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V.P. Shabayev · V.Yu. Smolin · V.A. Mudrik

Nitrogen fixation and CO_2 exchange in soybeans (*Glycine max* L.) inoculated with mixed cultures of different microorganisms

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Abstract N_2 fixation, photosynthesis of whole plants and yield increases in soybeans inoculated with mixed cultures of *Bradyrhizobium japonicum* 110 and *Pseudomonas fluorescens* 20 or *P. fluorescens* 21 as well as *Glomus mosseae* were found in pot experiments in gray forest soil carried out in a growth chamber. The effects of pseudomonads and vesicular-arbuscular (VA) mycorrhizal fungus on these parameters were found to be the same. Dual inoculation of soybeans with mixed cultures of microorganisms stimulated nodulation, nitrogenase activity of nodules and enhanced the amount of "biological" nitrogen in plants as determined by the ^{15}N dilution method in comparison to soybeans inoculated with nodule bacteria alone. An increased leaf area in dually infected soybeans was estimated to be the major factor increasing photosynthesis. *P. fluorescens* and *G. mosseae* stimulated plant growth, photosynthesis and nodulation probably due to the production of plant growth-promoting substances. Increasing phosphorus fertilizer rates within the range of 5–40 mg P 100 g^{-1} 1:1 (v/v) soil:sand in a greenhouse experiment led to a subsequent improvement in nodulation, and an enhancement of N_2 fixation and yield in soybeans dually inoculated with *B. japonicum* 110 and *P. fluorescens* 21. These indexes were considerably higher in P-treated plants inoculated with mixed bacterial culture than in plants inoculated with nodule bacteria alone.

Key words N_2 fixation · CO_2 exchange · *Glycine max* · *Bradyrhizobium japonicum* · *Pseudomonas fluorescens* · *Glomus mosseae* · Acetylene reduction activity · N dilution method

V.P. Shabayev (✉) · V.Yu. Smolin · V.A. Mudrik
Institute of Soil Science and Photosynthesis,
Russian Academy of Sciences, Pushchino,
Moscow Region, 142292 Russia

Introduction

Stimulation of plant growth in different non-legumes by plant growth-promoting rhizobacteria (PGPR) of the *Pseudomonas* genus has been reported (Alekseeva 1982; Kloepper et al. 1988; Mineev et al. 1991). Improved nodulation in common beans (Grimes and Mount 1984) and soybeans (Nishijima et al. 1988) inoculated with *Bradyrhizobium* and *Pseudomonas* has also been established. The largest clover yield and the best nodulation were observed when both *P. putida* and vesicular arbuscular (VA) mycorrhizal fungus *G. mosseae* were added to non-sterile soil (Meyer and Linderman 1986). Growth and yield responses in nodulated legume plants infected with VA mycorrhizal fungi have also been estimated (Barea et al. 1987; Vejsadová et al. 1989). Growth increases in nodulated and mycorrhizal legumes were accompanied by improved photosynthesis (Kucey and Paul 1982; Brown and Bethlenfalvay 1987), better nodulation and higher symbiotic N_2 fixation (Kucey and Paul 1982; Barea et al. 1987; Vejsadová et al. 1989), and enhanced P uptake in plants (Barea et al. 1987; Vejsadová et al. 1989).

In this study we assessed the comparative effects of PGPR of the *Pseudomonas* genus and VA mycorrhizal fungus *G. mosseae* on nodulated soybean plant growth, photosynthesis and N_2 fixation. The effects of increasing P fertilization rates on N_2 fixation and soybean yields were also studied on nodulated plants inoculated with pseudomonads.

Materials and methods

Pot experiments in a growth chamber

Two surface-sterilized seeds of *Glycine max* L. cv. Hadson were planted in plastic pots containing 1 kg soil. The plants were grown under light conditions of 30 klux with 12 h light and 12 h darkness with corresponding temperatures of 25° and 20 °C. The relative air humidity was maintained at 70–80%. $Ca(^{15}NO_3)_2$ with 17 atom% ^{15}N excess, KH_2PO_4 and K_2SO_4 was added to the soils at the rates of 8 mg NPK 100 g^{-1} soil, respectively, before sowing. Soils were watered up to 60% WHC with deionized water.

Experiment 1

In one treatment the 2-day-old seedlings were inoculated with a suspension of *B. japonicum* USDA 110 at a rate of 10^6 cells plant^{-1}. In another treatment a mixed culture of *B. japonicum* 110 and *P. fluorescens* 20 at a ratio of 5:1 was used. Treatments were replicated 4 times.

Experiment 2

In addition to the two treatments of experiment 1 a third treatment of mixed culture of *B. japonicum* 110 and *P. fluorescens* 21 was used in the same manner as above. In a fourth treatment *B. japonicum* 110 and *G. mosseae* were added. Mycorrhizal fungal inoculum, consisting of roots and adhering sand from oat previously infected with *G. mosseae* in sand nutrient culture, was placed in a layer 3 cm below the seeds. Treatments were replicated 6 times.

Pot experiment in a greenhouse

Two soybean plants cv. Mageva were grown in plastic pots containing 3 kg 1:1 soil:sand (v/v) mixture. P rates of 0, 5, 10, 20 and 40 mg 100 g^{-1} as KH_2PO_4 were added to the pots with a background of ^{15}N and K at rates of 20 mg 100 g^{-1} as $(^{15}NH_4)_2SO_4$ with 24, 3 atom% ^{15}N excess and K_2SO_4, respectively. In the first series, seeds were inoculated with *B. japonicum* 110 alone. In the second series, seeds were inoculated with a mixed bacterial culture of *B. japonicum* 110 and *P. fluorescens* 21 as described above. Treatments were replicated 5 times.

P. fluorescens 20 and *P. fluorescens* 21 were isolated from the oat rhizosphere. In experiment 2 in the growth chamber, transposon Tn5-labelled kanamycin- and streptomycin-resistant strains of pseudomonads were used. In all experiments root and adhering sand from non-mycorrhizal oat plants or autoclaved suspensions of bacteria were added to pots of uninoculated plants.

In order to estimate nodulation, pseudomonad populations in the roots and root infection by VA mycorrhizal fungus in the experiments in growth chamber, the plants were harvested from unlabelled ^{15}N pots at the beginning of pod setting. The populations of *P. fluorescens* 20 and *P. fluorescens* 21 in the rhizoplane were assessed by macerating the roots in 100 ml sterile water in a blender (Zvjagintchev et al. 1980) and by adding serial dilutions onto KB medium with kanamycin and streptomycin (200 µg/ml). The degree of mycorrhizal infection was assessed microscopically after clearing and staining 1-cm root samples taken prior to air drying with methyleneblue. Infection was expressed as a percentage of infected segments (Selivanov 1976).

In all experiments gray forest soil was obtained from a fallow field in the south of the Moscow region, Russia.

The total amount of N fixed in the plant was determined by the ^{15}N dilution technique (Rennie et al. 1978). Uninoculated soybean was used as the non-fixing reference plant.

Nitrogenase activity of nodules detached from roots was determined by acetylene reduction assay in a C_2H_2 atmosphere (10% v/v) immediately after harvesting. Acetylene reduction activity of intact plants on exposure in the C_2H_2 atmosphere (10% v/v) using propane as an internal standard in the greenhouse experiment was determined (Umarov 1976).

Photosynthetic activity was determined on the whole plants in the experiments in the growth chamber. To exclude CO_2 penetration from the root area and soil, the shoots were isolated from soil during measurement. The pot in which plants were growing was placed in a sealed chamber with a fan to provide a constant mix of air. Relative air humidity was maintained at a constant level by passage through a drier. Initially, CO_2 was injected into the chamber to a final concentration of 0.095%. The subsequent CO_2 depletion to 0.075% due to assimilation by plants during not more than 10 min of exposure was measured by an optical-acoustic CO_2 analyser "Infralyt" (Nema, Germany). Photosynthetic activity of the two plants in pot (F_1) and per unit (dm^2) leaf area (F_2) were calculated by Eqs. 1 and 2, respectively.

$$F_1 = \gamma \frac{\Delta CO_2 V 1000}{\Delta t\, 100} \ (\text{mg}\, CO_2\, \text{pot}^{-1}\, \text{h}^{-1}) \quad (1)$$

$$F_2 = \gamma \frac{\Delta CO_2}{\Delta t} \frac{V 1000}{100 S_L} \ (\text{mg}\, CO_2\, \text{dm}^{-2}\, \text{h}^{-1}) \quad (2)$$

where $\gamma = CO_2$ density (mg/dm^3), ΔCO_2 = difference of CO_2 concentration (v/v%), Δt = measuring time (h), V = chamber working capacity (dm^3) and S_L = leaf area (dm^2).

The N content and the atom% ^{15}N in plant material were determined as described elsewhere (Shabaev et al. 1991). The P content in plant material was determined after digestion of plant material in the mixture of H_2SO_4 and $HClO_4$ (10:1) using ascorbic acid as a reducer.

Results

Growth, N_2 fixation, CO_2 exchange and N yield of soybeans

Inoculation of soybeans with *B. japonicum* 110 alone in the absence of these bacteria in the study soil markedly increased the yield and N uptake by plants in the experiments carried out in a growth chamber (Table 1). The addition of *P. fluorescens* 20 or *P. fluorescens* 21 as well as *G. mosseae* to *B. japonicum* 110 enhanced grain weight by 14–17%. At the same time dual inoculation of soybeans with nodule bacteria and the above-mentioned microorganisms led to negligible or little change in total grain and shoot weight in both experiments compared with the application of nodule bacteria only. N uptake in nodulated soybeans infected with either *Pseudomonas* or *G. mosseae* was higher than in plants inoculated with only *B. japonicum* 110 by 13–28% and 18%, respectively.

The ^{15}N dilution method showed that mixed cultures of *B. japonicum* 110 and *P. fluorescens* 20 or *P. fluorescens* 21 increased the amount of N fixed in soybeans by 22–28% compared with pure culture of nodule bacteria (Table 1). The addition of *G. mosseae* induced N_2 fixation enhancement by 30%. However, the difference between the amount of atmospheric N in nodulated plants inoculated with each pseudomonad or mycorrhizal fungus was not significant.

Yield and N uptake increases in nodulated soybeans treated with bacteria of the *Pseudomonas* genus or the VA mycorrhizal fungus *G. mosseae* corresponded with significant nodule weight enhancement, ranging from 12 to 17% in comparison with the plants inoculated with the nodule bacteria alone (Table 1). *P. fluorescens* 20 and *P. fluorescens* 21 populations on plant roots ranged from $1.5 \cdot 10^4$ to $4.5 \cdot 10^4$ colony-forming units g^{-1} dry root, respectively. The incidence of *G. mosseae* infection in mycorrhizal roots was 47%.

Inoculation of soybeans with *Bradyrhizobium* alone did not always cause a significant rise in leaf area and CO_2 exchange of total leaf area (F_1) (Table 2). However, soybeans inoculated with each pseudomonad had a significantly larger leaf area and heightened CO_2 exchange per whole plant (F_1), except on the first measurement, in comparison with soybeans grown with nodule bacteria only. On average through the growth period photosynthetic ac-

Table 1 Soybean weight and N uptake by plants inoculated with different microorganisms. Pot experiments were carried out in a growth chamber

Treatment	Dry matter		Raw matter Nodules	N uptake Grain and shoots	N fixed in plants
	Grain (g pot^{-1})	Grain and shoots (g pot^{-1})	(g pot^{-1})	(mg pot^{-1})	(mg pot^{-1})
Experiment 1					
Uninoculated	2.09	5.02	–	97	–
B. japonicum 110	3.18	6.28	1.24	236	116
B. japonicum 110+ *P. fluorescens* 20	3.73	6.45	1.45	266	148
LSD (0.05)	0.49	0.58		27	15
Experiment 2					
Uninoculated	1.86	3.29	–	86	–
B. japonicum 110	2.21	3.99	1.03	101	27
B. japonicum 110+ *P. fluorescens* 20	2.57	4.40	1.18	126	34
B. japonicum 110+ *P. fluorescens* 21	2.59	4.44	1.20	129	33
B. japonicum 110+ *G. mosseae*	2.51	4.21	1.15	119	35
LSD (0.05)	0.27	0.41	0.08	16	6

Table 2 Photosynthesis activity in soybean plants inoculated with different microorganisms. Pot experiments were carried out in a growth chamber (S_L leaf area, dm^2, F_1, F_2 photosynthetic activity, mg CO_2 pot^{-1} h^{-1} and mg CO_2 dm^{-2} h^{-1}, respectively)

Treatment	14 days			28 days			38 days (budding-flowering)			Means[a]	
	S_L	F_1	F_2	S_L	F_1	F_2	S_L	F_1	F_2	F_1	F_2
Experiment 1											
Uninoculated	2.8	29	10	4.2	49	12	4.9	65	13	47	12
B. japonicum 110	3.0	32	11	4.4	55	12	6.1	70	11	52	12
B. japonicum 110+ *P. fluorescens* 20	3.4	39	12	5.7	66	12	6.8	84	12	63	12
LSD (0.05)	0.4	0.5	3	0.8	9	3	0.5	6	3	7	2
Experiment 2											
Uninoculated	1.4	20	14	3.2	44	14	4.2	64	15	43	14
B. japonicum 110	1.9	20	10	4.1	60	15	4.3	70	13	50	13
B. japonicum 110+ *P. fluorescens* 20	2.0	20	10	5.0	70	14	6.1	85	17	58	14
B. japonicum 110+ *P. fluorescens* 21	2.1	22	11	5.2	73	14	5.2	83	16	59	14
B. japonicum 110+ *G. mosseae*	2.0	20	10	4.3	65	15	5.1	81	16	55	14
LSD (0.05)	0.3	3	2	0.6	4	3	0.7	10	3	5	3

[a] Data are means calculated during the whole period of plant growth

tivity (F_1) of nodulated and *P. fluorescens* 20 inoculated plants was increased by 21% in the first experiment while application of *P. fluorescens* 20 or *P. fluorescens* 21 in the second experiment enhanced F_1 by 16–18%. The use of nodule bacteria with mycorrhizal fungus increased the mean photosynthesis of whole plants to a lesser extent, by 10%. Dual inoculation of soybeans with nodule bacteria and with each tested microorganism did not raise photosynthetic activity per unit leaf area (F_2), except for the third measurement in the second experiment.

Effect of increasing P levels on N_2 fixation, growth and N and P yield of soybeans

Nodulation was also enhanced by *P. fluorescens* 21 in the pot experiment carried out in the greenhouse (Table 3). These indexes depended considerably on P fertilizer level. P-treated soybeans with or without *P. fluorescens* 21 had more root nodules than non-P-treated plants. The number of nodules for each bacterial treatment was maximal at the 20-mg P rate. Only at the 20- and 40-mg P levels had significantly more nodules developed on the roots of plants grown with a mixed bacterial culture compared to nodule bacteria alone.

Table 3 Number, weight and nitrogenase activity of root nodules at the beginning of pod setting of soybeans (after 85 days) inoculated with *B. japonicum* 110 alone and with *B. japonicum* 110 and *P. fluorescens* 21. Pot experiment was carried out in a greenhouse

Treatment	P applied (mg 100 g^{-1} substrate)	Number of nodules (n pot^{-1})	Weight of raw nodules (g pot^{-1})	Nitrogenase activity of nodules (μm C_2H_4 h^{-1} pot^{-1})[a]
B. japonicum 110	0 (control)	45	3.1	0.01±0.01
B. japonicum 110+ *P. fluorescens* 21		38	3.1	0.01±0.00
B. japonicum 110	5	56	3.2	0.16±0.03
B. japonicum 110+ *P. fluorescens* 21		56	3.6	1.60±0.44
B. japonicum 110	10	59	3.2	1.12±0.15
B. japonicum 110+ *P. fluorescens* 21		58	4.0	1.50±0.13
B. japonicum 110	20	69	3.8	1.10±0.27
B. japonicum 110+ *P. fluorescens* 21		106	4.5	1.65±0.25
B. japonicum 110	40	55	3.9	5.85±0.68
B. japonicum 110+ *P. fluorescens* 21		66	4.9	3.32±2.11
LSD (0.05)		10	0.4	

[a] Means±SE of five replications

Table 4 Nitrogenase activity of intact soybean plants inoculated with *B. japonicum* 110 alone and with *B. japonicum* 110 and *P. fluorescens* 21. Pot experiment was carried out in a greenhouse

Treatment	P applied (mg 100 g^{-1} substrate)	24 days	47 days	69 days
		(μM C_2H_4 pot^{-1} h^{-1})[a]		
B. japonicum 110	0 (control)	0.389±0.060	0.101±0.020	0.001±0.00
B. japonicum 110+ *P. fluorescens* 21		0.417±0.129	0.093±0.009	0.001±0.00
B. japonicum 110	20	0.373±0.042	0.132±0.014	0.008±0.001
B. japonicum 110+ *P. fluorescens* 21		0.432±0.009	0.149±0.002	0.019±0.009

[a] Means±SE of three replications

Applications of 5 and 10 mg P 100 g^{-1} substrate did not lead to a change in nodule weight in singly inoculated plants. The highest nodule weights in soybeans inoculated only with *B. japonicum* 110 were found at 20 and 40 mg P. The nodule weights in soybeans infected with mixed bacterial culture were significantly higher than those in plants inoculated with only nodule bacteria at corresponding P levels. The nodule weights in dually infected plants were considerably increased by the first phosphorus dose, rising further after doubling and reaching a maximum at the 40-mg P level.

In the absence of phosphate fertilization the number and weight of nodules were the same in both bacterial treatments, and the nitrogenase activity of root nodules was extremely low. P fertilization greatly stimulated the nitrogenase activity of root nodules. Nitrogenase activity increased with increasing dose of phosphorus, in both singly and dually inoculated plants. In the treatment with *B. japonicum* 110 alone, nitrogenase activity of nodules began to increase at the second P rate in spite of the fact that the nodule weights did not grow. In contrast, both nitrogenase activity of nodules and their weight were significantly enhanced by mixed bacterial cultures at the first P level. It is interesting to note that N_2 fixation in dually infected plants at the 5-mg P rate was 1.4 times greater than those in plants inoculated only with nodule bacteria and receiving twice as much P. The subsequent doubling in phosphorus rates up to 20 mg was not accompanied by an enhancement in the nitrogenase activity of nodules in soybeans inoculated with either pure culture *B. japonicum* 110 or mixed bacterial culture, in spite of the increase in nodule weights. Only the highest level of phosphate fertilization in each of the bacterial treatments led to a further significant increase in nodule nitrogenase activity. The enhanced level of N_2 fixation at this P level in the treatment with *B. japonicum* 110 and *P. fluorescens* 21, in contrast to the application of only *B. japonicum* 110, was due to an increase in the nodule weight.

A higher level of nitrogenase activity in intact soybean plants grown with mixed bacterial culture was also found throughout for the entire growth period at the 20-mg P rate (Table 4).

In the absence of phosphate fertilization and at the 5-mg P level there were no significant differences in shoot

Table 5 Shoot dry weight, N and P uptake by soybeans inoculated with *B. japonicum* 110 alone and with *B. japonicum* 110 and *P. fluorescens* 21. Pot experiment was carried out in a greenhouse

Treatment	P applied (mg 100 g^{-1} substrate)	Dry matter (g pot^{-1})	N uptake (mg pot^{-1})	Atom ^{15}N excess[a]	^{15}N fertilizer uptake (mg pot^{-1})	P uptake (mg pot^{-1})
B. japonicum 110	0 (control)	11.6	317	3.09±0.33	40	62
	5	11.9	408	3.52±0.29	50	69
	10	10.5	389	3.62±0.30	58	88
	20	11.8	378	3.45±0.17	54	118
	40	12.6	411	3.50±0.40	59	143
B. japonicum 110+	0 (control)	10.6	381	3.47±0.18	55	70
P. fluorescens 21	5	12.6	425	3.22±0.37	57	71
	10	13.0	447	2.77±0.33	56	100
	20	14.3	448	3.01±0.22	57	115
	40	15.3	483	2.81±0.21	56	177
LSD (0.05)		1.8	49		4	18

[a] Means±SE of three replications

weight of singly and dually inoculated soybeans (Table 5). At the other corresponding P levels the shoot weights of plants grown with mixed bacterial culture were enhanced by 21–24%, respectively, compared with soybeans inoculated with nodule bacteria only.

With doubling amounts of P fertilization the yield of soybeans was not significantly raised by *B. japonicum* 110 alone. However, a subsequent increase in plant weight with doubling amounts of phosphorus, which reached a maximum at the highest P level in dually inoculated soybeans, was found.

Atom% ^{15}N excesses in shoot were decreased by mixed bacterial culture compared to only nodule bacteria at all P levels except the first one and control plants (Table 5). In dually infected plants these values were considerably lower in the range of 10–40 mg P 100 g^{-1} substrate than in the control. In contrast, atom% ^{15}N excesses in singly inoculated soybeans were enhanced or tended to increase under phosphate fertilization.

Increasing P rates did not affect soybean yields, but P application led to the enhancement of both total N and ^{15}N fertilizer uptakes by the shoot of plants inoculated with *Bradyrhizobium* only (Table 5). The subsequent doubling of P fertilization rates did not influence N uptake in singly infected soybeans but increased N uptake as well as increased yield in plants inoculated with a mixed culture of *B. japonicum* 110 and *P. fluorescens* 21 while not affecting the ^{15}N uptake in soybeans.

The P content of soybeans (mg pot^{-1}) increased with doubling phosphate levels in each microbial treatment (Table 5). The addition of *P. fluorescens* 21 to *B. japonicum* 110 did not have a significant influence on the P content of plants at all corresponding P rates except the highest one of 40 mg P 100 g^{-1} substrate. P uptake in plants grown with mixed bacterial culture and receiving this rate of phosphorus was significantly more than in plants infected with nodule bacteria alone.

Discussion

Dual inoculation of soybeans with *B. japonicum* 110 and PGPR of the *Pseudomonas* genus improved plant growth and photosynthesis, favoured better nodulation, and increased N_2 fixation and yield. This effect was the same as a simultaneous plant infection with nodule bacteria and VA mycorrhizal fungus *G. mosseae*. The data obtained by the ^{15}N dilution method show that growth and yield responses in nodulated soybeans to inoculation with bacteria of the *Pseudomonas* genus or *G. mosseae* resulted from the increase in "biological" nitrogen in plants. It seems likely that enhanced photosynthesis (F_1) in whole plants dually inoculated with nodule bacteria and each of the pseudomonads led to improved fixed C utilization by nodules that occured in nodulated and mycorrhizal legumes (Kucey and Paul 1982). An increase in the translocation of photosynthates to nodules was estimated to be the major factor enhancing N_2 fixation rates (Kretovich 1987). Since in our experiments the photosynthetic activity per unit (dm^2 of leaf area (F_2) did not increase in dually infected plants, the positive effect of PGPR and VA mycorrhizal fungus on plant growth and photosynthesis consisted of an increase in the leaf area that was probably conditioned by phytohormones produced by these microorganisms (Selivanov 1976; Přikryl et al. 1985) and by nutrient uptake increase in the plants as well (Meyer and Lindeman 1986).

One also may suggest the reverse sequence of these processes. Bacteria of the *Pseudomonas* genus and *G. mosseae* led to better nodulation with higher nitrogenase activity of nodules owing to the above-mentioned properties of these microorganisms, thus creating a large C sink, which could lead to improved CO_2 fixation rates of plants. Symbiotic N_2 fixation in legumes not only depends on supplying the photosynthates to nodules but also influences the photosynthesis and the distribution of photosynthates in legumes (Kretovich 1987; Imsande 1988). Our studies have shown improved nodulation and N_2 fixation in soybeans dually inoculated with *B. japonicum* 110 and each strain of *P. fluorescens* or *G. mosseae*. Enhanced

nodulation in mycorrhizal soybeans (Vejsadová et al. 1989) and in common beans and soybeans inoculated with bacteria of the *Pseudomonas* genus has been also established (Grimes and Mount 1984; Nishijima et al. 1988).

Our studies carried out in the greenhouse demonstrate the absence of weight gain in plants inoculated with only *B. japonicum* 110 in spite of the enhancement of nodule weight and their nitrogenase activity with doubling P. However, plant weight, nodulation and N_2 fixation with doubling P levels were significantly enhanced by nodule bacteria and pseudomonads compared with nodule bacteria alone. When applied with nodule bacteria, pseudomonads began to stimulate nodulation and N_2 fixation to a greater extent, while significantly increasing plant weight at minimal P levels. At the same time there was no difference in P uptake in singly and dually infected plants at any of the corresponding P rates, except the highest one. Enhanced nodulation and growth in plants inoculated with mixed bacterial culture of *Bradyrhizobium* and *Pseudomonas* may also be related to the formation of plant growth-promoting substances by pseudomonads.

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