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Plant Growth Regulatory Metabolites from Novel Actinomycetes

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Abstract. Metabolites from 796 isolates of aerobic actinomycetes were screened for plant growth regulatory properties using an algal bioassay. These included 266 isolates of *Streptomyces*, 28 unidentified actinomycetes, and 502 isolates of "novel" actinomycetes represented by 18 genera. Algal growth inhibition of ≥30% was observed with 60 isolates, 37 of which belonged to the genus *Streptomyces*. Among other inhibitors were 8 isolates of *Actinomadura*, 6 of *Actinoplanes*, 2 each of the genera *Thermomonospora*, *Streptoverticillium*, and *Promicromonospora*, and 3 unidentified. Metabolites from 70 isolates promoted algal growth by ≥20%. These included 13 isolates of *Micromonospora*, 11 of *Streptomyces*, 6 of *Nocardia*, 5 of *Actinomadura*, and 4 each of *Rhodococcus* and *Thermomonospora*. Sixteen unidentified isolates; 3 isolates of *Promicromonospora;* 2 isolates each of *Actinoplanes*, *Streptosporangium*, and *Oerskovia;* and 1 of "*Thermoactinomyces peptonophilus*-like" organism and *Saccharomonospora viridis* also promoted the algal growth by ≥20%. The plant growth inhibitory properties of 9 actinomycetes and the growth promoting properties of 6 were demonstrable during the secondary screening on higher plants using chemicals extracted from the culture broth. The metabolites from *Micromonospora, Nocardia, Rhodococcus, Streptosporangium*, and *Oerskovia* isolates were associated with plant growth promotion only; those from *Streptomyces* were most frequently involved with the growth inhibition.

Microorganisms constitute an integral part of the ecosystem and influence plant growth in many different ways. Their roles in the mineralization of complex organic molecules and nitrogen fixation is well documented (Waksman 1959, Becking 1982). Increased environmental considerations and awareness of the risks associated with the use of synthetic agrochemicals have stimulated greater interest in microorganisms as a potential source of safer pesticides.

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Several microbial products, such as cycloheximide, nigericin, geldanamycin, bialaphos, herbicidins, and herbimycin, inhibit the growth of plants, and their usefulness as selective herbicides or plant growth inhibitors is being evaluated (Sekizawa and Takematsu 1982, Heisey and Putman 1987). The plant growth stimulatory property of gibberellin, a product derived from the fungus *Gibberella fujikuroi,* suggests that microbial products can be equally useful as promoters. There is, however, a paucity of information concerning plant growth stimulatory properties of actinomycetes or their metabolic products. The results are presented here of a systematic search for microbial plant growth regulators, employing "novel" as well as commonly encountered actinomycetes. We proposed to ascertain if plant growth inhibitory and stimulatory properties are most frequently associated with specific groups of actinomycetes isolated from well-defined ecological niches.

Materials and Methods

Sample Collection and Isolation

Soil samples were collected by forcing sterile cork borers (No. 9, diameter 12 mm) into the ground and retrieving the soil from inside the borer into sterile containers. In all, 18 samples of soil from well-cultivated fields, indoor potted plants, parks, and lawns with and without fairy rings were examined. Fairy ring samples were collected from the center, where the grass was inhibited, and from the periphery, where the grass was relatively tall and luxuriant.

One gram of the soil sample was suspended in 9 ml sterile physiological saline (0.85% NaCl in distilled water), and serial 10-fold dilutions were prepared (up to 10^{-6}). One milliliter of the diluted suspension was mixed with 14 ml of the isolation media held at 45°C and poured into 100-mm Petri dishes. Two plates of each of the following media were used for every dilution (10^{-4} to 10^{-6}).

Starch-casein agar (Williams and Davies 1965). The medium was composed of starch 10.0 g, casein 0.3 g, KNO_3 2.0 g, K_2HPO_4 2.0 g, Mg $SO_4 \cdot 7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, bacto agar 18 g, and 1 L distilled water, pH 7.2. Nystatin, cycloheximide, polymyxin B sulfate, and penicillin solutions were added after autoclaving to give a final concentration of 50, 50, 1, and 1 μg/ml, respectively.

NZ amine agar. The medium contained NZ amine A (Schefield Chemicals, Norwick, NY, USA) 3 g, bacto agar 18 g, and 1 L tap water. Seven milliliters of bromcresol purple (0.04% in distilled water) was added after adjusting the pH to 7.2. Nystatin (0.03 g) and cycloheximide (0.5 g) (dissolved in dimethyl sulfonoxide and water, respectively) were added aseptically after autoclaving. Nystatin and cycloheximide are fungal inhibitors, and bromcresol purple restricts bacterial colonies.

Threonine agar. The medium was prepared by mixing 2 g threonine, 18 g bacto agar, and 1 L tap water. The pH was adjusted to 7.3 before autoclaving. Nystatin and cycloheximide solutions prepared as above were added after autoclaving.

Asparagine biphenyl agar. The medium was composed of 3 g asparagine, 1 ml each of 1 M $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$, 18 g bacto agar, and 1 L distilled water. The pH was adjusted to 7.2. One gram biphenyl dissolved in 10 ml ethanol was added after autoclaving. After the gel had solidified, the Petri dishes were sealed in polyethylene bags and incubated at 25°C. The plates were examined after 1 week. Petri dishes showing little or no growth were further incubated for 2–3 weeks. Well-isolated distinct colonies were transferred onto slanted YMG agar (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; bacto agar, 18 g; distilled water, 1 L; pH 7.2). After a suitable period of incubation at 25°C, the cultures were streaked on NZ amine agar (without bromcresol purple). The plates were sealed in a polyethylene bag and incubated at 25°C for 2–4 weeks. Microscopic morphology of the undisturbed growth in the Petri dishes was studied using long working distance objectives (see Starr et al. 1982). Chemotaxonomic analyses of the isolates, if required, were done according to the method described by Lechevalier and Lechevalier (1982). Species were identified in selected cases according to the method of Mishra and Gordon (1986) and Mishra et al. (1980).

Bioassays. After identification, batches of aerobic actinomycetes were grown in 500-ml baffle-bottomed Erlenmeyer flasks containing 100 ml liquid YMG or A-9 medium (peptone 5 g, glucose 10 g, molasses 20 g, distilled water 1 L) (Warren et al. 1955). Typically, A-9 was used for the fast growers, such as *Streptomyces, Nocardiopsis, Streptoverticillium,* and *Actinomadura* species, and the YMG medium was employed for *Micromonospora, Microbispora, Actinoplanes,* and *Nocardia* species and other microorganisms found to be fastidious or slow-growing. The flasks were placed on a rotary shaker at 200 rpm, 25°C, for 5–7 days (fast growers) or 10–14 days (slow growers).

The bioassays for plant growth regulatory properties were performed using an alga, *Chlamydomonas reinhardtii,* as an indicator. The algae were grown in sterile cultures on a shaker (with air supply) in a chemically defined medium (Wert et al., unpublished data). Actinomycete broth diluted 10- and 100-fold was added to 10 ml of the algal culture in a test tube and incubated in the growth chamber (light 200 $\mu E/S/M^2$) at 25°C for 24 h. The mixture was then centrifuged at 3000 rpm for 10 min, and the pellet suspended in 4 ml acetone and recentrifuged. Using the supernatant, the chlorophyll absorbance was read at 652 nm. The total chlorophyll equals the O.D. at 652 nm × 28.986 (μg/ml) × 5.0 μg chlorophyll per milliliter *Chlamydomonas* culture. The results obtained with the actinomycete broth were compared with those obtained with broth control (uninoculated medium) and analyzed statistically. In promising instances, the culture broth was extracted with n-butanol and dried in vacuo. The control broth was extracted in a similar manner. To confirm the results obtained with the crude broth, the extracted materials were dissolved in a suitable solvent (distilled water or acetone) and diluted to correspond to the original broth, and the bioassay was repeated.

During the follow-up study, cultures of promising isolates were grown in larger batches (400 ml medium in 2-L flasks). The culture broth together with the microbial growth was extracted with n-butanol and dried in vacuo. The residue was redissolved in distilled water or acetone, diluted in distilled water, and mixed with a surfactant (Surfel 0.1% v/v, Union Carbide), and known

Table 1. Inhibition or stimulation of growth of *Chlamydomonas reinhardtii* by metabolites from aerobic actinomycetes as measured by chlorophyll content.

Genera represented	Number of isolates examined	Number showing growth regulatory properties[a]	
		Inhibition (≥30%)	Promotion (≥20%)
Streptomyces	266	37 (14)	11 (4)
Micromonospora	134	0	13 (10)
Actinomadura[b]	59	8 (14)	5 (8)
Actinoplanes	46	6 (13)	2 (4)
Rhodococcus	26	0	4 (15)
Micropolyspora	24	0	0
Nocardia	22	0	6 (27)
Nocardiopsis[b]	22	0	0
Streptosporangium	22	0	2 (9)
Oerskovia	21	0	2 (10)
Thermomonospora	21	2 (10)	4 (19)
Thermoactinomyces[c]	19	0	1
Streptoverticillium	18	2 (11)	0
Saccharomonospora viridis	16	0	1
Promicromonospora	15	2 (13)	3 (20)
Dactylosporangium	11	0	0
Microbispora	11	0	0
Chainia	8	0	0
Pseudonocardia	7	0	0
Unidentified actinomycetes	28	3	16
Total	796	60	70

[a] Figures in parentheses indicate percentage of total isolates of that genus.
[b] Use of these generic names does not imply author's endorsement regarding their validity.
[c] "*Thermoactinomyces peptonophilus*-like" organisms.

amounts were sprayed on 7- to 10-day-old seedlings grown in a greenhouse in 18 × 12 cm styrofoam flats or 13-cm clay pots. After 2 weeks the shoots were harvested, dried, and weighed.

Results

Of the 796 isolates included in the present survey, 768 were identified to the generic level on the basis of their gross and microscopic morphology, occasionally aided by chemotaxonomic analyses. The identified isolates represented 19 genera of aerobic actinomycetes. Genus *Streptomyces* was represented by the largest number (33.4%), followed by *Micromonospora* (16.8%), *Actinomadura* (7.4%), and *Actinoplanes* (5.7%). The remaining 15 genera (Table 1) together accounted for 33% of the isolates. Most of the *Streptomyces* and *Actinomadura* were isolated on starch casein agar. The asparagine biphenyl medium facilitated greater isolation of *Micromonospora* and *Streptomyces* species of *S. hygroscopicus* type. The NZ amine agar picked up a wide

Table 2. Inhibition of the growth of several plant species by 9 isolates of actinomycetes.

Isolate number	Actinomycete	Percent growth inhibition (in algal bioassay)[b]	Effect on higher plants[a]	
			Species	Dry weight (% of control)
576	*Streptomyces* sp.	86	Flax	32
			Soybean	81
616	*S. hygroscopicus*	87	Flax	61
618	*Streptoverticillium* sp.	92	Flax	88
619	*S. hygroscopicus*	91	Flax	42
			Corn	70
27017	*Streptomyces* sp.	89	Flax	47
30001	*S. hygroscopicus*	98	Cucumber	84
30006	*S. hygroscopicus*	98	Corn	88
30099	*Actinomadura* sp.	95	Cucumber	71
39009	*Streptomyces* sp.	95	Cucumber	87

[a] All observations were significantly different from the control at the 1% level.
[b] Based on chlorophyll content.

spectrum of actinomycete genera, and several of the "rare" types were isolated on this and threonine agar.

Plant growth inhibition (≥30% as compared to controls) was observed with 60 isolates (8%) representing 6 genera (Table 1) and 3 unidentified actinomycetes. The percentages of *Streptomyces, Actinomadura, Actinoplanes,* and *Promicromonospora* isolates found to moderately inhibit the growth of *C. reinhardtii* were nearly the same, closely followed by *Streptoverticillium* and *Thermomonospora* species.

Twenty-one isolates, 10 of which were *Streptomyces,* inhibited algal growth by >70%. An inhibition of >90% was observed with 9 isolates represented by 4 isolates of *Streptomyces,* 2 each of *Actinoplanes* and *Actinomadura,* and 1 of *Thermomonospora* (which repeatedly caused nearly 100% inhibition of the algal growth). The 4 *Streptomyces* isolates in this category were subsequently identified as the "*hygroscopicus*" type. Six isolates that inhibited algal growth by 90% at 10^{-2} dilution promoted the growth of *C. rheinhardtii* by 10–25% at 10^{-4} dilution. Three of these isolates belonged to the genus *Streptomyces.*

During the follow-up study, 16 of 21 promising inhibitors were grown in large batches and extracted in n-butanol, and the crude extract was sprayed to drip on selected species of potted higher plants in the greenhouse. Extracts from 13 isolates induced an appreciable (statistically significant) loss in the dry weight of one or more plant species tested. Visible symptoms included necrosis or curling of leaves or shoots and reduced apical growth (Fig. 1). In some instances, signs of injury were less marked, but the plant growth was so drastically inhibited that the treated plants looked like dwarf cultivars (Fig. 2). The active component from 2 isolates was subsequently identified as cycloheximide, and one isolate produced a lesser known herbicide, nigericin (Heisey and Putnam 1986). Table 2 indicates the effect of extracts of the remaining 9 isolates on sensitive species of higher plants.

The culture broth from 70 isolates (9%) induced ≥20% increase in the

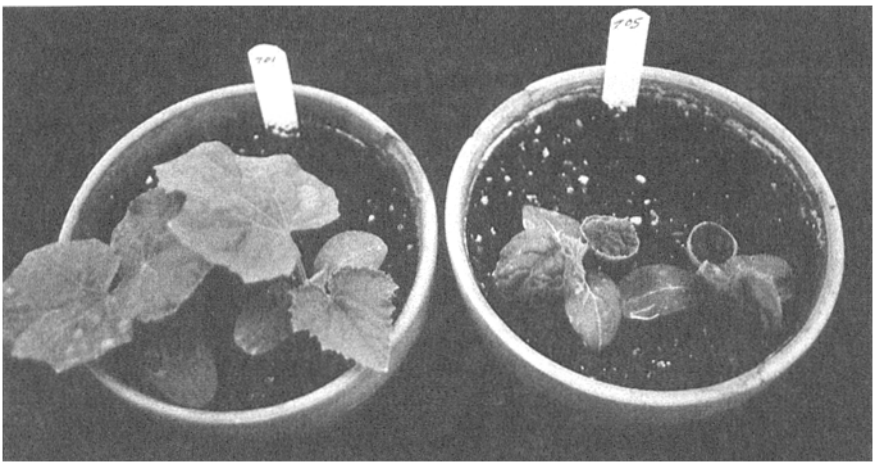

Fig. 1. Effect on potted cucumber of a plant growth regulatory substance from *S. hygroscopicus* strain 30001. Note the reduced growth, necrosis, and curling of leaves in the treated plants (right) as compared to the controls (left).

Fig. 2. Effect on potted corn of a substance derived from *S. hygroscopicus* strain 30006. Note reduced growth of the treated plants (2), as compared to the controls (1).

Table 3. Stimulation of the growth of some plant species by 6 isolates of actinomycetes.

Isolate number	Actinomycete	Percent growth increase (in algal bioassay)[b]	Effect on higher plants[a]	
			Sensitive species	Dry weight (% increase over control)
516	*Micromonospora* sp.	74	Corn	13
			Soybean	14
			Cucumber	17
533	*Nocardia* sp.	31	Tomato	11
538	*Thermomonospora* sp.	60	Soybean	18
			Cucumber	12
576	*S. hygroscopicus*	40	Soybean	15
580	*Micromonospora* sp.	48	Cucumber	20
			Sorghum	12
42002	*Actinomadura* sp.	52	Sorghum	12
			Cucmber	(23% decrease)

[a] All observations were significantly different from the control at the 1% level.
[b] Based on chlorophyll content.

growth of *C. rheinhardtii* in the primary screening (Table 1). Broth of 30 isolates increased algal growth by ≥50%. The identified actinomycetes represented 11 genera; among the most frequent promoters were isolates of *Nocardia* (27.2%) *Promicromonospora* (20%), *Thermomonospora* (19%), and *Rhodococcus* (15.3%). The frequencies of growth promotion by the isolates of *Micromonospora, Actinomadura, Streptosporangium,* and *Oerskovia* were comparable (8–10%). Percentages of the *Streptomyces* and *Actinoplanes* isolates with growth-promoting properties were the lowest (about 4%). Metabolites from a "*Thermoactinomyces peptonophilus*-like" organism and a *Saccharomonospora viridis* isolate also promoted the algal growth. Twenty of the promising isolates were regrown in larger batches and extracted with a suitable solvent, and known amounts were sprayed on higher plants, as described before.

The crude extract from 6 isolates showed a significant increase in the dry weight of one or more plant species tested (Table 3). One isolate (42022) induced an increase of 12% in the dry weight of sorghum but caused a 23% decrease in the dry weight of cucumber plants. The *Micromonospora* isolate 516 appeared to be the most promising growth promoter. A large batch was grown in YMG medium, extracted in methylene chloride-acetonitrile (3:1), and purified through silica gel flash column and high-performance liquid chromatography. The purified product, when tested on algae and higher plants, induced growth promotion comparable to that obtained with crude broth but at a low rate (50 ppm). Neither the crude extract nor the culture broth from the rest of the 20 isolates tested induced any significant increase in the dry weight of any of the tested plant species, although they repeatedly stimulated algal growth.

Discussion

Since the advent of the antibiotics era, actinomycetes have been screened mainly for the production of antimicrobial metabolites for possible use in medicine. Recent awareness that these microorganisms are capable of producing almost any desired substance and that microbial products are easily degraded in nature has triggered a worldwide interest in microbial products as possible substitutes for synthetic chemicals in agroindustries (Misato 1982, Omura 1986). A number of microbial products previously described as antibacterial or antifungal agents are also potent plant growth regulators or insecticides. For example, the insecticide valinomycin and the herbicide cycloheximide were initially discovered as antibacterial and antifungal substances, respectively (Wallen et al. 1950, Brockman and Schmidt-Kastner 1955). However, there have been only a few screening programs specifically directed at the discovery of plant growth regulators. The published reports suggest a greater emphasis on only one genus, *Streptomyces*, which is easy to isolate and easy to identify. The present study is unique in that it includes a large number of strains belonging to the other genera of actinomycetes commonly called "rare" or "novel" actinomycetes, because the techniques for their isolation and identification are not widely known. This has been possible through the use of selective media developed in our laboratory. Among other microbiologically interesting features of this study are the isolation of *Chainia* and *Promicromonospora* from the local soils. *Chainia* is known to occur in the arid or semiarid regions (Lechevalier 1981), and *Promicromonospora* has been reported from the fecal material of millipedes (*Diplopoda*) in Eastern Europe (Jager et al. 1983).

Except for the gibberellins produced by the fungus *Giberella fujikuroi* (Russell 1973) and helminthosporals produced by *Helminthosporium sativum* (Tamura et al. 1965), plant growth promoting properties of microbial products are little known. Present observations suggest that metabolic products from certain actinomycetes may promote plant growth. However, there seems to be a relationship between plant growth promotion or inhibition and the genera of actinomycetes. Isolates of *Nocardia*, *Micromonospora*, and *Rhodococcus*, for example, were found only as growth promoters. *Micromonospora* isolates are commonly present in the aquatic systems, and *Nocardia* and *Rhodococcus* are frequently isolated from well-cultivated fields (Lechevalier 1981, Mishra and Gordon 1986). The growth inhibitory properties of metabolites from *Streptomyces* were much more common than plant growth promotion properties. These observations raise the possibility that certain genera of soil microbes perform plant growth regulatory roles in the ecosystem. The data also suggest that in searches for bioactive compounds, more success might be achieved by focusing on selected genera for specific activities.

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