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Enumeration and Localization of N_2-Fixing Bacteria Associated with Roots of *Spartina alterniflora* Loisel

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Numbers and possible locations of N_2-fixing bacteria were investigated in roots of *Spartina alterniflora* Loisel, which support nitrogenase activity in the undisturbed native habitat. N_2-fixing bacteria were recovered in cultures both from *S. alterniflora* roots and from the surrounding sediment, and they formed a greater proportion of the bacteria recovered from root homogenates than from salt-marsh sediment. N_2-fixing bacteria were recovered in high numbers from the rhizoplane of *S. alterniflora* after roots were treated with 1 or 5% chloramine-T for 1 h or with 1% NaOCl for 1 or 2 h. Immersing *S. alterniflora* roots in 5% NaOCl for 1 h was more effective in distinguishing bacteria inside the roots since this treatment nearly eliminated N_2-fixing bacteria recoverable from the rhizoplane, although high numbers of N_2-fixing bacteria were recovered from homogenates of roots treated with 5% NaOCl for 1 h. However, this treatment was less effective with roots of *Zea mays* L. (Funks G4646) and *Sorghum bicolor* (L.) Moench (CK-60 A), indicating that techniques to surface sterilize roots should be evaluated for different plants. Bacteria were observed by light and electron microscopy inter- and intracellularly in the cortex and in the aerenchyma of *S. alterniflora* roots. This study clearly shows that bacteria, including N_2 fixers, colonize the interior of roots of *S. alterniflora* growing in a Chesapeake Bay, Maryland, salt marsh.

The roots of grasses have been suggested as suitable habitats for certain heterotrophic N_2-fixing bacteria (9, 10, 14, 21). Although it has been suggested that N_2-fixing bacteria colonize the interior of roots of grasses (2, 7, 11, 15–17, 19, 24), the methods used in those studies generally have not been adequately evaluated (21). Furthermore, those reports relate to studies in which C_2H_2 reduction by excised roots is detectable only after a prolonged delay (8 to 18 h). Van Berkum and Bohlool (21) have suggested that C_2H_2 reduction by excised roots detectable only after a prolonged delay does not indicate that grasses supported N_2 fixation before they were disturbed for experimentation. In contrast, roots of *Spartina alterniflora* Loisel reduce C_2H_2 in air immediately after excision from plants growing in a Chesapeake Bay salt marsh in Maryland (22). This observation has offered us the opportunity to enumerate and localize N_2-fixing bacteria associated with the roots of a grass which supports N_2 fixation in an undisturbed native habitat.

We first evaluated methods for surface sterilizing roots to enable us to distinguish the internally located bacteria when using classical bacterial enumeration techniques. In conjunction with light and electron microscopy, these techniques were used to investigate the sizes and locations of bacterial populations associated with the roots of *S. alterniflora*.

MATERIALS AND METHODS

Plants. Roots of *S. alterniflora* were collected from the same Chesapeake Bay, Maryland, brackish, irregularly flooded salt marsh as previously described (22). Plants of *Zea mays* L. (Funks G4646) and *Sorghum bicolor* (L.) Moench (CK-60A) were grown in field plots at the Beltsville Agricultural Research Station, Beltsville, Md., and the roots were collected from plants in the early flowering stage. C_2H_2 reduction by excised root samples was measured as described by van Berkum and Sloger (22).

Culture media and buffer solution. A heterotrophic nutrient (HN) medium used to estimate numbers of heterotrophic bacteria contained the following (in

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TABLE 1. MPN of heterotrophic and N_2-fixing bacteria recovered by shaking with glass beads or by homogenizing excised roots of *S. alterniflora* treated with chloramine-T or NaOCl

Treatment[a]	Titer (log MPN/g [fresh wt] root tissue) in culture medium[b]:					
	HN[c]		GMS[d]		MS[d]	
	Beads	Homogenates	Beads	Homogenates	Beads	Homogenates
Control	7.54	7.54	5.45	5.91	6.20	6.04
Chloramine-T						
1%, 1 h	4.96	6.54	4.38	4.63	4.23	4.59
5%, 1 h	4.73	5.73	3.20	4.85	3.52	4.96
NaOCl						
1%, 1 h	5.38	5.54	4.38	4.54	5.38	5.23
1%, 2 h	4.73	5.73	4.38	4.23	3.04	5.34
5%, 1 h	2.11	3.96	0.30	2.69	0.65	3.42

[a] Control roots were washed and immediately sampled. Treated roots were washed and then immersed in 70% ethanol for 5 min followed by immersion in either chloramine-T or NaOCl. Roots were then rinsed in sterile SPB.

[b] Least significant difference = 0.60 (P = 0.05). Numbers show MPN of bacteria released into sterile SPB when roots were shaken with glass beads for 15 min at 400 rpm (beads) and MPN of bacteria recovered from homogenized roots (homogenates).

[c] Growth was scored by visible turbidity within 14 days of incubation at 30°C.

[d] Criteria for a positive count were both visible turbidity and C_2H_2 reduction within 14 days of incubation at 30°C.

grams per liter): nutrient broth (Difco Laboratories, Detroit, Mich.), 8.0; Yeastolate (Difco), 2.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; KCl, 0.16; $CaCl_2$, 0.02; $FePO_4$, 0.5; agar, 1.75. The pH was adjusted to 7.0 before autoclaving. A nitrogen-deficient glucose mannitol-salts (GMS) medium used to enumerate facultatively anaerobic N_2-fixing bacteria was prepared by mixing the following autoclaved solutions: (i) glucose, 20 g; (ii) mannitol, 20 g; (iii) K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; and (iv) sodium thioglycolate, 0.3 g, each in 100 ml of distilled water; and (v) Tris, 6.0 g; yeast extract (Difco), 0.15 g; NaCl, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.015 g; $Na_2MoO_4 \cdot 2H_2O$, 0.004 g; distilled water, 600 ml. The pH of each solution was adjusted to 7.2 before autoclaving. A semisolid malate-salts (MS) medium, modified from that of McClung and Patriquin (13) by reducing the NaCl from 15.0 to 5.0 g/liter, was used to estimate the number of organic acid-utilizing N_2-fixing bacteria. Saline-phosphate buffer (SPB) contained (in grams per liter of distilled water): KH_2PO_4, 4.23; K_2HPO_4, 5.41; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02. The pH was adjusted to 7.0 before autoclaving. For experiments with *Z. mays* and *S. bicolor*, the NaCl concentration was reduced from 5.0 to 0.1 g/liter.

Preparation of samples for bacterial counts. Saltmarsh sediment was collected from a plant-free area adjacent to the zone of *S. alterniflora*. Roots of *S. alterniflora* were shaken to remove loose sediment, vigorously rinsed in adjacent creek water, and then excised from the plants. The sediment adhering to the roots after they had been shaken was considered to be rhizosphere sediment and was accumulated by decanting off the creek water in which the roots had been rinsed. Roots of *Z. mays* and *S. bicolor* were shaken to remove loose soil, washed vigorously in distilled water, and then excised from the plants.

Subsamples of the washed roots were immersed in 70% ethanol for 5 min and then were immersed in chloramine-T (Sigma Chemical Co., St. Louis, Mo.) or NaOCl. As controls, replicate root samples were immersed in sterile SPB. Treated and control roots were rinsed in three changes of sterile SPB, which together were equal in duration to the preceding treatments. The rinsed roots were separated into two subsamples to enumerate bacteria recoverable from rhizoplanes and from root homogenates.

To recover bacteria present in sediment, rhizophere sediment, and roots, weighed subsamples were homogenized in sterile SPB with a Virtis model 45 homogenizer at full speed for 1 min. Bacteria were recovered from root surfaces by shaking 2.0-g (fresh weight) subsamples with 3.0 g of glass beads (equal weights of beads 3 and 5 mm in diameter) in 20 ml of sterile SPB in 125-ml Ehrlenmeyer flasks at 400 rpm for 15 min on a New Brunswick Gyrotory shaker. The bacteria recovered in the SPB from the roots shaken with glass beads were defined, according to Watanabe et al. (24), as the rhizoplane population. In one experiment, subsamples of this rhizoplane population in SPB were homogenized to determine whether this treatment affected the recovery of bacteria.

Bacterial counts. To determine the most probable numbers (MPN) of culturable bacteria (3), decimal dilutions of homogenates and of supernatants from the bead treatments were prepared in sterile SPB and were used to seed tubes of HN, GMS, and MS media (five tubes each per dilution). The tubes of GMS medium were subsequently flushed briefly with filter-sterilized N_2 and were capped with serum stoppers. All tubes were incubated at 30°C, and visible turbidity in HN medium was scored after 14 days. The MPN of N_2-fixing bacteria were determined by the method of Patriquin and McClung (17), and C_2H_2 reduction was used to score positive counts.

Microscopy. Washed roots and roots treated with chloramine-T and NaOCl were incubated separately overnight at 30°C in SPB containing 1.0 g of 2,3,5-

TABLE 2. Recovery of N_2-fixing bacteria from excised roots of *S. alterniflora*, *Z. mays*, and *S. bicolor* treated with chloramine-T or NaOCl[a]

Treatment	Sample	Titer (log MPN/g [fresh wt] of root tissue)					
		S. alterniflora		*Z. mays*		*S. bicolor*	
		GMS	MS	GMS	MS	GMS	MS
Control	Beads	5.45	6.20	4.42	8.38[b]	4.20	8.38[b]
	Homogenates	5.91	6.04	4.63	8.20	4.96	8.20
Chloramine-T (1%, 1 h)	Beads	4.38	4.23	2.52	5.96	3.79	6.38[b]
	Homogenates	4.63	4.59	2.90	6.38[b]	4.23	5.73
NaOCl (5%, 1 h)	Beads	0.30	0.65	0.65	3.96	NG[c]	3.73
	Homogenates	2.69	3.42	1.81	4.83	0.30	3.91

[a] For details of experimental procedures, see Table 1, footnotes *a*, *b*, and *d*.
[b] Minimum number.
[c] NG, No growth.

triphenyltetrazolium chloride per liter and were then sectioned by hand for light microscopy.

Washed roots of *S. alterniflora* were cut into approximately 3-mm lengths and then were fixed and stored at 4°C in 2.5% (vol/vol) glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.3. The fixed roots were rinsed in fresh cacodylate buffer before and after postfixing for 4 h in 1% (wt/vol) OsO_4 at room temperature. The roots were then dehydrated through a graded series of ethanol and were transferred to propylene oxide before they were embedded in the low-viscosity resin of Spurr (20). Sections were cut with a Reichert OM 200 ultramicrotome. Thick (about 1 μm) sections were heat fixed to glass slides and were stained with toluidine blue-O or with methylene blue. Ultrathin sections were stained with uranyl acetate (either 4% [wt/vol] in ethanol or 7.7% [wt/vol] in water) and 2.7% (wt/vol) lead citrate (18) and were examined with a JEOL JEM-100 B transmission electron microscope operated at 80 kV.

RESULTS

Estimates of both the rhizoplane and total bacterial populations associated with roots of *S. alterniflora* were significantly reduced by treating roots with chloramine-T or NaOCl (Table 1). However, substantial numbers of bacteria were recovered from the rhizoplanes of roots treated with either 1 or 5% chloramine-T for 1 h or with 1% NaOCl for 1 or 2 h. In contrast, treatment with 5% NaOCl for 1 h reduced the recovery of rhizoplane N_2-fixing bacteria from 2.8×10^5 to 2.0 per g (fresh weight) as estimated in GMS medium and from 1.6×10^6 to 4.5 per g (fresh weight) as estimated in MS medium. The MPN of N_2-fixing bacteria recovered from roots of *S. alterniflora* homogenized after treatment with 5% NaOCl for 1 h were 5.0×10^2 and 2.6×10^3 per g (fresh weight) as estimated in GMS and MS media, respectively. The MPN of bacteria recovered in the sterile SPB from the bead treatment before and after homogenizing were statistically similar.

The total number of heterotrophic bacteria estimated with HN medium to be associated with the rhizoplane of roots of *S. alterniflora* was reduced from 3.5×10^7 to 1.3×10^2 by immersion of roots in 5% NaOCl for 1 h (Table 1). Tetrazolium reduction was absent at the surfaces of roots of *S. alterniflora* after this treatment. Bacteria were not recovered from the rhizoplane of *S. alterniflora* after roots had been immersed in 0.2% $HgCl_2$ for 15 min after the 5% NaOCl treatment. However, heterotrophic bacteria, including N_2 fixers, were recovered in low numbers from homogenates of roots of *S. alterniflora* which had been treated with NaOCl and $HgCl_2$.

The recoveries of rhizoplane bacteria were compared among *S. alterniflora* growing in a salt marsh and field-grown *Z. mays* and *S. bicolor* after treatments of the roots with chloramine-T or NaOCl. Large proportions of the bacterial

TABLE 3. MPN of heterotrophic and N_2-fixing bacteria recovered from sediment and from rhizosphere, rhizoplane, and roots of *S. alterniflora*[a]

Sample	Titer (log MPN/g [fresh wt] of sample) estimated in medium:		
	GMS	MS	HN
Salt-marsh sediment	4.73	4.23	7.20
Rhizosphere sediment	5.08	4.73	7.73
Washed roots			
Beads	5.54	5.96	7.49
Homogenates	5.38	5.73	7.85
NaOCl-treated roots (homogenates)	2.96	3.75	5.20

[a] For details of experimental procedures, see footnotes to Table 1.

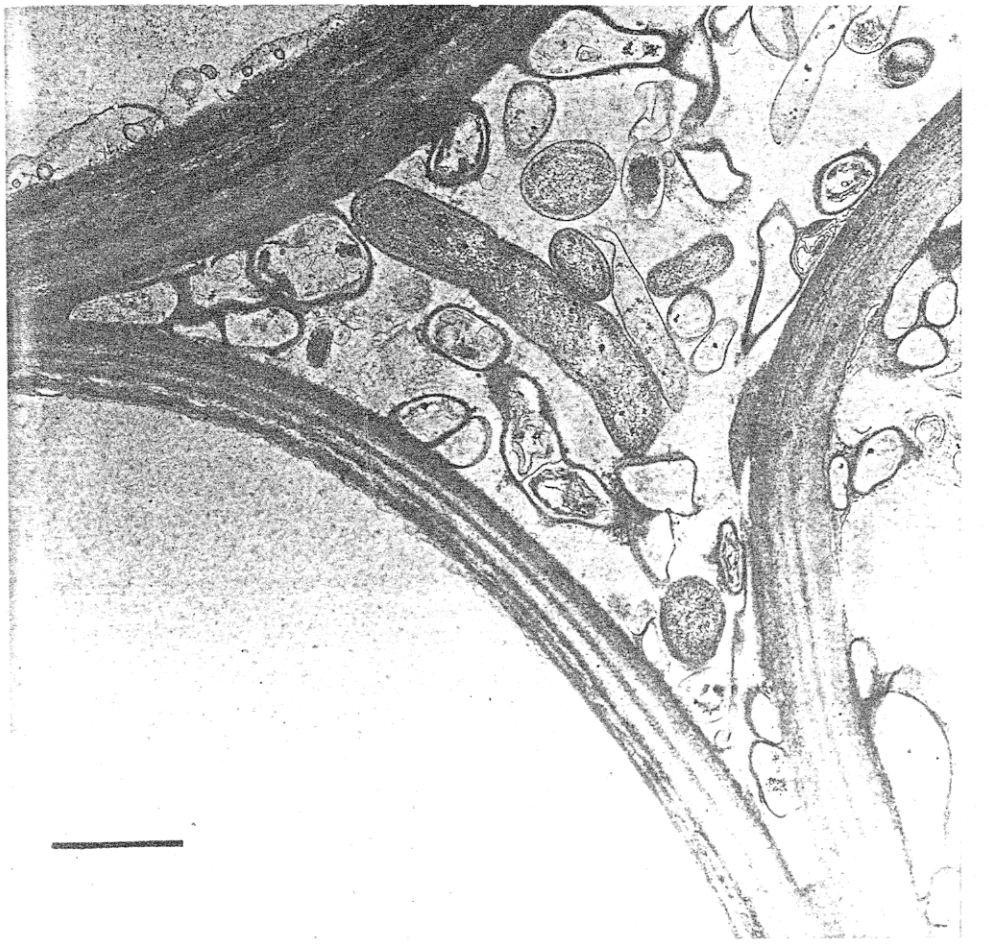

FIG. 1. Bacteria in intercellular space in outer cortex of roots of *S. alterniflora*. Bar, 1 μm.

populations, including N_2 fixers, were released from rhizoplanes when roots were shaken with glass beads after they had been treated with 1% chloramine-T for 1 h (Table 2). Treatment of the roots of these grasses with 5% NaOCl for 1 h reduced the recovery of the rhizoplane N_2-fixing bacteria culturable in GMS medium to less than five per g (fresh weight). The recoveries of the rhizoplane N_2-fixing population culturable in MS medium after treatment with 5% NaOCl for 1 h were reduced to 4.5, 9.2×10^3, and 5.4×10^3 bacteria per g (fresh weight) of roots of *S. alterniflora*, *Z. mays*, and *S. bicolor*, respectively. When expressed as a proportion of the total population of N_2-fixing bacteria as estimated in MS medium, this represents only 0.2% in the case of *S. alterniflora* but 13.5 and 65.8% in the cases of *Z. mays* and *S. bicolor*, respectively. In this study, roots of *S. alterniflora* supported appreciable rates of nitrogenase activity similar to those reported previously (22), but rates of acetylene reduction by roots of *Z. mays* and *S. bicolor* were negligible.

The MPN of N_2-fixing bacteria recoverable from salt-marsh sediment, rhizosphere sediment, rhizoplane, and root homogenates of *S. alterniflora* before and after treatment with 5% NaOCl for 1 h are presented in Table 3. The concentrations of bacteria in the rhizosphere were higher than in salt-marsh sediment by factors of 3.4, 2.2, and 3.2 as estimated with RN, GMS, and MS media, respectively. These differences, although perhaps indicating a slight rhizosphere effect, are not statistically significant and are much less pronounced than those observed in a Nova Scotian *S. alterniflora* stand

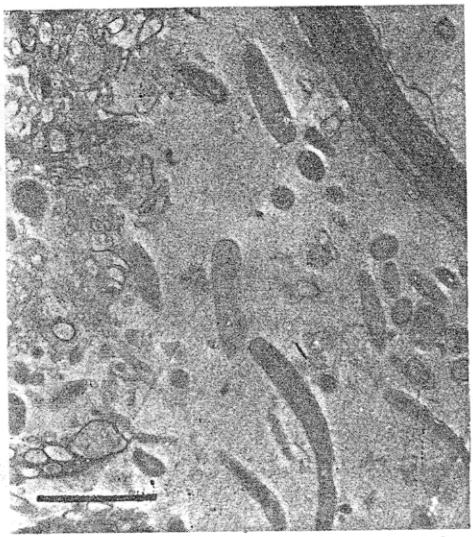

FIG. 2. Bacteria in cortical cells of *S. alterniflora* roots. Note vesiculate cytoplasm and lack of recognizable organelles. Bar, 2 μm.

(17). Exposure of roots of *S. alterniflora* to 5% NaOCl for 1 h decreased the number of bacteria subsequently recovered from root homogenates by factors of 440, 260, and 100 in HN, GMS, and MS media, respectively. N_2 fixers formed a greater proportion of the bacteria recoverable from NaOCl-treated roots (0.58 and 3.4% for GMS and MS media, respectively) than of those recoverable from untreated roots (0.34 and 0.76%) or from salt-marsh sediment or rhizosphere sediment (0.22 to 0.34 and 0.11%). These observations suggest that there is a relative enrichment of N_2 fixers, particularly those culturable in MS medium, in and on the roots of *S. alterniflora* and that this enrichment is most pronounced in the root interior.

Thin and ultrathin sections of roots of *S. alterniflora* revealed that the root interior had been colonized by bacteria. Bacteria were observed intercellularly in the outer cortex (Fig. 1) and also within cortical cells which either lacked cytoplasm or else contained numerous vesicles and no recognizable organelles (Fig. 2). Most cortical cells observed contained no bacteria, and many contained cytoplasm and appeared healthy. The majority of bacteria observed in the roots were in the aerenchymas, where they were predominantly associated closely with cell walls (Fig. 3). Areas of root branching appeared to have high incidences of internal bacterial colonization, consistent with observations in several grass species (16, 17).

DISCUSSION

We have enumerated and localized bacterial populations associated with roots of *S. alterniflora*, which support N_2 fixation when undisturbed in their natural habitat (22, 23). Techniques for surface sterilizing roots of *S. alterniflora* were evaluated first because the efficacy of these techniques has not been well documented. Knowledge of the success with which roots were surface sterilized is crucial for distinguishing bacteria cultured from internal or external locations (21). The technique most effective with roots of *S. alterniflora* was used together with bacterial counts and light and electron microscopy to estimate the sizes and determine the possible locations of bacterial populations.

Our results showed that treatment of *S. alterniflora* roots with 5% NaOCl for 1 h nearly eliminated the recovery of N_2-fixing bacteria from the rhizoplane. Because this treatment was less effective in reducing the recovery of N_2-fixing bacteria from the rhizoplanes of *Z. mays* and *S. bicolor*, we emphasize that techniques to surface sterilize roots must be evaluated before they are used with roots of different plants.

Tetrazolium reduction has been used to locate bacteria on and in roots (7, 11, 15–17), and its absence from root surfaces after chloramine-T treatment has been interpreted to indicate that roots had been surface sterilized (1, 11, 17). However, we found that bacteria could still be cultured from the rhizoplane of *S. alterniflora* that exhibited no tetrazolium reduction after treatment with 5% NaOCl for 1 h. Therefore, the use of tetrazolium reduction alone may not be adequate to evaluate the efficacy of techniques for surface sterilizing roots.

Chloramine-T (1%) has been commonly used in attempts to surface sterilize roots of grasses (1, 4, 5, 10, 15, 16). It has been recommended

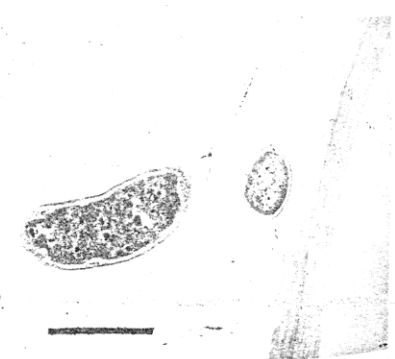

FIG. 3. Bacteria in aerenchyma (air space tissue) of *S. alterniflora* roots. Bar, 1 μm.

that roots of grasses be surface sterilized with 1% chloramine-T for 0.5 to 2.0 min to study endorhizosphere associations (5). Selective infection of roots of grasses by specific *Azospirillum* spp. has been suggested based on results of the surface sterilization of roots with 1% chloramine-T (1, 6). However, in our study, treatment with 1% chloramine-T for 1 h was relatively ineffective in preventing the recovery of N_2-fixing bacteria from the rhizoplanes of *S. alterniflora*, *Z. mays*, and *S. bicolor*. These results could be explained by incomplete or irregular penetration of the rhizoplane zone by chloramine-T. Alternatively, some bacteria at the root surfaces or within root tissues may be protected against the action of chloramine-T. Bacteria recovered in culture from chloramine-T-treated roots might well have originated at the root surface. Therefore, the recovery of bacteria from roots treated with chloramine-T does not necessarily demonstrate that bacteria had infected the roots.

We observed higher concentrations of bacteria in close association with roots of *S. alterniflora* than in the salt-marsh sediment. Also, the relative numbers of N_2-fixing bacteria were greater in and on roots than in sediment. Similar observations have been made with *S. alterniflora* growing in a salt marsh in Nova Scotia (17). In our study, the majority of bacteria recovered from the roots of *S. alterniflora*, including N_2 fixers, appeared to be associated with the rhizoplane. We have adopted an operational definition of rhizoplane bacteria as those released from roots by shaking with glass beads. This treatment may not have released all bacteria present at the root surface. It is also possible that this treatment damaged some cells at the root surface, possibly releasing bacteria from within the tissues as well as from the surface.

High numbers of bacteria, including N_2 fixers, were recovered in culture from homogenates, but not from rhizoplanes, of *S. alterniflora* roots after treatment with 5% NaOCl for 1 h. These data are evidence that N_2-fixing and other bacteria are located inside the roots of *S. alterniflora*. Our results may even underestimate the numbers of bacteria inside the roots because the NaOCl treatment which vastly reduced the rhizoplane population probably killed some bacteria that were actually inside the root. We substantiated the presence of bacteria inside *S. alterniflora* roots by light and electron microscopy, but we were unable to determine whether these were the bacteria that were recovered in culture and were able to fix N_2 in situ. Bacteria inside grass roots have been observed both in the presence and in the absence of extensive decomposition of the root tissue (4, 8, 11, 12, 16). In our study, internally located bacteria were in regions of roots where the outer cortex appeared to be intact and to contain healthy cells as well as in areas where the outer cortex was extensively damaged. Bacteria had colonized the aerenchyma, which may have provided an avenue for longitudinal colonization.

We were unable to surface sterilize the roots of *Z. mays* and *S. bicolor* with techniques described in this study, and consequently we could not deduce whether or not N_2-fixing bacteria had infected the roots of these plants. However, our findings clearly demonstrate that N_2-fixing bacteria are present inside and outside *S. alterniflora* roots which have been reported to support nitrogenase activity.

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