Population dynamics of endophytic bacteria in field-grown sweet corn and cotton

John A. McInroy and Joseph W. Kloepper

Abstract: Investigations were designed to gain fundamental information on the microbial ecology of endophytic bacteria in model dicotyledonous and monocotyledonous hosts. Population dynamics of indigenous endophytic bacteria in cotton (*Gossypium hirsutum* L. 'DES119') and sweet corn (*Zea mays* L. 'Silver Queen') stems and roots were studied in a 2-year field trial by quantifying culturable bacteria at intervals during the season on three media: R2A, medium SC, and tryptic soy agar. Population dynamics of endophytic bacteria inside cotton petioles and bolls were also determined in 1 year. Endophytes were recovered from sweet corn roots and stems at seedling emergence at mean population densities of 4 log (colony-forming units per gram fresh weight (cfu/g-fw)) for both seasons, and were present throughout most of the growing season at populations ranging from 4 to 6 log(cfu/g-fw) in 1990 and 4 to 7 log(cfu/g-fw) in 1991. Endophytic bacteria were also present at emergence in cotton roots and stems in 1991 but were not detected until 2 days after emergence in 1990. Endophytic populations in cotton roots ranged from 4 to 6 log(cfu/g-fw) for most of the growing season in 1990 and 1991, while populations in cotton stems fluctuated between 3 and 7 log(cfu/g-fw) during both seasons. In cotton petioles, mean populations generally ranged from 1 to 4 log(cfu/g-fw), while no endophytic bacteria were recovered from bolls (minimum detectable limit = 1.30 log(cfu/g-fw)). The relative contribution of seeds and soil as sources of endophytic bacteria recovered from inside plants was assessed using surface-disinfested seed in a potting mix or on water–agar. With sweet corn, the mean endophytic bacterial population in seedlings grown on water agar was below 2 log(cfu/g-fw), while with cotton the mean was 5 log(cfu/g-fw) 6 days after germination. Internal populations resulting from surface-disinfested seed planted in nonsterile potting mix were 6 log(cfu/g-fw) at 6 days after planting with corn but only 2 log(cfu/g-fw) with cotton. These results indicate that endophytic bacteria are natural inhabitants of internal regions of roots and stems and that the endophytes may arise from both seeds and soils.

Key words: cotton, sweet corn, endophytes, colonization.

Résumé : Des recherches ont été planifiées pour obtenir des informations de base sur l'écologie microbienne de bactéries endophytes chez des représentants modèles de monocotylédones et de dicotylédones. La dynamique des populations de bactéries endophytes indigènes des racines et des tiges du cotonnier (*Gossypium hirsutum* L. 'DES119') et du maïs sucré (*Zea mays* L. 'Silver Queen') a été étudiée durant 2 ans, dans des essais au chemp, par numération à intervalles au cours des saisons des bactéries viables sur trois milieux de culture : le R2A, le milieu SC et la gélose tryptique de soya. La dynamique des populations des bactéries endophytes dans les pétioles et les capsules du cotonnier a aussi été étudiée au cours d'une saison. Des endophytes ont été recouvrées de racines et de tiges de maïs sucré, au stade de plantule en émergence, à des densités moyennes de populations de 4 log(unités formatrices de colonies par gramme de poids frais (ufc/g-pf)) au cours des deux saisons et sont demeurées présentes durant la presque totalité des saisons, variant de 4 à 6 log(ufc/g-pf) en 1990 et 4 à 7 log(ufc/g-pf) en 1991. Les bactéries ont aussi été présentes dans les racines et les tiges du cotonnier en 1991 mais, en 1990, elles ne furent détectées que 2 jours après l'émergence. Dans les racines du cotonnier, les populations endophytes ont varié de 4 à 6 log(ufc/g-pf) durant presque la durée de la saison de croissance des années 1990 et 1991, tandis que dans les tiges elles ont fluctué entre 3 et 7 log(ufc/g-pf) durant les deux saisons. Dans les pétioles du cotonnier, les populations moyennes ont généralement varié de 1 à 4 log(ufc/g-pf), alors que dans les capsules de telles endophytes n'ont pas été détectées (limite minimale détectable = 1,30 log(ufc/g-pf)). La contribution relative des graines et des sols comme sources de bactéries endophytes à l'intérieur des plantes a été établie à l'aide de graines désinfectées en surface dans un mélange pour mise en pot ou sur de la gélose aqueuse. Chez le

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maïs, la population des endophytes dans les plantules croissant sur gélose aqueuse a été inférieure à 2 log(ufc/g-pf), tandis que chez le cotonnier la moyenne a été de 5 log(ufc/g-pf) 6 jours après la germination. Les populations internes résultant de graines désinfectées en surface dans un mélange non stérilisé de mise en pot ont été 6 log(ufc/g-pf) 6 jours après l'ensemencement du maïs, mais seulement de 2 log(ufc/g-pf) pour le cotonnier. Ces résultats indiquent que les bactéries endophytes sont des habitants naturels des régions internes des racines et des tiges et qu'elles peuvent provenir des graines comme des sols.

Mots clés : cotonnier, maïs sucré, endophytes, colonisation.
[Traduit par la rédaction]

Introduction

Owing to enhanced environmental awareness, research and development efforts on the use of biological alternatives for chemical pesticides for the control of crop diseases have increased (Dimock et al. 1989). One focus of biological control is the use of introduced antagonists; a major group of natural antagonists currently being investigated is plant-associated bacteria, including phylloplane and rhizosphere bacteria.

While several bacteria from the phylloplane have been shown to provide some biological disease control (Andrews 1990; Wilson and Lindow 1993), the vast majority of plant-associated bacterial biological control agents have arisen from the rhizosphere. The use of general rhizosphere bacteria, and specifically rhizobacteria (root-colonizing bacteria), for biological control of soilborne plant pathogens has been reviewed recently (Kloepper 1993; Schippers 1988; Weller 1988).

While most of the beneficial rhizobacteria studied to date colonize roots externally, some rhizobacteria also exhibit internal root colonization. Patriquin et al. (1983) have shown that *Azospirillum* spp. colonize plant roots externally and internally in various tissues, including the cortex and xylem. Specific bacteria, such as *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, and *Herbaspirillum rubrisubalbicans* (Gillis et al. 1991) have been shown to exist predominantly within plant tissue (Döbereiner 1993). These nitrogen-fixing bacteria have not been isolated from soil and are found in highest numbers in stems and leaves of sugar cane.

In addition to colonizing the interior of roots, nonpathogenic endophytic bacteria may colonize other regions of the plant. A number of reports since 1948 demonstrate that bacteria naturally inhabit healthy plant tissues, including fruits (Samish and Dimant 1959; Samish et al. 1961), tubers (Hollis 1951), stems (Fry and Milholland 1990), and roots (Philipson and Blair 1957). Mundt and Hinkle (1976) found endophytic bacteria within seeds and ovules of 25 of 27 plant species sampled, thus establishing the presence of endophytes prior to germination.

The internal tissues of plants may provide a more uniform and protective environment for potential biological control agents than the phylloplane, where exposure to ultraviolet radiation, rainfall, and temperature fluctuations negatively affect microorganisms or the rhizosphere, where a diverse microflora competes for nutrients. However, little is known at this time about the population dynamics of indigenous endophytic bacteria, it is not possible to predict how much competition an introduced endophyte may encounter.

The objectives of this study were to determine the stage in plant development when bacterial endophytes first become associated with roots, stems, petioles and fruiting structures; to determine the population dynamics of bacterial endophytes in stems and roots during the growing season; to compare populations in a model monocotyledonous plant, sweet corn (*Zea mays* L.), and a dicotyledonous plant, cotton (*Gossypium hirsutum* L.); and to determine if endophytes arise from both seed and soil/potting medium. A preliminary report of a portion of this project was published in abstract form (McInroy and Kloepper 1991).

Materials and methods

Media

Bacteria were enumerated on three different media: tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) was used to support the growth of a broad range of microorganisms; medium R2A (Difco Laboratories, Detroit, Mich.) was used for bacteria requiring a low level of nutrients (oligotrophs); and medium SC (Davis et al. 1980) was previously shown to support the growth of fastidious endophytic bacteria.

Field experiments

Cotton ('DES 119') and sweet corn ('Silver Queen') were planted in 1990 and 1991 in a fine, loamy, siliceous, thermic, Typic Hapludult soil at the E.V. Smith Research Center of the Alabama Agricultural Experiment station near Tallassee, Ala. Ten replicate blocks of nontreated seed were planted for each crop. Each replication consisted of a four-row plot, 7.5 m in length. Fertilization, weed control, and insect control were conducted according to recommendations of the Alabama Agricultural Experiment Station. In 1990, plants were sampled for stems and roots at emergence and 2, 7, 14, 21, 28, 42, 56, 70, and 112 days after emergence. In 1991, stems and roots of plants were sampled once prior to emergence, at emergence, and 7, 14, 28, 42, 56, and 70 days after emergence. At each sampling date, one randomly selected plant from each of the 10 replications was manually uprooted and transported at approximately 10°C to the laboratory.

Cotton petiole sampling in 1991 began with the development of the node-1 petiole (P1) 28 days after emergence and continued every 14 days thereafter until day 70. The same plants, which were sampled for stems and roots, as described above, were sampled for petioles giving 10 replicated samples. Three petiole samples were taken from each plant, with the exception of the first sampling date when only the P1 was taken. The three samples included P1; the last-formed leaf with a petiole of at least 2 cm in length (P3); and a petiole from a node equidistant to P1 and P3 (P2). Immature cotton bolls were also sampled in 1991, using the same plants sampled for stem, root, and petiole tissues, 10 replications in total. Sampling dates for cotton bolls were 42, 56, and 70 days after emergence.

Greenhouse experiments

Two experiments, each with three treatments, were conducted to determine the relative contribution of seeds and potting medium as sources of endophytic bacteria by examining endophytic populations from imbibition to emergence. For the first treatment, nonsterile seeds were planted in Promix soilless potting medium (Premier Peat Ltd., Rivière-du-Loup, Qué.) in 10 × 10 × 10 cm (L × W × D) pots, 1 seed/pot. The second treatment involved surface-disinfesting seeds with 1.05% sodium hypochlorite for 10 min, rinsing three times in sterile potassium phosphate buffer (pH 7.0), and planting in Promix potting medium. For the third treatment, surface-disinfested seeds were aseptically transferred to water–agar plates after rinsing with sterile water. Plates and pots were maintained in the greenhouse in a randomized complete block design. Pots were watered twice daily with sterile water. Ten replicative samples consisting of whole seedlings were taken at 0, 1, 2, 4, and 6 days after planting. Seeds on agar plates surrounded by visible bacterial growth were excluded from the study (sterile germination was achieved in at least 9 of 10 replications). Separate experiments were conducted for cotton and corn and each experiment was repeated once.

Sample preparation and surface sterilization

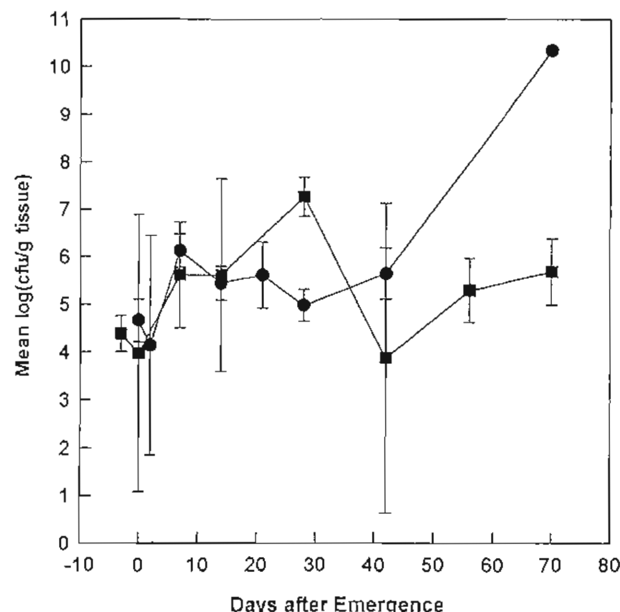
Individual plant samples were washed in running tap water to remove adherent soil. Sections, 2–3 cm in length, were excised with a flamed scalpel. Root sections were taken from 5–10 cm below the soil line and stem sections were taken from 5–10 cm above the soil line. To make immature boll samples easier to handle, the outer involucre and part of the soft fibrous tissue was excised. Whole seedlings were used from the greenhouse experiment.

All samples were blotted dry with a paper towel and weighed before processing. Stem, petiole, and boll samples were surface disinfested in 20% hydrogen peroxide for 10 min and rinsed four times with sterile 0.02 M potassium phosphate buffer (pH 7.0). Surface-disinfestation parameters for all tissues were optimized prior to experimentation. Root and seedling samples were surface disinfested with 1.05% sodium hypochlorite for 10 min and rinsed four times in buffer. To check for surface contamination, 0.1 mL of the final wash for each sample was transferred to 9.9 mL tryptic soy broth (TSB) and incubated at room temperature on a shaker (approximately 200 rpm) or spread plated onto TSA. After incubation at 28°C for 3 days, tubes were examined and samples with visible growth were not used in calculating means of population densities. This method was found to previously detect identical contamination percentages as placing samples directly in TSB for a few minutes or imprinting tissues directly on TSA plates. Each sample was triturated with a sterile mortar and pestle in 9.9 mL of the final buffer wash. Serial dilutions were made using phosphate buffer, as previously described, and plated with a spiral plater (Spiral Systems, Inc., Bethesda, Md.). Each dilution of every sample was plated on one plate each of TSA, R2A, and SC.

Growth conditions, bacterial counts, and data analysis

Agar plates were incubated at 28°C for 48–72 h except where noted. Colonies were counted with a laser colony counter (Spiral Systems, Inc.) and populations, expressed in colony-forming units (cfu) per millilitres, were calculated using the Bacterial Enumeration software (Spiral Systems, Inc.). All population data were transformed to log(cfu per gram fresh weight (cfu/g-fw)) prior to averaging. A zero value was used for populations below the minimum detection limit. Standard deviations were calculated for each data point.

Fig. 1. Populations of endophytic bacteria isolated on R2A medium from roots of field-grown sweet corn in 1990 (●) and 1991 (■). Error bars represent standard deviation of the mean of 10 replications.



Results

Field populations

Bacteria were recovered from surface-disinfested stems and roots of cotton and sweet corn during both growing seasons on all media. Populations from medium R2A and medium SC were significantly greater than populations on TSA ($P = 0.0001$), based on calculated standard deviations for each data point. Populations from medium R2A were not significantly different from medium SC ($P = 0.0001$). Plate counts from medium R2A were more accurately determined because of less colony overlap and smaller colony size and allowed for the growth of the same heterotrophic bacteria, based on identification that was previously reported (McInroy and Kloepper 1995). Data are therefore presented only from medium R2A.

Hydrogen peroxide was found to be more effective than ethanol and sodium hypochlorite for removing bacteria from the exterior surface of cotton petioles and bolls, and from stems of both sweet corn and cotton when a variety of concentrations were tested. An aqueous solution consisting of 20% hydrogen peroxide was found to be the most dilute solution that would eliminate surface bacteria at least 90% of the time. However, with roots and seedlings, a 20% aqueous solution of household bleach (1.05% sodium hypochlorite) gave similar results.

Mean total endophytic bacterial (TEB) populations of sweet corn roots (Fig. 1) and stems (Fig. 2) from the field showed that

Fig. 2. Populations of endophytic bacteria isolated on R2A medium from stems of field-grown sweet corn in 1990 (●) and 1991 (■). Error bars represent standard deviation of the mean of 10 replications.

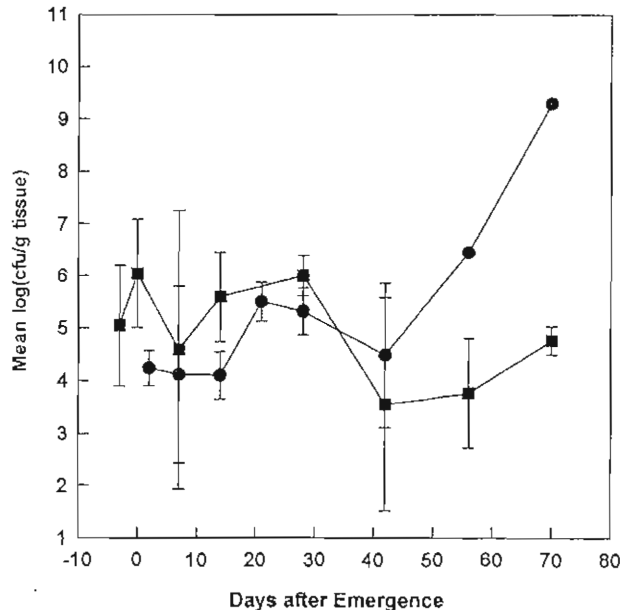
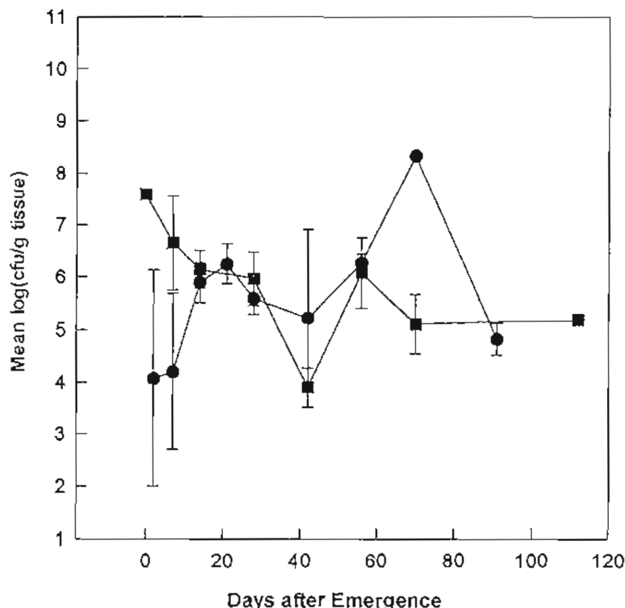
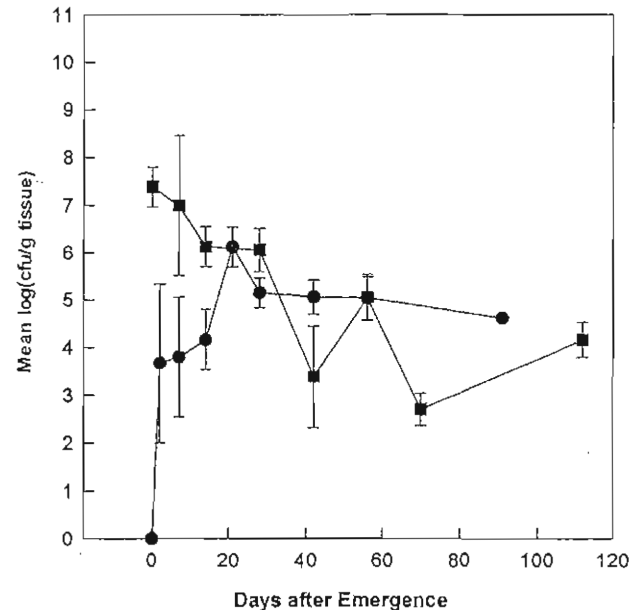


Fig. 3. Populations of endophytic bacteria isolated on R2A medium from roots of field-grown cotton in 1990 (●) and 1991 (■). Error bars represent standard deviation of the mean of 10 replications.



endophytic bacteria were present at emergence at 4 log(cfu/g-fw) for both seasons. Mean TEB populations in corn roots (Fig. 1) and stems (Fig. 2) in 1990 remained between 4 and 6 log(cfu/g-fw) for most of the growing season. These populations increased to 8 and 10 log(cfu/g-fw) postharvest. Mean TEB populations in 1991 ranged from 4 to 7 log(cfu/g-fw) for the entire growing season.

Fig. 4. Populations of endophytic bacteria isolated on R2A medium from stems of field-grown cotton in 1990 (●) and 1991 (■). Error bars represent standard deviation of the mean of 10 replications.



Endophytic bacteria were present at emergence in cotton roots in 1990 and 1991 (Fig. 3). In 1990, mean TEB populations from field-grown cotton roots were 4 log(cfu/g-fw) for the first week and from 5 to 8 log(cfu/g-fw) for the rest of the season. In 1991, mean TEB populations from cotton roots were 7 log(cfu/g-fw) during the 1st week and 4–6 log(cfu/g-fw) for the rest of the season. No populations were detected in cotton stems at emergence in 1990 (Fig. 4), but bacteria were present 2 days after emergence at 3 log(cfu/g-fw). Mean TEB populations in cotton stems in 1990 remained between 4 and 6 log(cfu/g-fw) for the rest of the season. In 1991, mean TEB populations in cotton stems were 7 log(cfu/g-fw) at emergence and 6–7 log(cfu/g-fw) for the 1st week. For the remainder of the season, populations in 1991 ranged from 3 to 6 log(cfu/g-fw) in cotton stems.

Data from 1991 showed that mean TEB populations from cotton petioles (Fig. 5) ranged from 1 to 4 log(cfu/g-fw) with the exception of the P1 petiole at 14 days, where the population was 6 log(cfu/g-fw). Mean TEB populations from P1 (2–4 log(cfu/g-fw)) were generally higher but not significantly different from P2 (1–3 log(cfu/g-fw)) and P3 (2–3 log(cfu/g-fw)). No bacteria were recovered from immature cotton bolls at any of the sampling dates. The minimum detectable limit was 20 cfu/g fw.

Greenhouse populations

Endophytic bacteria were recovered from all treatments in the greenhouse. Mean TEB populations in sweet corn seeds (Fig. 6) prior to germination in Promix were 5 log(cfu/g-fw).

Fig. 5. Populations of endophytic bacteria isolated on R2A medium from the petioles of field-grown cotton in 1991: P1 (●), P2 petiole (■), P3 (▲). Error bars represent standard deviation of the mean of 10 replications.

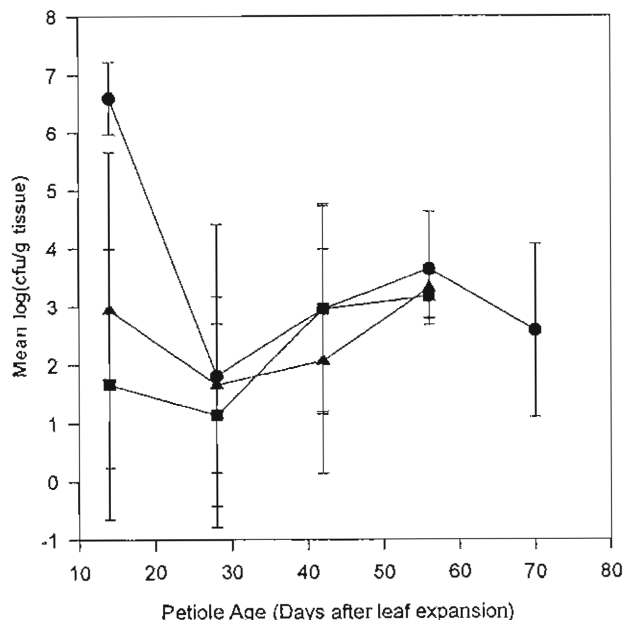
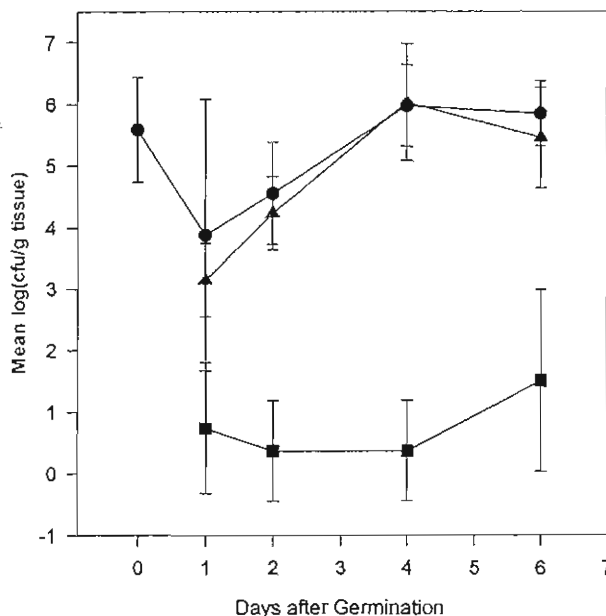


Fig. 6. Populations of endophytic bacteria isolated on R2A medium from germinating sweet corn seeds. Populations were determined from surface-disinfested seeds germinated in Promix (▲), surface-disinfested seeds germinated on water–agar (■), and nondisinfested seeds germinated in Promix (●). Error bars represent standard deviation of the mean of 10 replications.



These populations fluctuated between 3 and 6 log(cfu/g-fw) throughout germination as did populations from surface-disinfested sweet corn seeds germinated in Promix, although populations in these seeds were 1 log unit lower at 1 day after planting. In contrast, TEB populations of surface-disinfested sweet corn seeds germinated on water–agar were consistently below 2 log(cfu/g-fw).

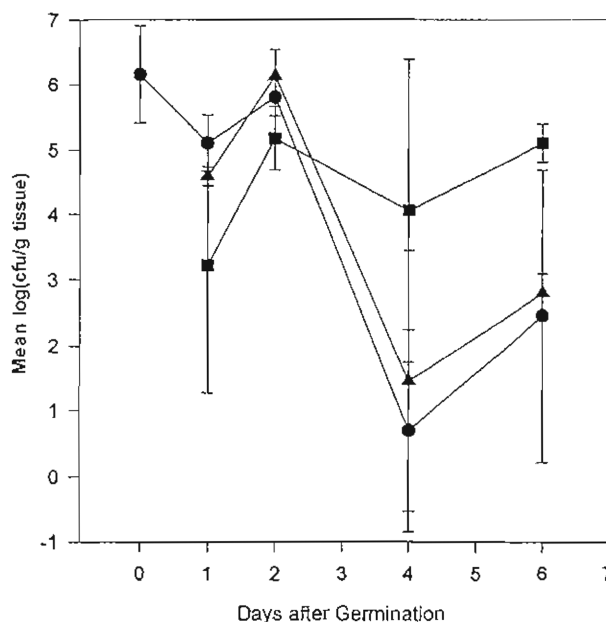
Mean TEB populations from cotton seedlings (Fig. 7) germinated in Promix were 5–6 log(cfu/g-fw) for the first 2 days of germination, but only 1–3 log(cfu/g-fw) from 4 to 6 days after planting. Mean TEB populations of surface-disinfested cotton seeds grown in Promix were similar to those of nondisinfested seeds. Mean TEB populations of surface-disinfested cotton seeds germinated on water–agar ranged from 3 to 5 log(cfu/g-fw) throughout germination.

Discussion

Stems and roots of healthy monocotyledonous and dicotyledonous plants were naturally colonized internally by endophytic bacteria at average populations of 3–7 log(cfu/g-fw) throughout two growing seasons. In the field, endophytes colonized plants early in the season, beginning prior to emergence, based on recovery from seedlings. The populations reported here agree with population ranges in previous studies of endophytes in sugar beet (Bugbee et al. 1975), lemon (Gardner et al. 1982), alfalfa (Gagné et al. 1987), and potato (DeBoer and Copeman 1974).

In the greenhouse trial, bacterial endophytes were recovered from surface-disinfested cotton and sweet corn seeds (Figs. 6 and 7) indicating that endophytic bacteria originate, in part,

Fig. 7. Populations of endophytic bacteria isolated on R2A medium from germinating cotton seeds. Populations were determined from surface-disinfested seeds germinated in Promix (▲), surface-disinfested seeds germinated on water–agar (■), and nondisinfested seeds germinated in Promix (●). Error bars represent standard deviation of the mean of 10 replications.



from seeds. This hypothesis has been discounted in the past (Gagné et al. 1987; Mundt and Hinkle 1976). However, it is also possible that these bacteria are merely thriving in the recesses created by the seed coat of the imbibing seed. When isolated on agar, bacterial endophytes were present in surface-disinfested cotton seeds at 3–5 log(cfu/g-fw), while in sweet corn populations were below 1 log(cfu/g-fw). The cotton seed coat is rough with deep grooves and partially open to the environment at the distal end during imbibition, and the perisperm is therefore in direct contact with the environment. In contrast, the surface of sweet corn seed is smooth and intact, providing a complete enclosure for the entire ovule. The action of the disinfesting agent may not reach bacteria that have colonized the deeper recesses of the seed-coat grooves.

TEB populations in cotton seeds germinated in Promix for 2 days were 3 log units less 2 days later, while TEB populations in seeds germinated on water–agar increased (Fig. 7). It is unclear from the experiments reported here why populations in cotton seeds in Promix decreased, while they increased in seeds on agar. One possible explanation is microbial competition. It is possible that on agar, seed endophytes develop into seedling endophytes, but in the rhizosphere, endophytes compete or change plant physiology so that all seed endophytes cannot survive. Constant moisture availability on water–agar may also affect endophytic density. It is also possible that differential diffusion of nutrients on agar and in the potting medium affected population densities of endophytes.

In the field trials, populations of bacterial endophytes tended to decrease acropetally. Root populations were generally slightly greater than stem populations and stem populations were higher than petiole populations. Two weeks after emergence, when P1 was proximally located to the main stem, the endophytic population there was similar to that of the stem population. However, as the plant grew and the distance between the P1 and base of the stem increased, so did the difference in colonization density (Figs. 4 and 5).

Since no endophytic bacteria were isolated from cotton bolls in this study, there is no evidence to suggest that bacteria can be transmitted from parent plant to seed. However, endophytes in cotton bolls may have been present below the minimum detectable level of the experimental design (1.30 log(cfu/g-fw)). Clark et al. (1947) isolated *Aerobacter cloacae* (*Enterobacter cloacae*), a contaminant of mature cotton bolls and a discoloring agent of cotton fiber, from mature cotton bolls; however, he was unable to isolate *A. cloacae* from the healthy interior tissues of immature bolls. Similarly, Misaghi and Donndelinger (1990) isolated *Erwinia* and *Bacillus* spp. from immature and mature cotton bolls.

The results presented here indicate that the interior regions of plants represent a major microbial habitat for plant-associated bacteria. To further understand bacterial colonization of internal plant tissues, isolated strains should be identified and associated with specific plant tissues. This information may help to explain the marked increase in sweet corn colonization that occurred in the field at the end of the 1990 growing season. It is possible that fluctuations in endophyte populations may be due to differential colonization by specific bacterial strains.

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