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Foliar Chlorosis in Symbiotic Host and Nonhost Plants Induced by *Rhizobium tropici* Type B Strains

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***Rhizobium tropici* CIAT899 induced chlorosis in the leaves of its symbiotic hosts, common bean (*Phaseolus vulgaris* L.), siratro (*Macroptilium atropurpureum* Urb.), and *Leucaena leucocephala* (Lam.) de Wit. Chlorosis induction by strains CIAT899 and CT9005, an exopolysaccharide-deficient mutant of CIAT899, required carbon substrate. When the bacteria were added at planting in a solution of mannitol (50 g/liter), as few as 10^3 cells of CIAT899 were sufficient to induce chlorosis in bean plants. All carbon sources tested, including organic acids and mono- and disaccharides, supported chlorosis induction. The addition of a carbon source did not affect the growth rate or the population density of CT9005 in the bean plant rhizosphere. Cell-free filtrates of cultures of CT9005 did not induce detectable chlorosis. All type B strains of *R. tropici* tested also induced chlorosis in common bean. Type A strains of *R. tropici* and all other species of bacteria tested did not induce chlorosis. Several lines of evidence indicated that nodulation was not required for chlorosis induction. Strain RSP900, a pSym-cured derivative of CIAT899, induced chlorosis in wild-type *P. vulgaris*. In addition, NOD125, a nodulation-defective line of common bean, developed chlorosis when inoculated with CIAT899, but did not develop nodules. CIAT899 consistently induced severe chlorosis in the leaves of the nonhost legumes alfalfa (*Medicago sativa* L.) and Berseem clover (*Trifolium alexandrinum* L.), and induced chlorosis in 29 to 58% of the plants tested of sunflower, cucumber, and tomato seedlings, but it did not induce chlorosis in the leaves of corn or wheat. Chlorosis induction in nonhost plants also required carbon substrate. The data are consistent with the hypothesis that *R. tropici* type B strains produce a chlorosis-inducing factor that affects a wide range of plant species.**

Nodule-forming bacteria in the family *Rhizobiaceae* typically participate in mutually beneficial relationships with their host plants. In several instances, however, rhizobia enter into relationships with plants that appear to have detrimental effects on the host. Some rhizobia induce disease-like symptoms in their legume hosts, including chlorosis induced in soybeans by *Bradyrhizobium japonicum* strains that produce rhizobitoxine (10, 16), leaf-roll in pigeon pea caused by *Rhizobium* sp. strain IHP324 (12), and chlorosis induced in beans by mutants of CIAT899, the type strain of *Rhizobium tropici* (18).

CIAT899, a *R. tropici* type B strain, is of interest because its tolerance to high temperatures, acidity, and high concentrations of aluminum may make it a suitable inoculant strain for tropical soils (7). The symbiotic hosts of *R. tropici* include common bean (*Phaseolus vulgaris* L.), siratro (*Macroptilium atropurpureum* Urb.), and *Leucaena* species (14). Initially, the strains that now compose *R. tropici* were grouped with the species *Rhizobium leguminosarum* bv. phaseoli (5, 13). *R. tropici* was recently separated from other bean-nodulating rhizobia on the basis of differences in host range, 16S rRNA sequences, and other traits (14). Strains of *R. tropici* have been further divided into two subgroups, types A and B, on the basis of several other phenotypes, including resistance to metals and antibiotics and the ability to metabolize certain carbon sources (14).

CIAT899 produces copious amounts of acidic exopolysaccharide (EPS) when grown on yeast extract-mannitol (YM) or BSM media (15, 18). Mutants of CIAT899 that are deficient in EPS production induce effective nodules on the roots of bean (15, 18) and siratro (15) and retain resistance to aluminum (11), but many EPS-deficient mutants are reduced in competitiveness relative to that of CIAT899 (15). We showed previously that EPS-deficient mutants of strain CIAT899 induce severe interveinal chlorosis in common bean and *Leucaena leucocephala* (18). In this report we show that CIAT899, the EPS-producing, wild-type strain also induces chlorosis if it is supplied with sufficient carbon substrate. In addition, we show that the ability to induce chlorosis is a property of a number of *R. tropici* type B strains and that *R. tropici* induces chlorosis in several dicot species that are not symbiotic hosts for *R. tropici*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain CT9005 is a Tn*5*-induced, EPS-deficient mutant of *R. tropici* CIAT899 (15, 18). CIAT899 was obtained from F. Bliss, Department of Pomology, University of California—Davis. All other *R. tropici* strains were provided by E. Martinez-Romero, Universidad Nacional Autonoma de México, Cuernavaca, Mexico, except strain RSP900, a derivative of CIAT899 cured of the Sym plasmid (25), which was provided by C. Quinto, Universidad Nacional Autonoma de México. Cultures of species of *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* were grown to stationary phase (3 to 4 days) in liquid YM medium (26) at 28°C on a rotary shaker. Cultures were maintained on solid YM or minimal medium (3) containing 15 g of agar per liter of medium or frozen to −80°C in liquid YM containing 10% dimethyl sulfoxide. Antibiotics were added to solid media at the following concentrations: spectinomycin, 200 μg/ml; kanamycin, 200 μg/ml.

Preparation of EPS. Crude EPS was prepared from cul-

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tures of CIAT899 grown in YM broth as previously described (18).

Plant growth conditions. Bean seeds were surface disinfected and planted in large test tubes containing sterile sand and vermiculite as described previously (1, 2). Unless otherwise noted, the bean cultivar WBR22-34 was used in all experiments (4). Seeds of WBR22-34 were obtained from K. Kmiecik, Department of Horticulture, University of Wisconsin—Madison. Seeds of bean cultivar RIZ30 and its nonnodulating mutant NOD125 (6, 21) were obtained from J. Kipe-Nolt, CIAT, Cali, Colombia, and J. C. Rosas, Escuela Agricola Panamericana, Zamorano, Honduras. Plants were watered with sterile distilled water. Seeds of *Leucaena leucocephala* and alfalfa were surface disinfected and scarified by immersion in 18 M sulfuric acid for 10 min and rinsed several times with sterile distilled water prior to planting. All other seeds were surface disinfected in the same manner as bean seeds, and all species were planted and maintained as described for bean plants. All plants were maintained in a growth chamber at 24°C with a 12-h photoperiod and a light intensity of 150 microeinsteins m^{-2} s^{-1}.

Chlorosis assays. To assay chlorosis on bean plants, bacteria of each strain were resuspended at a concentration of approximately 10^7 CFU/ml in water or a sterilized solution of mannitol (Sigma Chemical Co., St. Louis, Mo.), unless otherwise noted. One milliliter of inoculum per seed was applied at planting. Ten seeds were inoculated with each strain resuspended in either water or mannitol solution. Three to 4 weeks after planting and inoculation, the first trifoliolate leaves were examined for chlorosis. The severity of chlorosis in bean leaves was rated on a scale of 1 to 5, in which 1 represents no observed chlorosis and 5 represents severe chlorosis, as described previously (18). The leaflets on bean plants rated 5 were yellowish-white and usually smaller and more elongated than unaffected leaves. All chlorosis assays on bean plants were set up as completely randomized designs with 4 to 10 plants per treatment. Analysis of variance of chlorosis ratings was performed with the SAS statistics software package (24). In chlorosis assays on plant species other than bean, cells of strain CIAT899 were prepared as described above for beans. Plants other than bean were rated + or − for the occurrence of chlorosis, in which + indicates severe chlorosis in first or second true leaves and − indicates no chlorosis observed.

Bacterial growth in the bean rhizosphere. Bean seeds were surface disinfected and planted in large tubes as previously described (1, 2). Seeds were inoculated with 4×10^4 CFU of *R. tropici* CT9005 resuspended in 1 ml of either water or 10 g of mannitol per liter. At each time point, 10 plants from each treatment were gently removed from the tubes, and loose sand and vermiculite were removed by shaking the plants. Before emergence, the entire seedling was sampled. After emergence, the sample consisted of the entire root system, which was separated from the rest of the plant by cutting at the crown. Samples were placed in sterilized plastic 50-ml beakers containing 10 ml of sterile distilled water. The beaker was placed in a sonicator bath (Bransonic model 2200; Branson Ultrasonic Corp., Danbury, Conn.) for 30 s. After sonication, the roots with adhering particles were blotted with paper towels and weighed. The water in the beaker was then diluted and plated on solid YM medium containing spectinomycin and kanamycin. Colonies on YM plates were counted 2 to 3 days after plating.

TABLE 1. Effects of the concentration of mannitol in the inoculum on chlorosis severity in bean plants

Mannitol concn (g/liter)	Chlorosis[a] induced by following strain:	
	CIAT899	CT9005
0	1.0 ± 0.0 b	1.0 ± 0.0 b
1	1.0 ± 0.0 b	1.0 ± 0.0 b
2	1.0 ± 0.0 b	1.1 ± 0.1 b
5	1.0 ± 0.0 b	2.1 ± 0.2 b
10	1.9 ± 0.4 b	3.8 ± 0.4 a
25	3.9 ± 0.4 a	4.3 ± 0.4 a

[a] Plants were rated on a scale of 1 to 5 as described in Materials and Methods. Each value represents the mean chlorosis rating ± standard error of 8 to 10 plants. Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$) by Tukey's Studentized range test (honest significant difference = 1.17). Data are representative of three experiments.

RESULTS

Effect of culture components on chlorosis induction by *R. tropici*. We previously reported that EPS-deficient mutants of CIAT899, such as CT9005, induce severe chlorosis in host plants (18). In this study we found that wild-type *R. tropici* CIAT899 induced chlorosis when it was inoculated on seeds with mannitol (50 g/liter) (Table 1). CIAT899 required more mannitol than CT9005 to produce the same severity of chlorosis, and the severity of chlorosis induced by both CIAT899 and CT9005 was dependent on the amount of mannitol supplied at the time of planting (Table 1). The symptoms induced by CIAT899 were identical to those induced by strain CT9005 (18), including stunting and loss of apical dominance in some plants. In addition to mannitol, all of the other carbon compounds tested also supported the ability of CIAT899 and CT9005 to induce chlorosis. The bacteria induced chlorosis when supplied with mannose, glucose, sucrose, lactose, glycerol, inositol, sorbitol, citrate, succinate, or xylitol; solutions of these compounds without bacteria did not induce chlorosis and did not seem to adversely affect root growth (data not shown).

To determine whether chlorosis-inducing factors were released into the medium during culture growth, we prepared cell-free filtrates of cultures of EPS-deficient mutant CT9005 grown in YM broth and applied them to bean seeds at planting. We used CT9005 in this experiment because the viscosity of cultures of CIAT899 made it difficult to remove all bacteria from culture supernatants. Undiluted filtrates of CT9005 cultures grown in YM broth did not induce chlorosis, whereas whole CT9005 culture and washed CT9005 cells resuspended in mannitol or spent YM culture filtrate induced severe chlorosis (Table 2).

The addition of crude EPS from CIAT899 to suspensions of CT9005 did not inhibit chlorosis induction. CT9005 cells suspended in 10 g of mannitol per liter containing 2 mg of crude CIAT899 EPS per ml induced chlorosis in bean plants, with an average chlorosis rating of 3.4 ± 0.5. Cells of CT9005 suspended in 10 g of mannitol per liter without added EPS induced chlorosis in bean plants with an average rating of 3.7 ± 0.5; these two values did not differ significantly by Tukey's Studentized range test ($P \leq 0.05$) (24).

As few as 10^3 cells of CIAT899 added at planting in a 50-g/l mannitol solution induced chlorosis in 3-week-old bean plants. Plants inoculated with 1.6×10^3 cells of CIAT899 developed chlorosis with an average rating of 3.8 ± 0.5, whereas plants inoculated with 1.6×10^8 developed chlorosis with an average rating of 4.6 ± 0.3. These values did not

TABLE 2. Chlorosis induction in bean plants by cultures, filtrates, and resuspended cells of strain CT9005

Inoculum	Chlorosis rating[a]
CT9005 YM culture	4.7 ± 0.2 a
CT9005 culture filtrate (without cells)	1.0 ± 0.0 b
CT9005 cells resuspended in:	
CT9005 culture filtrate	4.8 ± 0.2 a
Mannitol, 10 g/liter	4.3 ± 0.8 a
Water	1.0 ± 0.0 b

[a] Each value represents the mean chlorosis rating ± standard error of 4 to 10 plants. Values followed by the same letter are not significantly different ($P \leq 0.05$) by Tukey's Studentized range test (honest significant difference = 0.97). Data are representative of three experiments.

differ significantly by Tukey's Studentized range test ($P \leq 0.05$) (24).

Mannitol in the inoculum did not affect the growth rate or population density of a chlorosis-inducing strain in the rhizosphere of bean plants (Fig. 1). When seeds were inoculated with approximately 4×10^4 CFU/ml in 10 g of mannitol per liter, the population density of CT9005 was approximately 1.6×10^8/g of root 22 days after planting, whereas CT9005 inoculated in water reached a final density of approximately 1.7×10^8 cells per g (fresh weight) of root 22 days after planting. In this experiment, plants inoculated with CT9005 in mannitol had an average chlorosis rating of 2.9 ± 0.5 after 21 days, whereas there was no chlorosis observed on plants that were treated with CT9005 cells resuspended in water.

Chlorosis induction by wild-type strains of *R. tropici* type B strains. We screened wild-type strains of several members of the *Rhizobiaceae* for the ability to induce chlorosis on beans when they were applied at planting in a solution of 50 g of mannitol per liter. Under these conditions, all of the *R. tropici* type B strains induced foliar chlorosis, although the chlorosis induced by strain BR862 was not significantly different from that of the water control (Table 3). In three experiments, BR862 resuspended in mannitol solution induced chlorosis (rating 2 or 3) in a total of 11 of 28 plants. In the same experiments, BR862 induced chlorosis in 0 of 29 plants when resuspended in water, suggesting that BR862 induces chlorosis by the same mechanism as other type B strains but less frequently and with less severity. None of the other species of bacteria tested induced chlorosis in bean plants when resuspended in mannitol solution. None of the bacteria tested, including the type B strains of *R. tropici*, induced chlorosis when resuspended in water alone (data not shown).

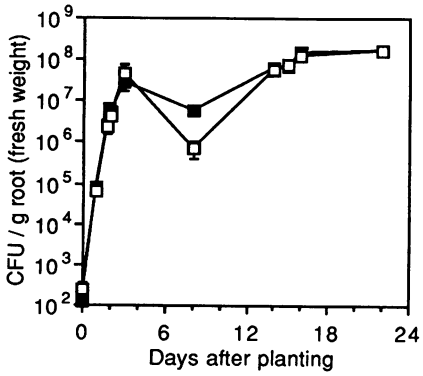

FIG. 1. Effects of mannitol on the growth rate and population density of *R. tropici* CT9005 in the bean plant rhizosphere. Bacteria were resuspended in water (□) or 10 g of mannitol per liter (■) prior to inoculation of seeds of bean cultivar WBR22-34. The experiment was performed as described in Materials and Methods. Each value represents the mean ± standard error of 8 to 10 measurements. Data are representative of two experiments.

TABLE 3. Ability of plant-associated bacteria to induce chlorosis in common bean

Strain or treatment	Chlorosis rating[a]	Source[b] or reference
R. tropici		
IAPAR 69	1.0 ± 0.0 g	14
BR436	1.0 ± 0.0 g	14
Type A		
C-O5-I	1.0 ± 0.0 g	14
BR842	1.0 ± 0.0 g	14
CFN299	1.0 ± 0.0 g	14
Type B		
BR847	3.2 ± 0.3 cd	14
BR850	4.8 ± 0.1 a	14
BR854	4.3 ± 0.3 ab	14
BR857	3.9 ± 0.4 bc	14
BR862	1.6 ± 0.2 fg	14
CIAT166	2.3 ± 0.4 ef	14
CIAT899	4.0 ± 0.4 abc	14
R. leguminosarum		
bv. phaseoli		
CE3	1.0 ± 0.0 g	17
KIM5s	1.0 ± 0.0 g	2
bv. trifolii USDA2046	1.0 ± 0.0 g	USDA
Rhizobium meliloti Rm2011	1.0 ± 0.0 g	9
B. japonicum USDA110	1.0 ± 0.0 g	USDA
Agrobacterium tumefaciens C58	1.0 ± 0.0 g	27
50 g of mannitol per liter (no bacteria)	1.0 ± 0.0 g	
Water	1.0 ± 0.0 g	

[a] Each value represents the mean chlorosis rating ± standard error of 8 to 10 plants. Values followed by the same letter are not significantly different ($P \leq 0.05$) by Tukey's Studentized range test (honest significant difference = 0.85). Each strain was grown to saturation and diluted 100-fold into 50 g of mannitol per liter before adding to seeds of bean cultivar WBR22-34 at planting. Data are representative of three experiments.

[b] USDA, *Rhizobium* culture collection, U.S. Department of Agriculture, Beltsville, Md.

Nodulation and chlorosis induction. We explored the role of nodulation in chlorosis induction with a nonnodulating host and a nonnodulating derivative of CIAT899. CIAT899 induced foliar chlorosis in nonnodulating bean line NOD125 (6, 21) and in its parent line, RIZ30 (Table 4). CIAT899 formed nodules on the root of one of eight NOD125 plants. This may be attributed to contamination of the NOD125 stock with a seed of the parental line; some leakiness of the NOD125 phenotype, which is not well characterized; or residual heterozygosity in the NOD125 population. All NOD125 plants inoculated with CIAT899, however, were chlorotic. The roots of RIZ30 and WBR22-34 plants were nodulated (Table 4). We also tested the chlorosis-inducing ability of RSP900, a Nod$^-$, pSym-cured derivative of CIAT899 (25). RSP900 induced chlorosis in the leaves of bean lines NOD125, RIZ30, and WBR22-34 and did not form nodules on the roots of any of the three cultivars (Table 4).

TABLE 4. Chlorosis induced by CIAT899 and RSP900 in wild-type and nodulation-defective cultivars of common bean

Bean cultivar	Strain	Chlorosis rating[a]	Nodule formation
WBR22-34 (Nod^+)	CIAT899 (Nod^+)	4.3 ± 0.4	+
WBR22-34 (Nod^+)	RSP900 (Nod^-)	3.8 ± 0.5	−
RIZ30 (Nod^+)	CIAT899 (Nod^+)	3.0 ± 0.5	+
RIZ30 (Nod^+)	RSP900 (Nod^-)	3.4 ± 0.4	−
NOD125 (Nod^-)	CIAT899 (Nod^+)	4.4 ± 0.4	−[b]
NOD125 (Nod^-)	RSP900 (Nod^-)	3.1 ± 0.5	−

[a] Values represent the mean ± standard error of 8 to 10 plants per treatment. Values do not differ significantly ($P \leq 0.05$) by Tukey's Studentized range test (honest significant difference = 1.93).
[b] Nodules found on one plant. All plants were chlorotic.

Chlorosis induction by CIAT899 in symbiotic host and nonhost species. Leaves of three symbiotic hosts of CIAT899, beans, siratro, and *Leucaena leucocephala*, showed interveinal chlorosis approximately 3 weeks after seeds were planted and inoculated with 10^7 cells of CIAT899 resuspended in 50 g of mannitol per liter (Table 5). CIAT899 also induced severe interveinal chlorosis in the first true leaves of alfalfa and Berseem clover and in some sunflower, cucumber, and tomato seedlings (Table 5). CIAT899 cells resuspended in water did not induce chlorosis in nonhost plants (data not shown). Chlorosis was not observed in the leaves of seedlings of corn or wheat inoculated with CIAT899 in mannitol solution. Mannitol solution alone did not induce chlorosis in any plant species tested (data not shown).

DISCUSSION

We have demonstrated that the ability to induce foliar chlorosis in bean plants is a property of several type B strains of *R. tropici*. A wide range of dicots were susceptible to chlorosis induced by *R. tropici*, including host legumes, legumes that are not symbiotic hosts for CIAT899, and dicot species that are not legumes.

Our data are consistent with the hypotheses that (i) type B strains of *R. tropici* produce a chlorosis-inducing factor in the rhizosphere of susceptible plants or that (ii) cells must make physical contact with the root in order to induce chlorosis. Cells of *R. tropici* induced chlorosis after they were washed and resuspended in solutions of carbon sources, indicating that culture supernatants were not required for chlorosis induction. Chlorosis induction required the presence of cells of *R. tropici* in the rhizosphere or inside the root, since cell-free bacterial culture filtrates did not induce chlorosis. These results may indicate that the synthesis of the chlorosis-inducing factor requires the presence of some compound(s) supplied by the plant, either as an inducer of gene expression or as a precursor that *R. tropici* converts to the chlorosis-inducing factor. If the plant does provide an inducer of the synthesis of a chlorosis factor, then *nodD*, which senses the inducers of *nod* genes (8), is not the bacterial sensor for the inducer, since strain RSP900 lacks a detectable *nodD*-containing DNA fragment (25) but did induce chlorosis. The ability of RSP900 to induce chlorosis without nodulating bean plants provides evidence that nodulation is not required for chlorosis induction, but this result does not rule out the possibility that chlorosis-inducing factors are also produced inside nodules. The ability of CIAT899 to induce chlorosis in several nonhost and nonlegume species further demonstrates that nodulation is not required for chlorosis induction and indicates that if a compound of plant origin is required for the bacteria to produce a chlorosis-inducing factor, the ability to make such an inducer must be widely distributed among dicots.

It is possible that bacterial culture conditions may not be conducive to the accumulation of the chlorosis-inducing factor. For example, chlorosis-inducing strains of *R. tropici* may require more than 3 days in broth culture to produce chlorosis-inducing factors, or the chlorosis-inducing factor may be unstable in culture. Experiments are in progress to distinguish among these possibilities. Other members of the *Rhizobiaceae*, the rhizobitoxine-producing strains of *B. japonicum*, induce chlorosis in their symbiotic legume hosts (10, 16, 20); however, we have found no evidence of rhizobitoxine in culture filtrates of chlorosis-inducing *R. tropici* strains by either chemical or enzymatic assays (19, 22, 23). However, rhizobitoxine may be produced by *R. tropici* type B strains under conditions other than those in broth culture.

The requirement for carbon in chlorosis induction is consistent with our previous finding that cultures of CIAT899 and CT9005 induce less chlorosis upon dilution and suggests an explanation for the difference in the severity of

TABLE 5. Chlorosis induced by CIAT899 in symbiotic host and nonhost plants

Plant species	No. of chlorotic plants/ no. of plants rated[a]	Germplasm source[b]
Common bean (*P. vulgaris* L.)[c]	20/20	K. Kmiecik
Leucaena leucocephala (Lam.) de Wit, PI 404037	6/13	USDA
Leucaena leucocephala (Lam.) de Wit, PI 435912	12/21	USDA
Siratro (*M. atropurpureum* Urb.)	19/20	M. Sadowsky
Alfalfa (*Medicago sativa* L.) cv. Iroquois	34/38	D. Viands
Berseem clover (*Trifolium alexandrinum* L.)	80/88	E. Triplett
Tomato (*Lycopersicon esculentum* L.) cv. Patio Hybrid	8/24	Olds Seed Co.
Cucumber (*Cucumis sativus* L.) cv. Salad Bush	7/24	Olds Seed Co.
Sunflower (*Helianthus annuus* L.) var. Massive Russian	7/12	Olds Seed Co.
Corn (*Zea mays* L.) var. A619	0/15	J. Coors
Wheat (*Triticum sativa* L.)	0/17	C. Smejkal

[a] Data are combined from two or three experiments per species.
[b] K. Kmiecik, Department of Horticulture, University of Wisconsin, Madison; M. Sadowsky, Department of Soil Science, University of Minnesota, St. Paul; D. Viands, Department of Plant Breeding, Cornell University, Ithaca, N.Y.; USDA, Beltsville, Md.; E. Triplett, Department of Agronomy, University of Wisconsin, Madison; Olds Seed Co., Madison, Wis.; J. Coors, Department of Agronomy, University of Wisconsin, Madison; C. Smejkal, Department of Plant Pathology, University of Wisconsin, Madison.
[c] A total of 18 cultivars were tested, including red, pinto, and black bean varieties. All cultivars tested were susceptible.

chlorosis induced by the two strains (18). In the earlier work, cultures of CIAT899 and CT9005 were diluted in water rather than fresh YM medium or mannitol solutions. Since we demonstrate here that, given a carbon source, relatively low populations of bacteria in the inoculum are sufficient to induce chlorosis, we believe that the decrease in severity observed when cultures were diluted in water was due to a dilution of free carbon remaining in the growth medium rather than a decrease in the number of bacteria applied. Since CIAT899 makes more EPS in broth culture than its EPS-deficient mutant CT9005 (15, 18), the spent growth medium of CIAT899 cultures may contain less carbon than cultures of CT9005, and as we show in this paper, CT9005 requires less carbon to induce symptoms of a given severity than CIAT899. Therefore, EPS production may decrease the severity of chlorosis induction by serving as a sink for available carbon in the rhizosphere, diverting carbon from the synthesis of a chlorosis-inducing factor. The inability of EPS from CIAT899 to inhibit chlorosis induction by CT9005 cells suspended in mannitol and the ability of several wild-type, EPS-producing strains of *R. tropici* to induce chlorosis when provided with sufficient carbon strongly suggest that EPS plays no direct role in preventing the development of chlorosis in bean plants or in preventing the bacteria from inducing these symptoms, as we previously suggested (18).

Although carbon is required for chlorosis induction, its role is unclear. Mannitol did not affect the growth of strain CT9005 in the rhizosphere, indicating that the availability of carbon did not limit growth in the rhizosphere. During the time after exponential growth in the bean rhizosphere, *R. tropici* may produce chlorosis-inducing compounds from secondary metabolic pathways that require an abundance of carbon, or an abundance of carbon in the rhizosphere may repress genes that prevent chlorosis induction. We know of no reports attributing chlorosis of beans in the field to the presence of type B strains of *R. tropici* in the rhizosphere, although it is conceivable that conditions in the field that lead to increased exudation of carbon from roots might provide conditions required for chlorosis induction.

The broad target range of the chlorosis-inducing factor makes it particularly interesting and potentially useful, since it might be a specific inhibitor of a plant enzyme, as are many of the phytotoxins produced by microorganisms. The chlorosis-inducing factor may provide a tool to study the biochemistry and physiology of plants, or it may be a model for pathogenesis and plant-bacterium signal exchange.

Chlorosis induction raises several questions about the evolution of *R. tropici*. Does chlorosis induction confer some selective advantage on *R. tropici*, either in the soil or in forming symbioses with its hosts? Is the ability to induce chlorosis vestigial, an important factor in some interaction with plants that is no longer important in the biology of *R. tropici*? Is chlorosis induction an important factor distinguishing type B strains from other strains of *R. tropici*? It will be interesting to determine whether *R. tropici* produces chlorosis-inducing factors under field conditions and, if so, whether they affect nitrogen fixation and overall plant and microbial health in the field.

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REFERENCES

1. **Araujo, R. S., J. Maya-Flores, D. Barnes-McConnell, C. Yokoyama, F. B. Dazzo, and F. A. Bliss.** 1986. Semienclosed cultures of bean plants (*Phaseolus vulgaris*) for enumeration of *Rhizobium phaseoli* by the most-probable-number technique. Appl. Environ. Microbiol. **52**:954–956.
2. **Beattie, G. A., M. K. Clayton, and J. Handelsman.** 1989. Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum* bv. *phaseoli*. Appl. Environ. Microbiol. **55**:2755–2761.
3. **Bergersen, F. J.** 1961. The growth of *Rhizobium* on synthetic medium. Aust. J. Biol. Sci. **14**:349–360.
4. **Bliss, F. A., P. A. A. Pereira, R. S. Araujo, R. A. Henson, K. A. Kmiecik, J. R. McFerson, M. G. Teixeira, and C. C. DeSilva.** 1989. Registration of five high nitrogen fixing common bean germplasm lines. Crop Sci. **29**:240–241.
5. **Brom, S., E. Martinez, G. Davila, and R. Palacios.** 1988. Narrow- and broad-host-range symbiotic plasmids of *Rhizobium* spp. strains that nodulate *Phaseolus vulgaris*. Appl. Environ. Microbiol. **54**:1280–1283.
6. **Davis, J. H. C., K. E. Giller, J. Kipe-Nolt, and M. Awah.** 1988. Non-nodulating mutants in common bean. Crop Sci. **28**:859–860.
7. **Graham, P. H., S. E. Viteri, F. Mackie, A. T. Vargas, and A. Palacios.** 1982. Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res. **5**:121–128.
8. **Györgypal, Z., B. K. György, and A. Kondorosi.** 1991. Transduction of plant signal molecules by the *Rhizobium* NodD proteins. Bioessays **13**:575–581.
9. **Hynes, M. F., R. Simon, P. Müller, K. Niehaus, M. Labes, and A. Pühler.** 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202**:356–362.
10. **Johnson, H. W., U. M. Means, and F. E. Clark.** 1958. Factors affecting the expression of bacterial-induced chlorosis of soybeans. Agron. J. **50**:571–574.
11. **Kingsley, M. T., and B. B. Bohlool.** 1992. Extracellular polysaccharide is not responsible for aluminum tolerance of *Rhizobium leguminosarum* bv. phaseoli CIAT899. Appl. Environ. Microbiol. **58**:1095–1101.
12. **Kumar Rao, J. V. D. K., P. J. Dart, and M. Usha Kiran.** 1984. *Rhizobium*-induced leaf roll in pigeonpea (*Cajanus cajan* (L.) Millsp.). Soil Biol. Biochem. **16**:89–91.
13. **Martinez, E., M. A. Pardo, R. Palacios, and M. A. Cevallos.** 1985. Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J. Gen. Microbiol. **131**:1779–1786.
14. **Martinez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41**:417–426.
15. **Milner, J. L., R. S. Araujo, and J. Handelsman.** 1992. Molecular and symbiotic characterization of exopolysaccharide-deficient mutants of *Rhizobium tropici* strain CIAT899. Mol. Microbiol. **6**:3137–3147.
16. **Minamisawa, K.** 1989. Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum*. Plant Cell Physiol. **30**:877–884.
17. **Noel, K. D., A. Sanchez, L. Fernandez, J. Leemans, and M. A. Cevallos.** 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. **158**:148–155.
18. **O'Connell, K. P., R. S. Araujo, and J. Handelsman.** 1990. Exopolysaccharide-deficient mutants of *Rhizobium* sp. strain CIAT899 induce chlorosis in common bean (*Phaseolus vulgaris*). Mol. Plant-Microbe Interact. **3**:424–428.
19. **O'Connell, K. P., and J. Handelsman.** Unpublished data.
20. **Owens, L. D., and D. A. Wright.** 1965. Production of the

soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. Plant Physiol. **40**:931–933.

21. **Pedalino, M., K. E. Giller, and J. Kipe-Nolt.** 1992. Genetic and physiological characterization of the non-nodulating mutant of *Phaseolus vulgaris* L. - Nod125. J. Exp. Bot. **43**:843–849.
22. **Peters, N. K. (Ohio State University).** 1990. Personal communication.
23. **Ruan, X., and K. Peters.** 1991. Rapid and sensitive assay for the phytotoxin rhizobitoxine. Appl. Environ. Microbiol. **57**:2097–2100.
24. **SAS Institute Inc.** 1988. SAS/STAT user's guide, release 6.03 ed. SAS Institute Inc., Cary, N.C.
25. **Vargas, C., L. J. Martinez, M. Megias, and C. Quinto.** 1990. Identification and cloning of nodulation genes and host specificity determinants of the broad-host-range *Rhizobium leguminosarum* bv. *phaseoli* strain CIAT899. Mol. Microbiol. **4**:1899–1910.
26. **Wacek, T. J., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. **16**:518–523.
27. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123**:255–264.