Paranodules and colonization of wheat roots by phytohormone producing bacteria in soil

N. Narula[1], A. Deubel[2], W. Gans[2], R.K. Behl[1], W. Merbach[2]

[1]Institute of Soil Science and Plant Nutrition, Faculty of Agriculture, Martin Luther University, Halle-Wittenberg, Halle, Germany
[2]Department of Microbiology, CCS Haryana Agriculture University, Hisar, India

ABSTRACT

Soil bacteria belonging to the genus *Azotobacter, Pantoea* and some unidentified soil isolates were tested *in vitro* for phytohormone production under laboratory and soil conditions. The German wheat variety *Munk* was inoculated by several soil bacteria with exogenously applied hormones (IAA, 2,4-D) and a flavonoid (naringenin) with a half of the amount of recommended doses of fertilizers under greenhouse conditions. Most of the soil bacteria tested were able to produce indole acetic acid (IAA), and stimulated a lateral root development and colonization by the addition of 2,4-D and IAA. A formation of paranodules on roots as a result of crack entry invasion was observed with 2,4-D as well as with IAA. We were able to reisolate the organism from the paranodules and could establish the same results. Analyses for root exudates and *in vitro* phytohormone production by various bacterial isolates were also carried out, revealing that 2,4-D can be replaced either by high IAA producing bacteria or by exogenous application of IAA. Bacterial survival in the rhizosphere as well as the root and shoot weight of wheat plants were positively affected also by the addition of IAA, 2,4-D and naringenin.

Keywords: paranodules; colonization; *Azotobacter chroococcum*; *Pantoea agglomerans*; wheat

Diazotrophic bacteria are important in agriculture as they are known to improve soil health and to increase crop productivity (Narula et al. 1991, Lakshminarayana 1993). A use of bioinoculants producing phytohormones is gaining importance around the globe as a means of sustainable crop production by utilizing the synergy between plants and microbes. It is generally believed that *Azotobacter* and *Azospirillum* enhance plant growth as a result of their ability to fix nitrogen. In many instances, nitrogen level does not increase appreciably upon the inoculation of these bacteria and growth promotion in such cases may be attributed to other mechanisms such as plant growth regulating compounds or phytohormones. Arshad and Frankenberger (1991) noticed this effect under nitrogen rich conditions. Many soil bacteria demonstrated a potential to promote plant growth and enhance plant yields. Production of plant hormones by *Azotobacter* (Lee et al. 1970, Nieto and Frankenberger 1989) and *Azospirillum* (Hubbell et al. 1979, Tien et al. 1979) as well as the concomitant changes in plant growth and development were observed. Out of the five major classes of hormones, indole acetic acid (IAA), gibberellic acid (GA_3), kinetin, abscisic acid and ethylene, *Azospirillum* is known to produce the first three kinds of phytohormones whereas *A. chroococcum* is known to produce indole acetic acid, gibberellic acid and cytokinnin (Crozier et al. 1988, Bottini et al. 1989, Cacciari et al. 1989). However, not much information is available about the system, nature and magnitude of phytohormone production in qualitative and quantitative terms. For this reason, the application of phytohormones microbiologically produced by plant growth promoting rhizobacteria (PGPR) or synthetic plant growth regulators is often recommended to improve the quality, yield and to alter plant life processes. Treatment with auxins showed to increase the colonization of roots by soil bacteria e.g., *Azospirillum* (Tchan et al. 1991, Kennedy and Tchan 1992). A change in morphology of roots to form paranodules following the addition of auxin analogues was reported. Sriskandarajah et al. (1993) reported on these structures with

A. brasilense (Sp7) partly due to the development of a protected niche. These paranodule structures derived from the induction of the initials of the lateral roots are quite dissimilar to root nodules particularly when colonized by non-symbiotic bacteria. Protoplasts of the bacteria (L-forms of *Azotobacter*, *Pseudomonas syringae* and *Bacillus polymyxa*) were suggested to have the ability to penetrate the cell wall and membrane structures of living plant cells and to colonize plant tissues (Cocking et al. 1990). Also some microorganisms colonize in the intercellular spaces as seen in apoplast of stem of sugarcane (Cocking 2003). Despite encouraging results with *Azospirillum*, *Azotobacter* etc., some controversy still exists about the mechanism of bacterial root interactions. Reports suggest that the colonization of these bacteria is caused by factors like N_2 fixation, siderophores, ammonia excretion, phytohormones (Lakshminarayana 1993) and antifungal properties etc. (Verma et al. 2001), collectively enhancing the root proliferation, increase in the lateral roots and root hair formation. Dobbelaere et al. (1999) suggested that plant growth substances are one of the key factors observed in plant growth promotion. Tien et al. (1979) reported that the morphological changes due to bacterial inoculations can be mimicked by applying a combination of plant growth substances.

Keeping all these factors in mind, we made an attempt to screen different soil bacteria for the production of various phytohormones, their colonization on German winter wheat (var. Munk) with or without exogenously applied natural and synthetic plant hormones and the concomitant changes in root morphology or formation of paranodules under controlled greenhouse conditions in addition to plant growth parameters. Natural hormone like IAA, synthetic-2,4-D (2,4-dichlorophenoxy acetic acid) and a flavonoid naringenin – a nodule activator, were used as exogenous hormones. 2,4-D is an auxin analogue and the flavonoid naringenin is the aglucon of naringenin that is found in flowers and grapefruits.

MATERIAL AND METHODS

Experiment 1. Bacteria preparation and phytohormone detection

The soil bacteria used in this study were isolated from geographically and climatically diverse locations: semiarid (India), Mediterranean (Spain) and temperate (Germany). These bacteria differ in their phenotypic characteristics (Table 1). The nitrogen fixing and other soil bacteria were maintained on Jensen's nitrogen free media (Jensen 1951) and LB media (Miller 1972).

For phytohormone detection, pure bacteria were cultured in the media at 30°C for 4–7 days under stationary conditions. Cells were centrifuged at 5000 *g* and the supernatant was used in triplicate to estimate various phytohormones by the method of Gransee and Wittenmeyer (2000).

To detect IAA, pure bacteria were cultured in Jensen media with 100 µg/ml of tryptophan for 7 days at 30°C under stationary conditions. Culture broth (2 ml) was removed at different time intervals and centrifuged at 7000 rpm for 2 min. IAA was determined in culture supernatant in triplicate by Salkowski's method (Tang and Bonner 1974).

Table 1. List of bacterial strains used

Isolates/Mutants	Phenotypic characteristics	Source
Azotobacter chroococcum Mac 27	mutant resistant to methyl ammonium chloride [an ammonia analoge (Mac)]	Department of Microbiology, Haryana, Agricultural University, Hisar, India
A. chroococcum MSX 9	mutant resistant to methionine sulfoximine (MSX)	Department of Microbiology, Haryana, Agricultural University, Hisar, India
A. chroococcum HR-23 & DR-26	wild type biotin producing strains	Department of Microbiology, University of Granada, Granada, Spain
Pantoea agglomerans	wild type strain	Department of Soil Science and Plant Nutrition, Halle, Germany
DF 23, D5, IS-1, IS-3, IS-10	unidentified soil isolates	Rhizosphere of eternal rye (*Secale cereal*) in long term experiments, Halle, Germany

Experiment 2. Phytohormone production by bacteria: inoculation on Petri dishes

Wheat seeds (var. Munk) were surface sterilized by washing with 70% ethanol for 3 min, rinsed three times with sterilized water, subsequently treated with 0.02% acidified mercuric chloride for 3–5 min and then washed 5 times with sterilized distilled water and germinated on 1% agar plates. Germinated seeds were treated with 10^9/ml of various bacterial cultures for 30 min and transferred on big agar plates. Afterwards, appointed amounts of IAA, 2,4-D and naringenin were separately added to the 10 µg/ml/seed.

A pure culture of each bacterium was grown at 30°C for 48–72 hrs until a 10^9 colony forming units (CFU)/ml cell concentration was achieved.

Experiment 3. Paranodulation by bacterial inoculation

Small pots were filled with soil and sand mixture in 1:1 ratio with 60 kg N and 60 kg P/ha basis. Seeds of the German wheat variety Munk were obtained from the Institute of Plant Breeding, Martin-Luther University, Halle (Germany). Seeds were surface sterilized as mentioned above and treated with a fully-grown inoculum (10^9/ml) of various bacteria, which was prepared as in experiment 2, along with phytohormone treatments. Treatment 1 comprised of bacterial cultures inoculation alone (where plants did not receive any hormones) while in treatments 2, 3, and 4 bacteria were inoculated in combination with exogenous application of IAA (10 ppm), 2,4-D (10 ppm) and naringenin (10 ppm), respectively. All the experiments were carried out in triplicate. Five seeds were planted in each pot in the greenhouse. After the emergence plants were thinned to three per pot and watered daily. Plants were harvested after 60 days of growth and analysed for fresh and dry plant weight, root biomass and nitrogen (N) and phosphorus (P) content. Survival rates of inoculants from the rhizospheric soil from all the treatments were determined.

Inoculation and hormone treatments were as follows: (1) uninoculated and untreated, (2) uninoculated + hormones and (3) inoculated + hormones.

Viable bacterial counts in rhizospheric soil from all the treated (phytohormones) and untreated roots inoculated with various bacteria were counted as described by Narula et al. (2002) using the most probable number technique. Soil adhering to roots was appropriately diluted and plated on Jensen's nitrogen free medium. Plates were incubated at 28–30°C and CFU/g was calculated.

The fresh weight of the plants (shoots and roots) was determined as soon as they were taken out of the pots (after 60 d). For dry weight determinations shoots and roots were dried at 80°C until the weight was constant.

To reisolate bacteria from paranodules, short and thick roots (sometimes paranodule) from both 60-day old inoculated plant from the pot experiment and 21-day old plants from Petri dishes were surface sterilized, crushed under aseptic conditions and diluted with water. The extract was streaked on Jensen media plates (Jensen 1951).

RESULTS

Experiment 1. Phytohormone production

Most of the cultures used in these studies showed an *in vitro* production of phytohormones like IAA. *A. chroococcum* strains produced various phytohormones as given in Table 2.

Experiment 2. Formation of paranodules in wheat roots by various soil microorganisms

There was a pronounced effect on the development and morphology of wheat roots resulting in a decrease in root length but an increase in root hairs. This was observed only in treatments with phytohormone producing bacteria and/or in combination with exogenous application of hormones. Maximum effect was percieved in the case of bacterial inoculation, along with 2,4-D and IAA treatments. Control (without inoculation) had a little effect of the exogenous phytohormones alone. This was observed visually, as well as under zoom microscope.

Experiment 3. Paranodulation by bacteria: inoculation in soil condition

As compared to uninoculated and untreated control (Figure 1), most of the treatments with bacteria and hormones resulted in thickening of roots as in Petri dish bioassay (Figures 2–6). Root elongation decreased while the number of root hair increased. But this effect was more pronounced in the case of bacterial inoculation along with

Table 2. *In vitro* phytohormone production (μM) by various soil bacteria

Mutant/Isolate		IAA	Gibberellic acid	Kinetin
		7d	14d	
Azotobacter chroococcum	Mac-27	10	–	+
	MSX-9	98	+	+
	HR-23	+	–	–
	DR-26	+	–	–
Pantoea agglomerans	D5	+	–	–
Unidentified	IS-1	+	ND	ND
	IS-3	+	ND	ND
	IS-10	+	ND	ND

hormones (Figure 7). Bacterial inoculants applied in combination with 2,4-D and IAA followed by naringenin were the most effective treatments (Figures 1, 7). Some of the most effective bacteria in terms of phytohormone production also formed one to two big nodule like structures in addition to small thick roots (Figures 8, 9).

Exogenous application of IAA, 2,4D and naringenin only (without inoculation) also affected root morphology (visual observation). Application of 10 ppm of exogenous phytohormones – natural (produced by the bacteria), synthetic (IAA, 2,4-D) and nod regulator (naringenin) – also decreased the root length. The number and length of root hair increased with exogenous phytohormone application as in the case of inoculated treatment. Effects of phytohormones were less pronounced in uninoculated treatments as compared to similar inoculated treatments.

As shown in Table 3, bacterial counts were different in various treatments. Survival count was in the range of 10^6 g/soil but most of it increased with the addition of 2,4-D and IAA followed by naringenin. Marked differences were observed between control (0.180×10^6) and the treatments without inoculation ($0.183–0.423 \times 10^6$). Maximum count was with IAA + MSX9 (12.240×10^6), followed by IS-10 (11.910×10^6), Mac27 (11.140×10^6) and *Pantoea* (4.855×10^6); in the case of 2,4-D the highest count was with Mac-27 (10.95×10^6), followed by *Pantoea* (5.157×10^6) and MSX9 (3.070×10^6), while it was low for isolate IS-10. Microbial number was also high with naringenin + Mac27 (10.060×10^6), followed by MSX9

Table 3. Survival rate of inoculated soil bacteria (1×10^6 cfu) along with exogenously applied IAA, 2,4-D and naringenin treatments in soil

Mutant/Isolate		Treatments			
		no hormone	IAA	2,4-D	naringenin
Azotobacter chroococcum	Mac-27	7.550	11.140	10.950	10.060
	MSX-9	8.770	12.240	3.070	8.820
	HR-23	0.248	0.156	0.105	0.338
	DR-26	0.358	0.485	0.696	0.820
Pantoea agglomerans	D5	3.320	4.855	5.157	3.450
Unidentified	IS-1	0.075	0.053	0.074	0.042
	IS-3	0.066	0.059	0.066	0.048
	IS-10	1.390	11.910	0.027	0.071
No inoculation		0.180	0.383	0.183	0.423
CD 5%		0.360	1.070	1.320	1.520

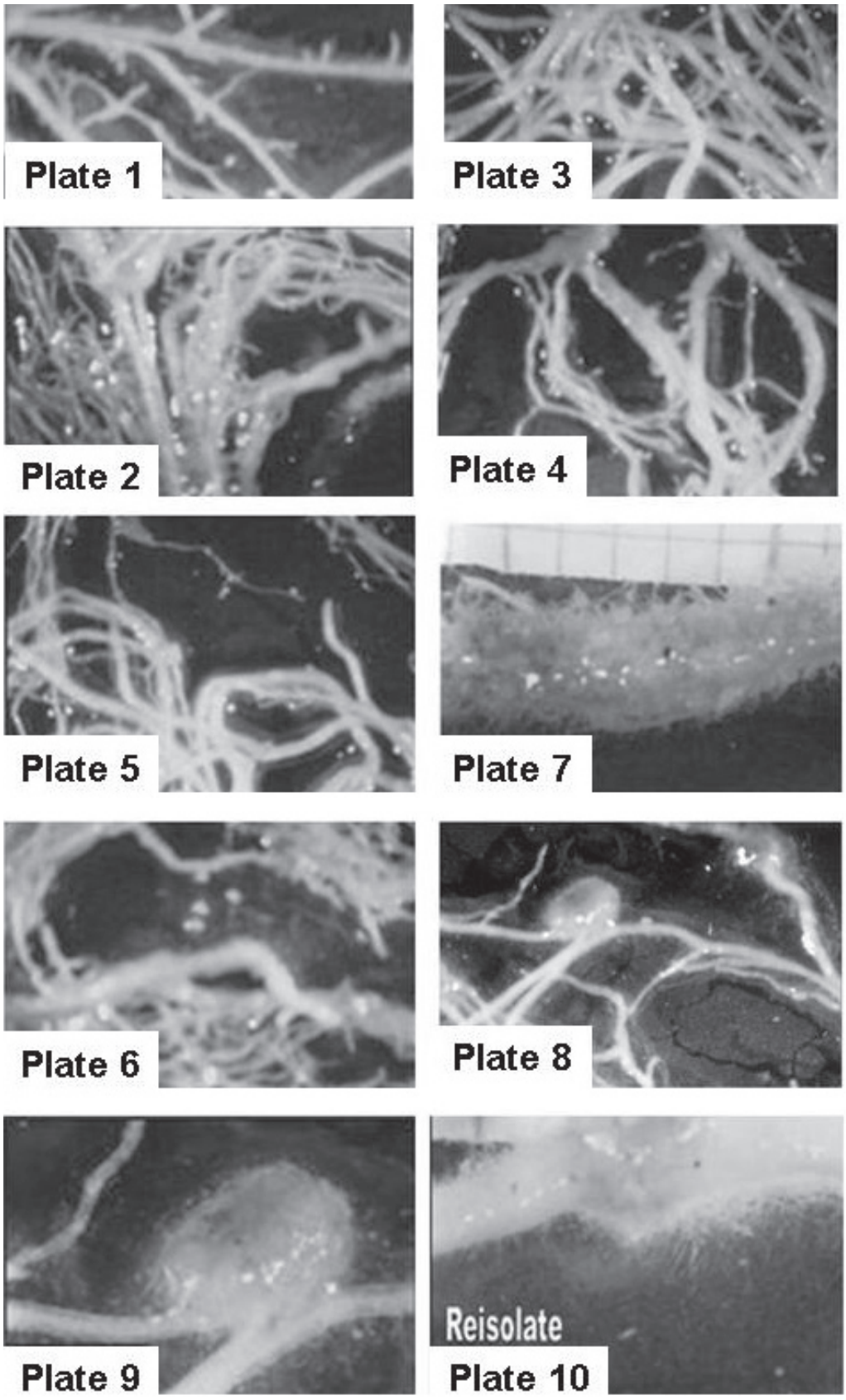

Figure 1. Zoom microscopic photographs of wheat roots in control and *Azotobacter* inoculation + hormone treatments plate 1: normal roots of wheat plants in control; plate 2: swollen roots – IS1 + IAA; plate 3: swollen roots – IS1 + naringenin; plate 4: swollen roots – IS1 + 2,4-D; plate 5: swollen roots – IS10 + IAA; plate 6: swollen roots – IS10 + 2,4D; plate 7: swollen roots – MSX9 + 2,4D; plate 8: paranodule formation – MSX9 + IAA; plate 9: paranodule formation – MSX9 + IAA; plate 10: reisolate from paranodule-tested

Table 4. Influence of exogenously applied hormones along with various inoculation on shoot and root weight (g)

Mutant/Isolate		Organ	Treatments			
			no hormone	IAA	2,4 D	naringenin
Control		SFW	0.573	0.578	0.484	0.659
		RFW	0.235	0.286	0.241	0.348
		SDW	0.062	0.059	0.024	0.071
		RDW	0.035	0.020	0.040	0.045
Azotobacter chroococcum	Mac 27	SFW	0.664	0.740	0.778	0.731
		RFW	0.360	0.392	0.518	0.418
		SDW	0.079	0.079	0.087	0.083
		RDW	0.082	0.059	0.065	0.082
	MSX 9	SFW	0.590	0.660	0.794	0.825
		RFW	0.305	0.430	0.541	0.510
		SDW	0.064	0.081	0.096	0.087
		RDW	0.045	0.066	0.070	0.011
	HR-23	SFW	0.710	0.744	0.813	0.633
		RFW	0.627	0.656	0.560	0.407
		SDW	0.085	0.089	0.097	0.074
		RDW	0.011	0.011	0.098	0.058
	DR-26	SFW	0.755	0.750	0.764	0.752
		RFW	0.612	0.655	0.606	0.636
		SDW	0.097	0.091	0.088	0.086
		RDW	0.014	0.087	0.100	0.083
Pantoea agglomerans	D5	SFW	0.670	0.827	0.857	0.800
		RFW	0.490	0.684	0.742	0.894
		SDW	0.086	0.103	0.105	0.107
		RDW	0.073	0.089	0.114	0.184
Unidentified	IS-1	SFW	0.740	0.790	0.844	0.545
		RFW	0.565	0.546	0.863	0.315
		SDW	0.102	0.096	0.099	0.072
		RDW	0.090	0.083	0.156	0.025
	IS-3	SFW	0.768	0.710	0.853	0.827
		RFW	0.484	0.504	0.670	0.617
		SDW	0.090	0.080	0.098	0.097
		RDW	0.082	0.074	0.010	0.028
	IS-10	SFW	0.725	0.785	0.600	0.614
		RFW	0.345	0.258	0.278	0.336
		SDW	0.077	0.077	0.066	0.067
		RDW	0.046	0.039	0.096	0.059
CD 5% (SFW)			0.080	0.090	0.070	0.020
CD 5% (RFW)			0.130	0.040	0.060	0.060
CD 5% (SDW)			0.008	0.001	0.089	0.004
CD 5% (RDW)			0.110	0.007	0.010	0.056

SFW = shoot fresh weight; RFW = root fresh weight; SDW = shoot dry weight; RDW = root dry weight; IAA = indole acetic acid

Table 5. Analysis of root exudates from wheat roots under the above mentioned chosen treatments

Mutant/isolate		Root exudate	Treatments			
			no hormone	IAA	2,4-D	naringenin
Azotobacter chroococcum	Mac-27	organic acids	malate fumarate	malate +2 un-peaks +3 un-peaks	malate succinate fumarate	malate citrate fumarate +4 un-peaks
		sugars	mannose arabinose	mannose arabinose	mannose arabinose	mannose arabinose
	MSX-9	organic acids	2 un-peaks	malate fumarate citrate succinate +3 un-peaks	malate fumarate +3 un-peaks	malate fumarate +2 un-peaks
		sugars	mannose arabinose	mannose arabinose	mannose arabinose	mannose arabinose glucose
Pantoea agglomerans	D5	organic acids	malate fumarate succinate citrate +3 un-peaks	fumarate +3 un-peaks	malate fumarate +6 un-peaks	malate fumarate +3 un-peaks
		sugars	mannose arabinose maltose	mannose arabinose	mannose arabinose	mannose arabinose
Unidentified	IS-10	organic acids	malate +3 un-peaks	malate fumarate +1 un-peak	malate fumarate oxalate 2-oxyhydrofuran +5 un-peaks	malate fumarate +2 un-peaks
		sugars	mannose arabinose	mannose arabinose	mannose arabinose	mannose arabinose
No inoculation		organic acids	malate fumarate succinate formate	malate fumarate +3 un-peaks	+4 un-peaks	malate citrate fumarate +2 un-peaks
		sugars	arabinose un-peaks:	mannose un-peaks	mannose	mannose

(8.820×10^6) and *Pantoea* (3.450×10^6). Thus it was evident that the Mac27, on an overall basis, had a maximum survival rate followed by MSX9. However, when treated with IAA, MSX9 showed a higher survival. *Pantoea* exhibited considerably lower survival rate as compared to Mac27 and MSX9 in all the treatments. *Azotobacter* strains IS-1, IS-3, IS-10, HR-23 and DR-26 had a much lower survival under control and treated conditions.

The influence of bacterial inoculation along with exogenous application of hormones (Table 4) on root and shoot biomass of wheat were also determined. The control (without any treatment) showed less shoot and root fresh weight, whereas the treatment only with various hormones increased root and shoot biomass. Nevertheless fresh and dry shoot and root weight increased significantly in treatments of bacterial inoculation with or without hormone application. Maximum fresh shoot weight was observed in the case of *Pantoea* Df/23 with IAA + bacterial treatment (0.827 g), followed by Mac27 (0.740) IS-1, IS-10 and DR-26. With 2,4-D, the highest shoot weight was in treatments with *Pantoea* (0.857), followed by IS-1 (0.844) and IS-3 and Mac27. Maximum root weight in IAA treatment was also observed in *Pantoea* (0.684), followed by HR-23 (0.656 g) and DR-26 (0.656 g). In the case of naringenin, shoot weight reached maximum with IS-3 (0.827 g), followed by MSX9 (0.825 g), DF/23 (0.800 g) and Mac27 (0.731 g), whereas root weight was highest with DF/23 (0.894 g), followed by DR-26 (0.636 g). Dry root and shoot weight followed the same trend with a few exceptions. Among hormones, naringenin showed the maximum effect on fresh and dry weight of shoot and roots followed by IAA. All the inoculants in control treatment showed their favourable effect on the increase of fresh and dry weight of shoot and roots. The increase in shoot and root weight was at par in strains IS-1, IS-3, IS-10, HR-23 and DR-26 and higher than that of MSX9, *Pantoea* and Mac27 which in turn were at par with each other for such effects. However, response of strains IS-1, IS-3, IS-10, HR-23 and DR-26 to added hormones was lower as compared to that of MSX9, *Pantoea* and Mac27.

Analyses of exudates from wheat roots (Table 5) revealed that sugars did not vary in qualitative terms and mannose and arabinose sugars were invariably found irrespective of treatment with bacterial strains individually or in combination with hormones, IAA, 2,4-D and naringenin. MSX9, however, revealed an additional presence of glucose in combination with naringenin while *Pantoea* in combination with IAA exhibited maltose. Likewise, organic acids malate and fumarate were present in root exudates in most treatments with bacterial strains. Mac27 in combination with 2,4-D and naringenin exhibited an additional presence of succinate and citrate, respectively. MSX9 in combination with IAA exhibited both succinate and citrate in root exudates. IS-10 revealed an additional presence of oxalate and 2-oxyhydrofuran in root exudates. Furthermore, 1–6 unidentified peaks were observed in different hormone producing bacterial strain treatment and bacterial strain + hormone combination treatment.

DISCUSSION

Phytoeffective interactions among plants, microorganisms and soil take place in the rhizosphere. Microorganisms mostly survive in the organic soil fraction depending on different factors such as a mode of crop, soil characteristics, growth promoting bacteria, or growth inhibiting root pathogens. This is partly reflected by different growth stimulation effects after inoculation. Auxins are growth regulators that are essential for plant growth. Some of the soil bacteria are reported to produce these growth hormones that affect the plant growth (Zimmer et al. 1988). The bacteria are known to change root morphology and increase their biomass enabling thus the roots to take up more soil nutrients. This type of bacteria can ameliorate the effects of nitrogen fixation.

Our studies dealt with the selection of phytohormone producing bacteria under laboratory conditions. Almost all the selected strains were able to produce IAA in the range of 10–98μM after 7 d. Additionally, MSX-9 was found to produce gibberellic acid and kinetin on 14 d, whereas Mac27 produced kinetin. However gibberellic acid and kinetin were, not detected in other strains (Table 2). Earlier, MSX-9 was found (Pathak et al. 1995) to produce all the three hormones (gibberellic acid, kinetin and IAA). The amount of auxins produced is generally low under cultural conditions as well. IAA is only produced in the presence of tryptophan (Tang and Borner 1974).

Crop yields increase with the inoculation of specific microbial preparations in many regions of the world. Growth promotion is attributed to plant growth regulating substances in rhizosphere besides nitrogen fixation. Even in rhizobia the fixation of nitrogen and its supply to the host was thought to be the only function of the nodules for many years. The hormone content of the root nodules gained an attention due to its formation and subsequent development in nodules (Nutman 1955). Hunter (1989) demonstrated that nodules containing large amounts of IAA in a culture also produced bacteroids with enhanced IAA producing capacity. It might be involved in several stages of symbiotic relationship (Hunter 1989). Several

diazotrophic bacteria are also able to synthesize phytohormones like substances. For example, gibberellic and cytokinnin production was reported in *Azospirillum* (Tien et al. 1979, Umali-Garcia et al. 1980) *Arthrobacter* (Caccciari et al. 1989) and *Azotobacter* (Pathak et al. 1995). Dobbelaere et al. (1997) observed that the effect of auxins produced by *Azospirillum* was further enhanced by adding tryptophan. Moreover, these effects could be mimicked by replacing *Azospirillum* cells with a specific concentration of IAA. Yegorenkova et al. (2001) studied the dynamics of adsorption of the nitrogen-fixing soil bacteria *Azospirillum brasilense 75*, 80 and Sp245 to the roots of seedlings of common spring wheat in relation to inoculum size, period of incubation with the roots and bacterial-growth phase. A possible mechanism of the mutual influence of bacteria and plants may involve key roles of wheat germ agglutinin present on the roots, and the polysaccharide-containing components of the *Azospirillum* capsule.

Several laboratories described the development of a laboratory model of associative nitrogen fixation known as paranodules (Kennedy and Tchan 1992, Kennedy 1994, Kennedy et al. 1997). 2,4-D is known to make a crack entry for bacteria. Lateral roots fail to extend, thus providing round nodular structures. This effect was reported as a concentration dependent. Also in our earlier experiments (unpublished data) we found paranodules on wheat roots (an Indian var. WH147) by crack entry of bacteria with the addition of 2,4-D. We extended our earlier studies to replace the use of a synthetic hormone such as 2,4-D that can cause problems to the cultivation of sensitive crops like cotton. Therefore, phytohormone producing bacteria along with the exogenous application of IAA and a naringenin of flavonoid were evaluated and compared with 2,4-D treatment so as to find an alternative for 2,4-D. Gopalsamy et al. (2000) reported that the addition of naringenin can enhance xylem colonization. We did experiments in Petri dishes as well as in small pots. There was thickening of the roots with a decrease in root length and an increase in root hair. But all such effects were more pronounced in the treatments with exogenous application of hormone along with bacterial inoculation (Figures 2–7). In the case of IAA producing bacteria an addition of 10 µg/ml, IAA was effective in the formation of paranodules. One or two big paranodules were observed (Figures 8–9). We were able to reisolate the organism from the paranodules and could establish similar results (Figure 10). Our results showed that high IAA producing bacteria with the addition of exogenous IAA have the same effect as 2,4-D. Also 2,4-D, naringenin and IAA provided improved colonization of bacteria. Kennedy et al. (1997) emphasized that these structures derived from the induction of the initials of the lateral roots are quite dissimilar to rhizobium nodules colonized by non-symbiotic bacteria. In this case, bacteria colonize usually the basal zone of intracellular paranodule.

We did not demonstrate the nitrogen fixation by these paranodules because our focus was on establishing an efficient colonization and observing changes in root morphology. However, Yu and Kennedy (1995) observed a nitrogenase activity in 2,4D induced root structures of *Azorhizobium*. Kennedy et al. (1997) reported that 10^7 azospirilla in wheat seedlings are active in nitrogen fixation. Sabry et al. (1997) showed that the wheat grown in pots and inoculated repeatedly with *A. caulinodans* colonized tissues at the point of emergence of lateral roots and appeared to contribute to significant amounts of fixed nitrogen to the plant.

Prayitno et al. (1998) conducted experiments to study interactions of rice seedlings with bacteria isolated from rice roots. Rice cv. Cairose, Pelde and LR-28 were root inoculated with strains of *Rhizobium leguminosarum* bv. *trifolii*, originally isolated from rice plants grown in Egypt or the Philippines. Different strains promoted, inhibited or had no effect on rice growth, and these effects were strongly influenced by the environmental growth conditions. They suggested that some of these rice-associating bacteria possess important genes that enhance their ability to intimately colonize niches on and within rice tissues, and to promote rice plant growth.

Yost et al. (1998) reported that methyl-accepting chemotaxis proteins (MCPs) play important roles in the chemotactic response of many bacteria. In this investigation oligonucleotide primers designed to amplify the conserved signalling domain of MCPs by PCR were used to identify potential MCP-encoding genes in *Rhizobium leguminosarum*. The results overall suggested that *R. leguminosarum* possessed mcp-like genes, and that at least some of them play a role in early steps in the plant-microbe interaction.

The probability of obtaining an effective associative system of cereals seems to be low at present. However, further intensification of research work on plant microbe interaction using tagged bacteria is required to trace bacterial path in the paranodules. Genetic tools such as PCR-based

marker analysis for the study of such parameters can provide an insight into the development of the symbiotic systems of cereals. This knowledge will aid in the development of technology that will harness a favourable plant-microbe interaction for sustainable wheat production.

REFERENCES

Arshad M., Frankenberger J.W.T. (1991): Microbial production of plant hormones. Plant Soil, *133*: 1–8.

Bottini R., Fulchieri M., Pearce D., Pharis R.P. (1989): Identification of gibberellins A1, A3 and iso-A3 in cultures of *Azospirillum lipoferum*. Plant Physiol., *90*: 45–47.

Cacciari D., Lippi D., Pietrosanti T., Petrosanti W. (1989): Phytohormone-like substances produced by single and mixed diazotrophic cultures of *Azospirillum* and *Arthrobacter*. Plant Soil, *115*: 151–153.

Cocking E.C. (2003): Endophyte colonization of plant roots by nitrogen fixing bacteria. Plant Soil, *252*: 169–175.

Cocking E.C., Al-Mallah M.K., Benson E., Davey M.R. (1990): Nodulation of non legumes by rhizobia. In: Gresshoff P.M., Roth E.C., Stacey G., Newton W.E. (eds.): Nitrogen Fixation-Achievements and Objectives. Chapman and Hall, New York: 813–823.

Crozier A., Arruda P., Jasmin J.M., Monterio A.M., Sandberg G. (1988): Analysis of indole-3 acetic acid and related indoles in culture medium and *Azospirillum brasilense*. Appl. Environ. Microbiol., *54*: 2833–2837.

Dobbelaere S., Croonenborghs A., Thys A., Broek A.V., Vanderleyden J. (1999): Phytostimulatory effect of *A. brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil, *212*: 155–164.

Gransee A., Wittenmayer L. (2000): Qualitative and quantitative analysis of water-soluble root exudates in relation to plant species and development. J. Plant Nutr. Soil. Sci., *163*: 381–385.

Hubbell D.H., Tien T.M., Gaskin M.H., Lee J. (1979): Physiological interaction in the *Azospirillum* grass root association. In: Vose P., Ruschel A.P. (eds.): Associative Symbiosis. CRC Press, the Netherlands: 1–6.

Hunter W.J. (1989): Indole-3-acetic acid production by bacteroids from soybean root nodules. Physiol. Plant., *76*: 31–36.

Jensen V. (1951): Notes on biology of *Azotobacter*. In: Proc. Soc. Appl. Bacteriol., *74*: 89–93.

Kennedy I.R. (1994): Auxin induced N_2 fixing associations between *Azospirillum brasilense* and wheat. In: Hegazi N.A., Fayez M., Monib M. (eds.): Nitrogen Fixation with Non-legumes. Am. Univ., Cairo: 513–523.

Kennedy I.R., Pereg-Gerk L.L., Wood C., Deaker R., Gilchrist, Katupitiya S. (1997): Biological nitrogen fixation in non-leguminous field crops. Facilitating the evolution of an effective association between *Azospirillum* and wheat. Plant Soil, *194*: 65–79.

Kennedy I.R., Tchan Y.T. (1992): Biological nitrogen fixation in non-leguminous field crops. Recent advances. Plant Soil, *141*: 93–118.

Lakshminarayana K. (1993): Influence of *Azotobacter* on nutrition of plant and crop productivity. In: Proc. Indian Nat. Sci. Acad., *B59*: 227–234.

Lee M., Breckenridge C., Knowles R. (1970): Effect of some culture conditions on the production of indole acetic acid and gibberellin like substances by *Azotobacter vinelandii*. Can. J. Microbiol., *16*: 1325–1330.

Miller J.H. (1972): Experiments of Molecular Genetics. Cold Spring Harbour Laboratory Press, New York: 352–355.

Narula N., Deubel A., Gransee A., Behl R.K., Merbach W. (2002): Impact of fertilizers on total microbiological flora in planted and unplanted soils of long term fertilization experiment. Arch. Acker- Pfl.-Bau Bodenkde, *48*: 171–180.

Narula N., Nijhawan D.C., Lakshminarayana K., Kapoor K., Verma O.P.S. (1991): Response of soil isolates and analogue resistant mutants of *Azotobacter chroococcum* on pearl millet [*Pennisetum typhoides* (Burn S & H)]. Indian J. Agr. Sci., *61*: 268.

Nieto K.F., Frankenberger W.T. (1989): Biosynthesis of cytokinins by *Azotobacter chroococcum*. Soil. Biol. Biochem., *21*: 967–972.

Nutman P.S. (1955): Study frame works for symbiotic nitrogen fixation. In: Newton W., Postgate J.R., Rodeiguej-Barrueco C. (eds.): Recent Development in Nitrogen Fixation. Acad. Press, London: 442–447.

Pathak D.V., Lakshminarayana K.L., Narula N. (1995): Analogue resistant mutants of *A. chroococcum* affecting growth parameters in sunflower (*Helianthus annus* L.) under pot culture conditions. In: Proc. Nat. Acad. Sci. (India), *18*: 203–206.

Prayitno J., Stefaniak J., Mclver J., Weinman J.J., Dazzo F.B., Ladha J.K., Barraquio W., Yanni Y.G., Rolfe R.G. (1998): Interactions of rice seedlings with bacteria isolated from rice roots. Aust. J. Plant Physiol., *26*: 521–535.

Sabry R.S., Saleh S.A., Batchelor C.A., Jones J., Jotham J., Webster G., Kothari S.L., Davey M., Cocking E.C. (1997): Endophytic establishment of *Azorhizobium caulinodans* in wheat. In: Proc. R. Soc. London Biol. Sci., *264*: 341–346.

Sriskandarajah S., Kennedy I.R., Yu D., Tchan Y.T. (1993): Effects of plant growth regulators on acetylene reducing associations between *A. brasilense* and wheat. Plant Soil, *153*: 165–177.

Tang Y.W., Bonner J. (1974): The enzymatic inactivation of IAA. Some characteristics of enzyme contained in pea seedling. Arch. Biochem., *13*: 11–25.

Tchan Y.T., Zeman A.M.M., Kennedy I.R. (1991): Nitrogen fixation in para-nodules of wheat roots by introduced free living diazotrophs. Plant Soil, *137*: 43–47.

Tien T.M., Gaskin M.H., Hubbel D.H. (1979): Plant growth substances produced by *A. brasilense* and their effect on the growth of pearlmillet (*Pennisetum americanum* L.). Appl. Environ. Microbiol., *37*: 1016–1024.

Umali-Garcia M., Hubbell D.H., Gaskins M.H., Dazo F.B. (1980): Association of *Azospirillum* with grass roots. Appl. Environ. Microbiol., *39*: 219–226.

Verma S., Kumar V., Narula N., Merbach W. (2001): Studies on *in vitro* production of antimicrobial substances by *A. chroococcum* isolates/mutants. J. Plant Dis. Prot., *108*: 152–165.

Yegorenkova I.V., Konnova1 S.A., Sachuk V.N., Ignatov V.V. (2001): *Azospirillum brasilense* colonisation of wheat roots and the role of lectin-carbohydrate interactions in bacterial adsorption and root-hair deformation. Plant Soil, *231*: 275–282.

Yost C.K., Rochepeau P., Hynes M.F. (1998): *Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins. Microbiology-Reading., *144*: 1945–1956.

Yu D., Kennedy I.R. (1995): Nitrogenase activity (C_2H_2 reduction) of *Azorhizobium* in 2,4D induced root structures of wheat. Soil Biol. Biochem., *27*: 459–462.

Zimmer W., Roeben K., Bothe H. (1988): An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*. Planta, *176*: 333–342.

Received on April 20, 2004

Corresponding author:

Dr. Neeru Narula, CCS Haryana Agriculture University, Department of Microbiology, Hisar-125004, India
fax: + 91 1662 234 952, e-mail: neerunarula@hau.ernet.in