THE PRESENCE AND POSSIBLE SIGNIFICANCE
OF THE ENDOPHYTIC BACTERIAL FLORA IN
SOLANUM TUBEROSUM L.

BY

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B.Sc. (Agr.), University of British Columbia, 1970

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
IN THE DEPARTMENT OF PLANT SCIENCE

We accept this thesis as conforming to the required standard.

THE UNIVERSITY OF BRITISH COLUMBIA
July, 1972
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Department of Plant Science

The University of British Columbia
Vancouver 8, Canada

Date July, 1972
ABSTRACT

The total number of viable bacteria found in *Solanum tuberosum* stems and tubers was found to vary from less than $1 \times 10^3$ to $4.7 \times 10^7$ cells per cm$^3$ in stems and from 0 to $1.6 \times 10^4$ cells per cm$^3$ in tubers. About 75% of both stems and tubers had populations at the lower end of the range but there was no significant correlation between bacterial counts in stems and tubers of the same plant. Bacteria found in potato tubers included species of *Micrococcus*, *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Agrobacterium*, and *Xanthomonas*. Also present were coryneforms and some others which were not identified to genus but were gram negative. Some of the coryneforms were morphologically indistinguishable from the bacterial ring rot organism (*Corynebacterium sepedonicum*) but non-pathogenic and biochemically different from it. About 5% of stem smears of healthy plants showed more than 30 gram positive rods per microscope field.

All the *Bacillus* spp., one *Pseudomonas* sp. and three unidentified species were found to inhibit *C. sepedonicum* in vitro. An antibiotic was partially purified from the *Bacillus* sp. showing the greatest amount of inhibition. This species was also antagonistic toward the following potato pathogens: *Pseudomonas solanacearum*, *Erwinia atroseptica*, *E. carotovora*, *Alternaria solani*, and *Phytophthora infestans*. Physical and chemical tests showed that both a lipid and a peptide antibiotic were involved in the antagonistic effect.
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ACKNOWLEDGMENTS

The author gratefully acknowledges the guidance and encouragement given during the research and writing of this thesis by Dr. R. J. Copeman.

Gratitude is extended to Dr. R. Stace-Smith, Canada Department of Agriculture, Research Station, Vancouver, who originally suggested the project and showed continual interest in it.

Thanks are also due for their helpful suggestions to the other members of my graduate committee which included Dr. V. C. Runeckles, Department of Plant Science; Dr. N. S. Wright, Canada Department of Agriculture; Dr. T. H. Blackburn, Department of Microbiology; and Dr. E. E. Ishiguro, Department of Microbiology.

Special thanks is extended to Dr. N. S. Wright for supplying the potato tubers used in this study and facilities for PVS testing.

Thanks also to Dr. J. Tremaine, Canada Department of Agriculture, Research Station, for doing the amino acid analysis; to Esther Lo and Beatrice Schroeder, also from the Canada Department of Agriculture, for operating the electron microscope; and to Randy Englar, Department of Soil Science for doing the I.R. spectrum.

Support for this work was from NRC operating grant A-6240 and grants from the UBC Committee on Research awarded to Dr. R. J. Copeman.
Part A
Endophytic Bacterial Flora in Solanum tuberosum

LITERATURE REVIEW

INTRODUCTION

At one time internal healthy plant tissue was thought to be sterile. However, since Perotti (45) found bacteria in healthy root tissue, this concept has changed. It is generally accepted that plant tissue may contain internal micro-organisms. These organisms have been labelled 'endophytes' or 'endophytic bacteria' (58). Endophytic bacteria are distinct from epiphytic bacteria which occur on the plant surface (36). The distinction between endophytes and epiphytes may at times be obscure. For example, when bacteria occur on leaf surfaces and in the substomatal chambers (4) the distinction is not sharp. However, bacteria occurring in the vascular systems of a stem (22), are definitely endophytes.

Presence of endophytic bacteria

The presence of non-pathogenic bacteria in apparently healthy stem and tuber tissue of Solanum tuberosum has previously been investigated (22, 38, 50, 56). Several genera were found to be present as determined by different biochemical reactions. Bacterium radiobacter (50), Aerobacter cloacae and Bacillus megaterium (22) were identified among the species present. Bacteria have also been obtained from potato seeds and seedlings, although their occurrence was not common (22).
Hollis (22) found the ratio of bacteria in xylem and phloem to be 21:1 in potato. He hypothesized that the bacteria are located in vessels and sieve tubes. However, Lutman and Wheeler (38) from histological observations suggested that they occur in the middle lamellae between cell walls.

Endophytic bacteria have also been reported in other crops. Approximately 40% of unblemished tomato fruits were found to contain internal micro-organisms (49). The genera encountered in the fruits included; Xanthomonas, Pseudomonas, Aerobacter, Escherichia, Micrococcus, Flavobacterium, and Corynebacterium. The number of organisms varied up to 10,000 per ml of fruit juice. Samish and Dimant (48) have shown that fresh, healthy cucumbers contain an endophytic bacterial population. They found an average of 4,000 bacterial cells per ml of cucumber juice. These micro-organisms were not identified. Bacteria have also been reported in the stems of Phaseolus vulgaris, and in the tap roots of Medicago and Melilotus spp. (50). In addition bacteria have been isolated consistently from storage organs of kohlrabi, red beet, turnip, sweet potato, and carrots (56). The presence of endophytic bacteria in carrots has been recognized as a problem in prolonged physiological experiments (25).

Mode of Entry

The manner by which the endophytic bacteria enter the plant tissues is still largely one of speculation. In those cases where bacteria are isolated from the fruit, flowers are a probable port of entry (39). This is substantiated by the recovery of greater numbers of bacteria from the blossom end as compared to the stem end in cucumbers (48). Moreover flowers have also
been known to act as a port of entry for some phytopathogenic bacteria e.g. *Erwinia amylovora*. Bacteria could enter the flower either through nectarthodes or along with pollen tubes.

Entry into roots and storage tissue probably occurs from the soil. Bacteria could enter through lenticels and wounds as has been shown for pathogenic organisms (53), or by direct penetration into the undifferentiated meristematic tissue as was hypothesized by Hollis (22). Once in the root system the organisms could move throughout the plant.

An attempt has been made to test the root entry hypothesis. Potato seedlings in sand culture were subjected to heavy suspensions of *Pseudomonas tabaci*. Subsequent attempts to isolate *P. tabaci* from the potato plants met with only limited success (22).

On vegetatively propagated crops bacteria may be transferred from year to year in the plant tissue as is *Erwinia atroseptica*, *E. carotovora* (21, 44) and *Corynebacterium sepedonicum* (58) in potato. Seeds may also act as carriers but probably are less effective than vegetative organs (22).

Significance as Latent Pathogenic Forms

Very little work has been done on what significance endophytic micro-organisms may have in the host plant. Some bacteria are able to multiply in non-host plants (24); thus their presence may have a greater effect on the host plant when their population increases.

*Erwinia atroseptica* and *E. carotovora* have been isolated from apparently healthy potato stems (44). Kennedy (33) has demonstrated that
Pseudomonas glycinea is present internally in field-grown soybean plants and seeds. Cameron (9) reported the presence of Pseudomonas syringae in healthy cherry trees. Keil and van der Zwet (32), moreover, isolated Erwinia amylovora from symptomless pear and apple tissue. In all these examples pathogenic species exist as latent forms or subclinical infections. Thus pathogenic forms may be part of the endophytic flora.

Significance in bacterial ring rot diagnosis

Diagnosis of bacterial ring rot of potato caused by Corynebacterium sepedonicum (Spieck. and Kotth.) Skapt. and Burkh. is based on the occurrence of a characteristic rot in vascular bundles of stems and/or tubers. Confirmation depends on finding large numbers of gram positive bacteria in smears from affected tissues (20). Strict control measures are applied in most areas because severe losses may result from infection. In British Columbia, for example, legislation provides disposal and cleanup procedures for local and imported lots in which the disease is found (Domestic Bacterial Ring Rot Act (8). Throughout Canada there is a zero tolerance for the disease in certified seed (Destructive Insect and Pest Act (10).

To avoid use of infected tubers for seed, gram-stained stem smears from selected plants are examined (3). If short, gram positive rods are found the plants are considered suspect. This scrutiny of "healthy" plants is based on the assumption that sub-clinical infection by C. sepedonicum may carry the organism from year to year in tubers. Walker (58) states that "it is possible for a tuber to carry the germs internally and produce symptomless plants and symptomless tubers although all three stages are infected."
However, no experimental evidence is cited. It is moreover assumed that other short gram positive rods are absent from potato stems, and that the endophytic flora in the stem reflects that in the tuber.

Effect of virus infection on host resistance

The effect of virus infection on the subsequent susceptibility to other diseases has been well documented. Geranium plants infected with any of four latent viruses are more susceptible to bacterial stem rot caused by *Xanthomonas pelargonii* (35). Similarly pea mosaic, alfalfa mosaic, bean yellow mosaic, and pea enation mosaic virus increase susceptibility to pea root rots caused by *Aphanomyces euteiches* or *Fusarium solani* (18). Sugar beets infected with beet mild yellowing virus are more susceptible to *Alternaria* sp. and *Erysiphe betae* (47). However, virus infection does not always render the plant more susceptible to subsequent infection. Susceptibility was reduced to *Cladosporium cucumerinum* infection by the cucumber mosaic virus (23), and chlamydospore germination of *Fusarium solani* f. sp. *cucurbitae* was decreased by squash mosaic, water melon mosaic and wild cucumber mosaic viruses (17). These are only a few examples from a large number of virus-fungus or virus-bacterium interactions reported.

Similar interactions in potato involving latent viruses and pathogenic fungi and bacteria have been reported. Potato plants infected with potato virus X were more resistant to subsequent infection with late blight (*Phytophthora infestans*) (41), and early blight (*Alternaria solani*) (42). Potato virus Y induced similar resistance to late blight (41) but decreased resistance to *Alternaria solani* (42). Jones et al (28) suggested that virus X
infected potatoes were more susceptible to *Fusarium* tuber rot. Ayers and MacKinnon (personal communication) failed to confirm this and their work indicated that X-free potatoes were more resistant to tuber rot. A varietal difference in response was found, however, and may account for the conflicting reports. A synergistic effect was observed on the potato plant when ring rot bacteria were inoculated into material infected with leafroll virus (43).

Since virus infection has an effect on potato diseases it seems reasonable that it may also have an effect on the endophytic bacterial flora.

Object of Project

This study was undertaken (i) to determine the bacterial population in *Solanum tuberosum*, (ii) to evaluate the effect of virus infection on this population, and (iii) to identify the coryneform bacteria found in healthy plants and compare them to *C. sepedonicum*.

MATERIALS AND METHODS

Plant Material

Two lots of *Solanum tuberosum* L. cv "Netted Gem" from the same parental clone were obtained from N. S. Wright (CDA, Vancouver). One lot was virus-free (VF), the other had been infected with potato virus X (PVX). Plants were grown in adjacent plots on the UBC campus. In late summer the plants were twice sprayed with Thiodan 4E for aphid control. In the fall stems and tubers were individually harvested and numbered. Stems were used immediately while the tubers were stored at 5 C for three weeks.
All plants in the virus-free plot were indexed for PVX and PVS to ensure that they had not been contaminated during the growing season. The PVX test was done by taking a small piece of tissue from each plant and grinding it with a few drops of water on a spot plate. The juice was then rubbed on *Gomphrena globosa* leaves with a square of foam rubber. The *Gomphrena globosa* plants had been trimmed to four leaves and dusted with carborundum (600 mesh Crystalon) prior to inoculation. Local lesions on the indicator plant denoted the presence of PVX.

PVS was indexed by tube precipitin serology. From each plant 0.25 gm of leaf tissue was ground in a hand tissue grinder with 2 ml Tris (0.01 M) buffered 0.85% saline at pH 7.4. The homogenate was centrifuged at 7,000g for 20 minutes. Two serial two-fold dilutions were made of the supernatant and 1 ml suitably diluted PVS antiserum was added to 1 ml of each dilution. The tubes were incubated for 2 hrs at 37 C in a water bath. Floculation, which indicates a positive test, was observed under transmitted light. One plant, found to be contaminated with PVS, was discarded.

Quantitative Assay

Stems were washed in a 10% solution of commercial bleach, rinsed and air dried. Stem smears were made by the method of Baribeau and Marcotte (3). The stems were cut with a scalpel and smeared on a slide along the complete width. Four smears were made per slide. Reed's modification of the gram stain was used (46). Slides were observed under oil immersion at 1250x and gram positive bacteria counted per microscope field. Fields were chosen at random in areas where bacteria were present. The mean of two fields in each smear was recorded.
For estimating the number of viable bacterial cells in the stem, 5 mm sections taken at the soil line were used. After surface sterilizing in a 10% solution of commercial bleach and rinsing 3 times in sterile distilled water (SDW), the stem sections were ground with 5 ml of SDW in a Virtis Model 45 homogenizer with the micro assembly at 32,000 rpm for 5 minutes. Volumes were brought up to 10 ml and serial dilutions made by the agar droplet method (51). The agar droplets were made from 4 drops of a Pasteur pipette. In this manner a drop of 0.097 ml was obtained which closely approximated 0.1 ml. Thus 0.1 ml of the homogenate was transferred to a molten agar tube containing 9.9 ml of media "NM" (30) in a 45 C water bath. Four serial dilutions were similarly made. Four replications of each dilution were placed on a petri dish (Fig. 1). Plates were incubated at 25C for 48 hrs. Microcolonies were counted under a dissecting microscope at 12 X magnification. Colony counting was facilitated by drawing a grid on a cover glass and placing it over the droplet to be counted (Fig. 2).

Tuber samples were obtained after surface sterilizing the tuber in 10% commercial bleach and rinsing 3 times in SDW. Plugs of tissue were extracted from the vascular ring at the stem end with a modified cork borer. The cork borer had a bolt which could be screwed in from the handle end to extrude the tuber plug. A specific number of turns extruded a measured length of plug which was subsequently sliced off with a sterile scalpel. The diameter and thickness of the discs so obtained were 10 mm and 3 mm respectively. They were homogenized and diluted in the same manner as the stem tissue.
Fig. 1 An agar droplet dilution plate showing four replications of four dilutions. From left to right the dilutions are $10^2$, $10^4$, $10^6$, and $10^8$. 
Fig. 2 An agar dilution droplet under the dissecting microscope. Note the grid on a glass coverslip superimposed upon the droplet to facilitate counting.
All procedures were carried out in a laminar air flow bench. To test for contamination, a control was run for every 9 samples. Controls consisted of sterile solidified pieces of 3% agar put through the same steps as the potato tissue. Only one of 20 controls contained two bacterial colonies.

Stem and tuber sections of equal size adjacent to the homogenized samples were taken for fresh and dry weight determinations. Dry weight was determined by drying to constant weight at 80 C. Diameter was also measured for the stem sections.

The bacterial numbers were calculated on a per volume basis. Analysis of variance was used to compare bacterial numbers in virus-free with virus-infected plants. The simple regression of bacterial numbers in stems on that in corresponding tubers was calculated. Both simple and multiple regression analyses were done on bacterial numbers using fresh and dry weights as independent variables. Sequential position in the rows was also used as an independent variable in the multiple regression because soil conditions varied along a row.

Qualitative Assay

In preliminary work pieces of potato tuber tissue were placed in M523 broth (29). After 24 hrs incubation, aliquots from the broth were streaked on to plates of the same media. Dissimilar colonies were isolated and retained for identification in pure culture on nutrient agar slants at 5 C.
In subsequent experiments only gram positive rods were retained. Potato stem and tuber tissue were surface sterilized and ground up as in the quantitative assay. The homogenate was streaked on to a medium which allowed rapid growth of *Corynebacterium* (54), a medium which was somewhat selective for soil *Arthrobacter* spp. (40), and a nutrient agar with sodium dichromate (1;20,000) to inhibit gram negative bacteria (54). Dissimilar colonies were isolated, stained and gram positive isolates retained for further study.

The following tests were performed for the identification of bacteria.

**Gram stain.** The method described by Bradbury (6) was used with the exception that 0.5% aqueous safranin was substituted for basic fuchsin.

**Acid FastStain.** The Ziehl-Neelson carbolfuchsin stain (19) was used to detect acid fastness. Methylene blue was used as the counter stain.

**Motility.** Motility was determined by both the hanging drop technique (52) and with Adler's semi-solid media (1).

**Flagellation.** Both electron microscope (EM) and light microscope techniques were used to determine number and location of flagella. Forty-eight hr cultures in nutrient broth were diluted 1:1 in SDW and the suspensions dropped on to grids previously coated with colodion and carbon films. The grids were stained with 20% phosphotungstic acid at pH 7.2, air dried and observed in a Phillips 200 microscope. The electron microscope was operated by technicians at the CDA Research Station, Vancouver. For the light microscope the silver impregnation stain (5) was used.

**Spore test.** The presence of spores was determined by heating a 2 ml aqueous cell suspension to 80 C for 15 min (6). Viability of the heated
suspension indicated the presence of spores. In all cases the conclusion was confirmed by observation using phase contrast light microscopy.

**Pigment production.** King's B medium (34) was used to test for the production of fluorescent pigment in *Pseudomonas* spp.

**Carbohydrate metabolism.** Peptone water containing 2 ml/l of a 1.6% alcoholic solution of bromocresol purple with the appropriate carbohydrate at a final concentration of 1% was used to detect acid production (52). Both glucose and lactose were used aerobically and anaerobically. Gas release was detected in the aerobic tubes by the insertion of a Durham tube. Anaerobic conditions were obtained by the addition of 0.3% agar (to prevent convection currents) and layering 1 cm sterile mineral oil on top of the medium after inoculation.

**Cellulose digestion.** Cultures were grown in peptone water containing a strip of Whatman #1 filter paper. Maceration of the filter paper indicated cellulose digestion (52). Cultures were incubated for 3 weeks at 25°C.

**Indole production.** The presence of indole was tested by shaking a 4-day-old culture grown in peptone water with 0.5 ml xylene. A few drops of Ehrlich's reagent was added and a pink color recorded as positive (12).

**Gelatin hydrolysis.** Cultures were grown on plates containing nutrient agar with 20% gelatin (W/V). Gelatin hydrolysis was observed after flooding the plate with a saturated ammonium sulfate solution. Formation of haloes around the colonies indicated a positive test (12).

**Nitrate reduction.** Cultures to be tested were grown for 3 days in peptone water with 0.1% KNO₃. The sulfanilic acid α-naphthylamine spot test method was used to test for the presence of nitrite (52). Further
reduction of nitrate was detected by the addition of a small amount (approx. 0.5 - 1 mg) of zinc dust which reduces nitrate to nitrite (12).

Pathogenicity tests. All isolates obtained were tested for pathogenicity in both potato and tomato. Stem inoculations were made by injecting aqueous cell suspensions (1 x 10^6 cells/ml) with a hypodermic syringe. Pathogenicity was indicated by chlorosis and marginal necrosis of leaflets and wilting of stems with the presence of a creamy exudate in the vascular tissue.

RESULTS

Quantitative Assay

Preliminary experiments suggested that there may be a difference between the endophytic bacterial population of VF and PVX plants. Subsequently 42 VF and 45 PVX plants were analyzed. Although the mean bacterial numbers (Table 1) were slightly higher for the VF plants, the difference was not significant at the 5% level.

Since no difference was found between VF and PVX plants the results were combined for the frequency distributions. As shown in Figure 3, the tubers were categorized into ten groups on the basis of the bacterial population which ranged from 0 to 1.6 x 10^4 cells per cm^3. Similarly, stems were placed in categories indicative of bacterial populations from less than 1 x 10^3 to 4.7 x 10^7 cells per cm^3. Most stems and tubers had bacterial populations toward the lower end of the scale but 25% contained more than 1.2 x 10^5 cells per cm^3 in stems and 50 cells per cm^3 in tubers.

There was no significant correlation between bacterial numbers in the stem with the corresponding tuber of the same plant. There was also no
Table 1. The viable endophytic bacterial population* in stems and tubers of *Solanum tuberosum*

<table>
<thead>
<tr>
<th></th>
<th>STEM</th>
<th>TUBER</th>
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<tr>
<td></td>
<td>VF*</td>
<td>PVX†</td>
</tr>
<tr>
<td>Mean</td>
<td>8.1 x 10^6</td>
<td>8.0 x 10^6</td>
</tr>
<tr>
<td>Median</td>
<td>4.1 x 10^6</td>
<td>9.7 x 10^5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.3 x 10^7</td>
<td>1.4 x 10^7</td>
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* Populations based on bacterial numbers per cm³ of tissue.
+ Based on 42 replications.
† Based on 45 replications.
Fig. 3 Frequency distributions of endophytic bacterial populations in stems and tubers of Solanum tuberosum.
significant correlation between bacterial numbers on a volume basis with fresh or dry weights in either stems or tubers. Nor was there a correlation between position in a row with bacterial number. The number of gram positive bacteria per microscope field from the stem smear is expressed as a frequency distribution in Figure 4. Most smears contained 15 or less gram positive bacteria per field. But of the 180 smears examined about 5% contained 30 or more and only 5 smears contained no bacteria.

Qualitative Assay

In the initial survey 67 isolates were obtained. Their tentative generic identification is given in Table 2. The largest number of isolates were gram positive cocci which lost the crystal violet stain quite readily. They always appeared singly or in irregular clusters. They grew well on agar containing 1:400,000 crystal violet and survived 60 C for 30 min. These characteristics plus the fact that they were obtained from a soil-plant environment led to the conclusion that they belong to the genus Microccocus (52).

The Pseudomonas isolates were identified on the grounds of fluorescence on King's B medium, oxidative glucose metabolism and the presence of polar flagella (7). The isolates that were gram positive or gram variable, aerobic and produced endospores were placed in the genus Bacillus (6).

Flavobacterium spp. were recognized by their yellow non-water soluble pigment, lack of motility, and lack of acid production from glucose (7). The Xanthomonas isolated was similar to the Pseudomonas spp. in all respects
Fig. 4 Frequency distribution of number of gram positive rods found in stem smears. Numbers are based on the average number of two microscope fields in which bacteria were visible.
Table 2. Generic identification of bacterial isolates from *Solanum tuberosum*

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. of isolates</th>
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<tr>
<td>Micrococcus</td>
<td>27</td>
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<tr>
<td>Pseudomonas</td>
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<tr>
<td>Bacillus</td>
<td>12</td>
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<td>Flavobacterium</td>
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<tr>
<td>Agrobacterium</td>
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<tr>
<td>Xanthomonas</td>
<td>1</td>
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<tr>
<td>Coryneforms</td>
<td>4</td>
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<tr>
<td>Other gram negative forms</td>
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except that it lacked a fluorescent pigment but had a yellow non-water soluble pigment (7). One isolate was placed in the genus *Agrobacterium*. It had peritrichous flagellation, fermentative glucose metabolism, and a colony and cell morphology which fitted the genus description (7). A number of other gram negative rods were isolated but could not be identified.

In the comprehensive study of gram positive rods, coryneform bacteria and *Bacillus* spp. could be isolated from both stems and tubers of VF and PVX plants with relative ease. The *Bacillus* spp. were not further considered. A total of 31 coryneform isolates were obtained. None of the isolates obtained was pathogenic on either potato or tomato. The biochemical characteristics of several isolates were identical. The characteristics of only the representative isolates are given in Table 3. Some of these isolates were morphologically indistinguishable from *Corynebacterium sepedonicum* (Fig. 5).
<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Motility</th>
<th>Acid from glucose</th>
<th>Acid from lactose</th>
<th>Acid Fast</th>
<th>Nitrate reduced</th>
<th>Gelatin Hydrolysis</th>
<th>Indole Product</th>
<th>Cellulose Digestion</th>
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<td>1</td>
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* Known culture of *Corynebacterium sepedonicum*
Fig. 5 Comparison of the morphology of *Corynebacterium sepedonicum* (A) with a non-pathogenic coryneform (B) isolated from *Solanum tuberosum*. (Phase contrast x5000).
DISCUSSION

The presence of endophytic bacteria in healthy potatoes has been previously demonstrated (22, 38, 50, 56). This study is the first attempt to quantify the endophytic bacterial flora. Since only aerobic conditions and one type of medium was used although all organisms from preliminary work grew readily on it, the true bacterial count may be somewhat higher than the results indicate if anaerobic and more fastidious organisms were present. Nevertheless, the bacterial counts should be a relatively accurate estimate of the endophytic bacterial distributions in a given population of *Solanum tuberosum*. The frequency distributions show that the majority of plants have a comparatively low bacterial population although about 25% have a higher one. Reasons for the large amount of variation is not known. Environmental factors such as external injury and the onset of senescence probably play an important role in predisposing the plants to bacterial colonization.

The recent development of virus-free potatoes presented a unique opportunity to study plants obtained from a sterile parental clone. Virus-free potatoes have been developed from the meristematic tips of axillary buds of heat-treated plants in sterile nutrient solution (55) and subsequently propagated by stem cuttings (11). Because the axillary buds remained sterile in culture it is assumed that the plants were initially sterile. Any bacteria in these plants must have entered during the growing season. This also supports the root entry hypothesis of Hollis (22). One would, therefore, expect the endophytic bacterial flora to comprise common soil inhabitants. This proved to be so in the general survey of bacterial genera (Table 2). Species of all
these genera are commonly found in soil (2). The isolates identified by other workers (22, 38, 50) is consistent with these results. They also obtained Bacillus and Arthrobacter (Bacterium) species.

Unfortunately the coryneform bacteria could not be identified as to genus. This is due mainly to the fact that habitat features prominently in the classification of members of this group (31). The isolated coryneforms were aerobes and from a plant - soil habitat. Therefore, they could possibly belong to the genera Arthrobacter, Brevibacterium, Cellulomonas, Corynebacterium, or Mycobacterium (7). The genera Cellulomonas and Mycobacterium could be excluded because the isolates did not digest cellulose and were not acid fast (15, 26). Some of the isolates (1, 4, and 12 in Table 3) produced a coccoid stage in older cultures and therefore probably belong to the genus Arthrobacter (13). The other isolates then should belong to either Brevibacterium or Corynebacterium.

Brevibacterium contains species of gram positive rods some of which have a coryneform morphology but fit into no other genus (27, 57). The genus Corynebacterium was first introduced to contain animal pathogenic forms such as the diphtheria bacillus (37), but plant pathogens and two saprophytic species have been included since then (14, 16). However, taxonomists agree that at least the plant pathogens require an appropriate new taxon because of their marked difference from the original type species (15, 57, 59). Thus the non-pathogenic coryneforms isolated from potato can not be placed in any genus until taxonomists compile sufficient information to classify the saprophytic coryneforms (57).
The presence of endophytic coryneform bacteria is especially significant in bacterial ring rot diagnosis by the stem smear test. One assumption upon which this test is based is that the bacterial flora in the stem reflects that in the tuber. However, this study has failed to find a statistically significant correlation between the number of bacteria in stems and tubers of the same plant.

Another assumption is that the presence of short gram positive rods in stem smears is indicative of infection by *C. sepedonicum*. In Canada, seed potatoes are considered suspect if any short gram positive rods are found in stem smears. This study has shown that gram positive rods, including those with dimensions of *C. sepedonicum*, can be found in almost all stem smears of healthy plants if a careful search is carried out (Fig. 4). When these gram positive rods were isolated in pure culture they were found to be non-pathogenic and biochemically different from *C. sepedonicum* (Table 3) although morphologically they were indistinguishable from the ring rot organism (Fig. 5). Some of the isolates represented by number 12 (Table 3) were biochemically similar to *C. sepedonicum* but had a coccoid stage in older cultures and thus probably belong in the genus *Arthrobacter*. These results explain why *C. sepedonicum* could not be isolated from and symptoms did not develop after several generations in potatoes suspected of having bacterial ring rot on the basis of the stem smear test.
SUMMARY

The endophytic bacterial population in *Solanum tuberosum* L. stems and tubers of both virus-free and virus-infected plants was studied. Bacteria were found in stems and tubers but there was a wide variation in occurrence between plants. No significant difference in bacterial population was found between virus-free and virus-infected plants, nor was there a significant correlation between populations in stems and tubers of the same plant.

Species of *Micrococcus*, *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Xanthomonas*, *Agrobacterium*, coryneforms, and other unidentified genera were found. Some of the coryneforms were morphologically indistinguishable from *Corynebacterium sepedonicum* but were found to be biochemically different. The results of this study questions the basic assumption made in the stem smear test for bacterial ring rot, namely that any gram positive rods in the smear suggests the presence of *C. sepedonicum*. 


Part B
The Inhibition of Potato Pathogens by an Endophytic Bacillus Sp.

LITERATURE REVIEW
INTRODUCTION

The presence of bacteria in healthy plant tissue has been well established (11, 19, 44, 45, 46, 56). However, almost no work has been done on the possible significance of the endophytic microflora. Endophytic bacteria could have diverse effects on the growth and development of the host plant. For example, bacteria may increase the absorption of ions by roots (3), increase IAA production (31, 60), affect phosphatase activity (42), and affect response to infection (35). Only the latter item lies within the scope of this review.

Biological control of plant diseases

Although biological control in plant pathology is not a new concept its actual application is almost negligible. The induction of resistance by heat-killed cells (26, 33), and similar induction by bacterial extracts has been reported (48). This has led to the speculation that saprophytic endophytes may also trigger such a resistance mechanism (16, 35).

At the present time biological control with antagonistic species has been investigated most extensively. Avirulent strains of phytopathogenic species may be antagonistic toward pathogenic strains and thus protect the host. Kelman and Averre (23) reported the influence of avirulent cells of Pseudomonas solanacearum on the severity of bacterial wilt. This antagonism
may in part be due to increased production of IAA by the avirulent cells to which the virulent cells are more sensitive (2). Similarly apple stem tissue becomes resistant to infection by Erwinia amylovora after being inoculated with an avirulent strain (16). Nelson and Semeniuk (38) report the inhibition of Corynebacterium insidiosum by an antagonistic variant, in this case an inhibitor substance was partially purified.

Antagonistic bacteria in the phyllosphere have also been investigated. This literature prior to 1965 has been reviewed by Leben (29). Epiphytic bacteria decrease plant disease losses by inhibiting the pathogenic organism and thus preventing infection. A short gram negative rod which produced an antibiotic on cucumber leaves was effective in control of cucumber anthracnose (Colletotrichum lagenarium) (28). Later it was also found to control early blight (Alternaria solani) of tomato and northern leaf blight (Helminthosporium turticum) of corn (30). Similarly, several epiphytic Bacterium isolates inhibited the fire blight organism, Erwinia amylovora (14). In the latter case increased acidity due to the antagonistic organism may have caused the protective effect. However, one of the Bacterium isolates did produce an antibiotic. Bacterial canker is inhibited by a saprophytic Erwinia sp. from the leaf surface of cherry trees (10). Some strains of Pseudomonas fluorescens are antagonistic toward P. phaseolicola (55). Several saprophytic bacteria from blight-infected rice leaves retarded symptom development when inoculated concurrently with Xanthomonas oryzae (49). Again, an antagonistic substance was detected but not characterized.

Not only are epiphytic bacteria effective in providing protection against bacterial diseases but also against fungal and viral infections. For
example, bacteria on the leaf surface inhibits germination of *Botrytis cinerea* spores on chrysanthemum (6) and lettuce (61). Other examples include the inhibition of *Rhizoctonia solani* on lettuce (61), *Uncinula necator* on grape (25), and *Botrytis cinerea* on beet leaves (5). Evidence of a non-pathogenic bacterium affecting a virus disease is reported in the *Bacillus uniflagellatus* - TMV interaction (34). This organism not only reduces TMV local lesions but also viral content.

The literature thus reveals that there are two principal ways in which bacteria may control plant diseases. The first is by activating or inducing a protective host response. The second is by actively inhibiting the pathogenic organism. Antibiotics are often implicated as the inhibitory agent.

Antibiotics in plant pathology

Species of *Bacillus* and *Streptomyces* are the chief source of antibiotics used in the control of plant disease. Although antibiotics theoretically could be as useful in controlling plant infections as they are in animal and human infections various practical considerations enter the picture. Plants have a type of circulatory system (8) but it is far from being as effective as that in higher animals in dispersing the antibiotic throughout the organism. Moreover there is a problem of rapid inactivation of the antibiotic once it has been applied to the plant (53). Despite these complications, however, there are many reports of potentially effective antibiotics.

Peach trees have been injected with oxytetracycline to control bacterial spot caused by *Xanthomonas pruni* (13). Streptomycin was shown to
be effective in control of halo blight (Pseudomonas phaseolicola) of beans (62), and bacterial wilt (Erwinia chrysanthemi) of chrysanthemums (43). Stem rust (Puccinia graminis) of wheat has been successfully controlled by cycloheximide (59) as was covered smut (18) and powdery mildew of roses (58). Vaneomycin was found to be effective against a wide range of plant pathogenic bacteria (36). Several Streptomyces antibiotics such as bleomycin, kasugamycin, bulgerin, and ablastmycin appear promising for the control of rice blast (Piricularia oryzae) and sheath blight (Pellicularia sasakii) (17, 40, 50, 51). In addition, viruses, such as tobacco mosaic virus and cauliflower mosaic virus, can be inhibited by appropriate antibiotics (20, 34, 37, 57).

Only a few of the antibiotics studied have found a commercial use. Streptomycin is used most widely. It is recommended for control of blackleg (Erwinia atroseptica) in potatoes in British Columbia (Vegetable Production Guide 1972 (1). It is also used alone or in mixtures with terramycin to control Erwinia amylovora in hawthorn, mountain ash, pear and other members of the rose family (41). Cycloheximide has been used to control white pine blister rust (53); powdery mildew of roses, phlox, and other ornamentals; azalea petal blight (Ovulinia azaleae); and several lawn grass diseases including Puccinia graminis f. agrostis and Curvularia lunata. Griseofulvin is used for the control of grey mold (Botrytis) of lettuce (15). And finally Bacticin is used successfully for elimination of crown gall tumors caused by Agrobacterium tumefaciens and olive knot tumors caused by Pseudomonas savastanoi (47).
Object of project

This study was undertaken to determine the possible role of endophytic bacteria in disease control of *Solanum tuberosum* L. and to elucidate the mechanism involved in any such role.

MATERIALS AND METHODS

Isolation of Bacteria and Demonstration of Antagonism

Endophytic bacteria were isolated from tubers of *Solanum tuberosum* and identified by the methods described (11). The 67 isolates obtained were tested for inhibitory properties against *Corynebacterium sepedonicum*. These tests were done on a lawn of *C. sepedonicum* made with a 1 ml suspension of approximately 1 x 10^6 cells. The test organisms were spotted on to the plate at four spots per plate. Media "NM" (22) was used in these experiments and plates were incubated at 25 C. A zone of no bacterial growth surrounding the test organism indicated an antagonistic effect.

One organism (Isolate 35) showing the greatest zone of inhibition was used for further study. To identify Isolate 35, dimensions and morphological characteristics were noted on gram-stained slides. Standard biochemical tests were performed by the methods previously reported (11). Inhibition was also tested in the same manner on the following potato pathogens: *Pseudomonas solanacearum*, *Erwinia carotovora*, *E. atroseptica*, *Alternaria solani*, and *Phytophthora infestans*. 
Separation of the Active Substance

Partial purification

A technique for the partial purification of the inhibitory substance was adapted from the method for isolating subtilin (21). Isolate 35 was grown in 500 ml Erlenmeyer flasks, each containing 100 ml of media NM, in still culture at room temperature. After 4 days, the pH was adjusted to 4.5 with conc. HCl and allowed to stand for 2 hrs at room temperature followed by centrifugation at 7,000 g for 30 min. Pellets were dried at 65 C and ground in a mortar and pestle with 1 gm of clean fine sand. To the powder was added 10 ml distilled water and 100 ml warm 95% ethanol. The suspension was again centrifuged at 7,000 g for 30 min and the supernatant dialysed against distilled water for 48 hrs. The resulting precipitate contained the active ingredient.

In later work the purification procedure was done more efficiently by omitting the drying step. Instead the first pellets were combined with an equal amount of distilled water (v/v) and ground intermittently for several minutes with a Polytron grinder. The pH of the mixture was adjusted to 2.0 with conc. HCl and allowed to stand at room temperature for 2 hrs. It was subsequently centrifuged at 7,000 g for 30 min and the pellet extracted with warm ethanol in the usual manner.

Extraction of lipids

The method for total lipid extraction was modified from Bligh and Dyer (7). To 100 mg of the partially purified antibiotic was added 0.4 ml
distilled water, 0.5 ml chloroform and 1.0 ml methanol. The suspension was homogenized for 2 min in a Virtis 45 homogenizer with the micro assembly. Another 0.5 ml chloroform was added and homogenized for another 30 sec. After being allowed to separate for a few minutes the chloroform layer containing the lipids was withdrawn with a Pasteur pipette.

Detection of carbohydrates

To test for the presence of carbohydrates in the partially purified antibiotic the anthrone reaction and acid hydrolysis were performed (54). The anthrone reaction was carried out with 40 μg of sample to which was added 5 ml of the anthrone reagent. The mixture was heated in a boiling water bath for 15 min and cooled. Absorbance was determined at 620 nm.

Acid hydrolysis was carried out in 6N HCl for 24 hrs in boiling water. Visible black or brownish-black humin is formed as the result of condensation between tryptophan and any carbohydrates under these conditions (32).

Column chromatography

The method of Bartlèy et al (4) was used for the columns of Sephadex LH-20. Sephadex LH-20 was stirred for at least 4 hr with aq. 90% (v/v) dimethylformamide and poured in a 1 cm diameter glass column and packed by gravity to a height of 16 cm. The column was then washed with aq. 90% (v/v) dimethylformamide for 6 hrs at a flow rate of 0.3 ml/min. Samples dissolved in the same solvent were added and 2 ml fractions collected.

The method used for the silicic acid columns was that of Dittmer and Wells (12). A 25 mg sample of the partially purified antibiotic was
transferred to a silicic acid column. The column had been prepared with 1 g silicic acid (100 mesh, Mallinckrodt) in chloroform. The column was washed with 20 ml chloroform at a flow rate of 0.06 ml/min followed by 20 ml methanol. The two fractions were collected separately in bulk.

Detection of the active components

Activity was detected in the antibiotic preparations by evaporating off the organic solvents in vacuo, suspending the dried substance in distilled water, autoclaving, and placing a drop of this suspension on a fresh lawn of C. sepedonicum. Plates were incubated at 25 C and a zone of inhibition around the antibiotic drop indicated activity.

To test for homogeniety of a preparation thin layer chromatography (t.l.c.) was used. Samples were spotted on Eastman Chromagram silica-gel sheets type 6061 and separated with butanol-acetic-acid-water (4:1:1), methanol-chloroform (4:1), or ethanol (99%). Dried sheets were sprayed either with 1% (w/v) ninhydrin in 70% ethanol or 50% (v/v) sulfuric acid and dried under an infrared heat lamp.

Spots on t.l.c. were assayed for activity by direct assay of scrapings or their eluates. The eluates were made by three washings of the t.l.c. scrapings with the methanol-chloroform solvent. The silica gel was removed in each case by centrifugation. The eluate was assayed for activity as described above.
Determination of Properties of the Antibiotic
Treatment with sodium dodecyl sulfate (SDS)

The antibiotic was solubilized in .001 M SDS. The solution was dialysed for 48 hrs to remove the SDS in distilled water at room temperature. The dialysate was subsequently assayed for activity. Controls consisted of SDS without antibiotic and antibiotic with distilled water instead of SDS.

Ultraviolet (U.V.) absorption spectrum

The U.V. absorption spectrum was determined in a 95% ethanol solution using a Perkin-Elmer Model 125 double beam spectrophotometer.

Infrared (I.R.) absorption spectrum

One mg of the antibiotic was ground with 100 mg KBr to a fine powder with a glass rod, compressed into a pellet, and dehydrated in vacuo. The absorption spectrum was scanned on a Beckman Infrared Spectrophotometer Model 20 A by Randy Englar (Department of Soil Science).

Amino acid analysis

A preliminary amino analysis was done on the partially purified antibiotic by Dr. J. Tremaine (CDA, Vancouver). The substance was hydrolysed with 6N HCl in a tube sealed under vacuum and heated to 107 C for 24 hrs. Subsequent analysis was done on a Beckman Model 120 B amino acid analyzer.
RESULTS

Of the 67 isolates obtained, 16 showed antagonism towards *Corynebacterium sepedonicum*. Twelve of these belonged to the genus *Bacillus*, one was a *Pseudomonas* sp. and the remaining three could not be identified to genus. Isolate 35, a *Bacillus* sp., showed the greatest amount of inhibition and was consequently chosen for further characterization of the inhibitory activity.

Isolate 35 was a gram positive motile rod. The vegetative cells varied in length from 2.8 - 4.0 μ and in width from 0.7 - 0.9 μ. Cells usually appeared singly but occasionally formed short chains. Spores formed readily on nutrient agar in 3 - 4 days at 25 C. The sporangia were not swollen. Spores (1.6 x 0.8 μ) were cylindrical and centrally or terminally located. The isolate did not grow under anaerobic conditions and grew very poorly in 7% NaCl. Acid was not produced from glucose or lactose. Tests for production of indole and reduction of nitrate were also negative. However, it hydrolysed starch and, to a lesser extent, gelatin. The colonies on nutrient agar were cream-colored, large, rough and irregular. Broth cultures formed a heavy firm pellicle with only slight turbidity. These characteristics indicate that isolate 35 was a strain of *B. subtilis*.

In addition to inhibiting growth of *C. sepedonicum* Isolate 35 also inhibited *Pseudomonas solanacearum*, *Erwinia atroseptica*, *E. carotovora*, *Alternaria solani*, and *Phytophthora infestans* (Fig. 1).

A metabolic by-product was implicated as the active agent when an agar disc aseptically removed from the inhibition zone was able to prevent growth of *C. sepedonicum*. Several purification techniques used for *Bacillus*
Fig. 1. The inhibition of several potato pathogens by a *Bacillus* sp. isolated from healthy potato tubers. A - *Corynebacterium sepedonicum*; B - *Erwinia atroseptica*; C - *E. carotovora*; D - *Pseudomonas solanacearum*; E - *Alternaria solani*. 
antibiotics were attempted. The method used for purifying subtilin (21) isolated an active fraction from the bacterial cells. One drop of a water suspension caused a 3 - 4 cm wide inhibition on an assay plate (Fig. 2).

The partially purified antibiotic was soluble in ethanol, methanol, butanol, propanol, and acetone, but insoluble in cyclohexane, benzene, and chloroform. Activity was not lost after autoclaving at 121°C for 15 min. The SDS treatment also did not destroy activity in four of five replications. The amino acids detected on one assay in the partially purified antibiotic is given in Table 1. Both tests for the detection of carbohydrates were negative. The U.V. and I.R. absorption spectra are given in Figures 3 and 4 respectively.

T.l.c. plates of the partially purified antibiotic showed three spots with all solvents. The Rf values were as follows: ethanol 0.23, 0.32, and 0.77; methanol-chloroform 0.49, 0.73, and 0.84; butanol-acetic acid-water 0.74, 0.79, and 0.83. The first two spots in all cases gave a positive ninhydrin reaction. The third was light yellow in color before developing and could be marked more easily after the H₂SO₄ spray. Because the methanol-chloroform solvent gave good separation most rapidly it was used in all subsequent tests.

Assay of eluates from the three spots on the t.l.c. plates showed that the spots at Rf 0.73 and 0.84 with the methanol-chloroform solvent had biological activity (Fig. 5). The latter spot showed the greatest amount of inhibition. Activity was not enhanced by combining any of the three eluates.
Fig. 2. The inhibition of *Corynebacterium sepedonicum* by the partially purified antibiotic from *Bacillus* sp. The plate shows three zones of inhibition each due to one drop of an aqueous suspension of the substance. The lower right hand area of the plate is the control area having been inoculated with a drop of sterile distilled water.
Table 1. Preliminary list of amino acids present in the partially purified antibiotic from an endophytic *Bacillus* sp.

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<td>Glutamic acid</td>
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<td>Iso-leucine</td>
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<td>Leucine</td>
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<tr>
<td>Lysine</td>
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<td>Phenyalanine</td>
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<td>Proline</td>
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<td>Serine</td>
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<td>Threonine</td>
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<tr>
<td>Tyrosine</td>
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<td>Valine</td>
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Fig. 3. The U. V. absorption spectrum of the partially purified antibiotic measured in 95% ethanol.
Fig. 4. The I. R. absorption spectrum of the partially purified antibiotic in a KBr pellet.
Fig. 5. Inhibition of growth of *Corynebacterium sepedonicum* by two active fractions from the partially purified antibiotic as separated on t.l.c. by methanol - chloroform. The upper zones of inhibition coincide with the eluate from spots at Rf 0.73 and lower zones from spots at Rf 0.84.
The other methods for separation of the three components were not successful. The active substance was present in the chloroform fraction of the lipid extraction procedure and in the methanol fraction of the silicic acid column. However, these fractions did not differ from the original purified preparations on t.l.c. Moreover, no separation was achieved with the Sephadex LH-20 column.
DISCUSSION

*Bacillus* spp. have been an important source of antibiotics. The organism used in this study also belongs to this genus. Isolate 35 is probably a strain of *Bacillus subtilis* although the great number of variations in the cultural characteristics exhibited by the various strains of this species makes positive identification difficult (9). This strain differed from the species description (9) of *B. subtilis* in that no acid was produced from glucose, nitrites were not produced from nitrates, and there was only poor growth in 7% NaCl. The negative test for acid from glucose, however, may be due to liberation of ammonia from peptone resulting in complete neutralization of the acid (52).

The inhibitory properties of this organism are due to two antibiotoic substances. The substance of Rf value 0.73 with the methanol-chloroform solvent, was ninhydrin positive and therefore indicated a peptide. The evidence from the U.V. and I.R. spectra and amino acid analysis also indicates a peptide. However, these results must be interpreted with caution due to the presence of another ninhydrin positive spot on t.l.c. plates of the partially purified substance. The amino acid analysis (Table 1) only indicates which of the amino acids may be present in the antibiotic. The large amount of leucine could account for the insolubility in water. The 280/260 absorption ratio in the U.V. spectrum was 0.93. It indicates the presence of the aromatic amino acids, tryptophan and tyrosine. The peaks between wave numbers 1800 and 1100 cm\(^{-1}\) in the I.R. spectrum are caused by the amide groups of the amino acids. I.R. spectra are often used to identify antibiotics. Although the active fractions were not obtained in a pure form it is interesting to
note that the spectrum was similar to blasticidin A (24), and plurallin (39). Both are wide spectrum peptide antibiotics obtained from species of Streptomyces.

The other active fraction is a lipid. The absorbance near 200 nm in the U.V. spectrum is probably due to this fraction. The lipid also explains why the SDS did not inactivate the partially purified antibiotic. The lipid was not identified but the results of the silicic acid column indicates a complex lipid because chloroform removes the simple and methanol the complex lipids (12).

The lipid extraction procedure (7) was unsuccessful because the partially purified antibiotic was insoluble in this solvent system. Sephadex LH-20 has been used to separate ethanol-soluble peptides (4); however, no separation of the partially purified antibiotic was achieved with it. Although the structure of the active components could not be determined due to difficulties in separating the components, it can be concluded that the inhibition is due to both a peptide and a lipid.

This is the first report of the production of an antibiotic by an endophytic micro-organism. Although in vivo activity has not yet been demonstrated, the organism has potential for disease control. As already noted, transport and rapid inactivation are unresolved difficulties in the use of antibiotics (53). However, an antibiotic-producing, endophytic population beneficial to the host solves both problems. The bacteria can move through the plant and can be a constant source of antibiotic. Such a system may not prevent initial infection but should prevent further progression of the pathogenic organism. The vegetative propagation of potatoes lends itself
very well to such a system. Once the desirable endophytic population has been established in a clone it could be maintained indefinitely through the tuber.

Such a symbiotic relationship has admittedly not been proven to be effective. However, we are only just beginning to explore the possibilities of biological control in plant pathology and this is definitely one which should not be ignored.

SUMMARY

Sixteen of 67 bacterial cultures isolated from healthy Solanum tuberosum plants were inhibitory toward Corynebacterium sepedonicum. A Bacillus sp. probably a strain of Bacillus subtilis was used for further characterization of the inhibitory activity. This species was also inhibitory toward the following potato pathogens: Pseudomonas solanacearum, Erwinia atroseptica, E. amylovora, Alternaria solani, and Phytophthora infestans. An antibiotic was partially purified. Thin layer chromatography followed by bioassay showed that two active substances were present. One fraction was identified as a peptide the other as a lipid.


