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Identification of plant growth hormones produced by bacterial isolates from rice, wheat and kallar grass

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Abstract

Identification and quantification of the plant growth hormones indoleacetic acid and gibberellic acid, produced by plant growth-promoting rhizobacteria (PGPR), was carried out by using high-pressure liquid chromatography (HPLC). The PGPR strains were isolated from roots of rice, wheat and kallar grass and belonged to the genera *Azoarcus*, *Azospirillum*, *Enterobacter*, *Pseudomonas* and *Zoogloea*. For these studies, bacteria were grown in liquid nitrogen free malate (NFM) or combined carbon medium (CCM) containing tryptophan and combined nitrogen. Some *Azospirillum* strains produced both indoleacetic acid and gibberellic acid, while none of the *Enterobacter* spp. tested produced these growth hormones. *Azoarcus* strain K-1 produced higher amounts of gibberellic acid and *Azospirillum* strain ER-2 produced higher amounts of indoleacetic acid. Indoleacetic acid production increased with the age of bacterial cultures while a decrease in the production of gibberellic acid was noted at later growth stages. Pure indoleacetic acid and gibberellic acid in the concentration range 1–2 µg/ml increased root area and plant biomass of rice and wheat. Among PGPR strains tested, *Pseudomonas* 96–51 and its extract containing growth hormones increased root area, root length and plant biomass of rice and wheat.

Introduction

Plant growth-promoting rhizobacteria live freely in the soil or develop an association with plants, resulting in improvement of plant growth. Extensive work during the past few years has shown that inoculation of cereals and forage grasses with *Azospirillum brasilense* improved plant growth and productivity (Okon, 1985). These bacteria provide nitrogen by biological nitrogen fixation and produce

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plant growth hormones, which enhance root development and improve mineral and water uptake by the system (Umali-Garcia *et al.*, 1980; Okon and Kapulnik, 1986). Different bacterial genera have been shown to possess the property of growth hormone production. *Azotobacter* species were reported to produce plant growth hormones in N-free media (Brown and Walker, 1970; Barea and Brown, 1974; Gonzalez-Lopez *et al.*, 1986). *Azospirillum* strains are also known to produce plant growth hormones such as auxins, gibberellins and cytokinins (Tien *et al.*, 1979; Hartmann *et al.*, 1983). Production of indoleacetic acid (IAA) has been reported in *Bradyrhizobium*, *Acetobacter*, *Alcaligenes*, *Enterobacter*, *Pseudomonas* and *Xanthomonas* (Fett *et al.*, 1987; Koga *et al.*, 1991; Minamisawa and Fukai, 1991; Fuentes-Ramirez *et al.*, 1993; Kobayashi *et al.*, 1993).

Colorimetric methods have long been applied for the detection of IAA produced by plants and microorganisms (Gordon and Weber, 1951; Hartmann *et al.*, 1983; Bric *et al.*, 1991; Malik *et al.*, 1994). Paper chromatography, thin-layer chromatography (TLC) and bioassays have been used for the identification of auxins, gibberellins and cytokinins (Lee *et al.*, 1970; Gonzalez-Lopez *et al.*, 1986; Harari *et al.*, 1989). The indoles, gibberellins and cytokinins were also identified by HPLC (Tien *et al.*, 1979; Crouch *et al.*, 1992; Costacurta *et al.*, 1994).

We have isolated several bacterial strains from soil and roots of kallar grass, wheat and rice. Using colorimetric methods, production of IAA by some of these strains has been reported earlier (Malik *et al.*, 1994). In the present study, production of IAA and gibberellic acid (GA) by PGPR strains, and their identification and quantification by HPLC, is reported. The effect of synthetic/pure phytohormones IAA and GA, and crude bacterial extracts containing these hormones, has been studied on rice and wheat.

Materials and methods

Identification of plant growth hormones by HPLC

Bacterial strains

A list of bacterial strains studied for growth hormone production is given in Table 1. These strains have been isolated from kallar grass, *Atriplex*, rice and wheat, and belonged to the genera *Azoarcus*, *Azospirillum*, *Enterobacter*, *Pseudomonas* and *Zoogloea* (Bilal and Malik, 1987; Bilal *et al.*, 1990; Malik *et al.*, 1991, 1994).

Growth media and growth conditions

The bacterial cultures were grown in CCM (Rennie 1981) or dye-free NFM (Okon *et al.*, 1977) liquid medium containing 1 g/L of NH_4NO_3 and 100 mg/L of tryptophan. Tryptophan was added as a precursor in IAA synthesis, while biotin and para-aminobenzoic acid (PABA) were eliminated from both the

Table 1. Production of plant growth hormones gibberellic acid and indoleacetic acid by different PGPR strains

Bacterial strain	Plant sp.	Gibberellic acid (µg/ml)		Indoleacetic acid (µg/ml)	
		10 days	22 days	10 days	22 days
Azoarcus K-1	Kallargrass	10*	–	7	8
Zoogloea Ky-1	Kallargrass	–	–	–	1
Azospirillum N-4	Rice	2	–	4	5
Azospirillum WRRN23	Rice	4	–	2	6
Flavobacterium 96–57	Rice	2	ND	ND	7
Pseudomonas 96–51	Rice	2	–	ND	15
Azospirillum ER-2	Wheat	–	–	ND	22
Azospirillum ER-20	Wheat	–	–	7	14
Azospirillum ER-201	Wheat	–	–	4	14
Azospirillum ER-24	Wheat	–	–	9	12
Enterobacter ER-23	Wheat	–	–	–	–
Enterobacter QH7	Wheat	ND	–	ND	–
E. agglomerans AX-12	*Atriplex* sp.	ND	–	ND	–

Quantities are given as µg/ml of the culture filtrate.
*= Production was also estimated at 5 days; –, not present; ND, not determined.

media. *Azospirillum* strains and *Azoarcus* K-1 were grown in NFM and *Enterobacter*, *Flavobacterium*, *Pseudomonas* and *Zoogloea* strains were grown in CCM. The 400 ml of CCM/NFM liquid medium was inoculated with 4 ml of overnight-grown culture. The cultures were harvested by centrifugation at 10,000 rpm for 10 min after 10 and 22 days.

Extraction process and identification of growth hormones by HPLC

The cell-free liquid culture medium was concentrated by freeze-drying up to 80 ml and the pH of the extract was adjusted to 2.8 with concentrated hydrochloric acid. Extraction with ethyl acetate was carried out as described by Tien *et al.* (1979). The ethyl acetate extract was evaporated to dryness and the residue was dissolved in one millilitre ethanol.

The samples were analysed on HPLC using Turbochom software (Perkin-Elmer, USA). A Licosorb-C18 column was used for elution of growth hormones. Methanol : acetic acid : water (30 : 1 : 70) was used as mobile phase at the rate of 1.5 ml/min.

For identification, 20 µl samples, filtered through a 0.45 µm filter, were injected into the column. The growth hormones were identified on the basis of retention time of the standard IAA and GA by using a refractive index detector (RI). The concentration of each acid was calculated on the basis of peak height and peak area.

Effect of pure IAA and GA, PGPR and extracts of PGPR on rice and wheat growth

Seeds of wheat variety PAK-81 and rice variety NIAB-6 were germinated on water agar plates and 2–5-day-old seedlings were transferred to glass tubes

(20 × 2 cm, length × diameter) containing vermiculite and nitrogen containing Hoagland solution (¼ strength). One seedling was grown in each tube. Different-concentrations of pure IAA and GA (0.25–4.0 µg/ml) were applied both on rice and wheat. Four seedlings were used for each treatment. The tubes were kept in a controlled-temperature room at 28 ± 2 °C with 16 h light and 8 h dark. Light intensity was 400 µE/m^2. The plants without inoculation/hormone treatment were used as control. The plants were harvested after 3 weeks and compared for their root area, root length and plant dry weight.

Bacterial strains, namely *Azoarcus* K-1, *Azospirillum* N-4 and *Pseudomonas* 96–51, and their extracts, were applied on both rice and wheat. For rice 1 ml of cell suspension (10^8–10^9 cells/ml) was used to inoculate each plant. For growth hormones the concentration of the extract in ethanol was calculated on the basis of IAA contents in the extract and this extract was applied to make a final concentration of 1, 2 and 4 µg/ml in each tube.

To study the effect of inoculation of PGPR and application of 2 µg/ml ethyl acetate extract of PGPR strains on wheat, seeds of variety Inqlab were grown in plastic buckets (1.5 kg capacity), half-filled with vermiculite, Hoagland liquid medium (750 ml) was provided in every bucket. Five millilitres of respective cell suspension (10^8–10^9 cells/ml) were inoculated into each bucket. The extract concentration 2 µg/ml was adjusted in the buckets, in the same way as for rice. Two buckets were used for every treatment and five plants were grown in each bucket. The plants without inoculation and without extract application were used as control.

Estimation of root area and plant dry weight

The root area and root length were measured with the help of a computer and a scanner by using the Root Image Analysis program; this program has been developed by Washington State University Research Foundation, Washington State University, USA. For plant dry weight, plants were kept at 70°C until no change in their weight was noted.

Results and discussion

Identification of growth hormones by HPLC

Gibberellic acid and indoleacetic acid were identified by HPLC on the basis of retention time of pure GA and IAA, which was 8.0 and 10.8 min, respectively, under the assay conditions (Figure 1). Out of 13 PGPR strains which were studied for growth hormone production, 10 produced both IAA and GA (Table 1). *Azoarcus* K-1 produced higher amounts of GA (10 µg/ml) as well as IAA (8 µg/ml). *Azospirillum* strain ER-2 also produced higher amounts of IAA, followed by *Pseudomonas* strain 96–51 and *Azospirillum* ER-20 and ER-201. *Enterobacter*

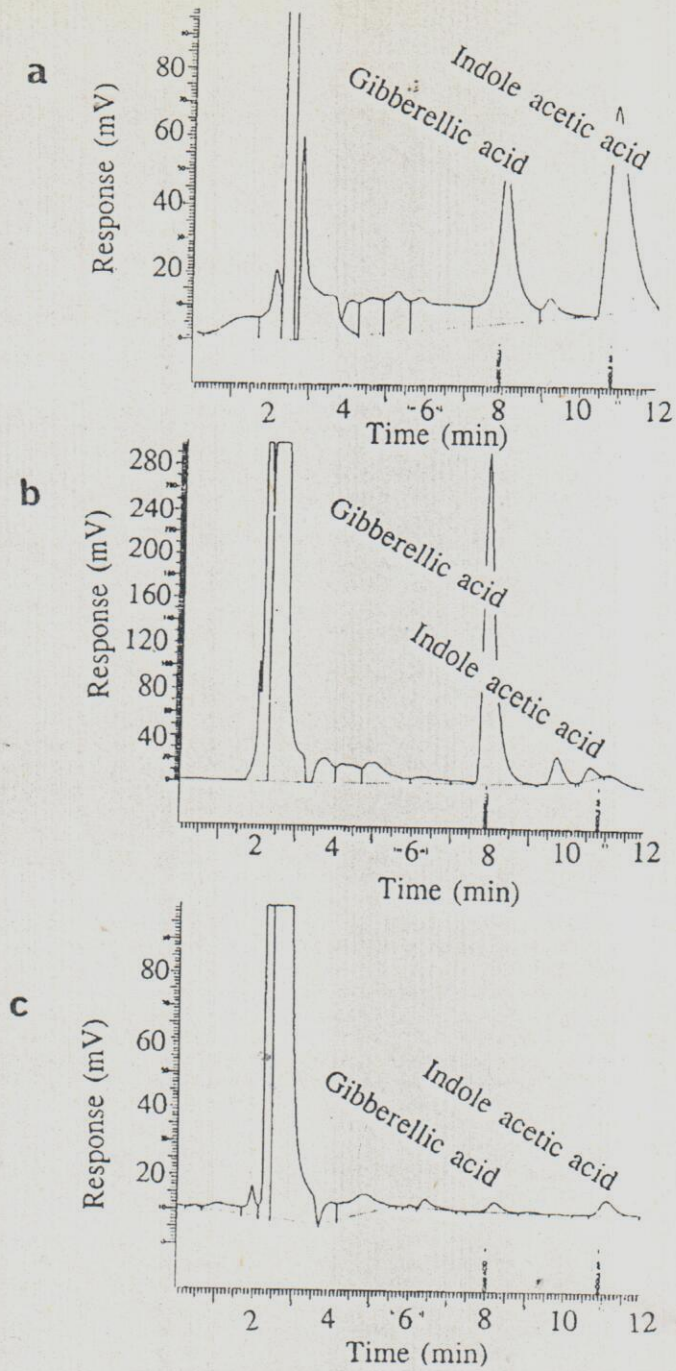


Figure 1. Identification of indoleacetic acid and gibberellic acid production by different PGPR strains by HPLC. Indoleacetic acid and gibberellic acid standards (**a**), *Azoarcus* K-1 (**b**) and *Flavobacterium* 96–57 (**c**).

strains QH7, ER-23 and Ax-12 did not produce IAA or GA (Table 1). GA production was higher at 10 days and IAA was maximum after 22 days of cultures growth (Table 1).

Azospirillum, *Azotobacter*, *Pseudomonas* and many other genera have already

been reported for production of phytohormomes (Reynders and Vlassak, 1979; Tien *et al.*, 1979). Production of IAA has been reported with and without tryptophan, under nitrogen fixing and non-fixing conditions and with and without combined nitrogen. Hartmann *et al.* (1983) described that there was no difference in IAA production by *Azospirillum lipoferum* under nitrogen-fixing and non-fixing conditions. It was also observed that IAA production increased in the presence of 0.1 mg/ml of tryptophan in the medium. In the present studies IAA and GA production was studied in the presence of combined nitrogen source because we have already reported that IAA production is increased in the presence of combined nitrogen (Malik *et al.*, 1994). However, Lee *et al.* (1970) reported that IAA production by *Azotobacter vinelandii* was reduced in the presence of combined nitrogen. Tien *et al.* (1979) reported that the combined nitrogen had little effect on the production of IAA. Gonzalez-Lopez *et al.* (1986) reported reduction in auxin production and increase in GA production (from 4 to 6 µg/ml) in the presence of combined nitrogen. Hartmann *et al.* (1983) described a 10-fold increase in excretion of IAA by *A. brasilense* in the presence of 10 mM ammonia.

In the present study in which HPLC was used, relatively lower levels of IAA production by PGPR strains were detected as compared to those reported previously (Malik *et al.*, 1994) in which the colorimetric method was employed. This indicates an overestimate of IAA production by the colorimetric method in which IAA conjugates like IAA alanine, IAA aspartate, IAA glycine and other compounds like indolebutyric acid, indolepyruvic acid and indolepropionic acid give a colour reaction with Salkowski reagent (Gordon and Weber, 1951).

The production of IAA was affected by the age of bacterial culture. GA production decreased with increase in incubation time (Table 1). Gonzalez-Lopez *et al.* (1986) also observed an increase in auxin production by bacterial culture for 3 days which became constant for up to 15 days. GA production was maximum after 7 days of growth and then became constant up to the 15th day.

It was observed that *Azospirillum* spp. produced up to 22 µg/ml of IAA in culture medium. Hartmann *et al.* (1983) observed up to 16 µg/ml of IAA in the culture medium. In the present study, considerable variation was observed in the production of GA by PGPR strains (Table 1). Vancura (1961) reported 20 µg/ml of GA3, and Brown and Burlingham (1968) described 0.03 µg GA3 equivalent/ml. *Azoarcus* strain K-1 produced up to 10 µg/ml of GA in culture medium. The other strains produced relatively lower amounts of GA. IAA and GA production was not detected in *Enterobacter* strains. Hennequin *et al.* (1966) also reported negative results for growth hormone production with some strains of *Azotobacter vinelandii* and *Azotobacter chroococcum*.

Effect of pure IAA and GA on rice and wheat growth

Different concentrations of GA and IAA were tested for their effect on rice and wheat growth. These hormones stimulated rice and wheat growth at their lower concentrations and inhibited growth at higher concentrations (Tables 2–5).

In rice 2 µg/ml of IAA and 1 µg/ml of GA produced a higher plant biomass

Table 2. Effect of indoleacetic acid on the growth of rice plant

Concentration of IAA (µg/ml)	Shoot dry weight (mg/plant)	Root dry weight (mg/plant)	Total plant dry weight (mg/plant)
0.0	23.1±2.7	5.2±1.2	28.5±2.5
0.5	18.6±3.3	6.0±1.0	24.6±1.6
1.0	21.4±3.7	7.1±1.1	28.8±2.2
2.0	25.1±1.5	8.7±0.9	34.0±3.5
4.0	21.5±2.1	7.3±0.9	29.0±2.9
8.0	21.1±2.3	6.9±1.0	27.8±3.6

The values given in the table are averages of six replicates; ± values indicate the standard deviation from means.

Table 3. Effect of different concentrations of gibberellic acid on rice growth

Concentration of GA (µg/ml)	Root area (cm^2)	Root length (cm)	Plant dry weight (mg)	Shoot length (cm)
Control	32^{bc}	264^{b}	270^{abc}	36^{d}
0.25	42^{a}	326^{a}	290^{ab}	43^{c}
0.50	39^{a}	331^{a}	280^{ab}	44^{c}
1.00	38^{ab}	330^{a}	330^{a}	48^{b}
1.50	28^{cd}	270^{b}	213^{c}	51^{b}
2.00	24^{d}	261^{b}	265^{bc}	52^{ab}
3.00	27^{cd}	288^{ab}	275^{abc}	48^{b}
4.00	25^{cd}	271^{b}	250^{bc}	56^{a}

Figures followed by the same letters are not significantly different at the 5% level as determined by the DMR test.

Table 4. Effect of different concentrations of indoleacetic acid on wheat growth.

Concentration of IAA (ppm)	Root area (cm^2)	Root length (cm)	Plant dry weight (mg)
Control	13.5^{a}	146^{b}	99^{ab}
0.25	12.4^{a}	112^{b}	79^{b}
0.50	ND	ND	92*
1.00	15.0^{a}	160^{ab}	91^{ab}
2.00	18.8^{a}	216^{a}	98^{ab}
3.00	19.8^{a}	217^{a}	107^{a}
4.00	13.9^{a}	175^{ab}	90^{a}

* Not included in statistical analysis. Figures followed by the same letters are not significantly different at the 5% level as determined by the DMR test

(Tables 2 and 3). Root area and root length were higher at 0.25–1.00 µg/ml of GA and the value for shoot height was higher at 4 µg/ml of GA (Table 3). In wheat a higher value for root area, root length and total plant biomass was recorded at 2–3 µg/ml of IAA. For GA, root area, root length and plant dry weight were higher at 0.5–1.5 µg/ml (Tables 4 and 5).

Similar effects of growth hormone application on pearl millet and *Panicum*

Table 5. Effect of different concentrations of gibberellic acid on wheat growth

Concentration of GA (µg/ml)	Root area (cm^2)	Root length (cm)	Plant dry weight (mg)	Shoot length (cm)
Control	11.7^{ab}	117^{b}	72^{b}	34^{a}
0.25	9.5^{ab}	113^{b}	65^{b}	34^{a}
0.50	14.5^{a}	167^{a}	90^{a}	31^{a}
1.00	11.5^{ab}	136^{ab}	72^{b}	33^{a}
1.50	14.0^{ab}	146^{ab}	73^{b}	31^{a}
2.00	11.3^{ab}	125^{ab}	59^{b}	33^{a}
3.00	9.0^{b}	116^{b}	59^{b}	33^{a}
4.00	8.8^{b}	100^{b}	40^{c}	31^{a}

Figures followed by the same letters are not significantly different at the 5% level as determined by the DMR test

miliaceum have been reported (Tien *et al.*, 1979; Harari *et al.*, 1989). In pearl millet total fresh plant biomass was higher at 0.01 µg/ml IAA in the medium as compared to when a 0.05 µg/ml concentration of IAA was used. For different concentrations of GA tested, the higher fresh biomass value was observed at 0.05 µg/ml (Tien *et al.*, 1979). It has been reported that root proliferation was maximum at 10^{-9} M IAA (Harari *et al.*, 1989).

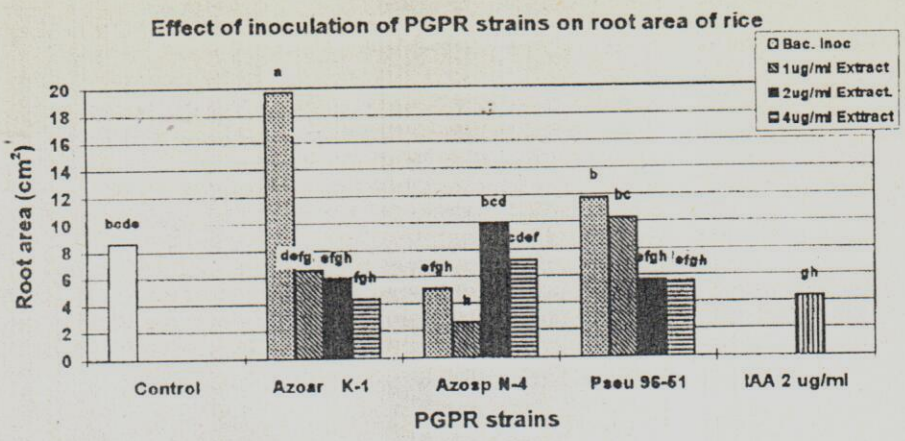
GA affected shoot formation as well as root area and root length of both rice and wheat (Tables 3 and 5). In rice the root formation was improved with a lower concentration of GA (1 µg/ml) but shoot length increased up to 4 µg/ml. There was a rapid increase in shoot length after 24 h of application of 1–4 µg/ml of GA which continued for 4–5 days (Table 3). In wheat the rate of increase in shoot length was slower as compared to that of rice plants, but wheat plants inoculated with GA were taller than the uninoculated wheat plants. The difference in shoot length of inoculated and uninoculated plants decreased with increase in the age of wheat plants (Table 5).

Effect of PGPR and extracts from PGPR strains on rice and wheat

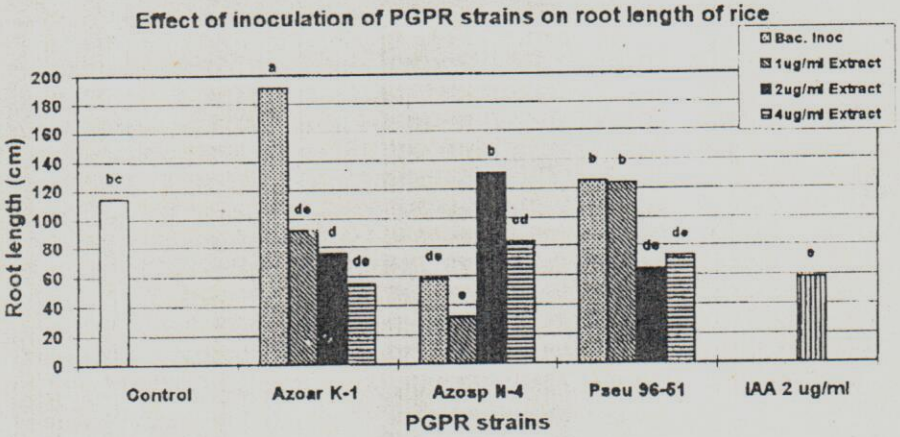
In rice, inoculation with *Azoarcus* K-1 increased the root area, root length and dry biomass as compared to uninoculated control (Figure 2). All three concentrations of extract of the *Azoarcus* K-1, equivalent to 1, 2 and 4 µg/ml of IAA, inhibited root area, root length and dry plant biomass. Inoculation with *Azospirillum* N-4 and its extracts, except 2 µg/ml, gave lower values for root area and root length as compared to control. The application of 2 µg/ml extract of *Azospirillum* N-4 increased the root area and root length. Plant dry weight values for N-4 inoculation and its extracts (1, 2 and 4 µg/ml) applications, were also lower than control. *Pseudomonas* strain 96–51 and its 1 µg/ml extract application increased root area, root length and plant dry weight while other concentrations (2 and 4 µg/ml) of the extract inhibited the growth (Figure 2).

For wheat, bacterial inoculation and 2 µg/ml extracts from different PGPR

(a)



(b)



(c)

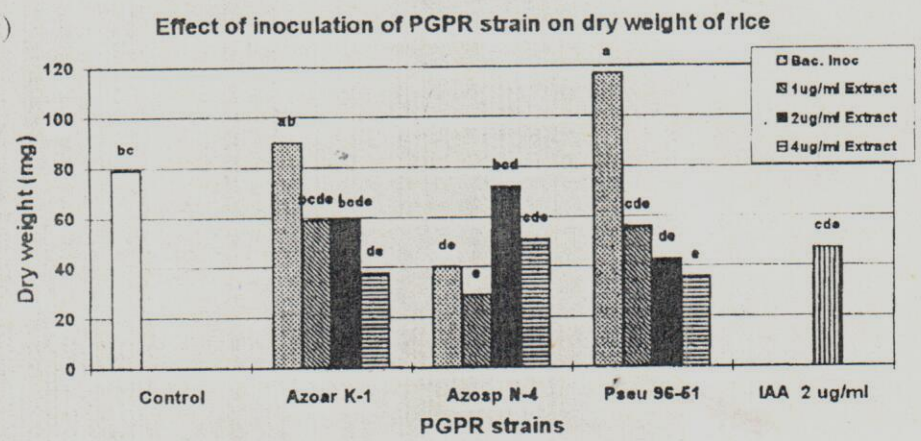


Figure 2. Effect of inoculation of PGPR strains and application of extracts from PGPR strains on root area (**a**), root length (**b**) and dryweight (**c**) of rice. Azoar = *Azoarcus* K-1, Azosp = *Azospirillum* N-4, Pseu = *Pseudomonas* 96-51. Bars followed by the same letters are not significantly different at the 5% level as determined by the DMR test.

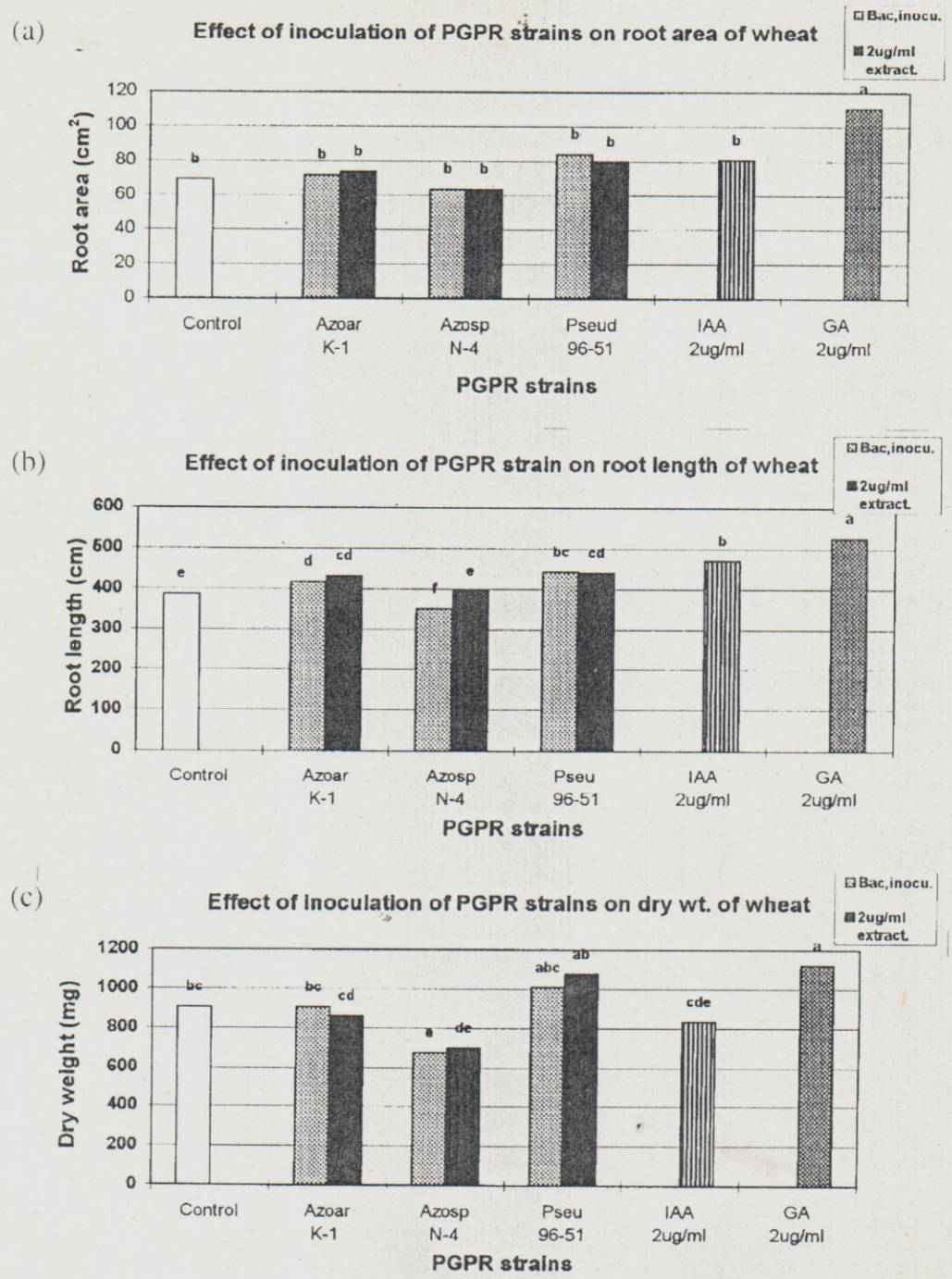


Figure 3. Effect of inoculation of PGPR strains and application of extracts from PGPR strains on root area (**a**), root length (**b**) and dry weight (**c**) of wheat. Azoar = *Azoarcus* K-1, Azosp = *Azospirillum* N-4, Pseu = *Pseudomonas* 96–51. Bars followed by the same letters are not significantly different at the 5% level as determined by the DMR test.

strains were applied (Figure 3). *Azoarcus* K-1 and its extract caused a minor increase in root area and root length as compared to control. The plant biomass values for K-1 inoculation and its extract were the same as for uninoculated control. For the treatment in which there was inoculation with *Azospirillum* N-4, the values for root area, root length and plant biomass were lower as compared to control. Application of the extract on wheat increased root length and inhibited root area and plant dry weight. Inoculation with *Pseudomonas* 96–51 and application of its extract increased root area, root length and plant biomass as compared to control (Figure 3).

The results of these studies in rice and wheat indicated that the bacterial inoculation or application of extract improved or inhibited plant growth. Similar effects have been observed by Tien *et al.* (1979) in pearl millet, and Harari *et al.* (1989) in *Panicum miliaceum*. The effects of plant growth may be due to single or combined action of growth hormones present in the extracts, and also depend on the relative concentrations of these hormones. Therefore, to understand the growth stimulation mechanism, in addition to correct identification and quantification of growth hormones, separation of growth hormones present in the extract, and study of different concentrations of each component is required. Work on these lines is in progress.

Conclusions

Azoarcus K-1, *Azospirillum* strains, *Zoogloea* Ky-1, *Flavobacterium* 96–57 and *Pseudomonas* 96–51 produced both indoleacetic acid and gibberellic acid in the liquid media. *Enterobacter* strains did not produce these hormones. A significant increase in root area as well as root and shoot length was observed in rice and wheat by application of lower concentrations (≤ 2 µg/ml) of indoleacetic acid and gibberellic acid. Inoculation with *Azoarcus* K-1 and *Pseudomonas* 96–51 increased root area, root length and total plant dry weight. The lower concentration of extracts from these PGPR strains which contained growth hormones (2 µg/ml from *Azospirillum* N-4 and 1 µg/ml from *Pseudomonas* 96–51) improved, and the rest of the higher concentrations inhibited plant growth.

Acknowledgements

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