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Herbaspirillum, an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench

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Abstract

Leaves of *Sorghum bicolor* were examined at 5 d and 14 d after inoculation with the N_2-fixing endophytic bacteria *Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans*. Plants inoculated with *H. rubrisubalbicans* expressed symptoms of 'red stripe disease' i.e. red stripes along the secondary veins of the leaf blade close to the inoculation point and spreading up the leaves. Infected leaves showed dense colonization by *H. rubrisubalbicans* in regions showing red stripe symptoms at 5 d after inoculation. The infection was confined within the vascular system, in particular, the protoxylem and associated lacunae, which were often completely filled with bacteria, with some of the latter expressing nitrogenase. The bacteria were recognized using *H. rubrisubalbicans*-specific antibodies and immunogold labelling, which also showed that the antibody reacted with material on the surface of the bacteria, and that this mucus was released into the lumen of the xylem. At 14 d after inoculation, disease symptoms were slightly more severe, with both meta- and protoxylem being even more heavily colonized in parts of the leaf showing red stripes. However, a strong host defence response was also apparent at this stage, with gums lining the walls of the vessels and enclosing the bacteria, although the latter were still actively dividing. At the edges of visible disease symptoms, plant gums filled the xylem; bacteria had formed distinct colonies within these gums, with some of the colonies associated with the xylem walls. Plants inoculated with *H. seropedicae* either did not express the disease or showed very mild symptoms close to the inoculation point. In the latter case, *H. seropedicae* were localized within protoxylem vessels and the metaxylem was partly occluded with plant-derived gums. By contrast with *H. rubrisubalbicans*, *H. seropedicae* was also localized in leaves at 14 d without disease symptoms and did not always appear to elicit a host response, i.e. they colonized the walls of metaxylem, with the xylem vessels themselves remaining unoccluded and largely free of gums. The fine line separating plant pathogens, endophytes and symbioses is discussed in light of these results.

Key words: *Herbaspirillum*, *Sorghum bicolor*, nitrogen fixation, endophyte, xylem.

Introduction

Members of the genus *Herbaspirillum* were described by Baldani *et al.* (1986, 1992) as being curvilinear rod-shaped, gram negative, microaerobic nitrogen-fixing bacteria associated (non-pathogenically) with roots of rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). Recently, based on molecular approaches, it has been proposed to include the misnamed phytopathogen '*Pseudomonas*' *rubrisubalbicans* in the genus *Herbaspirillum* (Gillis *et al.*, 1991; Baldani *et al.*, 1996). Both *H. rubrisubalbicans* and *H. seropedicae* are able to fix nitrogen, as demonstrated by acetylene reduction and $^{15}N_2$ incorporation evaluated in semi-solid JNFb medium without nitrogen (Pimentel *et al.*, 1991; Baldani *et al.*, 1992).

Herbaspirillum rubrisubalbicans (syn. *P. rubrisubalbicans*; Christopher and Edgerton, 1932) was described as a causal agent of 'mottled stripe disease' in sugar cane (*Saccharum officinarum*) and later was found within sorghum in Queensland, causing 'red stripe disease' (Hale and Wilkie, 1972*a*, *b*). However, there are no recent reports of red/mottled stripe disease in Brazilian sorghum

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and sugar cane fields, all agronomically important cultivars being resistant, even after artificial inoculation (Pimentel *et al.*, 1991; Olivares *et al.*, 1997). Nevertheless, field-grown Brazilian sugar cane and sorghum cultivars still contain large numbers of *Herbaspirillum*, and remain symptomless despite the phytopathogenic potential of the bacteria (Pimentel *et al.*, 1991; Olivares *et al.*, 1996). Indeed, studies on the ecology of *Herbaspirillum* have shown that the bacteria can be isolated from the interior of roots, stems and leaves of many symptomless graminaceous plants (Baldani *et al.*, 1992; Döbereiner *et al.*, 1994; Olivares *et al.*, 1996). Moreover, when *Herbaspirilla* are inoculated into native soil, there is a rapid reduction in their numbers, reaching values below the level of detection after only one month (Baldani *et al.*, 1992; Olivares *et al.*, 1996). Taken together, these characteristics suggest an endophytic interaction between *Herbaspirillum* and some Gramineae, including sorghum (Olivares *et al.*, 1996).

Recent reports of significant nitrogen fixation in some sugar cane varieties, as evaluated by N-balance and ^{15}N isotope dilution techniques (Lima *et al.*, 1987; Urquiaga *et al.*, 1992), were hypothesized to be due to colonization of the internal tissue of sugar cane by endophytic N_2-fixing bacteria (Boddey *et al.*, 1991, 1995; Döbereiner *et al.*, 1994; Boddey, 1995). The bacteria considered to be mainly responsible for the N_2 fixation are *Acetobacter diazotrophicus* and *Herbaspirillum* spp. (Döbereiner *et al.*, 1994; Boddey, 1995), all found in large numbers within Brazilian sugar cane cultivars with potential for N_2 fixation (Boddey *et al.*, 1991, 1995; Pimentel *et al.*, 1991; Reis *et al.*, 1994; Olivares *et al.*, 1996). As *Herbaspirillum* is also found in large numbers within other Gramineae, particularly sorghum and rice (Baldani *et al.*, 1986, 1992; Pimentel *et al.*, 1991; Olivares *et al.*, 1996), it has led to suggestions that it may also fix N_2 within other grasses, as well as in sugar cane (Döbereiner *et al.*, 1993, 1994).

Although the specific site(s) of N_2 fixation by *Herbaspirillum* in grasses has yet to be described, Olivares *et al.* (1993, 1977) have recently examined infection of leaves of mottled stripe disease-susceptible and mottled stripe disease-resistant sugar cane cultivars by *Herbaspirillum* spp., and the bacteria were found to colonize substomatal cavities, intercellular spaces in adjacent mesophyll, and particularly the xylem vessels. Intercellular spaces and xylem were also a favoured site of colonization by *Herbaspirillum* spp. in sugar cane roots (Olivares *et al.*, 1995) and *H.seropedicae* has been localized within intercellular cavities in rice seeds and roots (Baldani *et al.*, 1993). Previous attempts at artificial inoculation of sorghum with *Herbaspirillum* have shown that both *Herbaspirillum* species produced visible, but mild, disease symptoms in some cultivars, and that the bacteria could be reisolated from the third to fifth leaves above the inoculated leaf 60 d after inoculation, as well as be reisolated 10 cm below the inoculation point (Pimentel *et al.*, 1991). The latter study suggested that the bacteria were freely translocated within the leaves of compatible hosts like sorghum, sugar cane and rice, probably via the vascular tissue (Pimentel *et al.*, 1991; Baldani *et al.*, 1992; Döbereiner *et al.*, 1993, 1994), and was later supported by provisional structural studies (Olivares *et al.*, 1993, 1995) which showed that *Herbaspirillum* preferentially inhabits the vascular tissues of grasses. In the present study the colonization of vascular tissues in the leaves of a red stripe disease-susceptible cultivar of sorghum by *Herbaspirillum* spp. was examined in more detail, and compared to recent studies of the infection of sugar cane leaves by the same bacteria (Olivares *et al.*, 1993, 1997).

Materials and methods

Organisms, growth conditions, bacterial inoculation and phytopathogenic tests

Herbaspirillum rubrisubalbicans strains M4 (ATCC 19308), M1 (LMG 1278) and IBSBF175, which were originally isolated from sugar cane leaves in the USA and Mauritius, and *H. seropedicae* strains Z67 (ATCC 35892), Z78 (ATCC 35893) and HCC102, which were originally isolated from Brazilian rice roots, sorghum roots and sugar cane stems, respectively, were grown in nutrient broth medium (3 g l^{-1} beef extract, 5 g l^{-1} peptone) plus glycerol (10 g l^{-1}) at 30 °C, at 140 rpm for 24 h. Bacterial cells were harvested by centrifugation at 2000 *g* for 3 min. Inoculum was prepared by resuspending the pellet in a quarter strength solution (salts only) of NFb medium (Tarrand *et al.*, 1978; Pimentel *et al.*, 1991) and adjusted to an optical density of 10^8 cfu ml^{-1}. Sorghum variety BR303 (from EMBRAPA/CNPMS, Brazil) was grown in a greenhouse and inoculated, when 2 weeks old, with 0.5 ml of the inoculum using a hypodermic syringe according to Eira (1972). There were four replicates per treatment and the plants were incubated for 1 d in a dew chamber in order to facilitate colonization of bacteria. Inoculated plants were examined 5 d and 14 d after inoculation for symptoms of red stripe disease. The bacteria were re-isolated 5 d and 14 d after inoculation by excising small pieces of the leaf (0.5 cm^2) close to the inoculation point and transferred into JNFb (Baldani *et al.*, 1992) semi-solid medium without nitrogen. *Herbaspirillum* was recognized by formation of a typical pellicle in the medium, a curvilinear rod-shaped bacterium and typical dark green colonies in JNFb solid medium (Olivares *et al.*, 1996). Control plants were inoculated only with quarter strength (salts only) of NFb medium (Tarrand *et al.*, 1978).

Light and electron microscopy and immunogold labelling

Small pieces (0.1–0.3 mm^2) of sorghum leaves were taken from plants at 5 d and 14 d after inoculation, washed twice in phosphate buffer (50 mol m^{-3}, pH 7.0) and fixed in 5% glutaraldehyde (in 50 mol m^{-3} phosphate buffer, pH 7.0) for 24 h (followed by post-fixation in 1% osmium tetroxide for samples which were not to be used for immunogold labelling). Material was then prepared for light microscopy and transmission electron microscopy (TEM) according to James *et al.* (1994). Briefly, samples for light microscopy and TEM were rinsed in buffer, dehydrated in an ethanol series, infiltrated for 5 d in LR White acrylic resin (London Resin Company, UK)

followed by polymerization at 60 °C for 24 h. Ultrathin sections (70 nm) for TEM were collected on pyroxylin-coated nickel grids, and semi-thin sections (1–2 μm) were collected on gelatin-coated glass slides and either used for IGL or immediately stained with toluidine blue for conventional light microscopy.

Immunogold labelling (IGL) using polyclonal antibodies raised in rabbits against *H. seropedicae* strain Z67 and *H. rubrisubalbicans* strain M4 was used to determine whether the bacteria observed in the sorghum leaves were *Herbaspirillum*. The antibodies were raised by injecting rabbits intraveinally with 1 ml of 10^9 cells ml^{-1} of bacteria mixed 1 : 1 with Freunds adjuvant. The injections (without adjuvant) were repeated three times every 7 d and the first harvest of antisera was collected 25 d after the first inoculation, and henceforth collected at 7–10 d intervals. The antisera were tested using indirect ELISA according to Li and McRae (1992) against a wide range of N_2-fixing bacteria which are normally found in sorghum and sugar cane tissues (Döbereiner *et al.*, 1995), and negligible cross reactions were seen at a dilution 1 : 400 of the original antisera. Immunogold labelling with an antibody to nitrogenase component II raised in rabbits after isolation from *Rhodospirillum rubrum* (a kind gift from Dr PW Ludden, Madison, Wisconsin, USA) was used to determine expression of the nitrogenase enzyme complex by bacteria within the sorghum leaves.

The solutions and procedures used for IGL were as described in James *et al.* (1994). For IGL under the TEM, after incubation in a 1/400 dilution of the primary antibody (or 1/100 with antibodies to nitrogenase components) the sections were incubated in a 1/100 dilution of goat anti-rabbit antibodies conjugated to 15 nm gold particles (Amersham). For IGL under the light microscope the primary antibody dilutions were as for TEM, then the sections were incubated in a 1/100 dilution of goat anti-rabbit antibodies conjugated to 5 nm gold (Amersham) followed by silver enhancement using the IntenSE M kit (Amersham). In each IGL preparation, controls to recognize non-specific binding by the gold conjugated antibody to the sections, were run in parallel. These were (1) 'pre-immune' serum from the same rabbit before inoculation with *Herbaspirillum* spp., was substituted for the anti-*Herbaspirillum* primary antibodies; (2) normal rabbit serum (Sigma) was substituted for the primary antibody; (3) no primary antibody was used, the sections being incubated in blocking buffer alone for this step.

Results

Expression of red stripe disease symptoms and reisolation of bacteria

A small necrotic area was observed around the inoculation point on the leaf blade inoculated with *Herbaspirillum*; on leaves inoculated with *H. rubrisubalbicans* this area was red (Plate 1). Both *Herbaspirillum* species could be reisolated from the leaf tissue close to the inoculation point 5 d and 14 d after inoculation, and be cultured in N-free semi-solid JNFb medium. Symptoms of red stripe disease, however, developed only in the plants inoculated with *H. rubrisubalbicans*; the symptoms being characterized by the localized development of red stripes along the secondary leaf veins close to the inoculation point (Plate 1). These symptoms were similar to those reported by Hale and Wilkie (1972*b*) and Pimentel *et al.* (1991) with sorghum infected by '*Pseudomonas*' *rubrisubalbicans* (now renamed *H. rubrisubalbicans*; Baldani *et al.* 1996). At the time of the second harvest (14 d) the disease had reached its peak, and hence all stages of the disease could be observed (Plate 1) i.e. there was extensive host tissue degradation around and close to the inoculation point, with red stripes spreading along the leaves from the inoculation point. However, as *H. rubrisubalbicans* is only a weak pathogen of sorghum (Hale and Wilkie, 1992*b*; Pimentel *et al.*, 1991), the disease did not kill the infected plants, but shortened the lifetime of heavily diseased leaves by approximately half.

Plants infected with *H. seropedicae* generally did not develop typical symptoms of the disease, although small stripes (< 3 mm in length) were occasionally seen close to the inoculation point (not shown).

Localization of bacteria in plant tissues

Herbaspirillum rubrisubalbicans was easily seen within the leaf tissue from both harvests by sectioning material with visible red stripe disease symptoms (Plate 1). At 5 d, the bacteria could be seen in large numbers within protoxylem vessels and associated lacunae, often apparently completely blocking the vessels (Plates 2A, 3A). These bacteria were confirmed to be *H. rubrisubalbicans* by immunogold labelling with antibodies specific to this bacterium (Plates 2A, B, 3B). Moreover, the bacteria reacted positively with an antibody to nitrogenase component II in the 5 d harvest (Plate 2C), but not in the 14 d harvest (not shown). In contrast, *H. seropedicae* were observed in much smaller numbers than *H. rubrisubalbicans* at 5 d and were only visible in the few isolated areas of infected leaves that had visible disease symptoms (Plate 2D). As with *H. rubrisubalbicans* (Plates 2A, 3A), *H. seropedicae* were always seen in the xylem and never in the surrounding tissue (Plates 2D, 3C), and were confirmed to be *H. seropedicae* by immunogold labelling (Plates 2D, 3D).

Under the TEM, the close-packing of *H. rubrisubalbicans* in protoxylem/lacunae at 5 d was confirmed (Plate 3A). The bacteria were healthy in appearance, as were adjacent plant cells (Plate 3A). The surface of the bacteria reacted strongly to the *H. rubrisubalbicans*-specific antibody (Plate 3A, B) and the bacteria were surrounded by a mucus, presumably extracellular polysaccharide/lipopolysaccharide (EPS/LPS), which also reacted strongly with the antibody (Plate 3B). Gold labelled material (presumably EPS) was also present in the lumens of the vessels containing the bacteria (Plate 3B).

By contrast to *H. rubrisubalbicans* (Plates 2A, 3A), *H. seropedicae* colonies were not very dense, and did not completely occlude any of the vessels (Plates 2D, 3C, D), although a small amount of extracellular mucus was

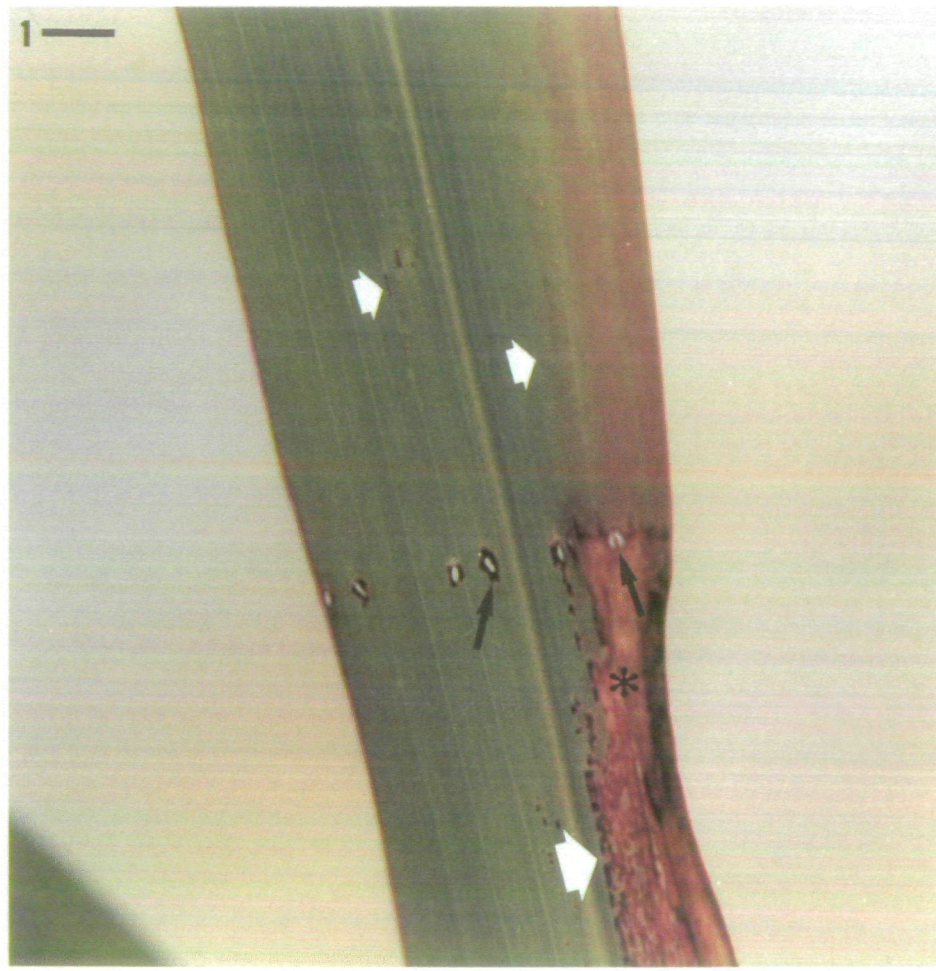


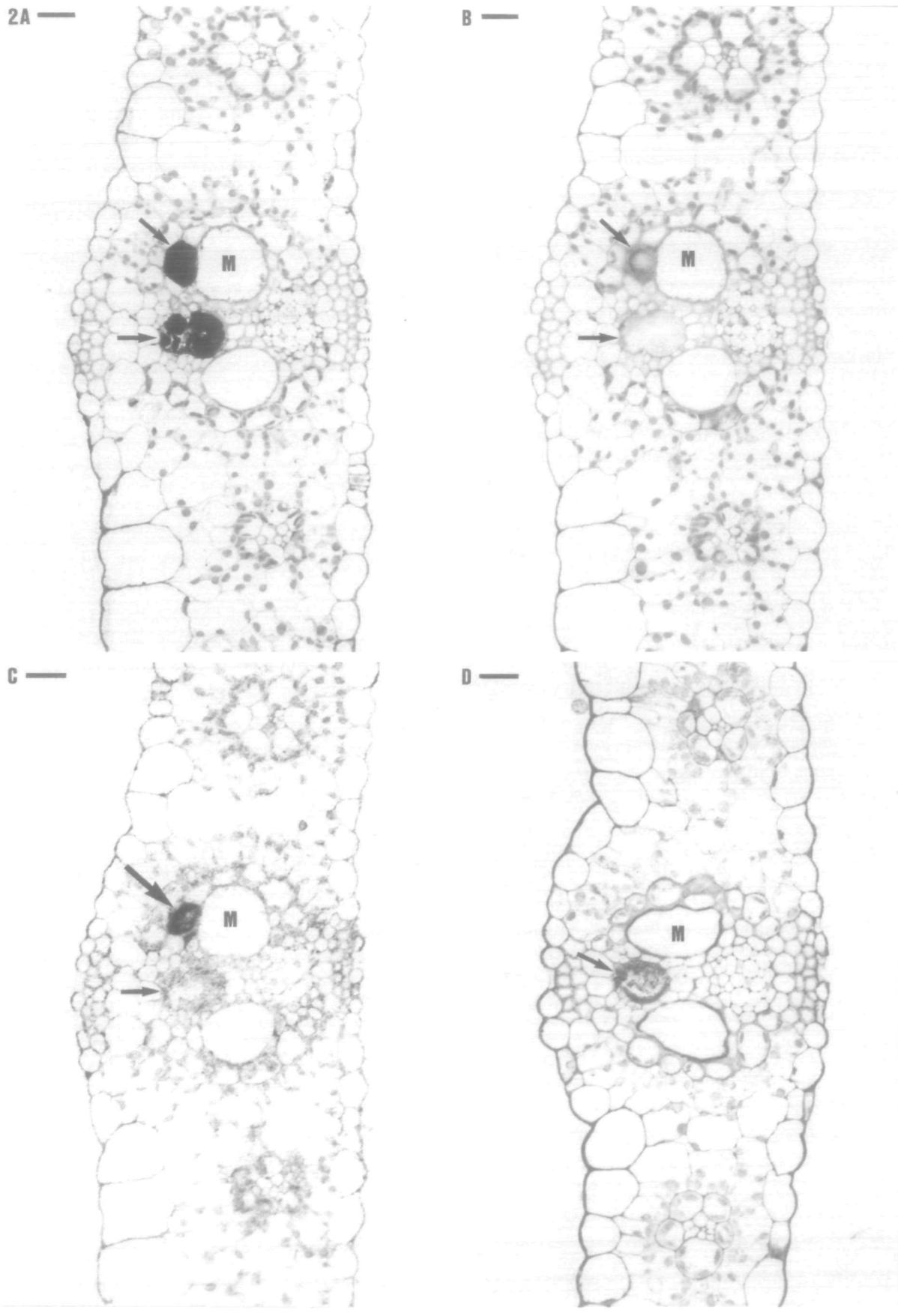
Plate 1. Sorghum (*Sorghum bicolor*) leaf showing red stripe disease symptoms at 14 d after inoculation by *Herbaspirillum rubrisubalbicans*. The points of inoculation are surrounded with red necrotic tissue (arrows), and red stripe symptoms can be seen above (small white arrows) and below them (large white arrow). The red stripes coincide with infection of the vascular traces. There is an extensive area of necrosis (*) immediately below some of the points of inoculation. Bar = 5 mm.

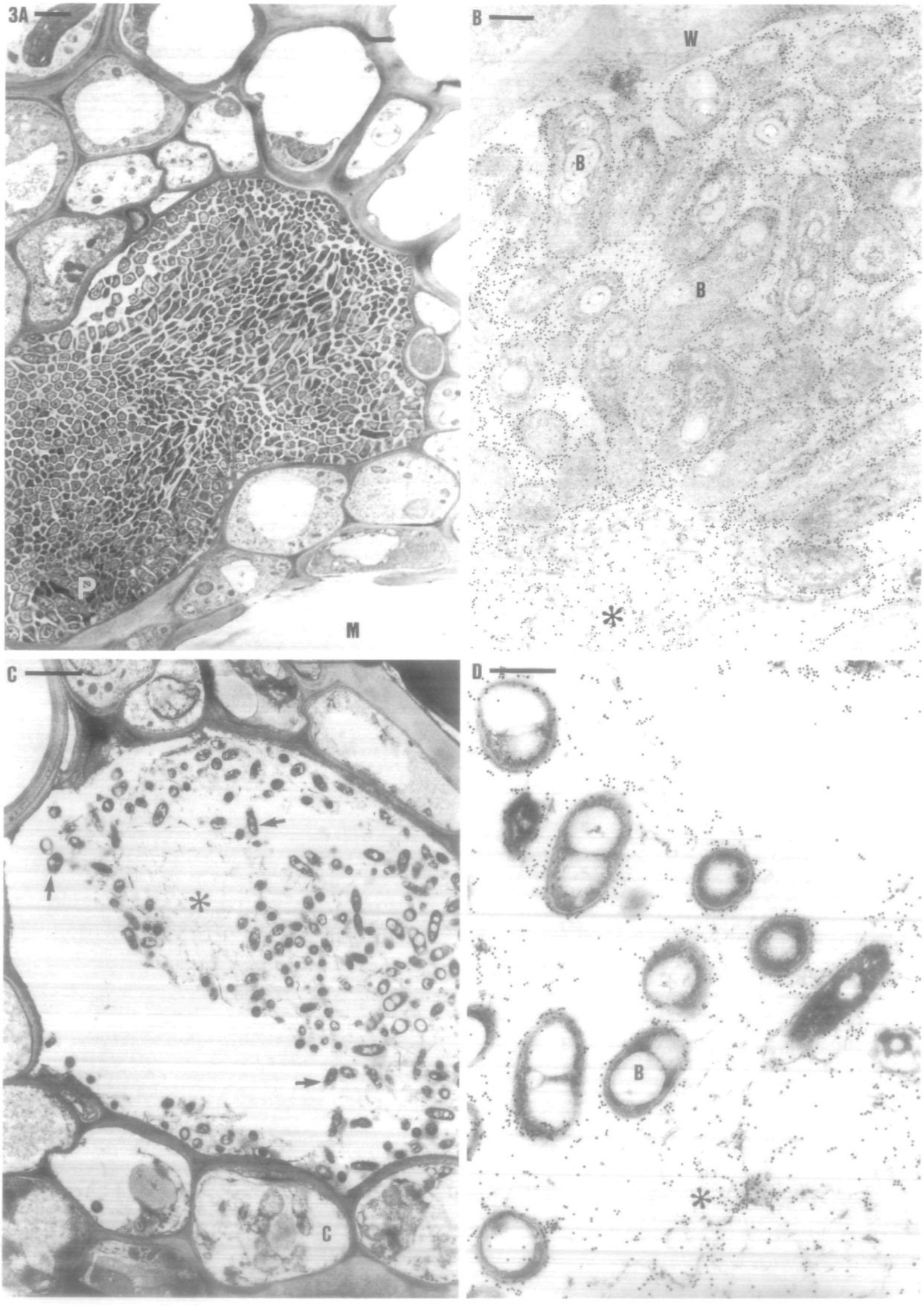
released by the bacteria into the lumens of the vessels (Plate 3C, D). Moreover, *H. seropedicae* did not react with the antibody to nitrogenase component II (not shown).

The metaxylem was left relatively uncolonized by both species of *Herbaspirillum* at the 5 d harvest (Plate 2), although the metaxylem adjacent to protoxylem colonized by *H. seropedicae* did sometimes contain gums of plant origin that stained green with toluidine blue, suggesting that they were phenolic compounds (not shown). These phenolic-containing gums were not apparent in vessels colonized by *H. rubrisubalbicans* until the 14 d harvest (Plates 4A, 5B).

At 14 d, the large-scale production of gums by the host at the edges of visible red stripe disease symptoms (Plate 1) appeared to confine *H. rubrisubalbicans* to large colonies within the vessels (Plate 4A, B), and presumably to slow down further spread of the disease in the xylem. Interestingly, the gum not only confined colonies to regions within the xylem, but also appeared to surround and define microcolonies of the bacteria (Plate 4A, B, C). Some of these colonies almost had the appearance of

Plate 2. (A) Section through a sorghum leaf 5 d after inoculation by *Herbaspirillum rubrisubalbicans*. This section was incubated in an antibody raised against *H. rubrisubalbicans*, followed by goat anti-rabbit gold plus silver enhancement, and shows that the bacteria (arrows) occupy the protoxylem and associated lacunae of the leaf, but have not infected any tissues other than the vascular system. Note that the metaxylem (M) is relatively free of bacteria. Bar = 20 μm. (B) Parallel section to A incubated in normal rabbit serum instead of anti-*H. rubrisubalbicans*. There is no gold labelling of the bacteria (arrows). M = metaxylem. Bar = 20 μm. (C) Parallel section to A incubated in an antibody raised against nitrogenase component II. The bacteria in the lacunae associated with the xylem are labelled (arrow) but those in the protoxylem are not (small arrow). M = metaxylem. Bar = 20 μm. (D) Section through a sorghum leaf 5 d after inoculation by *Herbaspirillum seropedicae*. This section was incubated in an antibody raised against *H.seropedicae*, followed by goat anti-rabbit gold plus silver enhancement. The bacteria (arrow) occupy the protoxylem, and the metaxylem (M) is apparently unoccupied. Bar = 20 μm.





being surrounded by a membrane, and within the colonies no host-derived gums were visible, and bacteria were obviously healthy (Plate 4B, C). The bacteria were also closely associated with the walls of the xylem, the microcolonies often being close to pits (Plate 4C), and they were also seen within a fibrillar matrix at junctions of meta- and protoxylem (Plate 4D).

Within heavily infected regions of leaves, particularly the areas close to the points of inoculation of *H. rubrisubalbicans*, the bacteria were allowed to grow apparently unchecked and thereby completely fill the vessels (Plate 5A, B). The bacteria often appeared to have a denser population within the protoxylem vessels compared to the metaxylem, which was slightly more diffuse and showed less intense staining (Plate 5A). However, as in the vessels at the edge of the symptoms, a host response could be seen, whereby the bacteria in the xylem were surrounded by phenolic-containing gums (Plate 5A, B). Nevertheless, despite this host defence response, the *H. rubrisubalbicans* still appeared healthy (Plate 5B), with some actively dividing (Plate 4C), and there were no obvious signs of attack by the plant on the bacteria. Considering that leaf necrosis was widespread (Plate 1) and that there were large numbers of bacteria in the vessels at this stage of the disease, it was interesting that the tissue adjacent to the infected xylem was still uninfected by either bacterium (Plates 4A, 5A, B, D).

Herbaspirillum seropedicae in 14 d plants were seen confined only to the walls of xylem vessels, leaving the lumen unoccluded (Plate 5D). Interestingly, in contrast to 5 d plants, host defence gums were not seen in vessels associated with or containing *H. seropedicae* at this harvest (Plate 5D).

Herbaspirillum spp. were not seen in, or on, any of the uninoculated control specimens from either cultivar, at either harvest (not shown).

Discussion

Red stripe disease

The results of Pimentel *et al.* (1991) were confirmed, i.e. that both *Herbaspirillum* spp. can cause symptoms of red stripe disease when inoculated artificially into sorghum leaves, with *H. rubrisubalbicans* being the more virulent bacterium. Olivares *et al.* (1997) have shown similar results with sugar cane, i.e. the formation of mottled stripe disease after inoculation of the leaves by *H. rubrisubalbicans*. However, there are important differences between the two grass species in their response to *Herbaspirillum* infection.

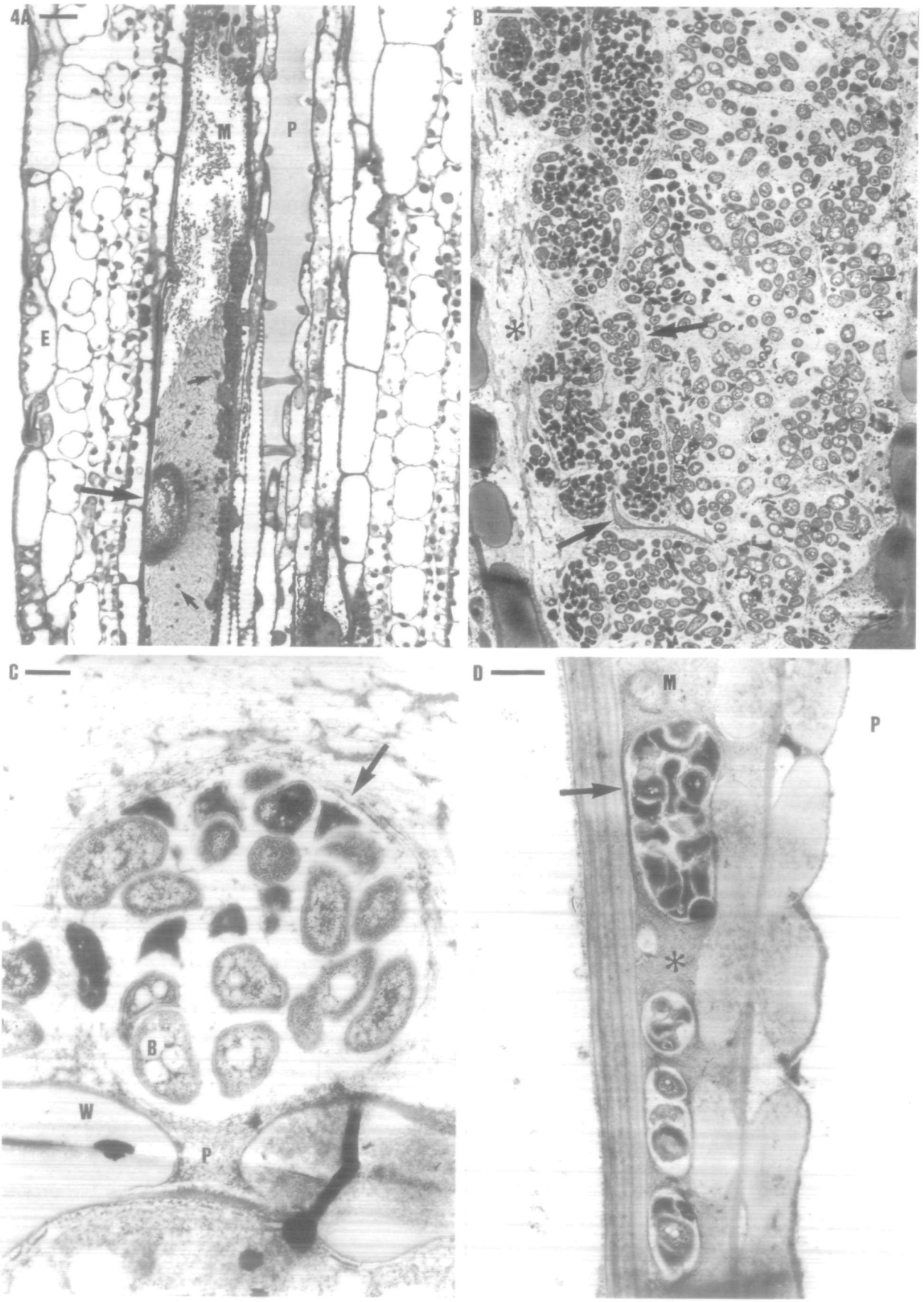
Firstly, *H. seropedicae* is capable of entering the leaves and causing mild (although infrequent) disease symptoms in sorghum but not sugar cane. Pimentel *et al.* (1991) previously reported that no cultivar of sugar cane expressed disease symptoms after inoculation of the leaves by *H. seropedicae*, and this has been recently confirmed by Olivares *et al.* (1997) who reported a hypersensitive response (HR) by sugar cane leaves to artificial inoculation of the bacterium, i.e. rapid (<48 h) host cell death around the point of inoculation (Sequeira *et al.*, 1977; McKhann and Hirsch, 1994). Indeed, Olivares *et al.* (1996) have recently found that, in the field, *H. seropedicae* only colonizes the stems and roots of sugar cane, but has not been isolated from the leaves.

The second difference between sorghum and sugar cane in their response to leaf infection by *Herbaspirillum* spp. is that neither species has been observed outside the vascular tissue of sorghum (this study; Olivares *et al.*, 1993), whereas in sugar cane leaves *H. rubrisubalbicans* escapes from the xylem to colonize the adjacent mesophyll (Olivares *et al.*, 1993, 1997).

The leaf stripes characteristic of the disease in sorghum (Hale and Wilkie, 1972*b*) probably correspond to colonization of the leaf vascular system by the bacteria; further leaf necrosis in the later stages of the disease (Plate 1) are probably due to the tissues adjacent to the blocked vessels being deprived of nutrients (Gross and Cody, 1985; Leigh and Coplin, 1992) rather than to direct pathogenic effects of the bacteria, as the latter were never observed outside xylem vessels. This contrasts with 'mottled stripe' disease in sugar cane leaves which have been inoculated with *H. rubrisubalbicans*. In sugar cane, the bacteria escaped from the xylem vessels to colonize the adjacent mesophyll and substomatal cavities, and actively attacked the host cells, causing the 'mottled' symptoms (Olivares *et al.*, 1993, 1997).

The colonization by *H. rubrisubalbicans* of xylem in sorghum leaves was similar to that reported in mottled stripe disease-susceptible sugar cane (cv. B-4362) leaves (Olivares *et al.*, 1993, 1997). In both sorghum and sugar cane cv. B-4362, tightly-aggregated *Herbaspirillum* cells filled the lumen of the protoxylem and associated lacunae

Plate 3. (A) Transmission electron micrograph (TEM) showing *Herbaspirillum rubrisubalbicans* packed into the protoxylem (P) and lacunae (L) of a sorghum leaf at 5 d after inoculation. The bacteria and host plant cells appear healthy and no bacteria have escaped from the vessels to colonize the adjacent tissue. M = metaxylem. Bar = 2 μm. (B) TEM of *H. rubrisubalbicans* in protoxylem of a sorghum leaf at 5 d after inoculation. The bacteria (B) are immunogold labelled with an antibody raised against *H. rubrisubalbicans*. Note that the uninfected lumen of the vessel is also filled with gold-labelled material (*). W = cell wall. Bar = 500 nm. (C) TEM of *H. seropedicae* in the protoxylem of a sorghum leaf at 5 d after inoculation. The bacteria (arrows) are less concentrated than *H. rubrisubalbicans* at 5 d (A). The bacteria appear to have released mucus into the vessel (*), and this reacted with an antibody specific to *H. seropedicae* (D). Note that the host cells (C) surrounding the protoxylem appear less healthy than those surrounding the protoxylem infected by *H. rubrisubalbicans* (A). Bar = 500 nm.



in the early stages of the disease, whereas the metaxylem remained largely uninfected. In later harvests, *Herbaspirillum* was observed to block much of the protoxylem completely, and the metaxylem was also extensively (though less densely) colonized. At the edges of the red/mottled stripe disease symptoms in sorghum and sugar cane cv. B-4362 the *Herbaspirillum* did not fill the vessels, but were embedded in the host-derived gums, within smaller microcolonies, often associated with the walls of the vessels, and apparently surrounded by a membrane. Similar *Herbaspirillum* microcolonies were also observed in the xylem of the very localized diseased parts of leaves of a disease-resistant sugar cane cultivar (SP 70–1143). However, unlike sorghum (this study) and sugar cane cv. B-4362 (Olivares *et al.*, 1993, 1997), in sugar cane cv. SP 70–1143 *H. rubrisubalbicans* were never observed to fill the vessels, but were always confined to microcolonies attached to the vessel walls, with the lumen of the vessel being filled with host-derived gums (Olivares *et al.*, 1993, 1997). Indeed, the limited disease symptoms exhibited by sugar cane cv. SP 70–1143 after infection by *H. rubrisubalbicans* (Olivares *et al.*, 1997) were very similar to those occasionally observed with sorghum infected with *H.seropedicae* (this study).

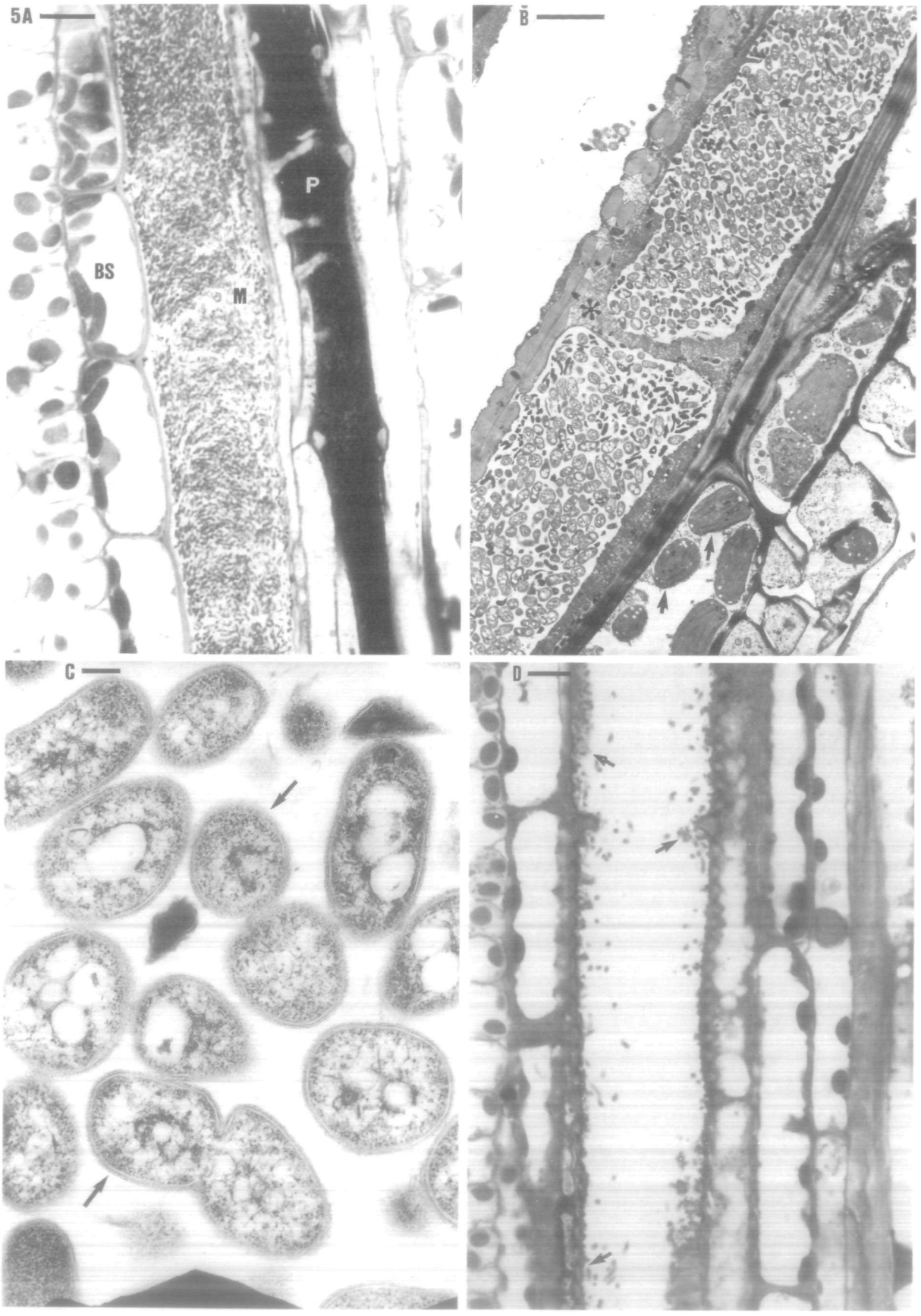
Bacterial microcolonies have also been observed in 'compatible' sugar cane infected by ratoon stunting disease (RSD), in which sugar cane xylem vessels infected by *Clavibacter xyli* subsp *xyli* suffered no visible damage to the walls of the vessels (Kao and Damann, 1980) and therefore, as was also shown with *Herbaspirillum*, successful colonization of the xylem of grasses can be achieved in a compatible plant-bacterial interaction. Indeed, xylem has already been suggested as a good location for N_2 fixation by endophytic diazotrophs (James *et al.*, 1994; Sprent and James, 1995), as evidenced by their regular discovery there (Patriquin and Döbereiner, 1978; Olivares *et al.*, 1993, 1995; James *et al.*, 1994; Hurek *et al.*, 1994; Hartmann *et al.*, 1995), and by the possibility that xylem vessels may be a suitable location for exchange of metabolites between host and endophyte, and may also allow for rapid intraxylem spread via the transpiration stream (James *et al.*, 1994; Sprent and James, 1995).

In common with sugar cane infected with *H. rubrisubalbicans* (Olivares *et al.*, 1993, 1997), bacteria within sorghum xylem vessels induced the production of plant-derived, phenolic-containing gums in the vessel lumens, presumably as part of the plant defence response to combat further bacterial colonization (Wallis, 1977; Djordjevic *et al.*, 1987; Bretschneider *et al.*, 1989). VanderMolen *et al.* (1977) proposed that the 'gels' and 'gums' produced as a host defence response originate from the expansion of primary walls and middle lamella constituents of the xylem vessel and are produced as a general response following infection in order to localize the pathogen in the vascular tissue. This mechanism for restricting further bacterial colonization of the xylem and surrounding mesophyll was reasonably effective in sorghum and also in the mottled stripe disease-resistant sugar cane cv. SP 70–1143 (Olivares *et al.*, 1997). However, host gums appeared not to be a particularly effective mechanism to defend against further invasion by *H. rubrisubalbicans* in the sugar cane cultivar B-4362, which is susceptible to mottled stripe disease (Olivares *et al.*, 1997). In the latter case, bacteria not only completely filled the xylem vessels, presumably severely impeding flow of fluids through them (Ouchi, 1983; Harrison and Davis, 1988), but also escaped to infect the adjacent mesophyll (Olivares *et al.*, 1997).

H. rubrisubalbicans in sorghum xylem vessels reacted strongly with an antibody raised against the bacteria and there was release of a considerable quantity of gold-labelled material into the lumen of the *H. rubrisubalbicans*-infected xylem vessels. The release of material containing bacterial antigens has also been reported in sugar cane xylem vessels and intercellular spaces infected with the same bacterium (Olivares *et al.*, 1993, 1997), in kallar grass infected with *Azoarcus* (Hurek *et al.*, 1994), and in plants infected with pathogenic *Pseudomonas* (Gross and Cody, 1985; Leigh and Coplin, 1992) and *Xanthomonas* (Boher *et al.*, 1995).

Exopolysaccharide (EPS) and lipopolysaccharide (LPS) have been determined as the main antigenic components of the purified sera used in the present study (Olivares, Baldani and Schloter, unpublished data), and are the likely location of an antibody-antigen reaction in most cases where antibodies have been raised against bacteria (Brewin *et al.*, 1986; Hurek *et al.*, 1994; James *et al.*, 1994). In the present study of sorghum, the *H. rubrisubalbicans* were possibly stimulated to produce EPS/LPS in order to avoid plant recognition and defence mechanisms against pathogenic bacteria (Sequeira *et al.*, 1977; Djordjevic *et al.*, 1987; Harrison and Davis, 1988;

Plate 4. These sections were taken from the edge of red stripe disease symptoms on sorghum leaves at 14 d after inoculation with *Herbaspirillum rubrisubalbicans*, i.e. coinciding with the areas marked with small white arrows on Plate 1. (A) Longitudinal section showing a metaxylem vessel (M) filled with host-derived gums which enclose large (arrow) and small (small arrows) colonies of the bacteria. The protoxylem vessel (P) in this section appears to be uninfected but is filled with host-derived gums. As at 5 d, the bacteria have not escaped from the vascular tissue. E = epidermis. Bar = 20 μm. (B) TEM showing *H. rubrisubalbicans* within metaxylem. The vessel appears to be filled with host-derived gums (*) which surround individual bacteria as well as discrete microcolonies of the bacteria. The latter appear to be surrounded by membranes (arrows). Bar = 2 μm. (C) Detail of an *H. rubrisubalbicans* microcolony associated with a pit (P) in the metaxylem wall (W). Note that the bacteria (B) appear healthy and have a 'membrane' surrounding them (arrow). Bar = 500 nm. (D) *H. rubrisubalbicans* within plant-derived gums (*) between the cell walls of protoxylem (P) and metaxylem (M) vessels. Bar = 1 μm.



Leigh and Coplin, 1992; Perotto *et al.*, 1994; McKhann and Hirsch, 1994). By comparison with *H. rubrisubalbicans*, *H. seropedicae* did not appear to produce much mucus (probably EPS) within sorghum xylem vessels, even when it caused slight disease symptoms on the leaves. This supports the view that *H. seropedicae* is not a virulent bacterium (Pimentel *et al.*, 1991), as high EPS production has been linked with virulence (Leigh and Coplin, 1992). Indeed, highly virulent strains of *Pseudomonas* are known to produce copious quantities of EPS, often sufficient to completely block xylem vessels (Gross and Cody, 1985; Leigh and Coplin, 1992).

Is Herbaspirillum *an endophyte?*

Herbaspirillum spp. have recently been confirmed by Olivares *et al.* (1996) to be endophytic, i.e. they do not survive in the soil (Baldani *et al.*, 1992; Olivares *et al.*, 1996), and are found only within tropical grasses that, in most cases, exhibit no symptoms of disease (Baldani *et al.*, 1992, 1993, 1996; Pimentel *et al.*, 1991; Olivares *et al.*, 1996). However, the fact that *Herbaspirillum* spp. will cause mild disease symptoms in some cultivars of sorghum and sugar cane (Pimentel *et al.*, 1991; Baldani *et al.*, 1996; Olivares *et al.*, 1996; this study) suggests that there is a fine line between endophytes and symbionts on the one hand and pathogens on the other. This is particularly well illustrated by examples from legume–*Rhizobium* interactions (Djordjevic *et al.*, 1987; Perotto *et al.*, 1994; McKhann and Hirsch, 1994).

Endophytic fungi and bacteria have been widely reported in grasses (Fisher *et al.*, 1992; McInroy and Kloepper, 1995). These bacteria appear to range from neutral symbionts through non-pathogenic saprobes to latent pathogens. However, obvious roles for most of these have yet to be found (Fisher *et al.*, 1992), and nitrogen fixation by bacteria within plants (e.g. legume–*Rhizobium* symbioses) remains as one of the few known mutualistic–symbiotic associations of bacterial endophytes. Nevertheless, although true symbioses between endophytic diazotrophic bacteria and grasses have yet to be properly demonstrated, it is evident that some of those associations involving grasses and *Herbaspirillum* spp./strains (Pimentel *et al.*, 1991; Olivares *et al.*, 1996; this study) are no longer pathogenic, but may be approaching full 'compatibility' with their hosts, i.e. symbiotic or mutualistic relationships may not have yet fully evolved but the partners may be evolving in that direction.

For example, the present study showed that *H. seropedicae* within leaves of sorghum rarely elicited a host response to their presence, i.e. formation of disease symptoms and hence can at least be termed endophytic, if not yet 'symbiotic'. This is also supported by work with *H. seropedicae* and sugar cane where Olivares *et al.* (1997) have shown that, although sugar cane leaves will have an HR if inoculated with the bacterium, *H. seropedicae* colonizes sugar cane stems and roots without any HR or disease formation (Olivares *et al.*, 1995, 1996, 1997), and possibly with some benefit to the host. Similar conclusions were made after work on sugar cane roots and stems infected by *A. diazotrophicus* (James *et al.*, 1994; Dong *et al.*, 1994) and on kallar grass and rice roots infected by *Azoarcus* (Hurek *et al.*, 1994), i.e. that a bacterium that has evolved to live within a particular location within a particular plant will not elicit a host defence response and hence can be termed an endophyte.

Potential for an N_2-fixing symbiosis

Along with other tropical grasses, such as sugar cane and rice (Eskew *et al.*, 1981; Boddey and Victoria, 1986; Lima *et al.*, 1987; Miranda *et al.*, 1990; Urquiaga *et al.*, 1992; Boddey *et al.*, 1995), sorghum has been shown to fix N_2 (Wani *et al.*, 1983; Giller *et al.*, 1984). In sugar cane, N_2 fixation has been attributed to the endophytes *A. diazotrophicus* and *Herbaspirillum* spp., which are the diazotrophic bacteria found in sufficient numbers to account for the observed fixation rates (Urquiaga *et al.*, 1992; Döbereiner *et al.*, 1993, 1994; James *et al.*, 1994; Dong *et al.*, 1994; Boddey, 1995). However, *A. diazotrophicus* has never been isolated from sorghum or rice (Reis *et al.*, 1994). Hence *Herbaspirillum* is a strong potential candidate for being responsible for at least some of the N_2 fixation in these grasses (Döbereiner *et al.*, 1995; Boddey *et al.*, 1995; Olivares *et al.*, 1996).

Although nitrogenase activity was not tested for in the present study, the expression of nitrogenase component II by *H. rubrisubalbicans* in sorghum leaves 5 d after inoculation means that it is reasonable to assume that conditions within the intraxylar colonies are, at least for a limited period, suitable for nitrogenase expression, and possibly function. This may be due to a localized reduction in pO_2 in the centre of large colonies of rapidly-

Plate 5. (A–C) These sections were taken from areas of sorghum leaves showing pronounced red stripe disease symptoms at 14 d after inoculation with *Herbaspirillum rubrisubalbicans*, i.e. coinciding with the area marked with a large white arrow on Plate 1. (A) Longitudinal section showing that xylem, particularly protoxylem (P), is very heavily colonized by bacteria at this stage of the disease. M = metaxylem. BS = bundle sheath cells. Bar = 10 μm. (B) TEM showing that the bacteria in the metaxylem are no longer confined to microcolonies as they were at the edge of disease symptoms (Plate 4A–C) but completely fill the vessels, with only a thin sheath of host-derived gums surrounding them (*). Note that the bacteria have still not colonized any of the tissue adjacent to the vessels. Moreover, the bundle sheath cells appear relatively healthy, with intact chloroplasts (arrows). Bar = 5 μm. (C) High magnification of healthy *H. rubrisubalbicans* within the metaxylem. Note that some are dividing (arrows). Bar = 200 nm. (D) Longitudinal section of protoxylem in a sorghum leaf at 14 d after inoculation with *Herbaspirillum seropedicae*. The bacteria are confined to the walls of the vessel (arrows) and the lumen of the vessel is unoccluded with either bacteria or host-derived gums. Bar = 10 μm.

respiring bacteria (Gallon, 1992) and by the fact that xylem vessels, by being surrounded by dense plant tissue, may also have an inherently low pO_2, especially when partly-filled with plant-derived gums (James *et al.*, 1994; Sprent and James, 1995). Moreover, free-living *Herbaspirillum* will express optimum nitrogenase activity under a higher pO_2 (3%) when grown in culture than related, root-associated diazotrophs such as *Azospirillum* (2%) (Baldani *et al.*, 1986; Döbereiner, 1992). Hence, inhibition of nitrogenase expression and function by oxygen (Gallon, 1992) may not be such a problem for the xylem-dwelling *Herbaspirillum* observed here and in sugar cane (Olivares *et al.*, 1993, 1997).

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References

Baldani JI, Baldani VLD, Seldin L, Döbereiner J. 1986. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *International Journal of Systematic Bacteriology* **36,** 86–93.

Baldani VLD, Baldani JI, Olivares FL, Döbereiner J. 1992. Identification and ecology of *Herbaspirillum seropedicae* and closely related *Pseudomonas rubrisubalbicans*. *Symbiosis* **13,** 65–73.

Baldani VLD, James EK, Baldani VLD, Döbereiner J. 1993. Colonization of rice by the nitrogen-fixing bacteria *Herbaspirillum* spp. and *Azospirillum brasilense*. In: Palacios R, Mora J, Newton WE, eds. *New horizons in nitrogen fixation*. Dordrecht: Kluwer Academic, 705.

Baldani JI, Pot B, Kirchhof G, Falsen E, Baldani VLD, Olivares FL, Hoste B, Kersters K, Hartmann A, Gillis M, Döbereiner J. 1996. Emended description of *Herbaspirillum*; inclusion of (*Pseudomonas*) *rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *International Journal of Systematic Bacteriology* **46,** 802–10.

Boddey RM. 1995. Biological nitrogen fixation in sugar cane: a key to energetically viable biofuel production. *Critical Reviews in Plant Science* **14,** 263–79.

Boddey RM, de Oliveira OC, Urquiaga S, Reis VM, Olivares FL, Baldani VLD, Döbereiner J. 1995. Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. *Plant and Soil* **174,** 195–209.

Boddey RM, Urquiaga S, Reis VM, Döbereiner J. 1991. Biological nitrogen fixation associated with sugar cane. *Plant and Soil* **137,** 111–17.

Boddey RM, Victoria RL. 1986. Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using ^{15}N labelled organic matter and fertilizer. *Plant and Soil* **90,** 265–92.

Boher B, Kpemoua K, Nicole M, Luisetti J, Geiger JP. 1995. Ultrastructure of interactions between cassava and *Xanthomonas campestris* pv. *manihotis*: cytochemistry of cellulose and pectin degradation in a susceptible cultivar. *Phytopathology* **85,** 777–88.

Bretschneider KE, Gonella MP, Robeson DJ. 1989. A comparative light and electron microscopical study of compatible and incompatible interactions between *Xanthomonas campestris* pv. *campestris* and cabbage (*Brassica oleracea*). *Physiological and Molecular Plant Pathology* **34,** 285–97.

Brewin NJ, Wood EA, Larkins AP, Galfre G, Butcher GW. 1986. Analysis of lipopolysaccharide from root nodule bacteroids of *Rhizobium leguminosarum* using monoclonal antibodies. *Journal of General Microbiology* **132,** 1959–68.

Christopher WN, Edgerton CW. 1932. Bacterial stripe diseases of sugar cane in Louisiana. *Journal of Agricultural Research* **41,** 259.

Djordjevic MA, Gabriel DW, Rolfe BG. 1987. *Rhizobium*-the refined parasite of legumes. *Annual Reviews of Phytopathology* **25,** 145–68.

Döbereiner J, Baldani VLD, Olivares FL, Reis VM. 1994. Endophytic diazotrophs: the key to BNF in gramineous plants. In: Hegazani NA, Fayez M, Monib M, eds. *Nitrogen fixation with non-legumes*. Cairo: American University in Cairo Press, 395–408.

Döbereiner J. 1992. History and new perspectives of diazotrophs in association with non-leguminous plants. *Symbiosis* **13,** 1–13.

Döbereiner J, Baldani VLD, Reis VM. 1995. Endophytic occurrence of diazotrophic bacteria in non-leguminous crops. In: Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M, eds. *Azospirillum VI and related microorganisms*. Berlin, Heidelberg: Springer-Verlag, 15–30.

Döbereiner J, Reis VM, Paula MA, Olivares FL. 1993. Endophytic diazotrophs in sugar cane, tuber plants and cereals. In: Palacios R, Mora J, Newton WE, eds. *New horizons in nitrogen fixation*. Dordrecht: Kluwer Academic, 671–6.

Dong Z, Canny MJ, McCully ME, Roboredo MR, Cabadilla CF, Ortega E, Rodes R. 1994. A nitrogen-fixing endophyte of sugarcane stems. *Plant Physiology* **105,** 1139–47.

Eira AP. 1972. Fatores que influem na triagem das variadades de cana-de-açúcar (*Saccharum* spp.) ao *Fusarium moliniforme* Sheldon, agente causal do 'Pokkah Boeng'. PhD thesis, FCMBB, Botucatu, Sao Paulo.

Eskew DL, Eaglesham ARJ, App AA. 1981. Heterotrophic $^{15}N_2$ fixation and distribution of newly-fixed nitrogen in a rice-flooded soil system. *Plant Physiology* **68,** 48–52.

Fisher PJ, Petrini O, Lappin Scott HM. 1992. The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytologist* **122,** 299–305.

Gallon JR. 1992. Reconciling the incompatible: N_2 fixation and O_2. *New Phytologist* **122,** 571–609.

Giller KE, Day JM, Dart PJ, Wani SP. 1984. A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$. *Journal of Microbiological Methods* **2,** 307–16.

Gillis M, Döbereiner J, Pot B, Goor M, Falsen E, Hoste B, Reinhold B, Kersters K. 1991. Taxonomy relationship between '*Pseudomonas*' *rubrisubalbicans*, some clinical isolates (EF group 1), *Herbaspirillum seropedicae* and '*Aquaspirillum*' *autotrophicum*. In: Polsinelli M, Materassi R, Vicenzini M, eds, *Nitrogen fixation*. Dordrecht: Kluwer Academic Publishers, 292–4.

Gross DC, Cody YS. 1985. Mechanisms of plant pathogenesis by *Pseudomonas* species. *Canadian Journal of Microbiology* **31,** 403–10.

Hale CN, Wilkie JP. 1972*a*. A comparative study of *Pseudomonas* species pathogenic to sorghum. *New Zealand Journal of Agricultural Research* **15,** 448–56.

Hale CN, Wilkie JP. 1972*b*. Bacterial leaf stripe of sorghum in New Zealand. *New Zealand Journal of Agricultural Research* **15,** 457–60.

Harrison NA, Davis MJ. 1988. Colonization of vascular tissues by *Clavibacter xyli* subsp. *xyli* in stalks of sugarcane cultivars differing in susceptibility to ratoon stunting disease. *Phytopathology* **78,** 722–7.

Hartmann A, Baldani JI, Kirchhof G, Assmus B, Hutzler P, Springer N, Ludwig W, Baldani VLD, Döbereiner J. 1995. Taxonomic and ecologic studies of diazotrophic rhizosphere bacteria using phylogenetic probes. In: Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M, eds. *Azospirillum VI and related microorganisms*. Berlin, Heidelberg: Springer-Verlag, 415–27.

Hurek T, Reinhold-Hurek B, Van Montagu M, Kellenberg E. 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *Journal of Bacteriology* **176,** 1913–23.

James EK, Reis VM, Olivares FL, Baldani JI, Döbereiner J. 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *Journal of Experimental Botany* **45,** 757–66.

Kao J, Damann KE. 1980. *'In situ'* localization and morphology of the bacterium associated with ratoon stunting disease of sugar cane. *Canadian Journal of Botany* **58,** 310–15.

Leigh JA, Coplin DL. 1992. Exopolysaccharides in plant-bacterial interactions. *Annual Review of Microbiology* **46,** 307–46.

Li R, McRae IC. 1992. Specific identification and enumeration of *Acetobacter diazotrophicus* in sugar cane. *Soil Biology and Biochemistry* **24,** 413–19.

Lima E, Boddey RM, Döbereiner J. 1987. Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N-aided nitrogen balance. *Soil Biology and Biochemistry* **24,** 413–19.

McInroy JA, Kloepper JW. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil* **173,** 337–42.

McKhann HI, Hirsch AM. 1994. Does *Rhizobium* avoid the host response? In: Dangl JL, ed. *Bacterial pathogenesis of plants and animals*. Springer-Verlag, 139–62.

Miranda CHB, Urquiaga S, Boddey RM. 1990. Selection of ecotypes of *Panicum maximum* for associated biological nitrogen fixation using the ^{15}N isotope dilution technique. *Soil Biology and Biochemistry* **22,** 657–63.

Olivares FL, James EK, Reis VM, Baldani VLD, Baldani JI, Döbereiner J. 1993. Colonization of vascular tissue by *Herbaspirillum* spp. in sorghum and sugar cane. In: *Proceedings of twenty third Brazilian congress of plant pathology*.

Olivares FL, dos Reis Jr FB, Reis VM, Baldani VLD, Baldani JI, Döbereiner J. 1995. Infection of sugar cane roots by the endophytic diazotrophs *Herbaspirillum seropedicae* and *H.rubrisubalbicans*. In: *Proceedings of international symposium on sustainable agriculture for the tropics–the role of biological nitrogen fixation*. Rio de Janeiro, 65–6.

Olivares FL, Baldani VLD, Reis VM, Baldani JI, Döbereiner J. 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems and leaves predominantly of Gramineae. *Biology and Fertility of Soils* **21,** 197–200.

Olivares FL, James EK, Baldani JI, Döbereiner J. 1997. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. *New Phytologist* **135,** (in press).

Patriquin DG, Döbereiner J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology* **24,** 734–42.

Perotto S, Brewin NJ, Kannenberg EL. 1994. Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. *Molecular Plant-Microbe Interactions* **7,** 99–112.

Pimentel JP, Olivares FL, Pitard RM, Urquiaga S, Akiba F, Döbereiner J. 1991. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. *Plant and Soil* **137,** 61–5.

Reis VM, Olivares FL, Döbereiner J. 1994. Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. *World Journal of Microbiology and Biotechnology* **10,** 101–4.

Sequeira L, Gaard G, De Zoeten GA. 1977. Interaction of bacteria and host cell wall: its relation to mechanisms of induced resitance. *Physiological Plant Pathology* **10,** 43–50.

Sprent JI, James EK. 1995. N_2-fixation by endophytic bacteria: questions of entry and operation. In: Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M, eds. *Azospirillum VI and related microorganisms*. Berlin, Heidelberg: Springer-Verlag, 15–30.

Tarrand JJ, Krieg NR, Döbereiner J. 1978. A taxonomy study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Canadian Journal of Microbiology* **24,** 967–80.

Urquiaga S, Cruz KHS, Boddey RM. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. *Soil Science Society of America Journal* **56,** 105–14.

VanderMolen GE, Beckman CH, Rodehorst E. 1977. Vascular gelation: a general response phenomenon following infection. *Physiological Plant Pathology* **11,** 95–100.

Wallis FM. 1977. Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. *Physiological Plant Pathology* **11,** 333–42.

Wani SP, Dart PJ, Upadhyaya MN. 1983. Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay. *Canadian Journal of Microbiology* **29,** 1063–9.