A streptomycete antagonist to *Phellinus weirii*, *Fomes annosus*, and *Phytophthora cinnamomi*[1]

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An actinomycete isolated from the rhizoplane of nitrogen-fixing nodules of *Ceanothus velutinus* was identified as a variety of *Streptomyces griseoloalbus*. *Streptomyces griseoloalbus* is a strong antagonist to three destructive root pathogens, *Phellinus weirii*, *Fomes annosus*, and *Phytophthora cinnamomi*, inhibiting all three on several culture media and preventing establishment of *F. annosus* on hemlock wood disks. The stability and longevity of the antimicrobial substance produced by it, its consistent effect on the pathogens on all substrates, its ability to colonize wood, and its ability to grow at 10°C suggest biological control possibilities for this organism in the Pacific Northwest.

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Une actinomycète isolée du rhizoplan des nodules fixatrices d'azote de *Ceanothus velutinus* a été identifiée comme une variété de *Streptomyces griseoloalbus*. *Streptomyces griseoloalbus* est un fort antagoniste pour trois pathogènes destructeurs de racines, *Phellinus weirii*, *Fomes annosus* et *Phytophthora cinnamomi*; il les inhibe tous les trois sur divers milieux de culture et empêche l'établissement de *F. annosus* sur des disques ligneux de pruche. La stabilité et la longévité de la substance antimicrobienne qu'il produit, son effet consistant sur les pathogènes dans tous les substrats, sa capacité de coloniser le bois et de croître à 10°C suggère que cet organisme offre des possibilités de contrôle biologique dans le nord-ouest du Pacifique.

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Introduction

A number of investigators have reported antagonism of fungi, actinomycetes, and true bacteria to root rot pathogens (Nelson 1972; Pedziwilk 1967; Pratt 1971; Hutchins, to be published). Inhibition of the growth of fungal pathogens by actinomycetes and true bacteria has been demonstrated by Broadbent et al. (1971). Among bacteria, mycolytic properties have been observed mainly in the genera *Bacillus*, *Pseudomonas*, and *Streptomyces*. Much of the reported inhibition is due to a response by the pathogen to an antimicrobial substance or antibiotic produced by the actinomycete or bacterium (Ballesta and Alexander 1972). Antibiotics are thought to be restricted to the rhizosphere where there is a higher concentration of roots and organic substances (Soulides 1969). Brian (1957) and Lingappa and Lockwood (1961) have also reported stable and continued antibiotic production by soil microorganisms isolated and cultured under laboratory conditions.

Recent work has demonstrated that biological control of root disease organisms in living trees may be possible by treating wounds with microorganisms or by artificial inoculation of soil microorganisms that can be stimulated to multiply and subsequently replace the established pathogen (Etheridge 1972). Actinomycetes have been found to inhibit the growth of *Fomes annosus* (Fr.) Cke., a destructive root rot pathogen of hardwoods and conifers in many parts of the world (Gunderson 1963; Nissen 1956). The fungus *Peniophora gigantea* (Fr.) Massee is a vigorous competitor and has been used successfully as a stump protectant on pines in Europe and the southeastern United States, but it does not satisfactorily inhibit the growth of *F. annosus* on western hemlock (*Tsuga heterophylla* Raf. Sarg.) in western North America (Wallis and Morrison 1975).

The most commonly used means of control of *F.*

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annosus root rot has been the application of chemicals to stump surfaces to prevent fungal colonization. Dry Borax, 10% zinc chloride, and 20% ammonium sulphamate are effective inhibitors (Wallis and Morrison 1975). Borax, however, fails to control the decay fungus during periods of high precipitation, and ammonium sulphamate and zinc chloride are relatively costly and toxic to man.

Phellinus weirii (*Poria weirii*) (Murr.) Gilb. is a serious root rot pathogen in the western conifer regions of North America where it causes considerable financial loss to the timber industry each year. *Phytophthora cinnamomi* Rands. is responsible for serious nursery loss and hardwood damage in many parts of the world as well as considerable financial loss in crop production of ornamental flowers, avocado, and pineapple (Malajczuk and Glenn 1978; Pegg 1976).

This report describes a *Streptomyces* repeatedly isolated from the rhizoplane of nitrogen-fixing nodules of *Ceanothus velutinus* Dougl. collected from central Oregon. This isolate produces a diffusable antimicrobial substance inhibitory to the growth of three important Northwest root rot fungi: *P. weirii*, *F. annosus*, and *P. cinnamomi*. This isolate is effective in culture media and colonizes and inhibits *F. annosus* on wood disks.

Methods and materials

Media

Each solid medium used for isolation and cultivation contained 1.5% agar. Glucose–nutrient agar (GNA) consisted of nutrient agar from Difco plus 1% glucose. Starch–casein agar (SCA) contained 1% soluble starch, 0.1% vitamin-free casein, and 0.05% K_2HPO_4 adjusted to pH 7.3. Malt–yeast–peptone agar (MYP) consisted of 3% malt extract, 0.5% peptone, and 0.1% yeast extract. MYP-B was a malt–yeast–peptone agar buffered to pH 5.8 with potassium phosphate.

Isolation and culture

Nitrogen-fixing nodules were excised from lateral roots of *C. velutinus* growing at a depth of 15 cm, placed in bags, and stored at 4°C until processed. Within 2 days of collection, nodules were separated from root tissue, washed in 1% Hyamine detergent for 20 min, and rinsed 3 times in sterile distilled water. After rinsing, nodules were either shaken for 8 min in 20% hydrogen peroxide and then rinsed in sterile water, or immersed in 1% mercuric chloride for 3 min followed by three rinses in sterile distilled water. After rinsing, nodules were transferred to the surface of GNA in petri dishes. The petri plates were incubated at room temperature (22–25°C) for 5 days. A *Streptomyces* with a distinctive diffusable pigment appeared among the several colonies of fungi and bacteria. This *Streptomyces* isolate was subcultured to GNA and SCA slant tubes and stored at 4°C for future studies.

Identification and taxonomy

The *Streptomyces* isolate was identified by the description and methods of Shirling and Gottlieb (Shirling and Gottlieb 1966, 1968; Shirling 1968) as modified by Kuster (1972) followed by comparisons with cultures from the American Type Culture Collection (ATCC). The criteria for identification were rate of melanin production, spore surface characteristics, morphology and color of aerial mycelium, color of substrate mycelium, number and kinds of soluble pigments, carbon utilization, and ability to fix atmospheric nitrogen as assayed by the acetylene reduction technique (Hardy et al. 1973).

Antagonistic determinations

Antagonism of the *Streptomyces* isolate against *F. annosus*, *P. weirii*, and *P. cinnamomi* was tested on MYP, MYP-B, and SCA by the cross-streak method (Johnson and Curl 1972). An agar plug from the margin of an actively growing fungal culture was placed on the agar surface opposite a streak of the *Streptomyces* isolate. The plates were examined at weekly intervals for a clear zone, devoid of fungal growth, indicative of inhibition between the organisms. Agar plugs of the fungal pathogen placed on the three media without the *Streptomyces* isolate served as controls. All plates were incubated at 26°C under dark conditions. The inhibition trials continued over a 6-month period (June to December, 1978), using cultures originally isolated in September, 1977. During this period, our isolate maintained its ability to inhibit the growth of the three pathogens under laboratory conditions.

The following procedure was used to determine if the *Streptomyces* isolate was able to colonize wood and antagonize *F. annosus* on this substrate. Stem disks, 7–7.5 cm in diameter and 2.5 cm in length, were cut from 11- to 13-year-old living western hemlock and immediately brought to the laboratory. Bark was removed and surfaces of the disks were sterilized for 1 h with ultraviolet light (254 nm). One flat surface of each disk was dipped in melted paraffin and placed downward on the bottom half of a sterile 50 mm × 90 mm glass petri dish. The nonparaffin-coated surface was brushed with a spore suspension (25 000 spores/mL liquid) of the *Streptomyces* isolate either in *Actinomyces* broth (Difco No. 9) or in water. Afterward, the cut surface of each disk was inoculated with a spore suspension of *F. annosus* in water. Disks treated with paraffin only, disks inoculated with spores of *F. annosus* but not with the *Streptomyces* isolate, and disks paraffin coated and treated with sterile distilled water were used as controls. Each treatment was replicated 10 times. Ten millilitres of sterile distilled water was poured into each petri dish to maintain a high relative humidity. A lid, which fit well but did not prevent gas exchange, was placed on each petri dish.

Disks were incubated at 22–24°C for 1 week and examined for the presence of mycelium and the *Oedocephalum* spore stage of *F. annosus*. Those disks that showed no signs of *F. annosus* were split; four chips from the split surface of one of the resulting halves and one from the upper surface of the disk were taken with a pair of chisel forceps and transferred to a medium selective for *F. annosus* (Kuhlman and Hendrix 1962).

Results and discussion

Taxonomy and identification

Spore chain morphology

Sporophores are flexed and included in the section Rectiflexibilis. The spore surface is smooth with about 50 spores per chain. Although spore production is generally good on oatmeal agar, the number of spores produced varies considerably on salts–starch agar and on yeast–malt agar.

Color characteristics

Aerial mycelium is white in mass on oatmeal agar, asparagine–glucose agar, yeast–malt agar, and salts–starch agar. The reverse of the colony is

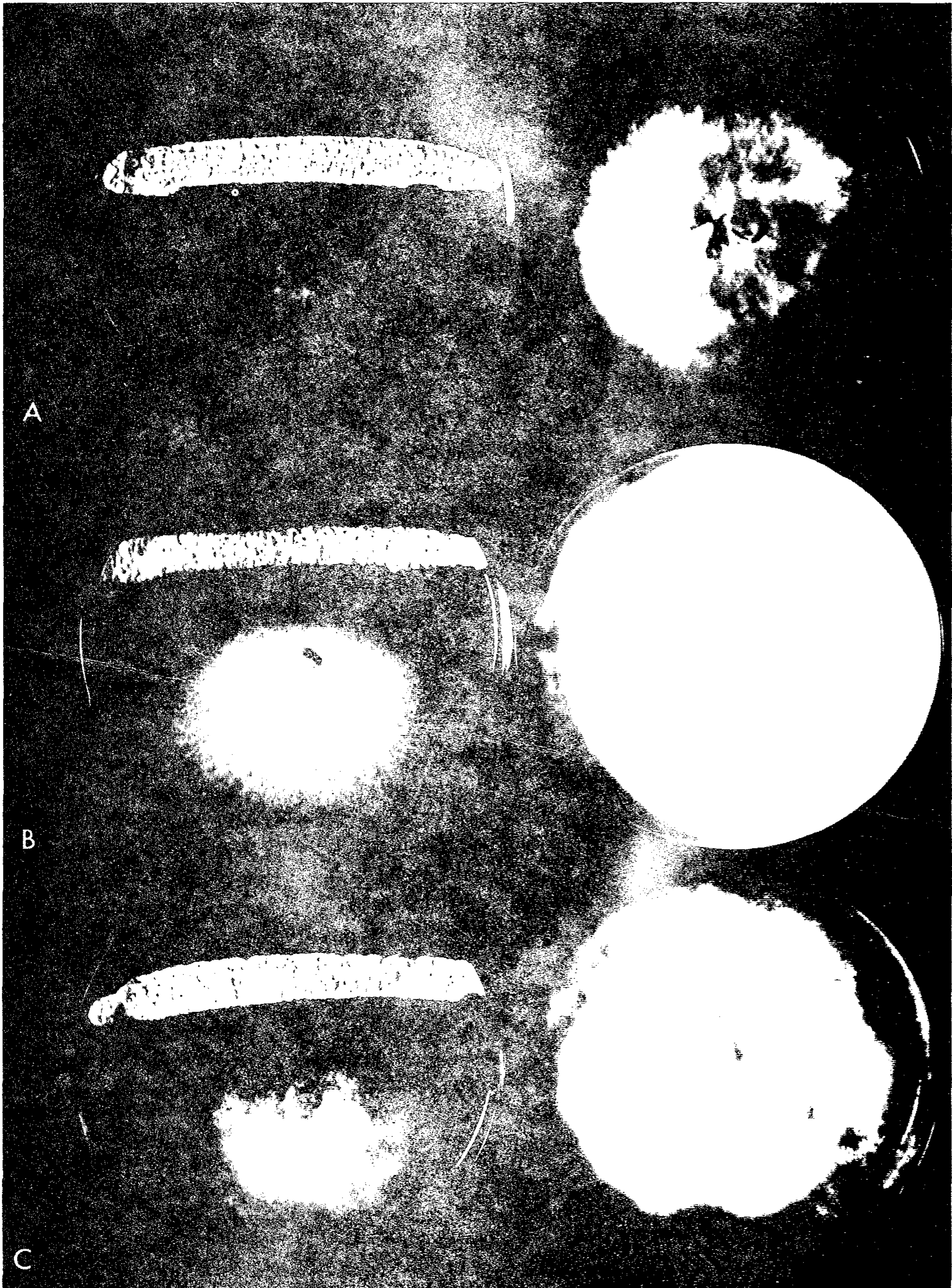

FIG. 1. Inhibition of three root rot pathogens by *Streptomyces griseoloalbus*. Two-week-old culture of *Phellinus weirii* and *S. griseoloalbus* on (A) malt–yeast–peptone agar, (B) malt–yeast–peptone agar buffered with potassium phosphate, and (C) starch–casein agar. Plates on right are the controls of *P. weirii*.

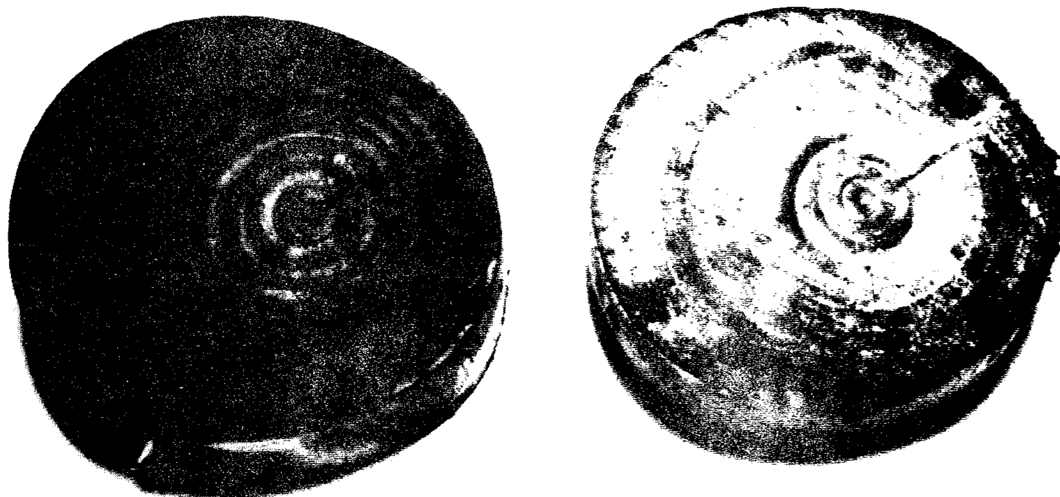

FIG. 2. Establishment of *Streptomyces griseoloalbus* on wood disk of western hemlock 2 weeks after being inoculated with liquid spore suspension. The disk on the left has not been inoculated with spores of *S. griseoloalbus*.

pigmented with a color varying from brownish–orange on oatmeal agar to bright orange–yellow on yeast–malt agar. Diffusion of the orange pigment around the colonies was evident on all media; however, production of the pigment decreases with repeated transfers. Melanoid pigments are not formed on peptone–yeast–iron agar or on tyrosine agar.

Carbon utilization

Good growth was observed on the following carbon sources: L-arabinose, sucrose, D-xylose, D-mannitol, D-fructose, rhamnose, raffinose, L-inositol, and glucose. No growth was observed on cellulose nor on the negative control (without a carbon source).

The isolate was unable to fix atmospheric nitrogen after a 2-week incubation period on a nitrogen-limiting medium (Hino-Wilson broth).

Antibiotic producing properties

This isolate produced a diffusable antimicrobial substance inhibiting *P. weirii*, *F. annosus*, and *P. cinnamomi* in culture media.

Temperature requirements

The optimum temperature for this isolate is 28°C on oatmeal agar with a maximum of 32°C, and a minimum of 10°C.

Based on the above and on the taxonomic descriptions of the type culture as described by Shirling and Gottlieb (Shirling and Gottlieb 1966, 1968, 1969; Shirling 1968), we have identified the *Streptomyces* isolate as a variety of *Streptomyces griseoloalbus* Kudrina.

The isolate was compared with *S. griseoloalbus* ATCC No. 23624. The two organisms were similar in most cultural and morphological properties but differed in several behavioral characteristics. Our isolate grew faster and produced aerial mycelium, spores, and pigments more rapidly than did the ATCC culture. For example, the ATCC culture required an incubation time of 2 weeks to produce aerial mycelium and spores compared with 5 days for our isolate. The ATCC organism would not grow at 10°C but did grow at 36°C, whereas our isolate would not tolerate temperatures above 32°C.

Antagonism toward root pathogenic fungi

Our isolate of *S. griseoloalbus* inhibited all three pathogens on MYP, MYP-B, and SCA agar (Fig. 1A, B, C). An inhibition zone, 10–19 mm depending on the medium used, was produced between this isolate and *P. weirii*. An orange–brown pigment produced by the isolate diffused throughout the medium but stopped at the edges of the fungal colony. A dark melanoid zone was produced on the reverse side of the fungal colony at the interface of the pigment and the fungal mycelium. Along this edge premature sporocarps, including a hymenial layer, were observed; however, no basidiospores were produced over a 2-month period. *Phellinus weirii* (T-55) did not produce basidiocarps on the control plates although we have observed it to do so under laboratory conditions within 2 months of incubation. *Phellinus* mycelium appeared similar on test and control plates.

A 10-mm inhibition zone was produced between our *S. griseoloalbus* isolate and *F. annosus* on SCA medium. An orange–brown pigment originating

from the *Streptomyces* colony diffused toward the fungus but stopped at the margin of the fungal colony. At this margin, the conidiophores grew back upon themselves, forming a tangled mass of convoluted hyphae. Control colonies did not exhibit this response. Conidia did not differ morphologically between the test and control plates.

Inhibition zones averaging 5 mm developed between our *Streptomyces* isolate and *P. cinnamomi*. A brown diffusable pigment produced by the isolate seemingly stimulated the production of chlamydospores or vesicles (Tucker 1931) where the pigment contacted the *Phytophthora* colony. These structures appeared as red protuberances under the agar surface. The red color, upon microscopic examination, was due to a pigmented granular material inside the hyphae. Neither pigmentation nor chlamydospore production occurred on the control plates.

When applied to the surface of the wood disks in Difco *Actinomyces* broth, our isolate of *S. griseoloalbus* grew rapidly over the surface without substantially altering the wood's properties or physical appearance, effectively preventing *F. annosus* from colonizing the disks (Fig. 2). Wood chips inoculum taken from split disks and disk surfaces failed to produce *F. annosus* colonies on the selective medium. *Fomes annosus* colonies did grow from the wood chip inoculum taken from the control disks. The actinomycete, however, was unable to retard development of *F. annosus* when applied as a water suspension. These results suggest that our *Streptomyces* isolate depends upon nutrients from the *Actinomyces* broth for growth and establishment on the wood. Continued survival on the wood and the noncollapsing appearance of the wood cells under microscopic observation suggest that our isolate was able to utilize nonstructural carbohydrates such as simple sugars which have been identified in wood (Smith and Zavarin 1960).

This organism not only produces an antimicrobial agent to retard the growth of *F. annosus* but possibly also inhibits the development of the pathogen by rapidly removing nonstructural carbohydrates from wood which seem to be necessary for rapid hyphal progression. The effectiveness of *S. griseoloalbus* as a stump protectant against *F. annosus* under field conditions is currently being investigated.

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