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A Mutant of *Azospirillum brasilense* Sp7 Impaired in Flocculation with a Modified Colonization Pattern and Superior Nitrogen Fixation in Association with Wheat

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We report here significant phenotypic and genetic differences between *Azospirillum brasilense* Sp7 and spontaneous mutant Sp7-S and their related properties in association with wheat. In contrast to the wild-type strain of Sp7, colonies of Sp7-S stained weakly with Congo red when grown on agar media containing the dye and did not flocculate in the presence of fructose and nitrate. Scanning and transmission electron micrographs showed clearly that the Sp7-S strain lacked surface materials present as a thick layer on the surface of the wild-type Sp7 strain. Different patterns of colonization on wheat roots between Sp7 and Sp7-S, revealed by in situ studies using *nifA-lacZ* as a reporter gene, were related to a large increase in nitrogenase activity (acetylene reduction) with Sp7-S in association with normal and 2,4-dichlorophenoxyacetic acid-treated wheat for assays conducted under conditions in which the nitrogenase activity of free-living *Azospirillum* organisms was inhibited by an excess of oxygen. Randomly amplified polymorphic DNA analysis indicated the close genetic relationship of Sp7-S to several other sources of Sp7, by comparison to other recognized strains of *A. brasilense*. Genetic complementation of Sp7-S was achieved with a 9.4-kb fragment of DNA cloned from wild-type Sp7, restoring Congo red staining and flocculation.

Azospirillum species are known to associate with many agriculturally important crops. Upon inoculation, bacteria adsorb to roots, proliferate on the surface, and may subsequently invade and colonize the internal tissues of roots. Promotion of plant growth and yield has been reported in some cases (20, 39). Studies by several investigators (9, 45, 47, 53, 54) have shown that the addition of small amounts of synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid can increase the colonization and nitrogen fixation of azospirilla, coincident with the formation of modified lateral root structures, previously termed *para*-nodules (22).

Factors controlling successful colonization of the roots by *Azospirillum* species are not yet fully understood, but it has been suggested that polar flagella, surface polysaccharides, and lectin-like proteins are involved in the process (for reviews, see references 10 and 31). In the *Rhizobium*-legume symbiosis, bacterial surface polysaccharides have been shown to play a role in defining plant-bacterial associations. β-Glucans, lipopolysaccharides, and exopolysaccharides are sometimes required for the formation of effective indeterminate nodules of legumes by the microsymbiont *Rhizobium* sp. (for a review, see reference 25). Similarly, in *Agrobacterium tumefaciens*, the production of periplasmic β-1,2-glucans is necessary for the attachment and subsequent tumor production on plants (38). Production of exopolysaccharides in azospirilla has been correlated with flocculation and the ability to develop into cyst-like forms and to bind calcofluor or Congo red (5, 7, 11, 33, 41). Acetylene reduction activity has been shown to decline concomitantly with cyst formation in cultures (5, 14, 37), but Berg et al. (6) have reported that cysts of *Azospirillum brasilense* that formed on plant tissue culture could fix nitrogen. Whether azospirilla synthesize polysaccharides that can act as determinants for specific interactions with host plants has not yet been resolved (10).

In this paper we report the comparison, based on the physiology, immunology, electron microscopy, and genetics, of two *A. brasilense* strains, referred to here as Sp7 and Sp7-S. Data indicate that Sp7-S is a spontaneous mutant of Sp7 that shows different surface polysaccharide characteristics. Differences in the patterns of colonization of wheat by the two strains, which are probably related to differences in nitrogenase activities, are reported.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Wild-type *A. brasilense* Sp7 was originally supplied by J. Döbereiner, Empresa Brasileira de Pesquisa Agropecuária, Rio de Janeiro, Brazil, to other laboratories and deposited at the American Type Culture Collection under no. 29145. The Sp7 strain maintained at the Institut Pasteur, Paris, France, was used as the reference strain in this study. The strain referred to here as Sp7-S was reisolated from an original Sp7 culture obtained from A. H. Gibson (Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia) by Y. T. Tchan (Department of Chemical Engineering, The University of Sydney, Sydney Australia). The Sp7-S strain of *A. brasilense*, designated Sp7, has been employed in this laboratory for studies of the association with wheat since 1989 (45, 47, 53, 54).

Complete medium for *Escherichia coli* was Luria-Bertani (LB). *Azospirillum*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Source or description	Reference
A. brasilense		
Sp7	C. Elmerich[a]	46
Sp7	A. H. Gibson[b]	46
Sp7	P. B. New[c]	46
Sp7-S	Referred to as Sp7 in previous studies (22, 45, 47, 53)	This work
Cd	P. B. New	14
Sp245	P. B. New	3
SpBr14	P. B. New	46
DN64	A. H. Gibson	17
A. lipoferum		
SpBr17	P. B. New	46
596	P. B. New	35
E. coli		
S17.1	*pro thi hsdR recA* (RP4-2-Tc::Mu::−Km::Tn*7* Tra^+ IncP)	44
TG1	Δ(*lac-pro*) *supE supF thi hsdD5* (F′ *traD36 lacI*^{q} Δ*lacZM15*)	50
Plasmids		
pVK100	Km^r Tc^r IncP Tra^-	23
pRK2013	Km^r Tra^+ ColE1 replicon	13
pAB576	*nifA-lacZ* transcriptional fusion, Tc^r Km^r, pVK100 derivative	27
pAB1066	Plasmid clone complementing Sp7-S, Tc^r, pVK100 derivative	This work
pAB1220	As pAB1066	This work
pAB1220-9	Contains 9.4-kb *Hin*dIII of pAB1220 subcloned into pVK100	This work
pD2	pLAFR1 derivative containing *R. meliloti exoB*, Tc^r	26
pD5	pLAFR1 derivative containing *R. meliloti exoD*, Tc^r	26
pD15	pLAFR1 derivative containing *R. meliloti exoC*, Tc^r	26
pD56	pLAFR1 derivative containing *R. meliloti exoBCDEF*, Tc^r	28
pD34	pLAFR1 derivative containing *R. meliloti exoA*, Tc^r	28
pAB154, pAB190, pAB290, pAB590, pAB890, pAB921, pAB958A, pAB1039, pAB1160, pAB1790	Set of recombinant plasmids containing p90 restriction fragments cloned into pVK100 or pLA29-17 vectors	36

[a] Institut Pasteur, Paris, France.
[b] Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia.
[c] Department of Microbiology, University of Sydney, Sydney, Australia.

sp. was grown on nutrient broth (Difco) as complete medium. Otherwise, *Azospirillum* sp. was grown on nitrogen-free semisolid malate medium (NFB) supplemented with 0.005 or 0.05% yeast extract as indicated (45) or in the minimal lactate medium containing ammonium chloride, or not, as required (18). Congo red was added to media at 37.5 μg/ml (final concentration) as described previously (5). Acidification of sugars in NFB and peptone-based liquid media, the lack of requirement for biotin, and the ability to use glucose as the sole carbon source under nitrogen-fixing conditions were tested as described by Tarrand et al. (46). Flocculation in the presence of 8 mM fructose and 0.5 mM KNO_3 was studied as described by Sadasivan and Neyra (41). Fluorescence with calcofluor was demonstrated as described by Michiels et al. (33). Tetracycline (TC) at 5 or 10 μg/ml and kanamycin at 20 μg/ml were added to culture media as required.

Transmission electron microscopy (TEM). Bacteria grown overnight in NFB were centrifuged to form a loose pellet and fixed in 2.5% glutaraldehyde in 0.05 M phosphate (pH 7.2) for 18 h at 4°C, washed in the same buffer, and postfixed in 1% OsO_4–buffer at room temperature for 1 h. The specimens were rinsed in distilled water, stained with 0.5% uranyl acetate for 30 min, and dehydrated through a graded acetone series, before being embedded in a low-viscosity epoxy resin. Thin sections were cut with a diamond knife on a Reichert-Jung Ultracut E ultramicrotome, stained sequentially with uranyl acetate and lead citrate, and examined with a Philips 400 transmission electron microscope.

Scanning electron microscopy (SEM). Root segments from inoculated plants were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h at room temperature, washed in the same buffer, and postfixed in 1% OsO_4–buffer for 1 h at room temperature. The specimens were washed thoroughly in distilled water, incubated in saturated thiocarbohydrazide for 20 min at 50°C, again washed in distilled water, and poststained in 2% aqueous uranyl acetate for 1 h at room temperature. The specimens were dehydrated through a graded ethanol series and critical point dried. The dried, mounted specimens were sputter coated with 2 nm of platinum and observed with a JEOL JSM 6000F scanning electron microscope operating at 15 kV or coated with 40 nm of platinum and observed with a JSM 35C scanning electron microscope operating at 25 kV.

Randomly amplified polymorphic DNA (RAPD) analysis. Total DNA from wild-type *A. brasilense* and *Azospirillum lipoferum* strains listed in Table 1 was extracted and amplified by PCR with primer RPO1 as the random primer (40). Primer RPO1 is 5′-AATTTTCAAGCGTCGTGCCA-3′ (20 nucleotides in length), corresponding to the sequence of a conserved domain within the *Rhizobium trifolii nifHDK* promoter. Amplification reactions were analyzed by agarose gel electrophoresis as described by Richardson et al. (40).

DNA techniques and plasmid construction. Plasmid and chromosomal DNA isolation, restriction analysis, hybridization by the method of Southern, transformation, and molecular cloning were performed by conventional techniques (43). Construction of a gene bank of *A. brasilense* Sp7 total DNA digested by *Hin*dIII into the broad-host-range cosmid vector pVK100 has been reported previously (8). To construct pAB1220-9, the 9.4-kb *Hin*dIII fragment from gene bank plasmid clone pAB1220 was purified from an agarose gel and subcloned into pVK100 vector. The ligation mixture was used to transform *E. coli* TG1 for TC resistance. Kanamycin-sensitive plasmid clones were then transferred by conjugation (see below) into strain Sp7-S to check for the phenotype on Congo red plates.

Conjugation and screening of *A. brasilense* gene bank. Plasmids in *E. coli* S17-1 were transferred into *Azospirillum* recipients by conjugation (44). In other cases,

triparental matings were performed by using pRK2013 as the mobilizing agent (13). Plasmid pAB576, which carries a *lacZ* fusion to the *nifA* promoter (27), was transferred by conjugation from strain S17-1 to Sp7 and Sp7-S as described by Katupitiya et al. (21).

Screening of the gene bank was performed by conjugation of 250 S17-1 donors containing the plasmid clones into strain Sp7-S as the recipient. Donors were grown in microculture containers, 10- to 20-μl aliquots of the culture were spotted with a multipoint inoculator onto nutrient agar plates, and the same volume of the recipient was also added to the spots. After overnight mating at 33°C, each mating mixture was reisolated onto selective media of minimal lactate ammonia medium plates containing TC and Congo red. The plasmid clones which gave transconjugants with red colonies were studied further.

Colonization of wheat roots. The number of azospirilla present in wheat roots was estimated by the most-probable-number technique, as described by Sriskandarajah et al. (45). Counts were made in NFB medium supplemented with TC (5 μg/ml), because the transconjugants which are resistant to TC were used to inoculate the plants.

The colonization pattern was investigated by in situ staining of roots with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) as described by Arsène et al. (1). Roots were taken from seedlings, treated with 2,4-D or not treated, and inoculated with Sp7 and Sp7-S containing pAB576. Samples were collected 3 days and 2 weeks after inoculation. After staining, specimens were examined and photographed by using a transmission light microscope (Olympus BHA) and an automatic Olympus camera.

ARA. The acetylene reduction assay (ARA) was performed with free-living cultures as well as in association with wheat plants. Ethylene formation was measured with a Shimadzu GC 8F gas chromatograph equipped with a flame ionization detector and 1-m Porapak T column.

Free-living cultures. The cultures were grown overnight in NFB supplemented with yeast extract (0.05%). The fully grown cultures were centrifuged, and the cells were washed in nitrogen-free minimal lactate medium. The cell suspensions were then adjusted to an A_{600} of 0.1 with nitrogen-free minimal lactate medium, and the ARA was carried out under argon with 0.5% oxygen (18), using 10 ml of the suspension in 50-ml conical flasks. Acetylene (10%) was added after 4 h of incubation with continuous shaking at 30°C. The rate of ethylene production was measured by taking gas samples 1 and 2 h after acetylene was introduced.

Plant assay. Wheat (cultivar Miskle) plants were grown in hydroponic medium under sterile conditions as described by Zeman et al. (54). After 1 week of growth, seedlings were inoculated with bacteria grown overnight in NFB medium supplemented with yeast extract (0.05%); some plants were treated with 2,4-D (0.7 ppm) prior to inoculation. The plants were then grown for another 2 weeks, and the ARA was performed (45, 54) by a technique that involves incubation with shaking in an aqueous medium at 0.025 atm (1 atm = 101.29 kPa) of oxygen, in order to prevent nitrogenase activity by free-living azospirilla external to the root tissue.

Immunodiffusion. Antisera against the two strains were prepared in rabbits by giving intramuscular injections. New Zealand White rabbits were used, and whole cells (10^9 to 10^{10}/ml) grown in NFB medium supplemented with NH_4Cl (0.1%) and washed in physiological saline (0.85% NaCl) were used as antigens, with Freund's adjuvant (1:1 [vol/vol]). A booster injection without Freund's adjuvant was given after 3 weeks, and 20 ml of blood was collected from the marginal ear vein after 1 month. Gel immunodiffusion tests were performed by the method described by Vincent (49).

RESULTS

Morphology and physiology. Although very similar in appearance in young cultures, the Sp7-S strain produced smooth, shiny colonies while wild-type Sp7 formed colonies that were drier in appearance when mature. When the two strains were grown on solid media containing Congo red, the colonies of strain Sp7 appeared red in color whereas colonies of Sp7-S remained pink. Although the two strains differed with respect to binding to Congo red, when grown on LB containing calcofluor white both produced equally bright fluorescence under UV.

Morphologically, cells of Sp7 and Sp7-S grown on nutrient broth, in minimal lactate medium, or in NFB medium supplemented with yeast extract (0.005%) were similar in form after 24 h of growth, both types being vibrioid. In contrast to the morphology of early growth, when the cultures were grown for more than 48 h, cells of Sp7 appeared as oval-shaped structures. The Sp7-S cells lengthened, and some were present as chains. This difference in morphology is shown in Fig. 1A and B. A transverse section examined by TEM revealed that the cells of strain Sp7 were surrounded by a thick, rough outer layer, which the Sp7-S strain lacked (Fig. 1E and F).

Identical results were obtained with the two strains in physiological tests regarding no requirement for biotin, lack of acidification from sugars in NFB medium, acidification from fructose in peptone medium, failure to use glucose as a growth substrate, and close similarity in growth rates on either nutrient broth or minimal lactate medium (ca. 0.6 divisions per h), indicating no major differences in the general metabolism of the two. However, Sp7-S failed to flocculate in the presence of fructose and nitrate (Fig. 2). It also failed to survive under conditions that allowed drying of a broth culture, in contrast to Sp7, which was readily resuscitated from 2-month-dried cultures, indicating a lack of cyst formation by Sp7-S.

The two strains also reacted similarly with the antiserum produced against the Sp7-S strain, forming two precipitation bands, with the bands formed by Sp7 and Sp7-S merging smoothly (data not shown). However, the strains reacted differently to the antiserum developed against wild-type Sp7; more precipitation bands formed than with Sp7-S antiserum, and the antigens of the two strains precipitated at different distances. This indicates that Sp7-S possesses surface antigens different from those of Sp7.

Genetic analysis. RAPD analysis of the strains was unable to distinguish Sp7 and Sp7-S (Fig. 3). The amplification profiles generated by PCR showed similar patterns for several isolates of Sp7 obtained from different sources. Amplification profiles obtained with strain Sp7-S and strain Cd, a strain probably derived from Sp7, were identical to that of Sp7 while the other unrelated strains of *A. brasilense* (Sp245, SpBr14, and DN64) and *A. lipoferum* (SpBr17 and 596) produced amplification profiles that were completely different from one another but unique to each strain (Fig. 3). This suggested that Sp7-S might have arisen after a spontaneous mutation of the original Sp7 culture. Over a 3 year period, no reversion of the phenotype of Sp7-S on Congo red has been observed, indicating that the mutation is very stable.

To characterize strain Sp7-S, different experiments based on genetic complementation were performed. It was previously reported that mutants of *Rhizobium meliloti* impaired in *exo* genes encoding exopolysaccharide production could be complemented by *Azospirillum* DNA regions (32). Two of these regions corresponding to *R. meliloti exoB* and *exoC* have been localized on the 90-MDa plasmid of strain Sp7 (30). It was worth attempting to assay whether Sp7-S could carry a mutation in the equivalent of an *R. meliloti exo* gene. A set of plasmids containing the *exo* genes of *R. meliloti* encoding the succinoglycan molecule (Table 1) was first introduced by conjugation into Sp7-S. None of the transconjugants obtained recovered the property of being stained by Congo red or of flocculating in fructose-containing medium. Similarly, with a set of plasmid clones overlapping the 90-MDa plasmid (Table 1), no complementation was detected. As a consequence of these negative results, a complementing clone was sought in the gene bank of total DNA of Sp7. By using the protocol described in Materials and Methods, 250 plasmid clones were introduced into Sp7-S, and two of them, pAB1066 and pAB1220, restored staining with Congo red. The two plasmids were purified from S17-1, and restriction analysis with *Hin*dIII was performed. pAB1066 contained three fragments with sizes of 11.5, 9.4, and 1.9 kb, while pAB1220 contained two fragments with sizes of 14 and 9.4 kb (data not shown). Because the two plasmids carried a 9.4-kb fragment, it was assumed that the fragment corresponded to the complementing region. This was verified by subcloning the fragment to yield pAB1220-9. Indeed, when introduced into Sp7-S, pAB1220-9 restored the red

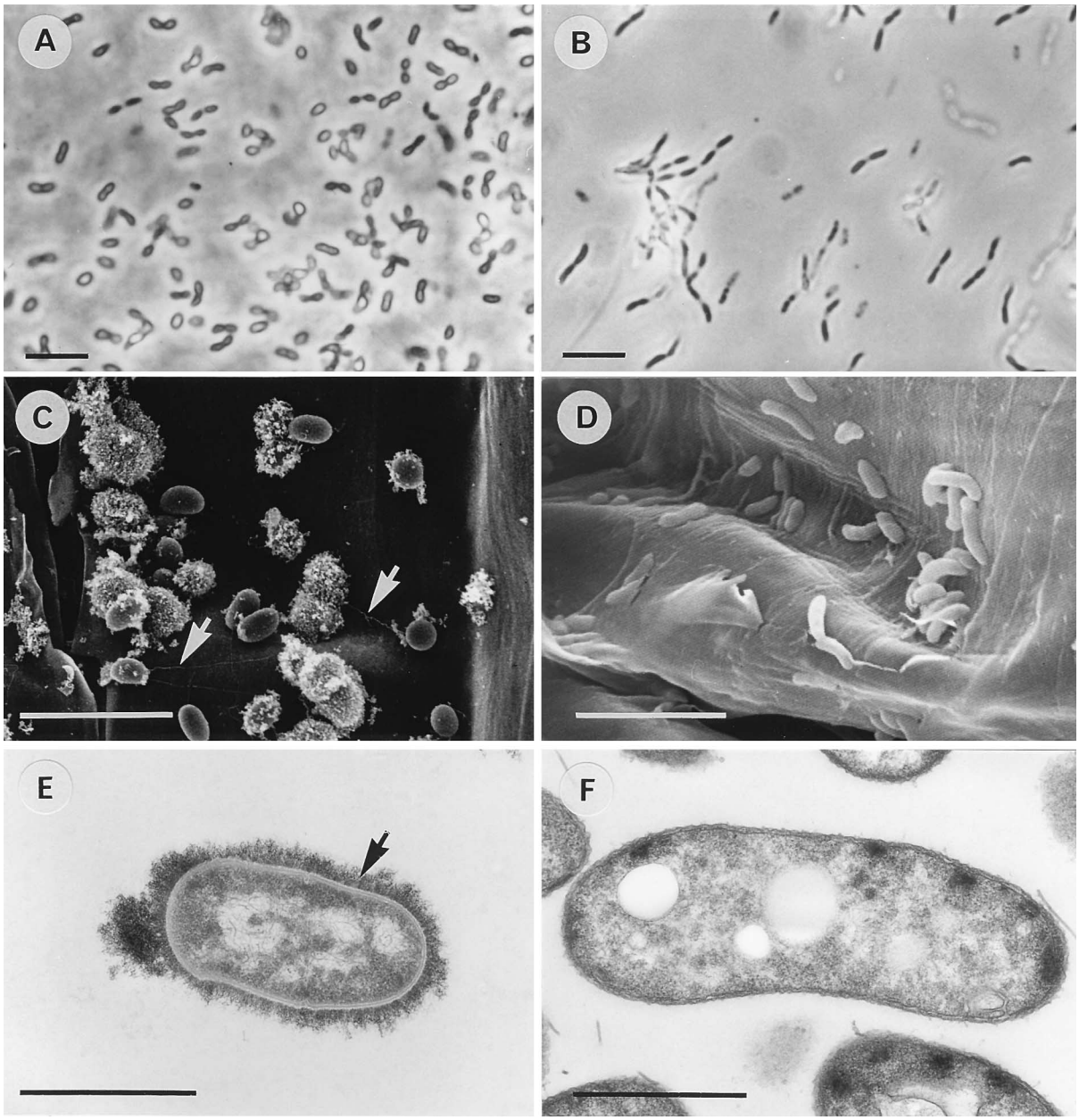

FIG. 1. Transmission and scanning electron micrographs of Sp7 and Sp7-S in free-living state and in association with root tissues. (A and B) Sp7 and Sp7-S cells, respectively, grown for 48 h in NFB semi-solid medium with yeast. (C) SEM of Sp7 on root surface. Some cells have thick walls and have changed to oval- or round-shaped structures or cysts. Arrows indicate polysaccharide fibrils. (D) SEM of Sp7-S on roots. Cells remained vibrioid and show the normal vegetative form. (E) TEM of transverse section of Sp7. The arrow indicates the thick exopolysaccharide layer around the cell. (F) TEM of transverse section of Sp7-S. The thick outer layer is not present.

phenotype, flocculation, and thick exopolysaccharide layer and SP7-S showed precipitation patterns similar to those of its parent strain in immunodiffusion (data not shown).

A subsequent control was performed by using the 9.4-kb fragment as a hybridization probe against total DNA of Sp7 and Sp7-S digested with different enzymes, including *Bgl*II, *Hin*dIII, and *Pst*I. Figure 4 shows that the probe revealed fragments with similar sizes in the two strains, with the three enzymes used. In particular, it revealed a *Hin*dIII fragment whose estimated size matched that of the probe used. Hybridization with a 2.5-kb *Sal*I fragment carrying *nifA* revealed *Eco*RI (5.6- and 3.6-kb) and *Sal*I (2.5-kb) fragments with similar sizes in the two strains (data not shown). Thus, in addition to the RAPD analysis described above, this confirmed that the two strains are very closely related and suggests that Sp7-S did not arise from a large deletion of the Sp7 genome.

Colonization of wheat roots. The colonization efficiencies of Sp7 and Sp7-S in plant roots with or without modified lateral root structures induced by 2,4-D were compared. The strains used for inoculation contained plasmid pAB576, which carries a *lacZ* fusion under the control of the *nifA* promoter. The *Azospirillum nifA* gene is expressed under all physiological conditions in the free-living state and also in association with plant tissue (1, 27). This enabled us to localize the bacteria on the

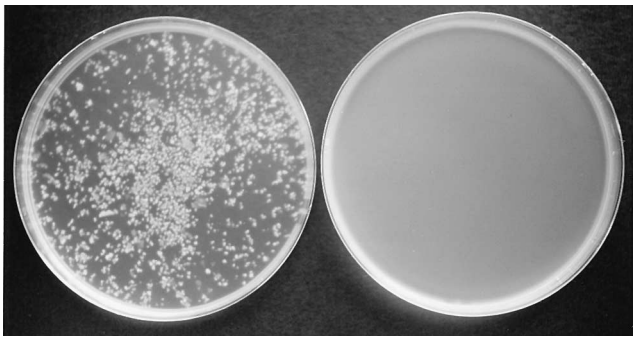

FIG. 2. Flocculation of Sp7 in broth containing fructose and nitrate (left). Sp7-S did not flocculate under the same conditions (right).

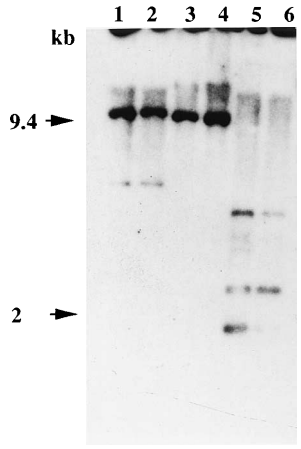

FIG. 4. Hybridization of total DNA of Sp7 (lanes 1, 3, and 5) and Sp7-S (lanes 2, 4, and 6) with 9.4-kb complementing fragment purified from pAB1220 as a probe. Digestion was done with *Bgl*II (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), or *Pst*I (lanes 5 and 6). The relative migrations of the 9.4- and 2-kb *Hin*dIII fragments of bacteriophage lambda DNA are indicated by arrows on the left (bands not shown).

root surface, after revealing the β-galactosidase activity in situ by using X-Gal as a chromogenic substrate, by examination with light microscopy. Plants inoculated with Sp7 and Sp7-S had similar numbers of associated bacteria (Fig. 5A). As also shown in Fig. 5A, 2,4-D-treated plants had more bacteria than the untreated plants, as reported previously (45).

Examination of segments of wheat roots stained with X-Gal revealed marked differences between the patterns of colonization for the two strains. The cells of Sp7 were distributed mainly on the root surface (Fig. 6A and C). However, in the plants treated with Sp7-S, there was very little or no surface colonization; most of the bacteria were present in the crevices surrounding the site of lateral root emergence (Fig. 6B and D) or sometimes in the first few layers of root cortical tissues. In 2,4-D-treated plants inoculated with Sp7-S, azospirilla were present in large numbers in cortical tissue around the base of the modified lateral root structures, which are formed because of the treatment with 2,4-D (Fig. 6F). In addition to the surface colonization, a similar pattern of colonization around the modified lateral root structures was observed for Sp7 (Fig. 6E) when the plants were treated with 2,4-D. These different patterns of colonization were established as early as 3 days after inoculation with azospirilla.

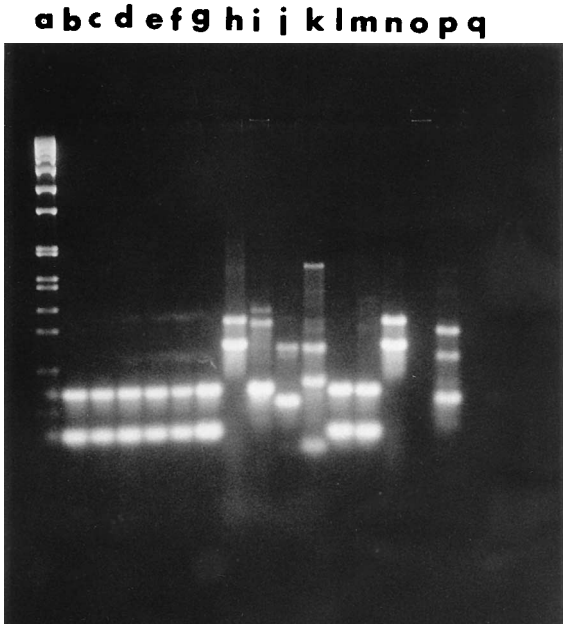

FIG. 3. PCR amplifications of *Azospirillum* DNA with the primer RPO1. Lanes: a, bacteriophage SPP-1 DNA digested with *Eco*RI (standard); b, Sp7-S; c, Sp7 from C. Elmerich; d, e, l, and m, Sp7 from A. H. Gibson; f, Sp7 from P. B. New; g, Cd; h and n, Sp245; i, SpBr14; j, SpBr17; k, 596; p, DN64; q, control. Lane o failed.

There were also clear differences in the morphological appearances of the two strains on wheat roots. Strain Sp7-S retained the normal curved rod shape of vegetatively grown azospirilla at all stages, while Sp7 on the surface of wheat roots was more ovoid in shape and showed thick walls (Fig. 1C and D), apparently tending to assume the morphology typical of cyst-like cells. Thin-section TEM of Sp7 bacteria confirmed the cyst-like structure, as shown in Fig. 1E. Fibril-like structures similar to those reported by Umali-Garcia (48) were observed for Sp7 but not for Sp7-S (Fig. 1C).

ARA. Measurement of nitrogenase activity in pure cultures of Sp7 and Sp7-S under conditions that allowed expression of *nif* genes indicated that there were no significant differences in fixing nitrogen (C_2H_4 formation) between the two strains in vitro in axenic cultures. However, when wheat seedlings were inoculated with the two strains, nitrogenase activity associated with oxygen-protected sites in the roots of the plants (45, 54) after 2 weeks was 6 to 10 times higher with Sp7-S than with Sp7 (Fig. 5B). The 2,4-D-treated plants showed significantly higher levels of activity than the untreated plants with either strain.

DISCUSSION

The data reported in this paper revealed a dramatic difference in the colonization properties of two *A. brasilense* strains and showed that Sp7-S most probably was derived from wild-type Sp7 after a spontaneous mutation. The following common properties support the hypothesis that Sp7-S is derived from Sp7: (i) Sp7-S is a pure culture which apparently arose after many reisolations from an initial culture received as Sp7; (ii) Sp7-S and Sp7 do not differ in their general nutritional properties or in growth rate, and they have the same vibrioid morphology and motility in young cultures; and (iii) more importantly, the two strains are genetically closely related, on the basis of the RAPD technique and hybridization analysis.

It is now established that the RAPD assay used with arbitrary primers is an effective tool for differentiating complex

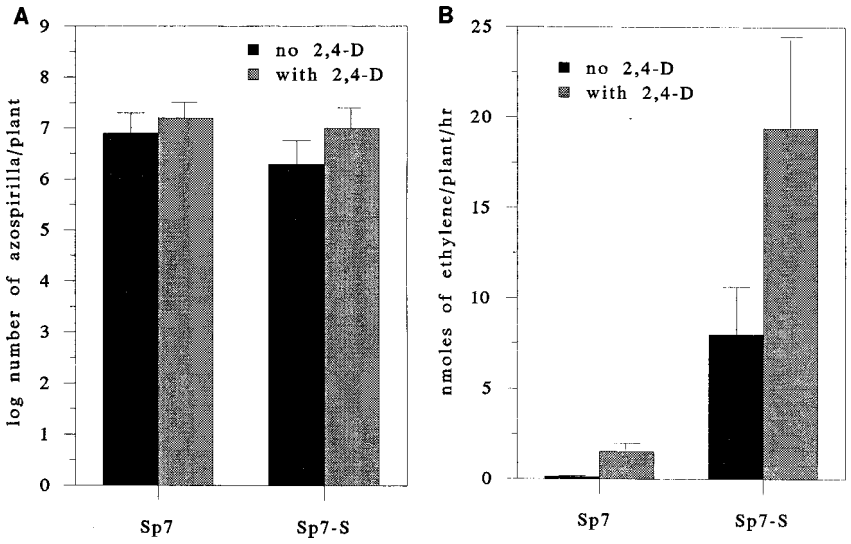

FIG. 5. Total number of bacteria (A) and acetylene reduction activity (B) associated with plants inoculated with Sp7 and Sp7-S, with or without 2,4-D treatment. For panel B, the assays were carried out in a modified atmosphere containing 2.5% oxygen. Data shown are averages for 4 (A) or 15 (B) plants, grown for 2 weeks after inoculation. Bars indicate standard errors.

genomes (51, 52), and it has been used to differentiate the genomes of a wide range of bacterial strains that are representative of diverse species (16, 19, 51), including *Azospirillum* species (15). Richardson et al. (40) showed that directed primers, such as RPO1, are suitable for differentiation of *Rhizobium* species to the strain level because of the unique amplification profiles generated by those primers. Indeed, the *R. trifolii* primer used here enabled a demonstration of similar PCR amplification products (Fig. 3) between Sp7 cultures from different sources (Table 1), Sp7-S and strain Cd, a pigmented strain reisolated from plants inoculated with strain Sp7 (14). In addition, a rapid procedure for plasmid extraction (27) has shown that Sp7-S has the 90- and 115-MDa plasmids found in Sp7 (data not shown). Hybridization of total DNA with a *nifA* probe and the cloned 9.4-kb fragment that restored phenotypic properties of the wild type in Sp7-S revealed fragments with similar sizes in both genomes. This is an additional finding that confirms the identity of the two strains.

At this stage, nothing can be said concerning the nature of the putative mutation of Sp7-S, except that it does not correspond to a large deletion or to a mutation on the 90-MDa plasmid. The fact that no genetic complementation was observed with the different plasmids carrying the *R. meliloti exo* genes cannot be interpreted yet, since this is a negative result and it is not known whether these genes are expressed in *Azospirillum* species.

Although nothing is known about the nature of the mutation, the data reported here support the hypothesis that exopolysaccharide production, and hence the flocculation process, is impaired in Sp7-S. At present, little is definitely known about these surface heteropolymers synthesized by the wild type. Del Gallo et al. (11) showed that the outer polysaccharides of azospirilla are organized into two distinct forms: tightly bound capsular polysaccharides and loosely attached slimy exopolysaccharides. They also showed that calcofluor-binding polysaccharides are found both in the capsular and exopolysaccharide fractions. Other investigators have used Congo red to reveal the capsular material (5, 7). Congo red is known to stain β-1,3- and β-1,4-glucans, including cellulose (34). The global chemical compositions of the exopolysaccharides have been examined (10, 12, 24) and shown to differ depending on the growth substrates utilized in the cultures (10). It is not known whether different types of molecules are produced, as suggested previously (10), or whether the polymer is a single molecule with different substitutions. Indeed, there is no clear indication that the fibrillar material, which anchors the bacteria on the root surface, is similar in composition to that produced during the flocculation process or to that surrounding colonies which is stained by Congo red and causes a fluorescent halo after binding calcofluor white.

It is remarkable, however, that the Sp7-S strain displays a pleiotropic negative phenotype, with respect to production of fibrillar material on the plant root, flocculation, and Congo red staining. These features are well correlated with the absence of capsular material surrounding Sp7-S and with the immunodiffusion analysis, which revealed differences in the surface antigens between the strains. It is also remarkable that Sp7-S complemented with the 9.4-kb cloned fragment recovered Congo red staining, flocculation, and immunological properties, showing similarity to its parent type strain.

Mutant strains impaired in calcofluor-binding and the flocculation process have been isolated after random Tn*5* mutagenesis (12, 33). These mutant strains carried the Tn*5* insertion in *Eco*RI fragments with different sizes, suggesting the existence of several loci governing both exopolysaccharide formation and flocculation in *Azospirillum* spp. (33). The Tn*5* mutant strains were derived from *A. brasilense* 7030, an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced streptomycin-resistant red-pigmented mutant of Sp7 which also lacks the 115-MDa plasmid (17). No differences in fluorescence with calcofluor were found between Sp7 and Sp7-S, suggesting that Sp7-S might be impaired in an unknown locus, different from the previously isolated mutants. Unfortunately, it cannot be known whether Tn*5* mutant strains stain with Congo red since 7030 is already red. The Tn*5*-induced mutant strains were impaired in their properties of anchoring on the root surface and did not bind as efficiently as the wild type during the early stage of colonization (29). By contrast, a Tn*5*-induced mutant of strain Cd which was deficient in flocculation was not found to be impaired in colonization efficiency on the host plant (2).

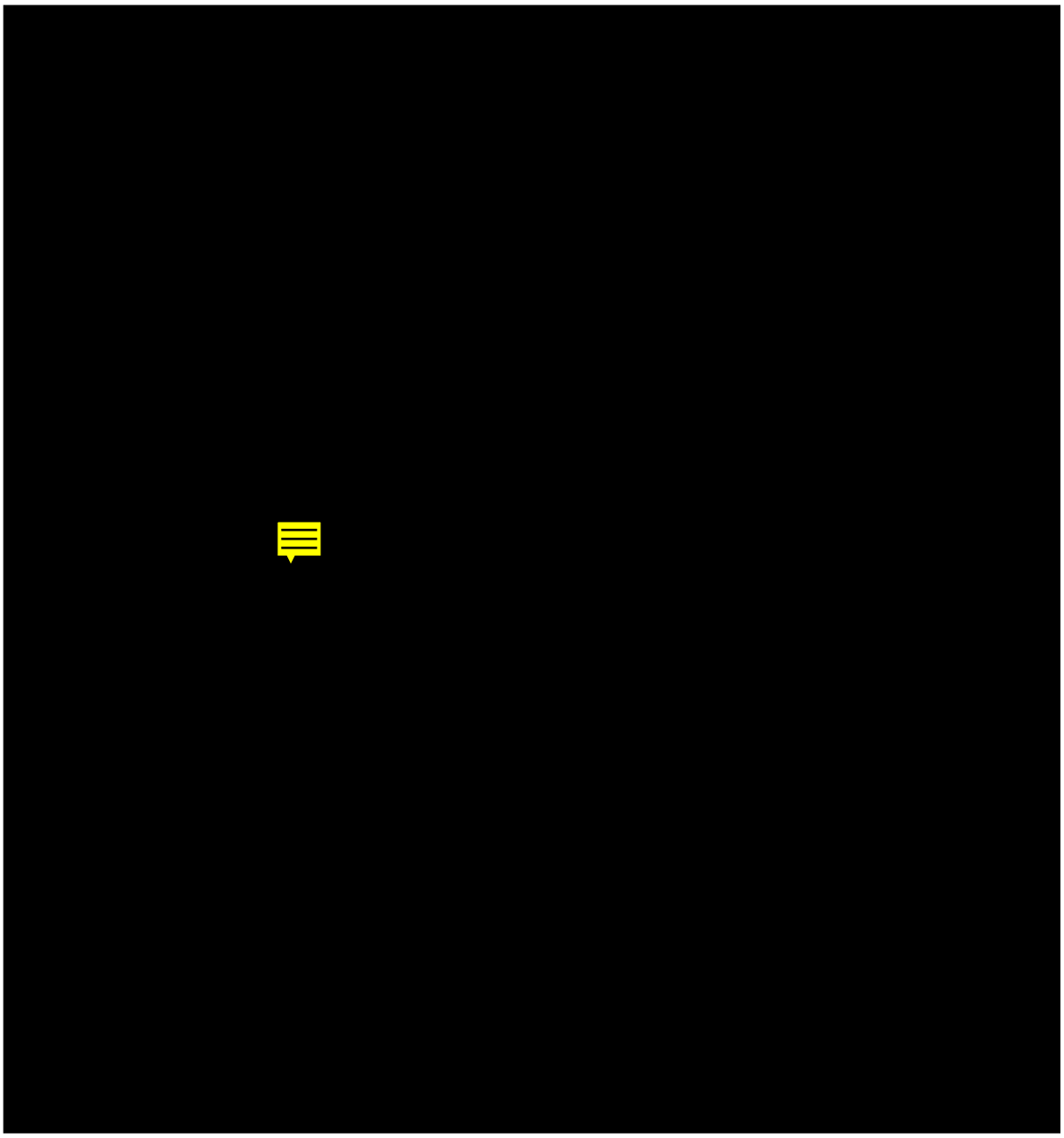

FIG. 6. X-Gal-stained wheat root segments inoculated with Sp7 and Sp7-S containing the *nifA-lacZ* fusion. β-Galactosidase activity is revealed in situ by the blue color. (A) Oval-shaped cells of Sp7 on root surface; (B) vibrioid-shaped cells of Sp7-S in crevice of lateral root emergence; (C) root fragment with dense surface colonization by Sp7; (D) root fragment with colonization around the lateral root base and little colonization of the surface by Sp7-S; (E) Lateral root modified by 2,4-D treatment (modified structures and root surface colonized by Sp7); (F) 2,4-D-treated root inoculated with Sp7-S. Bacteria are present mainly around modified root structures. Magnification, ×1,290 (A and B), ×60 (C, E, and F), and ×90 (D).

Similarly, other mutant strains of *A. brasilense* and *A. lipoferum* isolated as pale colonies on Congo red plates were not impaired in the ability to anchor to the root tissues (5).

In the present work no major differences were found in the number of Sp7 and Sp7-S bacteria associated with the root system after 2 weeks of incubation. Indeed, there is no proof that the accuracy of counting is the same for the two strains. It is possible that Sp7-S, which is not linked to the root tissue by fibrillar material, is more easily released from the plant tissue than Sp7. Despite this difficulty, the differences in the colonization properties between Sp7 and Sp7-S were clearly demonstrated by SEM, TEM, and in situ staining of the bacteria.

The wild-type strain (Sp7) was consistently present in large numbers on the surface of wheat roots, whereas relatively little surface colonization by the mutant (Sp7-S) was observed. The mutant strain was predominantly present in the crevices around the sites of lateral root emergence and showed limited attachment to the root epidermis.

The SEM observations of inoculated root tissues revealed that most wild-type cells present on the root surface had transformed to thick-walled cells with an ovoid shape, linked to the surface by fibrillar material, with a small proportion of cells remaining as thin-walled vegetative cells (Fig. 1C). Such forms of azospirilla, bearing thickened capsules, have been observed

previously in axenic associations with grass and other plant roots (4, 48). Transverse-section TEM clearly revealed the cyst-like structure of the bacteria, with thickened walls and important accumulation of polyhydroxybutyrate globules as reported for cyst-like structures occurring in free-living cultures (41, 42). In contrast, with Sp7-S, almost all the cells remained thin walled on root tissues.

These differences in morphology between the strains may account for the fact that Sp7-S can penetrate the basal region of the modified lateral root structures formed as a result of 2,4-D treatment (45) and thereby proliferate there more extensively than Sp7. The property of remaining in a vegetative form and not forming cyst-like cells by Sp7-S may be of significance in allowing the multiplication of cells internally and may also reflect a different metabolic property. It is known that cysts of azospirilla have increased resistance to environmental stress and remain metabolically dormant as long as the stress conditions persist, with no nitrogenase activity being exhibited (14, 37). This could explain why the wild-type Sp7 strain was less efficient than Sp7-S in fixing nitrogen in association with plants even though the number of wild-type azospirilla associated with the plants actually remained slightly larger than the number of azospirilla in plants inoculated with the mutant. However, the superficial location of wild-type Sp7 cells would also tend to produce low rates of nitrogen fixation as a result of inhibition by oxygen.

These findings open up possibilities for investigating the genetic basis of effective association and colonization and other mechanisms involved in *Azospirillum*-host plant specificity.

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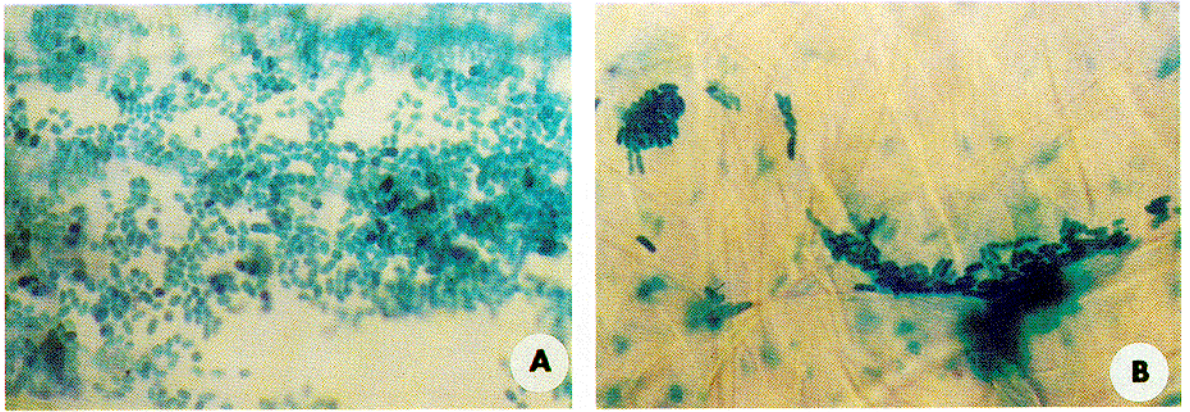
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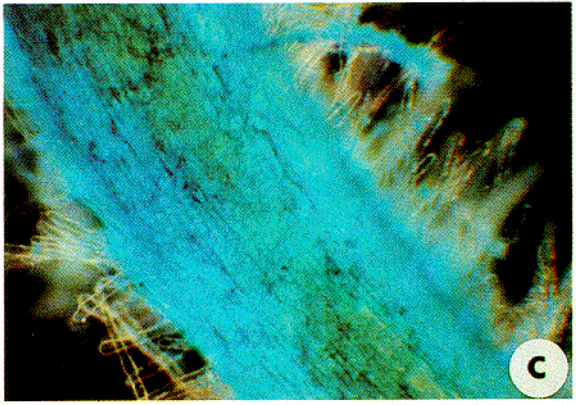
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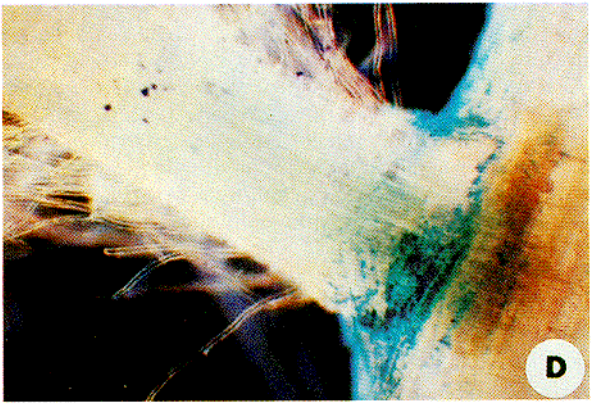
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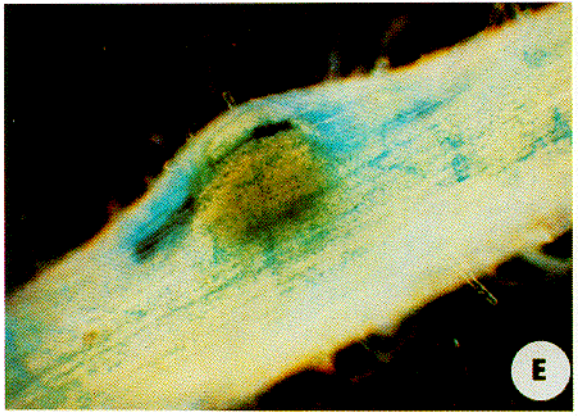
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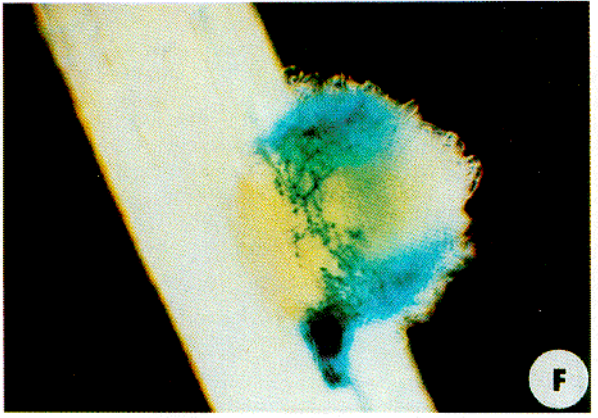
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