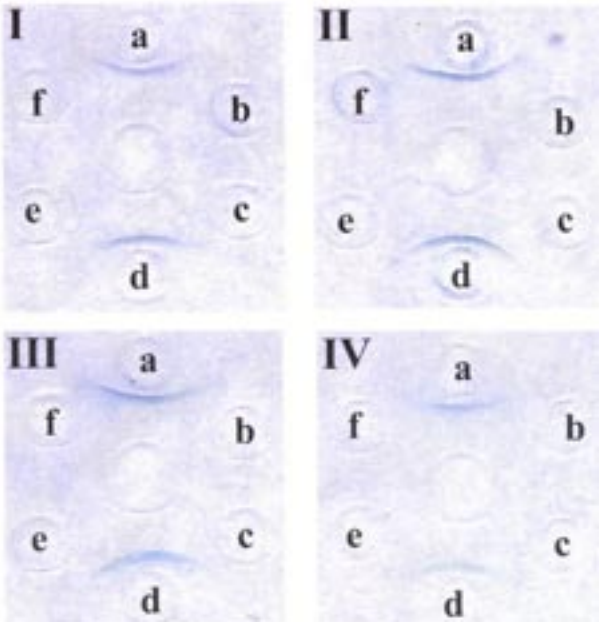

Figure 1b. Double Radial Immuno-diffusion cross reactivity test of serum (2S1B) with different bacterial strains (*A. chroococcum* Mac 27 1...1V a and d, *Klebsiella pneumoniae* CC2/17b, *K. ozaenae* CC12/13Ic, *Azotobacter vinelandii* Ie, *Agrobacterium radiobacter* VC IF, *Azospirillum brasilense* 29145 IIb, *Pseudomonas syingae/ atrofaciens* IIc, *Xanthomonas compestris* IIe, *Alcaligenes faecalis* IIf, *Bacillus polymyxa* IIIb, *Agrobacterium radiobacter* MS IIIc, K27 IIIe, *Pseudomonas putida* IIIf, *Klebsiella pneumonia* CC12/12 IVb, *Citobacter freundii* IVc, *Erwinia* herbicola 609 IVe, *Erwinia raphontici* 727 IVf; after 72 h

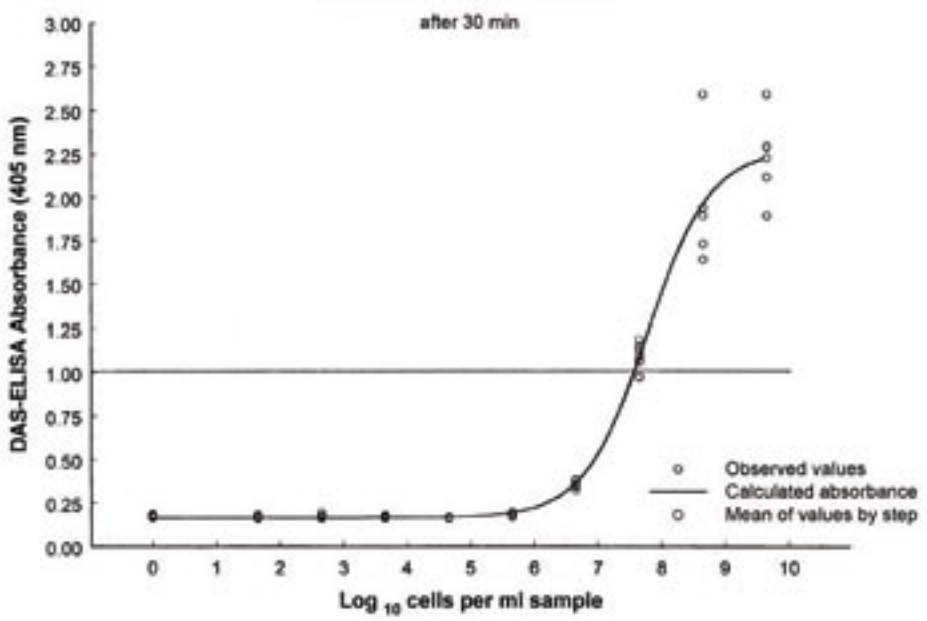

Figure 2. DAS ELISA calibration curve of *A. chroococcum* Mac 27

with FITC) was incubated on the slides over night at 6°C. Finally, the slides were rewashed with PBS-buffer, dried with tissue paper and prepared for fluorescence-microscopic observations.

Greenhouse experiments

Soil characteristics and plant experiments. Comparative effectivity of wheat inoculation with *A. chroococcum* strain and *P. agglomerans* under greenhouse conditions was investigated. Pots were filled with 1200 g of unsterilized soil and sand in 1:1 ratio. Used soil was sandy loam with 28% water holding capacity (WHC) and having 1.5% C, 0.12% N, pH 6.5. Samples were mixed with 200 mg K as K_2SO_4, 50 mg Mg as $MgSO_4$, 0.2 ml $FeCl_3$, 0.2 ml of micronutrient solution and 32 mg $CaCO_3$ per pot. Four different treatments comprising of phosphorus (P) and nitrogen (N) application, i.e. +P+N, –P+N, +P–N and –P–N, were given. A full dose of 60 kg/ha phosphorus as $(CaH_2PO_4)_2$ and 90 kg/ha nitrogen as NH_4NO_3 was applied. Seeds were treated with freshly grown cultures (10^9 cells/ml) of *A. chroococcum* and *P. agglomerans* and six replications were made. In this experiment, plant dry weight (dried at 80°C), N and P-uptake in shoots and survival rate of inoculated bacteria were determined in each treatment after harvest.

Total bacterial count. Samples of rhizospheric and non-rhizospheric soil were collected at harvest, diluted appropriately with normal saline (0.85% NaCl) and plated for viable counts by the drop method of Harris and Sommers (1968).

Statistical analysis. Linux based statistical programme was used for all the statistical analyses. Control treatments with corresponding inoculated (*A. chroococcum*) plants were compared using the non-parametric Mann Whitney-U test.

Colonization studies using DAS ELISA. Soil samples as well as root tips and basal roots were washed, macerated and mixed with PBS buffer in 1:3 ratio and then stored at –20°C. DAS ELISA test in roots (basal and tips) and soil from each treatment was performed for quantification of bacterial titre of *A. chroococcum* and *P. agglomerans.*

Tagging of immunoglobulins (IgG) with alkaline phosphatase. *A. chroococcum* was grown in glycerol-peptone media (Hirte 1961). Inoculated strain was detected using serological methods: polyclonal antibodies were produced in cooperation with the Immuno Globe Antikörpertechnik GmbH (D-63762 Großostheim) and the bacteria were detected using DAS ELISA (Clark and Adams 1977, Scholz et al. 1991, Remus et al. 2000). Antisera were tested with radial immuno-diffusion according to Ouchterlony (Ouchterlony 1962) and the serum charged with the highest antibody titre was selected for isolation of immunoglobulin (Figure 1) G-immunoglobulin (IgG) was isolated from serum by precipitation with $(NH_4)_2SO_4$ and ion exchange chromatography on DEAE cellulose. Alkaline phosphatase (specific activity 4600 μ protein at 25°C) was used as an enzyme for labelling of the second

Table 1. Colonization behaviour of *A. chroococcum* Mac 27 strain in wheat (GREIF) hydroponic experiment after various number of days

Sample		Population data of *A. chroococcum* in a hydroponic experiment					
Location	data type	0.5 h		7 days		28 days	
		control	inoculation	control	inoculation	control	inoculation
Medium	adsorption	0.136	0.178*	0.131	0.854*	0.127	1.06*
	titre	–	4.18×10^5	–	8.32×10^6	1.32×10^7	–
Root	adsorption	0.136	0.136	0.129	2.372*	0.137	2.521*
	titre	–	–	–	2.90×10^8	–	4.26×10^8
Shoot	adsorption	0.140	0.142	0.138	0.142	0.144	0.150
	titre	–	–	–	–	–	–

*denotes a significant difference between the control treatment and corresponding inoculated treatment done by the Mann-Whitney-U test ($P = 0.05$) (Wilcoxon test) – is non-significant difference between the control treatment and corresponding inoculated treatment done by the Mann-Whitney-U test ($P = 0.05$), the coefficient of variance of the titre values ranged from 28.44 to 89.1%

antibody. The substrate (4-nitrophenyl phosphate Na_2-salt) was added to the substrate buffer in the concentration of 0.8 mg/ml. The micro test plates were incubated with substrate buffer for 30 to 60 min at room temperature. The concentration of IgG and conjugate was 1.6 µg protein/ml of incubation reagent. The calibration of ELISA method was carried out with PBS and bacterial suspension at various dilutions (Figure 2). The specificity of IgG was tested in DAS ELISA with *Azotobacter* spp. We could only find a specific antigen-antibody reaction in *A. chroococcum*. The population density of the bacteria was expressed as cells/ml sample (medium) and as cells/g soil or root macerate.

Non-linear regression analysis of DAS ELISA standard curve. To enumerate inoculated bacteria in samples, a logistic functional model suggested by Kvalseth (1985) was used. The coefficient of determination (R^2–1), residual sum of squares/corrected sum of squares in the non-linear regression analysis of DAS ELISA were estimated for standard curve as given below.

$$A = \alpha/[1 + e^{(\beta + \gamma + C)}] + \delta$$

where: A = absorbance; C = log 10 cells/ml: α, β, γ, δ; ε = Euler's number

With the transformed equation the DAS ELISA absorbance values and a special dilution factor of correction (soil, root, or shoot macerate was resuspended in phosphate buffer for bacterial titre of each single inoculated treatment) was calculated. Geometric mean of bacterial titre of each sample in hydroponic experiment (Table 1) and in soil (Table 2) were calculated.

RESULTS AND DISCUSSION

An immuno-fluorescence microscopic study of squeezed roots of hydroponically grown plants was performed to test the ability of *A. chroococcum* to colonize the root surface of wheat plants and the results showed cells scattered on the roots (Figure 3). Bacteria were clearly detectable after 7 days of inoculation. Our results also showed that the bacteria colonized the medium and the roots densely after 7 days of inoculation. The population of bacterial strain in the medium reached to 1.32×10^7 cells/ml while on the roots, the colonization amounted to 1.12×10^8 cells/ml sample (geometrical mean) after 28 days (Table 1). No bacterial colonization of shoots by *A. chroococcum* was detected. These observations encouraged us to design further studies on colonization behaviour.

Colonization of inoculated bacteria on wheat under greenhouse conditions

No bacteria were detected in the uninoculated controls using DAS ELISA. The respective absorbance (measured values) of all inoculated treatments was significantly higher than that of controls. The

Table 2. Colonization behaviour of *A. chroococcum* Mac 27 strain in wheat in unsterile soil after 60 days of seed inoculation (10^9cells/ml)

Sample Location	data type	Population data of *A. chroococcum* in soil							
		–P–N		–P+N		+P–N		+P+N	
		control	inoculation	control	inoculation	control	inoculation	control	inoculation
Soil	adsorption	0.177	0.535*	0.163	0.573*	0.173	0.541*	0.169	0.610*
	titre	–	3.58×10^7	–	4.07×10^7	–	3.71×10^7	–	4.75×10^7
Root tip	adsorption	0.189	0.352*[a]	0.189	0.546*[a]	0.185	0.363*[a]	0.189	0.530*[a]
	titre	–	3.99×10^7	–	3.07×10^7	–	1.66×10^7	–	3.65×10^7
Root basal	adsorption	0.183	0.546*[b]	0.175	0.717	0.178	0.639*[b]	0.185	0.575*[a]
	titre	–	3.99×10^7	–	6.0×10^{7a}	–	5.4×10^7	–	4.37×10^7

*denotes a significant difference between the control treatment and corresponding inoculated treatment done by the Mann-Whitney-U test ($P = 0.05$) (Wilcoxon test); [a, b]denotes different letters showing a significant difference between root tip sample and root basal sample of different fertilizer combination done by the Mann-Whitney test ($P = 0.05$); the coefficient of variance of the titre values ranged from 12.45 to 90.74%

enumeration of *A. chroococcum* using DAS ELISA showed that the rhizospheric soil and roots were colonized similarly. A significant difference for titre in basal part and tip of the root was found in the treatments without any mineral fertilizers (–P–N) followed by P (+P–N). On the other hand, non-significant differences of titre were observed in root basal part and tip in the treatments with both mineral fertilizers (+P+N) and with nitrogen –P+N. In contrast to root tips, where a significant difference was found, differences between bacterial titre in soil and root base in different nutrient treatments were not found (Table 2).

Plant weight and nutrient uptake

Total dry weight of the plants increased in all the inoculation and nutrient treatments compared to the control (Table 3). Maximum dry matter was obtained in *A. chroococcum* inoculation treatments without P and N (130%) and with P (128%) treatments. A similar trend was observed in the case of *P. agglomerans* (119%, 122%). Plant dry matter increased with bacterial inoculation (Table 3).

Inoculation with *P. agglomerans* resulted in a higher nitrogen percentage in shoots in all the treatments with mineral fertilizers +P–N, +P+N,

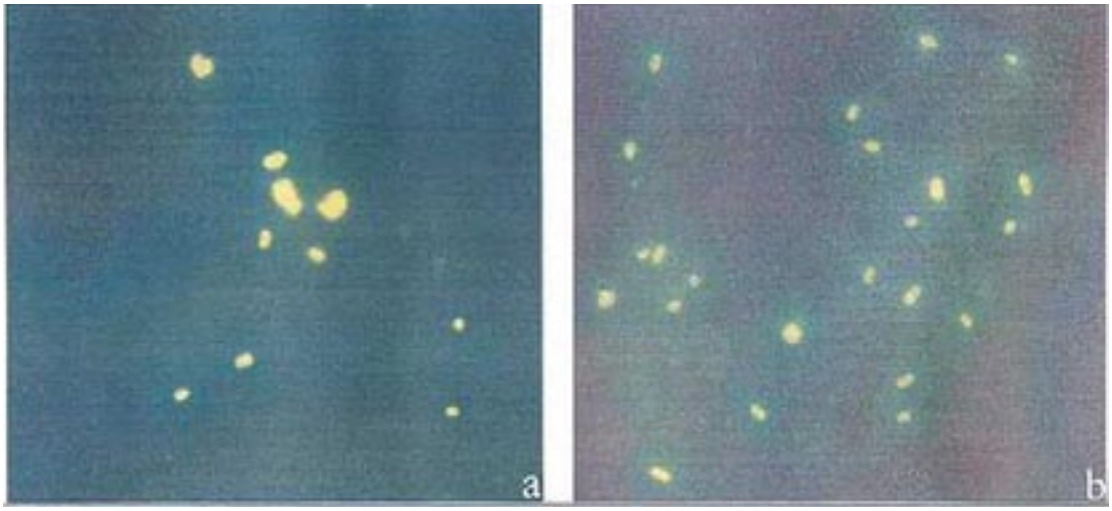

Figure 3. Detection of *A. chroococcum* Mac 27 by use of immuno-fluorescence microscopy (indirect immunological detection with Anti Mac 27 rabbit IgG and Anti-Rabbit-Goat IgG labeled with FITC) in pure bacteria suspension (a) and in a root squeeze-section of wheat-hydroponic inoculation experiment, 7 days after medium inoculation (b)

Table 3. Influence of bacterial inoculation (*P. agglomerans*; *A. chroococcum)* on total dry matter (g) and P uptake (per mg shoot) in the wheat plants

Treatments	Total dry matter (g)	% dry matter	Total P uptake (mg P in total shoot)	% P
Control				
–P–N	0.38	100	1.50	100
+P–N	0.45	100	2.95	100
–P+N	0.51	100	1.62	100
+P+N	0.61	100	3.87	100
P. agglomerans				
–P–N	0.45	119	1.73	115
+P–N	0.55	122	3.50	119
–P+N	0.40	78	1.52	94
+P+N	0.65	106	3.91	101
A. chroococcum				
–P–N	0.49	130	1.79	119
+P–N	0.57	128	3.49	118
–P+N	0.43	83	1.77	109
+P+N	0.49	80	3.02	78

Data are the average of three replicates

and –P+N (106–112%). In the case of *A. chroococcum*, nitrogen percentage in shoots was higher (Figure 4) in presence of both mineral fertilizers +P+N (119%), followed by –P–N (113%), –P+N (112%), and +P–N (105%).

Bacterial counts

Bacterial count was the highest when inoculated with *A. chroococcum* (1.49×10^5 cfu/g soil) in presence of the mineral nutrients (+P+N), followed by *P. agglomerans* (1.4×10^5). With nitrogen only (–P+N) *A. chroococcum* had higher survival rate (1.19×10^5 cfu/g soil), as well. In *P. agglomerans,* the maximum (1.23×10^5 cfu/g soil) was reached when only P was added (+P–N). The survival rate was much lower in the absence of both minerals when inoculated with both Mac 27 and *P. agglomerans* (5.7×10^4 and 6.7×10^4 cfu/g soil), respectively (Figure 5).

There was an overall increase in the bacterial count in rhizosphere soil with all the mineral treatments compared to the bulk soil. The maximum count was observed with *A. chroococcum* inoculation (4.64×10^5 cfu/g soil) followed by *P. agglomerans* (2.06×10^5 cfu/g soil) in the presence of +P+N (Figure 5). The maximum survival rate was observed for *P. agglomerans* (3.72×10^5 cfu/g soil) in the presence of N, while it was 2.86×10^5 cfu/g soil with *A. chroococcum* in the presence of P. These results indicate that *A. chroococcum* colonized in medium and in the roots more intensely than *P. agglomerans*.

DAS ELISA under hydroponic conditions

Colonization of wheat shoots by *A. chroococcum* could not be detected in our hydroponic experiment. In contrast, wheat shoots colonization by *P. agglomerans* was described previously by Ruppel et al. (1992) and Remus et al. (2000). The colonization study in an unsterile soil showed that *A. chroococcum* Mac 27 colonised the rhizosphere soil and the roots (tip and basal) 60 days after the seed inoculation. This indicates a high competitive ability of the bacterial strain. For some *Enterobacteria*, a stronger colonization of roots than that of rhizosphere soil was described by Rattray et al. *(*1995); however, in our studies *A. chroococcum* colonized the rhizosphere soil and the roots equally.

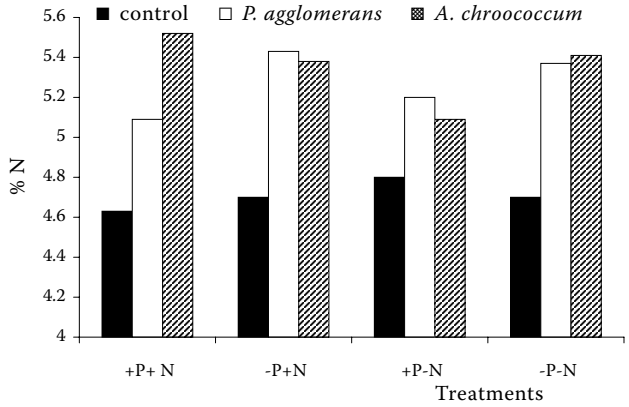

Figure 4. Influence of bacterial inoculation on nitrogen contents of wheat shoots (8 weeks old) at different fertilization levels

Plant weight and nutrient uptake under hydroponic conditions

There was an overall increase in plant dry matter (30% in *A. chroococcum*) in the presence of phosphorus. But in the presence of nitrogen only, a decrease in plant dry matter was observed both in *A. chroococcum* and *P. agglomerans* (Table 3). Jiang and Sato (1994) also observed that along with microorganisms, super phosphate was beneficial for plant growth in regard to length and dry weight of plant shoot. They further mentioned that the changes in bacterial populations and growth of wheat focus on the differences in phosphate fertilization. Reasons of this increase could be that microorganisms, in turn, affect the plants either directly via various metabolites or by modifying the soil environment. The influence of microorganisms seems to play a key role for the growth, development and physiological state of the plants. Pandey and Kumar (1989) and Wani et al. (1989) also observed an increase in shoot weight and yield even in the presence of farmyard manure and N, P, K fertilizers along with inoculation. The reason for the increase was however attributed more to growth-promoting substances, which help the plant in N assimilation, rather than to appreciable nitrogen fixation. In our studies, a non-significant difference was observed in dry matter in presence of the minerals (+P+N) in *P. agglomerans*.

P percentage (Table 3) in shoots increased in the presence of N in the treatments with inoculation (10–32%). Plant adaptations allowing for an improved growth in low P soils are related (1) to the ability of the plant to take up more P from a deficient soil, to produce more dry matter for a given quantity of P, or to bring changes in root physiology which allow more uptake of P at lower concentrations in the soil solution, or from insoluble inorganic or organic forms (Marschner et al. 1997); or (2) to the development of a more extensive root system in association with P solubilizers (McCully and Canny 1988)

The maximum increase in plant N was with *A. chroococcum* (19%), followed by *P. agglomerans* (10%), showing that bacteria fix nitrogen and provide plants with some nitrogen, too. The increase

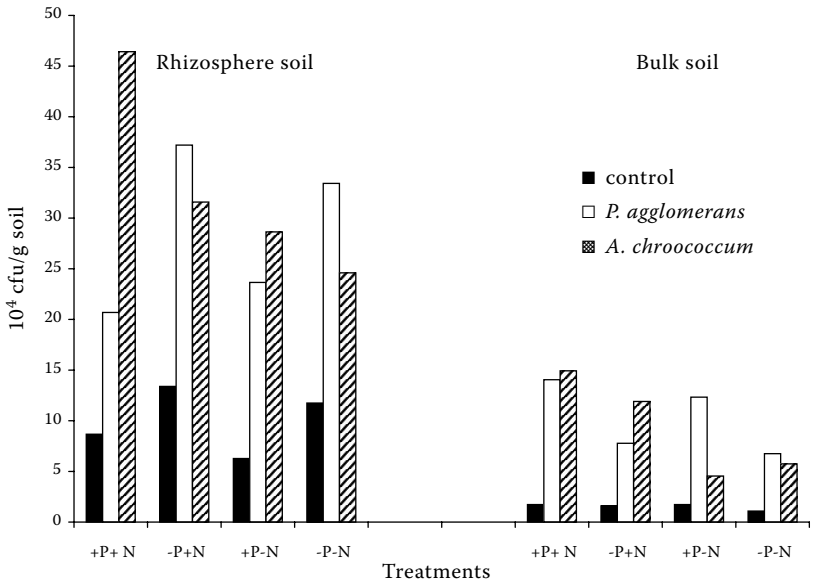

Figure 5. Influence of bacterial inoculation on total microbial counts in rhizosphere and bulk soil of wheat (8 weeks old) at different fertilization levels

in nitrogen percentage was observed mostly with *A. chroococcum* and *P. agglomerans* in the absence of minerals. It was hypothesized earlier that root-associated diazotrophs fix atmospheric nitrogen but translocate only little of this fixed nitrogen from cells to the host plant (Christiansen-Weniger et al. 1992). In few cases, however, there is strong evidence of nitrogen-fixing bacteria contributing to the nitrogen accumulation in the plants (Boddey and Dobereiner 1988). In previous studies (Lakshminarayana 1993) *A. chroococcum* was found to fix nitrogen as assessed by acetylene reduction assay (ARA) to the range of 613–2184 nmoles/h/mg dry weight of cells. Nitrogen percentage in bulk soil was much lower in the inoculated samples compared to the control irrespective of the presence or absence of minerals (Figure 4).

Van Berkum and Bohlool (1980) reported that diazotrophic bacteria in the rhizosphere utilise the products of nitrogen fixation for their own growth but do not release it while they are alive, the other reason could be the production of phytohormone in the rhizosphere by the microorganisms (Zimmer and Bothe 1988, Zimmer et al. 1988). Also *P. agglomerans* and *A. chroococcum* were able to produce phytohormones in pure culture (Scholz-Seidel and Ruppel 1992, Höflich et al. 1994, Pathak et al. 1995, Kumar and Narula 1999).

There was a significant difference between the microbiological counts in rhizosphere and bulk soils (Figure 5). The count of *A. chroococcum* and *P. agglomerans* in rhizosphere and bulk soil was 10^4 and 10^5/g soil in individual treatments, respectively. This might be probably due to the root exudates that help in modification of soil environment and proliferation of bacteria (Parke 1991, Christiansen-Weniger et al. 1992, Jiang and Sato 1994, Baldani et al. 1997), and improve the wheat growth. Variable quantities and compositions of root exudates of different plant species could be responsible for specific colonizations. In bulk soil, the presence of autochthonous microorganisms containing a considerable number of nitrogen-fixing bacteria might also have contributed as the experiment was done in unsterilized soils under greenhouse conditions. Bahme and Schroth (1987) reported a decline in the recoverable populations of introduced bacteria over time even if the organisms were considered to be good colonizers. This might result from the addition of much greater populations than the actual capacity of roots.

It can be observed generally that root colonization by inoculated bacteria improves crop productivity. However, wheat varieties as well as bacterial strains were found to show differences in survival of *A. chroococcum* (Behl et al. 2003, Singh et al. 2004, Narula et al. 2006). Therefore, plant-microbe interaction studies remain important. We must take into account the establishment of these bacteria on or in the roots and in the rhizosphere, and serological methods (ELISA) are generally suitable for such studies. We observed the higher bacterial numbers compared to control with DAS ELISA when the experiment was done under controlled conditions. Significant differences in bacterial titre were observed in root tips compared to basal parts in the inoculated plants but not in all treatments. *A. chroococcum* successfully colonized wheat roots and was found scattered around the roots as observed by immuno-fluorescence microscopy by hydroponic experiment. Results obtained in the present study are in line with the earlier reports on DAS ELISA on *P. agglomerans* colonization on wheat (Ruppel et al. 1992, Remus et al. 2000). A significant progress was made in root colonization studies but the characterization of microorganisms and plants, management of rhizosphere soil micro flora and better methods to measure microbial competition require more attention. Lower bacterial count in a plate count determination method compared to DAS ELISA is attributable to the fact that plate dilution method determines only about 20% of total bacteria. Our study suggests that immunofluorescence and DAS ELISA could be used as potential techniques for investigating colonization behaviour of bioinoculants.

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