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Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control

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Abstract

Changes in microbial communities associated with nematode control were studied by comparing population numbers of fungi and bacteria in the soil and in internal root tissues (endorhiza) in non-amended and chitin-amended soils. Addition of chitin to soil at 1% (w/w) eliminated plant-parasitic nematodes in a first planting of cotton cv. 'Rowden' and significantly reduced *Meloidogyne incognita* infestation in a second planting, confirming long-term nematode suppressiveness induced by this organic amendment. The chitin amendment was associated with an increase in fungal and bacterial populations, especially those with chitinolytic activity. The bacterial communities of soil, rhizosphere and endorhiza were assessed by examining the taxonomic diversity of recoverable bacteria based on identification with fatty acid analysis of sample sizes of 35 soil and rhizosphere bacteria and 25 endophytic bacteria. All major bacterial species which formed at least 2% of the total population in non-amended soils and rhizospheres also occurred with chitin amendment. In contrast, chitin-amended soils and rhizospheres yielded several species which were not found without chitin amendment, including *Aureobacterium testaceum, Corynebacterium aquaticum* and *Rathayibacter tritici. Burkholderia cepacia* was recovered from both amended and non-amended soils and rhizospheres, but it was most abundant with chitin amendment at the end of the first cotton planting. Soil was probably the major source for bacterial endophytes of cotton roots, since nearly all endophytic bacteria were also found in the soil or rhizosphere. However, two dominant genera in the soil and rhizosphere, *Bacillus* and *Arthrobacter,* were not detected as endophytes. Chitin amendment exhibited a further specific influence on the endophytic bacterial community; *Phyllobacterium rubiacearum* was not a common endophyte following chitin amendment, even though chitin amendment stimulated its populations in non-planted soil. *Burkholderia cepacia,* found in similar numbers in the soil of both treatments, was the dominant endophyte in plants grown in chitin-amended soil but rarely colonized cotton roots grown in non-amended soil. These results indicate that application of an organic amendment can lead to modifications of the bacterial communities of the soil, rhizosphere and endorhiza.

Keywords: Chitin amendment; Endophytic bacteria; *Gossypium hirsutum; Meloidogyne incognita;* Rhizosphere bacteria

Introduction

Naturally-occurring nematode suppressiveness has been reported for several agricultural systems (Stirling et al., 1979; Kerry, 1982; Kluepfel et al., 1993), but suppressiveness can also be induced by crop rotation with antagonistic plants such as switchgrass *(Panicum virgatum)* (Kokalis-Burelle et al., 1995) and velvetbean *(Mucuna deeringiana)* (Vargas et al., 1994) or organic amendments including pine bark (Kokalis-Burelle et al., 1994), hemicellulose (Culbreath et al., 1985) and chitin (Mankau and Das, 1969; Spiegel et al., 1986; Rodríguez-Kábana and Morgan-Jones, 1987). A major component of the suppressiveness of chitin amendments is believed to be biotic and several reports confirm increased numbers of nematode antagonistic microorganisms associated with chitin-induced sup-

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pressive soils (Godoy et al., 1983; Rodríguez-Kábana et al., 1984). Extensive work has been done over the past years on fungi associated with chitin amendments (Godoy et al., 1983; Rodríguez-Kábana et al., 1984); however, information on bacterial community structure and the role of bacteria in chitin-induced suppressiveness is still very limited. We chose chitin amendments as a model system to study the effect of suppressiveness on bacterial diversity in the soil and endorhiza. Endophytic bacteria were included in this study because they colonize the same root tissues as sedentary plant-parasitic nematodes. This association of endophytic bacteria with nematodes throughout the nematode life cycles makes these bacteria excellent candidates for biocontrol strategies.

Chitin is a structural component of some fungi, insects, various crustaceans and nematode eggs. In egg shells of tylenchoid nematodes, chitin is located between the outer vitelline layer and the inner lipid layer and may occur in association with proteins (Bird and Bird, 1991). The breakdown of this polymer by chitinases can cause premature hatch, resulting in fewer viable juveniles (Mercer et al., 1992). In the soil, chitinases are produced by some actinomycetes (Mitchell and Alexander, 1962), fungi (Mian et al., 1982) and bacteria (Ordentlich et al., 1988; Inbar and Chet, 1991), but chitinases are also released by many plants as part of their defense mechanism against various pathogens (Punja and Zhang, 1993) and plant-parasitic nematodes (Roberts et al., 1992). Chitinases depolymerize the chitin polymer into *N* -acetylglucosamine and chitobiose. Further microbial activity results in the deamination of the sugar and accumulation of ammonium ions and nitrates (Rodríguez-Kábana et al., 1983). Nematicidal concentrations of ammonia in association with a newly formed chitinolytic microflora are believed to cause nematode suppressiveness (Mian et al., 1982; Godoy et al., 1983). Benhamou et al. (1994) have shown that chitosan, the deacetylated derivative of chitin, induces systemic plant resistance against *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomato when applied as a seed treatment or soil amendment. This suggests that plant defense mechanisms might contribute to the overall nematode suppression. Our objective was to determine if the chitin-mediated suppression of plant parasitic nematodes is related to changes in bacterial communities in soils, rhizospheres or within cotton roots.

Material and methods

2.1. Greenhouse studies

The effect of chitin on *Meloidogyne incognita* was studied under greenhouse conditions using soil from the E.V. Smith Research Center, Plant Breeding Unit of the Alabama Agricultural Experiment Station near Tallassee, Alabama, USA. The soil, which was collected from a cotton field at the end of the season, was a sandy loam with pH = 6.5 and organic matter content < 1% (w/w). The soil was naturally infested with *M. incognita, Hoplolaimus* spp. and *Paratrichodorus* spp. at populations of 3, 7 and 6 nematodes 100 cm^{-3}, respectively, and with free-living species at 44 nematodes 100 cm^{-3}. This field soil was mixed 1: 1 (w/w) with sand, hereafter referred to as soil. Crustacean chitin flakes (United States Biochemical Corporation, Cleveland, OH) were ground to pass a 2 mm sieve and mixed into half of the soil to give a final concentration of 1% (w/w). Chitin-amended and non-amended soil were transferred into 1000 cm^{-3} cylindrical pots and maintained moist at about 60% field capacity for 3 weeks in a greenhouse at 20-30°C to allow decomposition of the chitin. The total number of pots filled with either non-amended soil or chitin-amended soil was further divided into two sets of equal numbers with one half of the pots of each treatment seeded with cotton *(Gossypium hirsutum* L. cv. 'Rowden') at 3 seeds per pot. The seedlings were thinned to 1 plant pot^{-1} after emergence. The experiment consisted of four treatments: non-amended soil without cotton, non-amended soil planted with cotton, chitin-amended soil without cotton, and chitin-amended soil planted with cotton. The experiment was set up as a randomized complete block design with eight replications. All replications were used for recording plant growth and nematode infestation. To describe soil physical and chemical properties as well as microbial populations, four replications were sufficient to demonstrate significant differences, so the first four replications of each treatment were used. Plants were fertilized in alternate weeks with 15 ml of Peters' fertilizer (20-20-20) solution (Scotts-Sierra, Marysville, OH).

At 54 d after chitin amendment, the complete cotton plants were harvested. Plant fresh weight was recorded and the roots were processed for isolation of endophytic bacteria. The remaining soil was placed back into the pots and replanted with cotton cv. 'Rowden'. Due to low numbers of plant-parasitic nematodes in the initial soil of the first cotton crop, all pots were inoculated with approximately 3,000 eggs of *M. incognita* 8 d after planting using the alginate film method described by Rodríguez-Kábana et al. (1994). Four films of approximately 750 eggs $film^{-1}$ were buried around the cotton root at about 1 cm depth and 1 cm distance from the tap root. For the films, eggs were extracted from galled cotton roots by the NaOCl-method (Hussey and Barker, 1973). Plants were fertilized once with 15 ml Peters' solution 3 weeks after planting and the experiment was terminated 6 weeks after the second planting. Plant growth was recorded

and the gall index determined using 0-10 scale with 0 = no galls and 10 = maximum gall infestation (Zeck, 1971). The experiment lasted for 96 d from the time of chitin application to the final evaluation of the second cotton crop.

The experiment comprised four sampling times: d 0 (before chitin amendment), d 21 (before planting), d 54 (end of first cotton planting) and d 98 (end of second cotton planting). Soil and rhizosphere samples of various size were taken to measure pH and to estimate nematode, fungal, bacterial and chitinolytic populations as well as bacterial diversity at each sample time. The term rhizosphere was used for soil thoroughly rooted with cotton. After 54 d, shoot and root fresh weight, numbers of galls and population sizes of indigenous fungal, bacterial and chitinolytic endophytes as well as endophytic bacterial diversity were determined. At the final sampling, only plant growth and gall index were recorded.

2.2. Soil pH and nematode populations

For each sampling, soil was transferred into 4-1 capacity polyethylene bags, thoroughly mixed and aliquots were taken to measure the different variables. Soil pH was determined in a suspension of 10 g moist soil and 10 ml demineralized water after shaking for 30 min. For nematode counts, 100 cm^{-3} soil aliquots were incubated for 72 h using a modified Baerman technique (Rodríguez-Kábana and Pope, 1981). The numbers of nematodes were determined for the main genera of plant-parasitic nematodes and free living nematodes.

2.3. Soil and rhizosphere microbial populations

Soil microbial populations were determined at d 0, d 21 and d 54 and rhizosphere populations at d 54. Soil (10 g) was added to 100 ml 20 mM sterile potassium phosphate buffer, pH = 7.0. The flasks were shaken on an orbital shaker at 200 rpm for 30 min. A serial dilution was prepared and 52 µl aliquots of dilutions 10^{-2}, 10^{-4} and 10^{-6}were plated on three media using a spiral plater (Spiral System, Cincinnati, OH). The media were: 5% strength tryptic soy agar (5% TSA) (Difco Laboratories, Detroit, MI) containing 150 mg cycloheximide 1^{-1} to support the growth of a broad range of bacteria; Ohio agar (Johnson and Curl, 1972) containing 150 mg streptomycin 1^{-1} for fungal populations and chitin agar containing 0.2% colloidal chitin (Godoy et al., 1982) to estimate total chitinolytic microorganisms. Only microorganisms producing a clearing zone around the colony were counted as chitinolytic.

2.4. Plant preparation and surface-disinfestation

Fresh weights of cotton roots and stems were recorded and the roots were washed with tap water. The roots were then surface-disinfested in a mixture of 1.05% sodium hypochlorite (Clorox@ household bleach diluted 1:5 with tap water) and 0.1% Tween 20 (Fisher Biotech, Fair Lawn, NJ) for 60 s followed by three rinses in sterile phosphate buffer. The whole root system was imprinted on full strength TSA to check for root surface contamination. If microbial growth on the TSA check occurred within 48 h, the surface-disinfestation was considered as incomplete and the samples were rejected. Following the sterility check, roots were triturated in 5 times the root weight in sterile phosphate buffer (w/v) with mortar and pestle. The macerate was serially diluted in sterile H_20 and dilutions $l0^o$, 10^{-2} and $l0^{-4}$ were plated on each of the three media indicated above. Agar plates were incubated at 27°C for 48 h (5% TSA, Ohio agar) or 96 h (chitin agar), respectively.

2.5. Bacterial diversity

Single colonies were selected from 5% TSA plates by starting at the outside of the plate and transferring each colony along the spiral line onto fresh TSA plates until a total of 50 colonies was taken per sample. The bacteria were incubated at 27°C and bacterial purity was checked visually after 48 h. One loop with approximately 50 mg bacterial cells was then transferred into 1.2 ml vials containing 0.8 ml sterile tryptic soy broth (TSB) (Difco, Detroit, MI) and 0.2 ml glycerine. The samples were stored in a Nalgene@ cryobox at -80°C until processed for identification. For bacterial diversity, the first 35 colonies per plate from soil samples were identified, while for endophytes, the first 25 colonies per plate were identified. Using rarefraction analysis, these numbers were previously reported as sufficient for community studies at the genus level (Mahaffee and Kloepper, 1997). Bacterial identification was based on analysis of fatty acid methyl-esters (FAMEs) of total cellular fatty acids (Sasser, 1990). The extraction procedure was as described by McInroy and Kloepper (1995) and samples were processed with the microbial identification system (MIS) of MIDI (Newark, DE). FAME peaks were identified by the MIS software and bacterial isolates were identified using the MIS 'Aerobe Library' (Version 3.7). Bacterial strains with a similarity index below 0.100 were considered unidentified. Bacterial diversity was characterized at the genus level with two indices: richness (total number of genera) and Hill's diversity number Nl which combined richness and evenness (Ludwig and Reynolds, 1988).

Table 1
Effect of 1% chitin in soil on plant growth and *Meloidogyne incognita* infestation of the first cotton planting 54 d after amendment

Treatment	Shoot fresh weight (g)	Root fresh weight (mg)		Number of galls plant
Control	1.75 a	730 a	2.40 b	8 a
Chitin	1.84 a	320 b	5.75 a	0 b
LSD	0.44	130	0.80	3.3

Means with the same letter are not significantly different at $P = 0.05$, $n = 8$. Number of replicates = 8.

2.6. Statistical analysis

Bacterial and fungal population numbers were transformed to log,, cfu g^{-1} soil or root, respectively. Statistical analysis was performed using SAS general linear models and LSD procedures (SAS, Cary, NC). Unless otherwise stated, differences referred to in the text were significant at $P = 0.05$.

3. Results

There were no differences in shoot fresh weights of cotton plants of the first planting grown in soil amended with 1% chitin and those grown in non-amended soil (Table 1). Nevertheless, root fresh weight was significantly reduced for plants grown in chitin-amended soil averaging 320 mg $plant^{-1}$ compared to 730 mg $plant^{-1}$ for cotton grown in non-amended soil. The shoot to root ratio was significantly higher for plants grown in chitin-amended soil (5.75) than in non-amended soil (2.40).

The initial nematode population was 16 plant-parasitic and 44 free-living nematodes 100 cm^{-3} soil (Table 2). Individual plant-parasitic nematodes were observed throughout the experiment in the non-amended soil but not in chitin-amended soil. Some *Meloidogyne* spp. galls were formed on plants from non-amended soil and no galls were observed in cotton roots from chitin-amended soil (Table 1). Populations of free-living nematodes were significantly higher in chitin-amended soil than in non-amended soil.

Soil-pH was initially 7.3 and it changed by d 21 to 6.9 and 6.9 for non-amended and chitin-amended soil, respectively. At d 54, pH ranged from 6.6-6.9 in non-amended soil and between pH 4.9-5.0 for chitin-amended soil.

The second cotton planting was set up to evaluate long-term effects of chitin amendment on nematode suppression. To determine suppressiveness, the soil of each of the four treatments was challenge-inoculated with *M. incognita* eggs. Cotton grown in chitin-amended soil previously planted with cotton yielded the highest shoot weight (4.99 g) (Table 3) whereas shoot weight in non-amended soil was significantly lower for both, previously non-planted (3.75 g) and planted (2.41 g) soil. Both treatments lacking cotton crops in the first planting resulted in the lowest root weight, with 2.24 g for non-amended soil and 2.06 g for chitin-amended soil. The shoot to root ratio was lowest for cotton grown in non-amended soil previously planted with cotton (0.83) and highest for cotton grown in chitin-amended soil previously without cotton (2.08).

Chitin amendment significantly reduced the gall index from 3.71 and 3.28, respectively, for cotton

Table 2
Effects of 1% chitin amendment to soil on cotton cv. 'Rowden' on soil nematode populations

Nematode taxon	Number of nematodes 100 cm^{-3} soil of indicated treatment[a] over time						
	d 0	d 21	d 54				
			3	1	2	3	4
Meloidogyne	3	0	0	0	0	0	0
(Para-) Trichodorus	6	1	0	0	4	0	0
Hoplolaimus	7	4	0	0	0	0	0
Tylenchorhynchus	0	0	0	0	1	0	0
Free-living species	44	33	1814	4	30	3424	4338
Total	60	38	1814	4	35	3424	4338

[a] Treatments were: 1 = non-amended soil without cotton, 2 = non-amended soil with cotton, 3 = chitin-amended soil without cotton, and 4 = chitin-amended soil with cotton.

Table 3
Effects of 1% chitin in soil on plant growth and gall index of *Meloidogyne incognita* infestation of the second cotton planting 96 d after amendment

Previous treatment[a]	Shoot weight (g)	Root weight (g)	Shoot to root ratio	Gall index[b]
-chitin, -cotton	3.75 b	2.24 bc	1.78 ab	3.71 a
-chitin, + cotton	2.41 c	2.85 ab	0.83 c	3.28 a
+ chitin, -cotton	4.27 ab	2.06 c	2.08 a	0.75 b
+ chitin, + cotton	4.99 a	3.22 a	1.64 b	0.86 b
LSD	1.14	0.64	0.44	2.10

[a] In the second cotton planting all treatments were planted with cotton. However, to compare date with the first cotton planting, this table lists the treatments as described for the first cotton planting.[b]Gall index on a scale from 0-10 with 0 = no galls and 10 = maximum number of galls (Zeck, 1971).Means with the same letter are not significantly different at $P = 0.05$, $n = 8$.

grown in non-amended soil previously non-planted and planted with cotton, to 0.75 and 0.86 for cotton grown in chitin-amended soil (Table 3).

Amendment of soil with 1% chitin generally resulted in significant increases in soil populations of total culturable aerobic bacteria on 5% TSA, fungi on Ohio agar, relative to populations in non-amended soil (Table 4). In addition, bacterial and fungal populations of the rhizosphere and inside roots, as detected on 5% TSA and Ohio agar, were significantly higher, following chitin amendment, at 21 and 54 d after planting. Numbers of chitinolytic microorganisms in soil and rhizosphere samples were also significantly enhanced by chitin amendment.

The total spectrum of soil bacteria isolated from chitin-amended and non-amended soil is shown in Table 5. Gram-positive bacteria accounted for 73.8% of the total number of species from the test soil (d 0) and remained dominant in non-amended soil at d 21 and d 54. In contrast, in chitin-amended soil, Gram-positive bacteria were less frequent than Gram-negative bacteria at 21 and 54 d. Most species occurred in low numbers throughout the experiment, regardless of treatment or sampling time. Among the predominant taxa, some effects of chitin-amendment were noted. *Arthrobacter* and *Bacillus* were the two dominant Gram-positive bacteria in the test soil (d 0) and in the non-amended soil at d 21; however, their frequency of recovery was greatly reduced at d 21 in chitin-amended soil. The frequency of *Arthrobacter* at d 54 in soils and rhizospheres was about 90% less from chitin-amended than from non-amended treatments. Chitin amendment also led to a decreased frequency of recovering *Burkholderia* spp from soil at d 21 and 54, compared to the non-amended soil; however, rhizosphere populations were higher from chitin-amendments than from the non-amended treatment. Some species which contributed at least 5% of the total population were only recovered following chitin-amendment, including *Aureobacterium testaceum*, *Corynebacterium aquaticum* and *Rathayibacter tritici*, while all species found in non-amended soil were also recovered from chitin-

Table 4
Effect of 1% chitin amendment on microbial populations of soil, rhizosphere and endorhiza

Sample time (d)	Sample (S, R, E)[a]	Chitin amendment (+ or -)	log cfu g^{-1}		
			5% TSA	Ohio agar	chitin agar
d 0	S	-	6.39	3.74	5.29
d 21	S	-	6.83 b	3.67 b	5.66 b
	S	+	8.29 a	4.59 a	7.14 a
d 54	S	-	6.38 b	3.80 b	5.51 b
	R	-	6.39 b	3.90 b	5.51 b
	S	+	7.11 a	6.17 a	6.55 a
	R	+	7.10 a	6.17 a	6.84 a
	E	-	5.43 b	1.67 b	1.41[b]
	E	+	5.74 a	3.36 a	2.87

[a] S = non-planted soil; R = rhizosphere soil; E = endorhiza. [b]Data were not statistically analyzed, since chitinolytic bacteria were only observed in 3 out of 8 roots for the control and 5 out of 8 roots grown in chitin-amended soil; presented is the average chitinolytic population for those samples from which chitinolytic microorganisms were recovered.Means with the same letter within the same sampling time are not significantly different at $P = 0.05$, $n = 8$.

Table 5
Temporal changes in the isolation frequency[a] (%) of indigenous soil bacteria affected by chitin amendment (1 %) and planting with cotton cv. 'Rowden'

Bacterial species	EBC[b]	Sample (S, R)[c] (Chitin amendment, + /-)						
		day 0	day 21		day 54			
		S -	S -	S +	S -	R -	S +	R +
Gram-positive								
A. globiformis		11.5	4.4	3.2	7.7	6.1	0.7	
A. ilicis		9.0	17.8	7.8	17.5	19.6		
Arthrobacter, total		23.0	22.9	13.4	31.5	28.5	0.7	2.1
Aureobacterium testaceum							3.5	12.9
Bacillus megaterium		17.2	9.6	0.8	7.7	5.4	11.3	9.3
B. pumilus		9.0	8.9	0.8	8.4	4.7	2.8	2.1
Bacillus, total		36.0	23.5	1.6	19.6	17.6	15.5	16.4
Corynebacterium aquaticum				1.6			5.6	7.1
Micrococcus luteus	EBC	0.8	0.7			2.7		
Mycobacterium parafortuitum		0.8						
Paenibacillus polymyxa		1.6	3.7		5.6	4.1	0.7	1.4
Paenibacillus, total		5.7	7.4	2.4	9.8	8.2	3.5	2.1
Rathayibacter tritici				1.6			9.2	22.9
Subtotal		73.8	61.5	28.3	65.7	62.1	40.8	72.9
Gram-negative								
Agrobacterium radiobacter	EBC			7.9	1.4	2.0	1.4	1.4
Burkholderia cepacia	EBC		1.5	1.6	2.1	4.1	7.0	12.9
B. pickettii	EBC	0.8	6.7	2.4	10.5	2.7	0.7	
Burkholderia, total		0.8	8.2	4.0	13.3	7.5	7.7	12.9
Cytophaga johnsonae			0.7	9.5				
Phyllobacterium rubiacearum	EBC	1.6	0.7	5.5		4.7	0.7	2.1
Pseudomonas chlororaphis		1.6	3.0	2.4	2.8	2.7		
Pseudomonas, total		3.3	5.9	9.5	4.2	3.4		
Variovorax paradoxus			10.4	14.2	2.8	3.4	0.7	
Vibrio cholerae							6.3	
Xanthomonas campestris	EBC					2.0	0.7	4.3
Subtotal		9.8	28.1	58.3	25.2	25.7	50.7	20.7
Unidentified		16.4	10.4	13.4	9.1	12.2	8.5	6.4
Grand total		100	100	100	100	100	100	100
Number of species		31	32	31	32	36	39	22

[a] Isolation frequency per treatment is based on 35 identified isolates per replication and 4 replications per treatment. Predominant species are those isolated at > 5% frequency. Less frequently isolated Gram-positive species include *Arthrobacter citreus, A. nicotianae, A. pascens, A. protophormiae, A. saperdae, A. uratoxydans* and *A. viscosus; Aureobacterium barkeri, A. seperdae* and *A. liquefaciens; Brevibacillus brevis; Bacillus cereus, B. chitinosporus, B. circulans, B. freudenreichii, B. laterosporus, B. longisporus, B. mycoides, B. pumilus, B. sphaericus* and *B. thuringiensis; Brevibacterium helvolum; Cellulomonas biazotae, C. cartae,* C. *fimi,* and C. *gelida; Clavibacter michiganensis; Corynebacterium bovis; Curtobacterium flaccumfaciens; Gluconobacter asaii; Kurthia gibsonii; Microbacterium imperiale; Micrococcus kristinae; Paenibacillus pabuli; Rhodococcus chubuensis* and *Staphylococcus aureus.* Less frequently isolated Gram-negative species include *Acidovorax avenae* and *A. facilis; Actinobacillus lignieresii; Alcaligenes eutophus* and *A. xykisixydans; Azospirillum brasilense; Chryseobacterium balustinum* and *C. meningosepticum; Citrobacter freundii; Comamonas acidovorans* and *C. testosteroni; Enterobacter asburiae* and *E. cancerogenus; Erwinia amylovora* and *E. chrysanthemi; Escherichia coli; Flavobacterium aquatile* and *F. yabuuchiae; Kluyvera cryocrescens; Methylobacterium mesophilicum* and *M. zatmanii, Ochrobactrum anthropi; Phyllobacterium rubiacearum* and *P. myrsinacearum; Pseudomonas cichorii, P. fluorescens, P. marginalis, P. putida, P. saccharophila* and *P. savastanoi; Ralstonia solanacearum; Rhodobacter adriaticus; Sphingomonas capsulata; Vibrio fluvialis;* and *Xanthobacter agilis.* [b]EBC = endorhiza bacterial community. [c]S = nonplanted soil; R = rhizosphere.

amended soil. Analysis of bacterial diversity (Table 6) using measurements of richness and diversity, revealed that chitin-amendment resulted in significantly more genera at 54 d than non-amendment. Diversity, measured by the N1 index, was significantly higher in chitin-amended treatments than non-amended at d 21.

The range of endophytic bacteria isolated from surface-disinfested cotton roots is presented in Table 7. Except for one strain, all isolated bacteria belonged to

Table 6
Effect of chitin amendment (1%) on soil bacterial diversity at three sampling times and the endophytic population of cotton roots at the third sampling time

Index	Treatment	Day 0	Day 21	Day 54	Endophytes
Richness[a]	non-amended	7.75	10.00 a	14.50 b	4.00 a
	chitin-amended		11.75 a	17.50 a	3.75 a
Diversity N1[b]	non-amended	5.24	7.02 b	8.29 a	2.48 a
	chitin-amended		9.50 a	12.59 a	2.53 a

[a] Richness is a measure for number of genera.[b]Diversity Nl (modified by Simpson *in* Ludwig and Reynolds, 1988) is a measure for abundant taxa.Means with the same letter were not significantly different at $P = 0.05$, $n = 4$.

Gram-negative species. The endophytic bacterial community of cotton grown in non-amended soil was dominated by *Phyllobacterium rubiacearum* which accounted for 61% of the total population. Other strains with significant occurrence were *Burkholderia cepacia (9%), B. pickettii* (9%) and *Phyllobacterium myrsinacearum* (8%). In contrast, the endophytic population isolated from cotton roots grown in chitin-amended soil was dominated by *B. cepacia* with 73% of the recovered population. The number of bacterial species within one plant sample ranged from 2 to 8 species (data not shown); however, all samples were dominated by one strain with a minimum isolation frequency of at least 44% of the total population. Differences in the richness and diversity of bacterial endophytes between cotton grown in non-amended and chitin-amended soil were not significantly different (Table 6).

4. Discussion

Our results indicate that amendment of soil with chitin caused soil suppressiveness to plant parasitic nematodes and resulted in changes in the bacterial communities of the soil, rhizosphere and endorhiza.

Table 7
Effect of chitin amendment on endophytic bacterial communities in cotton roots

Bacterial taxon	Also found in		Mean % of total isolates identified as the indicated taxon[a]	
	soil	rhizosphere	non-amended soil	chitin-amended soil
Gram-positive				
Micrococcus luteus	+	+	1	0
Subtotal			1	0
Gram-negative				
Agrobacterium radiobacter	+	+	2	6
Bordetella bronchiseptica	–	–	0	1
Burkholderia cepacia	+	+	9	73
B. gladioli	–	–	1	0
B. pickettii	+	+	9	0
Burkholderia, total			19	73
Comamonas acidovorans	+	–	4	0
Enterobacter asburiae	+	–	0	3
E. cloacae	–	–	0	1
E. taylorae	+	–	0	3
Kluyvera cryocrescens	+	–	0	2
Phyllobacterium myrsinacearum	+	–	8	0
P. rubiacearum	+	–	61	7
Phyllobacterium, total			69	7
Pseudomonas saccharophila	+	–	0	1
Salmonella spp.	+	–	0	3
Xanthomonas campestris	+	+	1	0
Subtotal: identified taxa			96	100
Subtotal: unidentified taxa			4	0

[a] Isolations were made from surface-disinfested cotton roots grown in non-amended and chitin-amended (1%) soil 54 d after chitin amendment. $n = 4$.

We suggest that bacterial species, especially endophytes, which were exclusively deleted or specifically promoted by the chitin amendment might contribute to the observed suppressiveness of *M. incognita*. Although bacterial isolation on 5% TSA might represent only a small portion of the total soil and endophytic bacterial community (Bell et al., 1995), these bacteria can be easily cultivated and applied in biocontrol strategies, thus justifying our main interest in this particular group of bacteria.

Bacterial populations in amended soil were 30-fold (d 21) and 5-fold (d 54) higher than in the control and for fungal populations these differences were 8-fold (d 21) and 200-fold (d 54) (Table 4). This supports the previously published suggestion that bacteria responded much faster than fungi to the altered environmental conditions by degrading the chitin polymer (Rodríguez-Kábana et al., 1984). Following chitin-amendment, numbers of chitinolytic microorganisms were especially favored due to the chitin amendment (Table 4). Similar results were reported by Godoy et al. (1983) and Spiegel et al. (1987). Effects of chitin-amendment on bacterial communities were also expressed by changes in the frequency of isolating some species, several of which were exclusively recovered from chitin-amended soil, including *A. testaceum, C. aquaticum* and *R. tritici*, all belonging to the coryneform group of Gram-positive bacteria. A similar increase in coryneform bacteria was also reported for the rhizosphere of nematode-antagonistic sword bean *(Cannavalia ensiformis)* and velvetbean *(Mucuna deeringiana)* when compared with the rhizosphere of nematode-susceptible soybean *(Glycine max)* (Kloepper et al., 1991). The role of coryneform bacteria in nematode suppressiveness is unknown, but the antagonistic potential of this group of microorganisms has already been demonstrated for fungal (Fernando and Linderman, 1995) and bacterial (Gamard and deBoer, 1995) pathogens.

While changes in the soil bacterial community following chitin amendment were expected, interestingly, the endophytic bacterial community of cotton was also strongly affected. *Burkholderia cepacia* was the predominant species isolated from surface-disinfested cotton roots grown in chitin-amended soil, whereas the endophytic bacterial spectrum of cotton roots grown in non-amended soil was dominated by *Phyllobacterium rubiacearum.* The predominance of just one species in both treatments lead to an overall low bacterial diversity within the endorhiza. With few exceptions, all endophytic species were also found in the soil, supporting the hypothesis that soil organisms are a major source for internal colonization (Hallmann et al., *1997).* However, not all soil microorganisms can become endophytes as clearly demonstrated by the absence of Gram-positive bacteria within the roots. The internal environment of plant tissue is different from the soil and might not meet the requirements of those non-colonizing bacteria. The plant itself also seems to control internal colonization by excluding potential endophytic colonizers from penetration under certain circumstances. *P. rubiacearum* was more abundant in chitin-amended soil than in non-amended soil at planting time 21 d after chitin application, but was predominantly isolated from cotton roots grown in non-amended soil and rarely found in cotton roots grown in chitin-amended soil. In contrast, *B. cepacia* reached similar population numbers in the soil of both non-amended and chitin-amended soil but was predominantly isolated from cotton grown in chitin-amended soil where it accounted for 73% of the isolated internal population. These observations cannot be explained by the bacterial source in the soil alone and therefore strongly suggest specific plant-regulated selectivity of bacterial colonization. It is probable that the chitin amendment led to alterations in plant physiology and biochemistry which, in turn, explains differences in endophytic bacterial communities. Evidence for chitin-induced physiological changes in plants has been reviewed by Lafontaine and Benhamou (1996). They reported that amendment with chitosan, a derivative of crab shell chitin, caused protection of tomato against the pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* through induction of physiological and structural changes in the host plant (Benhamou et al., 1994).

Interestingly, *B. cepacia* was present in higher numbers in the soil, rhizosphere and endorhiza of cotton grown in chitin-amended soil. The role of *B. cepacia* in nematode suppressiveness can only be speculated. However, increased populations of *B. cepacia* have been reported for plant systems antagonistic to plant-parasitic nematodes (Vargas, 1995, unpublished Ph.D. thesis, Auburn University). Following intensive screening of endophytic bacteria to control root-knot nematodes, we found 5 out of 12 tested *B. cepacia* strains isolated from surface-disinfested cotton roots to give a 30-50% control of *M. incognita* on cotton (unpublished data). Plant beneficial effects provided by *B. cepacia* are widely reported, emphasizing the enormous potential of this species in plant-microbe interactions (De Freitas and Germida, 1991; Parke et al., 1991; Hebbar et al., 1992; Chiarine et al., 1994).

Larger microbial populations recovered from within roots grown in chitin-amended soil than in non-amended soil is somewhat surprising, since the cotton roots did not show any presence of plant-parasitic nematodes which are known to increase endophytic populations (Hallmann et al., 1998). It is assumed that either the initial nematode inoculum potential in the non-amended soil was too low to significantly increase internal populations of microorganisms or, due to the

larger bacterial populations in chitin-amended soil than in non-amended soil, more microorganisms penetrated the root tissue.

Chitin-induced nematode suppressiveness was documented by Mian et al. (1982) and Spiegel et al. (1986) and was confirmed in our studies for *Meloidogyne incognita* in cotton. Following inoculation of the soil with 3,000 eggs of *M. incognita* to the second cotton planting 62 d after chitin amendment, the nematode suppressiveness was still present demonstrating its long-lasting effect. As reported by Spiegel et al. (1986) and Rodríguez-Kábana and Morgan-Jones (1987), it is not unusual to obtain better nematode control in the second crop following chitin amendment than in the first crop and this might be due to more bacteria, actinomycetes and nematode parasitizing fungi in the amended soil. In contrast to plant-parasitic nematodes, populations of free-living nematodes increased up to 100-fold at day 54 by the chitin amendment when compared with non-amended soil.

Our results reported here indicate that chitin amendment resulting in suppression of plant-parasitic nematodes is not only associated with microbial changes in the soil and rhizosphere but also with changes in the bacterial community within the plant tissue. This strongly suggests that the physiology of a plant growing in chitin-amended soil is different from one grown in the absence of chitin. Hence, it may be possible to use organic amendments to manipulate the soil microflora and induce desired changes in the endophytic microflora.

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