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Endophytic bacteria in *Coffea arabica* L.

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Eighty-seven culturable endophytic bacterial isolates in 19 genera were obtained from coffee plants collected in Colombia ($n = 67$), Hawaii ($n = 17$), and Mexico ($n = 3$). Both Gram positive and Gram negative bacteria were isolated, with a greater percentage (68%) being Gram negative. Tissues yielding bacterial endophytes included adult plant leaves, various parts of the berry (e.g., crown, pulp, peduncle and seed), and leaves, stems, and roots of seedlings. Some of the bacteria also occurred as epiphytes. The highest number of bacteria among the berry tissues sampled was isolated from the seed, and includes *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*. This is the first survey of the endophytic bacteria diversity in various coffee tissues, and the first study reporting endophytic bacteria in coffee seeds. The possible role for these bacteria in the biology of the coffee plant remains unknown.

The importance of coffee (*Coffea arabica* L. and *Coffea canephora* PIERRE ex FROEHNER; Fa. Rubiaceae) as an agricultural commodity throughout the world cannot be understated: its retail value of US $70 billion (OSORIO 2002) surpasses the value of total US agricultural exports for 2003, which was forecast at US $57.5 billion (WHITTON and CARTER 2002). Even though an extensive amount of literature exists on all aspects of coffee production and utilization, very little is known about microorganisms associated with the coffee plant, and specifically, about coffee endophytes. For this paper, we will use WILSON's (1995) definition of endophytes as "fungi or bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease".

To our knowledge, only two papers have reported the presence of bacterial endophytes from coffee tissues: JIMÉNEZ-SALGADO *et al.* (1997) reported *Acetobacter diazotrophicus*, a nitrogen-fixing bacterium isolated from coffee roots collected in Mexico, and SAKIYAMA *et al.* (2001) reported several species of *Paenibacillus* isolated from coffee berries sampled in Brazil. Other members of the Rubiaceae, to which coffee belongs, have been shown to have bacterial endophytes (GORDON 1963, VAN OEVELEN *et al.* 2002).

Coffee endophytes are currently being studied at the U. S. Department of Agriculture, Agricultural Research Service. Our coffee research program involves the development of innovative biological control methods aimed at reducing damage caused by the coffee berry borer (*Hypothenemus hampei* (FERRARI); Coleoptera: Curculionidae), the most serious pest of coffee throughout the world. This research includes the inoculation of coffee plants with the fungal insect pathogen *Beauveria bassiana* (BALSAMO) VUILLEMIN (Ascomycota: Hypocreales), with the objective of having it function as a fungal endophyte that might control the coffee berry borer. As part of this research, we have been conducting a survey of fungal endophytes in coffee plants, and detected bacterial growth associated with some of the tis-

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sues sampled. Here, we report on bacterial endophytes in coffee plants collected in Hawaii, Mexico and Colombia. Results on fungal endophytes will be reported elsewhere (VEGA *et al.*, in preparation).

Materials and methods

Coffee tissues (stems, leaves, berries) from *Coffea arabica* L. growing in the field were collected on 3 Hawaiian islands (Oahu, Hawaii, and Kauai – January and March 2003), one location in Mexico (Cacahoatán, Chiapas – February 2003), and one location in Colombia (Centro Nacional de Investigaciones de Café, CENICAFÉ, Chinchiná – July 2003). Different parts of the berry were sampled: crown, peduncle, pulp, and seed; leaves, stems and roots from seedlings originating in Hawaii were also sampled. Tissues were individually washed in running tap water and moved to the laminar flow hood where sections were cut with a sterile scalpel. These sections were surface-sterilized by dipping in 0.525% sodium hypochlorite for 2 min, 70% ethanol for 2 min, and rinsed in sterile distilled water followed by drying on sterile tissue paper. The edges of each sampled tissue were cut off with a sterile scalpel and discarded and six subsamples of the remaining tissue measuring approximately 2 × 3 mm were individually placed in 5 cm dia. petri dishes containing yeast malt agar (YMA; Sigma Y-3127, Sigma-Aldrich Co., St. Louis, MO) to which 0.1% stock antibiotic solution was added. The antibiotic stock consisted of 0.02 g of each of three antibiotics (tetracycline, streptomycin and penicillin) dissolved in 10 ml sterile distilled water, followed by filter sterilization through a 0.2 µ filter (Nalgene Disposable Filterware, Nalge Nunc International Rochester, NY); from this, 1 ml was added to each liter of media. Plates were incubated at room temperature (22 ± 3 °C). Bacterial growth in YMA was observed 4–5 days after incubation. Single colonies were selected based on morphology characteristics and appearance and they were subsequently re-isolated and plated in nutrient agar media (Becton Dickinson, Sparks, MD) and incubated at 27 ± 2 °C for 24–48 h.

To assess whether the method used to surface sterilize tissues – which is aimed at surface sterilization for fungal endophyte isolation - would be sufficient to eliminate bacteria, we individually assayed the sterile distilled water in which tissues were washed after being placed in bleach and ethanol and plated aliquots; this resulted in no bacterial growth in YMA without antibiotic. We also plated aliquots from washes prior to surface-sterilizing to determine whether there were bacteria on the tissue surface, with positive results.

To assess for the presence of epiphytic bacteria, coffee berries and leaves from six individual trees were collected in Colombia. Two leaves and two berries from each tree were individually placed in sterile bottles into which 100 ml of sterile water plus 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, MO) were added. The samples were vortexed for five minutes followed by the preparation of serial dilutions from which 1 ml was plated on the surface of YMA without antibiotic. The aliquots were spread over the media and the plates were incubated a 27 ± 2 °C for three days.

Single colony bacteria were isolated based on morphology characteristics and appearance and streaked in nutrient agar media (Becton Dickinson, Sparks, MD), for subsequent identification based on fatty acid analysis using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) and Sherlock software (MIDI, Inc., Newark, DE, USA) as described by Buyer (Buyer 2003). Cut-off values for MIDI matches were as follows: <0.2 unknown, ≥0.2 genus level, ≥0.5 species level (BUYER 2002).

Results and discussion

Eighty-seven species of endophytic bacteria were isolated from coffee tissues collected in Colombia ($n = 67$), Hawaii ($n = 17$), and Mexico ($n = 3$) (Table 1). Endophytic bacteria were isolated from the crown, peduncle, pulp and seed of coffee berries, from leaves of adult trees, and from leaves, stems and roots of seedlings (Table 1). Nine genera of epiphytic bacteria were isolated from leaves and berries collected in Colombia (Table 2). In total, 22 genera were identified and from these, 15 (68%) were Gram negative bacteria (Table 2).

Table 1
Number of bacterial endophytes isolated in various coffee tissues collected in Hawaii, Colombia, and Mexico

Plant tissue		Bacterial endophytes	
		#	%
Berry	Crown	2	2.3
	Pulp	7	8.0
	Peduncle	1	1.2
	Seed	28	32.2
Adult plant	Leaves	10	11.5
Seedlings	Leaves	5	5.8
	Stems	13	14.9
	Roots	21	24.1
Total		87	100

Our sterilization protocol was originally aimed at fungal endophytes (ARNOLD *et al.* 2001); bacterial growth came as a surprise to us, considering we were using a weak antibiotic concentration in the media and that only two endophytic bacteria had been previously reported in coffee. Nevertheless, similar surface sterilization techniques as those used by ARNOLD *et al.* (2001) have been reported by COOMBS *et al.* (2004) and MOCALI *et al.* (2003) for the isolation of bacterial endophytes.

Even though at present we do not know what role the endophytic bacteria in coffee might have, reported functions for endophytic bacteria include (1) promotion of plant growth and increased disease resistance (STURZ *et al.* 1997, 1999, CHANWAY 1998, WELBAUM *et al.* 2004); (2) N_2-fixation, e.g., sugarcane (NOGUEIRA *et al.* 2001, BODDEY *et al.* 2003) and maize (ESTRADA *et al.* 2002); and (3) protection against plant pathogenic microorganisms via production of antibiotics (BROADBENT *et al.* 1971, WELLER 1988, STURZ *et al.* 1999) or synthesis of secondary metabolites (ASSIS *et al.* 1998, LONG *et al.* 2003).

We detected *Burkholderia cepacia, B. gladioli, B. glathei* and *B. pyrrocinia* (Table 2) as coffee endophytes, and *B. gladioli* as an epiphyte in Colombia. ESTRADA-DE LOS SANTOS *et al.* (2001) detected *Burkholderia* sp. in the coffee rhizosphere in Mexico and CASTRO and RIVILLAS (2000) have reported the use of *B. cepacia* for the control of *Rosellinia bunodes* (collar rot) in coffee plants. The genus *Burkholderia* was erected from seven *Pseudomonas* species (YABUUCHI *et al.* 1992), and members of the *B. cepacia* complex (seven closely related species) have been reported in soil environments (MCARTHUR *et al.* 1988), as a plant pathogen (GONZÁLEZ *et al.* 1997), and as a N_2-fixing maize endophyte in Mexico (ESTRADA *et al.* 2002), as well as a sugarcane endophyte in South Africa, where in vitro tests revealed antifungal activity against *Ustilago* and *Fusarium* (VAN ANTWERPEN *et al.* 2002).

An endophyte we detected in coffee seeds, *Stenotrophomonas maltophilia*, has been successfully used for control of leaf spot (*Bipolaris sorokininana*) on tall fescue (ZHANG and YUEN 1999), and has also been reported in potatoes (GARBEVA *et al.* 2001, KRECHEL *et al.* 2002). *Methylobacterium radiotolerans*, detected in coffee pulp, has been successfully used for *Fusarium* control in black pepper seedlings (BENCHIMOL *et al.* 2000). *Pantoea agglomerans*, detected in adult plant coffee leaves and coffee seeds, has been reported in rice seeds (VERMA *et al.* 2001), maize (RIGGS *et al.* 2001), potatoes (KRECHEL *et al.* 2002), citrus (ARAUJO *et al.* 2001), red clover (STURZ *et al.* 1997) and peas (ELVIRA-RECUENCO and van VUURDE 2000) and has also been used for the control of *Penicillium digitatum* and *Penicillium italicum* in citrus (POPPE *et al.* 2003). *P. agglomerans* has also been reported as a N_2-fixer in the gut of termites (POTRIKUS and BREZNAK 1977).

Table 2
Number of bacterial endophytes in coffee tissues collected in Hawaii (H), Colombia (C) and Mexico (M), and bacterial epiphytes in coffee leaves and berries collected in Colombia

Bacterial Identification	Endophytes									Epiphytes (Colombia)	
	Gram	Berries				Adult Plant Leaves	Seedlings				
		Crown	Pulp	Peduncle	Seed		Leaves	Stems	Roots	Leaves	Berries
Bacillus sp.	+				1H				2C		
Bacillus cereus – GC subgroup A	+						1C				
Bacillus megaterium – GC subgroup A	+					1H					
Bacillus subtilis	+	1H									
Burkholderia sp.	–		1C		1C			3C	2C	2	
Burkholderia cepacia – GC subgroup B	–							1C	1C		
Burkholderia gladioli – GC subgroup A	–				1C						
Burkholderia gladioli – GC subgroup B	–				1C					3	
Burkholderia glathei	–								1C		
Burkholderia pyrrocinia	–								1C		
Cedecea sp.	–					1C					
Cedecea davisae	–										1
Chromobacterium sp.	–					1C			1C		
Clavibacter michiganense insidiosum	+				2M						
Curtobacterium sp.	+							1C			
Curtobacterium flaccumfaciens-flaccumfaciens	+				1C	1C				2	1
Curtobacterium flaccumfaciens oorti	+									1	
Curtobacterium flaccumfaciens-poinsettiae	+				1C						
Enterobacter asburiae	–								1C		
Enterobacter cancerogenus	–										1
Enterobacter gergoviae	–					1C					
Escherichia vulneris	–				1C						
Gordona sp.	+		1H								
Klebsiella planticola	–								1C		
Klebsiella pneumoniae-ozaenae	–								1C		
Klebsiella trevisanii	–						1C				
Kocuria kristinae	+									1	
Methylobacterium radiotolerans	–		1C								

Micrococcus sp.	+		1H	1M	1H							
Pantoea agglomerans	–			1H, 1C	1C		1C				6	
Pseudomonas sp.	–						1C	6C				
Pseudomonas chlororaphis	–				1C		1C					
Pseudomonas putida	–				1C			1C				
Pseudomonas putida biotype A	–		1C	1C								
Pseudomonas putida biotype B	–			1C								
Rhodococcus equi – GC Subgroup A	+		1H									
Salmonella typhimurium – GC subgroup B	–	1H										
Serratia liquefaciens	–			1C					2	2		
Stenotrophomonas sp.	–				1C			1C				
Stenotrophomonas maltophilia	–		1H	4H, 1C		1C						
Variovorax paradoxus – GC subgroup A	–						1C					
Xanthomonas sp.	–										1	
Yersinia frederiksenii	–										1	
Unknown	+		1H	1H		1C		2C				
Unknown	–			4C			4C		7	1		
No matches found				1H, 2C		1C						
Bacteria detected		2	7	1	28	10	5	13	21	18	14	

Klebsiella pneumoniae, detected in the roots of coffee seedlings, has been reported as an endophyte in maize (CHELIUS and TRIPLETT 2000) and has also been found to have high colonization levels that enhance plant growth when applied to the rhizosphere of *Arabidopsis* and *Triticum* (DONG *et al.* 2003). *Curtobacterium flaccumfaciens*, detected as an endophyte in coffee seeds and leaves as well as an epiphyte, has been reported as endophytic bacteria in potatoes (KRECHEL *et al.* 2002). *Bacillus megaterium* and *Pseudomonas putida*, detected in various coffee tissues, have been reported as endophytes in potatoes (KRECHEL *et al.* 2002) and oaks where *P. putida* has been further tested to control oak wilt (BROOKS *et al.* 1994). *Bacillus subtilis*, detected in the crown of the coffee berry, has been reported as a chestnut endophyte (WILHELM *et al.* 1998). The two endophytic bacteria (*Acetobacter diazotrphicus* and *Paenibacillus*) previously reported in coffee (JIMÉNEZ-SALGADO *et al.* 1997, SAKIYAMA *et al.* 2001) were not isolated in this study.

Detailed information on the biodiversity and role of bacterial endophytes in specific crops could lead to their use as vectors for specific genes of interest. For example, two sugarcane N_2-fixing endophytic bacteria, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae*, have been successfully used as vectors for the *cry3A* gene from *Bacillus thuringiensis* (FALCÃO SALLES *et al.* 2000). Similarly, *Stenotrophomonas maltophilia* has been used as a vector for cloning the chitinase gene and enhancing its enzymatic activity (KOBAYASHI *et al.* 2002).

Even though the initial medium we used in this study contained antibiotics, many bacteria are known to be antibiotic resistant, among these some we isolated from coffee, e.g. *Stenotrophomonas maltophila* (ALONSO and MARTÍNEZ 1997), *Burkholderia cepacia* complex (DE SOYZA *et al.* 2001) and several species of *Pseudomonas* (MCINROY *et al.* 1996, NAIRN and CHANWAY 2002). This antibiotic resistance explains the growth observed in the antibiotic-containing medium we used.

Our survey in Hawaii, Mexico, and Colombia has revealed for the first time the presence of a wide diversity of bacterial endophytes in various coffee tissues, including the seed. Out of 9 genera of bacterial epiphytes isolated from coffee leaves and berries collected in Colombia (Table 2), six were also found as endophytes (*Burkholderia*, *Cedecea*, *Curtobacterium*, *Enterobacter*, *Pantoea,* and *Serratia*); only three genera were only found as epiphytes (*Kocuria*, *Xanthomonas*, and *Yersinia*). Whether coffee epiphytes can become endophytes remains to be determined.

Our endophytic bacteria results are likely a vast under sampling due to the fact that the media we used contained antibiotics and was originally intended for fungal endophyte isolation. In addition, it is very likely that there are non-culturable endophytic bacteria in coffee tissues that could be detected using molecular methods; CHELIUS and TRIPLETT (2001) reported that only 48% of the bacterial endophytes in maize that were identified using molecular methods were actually culturable. Future studies in our laboratory will use non-selective bacterial media to isolate endophytic bacteria from coffee tissues, and molecular methods to identify them. This will result in a more detailed view of the bacterial diversity occurring in various coffee tissues.

It is important to note that even though many of the bacteria detected in this study are of clinical interest in human medicine, e.g., *Burkholderia* spp. (JIAO *et al.* 2003, BRISSE *et al.* 2004, MANZAR *et al.* 2004, FOLEY *et al.* 2004), *Enterobacter asburiae* (STEWART and QUIRK 2001), *Enterobacter gergoviae* (GANESWIRE *et al.* 2003), *Klebsiella trevisanii* (FRENEY *et al.* 1986), *Escherichia vulneris* (BRENNER *et al.* 1982, AWSARE and LILLO 1991), *Micrococcus* and *Kocuria* (SZCZERBA 2003), *Pantoea agglomerans* (DE CHAMPS *et al.* 2000, KRATZ *et al.* 2003), and *Stenotrophomonas maltophilia* (KOSEOGLU *et al.* 2004), these would certainly not be present in roasted coffee.

Our results raise various areas in need of research: Do endophytic bacteria in coffee interact with their host plant? Do they interact with endophytic fungi? Do they produce metabolites? How do they enter the plant? Do they persist in time? Can they be vertically transmitted? Future studies in our laboratory will address some of these questions.

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