5-1992

Establishment of a pure culture line for a bacterium which stimulates adventitious rooting in seedling explants of slash pine and white pine

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Establishment of a pure culture line for a bacterium which stimulates adventitious rooting in seedling explants of slash pine (*Pinus elliottii*, Engelm.) and white pine (*Pinus strobus*, L.)

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Tennessee Scholars Program
Senior Research Project

Spring 1992

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ABSTRACT

A pure culture line was established for a bacterium which stimulates adventitious rooting in seedling explants of slash pine (*Pinus elliottii,* Engelm.) and white pine (*Pinus strobus*, L.). The pure culture line was designated RSB. Protocols for maintaining this culture line were developed. The ability of RSB to stimulate rooting was verified using *Pinus strobus* seedling explants.

Further studies were conducted on the RSB culture line. Growth of this organism was tested on several solid media as well as in liquid media. A sterile filtrate from liquid culture of the bacterium was tested for the ability to stimulate rooting in *Pinus elliottii* explants, but that experiment produced no rooting.

Cultures of the bacterium were sent to other laboratories for identification. No conclusive identification was produced, but the best match appears to be *Flavobacterium breve.*

INTRODUCTION

Methods for *in vitro* propagation of forest trees provide clonal stocks for a variety of research and commercial uses (Zobel and Talbert 1976, Mott et al. 1977, Ahuja 1987, Timmis et al. 1987, Schlarbaum 1988). The ability to promote *in vitro* root organogenesis is of general interest in plant tissue and organ culture, both as a means of studying

organogenesis and because a root system must eventually develop if a plantlet is to survive after transfer to soil (Burns 1990). Spontaneous rooting in *Pinus* cultures is generally rare (Mohammed and Vidaver 1988). Hormonal treatments have been used to promote adventitious rooting in explants of white pine (*Pinus strobus*, L.) and slash pine (*Pinus elliottii*, Engelm.) (Mohammed and Vidaver 1988, Burns 1990). However, these methods of promoting root organogenesis often demonstrate poor root morphology, including little branching and poor root hair formation (Mohammed and Vidaver 1988).

Previous studies of root organogenesis in *Pinus elliottii* have indicated that a bacterial contaminant promotes adventitious root formation (Burns 1990), but pure culture lines of the bacterium involved were not attained. Bacterial influence on plant growth and development *in vitro* has been demonstrated in other instances (Frommel et al. 1991), but Burns was the first to note such a relationship for a *Pinus* species. Exploration of such bacterial influences requires a stable source of the bacteria involved. Such a source is provided by establishing and maintaining pure culture lines.

This study presents the methods used to establish and maintain a pure culture line of the bacterium which stimulates rooting in *Pinus*

elliottii explants. This culture line is designated RSB. The ability of cells in the RSB culture line to enhance rooting of *Pinus strobus* explants demonstrates that RSB retains the root-stimulating phenotype, and furthermore shows that the effect of the bacterium on adventitious rooting is not limited to one species.

MATERIALS AND METHODS

Media

Enhanced Woody Plant Medium was used as the primary culture medium for the bacterium. This is a synthetic medium supplemented with sucrose and casein hydrolysate. The components are listed in Appendix B. The liquid form of this medium was designated WPM, while the agar-containing solid form was designated WPA. Other media used were Nutrient Agar (Difco™), Trypticase Soy Broth Agar (TSBA), Difco™ Lowenstein-Jensen slants, Gresshoff and Doy (GD) medium plus 1.5% agar and 0.5% charcoal (Sommer et al. 1975), and potato cube medium (PCM). Potato cube medium was produced by cutting potatoes into roughly 1 cm^3 cubes, placing them in distilled water, and autoclaving. All media were

sterilized by autoclaving at 15 lb/in^2 and 121°C for 20 minutes. Agar plates for streaking were prepared by pouring liquified agar medium into 100x20 mm petri dishes. Culture tubes for seedling cultures were prepared by pouring liquified agar medium into the tubes and then slanting the tubes so that the medium solidified in a slant. These tubes contained GD medium plus 1.5% agar and 0.5% charcoal. All pouring was conducted in a sterile, laminar flow hood.

Culturing techniques

Subculturing was performed on WPA using a t-streak technique as a means of obtaining isolated colonies (Claus 1988). Sterile 10 μl inoculating loops were utilized for transferring and streaking bacteria. Cultures were incubated at 27 ± 2°C in a Scientific Systems™ double door growth cabinet.

The pure culture line was established by subculturing using an isolated colony as the source for the bacterial streak. The culture line was maintained in a generational manner by producing fresh subcultures every one to two weeks. Each group of subcultures was produced using

confluent colonies from the most recent uncontaminated plate obtained from the main culture line.

Frozen stocks stored at -70°C were produced from bacterial cultures growing both in WPM liquid culture and on WPA agar plates using 15% glycerol following a protocol modeled after that presented in Molecular Cloning: A Laboratory Manual (Sambrook et al. 1989). This technique used 0.15 ml of sterile glycerol added to 0.85 ml of liquid bacterial culture. The liquid bacterial culture was obtained while the bacteria were still growing rapidly but after a high titer had been achieved (48 hrs after inoculation). The bacteria and glycerol were added to a labeled tube which was then sealed air-tight and vortexed. The tube was then placed immediately into a freezer at -70°C, or alternatively it was packed in dry ice and then placed in the freezer. The bacteria were recovered either by scraping the surface of the frozen culture with a sterile needle or by allowing the culture to thaw and using a sterile loop. Either way, the bacteria were transferred onto a culture plate by streaking onto the surface, using the standard t-streaking technique, followed by incubation at 27°C.

Source of the bacterium

The bacterium was initially isolated by Burns from an in vitro culture of a *P. elliottii* hypocotylary seedling explant growing on GD medium containing agar and 0.5% charcoal (Burns 1990). Bacteria from these original isolates were maintained on WPA culture plates and used to inoculate more *P. elliottii* explants. Such an explant-bacteria culture was used as the source of bacteria for establishing a pure culture line. The explant used was exhibiting adventitious rooting and was growing on half-strength GD medium plus 1.5% agar and 0.5% charcoal in 20x100 mm culture tubes.

Identification of the bacterium

Subcultures from the RSB culture line were sent to three different commercial laboratories for identification. These were Microcheck (Northfield, Vermont), Presque Isle Cultures (Presque Isle, Pennsylvania), and the American Type Culture Collection (Rockville, Maryland). Microcheck analyzed the fatty acid profile of the cultures and compared those profiles with other strains in a databank. The other companies

relied on a more comprehensive battery of biochemical and physiological tests to determine identity.

Plant sources for rooting experiments

Two seedling age groups of *Pinus strobus* were used to look for signs of the effect of seedling age on adventitious rooting. These groups were planted on 22 August 1991 and 4 September 1991, respectively. Each group was harvested for culturing on 5 November 1991, making the older group 75 days past planting (dpp) and the younger group 62 dpp when harvested.

Several genotypes of *Pinus elliottii* seedlings were used as a source of explants for studying the effect of RSB-conditioned medium on adventitious rooting. These plants were 120 dpp when they were harvested for culturing on 27 February 1992.

Surface sterilization of plants

Groups of about fifty seedlings were cut roughly 4 cm below the stem apex and placed in approximately one liter of double distilled water

containing a few drops of Tween-20™. The plants were agitated to wet as much of the plant as possible. Dead plant parts were trimmed away and the plants were moved to a sterile laminar flow hood, where sterilization dips were performed. All dips were in 250 ml of aqueous solutions contained within 10x8 cm petri dishes with lids. Water for these solutions was double distilled and was sterilized by autoclaving. Ethanol was filter sterilized using a 0.22 μm filter membrane, with the entire filter apparatus sterilized by autoclaving prior to filtering. Commercial bleach (sodium hypochlorite solution) was assumed to be sterile.

The dips were performed as follows: a group of no more than fifteen plants was placed in a sterile 10x8 cm petri dish and immersed in a 70% ethanol dip for 30 seconds while stirring continuously using flame-sterilized tweezers; the ethanol solution was drained and replaced with a 20% bleach solution for 5 minutes, with stirring for 30 seconds at the start, then at 2.5 minutes, and also at 4.5 minutes; the bleach solution was then drained and another 70% ethanol immersion was performed for 30 seconds with stirring; then the ethanol solution was drained and replaced with water. The explants were stirred for about 15 seconds and then left in the water for 15-30 minutes. The explants were then placed on sterile 14x2 cm petri plates within the laminar flow hood and dried for

about ten minutes. Next, the explants were cut with a flame-sterilized blade. Cuts were made roughly 1.5-2.5 cm below the stem apex. These explants were then ready for treatment and placement in culture tubes.

Procedures for testing the effect of the bacterium on rooting

White pine seedling explants ready for culturing were treated with bacteria by dipping the bottom of the stem in a bacterial colony on a WPA streak plate from the RSB pure culture line. Two different culture ages were used, plates streaked 19 days before explant culturing and plates streaked 5 days before explant culturing. Controls were produced by dipping into WPA on a sterile culture plate. The explants were then placed in a culture tube containing half strength GD medium plus 1.5% agar and 0.5% charcoal, and the culture tubes were sealed with parafilm. Plants were incubated at 27 ± 2°C with a photoperiod of 16 hr light followed by an 8 hr dark period. Rooting was scored after 84 days by gently removing the plants from the culture tubes. Cultures with contaminants were not scored.

Slash pine seedling explants ready for culturing were used to test the ability to stimulate adventitious rooting using filter-sterilized liquid

medium (WPM) in which RSB had been growing. The RSB filtrate was obtained by first centrifuging several five-day-old 50 ml liquid cultures of RSB at 27,000 g for fifteen minutes and pooling the supernatants, repeating the centrifugation, and finally producing sterile filtrate by passing the supernatant through a 0.22 μm filter. Equal proportions of the sterile RSB filtrate and sterile full strength GD medium with 3% agar and 1% charcoal were mixed to obtain the medium for the explant cultures. The explants were incubated under the same conditions as in the white pine experiment, except rooting was scored 56 days after culturing. Again, cultures containing contaminants were not scored.

Data analysis

Analysis of adventitious rooting was performed using chi-square tests for independence of rooting. Three such analyses were performed with different conditions serving as the independent variable; one with regard to presence or absence of bacterial inoculation, another with respect to seedling age at the time of culturing, and the third in relation to bacterial culture age at the time of inoculation. In addition, length of the longest root and number of observable secondary roots on the longest

root were compared by treatment type using SAS™ for personal computers (SAS Institute Inc., version 6.04, 1990) to perform several analyses of variance.

RESULTS AND DISCUSSION

Robert Koch, working in the late 1800s, developed rules for establishing the specificity of a pathogenic microorganism. Koch's postulates serve as a cornerstone of medical microbiology. However, they do not directly address microbial involvement in non-pathogenic relationships. There is no compelling reason to restrict their use to medical applications, so a modification of Koch's postulates was used to provide a general means of demonstrating that a microbial species (or strain) is the causative agent of a particular effect on other organisms. These modified postulates are: 1) strong association of the microbial species with organisms exhibiting the effect; 2) cultivation of the microbial species in pure culture; 3) inoculation of the environment of test organisms with microbes from the pure culture and demonstration of the effect in those test organisms; 4) reisolation of the microbial species from the environment of affected test organisms. The overall goal of this

study was the application of these postulates to the bacterium which had been demonstrated by Burns (1990) to promote adventitious rooting in *Pinus elliottii* explants. The first three of these postulates had been satisfied by Burns, but the lack of a surviving pure culture from that work led to the need to reestablish and maintain pure cultures of the bacterium. The primary concern of this study was demonstrating reisolation of the microbial species as stated in the fourth postulate and using that reisolation to produce a constant source of the bacterium in pure culture.

Establishing a pure culture line of the bacterium

The previous ongoing cultures of this bacterium had been stored on refrigerated WPA plates, but these plates exhibited confluent colony growth and signs of a mixed culture. This interpretation is based on the appearance of a thin, whitish substance surrounding a thicker, yellowish growth on the surface of the agar. The yellowish organism was considered to be the best candidate for the root-stimulating bacterium since the original root-stimulating bacteria were described as mustard yellow and subsequent rooting experiments had used mustard yellow bacteria (Burns 1990). An example of such bacteria growing in a tissue

culture vessel with a *Pinus* species is provided by Figure 1. Attempts to use these previous ongoing cultures as a source of the organisms for a pure culture line were eventually successful, but several subcultures of apparently isolated colonies were required to remove the whitish contaminant. Despite these problems, the previous ongoing cultures had been used successfully in rooting experiments.

FIGURE 1: Culture tube with a *Pinus* sp. demonstrating the growth of mustard yellow bacteria (this culture was inoculated before the RSB pure culture line was established).

The main pure culture line was established using bacteria obtained directly from a *P. elliottii* seedling explant culture which had been inoculated with bacteria from previous ongoing cultures. This explant exhibited adventitious

rooting. Enhanced WPA streak plates were inoculated using this source, and an isolated colony from one of these plates was subcultured to

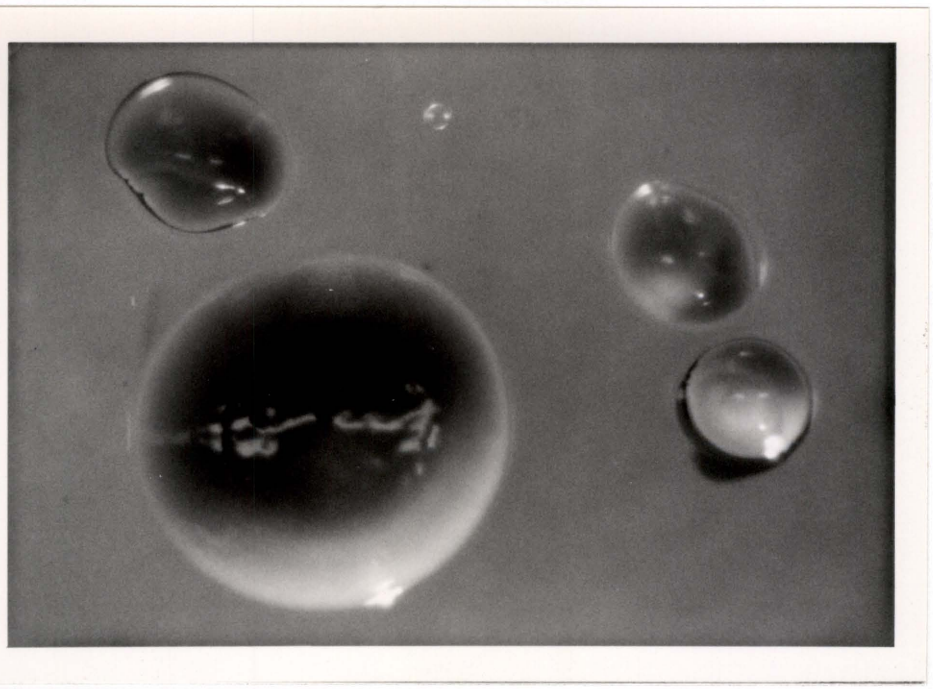

FIGURE 2: Close-up of isolated RSB colonies.

establish the pure culture line.

The subcultures in the pure culture line contained colonies which had the following characteristics: generally circular, convex, smooth, slightly shiny, yellow, and with a cheesy texture. Figure 2 is a photograph of such colonies. The culture lines were maintained using confluent colonies to subculture the next generation. Results were consistent for each plate, suggesting that each plate had the same species of bacterium.

The practical criteria for judging a culture to be pure is that all plates in the culture line have only one colony type and that cells from these cultures show the same Gram reaction and microscopic appearance (Claus 1988). This requires that the culture line start with an isolated colony and that the consistency be demonstrated for several subsequent generations of plates. Although Gram staining and microscopic viewing were not performed on each generation of plates, a visual inspection of the plates provided enough confidence in culture purity to allow proceeding with assaying the main culture line for the ability to enhance rooting in *Pinus* seedling explants. This experiment will be described in detail later, but it is important to note that the bacterial cultures did significantly enhance adventitious rooting. This culture line has been designated RSB for root-stimulating bacterium. All subcultures from this culture line carry the RSB designation. In addition, the main culture line is labelled "D" for direct isolate source. Cultures in the main pure culture line are also marked with a number to indicate subculture generation and an additional letter to describe the inoculation source (A for an isolated colony and B for confluent colonies). For example, the culture plate inoculated with isolated colonies from the tenth generation in the main culture line is designated RSB D11.A. All bacterial cultures and

experiments since the establishment of this main pure culture line have utilized the RSB D line as the ultimate source of bacteria. This includes the frozen stocks which have been produced to allow indefinite storage of bacteria from the culture line.

The purity of the RSB culture line was assumed based on characteristics visible without magnification. This assumption is most likely valid but is not necessarily so. For example, if there are two types of bacteria which depend on each other to be able to grow on WPA, then colonies will form only with both cell types present. Microscopic characterizations and staining techniques should be utilized to determine if such a situation is present in the RSB line. However, if such a situation were to be present it would be favorable to maintain the mixed culture line, since such a mixed line would be needed for the production of the colonies which have been tested for rooting ability. While each type of bacterium present would need to be assayed for the presence of the root-stimulating phenotype, it would be difficult to establish pure cultures is such a case. Therefore, the current culture line provides the best reproducible unit for demonstrating the microbial enhancement of adventitious rooting in *Pinus* cultures. Such a reproducible unit fits a

functional definition of culture purity with regard to exploring causative effects in the spirit of Koch's postulates.

Maintenance of the pure culture line

Maintenance of the culture line required the establishment of reasonable criteria for subculturing. The procedure would place a burden on time and resources if each subculture in a generation were further subcultured. However, enough cultures need to be maintained to ensure the survival of the culture lines. A compromise was reached which minimized the amount of plates used for each generation while still allowing for proper testing of culture purity at each generation. This protocol involved the use of sister cultures, which are different subculture plates inoculated with bacteria from the same plate in the previous generation. A pair of sister cultures in each generation represented each culture line being maintained. One of these was produced from an isolated colony, while the other was inoculated with bacteria from a confluent region on the parent plate. The sister plate with the confluent inoculum was used as the parent plate for the next generation. This enabled the observation of a culture from an isolated

colony in each generation while protecting the culture line from mutations that might be present in isolated colonies. Transfers were performed every week to two weeks, and the number of generations from the original isolated colony source were recorded as part of the identification of each plate. In the rare event of culture contamination, the most recent uncontaminated generation was used again to reestablish the culture line. The older generation plates were sealed with parafilm after the transfer to the next generation.

The main culture line used sister plates of the culture line which was tested in the rooting experiment described in the next section. This culture line was selected because it was the one tested for the ability to stimulate rooting and because the source of the isolated colony for this cell line was a culture produced from a rooting slash pine explant. This culture line holds the best chance of maintaining the ability to enhance adventitious rooting of pine explants, and its reisolation, combined with demonstrating the ability of cells in this culture line to elicit adventitious rooting, fulfills the last postulate for demonstrating the cause of an effect by a microorganism.

In addition to the main culture line, at least one other culture line has always been maintained to provide further checks on the consistency

of the colonies. However, these other culture lines were often abandoned after several generations as other source lines were initiated. The only continuous cell line is the main pure culture line, and all current cultures are derivatives of that cell line.

Growth of the bacterium on other media

Growth of cells from the RSB culture line was tested in different media and under different conditions. The bacterium grew poorly or not at all on nutrient broth agar and trypticase soy broth agar (TSBA). Slants of Lowenstein-Jensen medium supported a level of growth similar to that seen on Enhanced WPA, a result which was also demonstrated by Burns (1990). Liquid potato medium (PCM) and liquid Enhanced WPM supported growth to a visibly high titer within a few days if the culture was continually shaken at about 130 ± 10 rpm within a 27 ± 2°C incubator. The protocol established for liquid cultures required the use of 50 ml of media in a graduated 125 ml flask. Aseptic techniques were used to prevent contamination of the culture, and the inoculation was performed in the sterile hood. The inoculum source was usually isolated RSB colonies picked up with a sterile 10 μl loop. These liquid cultures were then

placed on a Junior Orbit Shaker (Lab-Line) within the incubator and the shaker was turned up to 130 rpm on the continuous setting.

Liquid cultures are of particular interest as a means of assaying metabolites produced by RSB for their ability to enhance adventitious rooting. There is evidence that RSB conditions the media in which it grows such that the media itself can stimulate rooting (Burns 1990), but there were problems with the initial experiments. No successful liquid cultures of the bacterium had been established prior to the success with the RSB pure culture line, so the previous experiment required a complicated procedure to derive eluate from a solid medium. In addition, all of the cultures were contaminated with a white microbe which was able to pass through a 0.22 μm filter; this contaminant is probably the same as that in the mixed culture plates used in the early steps of establishing a pure culture line. The results of the experiment using an eluate from a solid medium were inconclusive due to this contamination, but they did suggest that the rooting activity may be due to soluble substances released into the medium by RSB (Burns 1990). However, an experiment using liquid cultures from the RSB culture line failed to produce any positive rooting response.

Production of frozen stocks of RSB

The ultimate reason for establishing and maintaining a culture line of RSB is to ensure the availability of RSB for future experiments. This requires a means of keeping the cell line alive indefinitely in a manner which effectively eliminates the risk of the properties of the cell line changing. Beyond the initial step of establishing the cell line, the use of isolated colonies in the main cell line is not wise because such isolated colonies may be derived from a mutant, changing the characteristics of the strain. However, each transfer involves a risk of losing an important genotype even with the use of confluent colonies as a source. The best check on this is to periodically test the cell line for the ability to enhance rooting, but it takes weeks to get the results of these experiments. So far the cell line has not lost its ability to confer the rooting response, but if this were to occur another main culture line would need to be established. The best source for such a culture line would be frozen stocks of the bacterium produced at a time when the culture line was known to enhance rooting response. If bacteria can be recovered from such frozen stocks, these cultures provide a means of indefinite storage with the bacteria in essentially a state of suspended animation (Sambrook et al. 1989).

Establishment of such frozen stocks would provide the ultimate resource for maintaining the RSB line.

A simple technique for frozen storage appears to have been successful in preserving viable RSB cells. The bacteria were suspended in a 15% glycerol solution and stored at -70°C. The ability to recover RSB from such a frozen stock was demonstrated, and the visible characteristics of the culture plates were consistent with what is expected for RSB. However, the ability of such a recovered culture to enhance adventitious rooting was not tested.

Attempts to identify the bacterium

The identity of the bacterium is still in question, and it is likely that RSB represents at least a unique strain if not a previously undescribed species. An initial attempt to identify the bacterium had used an API™ Rapid NFT biochemical and assimilation test kit for gram negative bacteria (Burns 1990). This putative identification was *Pseudomonas vesicularis* (Büssing, Döll, Freytag). However, the lack of a pure culture line made this identification questionable. Furthermore, a type culture of *Pseudomonas vesicularis* did not exhibit the root-

stimulating phenotype and produced colonies on WPA which did not look the same as RSB colonies (O.J. Schwarz, pers. comm.).

Streak plates of from the RSB pure culture line were sent to three companies which perform identifications for a fee. Microcheck, Inc., performed a fatty acid profile analysis of the bacterium grown on TSBA. This procedure was modified from normal protocol due to the poor growth of RSB on TSBA, and this modification may have prevented detection of a true match for RSB. The fatty acid profile was compared to over 7000 strains in a database and similarity indices were derived for possible matches in the database. The only match was *Pseudomonas saccharophila*, with a similarity index of 0.053. A similarity index of 1.0 is an exact match, while a similarity index under 0.1 places the genus match in question. Thus, this analysis did not provide an adequate confidence level for identification. However, the previous identification of *P. vesicularis* was placed in question since that species is represented in the Microcheck database but does not match RSB.

The second company contracted to identify the bacterium was Presque Isle Cultures, which examined the morphology and growth characteristics of two RSB cultures. Their results showed that the bacterium is a slender, Gram negative rod which grows at 30°C but not at

37°C. A phenol red glucose test of each culture gave slight acid results. Tests for use of H_2S and Litmus milk were negative, as were tests for starch hydrolysis and conversion of NO_3 to NO_2. A test for production of indole was negative for one culture and weak for the other. Based on these results, both cultures were identified by Presque Isle Cultures as *Flavobacterium breve*. The 1984 edition of *Bergey's Manual of Systematic Bacteriology* recognizes the type culture for this species as strain CL88/76 of Holmes et al. (1978). This strain was isolated from a bronchial secretion and does appear to be a good match for RSB, except that it will grow at 37°C (Holmes et al. 1978). Also, *F. breve* is present in the Microcheck database but did not match our bacterium in Microcheck's analysis. Several more biochemical tests should be performed to explore the similarity of the RSB strain to the *F. breve* type culture.

The American Type Culture Collection (ATCC) was also contracted to identify RSB. Difficulties in subculturing the bacterium onto several media used in biochemical tests prevented ATCC from identifying the bacterium.

The effect of RSB on Pinus strobus *seedling explants*

An example of a slash pine seedling which exhibited adventitious rooting in the presence of the bacterium is provided in Figure 3. This

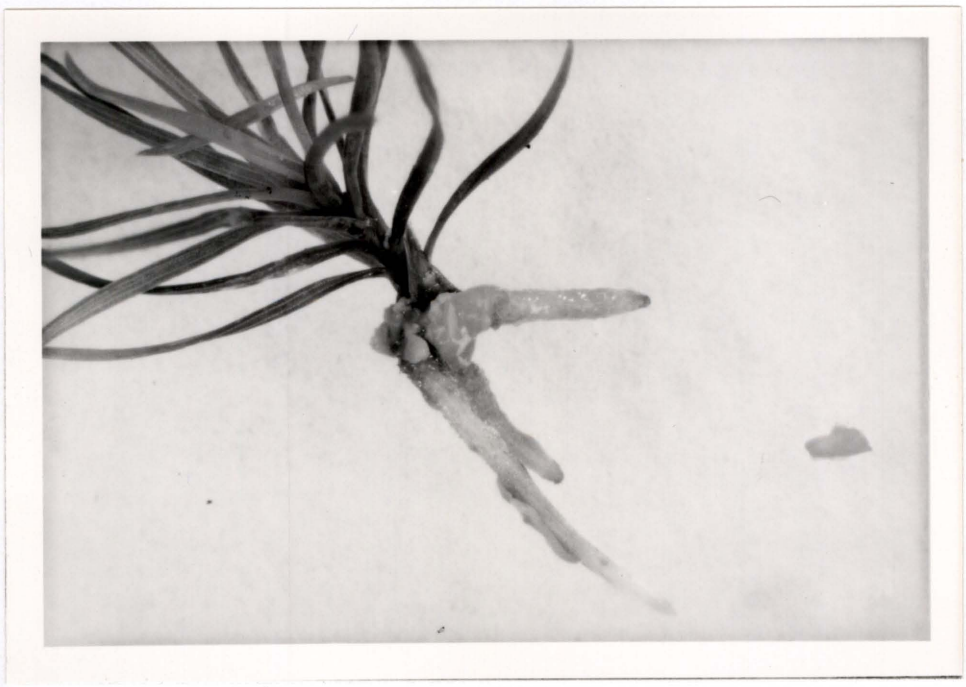

FIGURE 3: *Pinus elliottii* seedling explant with adventitious roots and mustard-yellow bacteria along the roots.

culture was from an experiment performed before the pure culture line was established. Similar results were obtained using the RSB pure

culture line to stimulate adventitious rooting in *Pinus strobus* (Figure 4). A summary of these results is presented in Appendix A. The frequencies

FIGURE 4: Eight *Pinus strobus* seedling explants 84 days after culturing. Note the thick callus present in the top four, which are all control, and the different levels of adventitious root development in the bottom four, which were all inoculated with RSB.

of adventitious rooting in the white pine experiment are presented in Table 1. Rooting was scored as absent or present for each explant regardless of root quality, size, or quantity. These results were crosstabulated in 2 x 2 contingency tables for tests for independence (Sokal and Rohlf 1981, Walpole and Myers 1989). Inoculation with bacteria versus no inoculation, age of seedling at explant culturing, and

age of bacterial culture at explant culturing were all tested. These contingency tables are given together as Table 2, and the results of the tests are given as Table 3. The test for independence uses the formula

$$X^2 = \sum_i \{(o_i - e_i)^2/e_i\}$$

and has degrees of freedom

$$df = (\#rows - 1) \times (\#columns - 1).$$

The probability of independence is obtained from a table relating the X^2 value and df to the probability (Walpole and Myers 1989). The null hypothesis is that the events are independent; that is, that adventitious rooting is not dependent on the treatment (e.g. bacterial inoculation). The null hypothesis is generally rejected if $P<0.05$. Rooting appears to be strongly dependent on bacterial inoculation but independent of seedling age at time of planting or of the age of the bacterial culture used for inoculation. Based on this evidence, the RSB pure culture line has the phenotype for stimulating adventitious rooting. Furthermore, since

TABLE 1

Rooting Frequencies by Treatment Groupings of White Pine Seedling Explants

Grouping	#not rooting/total (% not rooting)	# rooting/total (% rooting)
Control, 62 dpp	9/9 (100%)	0/9 (0%)
Control, 75 dpp	14/14 (100%)	0/14 (0%)
5 day bact, 62 dpp	5/14 (35.7%)	9/14 (64.3%)
5 day bact, 75 dpp	5/14 (35.7%)	9/14 (64.3%)
19 day bact, 62 dpp	4/9 (44.4%)	5/9 (55.6%)
19 day bact, 75 dpp	5/15 (33.3%)	10/15 (66.7%)

the seedlings in this experiment are from white pine while the bacterium was originally isolated as a cause of rooting in slash pine, this stimulatory ability is not specific for one *Pinus* species.

TABLE 2

2 x 2 Contingency Tables for Observed Rooting Frequencies of White Pine Explants with Various Treatments as the Independent Variable*

	Bacterial Inoculation		
Presence of Rooting	Control	Experimental	Total
Absent	23 (12.9)	19 (29.1)	42
Present	0 (10.1)	33 (22.9)	33
Total	23	52	75

	Seedling Age*		
Presence of Rooting	62 dpp	75 dpp	Total
Absent	9 (8.4)	10 (10.6)	19
Present	14 (14.6)	19 (18.4)	33
Total	23	29	52

	Age of Bacterial Source*		
Presence of Rooting	5 Days	19 Days	Total
Absent	10 (10.2)	9 (8.8)	19
Present	18 (17.8)	15 (15.2)	33
Total	28	24	52

*Only explants inoculated with bacteria were included.
dpp = explant age in days past planting at the time of culturing
( ) = expected values
Control = explants touched to a bacteria-free agar plate
Experimental = explants touched to a bacteria colony on an agar plate

TABLE 3

Chi-square Tests for Independence for Rooting Frequency of Explants Under Various Treatments After 84 Days

Treatment	n	df	X^2 Value	P	Accepted Hypothesis
Bacteria	75	1	26.065	$P \leq 0.001$	H_1
Plant Age	52*	1	0.119	$P \geq 0.70$	H_0
Bacteria Age	52*	1	0.018	$P \geq 0.80$	H_0

*Only explants inoculated with bacteria were included.

n = sample size
df = degrees of freedom
P = probability of independence
H_0: Rooting is independent of the treatment.
H_1: Rooting is not independent of the treatment.

Besides presence or absence of rooting, the number of primary roots, lengths of primary roots, and number of secondary roots were recorded.

FIGURE 5: Close-up of a *Pinus strobus* seedling explant 84 days after culturing exhibiting multiple adventitious roots as well as abundant secondary rooting.

An example of an explant exhibiting multiple adventitious roots as well as numerous secondary roots is shown in Figure 5. The root lengths for the longest adventitious root and number of secondary roots for these longest primary roots are summarized in Tables 4 and 5, respectively. Statistical significance of the differences in average root lengths were calculated using a comparison or contrast in treatment means test. The same was true for the number of secondary roots. These are presented as Tables 6 and 7,

TABLE 4

Average root lengths

Group no	N*	Length (mm) Mean ± SD
C(75)	0	0
C(62)	0	0
N(75)	11	19 ± 11
N(62)	15	29 ± 16
O(75)	16	37 ± 20
O(62)	11	18 ± 16

*number rooting in group

TABLE 5

Average number of secondary roots per primary root

Group no.	number ± SD
C(75)	0
C(62)	0
N(75)	7.8 ± 4.8
N(62)	7.0 ± 4.6
O(75)	10.6 ± 4.1
O(62)	4.2 ± 3.6

C(75) = Control, seedling 75 days past planting (dpp) when cultured
C(62) = Control, seedling 62 dpp
N(75) = Inoculation with 5-day-old bacterial culture, 75 dpp
N(62) = Inoculation with 5-day-old bacterial culture, 62 dpp
O(75) = Inoculation with 19-day-old bacterial culture, 75 dpp
O(62) = Inoculation with 19-day-old bacterial culture, 62 dpp

TABLE 6

Data Analysis of Root Length
(General Linear Models Procedure)

Contrast Groups	DF	Contrast SS	F	Pr > F
3 vs 4	1	1143	6.33	0.0205
3 vs 5	1	2439	13.50	0.0015
3 vs 6	1	112	0.62	0.4398
4 vs 5	1	270	1.49	0.2357
4 vs 6	1	433	2.40	0.1372
5 vs 6	1	1258	6.97	0.0157
Plant age:				
62 dpp vs 75 dpp	1	6.11	0.03	0.8559
Bacteria:				
5 days vs 19 days	1	349	1.93	0.1797

TABLE 7

Data Analysis of Number of Secondary Roots
(General Linear Models Procedure)

Contrast Groups	DF	Contrast SS	F	Pr > F
3 vs 4	1	0.193	0.02	0.8953
3 vs 5	1	121	11.17	0.0033
3 vs 6	1	3.026	0.28	0.6035
4 vs 5	1	120	11.05	0.0034
4 vs 6	1	4.83	0.44	0.5126
5 vs 6	1	144	13.27	0.0016
Plant age:				
62 dpp vs 75 dpp	1	71.96	6.62	0.0181
Bacteria:				
5 days vs 19 days	1	34.23	3.15	0.0911

3 = 75 dpp, 5 days
4 = 62 dpp, 5 days
5 = 75 dpp, 19 days
6 = 62 dpp, 19 days

days = age of bacterial culture used
dpp = age at culturing in days past planting
DF = degrees of freedom
F = value of F statistic
Pr > F = probability of error in rejecting null hypothesis

respectively. These comparisons only considered rooted plants, so some groups had very small sample sizes. The reliability of the contrast statistics are therefore limited. Tests with larger sample sizes should be conducted to fully explore these relationships. The most likely relationship is between age of seedling at the time of culturing and the amount of secondary rooting at day 84 after culturing. The amount of secondary rooting (as well as root length) could be related to the time at which root organogenesis is triggered.

The effect of a sterile filtrate of RSB liquid culture on rooting

As mentioned earlier, rooting of slash pine seedling explants was not produced from a sterile filtrate of RSB liquid culture, even though a similar experiment by Burns (1990) did produce rooting. There could be several reasons for this discrepancy. The procedures for the experiments were different; Burns did not use liquid cultures but instead used eluates derived from bacterial cultures grown on agar-solidified GD medium with charcoal. In addition, the entire group in Burn's experiment was contaminated with a white microorganism, thought most likely to be mycoplasma (Burns 1990), and it is possible that these organisms were

able to stimulate adventitious rooting. These differences complicate comparison of the experiments. In addition, if the bacterium does induce adventitious rooting by conditioning the medium, then such conditioning could be dose-dependent, and the filtrate experiment may have had either too much or too little of the root-stimulating factors. Another possibility is that the bacteria may only release root-stimulating factors when grown on GD medium or on agar; this would not be inconsistent with Burn's results. Also possible is that something produced by the plant stimulates release of the conditioning substance by the bacteria; this appears inconsistent with Burn's results, but the contamination in his experiment clouds the issue. Another alternative is that the bacteria need to be in direct contact with the plant in order to stimulate rooting. Again, this appears inconsistent with Burn's results but cannot be ruled out due to the contamination problem in Burn's experiment. More refined investigations into the possibility of stimulation of adventitious rooting due to medium conditioning by RSB should be pursued.

CONCLUSIONS

A generalization of Koch's postulates served as a guide for demonstrating an effect of a microorganism on adventitious rooting in *Pinus* explants. These generalized postulates are: 1) show a strong association of the microbial species with organisms exhibiting the effect; 2) cultivate the microbial species in pure culture; 3) inoculate the environment of test organisms with microbes from the pure culture and demonstrate the effect in those test organisms; and 4) reisolate the microbial species from the environment of affected test organisms. The association of adventitious rooting in *Pinus elliottii* explants with a mustard yellow microbial growth was noted by Burns (1990). Cultures of this microorganism were established by Burns, and these were used as a source of inoculum in cultures of other *Pinus elliottii* explants. A pure culture line was established from bacteria reisolated from such a test explant which exhibited the rooting effect. The identity of the organism was demonstrated by comparisons with the characteristics noted for the organism by Burns and by demonstration of the ability to stimulate rooting in *Pinus strobus* seedling explants. This ability needs to be demonstrated again using *Pinus elliottii* seedling explants, which were

the original source of the bacterium, and should be tested with other *Pinus* species.

The pure culture line for the root-stimulating bacterium, RSB, was established and maintained using a streaking method which allowed the production of both isolated and confluent colonies. The purity of the culture line was demonstrated by several generations of culture plates grown at 27°C with no changes in the appearance of the colonies. The RSB pure culture line was maintained using confluent colonies to help ensure preservation of the root-stimulating phenotype. In addition, the ability to recover bacteria stored at -70°C may provide a means of indefinite storage of RSB stock cultures. However, the ability of recovered RSB to stimulate rooting must be demonstrated.

There is a slight possibility that the cell line is still a mixed culture, but the uniformity of colony phenotype implies that if this is the case then the different organisms present must need each other in order to grow and form a visible colony. In view of this possibility, it may be best to consider RSB to be a colony-forming unit rather than a single species. Cells should be examined by staining techniques and microscopy to determine the purity of the culture line. If a mixed culture were to be present, then it would be desirable to preserve the mixed culture unit

intact as it is now to ensure the growth of the cell line as well as to try to isolate and test the component organisms in the culture.

Reproduction of experiments relies on a consistent culture line. The establishment and preservation of the RSB culture line provides a stable source of bacteria for further studies. With this source the identity of the bacterium can be explored in more detail. This should include a more detailed comparison of the RSB culture line with the *Flavobacterium breve* type strain, and a test of the type strain for the ability to stimulate adventitious rooting. Also, further research into the details of the interaction between RSB and *Pinus* explants can proceed. The roots induced by RSB demonstrate better morphology than those developed using hormonal treatments, so the use of RSB to stimulate rooting holds the promise of improving root organogenesis for *Pinus* explants.

ACKNOWLEDGMENTS

This research would not have been possible without support in several areas. Direct support for the project was provided by Dr. Otto Schwarz, who served as project mentor and supervisor, while Dr. Howard Adler provided advice for working with bacterial cultures. Sridhar Venkataraman and Cynthia Bowling assisted with the statistical analysis. Laboratory and culture facilities were provided by the Botany Department of the University of Tennessee, Knoxville. Countless bits of advice and favors were provided by others working in the Plant Tissue Culture Laboratory, especially Lynn Atkins, and by those working in the laboratories of Dr. Schwarz and Dr. Beth Mullins.

I have had the good fortune of working with several outstanding scientists while at the University of Tennessee, including Dr. Soren Sorensen, Dr. Susan Reichert, Dr. Howard Adler, and of course Dr. Otto Schwarz. Their aid has been invaluable in showing me what it means to be a true scientist as well as in teaching me how to design meaningful experiments.

I would also like to thank the University of Tennessee Honors Program, the Martin Marietta Scholarship Corporation Foundation, the

National Merit Scholarship Foundation, the University of Tennessee Alumni Association, the Tennessee Student Assistance Corporation, the Reeder-Siler Scholarship Fund, the University of Tennessee College of Liberal Arts, my parents Tony and Linda Bowling, and my wife Cynthia Bowling for providing funding for five years of undergraduate study at the University of Tennessee.

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APPENDIX A: Summary of data for *Pinus strobus* explant cultures

Treatment	Plant Age Group	Bacteria Age Group	ID #	Rooting	#roots	Longest root length
Control	75 dpp	NA	1	no	NA	NA
Control	75 dpp	NA	2	no	NA	NA
Control	75 dpp	NA	3	no	NA	NA
Control	75 dpp	NA	4	no	NA	NA
Control	75 dpp	NA	5	no	NA	NA
Control	75 dpp	NA	6	no	NA	NA
Control	75 dpp	NA	7	no	NA	NA
Control	75 dpp	NA	8	no	NA	NA
Control	75 dpp	NA	9	no	NA	NA
Control	75 dpp	NA	10	no	NA	NA
Control	75 dpp	NA	11	no	NA	NA
Control	75 dpp	NA	12	no	NA	NA
Control	75 dpp	NA	13	no	NA	NA
Control	75 dpp	NA	14	no	NA	NA
Control	75 dpp	NA	15	cont	NA	NA
Control	75 dpp	NA	1	cont	NA	NA
Control	62 dpp	NA	2	cont	NA	NA
Control	62 dpp	NA	3	no	NA	NA
Control	62 dpp	NA	4	no	NA	NA
Control	62 dpp	NA	5	cont	NA	NA
Control	62 dpp	NA	6	no	NA	NA
Control	62 dpp	NA	7	no	NA	NA
Control	62 dpp	NA	8	no	NA	NA
Control	62 dpp	NA	9	no	NA	NA
Control	62 dpp	NA	10	cont	NA	NA
Control	62 dpp	NA	11	no	NA	NA
Control	62 dpp	NA	12	no	NA	NA
Control	62 dpp	NA	13	cont	NA	NA
Control	62 dpp	NA	14	cont	NA	NA
Control	62 dpp	NA	15	no	NA	NA
Experimental	75 dpp	5 days old	1	yes	2	21 mm
Experimental	75 dpp	5 days old	2	no	NA	NA
Experimental	75 dpp	5 days old	3	cont	NA	NA
Experimental	75 dpp	5 days old	4	no	NA	NA
Experimental	75 dpp	5 days old	5	yes	1	14 mm
Experimental	75 dpp	5 days old	6	no	NA	NA
Experimental	75 dpp	5 days old	7	yes	1	11 mm
Experimental	75 dpp	5 days old	8	yes	2	35 mm
Experimental	75 dpp	5 days old	9	yes	1	39 mm
Experimental	75 dpp	5 days old	10	yes	1	8 mm
Experimental	75 dpp	5 days old	11	yes	1	28 mm
Experimental	75 dpp	5 days old	12	no	NA	NA
Experimental	75 dpp	5 days old	13	no	NA	NA
Experimental	75 dpp	5 days old	14	yes	1	15 mm
Experimental	75 dpp	5 days old	15	yes	1	9 mm
Experimental	62 dpp	5 days old	1	yes	2	12 mm
Experimental	62 dpp	5 days old	2	cont	NA	NA
Experimental	62 dpp	5 days old	3	yes	1	52 mm
Experimental	62 dpp	5 days old	4	yes	1	35 mm
Experimental	62 dpp	5 days old	5	yes	1	55 mm
Experimental	62 dpp	5 days old	6	yes	3	44 mm
Experimental	62 dpp	5 days old	7	no	NA	NA
Experimental	62 dpp	5 days old	8	yes	1	29 mm
Experimental	62 dpp	5 days old	9	yes	2	48 mm
Experimental	62 dpp	5 days old	10	no	NA	NA
Experimental	62 dpp	5 days old	11	no	NA	NA
Experimental	62 dpp	5 days old	12	yes	2	40 mm
Experimental	62 dpp	5 days old	13	no	NA	NA
Experimental	62 dpp	5 days old	14	no	NA	NA
Experimental	62 dpp	5 days old	15	yes	2	33 mm
Experimental	75 dpp	19 days old	1	yes	2	16 mm
Experimental	75 dpp	19 days old	2	no	NA	NA
Experimental	75 dpp	19 days old	3	yes	3	43 mm
Experimental	75 dpp	19 days old	4	no	NA	NA
Experimental	75 dpp	19 days old	5	yes	1	29 mm
Experimental	75 dpp	19 days old	6	yes	1	25 mm
Experimental	75 dpp	19 days old	7	yes	3	60 mm
Experimental	75 dpp	19 days old	8	yes	2	46 mm
Experimental	75 dpp	19 days old	9	yes	1	94 mm
Experimental	75 dpp	19 days old	10	no	NA	NA
Experimental	75 dpp	19 days old	11	yes	1	31 mm
Experimental	75 dpp	19 days old	12	yes	1	48 mm
Experimental	75 dpp	19 days old	13	no	NA	NA
Experimental	75 dpp	19 days old	14	no	NA	NA
Experimental	75 dpp	19 days old	15	yes	1	34 mm
Experimental	62 dpp	19 days old	1	no	NA	NA
Experimental	62 dpp	19 days old	2	cont	NA	NA
Experimental	62 dpp	19 days old	3	cont	NA	NA
Experimental	62 dpp	19 days old	4	yes	3	52 mm
Experimental	62 dpp	19 days old	5	cont	NA	NA
Experimental	62 dpp	19 days old	6	no	NA	NA
Experimental	62 dpp	19 days old	7	no	NA	NA
Experimental	62 dpp	19 days old	8	yes	1	40 mm
Experimental	62 dpp	19 days old	9	no	NA	NA
Experimental	62 dpp	19 days old	10	cont	NA	NA
Experimental	62 dpp	19 days old	11	yes	1	35 mm
Experimental	62 dpp	19 days old	12	yes	3	10 mm
Experimental	62 dpp	19 days old	13	yes	3	12 mm
Experimental	62 dpp	19 days old	14	cont	NA	NA
Experimental	62 dpp	19 days old	15	cont	NA	NA

APPENDIX B: Media components

Enriched Woody Plant Agar (Enriched WPA)

Component	Info	Amount
WPM Macro 5X	stock solution	100 ml
MS Micro full strength	stock solution	5.0 ml
GD Fe stock	stock solution	5.0 ml
meso-Inositol		0.05 g
Nicotinic acid solution	0.1 mg/ml	2.5 ml
Pyridoxine HCl solution	0.1 mg/ml	2.5 ml
Thiamine HCl solution	0.1 mg/ml	0.5 ml
Glycine solution	2.0 mg/ml	0.5 ml
Sucrose		10.0 g
Casein Hydrolysate	(enzymatic)	1.0 g
Agar		7.5 g
add dd H_2O		to 500 ml

WPM Macro 5X

Component	Amount
NH_4NO_3	2.00 g
$CaCl_2 \cdot 2H_2O$	0.48 g
$MgSo_4 \cdot 7H_2O$	1.85 g
KH_2PO_4	0.85 g
$Ca(NO_3)_2 \cdot 4H_2O$	2.78 g
K_2SO_4	4.95 g
add dd H_2O	to 1.0 l

Appendix B (continued):

MS Micro Full Strength

Component	Info	Amount
H_3BO_3		0.62 g
$MnSO_4 \cdot H_2O$		1.69 g
$ZnSO_4 \cdot 7H_2O$		0.86 g
KI		0.083 g
$Na_2MoO_4 \cdot 2H_2O$		0.025 g
$CuSO_4 \cdot 5H_2O$ solution	2.5 mg/mL	1.0 ml
$CoCl_2 \cdot 6H_2O$ solution	2.5 mg/mL	1.0 ml
add dd H_2O		to 1.0 l

GD Fe Stock

Component	Amount	Directions
$FeSO_4 \cdot 7H_2O$	2.78 g	dissolve in 500 ml dd H_2O
Na_2EDTA	3.73 g	dissolve in 500 ml dd H_2O

Heat Na_2EDTA solution until boiling and add to $FeSO_4$ solution; then boil for one hour and add distilled/deionized H_2O to a final 1 liter volume.