Spatial Colonization Patterns and Interaction of Bacteria on Inoculated Sugar Beet Seed

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ABSTRACT

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Development and spatial distribution of microcolonies of *Pseudomonas* spp. and *Bacillus subtilis* GB03 inoculated singly and in combination on sugar beet (*Beta vulgaris*) seed were observed with a scanning electron microscope (SEM). SEM examination of seed directly after inoculation with *Pseudomonas* strain 33-2 or ML5 at population densities of approximately 10^4 cfu per seed revealed a random distribution of individual cells. By 24 h, when population densities had reached the stationary phase (approximately 10^6 cfu per seed), microcolonies had developed in a random pattern over the seed surface. However, even at these populations, only 10–40% of the seed surface was colonized. Most microcolonies developed as separate entities on the indented surface of cells of the perianth and the operculum. The colonization patterns at 48 h were similar to those at 24 h, except that the colonies were larger. Since the number of cfu measured by dilution plating (detectable population) was similar at both time periods, it was assumed that many cells were dead or dormant in the larger microcolonies. The spatial colonization patterns were entirely different, depending on the density of the initial inoculum. The entire seed surface was covered when sufficient inoculum was applied to attain a detectable population size of approximately 10^7 cfu per seed. Yet, even when the detectable population size increased to 10^7 cfu per seed following growth from an initial inoculum density of 10^4 cfu per seed, only 40–50% of the seed surface was colonized. This indicates the need for differentiating among live, dormant, and dead cells. The spatial colonization pattern of strain GB03 differed greatly from *Pseudomonas* strains. At temperatures favoring its growth, microcolonies of GB03 were located primarily near the basal pore of the seed, whether inoculated singly or coinoculated with *Pseudomonas putida* 33-2. In coinoculations, few microcolonies of 33-2 developed near the basal pore. However, this localized interaction could not be detected by dilution plating of bacteria from the spermosphere, demonstrating the difficulty of determining microbial interactions without visual examination. The overall conclusion from the study was that the spatial distribution patterns of developing and established microbial colonies of pseudomonads are such that little direct interaction occurs in the spermosphere unless massive amounts of inocula are present, and that availability of nutrients is the limiting factor in population size. An interaction was detected with GB03 only at 37 C.

Little is known about the spatial relationships of bacterial microcolonies developing on germinating seeds or roots. This information would help our understanding of how microorganisms interact in natural ecosystems or when inoculated together into plant parts. Moreover, there is a poor understanding of the physical relationships between antagonists and pathogens. For example, in most reports on microbial interactions in the spermosphere and rhizosphere, the assumption is made that there is direct physical contact or a direct interaction because of such processes

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as antibiosis and competition for nutrients. This assumption is usually based on data showing that the presence of one organism (antagonist) caused reduced infection of plant parts by a pathogen or a reduction in the pathogen's population size. However, indirect processes may also be involved. These include the possibility that the antagonist induced host plant resistance (21,22), or that the antagonist affected the activities and population sizes of other organisms, which then interacted with the pathogen. Furthermore, scanning electron micrographs of the surfaces of plant organs often indicate that large areas are not colonized by microorganisms (17). This raises the possibility that there is less direct interaction than often thought, and that colonization is restricted to certain sites.

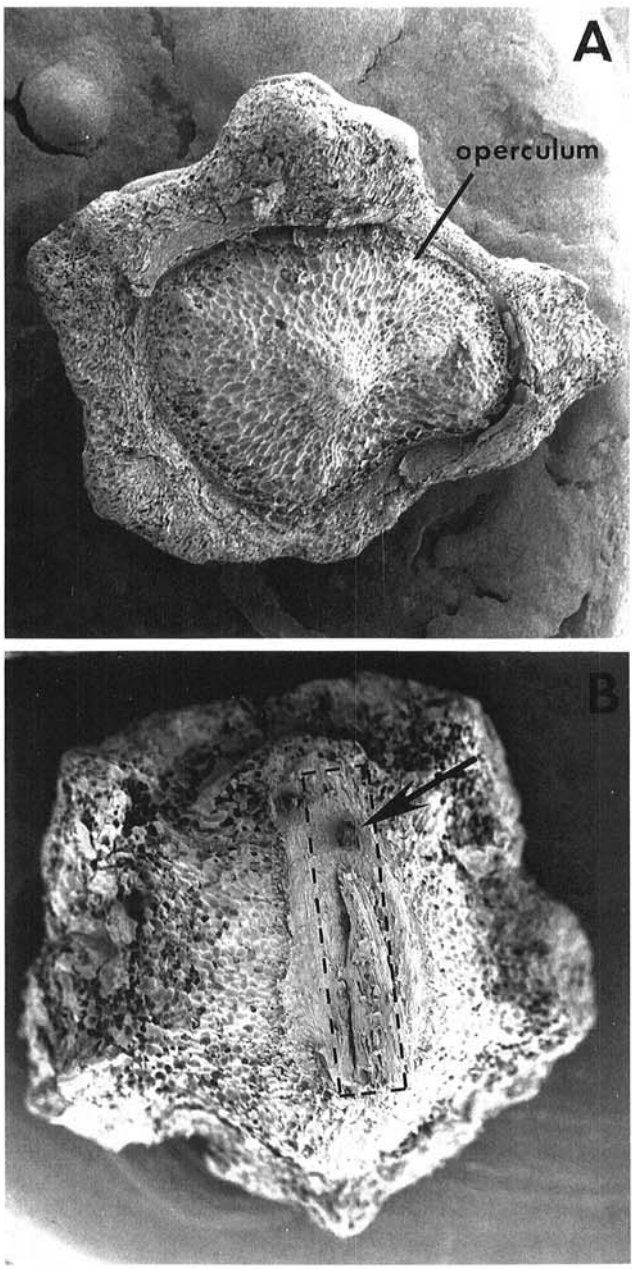

Fig. 1. Close-up photograph of sugar beet seed. The superior surface, **A,** consists of the operculum, representing the hardened upper surface of the ovary and surrounding remnants of the perianth (×19). The basal surface, **B,** consists of cells similar to those of the operculum except in the region of the basal pore (arrow), through which vascular tissue of the peduncle passes to the funiculus (×21.5). In the seeds observed with a dissecting microscope, the peduncle was usually detached from the basal pore. The rectangle indicates the area where microcolonies of GB03 were found. Exact locations of microcolonies were not shown.

Early studies using the light microscope indicated that bacteria exist as individual microcolonies in rhizosphere soil (9). Rovira et al (18) reported that only 4–10% of the root surface was colonized by bacteria. Bacterial microcolonies on roots were seldom closer than a few micrometers from each other (20) and occurred primarily at sites of root exudation (17). It would be expected that interactions among microorganisms would be greatest at sites of root exudation where substrates are present (2).

The spermospheres of sugar beet (*Beta vulgaris* L.) seed are ideal for studying the spatial relationships of microorganisms. The experimental system includes conditions that simulate those in nature (7,8,14). There is little or no difference in moisture potential among replicate experiments, and population dynamics can be followed on an hourly basis. The experiment is completed by the time the seed has germinated and root growth has begun. During root growth, considerable variation in moisture potential occurs among replicate experiments, thus making it difficult to obtain meaningful data on population dynamics of the rhizoplane and rhizosphere. The anatomy of the sugar beet seed enables colonization to be studied from surface to internal tissues. The true seed is surrounded by a pericarp, which consists of the operculum, receptacle tissue, and calyx remnants of the perianth. This entire structure is termed a glomerule. Vascular tissue of the peduncle passes through the basal pore located on the basal surface to the funiculus, which attaches the ovule to the ovary. The superior surface is covered by an operculum, or ovary cap. The operculum is delimited by a zone that dehisces when the radicle expands (3,4,19).

The pericarp is generally colonized by both bacteria and pathogens such as *Pythium ultimum* Trow (14). The true seed is a source of nutrients, which are released primarily through the basal pore (15). Large amounts of nutrients allow population densities of *Pseudomonas* strains in the spermosphere of sugar beet to reach a maximum (plateau) of 10^6–10^7 cfu per seed (8). When the total surface area of the seed is considered, it appears that large areas are not colonized. This is based on the rough calculation that one cell occupies an area of approximately 0.5 μm^2

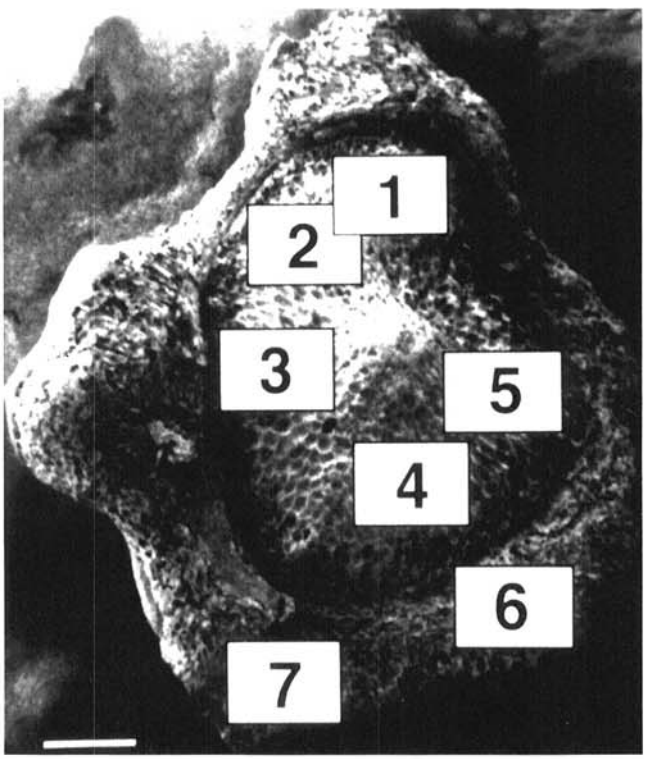

Fig. 2. Areas photographed to illustrate the distribution of microcolonies of *Pseudomonas putida* 33-2 on the spermoplane. Numbers represent selected areas on the spermoplane shown in Figure 3. Seed inoculated with 33-2 was incubated in sterile moist sand at 16 C for 24 h. Bar represents 500 μm (×20).

and that the maximum population of 10^7 bacteria on the spermoplane would occupy about 5 mm^2. However, the surface area of a typical decorticated seed is about 20–25 mm^2. The question thus arises as to why a larger area is not colonized. It is possible that nutrients may not be evenly distributed around the pericarp and that some sites are more amenable for colonization than others.

Previous studies on population dynamics of pseudomonads in the spermosphere suggest that there is little initial competition for nutrients and that different populations of bacteria grow inde-

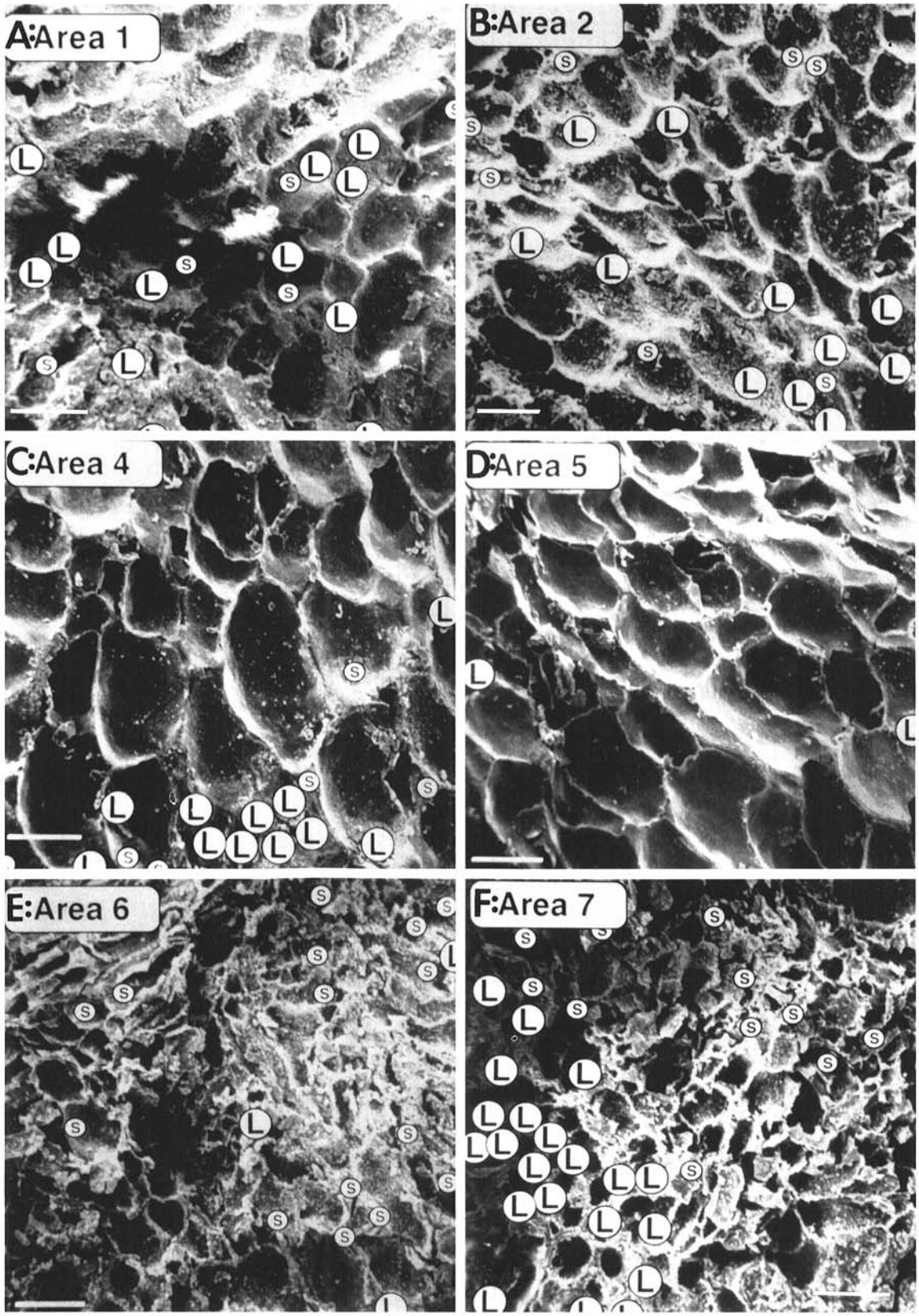

Fig. 3. Locations of microcolonies of *Pseudomonas putida* 33-2 in selected areas on the spermoplane shown in Figure 2. Locations of larger microcolonies consisting of hundreds to thousands of cells and locations of smaller microcolonies with approximately 50 to a few hundred cells are indicated by circles with capital letters "L" and "S," respectively. The micrographs **A, B, C, D, E, and F,** are magnifications of the areas 1, 2, 4, 5, 6, and 7, respectively, shown in Figure 2. Bars represent 50 μm (×200).

pendently until they reach densities of $>10^6$ cfu per seed (7). At these populations, competition for nutrients appears to be an important factor in bacterial growth. Infection studies with *P. ultimum* also suggested that populations of antagonistic bacteria at approximately 10^4 cfu per seed were not sufficient to protect them from infection (7). Visual information on locations of developing bacterial microcolonies may help explain the basis for microbial interactions or lack thereof reported by Fukui et al (7). However, considering what is known about the colonization of plant parts by microorganisms, it is unlikely that direct contact occurs unless very large amounts of inocula are used to saturate plant surfaces.

The scanning electron microscope (SEM) has been used effectively to observe bacterial populations on leaves (5,13), buds (11), and roots in soil (1,17). It should also be useful for detecting microcolonies at different sites on the spermoplane. Fixation of seed by freeze-drying prevents proliferation of bacteria on the spermoplane during the processing necessary for scanning microscopy. SEM also reveals morphological characteristics of bacterial cells, thus allowing colonies with different characteristics to be distinguished.

This study describes the development and distribution of microcolonies of bacteria shown to be antagonistic to soilborne fungal pathogens on sugar beet spermoplanes (7,8,14). Growth of two morphologically different bacteria, *Pseudomonas* spp. and *Bacillus subtilis,* were studied using SEM to examine population growth and colony proliferation on the spermoplane. A preliminary report was published elsewhere (6).

MATERIALS AND METHODS

Seed. The term seed as used in this paper refers to the sugar beet fruit consisting of a single true seed surrounded by the pericarp with traces of perianth calyx tissue. Most of the outer corky perianth had been removed. Sugar beet seed cv. USH11 (Holly Sugar Co., Colorado Springs, CO), size 8–9, was used in all experiments. Seed was surface-sterilized by soaking in 25% commercial-grade bleach (5.25% sodium hypochlorite) for 5 min, followed by thorough washing under running tap water and air-drying aseptically overnight.

Bacterial strains. Strain 33-2 of *Pseudomonas putida* (12) was from Esso Ag. Biologicals, Saskatoon, Canada (formerly Allelix, Inc., Ontario, Canada) courtesy of J. W. Kloepper. Strain ML5 of *Pseudomonas fluorescens-putida* was isolated from the spermosphere of sugar beet in California (14). Strain GB03 of *B. subtilis* was obtained from Gustafson, Inc., Dallas, Texas, courtesy of D. A. Kenney.

Inoculum preparation and seed inoculation. Each *Pseudomonas* strain was grown on King's medium B (KB) (10) for 48 h at 28 C. Strain GB03 was grown on tryptic soy agar (TSA) for 48 h at 37 C. The resulting bacterial lawn was suspended in equal volumes (1.5 ml each) of 1.0% hydroxypropyl methylcellulose (Methocel HG, Dow Chemical Company, Midland, MI) and 0.1 M $MgSO_4$. To inoculate seed with bacterial cells at a high density (10^6–10^7 cfu per seed), 1.5 ml of the bacterial suspension and 5 g of sugar beet seeds were mixed in a small plastic bag (14). A 200-fold dilution of the methylcellulose-$MgSO_4$ mixture was made from the dense bacterial suspension to reduce the initial inoculum density to approximately 10^4 cfu per seed. A portion (1.5 ml) of this dilution was used to inoculate seeds. For coinoculations, a 100-fold dilution was prepared first for each strain, and equal volumes from the two dilutions then were mixed to make up 1.5 ml of suspension for seed inoculation. All inoculated seeds were air-dried at room temperature (22–26 C) overnight before planting.

Enumeration of bacterial population density on seed. The method of Osburn et al (14) was used to incubate seeds. Briefly, 5.5-cm-diameter brass rings and -50 J/kg ($= -0.5$ bar) ceramic pressure plates were disinfested by soaking overnight in 70% ethanol before use. Seeds were planted approximately 1 cm deep in autoclaved sand (Monterey sand, size 60, RMC Lonestar, Pleasanton, CA) placed in the rings (10 seeds per ring) on the pressure plate. The sand was moistened to saturation with sterilized distilled water and adjusted to -15 J/kg matric potential using the pressure plate extraction system. After equilibration for 3–4 h, the rings were transferred onto lids of plastic petri dishes and covered with plastic bags to maintain constant moisture. Seeds inoculated with *Pseudomonas* spp. were incubated for 24 or 48 h at 16 C. Seeds inoculated with GB03 either singly or in combination with strain 33-2 were incubated for 48 h at 16 and 37 C. Although 37 C is an abnormally high temperature to use compared to natural soil temperatures, preliminary experiments indicated that at lower temperatures, pseudomonads totally prevented colonization of GB03.

Growth of GB03 and 33-2 on the spermosphere were analyzed by measuring the population density before planting and at 12-h intervals for 48 h. Five seeds were randomly recovered from each ring, suspended in 5 ml of 10 mM sterile phosphate buffer (pH 6.9), and vortexed for 1 min to remove bacteria from seed. The population density was determined by dilution plate counting

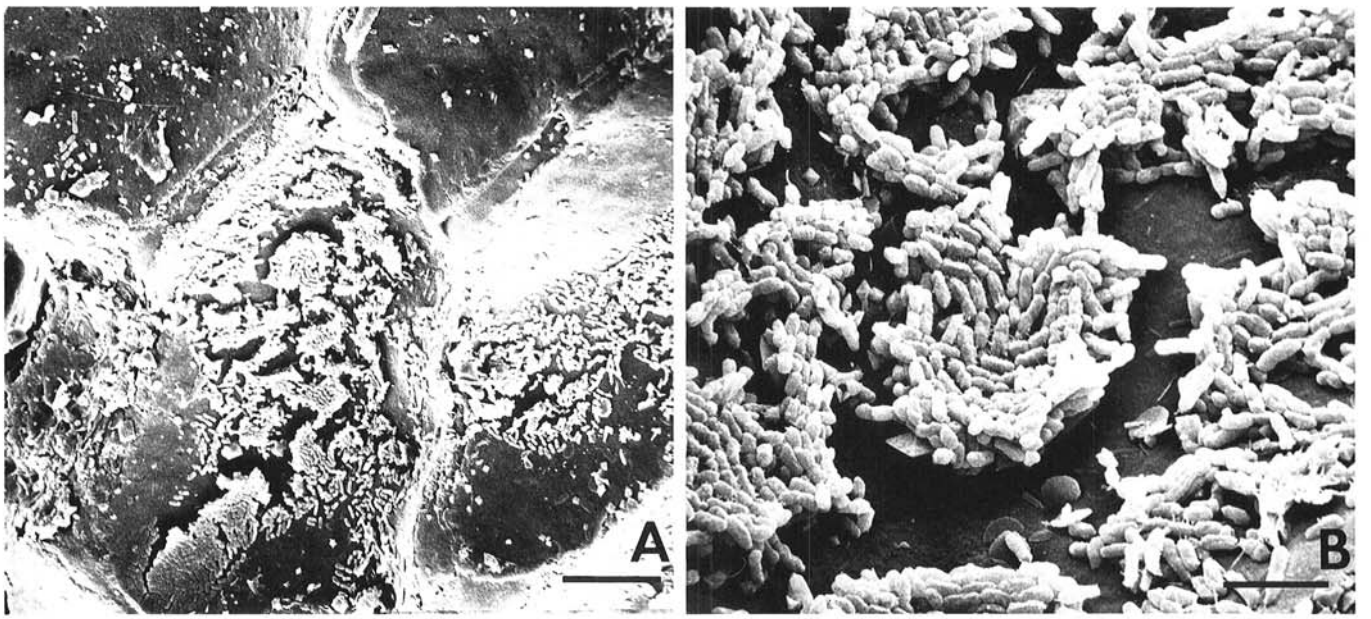

Fig. 4. Microcolonies of *Pseudomonas putida* 33-2 on the spermoplane of sugar beet after incubation in moist sterile sand for 24 h at 16 C. **A,** ×515. Bar represents 10 μm. **B,** ×3,190. Bar represents 3 μm.

on KB and half-strength TSA for *Pseudomonas* spp. and GB03, respectively. The densities of bacteria were expressed as an average of four replicates, each consisting of five seeds.

Fixation of seeds for observation under SEM. Seeds were removed gently from the sand at the end of incubation and immediately frozen by dipping into liquified freon gas (Freon 22, Matheson, Newark, CA) that had been cooled to −80 C in a liquid nitrogen bath, and transferred into liquid nitrogen. The frozen seeds were dehydrated overnight with a freeze drier (E5300, Polaron Instruments, Inc., Doylestown, PA) and sputter coated with a thin layer of platinum or gold using the SEM Coating System (Polaron Equipment Ltd., Watford, England). Fixed seeds were observed by scan field emission microscopy (DS-130, International Scientific Instrument [Topcton], Pleasanton, CA). Magnifications of up to 5,000× were used for microscopic observations. The population densities of bacteria on seed were determined with a separate subset of incubated seeds.

Observation and distribution of microcolonies on the spermoplane. The distribution of microcolonies was examined on seed inoculated with 33-2 and ML5 at approximately 10^4 cfu per seed after incubation in moist, sterile sand for 24 and 48 h. Photomicrographs (approximately 200×) were taken at several randomly selected areas, particularly on and near the operculum. Each area then was scanned at higher magnification to locate microcolonies. Large colonies consisting of hundreds to thousands of cells and smaller colonies with 50 to a few hundred cells were indicated with circles and capital letters L and S, respectively. Small aggregations of cells or scattered individual cells were not marked. Various areas of seed were observed, but photomicrographs were not always taken because the polygonal structure of seed made focusing difficult. Microscopic observations also were done with seeds inoculated with bacteria at approximately 10^7 cfu per seed.

To determine whether patterns of spatial colonization differ between two bacterial strains and whether colonization by one strain influences colonization of the other, two bacterial species with different cell morphologies, 33-2 and GB03, were inoculated onto seed singly and in combination at approximately 10^4 cfu per seed. Microscopic observation of several seeds was done after incubation in moist sterile sand for 48 h. One representative seed was used for taking photomicrographs.

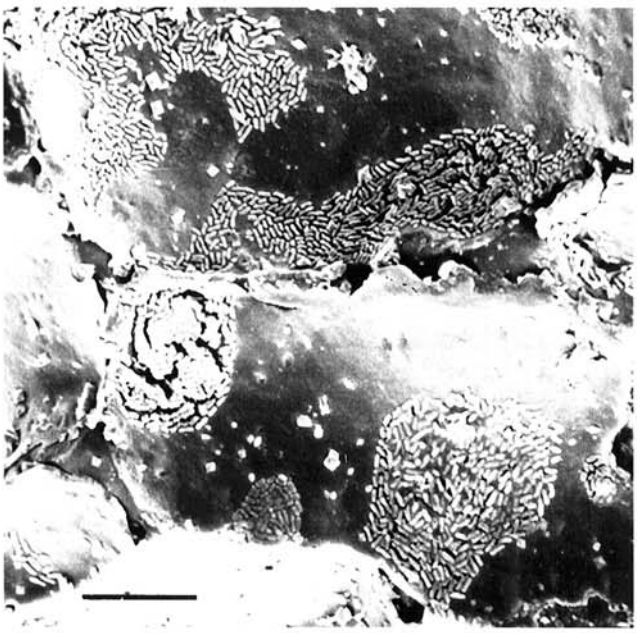

Fig. 5. Microcolonies of *Pseudomonas fluorescens-putida* ML5 on the spermoplane of sugar beet after incubation in moist sterile sand for 24 h at 16 C. Bar represents 8 μm (×1,280).

RESULTS

Proliferation and distribution of *Pseudomonas* strains on the spermoplane of sugar beet seed. A photomicrograph of the sugar beet seed is shown in Figure 1. Population densities of 33-2 and ML5 on seed were 2.1×10^4 and 1.5×10^4 cfu per seed, respectively, at planting time, and 1.0×10^6 and 1.1×10^6 cfu per seed, respectively, after incubation for 24 h at 16 C. Population densities of these strains did not increase after 24 h. By 4 h, many scattered, individual, randomly distributed bacterial cells were observed by SEM over the entire seed surface, but neither microcolonies nor large clusters of cells were detected with either strain. At 24 h, when the stationary phase was reached, microcolonies were found over the entire spermoplane, but their distribution and size were variable. Only 10–40% of the seed surface was colonized by ML5 or 33-2. Figures 2 and 3 show the respective general and detailed locations of microcolonies in several areas on the spermoplane. The largest number of colonies was observed in area 7 (Fig. 3F),

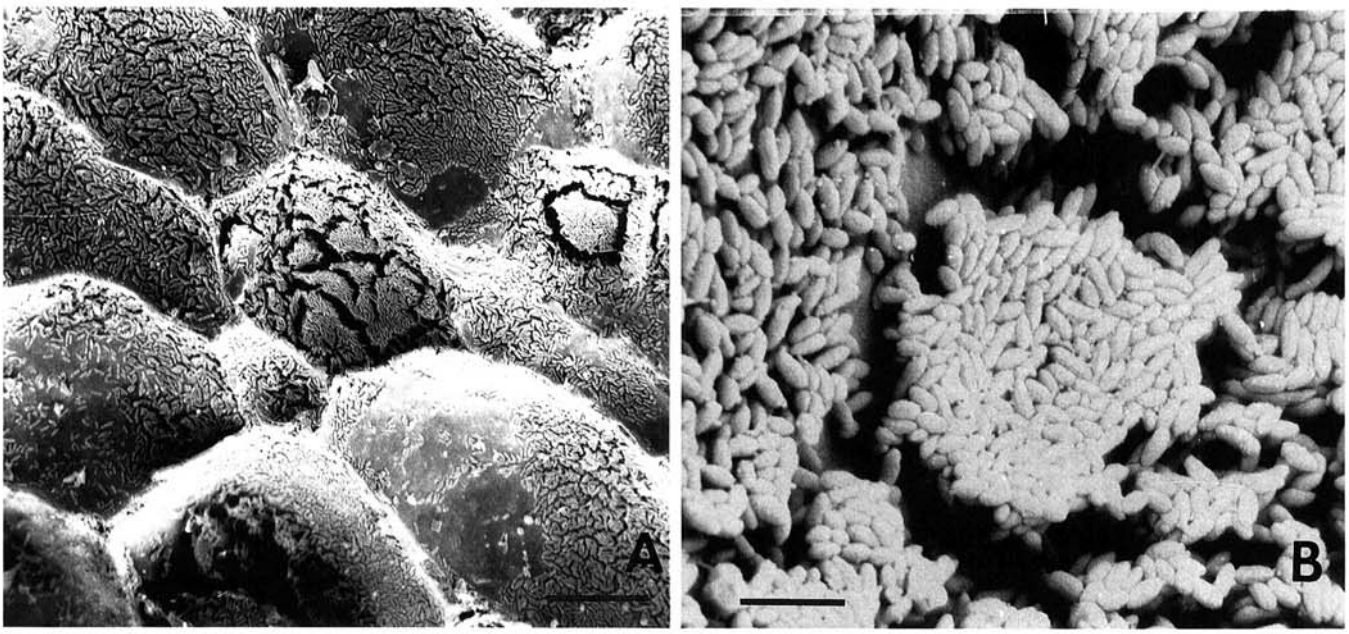

Fig. 6. Microcolonies of *Pseudomonas putida* 33-2 on the spermoplane of sugar beet after incubation in moist sterile sand for 48 h at 16 C. **A,** ×560. Bar represents 20 μm. **B,** ×3,520. Bar represents 3 μm.

whereas only a few colonies were seen in area 5 (Fig. 3D). A photomicrograph of area 3 is not shown, since no microcolonies were found. Many microcolonies were found at the periphery of the operculum and were >20–50 μm apart. Photomicrographs of microcolonies of 33-2 and ML5 that developed on the operculum are shown in Figures 4 and 5, respectively. Microcolonies were not detected on nontreated seed. Microcolonies were also found on the side of the seed containing perianth cells. The distribution was similar to that found on the operculum. Photomicrographs at higher magnifications showed that 33-2 was still undergoing some cell division at 24 h (Fig. 4B), whereas few cells were seen dividing at 48 h (Fig. 6B). By 48 h, microcolonies of 33-2 were larger than those at 24 h and were often fused with neighboring microcolonies across several cells of the operculum or perianth tissues (Fig. 6A). Most of the seed surface was still not colonized by the bacteria (photomicrographs not shown). Greater surface coverage by bacterial cells was attained only when a high initial inoculum density was applied to seed. Most of the seed surface was covered with bacterial cells (similar to the isolated area shown in Figure 6A) when seed were inoculated with 33-2 or ML5 at approximately 10^7 cfu per seed and incubated for 4, 12, and 24 h. At this inoculum density, there was no difference in the distribution or spatial relationships of microcolonies and population densities of the strains at any time interval. It was not possible to differentiate between developing colonies and dead cells delivered in the original inoculum. The same colony patterns were observed with ML5 at all incubation time periods and inoculum densities.

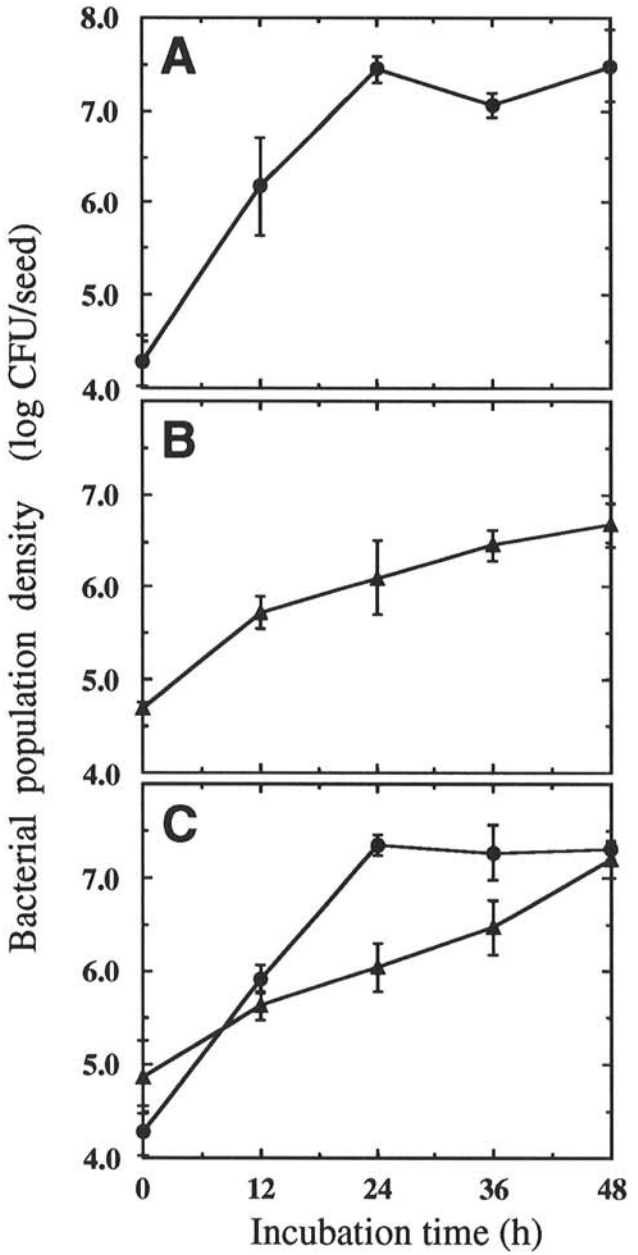

Fig. 7. Population growth of *Pseudomonas putida* 33-2 and *Bacillus subtilis* GB03 inoculated singly and together onto seed incubated in moist sand at 37 C. Circles and triangles indicate the population densities of 33-2 and GB03, respectively. Mean values from two repeated experiments were presented in the figure. The bars represent standard errors. **A,** 33-2 inoculated singly; **B,** GB03 inoculated singly; and **C,** 33-2 and GB03 inoculated in combination.

Proliferation and distribution of microcolonies of *B. subtilis* GB03 inoculated singly and in combination with 33-2 on the spermoplane. GB03 was such a poor colonizer of sugar beet seed at 16 C that little data could be obtained on colonization patterns, growth rates, or its interaction with 33-2. At 16 C, only a few scattered cells could be found on seed and only in the area near the basal pore. When GB03 and 33-2 were inoculated singly at 37 C, 33-2 reached the stationary phase at 24 h, the same as at 16 C, whereas GB03 reached the stationary phase at 48 h (Fig. 7A and B). At 37 C, the maximum population size of 33-2 was about 50-fold greater than was detected at 16 C. Even here, only 40–50% of the surface area of the seed was colonized. The population densities of both strains were approximately 10^7 cfu per seed by 48 h.

The spatial colonization pattern of GB03 differed greatly from that of *Pseudomonas* strains. In single-strain inoculations, microcolonies occurred primarily near the basal pore of the seed (Fig. 8). Occasionally, microcolonies developed near the gap between the operculum and the perianth, which is produced when the radicle emerges. The microcolonies consisted of loosely aggregated, elongated chains of cylindrical rods with club-head-like ends; and they were more than twice as long as those of pseudomonad strains (Fig. 8A and B). Many microcolonies were fused to each other and occupied the indented surfaces of several cells of the perianth. In dual inoculations, it was easy to differentiate the *Bacillus* strain from pseudomonads because of the distinct colony and cell morphologies.

The spatial colonization patterns of GB03 in dual-strain inoculations with 33-2 were similar to the patterns in single-strain inoculations. The rectangle in Figure 1 indicates the general area where microcolonies of GB03 were found. Microcolonies of GB03 occurred primarily near the basal pore, as observed with single-strain inoculations. Some microcolonies also were found near the detached peduncle. However, none were detected in other areas of the seed. Colonization of GB03 appeared to inhibit the development of 33-2 near the basal pore of seed. Microcolonies of 33-2 were located around and near the basal pore when inoculated singly, but did not develop near microcolonies of GB03 when coinoculated. Only small groups of cells and scattered individual cells of 33-2 occurred in the basal pore area. However, microcolonies of 33-2 were distributed randomly on the other parts of seed, as found on seed inoculated singly with 33-2.

DISCUSSION

SEM examination of the spermoplane of germinating sugar beet seeds provided data explaining why there was an apparent lack of interaction between pseudomonads following their coinoculation at population densities of 10^4 cfu per seed despite in vitro studies showing that many strains were inhibitory to each other (7). At these initial densities, it was difficult to locate bacterial cells with SEM on seed surfaces. By the time the stationary phase was attained, only 10–40% of the seed surface was colonized. No differences were detected in the spatial relationships of microcolonies in seeds that were inoculated with different *Pseudomonas* strains. Most of the microcolonies were 20–50 μm apart. At these distances and population densities, there probably was little or no competition for nutrients during the exponential growth phase. This theory is supported by the growth patterns of bacterial strains inoculated singly and in combination (7). In addition, it is not likely that antibiosis would come into play, since the stationary

phases of coinoculated pseudomonad strains are reached at approximately the same time for most of the tested strains (7). Secondary metabolites involved in antibiosis generally are produced at the stationary phase.

We suggest that the seeds were unevenly colonized by bacteria because many sites were not suitable for bacterial growth. Part of the reason may be that nutrients are not uniformly distributed in the pericarp. This conclusion is based on findings (7) that when two near-isogenic strains (a wild type strain R20 of *Pseudomonas putida* and a spontaneous rifampicin-resistant mutant of the same strain) were coinoculated at low and high inoculum densities, the high-inoculum strain inhibited the growth of the other. Presumably, this was because the high-inoculum strain occupied most of the available sites for colonization.

Further evidence supporting the uneven distribution of exudates in the pericarp comes from the observation that, at both 16 and 37 C, *B. subtilis* strain GB03 only colonized the pericarp in the region near the basal pore. The basal pore of sugar beet seed, filled with loose cells characteristic of dead parenchyma tissue, is the route for oxygen and water uptake by the true seed. It also serves as the route for exudate from the true seed to the seed surface. In contrast, the operculum and other walls surrounding the fruit cavity protect the true seed and are heavily lignified and impervious to water (15). Other nutrients in the pericarp are probably remnants of dead tissues. It is unknown why GB03 primarily colonizes areas near the basal pore, while pseudomonads colonize the entire pericarp. It may be that the pseudomonads are more nutritionally versatile than *B. subtilis* and multiply in regions where nutrients are more limiting. It is apparent from these and other studies (R. Fukui, *unpublished*) that GB03 is a poor colonizer of sugar beet seed, at least at normal soil temperatures and in the time period of the experiments. Only at temperatures deemed excessive for a soil environment (37 C) could growth and developing colonies easily be detected on sugar beet seed. Still, it is noteworthy that at 37 C GB03 inhibited colonies of 33-2, which could be seen microscopically but not detected by making dilution plate counts. Individual growth rates and population sizes of the two strains were the same whether they were inoculated singly or together. Despite the relative extreme temperature conditions of the experiments, these data illustrate two very important points: that antagonism may only occur under certain environmental conditions, especially if microorganisms have quite different temperature optima; and that antagonism among seemingly compatible organisms may still occur at microsites, escaping detection by the usual dilution plate assays. Data from gross monitoring of population sizes provide little physical detail of how pathogens and their antagonists interact at infection courts. This was further exemplified when relating the dynamics of bacterial growth on the spermoplane with concomitant SEM micrographs of developing colonies. For example, with 33-2, the stationary phase was reached at 24 h at both 16 and 37 C, and there were many microcolonies. At 48 h, the microcolonies were considerably larger, but the total population density as determined by plate counts was about the same. Presumably, the colonies were expanding at the perimeters while dying in the centers. Previous findings suggest that cell growth in colonies on the surface of agar media occurs only at the edges (16).

The inability of SEM to differentiate between live and dead cells was evident when the number of recoverable cells was compared with spatial colonization patterns on the spermoplane. For example, when seed was inoculated with an inoculum sufficient to attain a recoverable population of approximately 10^7 cfu per seed, SEM indicated that the entire seed surface was covered with bacteria at 6- to 24-h time intervals. However, entirely different patterns were seen with SEM when the initial population density was 10^4 cfu per seed. In this case, even though the final population density was 10^7 cfu per seed, only 40–50% of the surface was colonized. Thus, many bacterial cells on the sugar beet seed must have been dead or nonculturable when using high-density inocula.

Seeds, as viewed by SEM, appear to provide an enormous surface area for colonization by bacteria and fungi. However, these studies with bacteria indicated that certain sites are more habitable than others for both *Pseudomonas* strains and *B. subtilis,* and that the availability of nutrients appears to be the primary factor limiting population size. The overall conclusion is that large areas of the seed are not occupied by bacteria unless very high density inocula are used, and even then it is questionable if all the cells are actively metabolizing. Many may be dead or dormant. Although we have not examined the location and infection sites of *P. ultimum* by SEM concomitantly with bacterial colonization of the seed surface, it is likely that many sites remain available for colonization and penetration by the fungus. This may explain why bacterial biological control agents generally fail to protect seeds from infection by *P. ultimum* when the soil contains a high inoculum density of the pathogen (8).

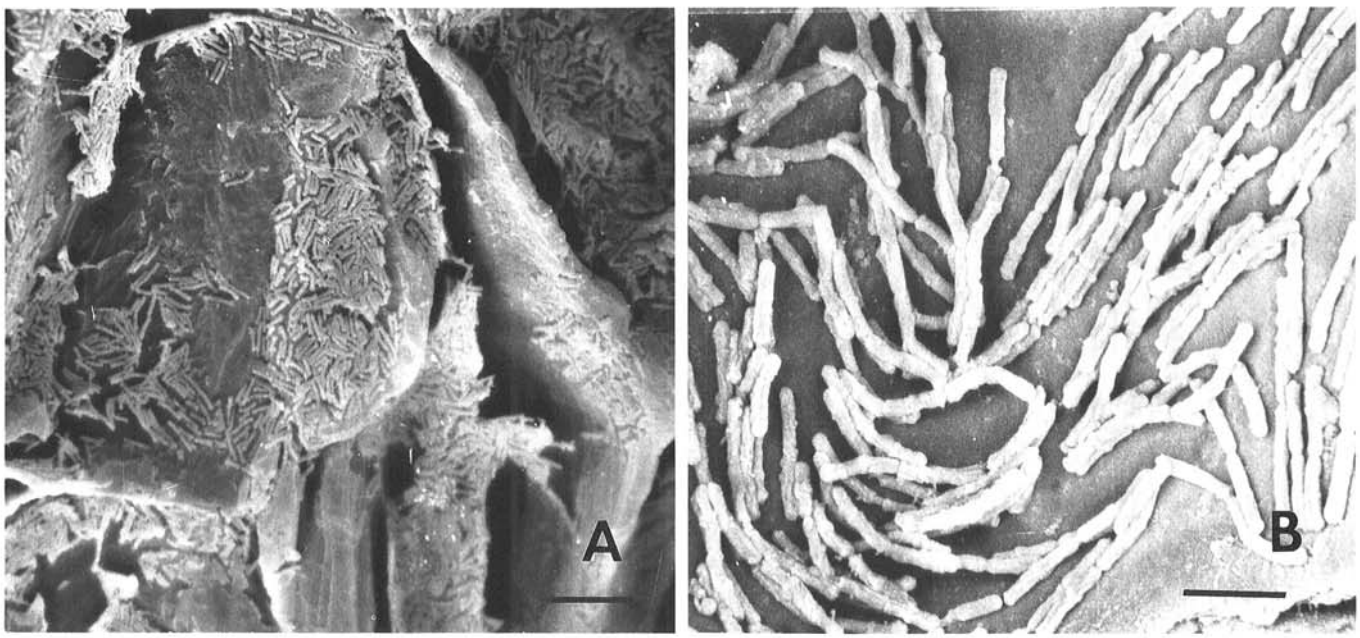

Fig. 8. Microcolonies of *Bacillus subtilis* GB03 on the spermoplane of sugar beet after incubation in moist sterile sand for 48 h at 37 C. **A,** ×1,080. Bar represents 10 μm. **B,** ×3,540. Bar represents 3 μm.

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