Microbiology, Vol. 74, No. 1, 2005, pp. 46–53. Translated from Mikrobiologiya, Vol. 74, No. 1, 2005, pp. 55–62.

EXPERIMENTAL ARTICLES

Auxin Production by Bacteria Associated with Orchid Roots

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Received March 16, 2004

Abstract—Bacteria associated with the roots of greenhouse tropical orchids were shown to produce indole-3-acetic acid (IAA) and to excrete it into the culture liquid. The presence and activity of IAA were demonstrated colorimetrically, by thin-layer chromatography, and by biotests. The associated bacteria varied in their ability to excrete indole compounds (1–28 μg/ml nutrient broth). Addition of tryptophan to the growth medium enhanced phytohormone production. Upon addition of 200 μg/ml tryptophan, the bacteria isolated from *Dendrobium moschatum* roots (*Sphingomonas* sp. 18, *Microbacterium* sp. 23, *Mycobacterium* sp. 1, *Bacillus* sp. 3, and *Rhizobium* sp. 5) produced 50.2, 53.1, 92.9, 37.6, and 60.4 μg IAA/ml, respectively, while the bacteria isolated from *Acampe papillosa* roots (*Sphingomonas* sp. 42, *Rhodococcus* sp. 37, *Cellulomonas* sp. 23, *Pseudomonas* sp. 24, and *Micrococcus luteus*) produced 69.4, 49.6, 53.9, 31.0, and 39.2 μg IAA/ml. Auxin production depended on cultivation conditions and on the growth phase of the bacterial cultures. Treatment of kidney bean cuttings with bacterial culture liquid promoted formation of a "root brush" with a location height 7.4- to 13.4-fold greater than the one in the control samples. The ability of IAA-producing associated bacteria to act as stimulants of the host plant root development is discussed.

Key words: orchids, associated bacteria, indole-3-acetic acid (IAA).

Bacteria colonizing plant roots are in many cases beneficial for plant growth, development, and productivity. The synthesis of phytohormones (auxins, gibberellins, and cytokinins) by such associated microorganisms is believed to be one of the major forms of host plant–microbial interactions [1, 2].

Among auxins, the phytohormones widely present in nature, indole-3-acetic acid (IAA) is the most active. It is responsible for division, expansion, and differentiation of plant cells and tissues. Promotion of the processes of xylem and root formation is the most pronounced among the diverse effects of this phytohormone [3].

The ability for auxin production has been found among various phototrophic and heterotrophic bacteria, not only pathogenic or symbiotic species but also free-living microorganisms not associated with plants [1, 2, 4]. IAA synthesis was reported for the representatives of the genera *Pseudomonas, Agrobacterium, Azospirillum, Azotobacter, Alcaligenes, Enterobacter, Acetobacter, Rhizobium, Bradyrhizobium, Bacillus, Arthrobacter, Herbaspirillum, Xanthomonas, Klebsiella, Methylobacterium, Methylovorus, Aminobacter*, and *Paracoccus* [1, 2, 4–10]. *Achromobacter, Flavobacterium, Rhodococcus, Acinetobacter, Corynebacterium*, and *Micrococcus* also produce this plant growth stimulator [11–13].

Wilkinson and coworkers isolated from the roots of wild terrestrial Australian orchids associated endotrophic bacteria, some of which (belonging to the genera *Pseudomonas, Bacillus*, and *Xanthomonas*) produced IAA [9]. We also earlier demonstrated abundant bacterial colonization of the substrate and aerial roots of tropical terrestrial and epiphytic orchids grown in a greenhouse [14, 15]. Our research in this field was stimulated by fragmentary data on the ability of orchid-associated bacteria to produce phytohormones and on the effect of such microorganisms on plant development under natural and artificial conditions of cultivation. The aim of the present work was to study the ability of bacteria colonizing the roots of greenhouse tropical orchids to produce auxins.

MATERIALS AND METHODS

In the present work, 42 bacterial strains were used that had been previously isolated by us [14, 15] from the rhizoplane of greenhouse orchids: the ground *Calanthe vestita* Lindl. var. *rubro-oculata* and the epiphytic *Acampe papillosa* (Lindl.) Lindl. and *Dendrobium moschatum* (Buch.–Ham.) Swartz. Bacteria were grown in liquid cultures on nutrient broth and on synthetic Czapek medium with glucose. For the study of the effect of tryptophan on IAA production, 50, 100, or 200 μg/ml tryptophan was added to the Czapek medium. A synthetic medium was chosen for this purpose because it did not contain any substances of unde-

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0026-2617/05/7401-0046

fined composition or growth factors, which could have directly or indirectly affected IAA synthesis. Moreover, the medium contained nitrate as the nitrogen source since auxin synthesis is known to be inhibited by ammonium ions [8, 10]. We also took into consideration reports on auxin production by bacterial cultures reaching its maximum at 30°C under intense aeration [5, 9, 16]. In the present work, the inoculum was grown for two days on the same medium as was used for the experiment and then introduced in a dose of 5–10% into 20 ml of medium in 50-ml flasks. Cultivation was performed in the dark at 30°C on a shaker (180 rpm). The optical density (OD_{540}) of the cell suspension was determined on an FEK 56M photoelectric colorimeter in 0.5-cm cuvettes. Uninoculated medium (nutrient broth or Czapek medium) was used as the control.

The concentration of IAA was determined colorimetrically on an Ultrospec II spectrophotometer (LKB Biochrom, England) with the Sal'kovskii reagent according to the common procedure [17]. Uninoculated medium with the reagent added was used as the control.

The biological activity of IAA was evaluated using a biotest determining the stimulating effect on the rooting of kidney bean (*Phaseolus vulgaris*) cuttings as described earlier [18]. Heteroauxin solution (50 μg/ml IAA (ICN, Germany)) and bacterial culture liquids (CLs) were used in the experiment. Bacterial cultures were grown on Czapek synthetic medium with 200 μg/ml tryptophan until the maximum IAA concentration in the medium was reached. Bacterial cells were removed from CL by centrifugation. Cuttings immersed in sterile tap water or in uninoculated Czapek medium were used as the control. The experiment was performed during the spring–summer period. The height of the stem on which the roots were formed was used as the criterion of growth stimulant activity.

Extraction and identification of indole compounds by thin-layer chromatography were performed in accordance with published procedures [5, 18]. Chromatography was performed in a chloroform–ethyl acetate–formate system (50 : 40 : 10 vol/vol/vol) on silica gel–covered plates with UV indicator (Sorbfill, Russia). The plates were subsequently investigated under UV illumination to mark the zones that were close in R_f to the position of the standard, IAA (ICN, Germany). For better separation, the spots were eluted with ethyl acetate for secondary chromatography in another solvent system, also used for identification of indole compounds, isopropanol–10% ammonium hydroxide in a ratio of 10 : 2 (vol/vol).

All experiments were performed in three to five replicates. Experimental data were treated using accepted methods of mathematical statistics.

RESULTS

A full-value natural medium, nutrient broth, was chosen for determining bacterial ability to synthesize IAA and for selection of the most active producers. Our experiments revealed both strains synthesizing ca. 10–28 μg/ml and less active strains producing about 1 μg/ml (Table 1). Bacteria belonging to one genus produced different amounts of auxins (Table 1). Variation in phytohormone production within a bacterial genus and even within a species is in agreement with data presented by other authors [8, 19].

The most active IAA producers isolated in this work belonged to the genus *Pseudomonas*. Many pseudomonads isolated from soil and the rhizosphere are known to be active IAA producers [8, 11]. Bacteria of other genera, *Rhodococcus, Rhizobium, Arthrobacter, Bacillus, Xanthomonas, Flavobacterium, Micrococcus,* and *Mycobacterium,* were also capable of IAA synthesis; this is in accordance with published data [2, 11–13].

The highest level of IAA accumulation in the culture liquid of the bacteria studied was found during the early or late stationary growth phase. The results on the dynamics of growth and IAA production by some of the active bacterial cultures are presented in Fig. 1. Other researchers have obtained similar results with bacteria of the genera *Pseudomonas, Azospirillum, Arthrobacter*, and *Agrobacterium* [5, 6, 16, 19].

It is known that tryptophan is the main precursor in IAA synthesis by microorganisms and enhances the production of this phytohormone [6–8, 13, 16]. In order to determine the effect of this amino acid on auxin synthesis by the bacteria studied, the most active microbial IAA producers isolated from substrate and aerial orchid roots were selected. Experimental results are presented in Table 2 (for more convenient comparison with published data, the amount of IAA produced was recalculated per milliliter medium and unit OD).

Bacterial IAA synthesis on Czapek medium without tryptophan was shown to be substantially lower than on nutrient broth (Tables 1, 2). On Czapek medium, IAA production was 0.3–6.6 μg/ml, while on nutrient broth it was 4.8–28.1 μg/ml. These results indicate that the synthetic medium lacked the nutrients required for optimal growth and development of bacterial cultures and for IAA biosynthesis.

An increased tryptophan concentration was shown to enhance the amount of IAA excreted into the culture liquid (Table 2); at 50 μg/ml, however, tryptophan did not enhance IAA biosynthesis by certain bacteria, e.g., *Microbacterium* sp. 23, *Rhizobium* sp. 5, *Bacillus* sp. 12, and *Bacillus* sp. 3. At 200 μg/ml, the stimulatory effect of tryptophan was the highest; bacteria isolated from *D. moschatum* roots (*Sphingomonas* sp. 18, *Microbacterium* sp. 23, *Mycobacterium* sp. 1, *Bacillus* sp. 3, and *Rhizobium* sp. 5) synthesized 50.2, 53.1, 92.9, 37.6, and 60.4 μg IAA/ml, respectively, and bacteria isolated from *A. papillosa* (*Sphingomonas* sp. 42, *Rhodococcus* sp. 37, *Cellulomonas* sp. 23*, Pseudomonas* sp. 24, and *Micrococcus luteus*) produced 69.4, 49.6, 53.9, 31.0, and 39. 2 μg IAA/ml. Generally, introduction of 200 μg/ml tryptophan into the growth

Table 1. IAA production by associated bacteria on nutrient broth

Orchid species	Root type	Bacterial culture	IAA, μg/ml	IAA, μg per unit OD
Calanthe vestita	Substrate	*Mycobacterium* sp. 1	2.37*	1.9
		Arthrobacter sp. 4	1.18	2.7
		Bacillus sp. 12	2.33	1.0
		Pseudomonas sp. 5	4.70	2.2
		Pseudomonas sp. 10	5.60	4.8
		Pseudomonas sp. 22	6.60	8.5
Dendrobium moschatum	Aerial	*Xanthomonas* sp. 16	1.64	1.2
		Xanthomonas sp. 21	2.07	1.4
		Bacillus sp. 24	3.62	2.7
		Rhodococcus sp. 25	0.50	1.1
		Sphingomonas sp. 18	28.1	17.7
		Microbacterium sp. 23	17.4	5.0
		Flavobacterium sp. 17	1.50	3.3
	Substrate	*Pseudomonas* sp. 50	2.30	1.8
		Bacillus sp. 3	6.30	7.5
		Mycobacterium sp. 1	7.50	2.8
		Rhizobium sp. 5	22.40	11.7
		Pseudomonas sp. 60	2.66	2.5
		Pseudomonas sp. 65	3.10	1.5
		Rhodococcus sp. 3	0.81	0.5
		Pseudomonas sp. 60	1.48	0.8
		Rhodococcus sp. 6	3.37	2.6
		Rhodococcus sp. 8	3.70	2.5
		Pseudomonas sp. 62	10.25	17.1
		Pseudomonas sp. 63	2.96	3.0
		Pseudomonas sp. 64	1.55	2.0
Acampe papillosa	Aerial	*Pseudomonas* sp. 6	1.78	3.9
		Sphingomonas sp. 42	11.80	10.0
		Pseudomonas sp. 11	3.22	1.7
		Pseudomonas sp. 43	2.20	2.8
		Rhodococcus sp. 36	2.18	2.9
		Micrococcus sp. 8	1.52	1.6
		Micrococcus sp. 7	2.15	2.1
		Micrococcus luteus	27.97	8.8
		Rhodococcus sp. 37	5.73	14.3
		Rhodococcus sp. 21	1.96	2.6
		Bacillus sp. 12	4.25	13.3
	Substrate	*Rhodococcus* sp. 16	0.53	0.2
		Rhodococcus sp. 28	1.92	2.0
		Mycobacterium sp. 25	0.70	0.8
		Pseudomonas sp. 24	10.70	23.3
		Cellulomonas sp. 23	4.81	6.5

* Standard deviation values were ±(0.05–0.2).

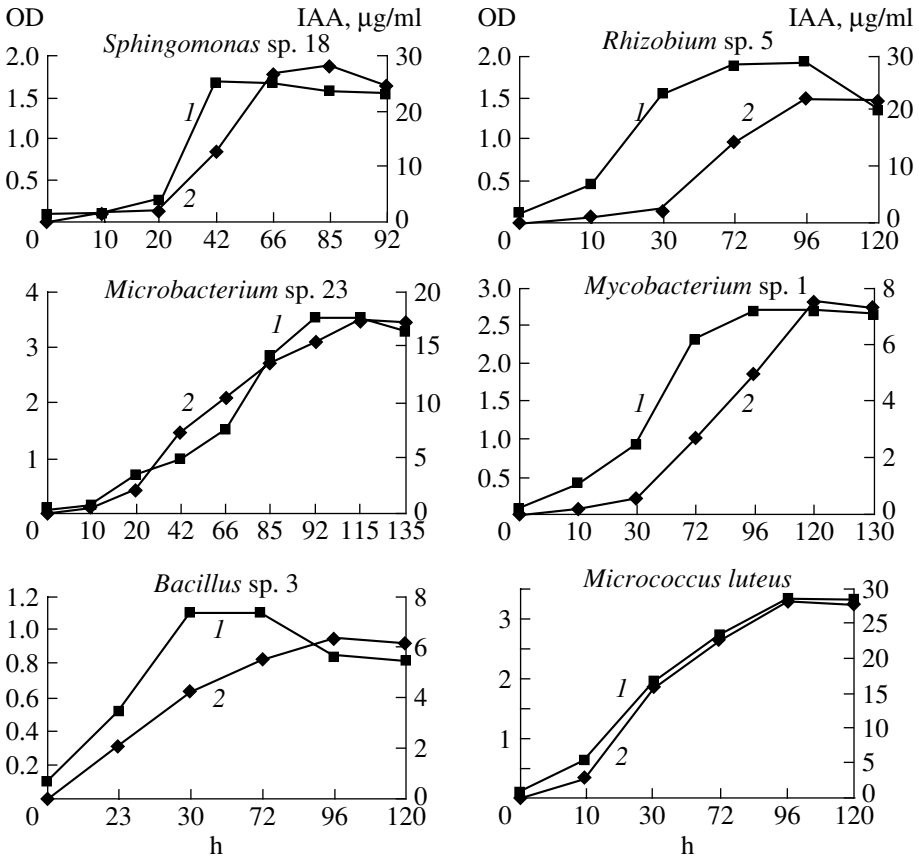

Fig. 1. Dynamics of (*1*) growth and (*2*) IAA production in bacterial cultures.

medium increased the amount of excreted auxins by 30- (*Sphingomonas* sp. 18, *Mycobacterium* sp. 1, *Rhizobium* sp. 5, *Cellulomonas* sp. 23, and *Micrococcus luteus*) to 170-fold (*Pseudomonas* sp. 24). This finding confirms the supposition that tryptophan is the auxin precursor in these bacterial cultures. The highest stimulatory effect (three- and eightfold) was recorded for two *Bacillus* species.

It has been previously reported that, in response to the addition of 200–500 μg/ml of tryptophan, methylobacteria and representatives of the genera *Arthrobacter* and *Pseudomonas* synthesize auxins within the ranges of 5–15, 20–25, and 90–120 μg IAA/ml [8, 10, 13]. Comparing our results with the published data, it may be concluded that bacteria associated with the epiphytic orchids are capable of efficient tryptophan utilization and its transformation to auxins.

Root exudates of higher plants are known to be the source of tryptophan in the rhizosphere [7]. Using the tryptophan of root exometabolites, epiphytic microflora can change the hormonal state of the rhizosphere by transforming this amino acid to IAA, which can in turn influence the host plant. We have demonstrated the stimulatory effect of IAA on the formation of the plant root system in a biotest with kidney bean cuttings; under the influence of IAA, the root formation increased significantly (Table 3, Fig. 2). Treatment of the cuttings with bacterial culture liquid enhanced formation of the "root brush," located 7.4–13.4 times higher than in the control samples.

In no case did the culture liquid contain inhibitory substances or cause death of the cuttings. For all the bacteria studied, except *Bacillus* sp. 12, *Rhodococcus* sp. 37, and *Mycobacterium* sp. 1, the height of root formation was demonstrated to be greater than for the cuttings treated with 50 μg/ml auxin (Table 3; Fig. 2). The IAA content of the culture liquid of these microorganisms was 30–47 μg IAA/ml. The biological activity of the culture liquid is specific; plant root formation occurred differently even in the case of microorganisms excreting similar auxin concentrations. This may indicate the presence of other plant growth stimulators or other indole compounds in the culture liquid similar to IAA in composition but differing in activity; this finding is in accordance with data published elsewhere [20].

Chromatographic analysis of indole compounds obtained from bacterial culture liquids confirmed the presence of IAA with R_f identical to the R_f of the standard. These experimental results are presented in Table 4. According to the chromatography in the first solvent system, some of the spots in *Rhizobium* sp. 5, *Cellulomonas* sp. 23, *Bacillus* sp. 3, and *Mycobacterium* sp. 1

Table 2. IAA production by bacteria on Czapek medium as dependent on tryptophan concentration

Orchid species	Bacterial culture	Tryptophan, μg/ml medium	IAA, μg/ml	Maximal increase of IAA synthesis, times	IAA, μg per unit OD	Maximal increase of IAA synthesis, times
D. moschatum	*Sphingomonas* sp. 18	0	1.8*	28	0.7	31
		50	17.4		7.1	
		100	38.4		14.6	
		200	50.2		21.4	
	Microbacterium sp. 23	0	2.5	21	0.3	60
		50	3.1		0.9	
		100	4.6		5.7	
		200	53.1		18.0	
	Mycobacterium sp. 1	0	3.1	30	0.8	29
		50	34.0		8.1	
		100	86.1		21.0	
		200	92.9		23.2	
	Bacillus sp. 3	0	6.6	6	4.5	8
		50	13.2		11.6	
		100	28.4		24.3	
		200	37.6		36.3	
	Rhizobium sp. 5	0	1.5	34	0.7	31
		50	2.8		1.2	
		100	16.3		6.8	
		200	60.4		21.6	
A. papillosa	*Sphingomonas* sp. 42	0	1.1	63	0.5	75
		50	17.5		7.0	
		100	43.4		20.4	
		200	69.4		37.3	
	Rhodococcus sp. 37	0	0.4	124	1.1	52
		50	6.2		25.7	
		100	22.7		36.0	
		200	49.6		57.7	
	Cellulomonas sp. 23	0	1.0	54	0.7	29
		50	19.9		8.3	
		100	35.6		12.8	
		200	53.9		20.6	
	Pseudomonas sp. 24	0	0.3	103	1.0	172
		50	14.2		78.7	
		100	21.2		109.9	
		200	31.0		172.4	
	Bacillus sp. 12	0	1.9	4	2.4	3
		50	1.9		2.7	
		100	3.7		3.1	
		200	8.4		7.3	
	Micrococcus luteus	0	1.0	39	4.9	32
		50	13.4		23.3	
		100	25.3		74.4	
		200	39.2		156.9	

* Standard deviation values were ±(0.07–0.15).

Table 3. Effect of bacterial culture liquids on root formation by kidney bean cuttings

Bacterial culture	IAA in culture liquid, μg/ml	Height of root formation	
		mm	Increase, times
Rhizobium sp. 5	37.9	57 ± 3.2	11.4
Sphingomonas sp. 42	30.6	70 ± 3.8	14.0
Sphingomonas sp. 18	42.6	67 ± 3.4	13.4
Micrococcus luteus	31.2	66 ± 3.1	13.2
Rhodococcus sp. 37	19.4	46 ± 2.5	9.2
Microbacterium sp. 23	46.6	62 ± 3.5	12.4
Cellulomonas sp. 23	45.0	58 ± 3.4	11.6
Bacillus sp. 3	32.9	60 ± 2.9	12.0
Mycobacterium sp. 1	37.6	47 ± 3.4	9.4
Pseudomonas sp. 24	30.7	57 ± 3.2	11.4
Bacillus sp. 12	8.4	35 ± 2.2	7.0
IAA (50 μg/ml)	–	57 ± 2.7	11.4
Czapek medium	–	5 ± 0.45	–
Tap water	–	5 ± 0.45	–

samples did not exactly coincide in R_f with IAA. According to repeated chromatography, however, this factor was identical for all the bacteria studied. Additional spots were found (shown in parentheses in Table 4) corresponding to other indole compounds in the microbial culture liquids. Repeated chromatography also enabled us to separate the closely related indole compounds that formed single spots during the first chromatographic analysis. For instance, after elution of the compounds with $R_f = 0.89$ from the *Rhodococcus* sp. 23 sample, three indole compounds were revealed, with R_f values of 0.82 (IAA), 0.90, and 0.85.

It may therefore be concluded that bacteria associated with greenhouse tropical orchids produce and excrete auxins into the culture liquid and that this biosynthesis is enhanced by the addition of tryptophan. The amount of auxins produced varies within a bacterial genus. IAA synthesis depends on the growth medium composition, on the growth phase, and on the concentration of exogenous tryptophan.

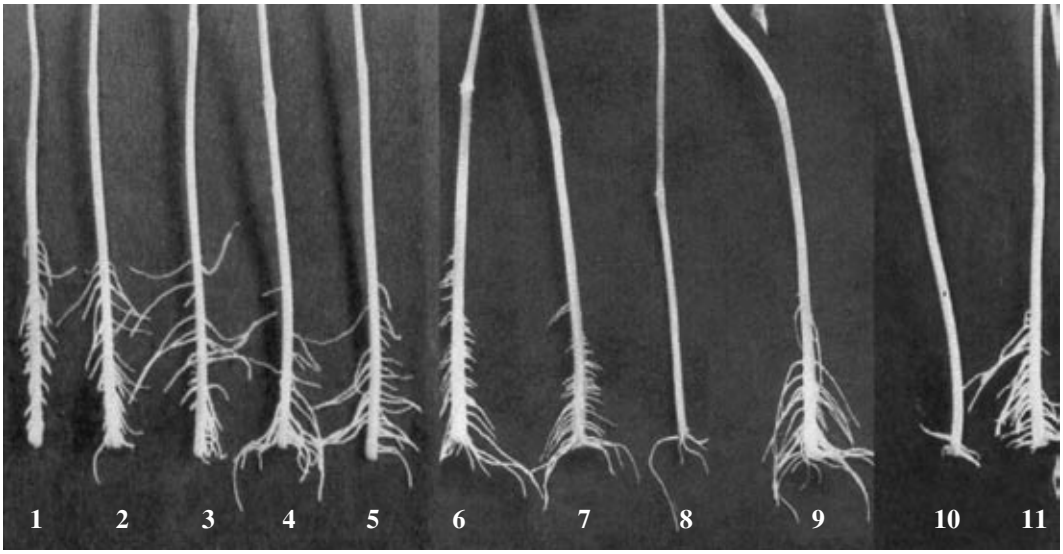

Fig. 2. Effect of bacterial culture liquids on root formation by kidney bean cuttings: (1) *Micrococcus luteus*; (2) *Pseudomonas* sp. 24; (3) *Bacillus* sp. 12; (4) *Microbacterium* sp. 23; (5) *Cellulomonas* sp. 23; (6) *Sphingomonas* sp. 18; (7) *Rhizobium* sp. 5; (8) tap water; (9) auxin solution (50 μg/ml); (10) Czapek medium; (11) *Rhodococcus* sp. 37.

Table 4. R_f values for the standard (IAA) and for the experimental samples after (1) chromatography and (2) repeated chromatography

Bacterial culture	1		2	
	R_f, IAA	R_f, experiment	R_f, IAA	R_f, experiment
Rhizobium sp. 5	0.89	0.88 (0.60)	0.82	0.82 (0.90, 0.80)
Sphingomonas sp. 42	0.94	0.94 (0.89, 0.76, 0.65)	0.86	0.86 (0.91, 0.43)
Sphingomonas sp. 18	0.84	0.84 (0.74, 0.53)	0.82	0.82 (0.91, 0.48)
Micrococcus luteus	0.87	0.87 (0.50)	0.86	0.86 (0.90, 0.58, 0.50)
Rhodococcus sp. 37	0.94	0.94 (0.88, 0.63)	0.82	0.82 (0.85)
Microbacterium sp. 23	0.89	0.89 (0.84, 0.61)	0.82	0.82 (0.90, 0.85)
Cellulomonas sp. 23	0.84	0.83 (0.9, 0.72, 0.53)	0.86	0.86 (0.90, 0.41)
Bacillus sp. 3	0.84	0.83 (0.74, 0.55)	0.82	0.82 (0.91)
Mycobacterium sp. 1	0.84	0.83 (0.53, 0.40)	0.86	0.86 (0.76, 0.41)
Pseudomonas sp. 24	0.94	0.94 (0.90, 0.7, 0.63)	0.82	0.82 (0.90, 0.50)
Bacillus sp. 12	0.94	0.94 (0.88, 0.73)	0.86	0.86 (0.89, 0.76, 0.70, 0.55)

Note: In parentheses are the R_f values of other indole compounds (of yet undetermined nature) present in bacterial culture liquids.

We have earlier demonstrated that many of the associated bacteria studied possess a highly pronounced intercellular matrix. Since accumulation of nutrients, ions, and signal molecules is one of its functions, it may possibly accumulate phytohormones as well, thus increasing their concentration and increasing their availability for plants. The beneficial effect on plants of the phytohormones excreted by the bacteria studied in this work is confirmed by enhanced root formation in kidney bean cuttings and by the absence of any inhibiting or suppressing effects. Associated bacteria may have a similar effect on mature orchids, stimulating their root formation. This problem, however, requires further and more detailed study.

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