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Research Article

RESEARCH ARTICLE

Characterization of endophytic bacteria from cucurbit fruits with potential benefits to agriculture in melons (*Cucumis melo* L.)

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One sentence summary: Diversity and localization of endophytes living inside cucurbit fruits were explored and it was found that some of these bacteria may be used for improving agricultural practices in melons.

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ABSTRACT

Endophytes are microorganisms that mainly colonize vegetative parts, but are also found in reproductive and disseminating organs, and may have beneficial characteristics. To identify microorganisms associated with the agriculturally important family, Cucurbitaceae, endophytes were initially determined in fruits of *Cucumis melo Reticulatus* Group 'Dulce' by a cultivation-independent approach based on fluorescence *in situ* hybridization using double labeling of oligonucleotide probes. *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* were localized inside the fruits. Culturable bacteria were further isolated and identified from fruit tissues of 'Dulce', from fruits of other cultivated and wild-field-grown Cucurbitaceae, and from wild fruits growing under natural conditions. Low densities of culturable bacteria were detected in the investigated fruits, especially in four out of the five wild species, regardless of their growing environment. Substantial differences were observed between the wild and cultivated cucurbit taxa in regard to the number of colonized fruits as well as the type of endophytes. *Bacillus* was the most dominant genus of endophytes colonizing fruits of Cucurbitaceae. The antagonistic effects of isolated endophytes were assessed against cucurbit disease agents in dual-culture assays. Several bacterial isolates exhibited antagonistic properties against the tested plant pathogens. The identified bacteria may be useful for protecting plants not only in the field, but also for post-harvest.

Keywords: Cucurbitaceae *Reticulatus* Group; 'Dulce'; DOPE-FISH; soil-borne pathogenic fungi

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INTRODUCTION

Bacterial endophytes are part of the plant-associated microbiota and have been known for over 125 years (Compant, Sessitsch and Mathieu 2012). These bacteria have established intimate interactions with their host (Assmus *et al.* 1995; Hallmann *et al.* 1997; Hardoim, van Overbeek and van Elsas 2008; Ryan *et al.* 2008). Some of them can show plant growth-promoting effects (Bent and Chanway 1998; Hardoim, van Overbeek and van Elsas 2008), accelerate seedling emergence (Long, Schmidt and Baldwin 2008) and protect plants from biotic and abiotic stresses under different environmental conditions (Sessitsch *et al.* 2002; Mitter *et al.* 2013), and therefore they are often listed among microorganisms with potential agricultural benefits (Compant *et al.* 2005; Berg 2009; Compant, Clément and Sessitsch 2010; Mitter *et al.* 2013).

Bacterial endophytes have been reported to be highly diverse, comprising a range of different bacterial taxa in various plant species, as recently described by culture-dependent and -independent approaches (James *et al.* 1994; Lodewyckx *et al.* 2002; Rosenblueth and Martínez-Romero 2006). Most endophytes, particularly those colonizing roots and stems, seem to originate from the rhizosphere and colonize plant organs as part of their life cycle and are able to move systematically within the plant (Gray and Smith 2005; Hallmann and Berg 2006; Rosenblueth and Martínez-Romero 2006), while others are restricted to below ground parts of the plants (Hallmann *et al.* 2001; Compant *et al.* 2011). Recent studies have shown that plant reproductive and disseminating organs can host endophytic bacteria (reviewed in Compant, Clément and Sessitsch 2010; Truyens *et al.* 2014). Distinct microbial communities have been found albeit in low densities, in plant organs, such as flowers, fruits and seeds (Compant, Clément and Sessitsch 2010; Compant *et al.* 2011; Fürnkranz *et al.* 2012). These endophytes may derive from the rhizosphere, but also from the anthosphere or the carposphere environments (Compant, Clément and Sessitsch 2010; Compant *et al.* 2011). Examples of studies related to endophytes in such organs include reports of bacteria in the seeds of Norway spruce and tobacco, as well as bacteria associated with caryopses of rice and maize (Cankar *et al.* 2005; Okunishi *et al.* 2005; Mastretta, Taghavi and van der Lelie 2009; Johnston-Monje and Raizada 2011), or in the seeds and fruit flesh of grapes, Styrian oil pumpkin and papaya (Compant *et al.* 2011; Fürnkranz *et al.* 2012; Krishnan *et al.* 2012). The bacterial genera *Pseudomonas* and *Bacillus* have been isolated, for instance, from the interior of flowers, fruits and seeds of grapevine (Compant *et al.* 2011), whereas in papaya *Bacillus* has been found as the predominant species in the fruits, along with *Kocuria*, *Acinetobacter* and *Enterobacter* species (Shi *et al.* 2010; Krishnan *et al.* 2012). Although these and other studies have demonstrated the presence of endophytic bacteria in plant reproductive and disseminating organs, we still have limited understanding of the diversity, functionalities, colonization routes and identities of those endophytes in various plant species.

Fürnkranz *et al.* (2012) reported that the seeds and flowers of the Styrian oil pumpkin contain bacteria with antagonistic activity against pumpkin pathogens. Pumpkin belongs to the Cucurbitaceae that is a large family of commercially important crops such as melon, cucumber, squash and watermelon and is one of the broadest families in terms of natural genetic variability. Each species comprises a broad range of genotypes, wild and cultivated, that differ in a large number of traits. Information on endophytic bacteria in different fruits of cucurbits is, however, still lacking. One Cucurbitaceae member is *Cucumis melo Reticulatus* Group, 'Dulce', a common cultivated melon with fleshy fruits and a pericarp that surrounds and protects the seeds. This niche might be an interesting habitat for endophytes with yet undetermined characteristics in regard to promoting plant growth or health. Therefore, the aim of this study was to analyze bacterial endophytes associated with the fruits of *C. melo Reticulatus* Group, 'Dulce' by culture-independent double-labeled oligonucleotides-fluorescence *in situ* hybridization (DOPE-FISH) approach using 16/23S rRNA-directed oligonucleotide probes for different bacterial families and groups, labeled with diverse fluorescent dyes and applying digital imaging confocal laser-scanning microscopy, as well as by isolating and characterizing culturable bacterial endophytes. We further isolated bacterial endophytes from fruits of different Cucurbitaceae members, comprising cultivated and wild species, and tested whether they are able to antagonize relevant cucurbit pathogens.

MATERIALS AND METHODS

Plant material and sampling procedure

The cultivated melon *C. melo Reticulatus* Group, 'Dulce' was used in this study for microscopic analysis of fruit-associated microbiota as well as for the isolation of bacterial endophytes. Other wild and cultivated members of the family Cucurbitaceae were additionally selected, based on their array of genetic and morphological traits (Table 1), in order to isolate further bacterial endophytes with potential benefit for plant improvement. For this study, the cultivated melon *C. melo Reticulatus* Group, 'Dulce', its wild relatives *C. melo Momordica* Group, 'PI414723' (Harel-Beja *et al.* 2010) and *C. callosus,* the cultivated watermelon *Citrullus lanatus* 'Early moonbeam' and its wild relative *Ci. colocynthis* were sampled. They derived from an experimental field, where they were grown from seeds originated from the Newe Ya'ar collection (Burger *et al.* 2006). Field-grown taxa were planted in April 2012 in an experimental plot at Newe Ya'ar Research Center in the Jezreel Valley of Israel (32°42′30.7″N 35°10′47.7″E). The wild, naturally growing species *Ecballium elaterium* (squirting cucumber) and *Bryonia cretica* (bryony) were additionally sampled for this study and derived from their natural habitats in different sites along the northern valleys of Israel: Afula (32°37′09.2″N 35°18′35.0″E) and Kfar Yehezkel (32°33′43.6″N 35°20′59.1″E) for *E. elaterium*; and Shluhot (32°28′17.7″N 35°29′04.8″E) and Nir David (32°30′23.2″N 35°27′34.1″E) for *B. cretica* (Table 1). Soil type at Newe Ya'ar and *E. elaterium* sampling sites was brown silty clay, containing 53–60% clay, 23–33% silt and 14–16% sand, on a dry-weight basis. The $CaCO_3$ content was 8.9–11% with 2% organic matter and pH 7.6. At *B. cretica* sampling sites, the soil type was gray silty clay, containing 45% clay, 31% silt and 23% sand, on a dry-weight basis and the $CaCO_3$ content was 42.7% with 1.75% organic matter and pH 7.6. Climate zones where the plants were grown are Mediterranean climatic conditions (with a mean annual rainfall > 500 mm) at Newe Ya'ar and the *E. elaterium* sampling sites, and semi-arid (300–500 mm) at the *B. cretica* sampling sites.

Field-grown plants were grown for about 3 months, according to common commercial practices under open-field conditions, including herbicide, fungicide and pesticide applications following standard plant protection protocols. All planted species were fertilized with 6 kg of net nitrogen ($N\text{-}NO_3$, $N\text{-}NH_4$) and 6 kg of net P_2O_5 per 0.1 hectare of land. Flowers were open pollinated and tagged at anthesis. Fully developed fruits were harvested from the middle of July (*C. melo Momordica* Group, 'PI414723') to the end of August (*Ci. colocynthis*), depending on the maturation status of

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Table 1. Species specification and seed origin.

Plant taxon	Common name	Reasons for selection	Origin
Cucumis melo Reticulatus Group, 'Dulce'	Melon	Cultivated	USA
Cucumis melo Momordica Group, 'PI414723'	Melon	Wild, field-grown	India
Cucumis melo (=*C. callosus* or *C. trigonus*)	Wild melon	Wild, field-grown Naturally grown in Israel (rare)	Israel
Citrullus lanatus 'Early moonbeam'	Watermelon	Cultivated	USA
Citrullus colocynthis	Colocynth	Wild, field-grown Locally grown in Israel, desert area	Israel
Ecballium elaterium A. Rich	Squirting cucumber	Wild Naturally grown in Israel, collected from natural environments	Israel
Bryonia cretica	Bryony	Wild Locally grown in Israel, collected from natural environments	Israel

each species. Fruits from naturally growing plants (*E. elaterium* and *B. cretica*) were collected twice at the beginning (May) and end (September) of the 2012 growing session. Between 10 and 20 fruit samples were collected from each species.

Preparation of fruits

Fruits of all plants were harvested upon ripening and kept at room temperature (20°C) for no more than 5 days until processing. To avoid contamination by environmental bacteria, fruits of field-collected species were thoroughly washed with soap and water, surface-sterilized for 5 min with 70% ethanol and left to dry. Surface sterility was verified by plating samples of the fruit surface on agar medium. In the naturally growing species, this sterilization step did not eliminate all surface-associated bacteria, and thus the fruits were also immersed in 1% sodium hypochlorite solution (5 min), followed by rinsing with sterile distilled water three times to complete the surface sterilization.

Confocal laser-scanning microscopy with DOPE-FISH and quantification of the different bacterial taxa

For cultivation-independent DOPE-FISH and CLSM-microscopy analysis, fruit samples of 'Dulce' grown in the field were used. Flesh tissues and placenta (seed cavity without seeds) were cut in small parts (0.5 cm-long sections), fixed overnight at 4°C in a paraformaldehyde solution (4% in PBS, pH 7.2) and rinsed twice in PBS. Samples were then treated with a lysozyme solution (1 mg mL^{-1} in PBS) for 10 min at 37°C and dehydrated in an ethanol series (25, 50, 75 and 99.9%; 15 min each step). DOPE-FISH was carried out using probes from Genecust (Luxembourg) labeled at both the 5′ and 3′ positions. An EUBmix (equivalent mixture of EUB338, EUB338II, EUB338III) coupled with a FLUOS fluorochrome (Amann *et al.* 1990; Daims *et al.* 1999) and a *Firmicutes* probe (LGC; Küsel *et al.* 1999) as well as *Alpha-*, *Beta-* and *Gammaproteobacteria* probes (ALF1B, BET42a, GAM42a; Manz *et al.* 1992) or *Actinobacteria* (HGC) coupled with Cy5 were used. NONEUB probe (Wallner, Amann and Beisker 1993) coupled with Cy5 or FLUOS was also used independently as a negative control. All probe characteristics can be found in Table 2. Hybridization was carried out at 46°C for 2 h with 10–20 μL solution (containing 20 mM Tris-HCl pH 8.0, 0.01% w/v SDS, 0.9 M NaCl, formamide at the concentration suited to the probe and 10 ng μL^{-1} of each probe) applied to each plant sample and placed on slides in a 50 mL moist chamber (also housing a piece of tissue imbibed with 5 mL hybridization buffer). Washing was conducted at 48°C for 30 min with a post-FISH pre-warmed solution containing 20 mM Tris-HCl pH 8.0, 0.01% (w/v) SDS, 5 mM EDTA pH 8.0 and NaCl at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before air-drying for at least 1 day in the dark. The samples were observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G)laser FV10-LAHEG230–2). X, Y and Z pictures were taken at 405, 488 and 633 nm and then merged (RGB) using Image J software (Schneider, Rasband and Eliceiri 2012). Z Project Stacks was used to create the pictures (as described in Campisano *et al.* 2014). Whole pictures were sharpened (due to the convolution process in the microscope), and the light/contrast balance improved to see better image details as seen when samples were observed in the dark conditions under the microscope. Following confocal microscopy, analysis was done on each bacterial taxon and quantification was carried out using Image J software (Schneider, Rasband and Eliceiri 2012) by measuring the different RGB channels and by using normalization of data and Student *t*-test.

Enumeration and isolation of bacterial endophytes from cucurbit fruits

For CFU counting and isolation of bacterial endophytes, 'Dulce' fruits were initially used. Additional experiments were carried out with the wild and commercial-related species described in Table 1. All fruits were cut under aseptic conditions in a laminar flow hood. Bacteria were enumerated and isolated from fruit flesh (20 g) or from the content of the seed cavity corresponding to placenta tissues, which contains liquids with gelatinous tissue (5 g) (Burger 2000). Fruit flesh was homogenized in a sterile 0.85% NaCl solution (1:5, w/v), and samples were macerated using a mortar and pestle and further homogenized by vortexing at high speed for 1 min. The seed cavity was collected in a plastic tube and shaken for 1 h at 250 rpm on a rotary shaker. Homogenates were filtered through a double layer of sterilized miracloth, and then transferred to a clean centrifugation tube. A solution of each sample was then plated in triplicate on tryptic soy agar (TSA) and Reasoner's 2A agar (R2A) medium (Difco). Plates were incubated for 5 days at 28°C. All colonies were counted (142 colonies), and those that exhibited different morphologies were picked from each plate for further analysis, of them a total of 57 colonies were sequenced and analyzed. Differences in rate of populated fruits were calculated using R-version 3.1.0, Fisher's exact test with significance of $P = 0.0007$. Statistical analysis of bacterial counts was performed using one-sided Wilcoxon test

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Table 2. Probes used for DOPE-FISH microscopy according to Loy *et al.* (2007).

Probe	Accession number	Target	% Formamide	References
EUB338I	pB-00159	16S rRNA—90%/169389 most bacteria and 0 hit non-target groups	0–50	Amann *et al.* (1990)
EUB338II	pB-00160	16S rRNA—69%/1214 *Planctomycetes* and 83 hits non target groups	0–50	Daims *et al.* (1999)
EUB338III	pB-00161	16S rRNA—93%/943 *Planctomycetes* and 913 hits non target groups (Chloroflexi and *Insertae sedis* OP10 (32/81)-OP11(32/132))	0–50	Daims *et al.* (1999)
NONEUB	pB-00243	Control probe complementary to EUB338	0–50	Wallner *et al.* (1993)
LGC	pB-01040	16S rRNA—*Firmicutes*	20	Küsel *et al.* (1999)
ALF1B	pB-00017	16S rRNA—68% *Alphaproteobacteria*, some *Deltaproteobacteria*, *Spirochetes*, *Verrumicrobia*, *Nitrospira*	20	Manz *et al.* (1992)
BET42a	pB-00034	23S rRNA—*Betaproteobacteria*	35	Manz *et al.* (1992)
GAM42a	pB-00174	23S rRNA—*Gammaproteobacteria*	35	Manz *et al.* (1992)
HGC69A	pB-00182	23S rRNA—*Actinobacteria*	25	Roller *et al.* (1994)

($P < 0.05$) conducted by R platform (R Development Core Team 2009).

Identification of culturable endophytic bacterial communities in cucurbit fruits

Isolated endophytes were identified by PCR using the general 16S rRNA gene primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg *et al.* 1991). Reactions were performed in a 25 μL volume containing a streak from each of the colonies, 10 pmol of each primer, 0.2 mM dNTPs, 1X Red *Taq* buffer and 1 unit of Red*Taq* DNA polymerase (Sigma). PCR products were stained with SafeView (NBS Biologicals) and visualized on a 1.2% agarose gel. Sterile water was used as a negative control. PCR products were cloned into the pGEM T-Easy plasmid vector (Promega) and transformed into competent *Escherichia coli* strain DH5-α cells. Two colonies from each plate were randomly picked and sequenced. These sequences were compared to known sequences in the NCBI database using BLAST. The 16S rRNA gene sequences of bacteria isolated which were determined in this study were deposited in the GenBank database (GenBank KM670338–KM670394).

Screening bacterial isolates for antifungal activity

To test for possible antagonistic activities, all identified bacterial endophytes were screened for their influence on four soil-borne melon-pathogenic fungi: *Macrophomina phaseolina, Fusarium oxysporum* f. sp. *melonis* races 1 and 2 (Cohen *et al.* 2002) and *F. oxysporum* f. sp. *radicis-cucumerinum* (Vakalounakis 1996). The four pathogenic fungi were routinely grown on potato dextrose agar (PDA, Acomedia) as described previously (Cohen *et al.* 2002). Antagonism was tested *in vitro* according to a previously described dual-culture assay (Berg *et al.* 2005). Briefly, bacteria were transferred to a fresh TSA plate and incubated at 28°C for 24 h. The bacterial cells were then streaked in a cross shape at the center of a PDA plate, creating four open quadrants. The PDA plates were incubated at 28°C for 72 h to allow bacterial growth. Two 5 mm-diameter agar disks from a 1-week-old actively growing fungal culture were placed in the center of each quadrant. The plates were incubated at 25°C for 2 to 6 days, until the mycelium of the control plate (without bacteria) fully covered the plate, and then the radius of the growth zone of each fungus was measured. Isolates were tested in triplicates and each experiment was repeated at least three times.

Screening bacterial isolates for antibacterial activity

Antibacterial activity was assessed by dual-culture assay (Fürnkranz *et al.* 2012) with the plant pathogen *Pseudomonas syringae* pv. *lachrymans*, the causal agent of angular leaf spot on cucurbits (Morris *et al.* 2000). Fresh *P. syringae* culture was grown to an OD_{600} of 0.6 in tryptic soy broth medium. Then, 100 μL of *P. syringae* culture was inoculated in 1 mL of 0.7% agar (cooled to 50°C), mixed thoroughly and poured over solidified 50 mm TSA plates. Plates were allowed to solidify and then each of the identified bacterial isolates from the fruits was streaked on these treated plates. Plates were incubated at 28°C and after 5 days, the presence or absence of clearing zones surrounding the tested strains (due to growth inhibition of the pathogen) was assessed.

RESULTS

Visualization and quantification of endophytic bacteria inside fruit flesh and placenta of *C. melo Reticulatus* Group, 'Dulce'

Using DOPE-FISH microscopy and different probes coupled with different fluorochromes, bacteria were localized in the flesh tissues and placenta of fruits of *C. melo Reticulatus* Group, 'Dulce' (Figs 1 and 2). Single bacterial cells or clusters can be easily seen in the mesocarp as well as in some specific places as xylem elements of cells using EUB mix probes (Fig. 1A and B). Autofluorescing microbes were additionally detected in the whole study, probably resulting from a color combination between blue light (Fig. 1A, B and G) and pink (Fig. 1C–E). Use of NONEUB probe confirmed that microbes with natural autofluorescence can be detected in the mesocarp of the fruits (Fig. 1D). Use of specific probes allowed to detect *Firmicutes* as yellow/orange color (corresponding to the use EUBmix and LGC probe, green and red

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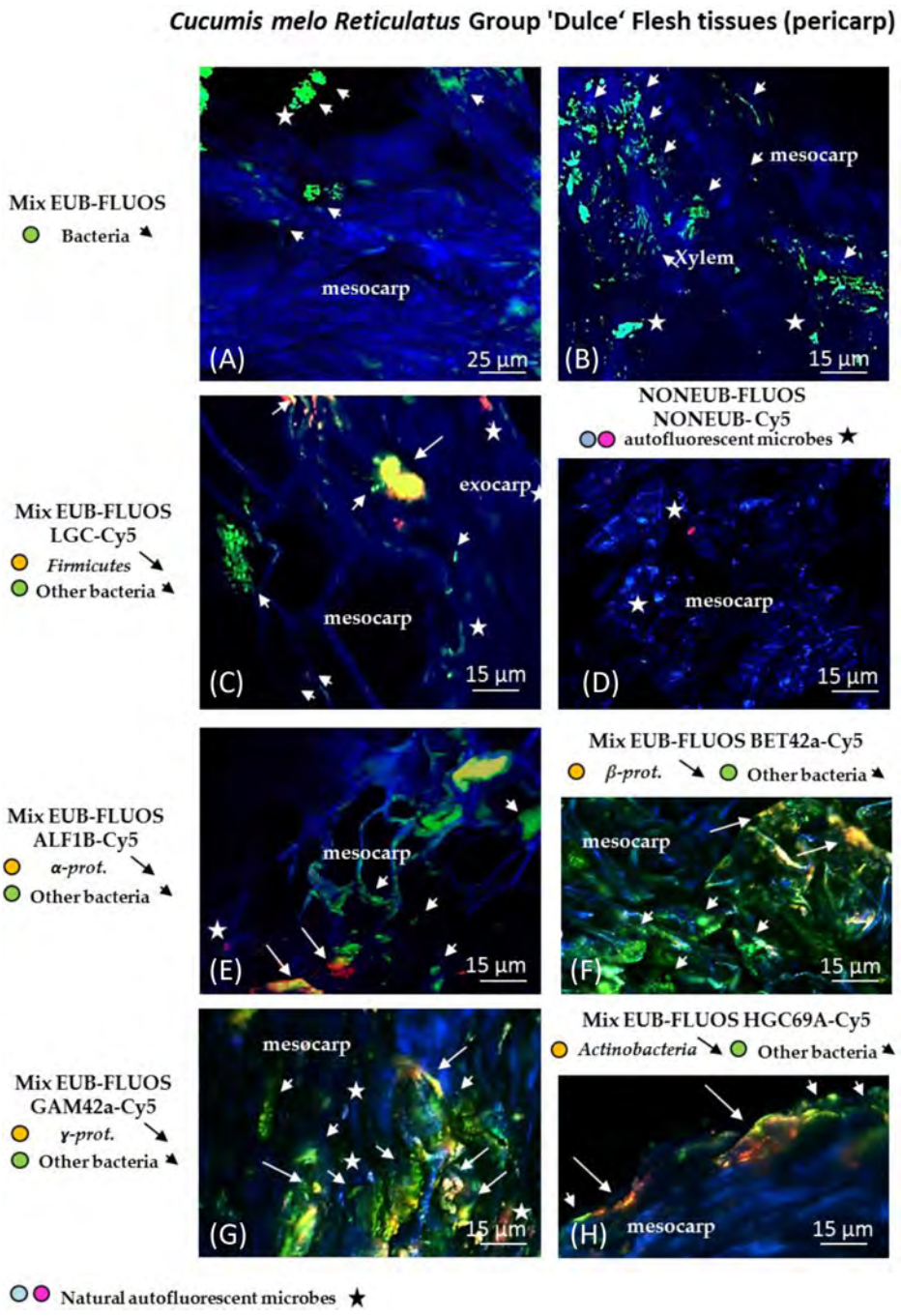

Figure 1. Confocal laser-scanning micrographs of DOPE-FISH results of fruit flesh of *C. melo Reticulatus* Group, 'Dulce' showing the presence of bacterial taxa using probes targeting all bacteria, *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* as well as NONEUB probe showing the presence of bacteria in the mesocarp (flesh part) in presence of other microbes with natural autofluorescence.

respectively and RGB merged) in the limit of the mesocarp/exocarp as microcolonies (Fig. 1C). Microphotographs resulting from confocal microscopy further showed that *Alphaproteobacteria* (Fig. 1E), *Betaproteobacteria* (Fig. 1F) and *Gammaproteobacteria* (Fig. 1G) are inhabitants of the mesocarp of the fruit flesh together with *Actinobacteria* (Fig. 1H). In the placenta tissues, the use of different probes allowed to detect *Alphaproteobacteria* (Fig. 2A), *Betaproteobacteria* (Fig. 2B), *Gammaproteobacteria* (Fig. 2C), *Firmicutes* (Fig. 2D) as well as *Actinobacteria* (Fig. 2E) in comparison to the application of NONEUB probe as negative FISH control (Fig. 2F). The different taxa inhabiting fruit flesh and placenta of the fruits were quantified by excluding microbes with natural autofluorescence and by pixels counts (Fig. 3). *Alphaproteobacteria* together with some *Deltaproteobacteria* and *Spirochetes* were detected as 2% of the fruit flesh bacterial microbiome, *Beta-* and *Gammaproteobacteria* accounted for 11 and 9%, respectively, while 32% *Firmicutes* and 18% *Actinobacteria* were detected (Fig. 3). Other bacteria not detected with the probes used were about 28% (Fig. 3). In placenta tissues of the fruits, *Alphaproteobacteria* together with some *Deltaproteobacteria*

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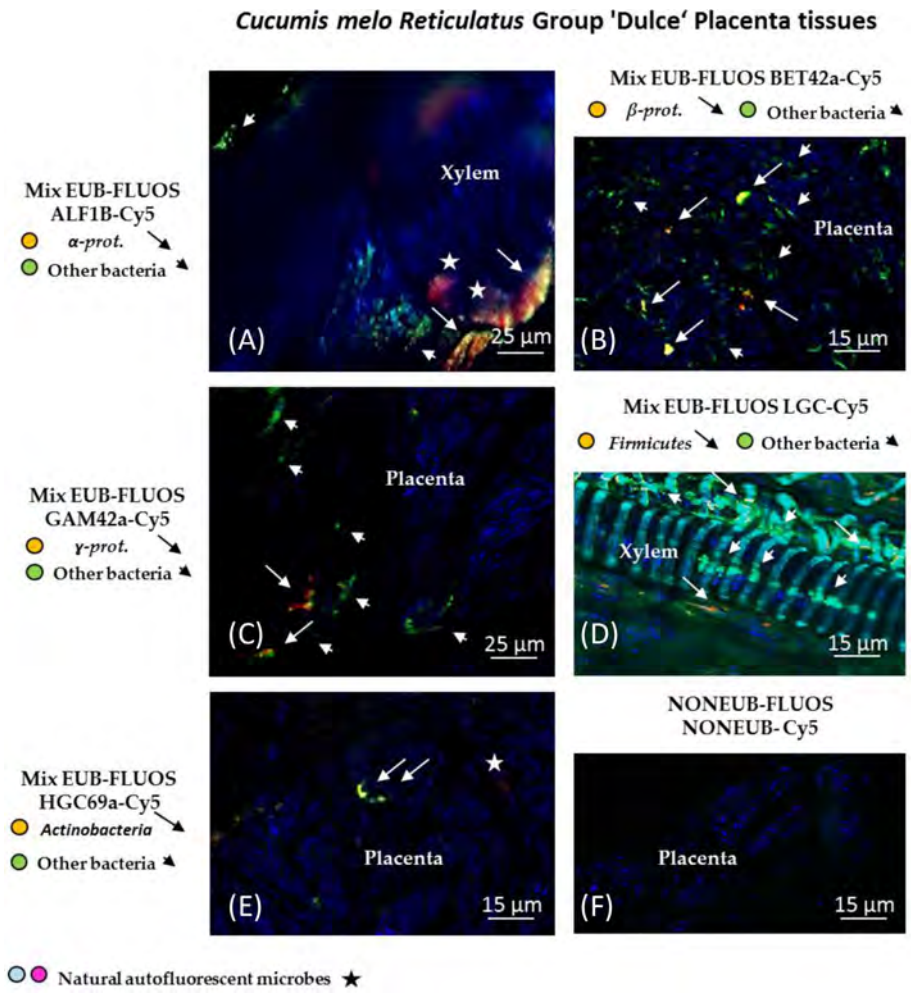

Figure 2. Confocal laser-scanning micrographs of DOPE-FISH results of placenta tissues of *C. melo Reticulatus* Group, 'Dulce' showing the presence of *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria*. NONEUB probe was also used as a negative control. Other microbes with natural autofluorescence were also detected.

and *Spirochetes* were 4%, *Betaproteobacteria* 10%, *Gammaproteobacteria* 15%, *Firmicutes* 18%, *Actinobacteria* 32% and other bacteria were about 21% (Fig. 3). The differences in the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* were statistically significant between the fruit flesh and the placenta tissues of the investigated fruits ($P < 0.05$).

Enumeration of bacterial endophytes associated with fruits of *C. melo Reticulatus* Group, 'Dulce' and from other cucurbit taxa

Analysis of culturable bacterial endophytes occupying the fruit endosphere was carried out with 'Dulce'. Most of the fruits (70%) were colonized (Fig. 4A). The average number of culturable bacteria in the positive fruits was 2.46 ± 0.05 $\log_{10}$ CFU g^{-1} (Table 3). For comparison, analysis of culturable endophytic bacteria occupying the fruit endosphere of other cucurbit taxa was carried out too. In the wild melon fruits, *C. melo Momordica* Group, 'PI1414723', grown in the same field, only 20% of the fruits were colonized by culturable bacterial endophytes, and the average number of culturable bacteria in the positive fruits was 1.38 ± 0.13 $\log_{10}$ CFU g^{-1} ($P < 0.05$) (Table 3). Looking at all investigated taxa of field-grown plants, it can be concluded that significant differences were observed between the wild and cultivated plants, in both the amount of populated fruits ($P < 0.05$; Fig. 4A) and bacterial numbers in the positive fruits ($P < 0.05$; Table 3). Up to 20% of the fruits collected from field-grown wild plant genotypes were inhabited with endophytes, while most of the fruits of cultivated genotypes (60–70%) were colonized by culturable endophytic bacteria (Fig. 4A). The numbers of CFU per gram in the positive wild fruits were at least 7-fold lower than those in the cultivated species, even though they had been grown in the same site and under the same conditions (Table 3).

In contrast to the other cucurbit species tested, *C. melo* types (*Reticulatus* Group, 'Dulce' and *Momordica* Group, 'PI414723') showed a clear distinction between the seed cavity surrounding the seeds and the fruit flesh. The soft tissue enveloping the seeds contained a higher number of CFU per gram, whereas the fruit flesh was only poorly colonized ($P < 0.05$; Fig. 4B). This indicates that, at least in these two cucurbit species, most of the culturable endophytes isolated from fruits were located in the seed cavity, on and around the seeds.

Unlike the field-grown wild plants, bacterial abundance in fruits from the two wild genotypes growing in their natural habitat (*E. elaterium* and *B. cretica*) did not show a notable trend, and they even differed significantly ($P < 0.05$). In the latter, bacteria were isolated from only 2 out of 15 fruits (Fig. 4A) at 1.75 ± 0.05 $\log_{10}$ CFU g^{-1} (Table 3), results resembling those obtained from the wild genotypes growing in the field, while all of the *B. cretica*

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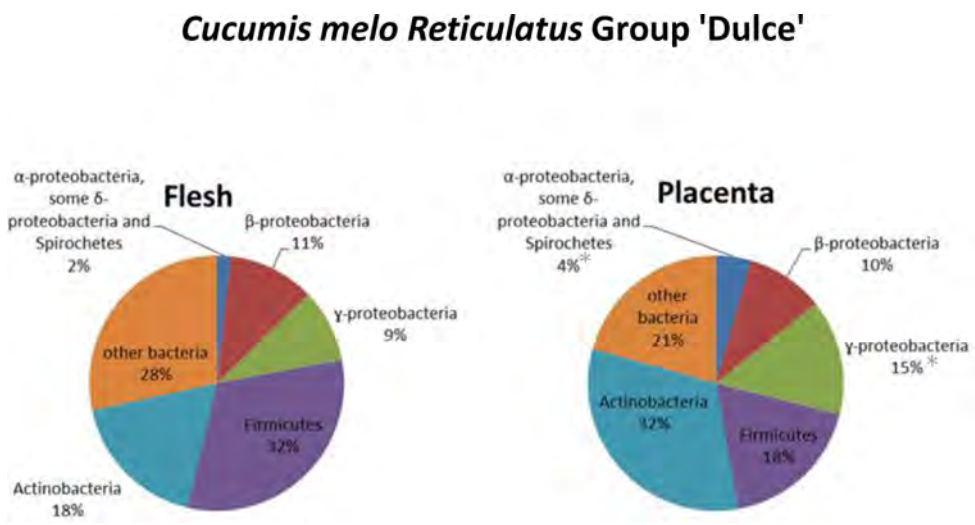

Figure 3. Quantification of bacterial taxa after DOPE-FISH of *C. melo Reticulatus* Group, 'Dulce' fruits showing distribution of the main bacterial groups in fruit flesh or placenta tissues. Significant differences between fruit flesh and placenta tissues ($P < 0.05$) are marked with an asterisk.

fruits sampled (100%) were colonized by culturable bacterial endophytes (Fig. 4A) and showed highest colonization levels (3.00 $\pm$ 0.06 $\log_{10}$ CFU g^{-1}) (Table 3).

Culturable endophytic bacterial communities

After plate counting, 57 isolates were selected for further analysis (Table 4). These culturable endophytes were identified to the species or genus level by analysis of ca. 1400 bp of their 16S rRNA genes. In 'Dulce', mainly *Firmicutes*, *Actinobacteria* and *Alphaproteobacteria* were isolated (Table 3). The same phyla and classes were observed in the other field-grown fruits too, but in fruits from the wild genotypes growing in their natural habitat *Beta-* and *Gammaproteobacteria* were also identified (Table 3). In most cucurbit fruits of different taxa, bacterial communities were dominated by *Firmicutes* with the genus *Bacillus* being most prominent, with the exception of *E. elaterium*, in which none of the positive fruits contained *Firmicutes* (Table 4). When present, *Bacillus* species accounted for more than 50% of the bacteria isolated from endophyte-containing fruits. The most common *Bacillus* species isolated were *Bacillus subtilis*, *B. licheniformis* and *B. amyloliquefaciens*. In *E. elaterium*, *Burkholderia* species were the most dominant bacteria (Table 4). Overall, taxa with higher CFU counts also showed higher diversity, as seen for 'Dulce', *C. lanatus* and *B. cretica* (Table 4); however, due to the rather low numbers of endophytes obtained per plant species, statistical testing was not possible.

Antagonistic activity of selected endophytes toward pathogens

Forty-eight isolates that were able to grow on PDA or TSA were screened for their antifungal and antibacterial activities by measuring their ability to inhibit the growth of five cucurbit pathogens, including four fungi and a bacterium. Diverse antibacterial and antifungal activities were observed among endophytes, which originated from different sources (Table 5). The *Bacillus* strains exhibiting highest homology with *B. subtilis*, *B. amyloliquefaciens* and *B. cereus* and a *Streptomyces* strain showed the strongest activity against all five investigated pathogens. Others, such as *B. safensis*, showed no activity. Isolates related to *B. licheniformis* and *B. megaterium* had only antibacterial activity against *Pseudomonas*, and some isolates of *Bacillus* and *Massilia* had only antifungal activity. Although antagonistic effects were observed in isolates from both cultivated and wild taxa, a stronger and more frequent activity was detected in bacteria isolated from field-grown fruits. The frequency of isolates with a wide range of antimicrobial activity was highest in *C. melo Reticulatus* Group, 'Dulce', whereas a high frequency of antibacterial activity was observed in 'Dulce', *C. colocynthis*, *C. lanatus* and *B. cretica*. However, statistical testing was not possible due to the low numbers of each strain associated with each plant.

DISCUSSION

In this study, we analyzed the bacterial endophytes associated with fruits of 'Dulce'. Using a culture-independent approach, we determined that *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes*, *Actinobacteria* and other unidentified bacteria can thrive as endophytes inside the mesocarp as parts of fruit flesh as well as inside placenta surrounding the seeds. Microscopic analysis showed which niches can be colonized by members of these taxa and suggested that endophytes were likely active, as they were detected by RNA-targeting FISH analysis. However, only bacteria with high ribosome content can be detected and usually spores or resting cells cannot be stained. In addition, the permeability of cells after a PFA-fixation step may not be enough to allow fluorescing oligonucleotides probes to enter bacterial cells. Therefore, not all bacteria can be visualized with the FISH technique. Microbial composition differs in fruit parts such as fruit flesh and placenta of 'Dulce'. This cucurbit line allowed also cultivation of some endophytes, but not all endophytes observed by microscopic analysis were detected by cultivation, as only *Alphaproteobacteria*, *Firmicutes* and *Actinobacteria* were able to grow. These classes accounted for about 50% of the bacteria observed in the microscopic images.

We further compared the culturable abundance of endophytic bacteria associated with fruits from different cultivated or wild cucurbit plants grown in the field or under natural conditions and showed differences among plant samples. We found very low densities of culturable bacteria (less than 10^3 CFU g^{-1}) in the different cucurbit fruits, including 'Dulce'. In addition, species diversity was low in the positive fruits. Endophytes

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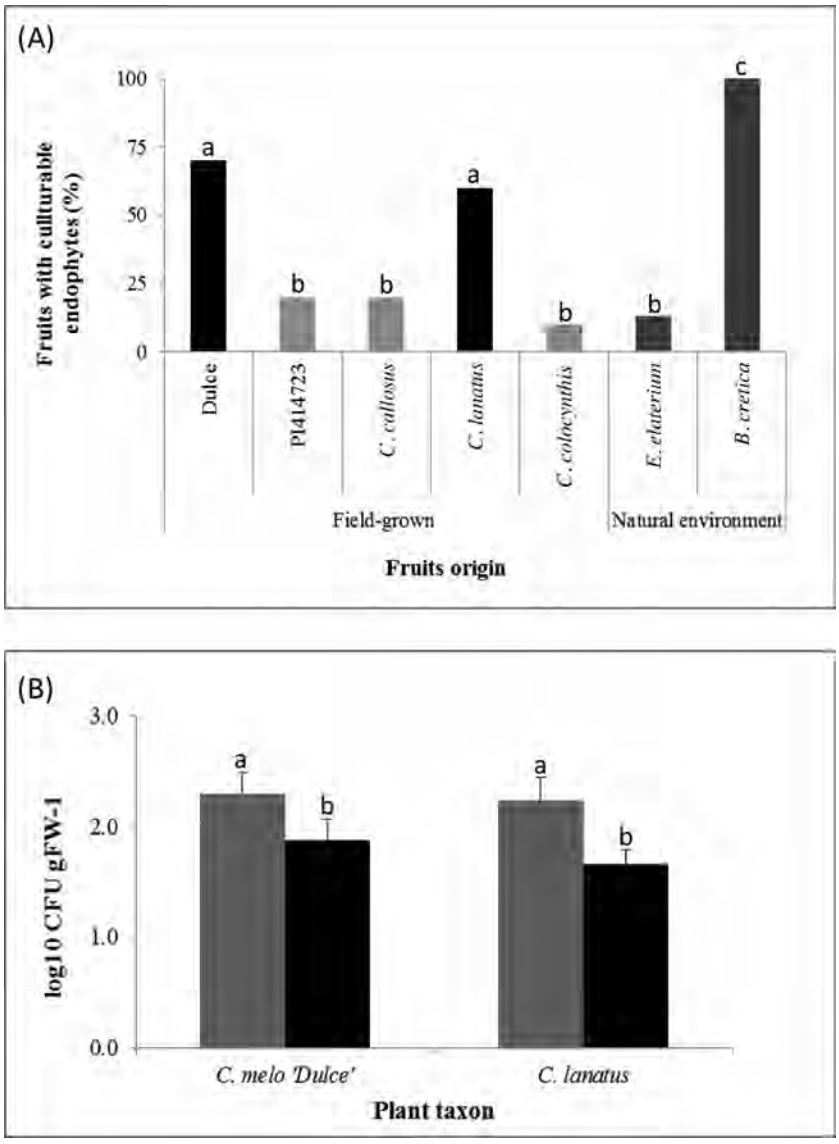

Figure 4. Endophytic colonization of cucurbit fruits. (**A**) Percentage of fruits from which endophytic bacteria could be isolated. Black bars—cultivated field-grown cucurbit fruits; light gray bars—wild-field-grown cucurbit fruits; dark gray bars—fruits collected from their natural environment. (**B**) Number of bacteria in CFU obtained from the seed cavity (or placenta tissues surrounding the seeds) (gray bars) and fruit flesh (black bars) of *C. melo Reticulatus* Group, 'Dulce' and *Ci. lanatus*. Columns assigned with a different letter indicate significant differences between bacterial counts ($P < 0.05$).

colonizing the reproductive and disseminating organs (flowers, fruits and seeds) have been only scarcely investigated, probably because their densities (and in particular those of culturable bacteria) are low as compared to those in other plant parts, such as roots or even leaves (Hallmann *et al.* 2001; Rosenblueth and Martínez-Romero 2006; Compant *et al.* 2011). However, such endophytes are of particular interest because they may have high potential to benefit the host plant by promoting its germination and growth or by suppressing pathogens, and because they may undergo a vertical transfer through the generations. The low densities of culturable bacteria found in the investigated fruits are in agreement with other investigated fruits of plants such as cotton, papaya, pumpkin and grapevine (Misaghi and Donndelinger 1990; Compant *et al.* 2011; Fürnkranz *et al.* 2012).

Bacterial distribution was uneven among fruits of the same cucurbit species grown in the same field. From about 50% of the investigated fruits, we were not able to isolate bacterial endophytes. It should be noted that numbers of culturable endophytes from the positive fruits were very low, usually just above the detection level. In addition, many bacteria are difficult to culture because of unknown cultivation requirements or they may enter a viable but non-culturable state. Indeed, as mentioned above, using microscopic analysis we showed that different bacteria can inhabit plant tissues of 'Dulce' fruits, and that only some of them can be culturable under the conditions applied in this study. Similarly, Fürnkranz *et al.* (2012) identified *Pseudomonas* and *Bacillus* as endophytes in pumpkins using cultivation-independent approaches and showed higher diversity.

It is assumed that endophytes inhabiting naturally growing fruits differ from those found in an agricultural field environment. On the one hand, plants grown in the field are exposed to fertilization and to different chemicals and treatments that may influence microbial number and composition.

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Table 3. Abundance of culturable bacteria and bacterial phyla and classes in different cucurbit taxa.

Plant taxon	n[a]	Abundance[b] [Log_{10} CFU (g FW)$^{-1}$]	Bacterial phyla and classes isolated from fruits
Cucumis melo Reticulatus Group, 'Dulce'	7/10	2.46 ± 0.05^{A}	*Firmicutes*, *Alphaproteobacteria*, *Actinobacteria*
Cucumis melo Momordica Group, 'PI414723'	2/10	1.38 ± 0.13^{B}	*Firmicutes*, *Actinobacteria*
Cucumis callosus	2/10	1.48 ± 0.23^{B}	*Firmicutes*, *Alphaproteobacteria*
Citrullus lanatus	6/10	2.37 ± 0.06^{A}	*Firmicutes*, *Alphaproteobacteria*, *Actinobacteria*
Citrillus colocynthis	1/10	1.50^{B}	*Firmicutes*
Ecballium elaterium	2/15	1.75 ± 0.05^{A}	*Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*
Bryonia cretica	20/20	3.00 ± 0.06^{C}	*Firmicutes*, *Betaproteobacteria*, *Gammaproteobacteria*

[a]Number of fruits from which endophytes were isolated out of total fruits sampled.
[b]Mean of positive fruits only. Numbers assigned with a different letter (A, B, C) indicate significant difference between bacterial counts ($P < 0.05$).
FW, fresh weight.

Table 4. Endophytic bacteria isolated from fruits of seven cucurbit accessions (taxa).

Closest relative (accession number)	% Identity[†]	Number of isolates	CmD[a]	CmPI[a]	Cuc[a]	Cil[a]	Cic[a]	Ece[a]	Brc[a]
Acinetobacter sp.	99	1				1			
Alloactinosynnema sp.	94	1				1			
Bacillus amyloliquefaciens	100%	5	5						
Bacillus brevis	99	1	1						
Bacillus cereus	99	1							1
Bacillus koreensis	e.g. >99%	2	2						
Bacillus licheniformis	e.g. >98%	5			1	2			2
Bacillus megaterium	98	1					1		
Bacillus pumilus	96	1	1						
Bacillus safensis	97	1					1		
Bacillus sp.	e.g. >97%	7	2	2		1			2
Bacillus subtilis	e.g. >99%	8	2	1	1	2			2
Bacillus vallismortis	87	1				1			
Brevibacillus	100	1							1
Brevibacterium sp.	94	1				1			
Brevundimonas sp.	97	1			1				
Burkholderia sp.	e.g. >100%	4						4	
Massilia sp.	99	1							1
Micrococcus sp.	e.g. >98%	2				1			1
Paenibacillus tylopili	98	1					1		
Pantoea agglomerans	100	1							1
Pantoea ananatis	99	1							1
Pseudomonas otitidis	99	1							1
Pseudomonas sp.	97	1						1	
Rhodobacter sp.	99	1	1						
Sphingomonas mathurensis	100	1				1			
Sphingomonas sp.	100	1			1				
Streptomyces sp.	99	1	1						
Tsukamurella sp.	94	1		1					
Vibrio cholerae	100	1							1
Xanthobacter sp.	99	1						1	
Total number of isolates		57	15	4	4	11	3	6	14

[a]Cucurbit abbreviations; *Cucumis melo* Reticulatus Group, 'Dulce' (CmD), *Cucumis melo* Momordica Group, 'PI414723' (CmPI), *Cucumis melo* (=*Cucumis callosus*) (Cuc), *Citrullus lanatus* (Cil), *Citrullus colocynthis* (Cic), *Ecballium elaterium* (Ece), *Bryonia cretica* (Brc).

On the other hand, natural plants are exposed to stresses such as drought and low nutrient status. Our results, however, showed that similar bacterial numbers can be isolated from fruits of one wild species, *E. elaterium*, and field-grown fruits. The other wild species, *B. cretica*, had at least one order of magnitude more bacteria than all other cucurbit species, and all of its tested fruits were positive. Moreover, the studied taxa belonged to two tribes: *C. melo*, *C. Lanatus* and *C. colocynthis* are members of the Benincaseae, whereas *E. elaterium* and *B. cretica* belong to the Bryonieae (Schaefer and Renner 2011). Neither bacterial number nor bacterial identity was visibly correlated with the taxonomy of the tested fruits. It can thus be seen that the division between the two environments with regard to endophyte numbers is not straightforward, and may therefore be influenced by other

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Table 5. Antifungal and antibacterial activities of isolated endophytes against *Macrophomina phaseolina* (Mac), *Fusarium oxysporum* f. sp. melonis races 1 and 2 (FOM 1 and FOM 2, respectively), *F. oxysporum* f. sp. *radicis-cucumerinum* (Forc) and *Pseudomonas syringae* (P.s.).

		Antagonistic activity against*				
Plant samples	Closest relative (16S rRNA analysis)	Mac	FOM 1	FOM 2	Forc	P.s.
Cucumis melo ReticulatusGroup, 'Dulce'	*Bacillus* sp.	3	3	3	3	3
	Bacillus amyloliquefaciens	3	3	3	3	2
	Bacillus amyloliquefaciens	3	3	3	3	2
	Bacillus amyloliquefaciens	3	3	3	3	3
	Bacillus amyloliquefaciens	3	3	3	3	3
	Bacillus amyloliquefaciens	1	1	1	0	1
	Bacillus pumilus	1	1	1	0	2
	Bacillus sp.	0	0	0	0	0
	Bacillus koreensis	3	1	2	2	2
	Bacillus brevis	3	2	2	2	1
	Bacillus subtilis	3	3	3	3	2
	Bacillus subtilis	0	0	0	0	0
	Streptomyces sp.	3	3	3	3	3
Cucumis melo Momordica Group, 'PI414723'	*Bacillus* sp.	0	0	0	0	0
	Tsukamurella sp.	0	0	0	0	0
	Bacillus subtilis	3	3	3	3	3
	Bacillus sp.	1	1	1	0	0
Cucumis callosus	*Bacillus subtilis*	3	3	2	3	2
	Bacillus licheniformis	0	0	0	0	1
	Sphingomonas sp.	0	1	0	0	0
Citrullus lanatus	*Acinetobacter* sp.	0	0	0	0	0
	Sphingomonas mathurensis	0	0	0	0	0
	Bacillus licheniformis	0	0	0	0	3
	Bacillus licheniformis	0	0	0	0	3
	Bacillus subtilis	3	2	2	2	1
	Brevibacterium sp.	0	0	0	0	0
	Micrococcus sp.	0	0	0	0	1
	Bacillus sp.	2	0	1	0	3
	Bacillus vallismortis	3	2	2	3	1
	Bacillus subtilis	3	1	1	0	3
Citrullus colocynthis	*Bacillus megaterium*	0	0	0	0	3
	Bacillus safensis	0	0	0	0	1
	Paenibacillus tylopili	0	0	0	0	–
Ecballium elaterium	*Xanthobacter* sp.	–	–	–	–	0
	Pseudomonas sp.	–	–	–	–	0
Bryonia cretica	*Bacillus subtilis*	3	2	2	2	3
	Bacillus cereus	3	3	3	3	3
	Pantoea agglomerans	0	0	0	0	0
	Bacillus subtilis	3	2	2	2	2
	Brevibacillus	1	0	0	0	1
	Bacillus sp.	0	0	0	0	1
	Pseudomonas otitidis	0	0	0	0	0
	Micrococcus sp.	0	0	0	0	0
	Bacillus licheniformis	0	0	0	0	3
	Bacillus licheniformis	0	0	0	0	3
	Pantoea ananatis	0	0	0	0	0
	Bacillus sp.	0	0	0	0	0
	Massilia sp.	0	2	2	1	0

*Inhibition index (mm): 0–: no inhibition; 1–: 1–5 mm; 2–: 6–10 mm; 3: –>10 mm.

factors such as insects, soil content or the climate, an issue that requires further investigation.

In field-grown fruits, the number of bacteria in the cultured taxa was significantly higher than the levels associated with the wild cucurbits. In addition, the percentage of endophyte-free fruits was higher in wild taxa versus cultivated ones. Since all field-grown plants were subjected to the same conditions, including the same soil and climate, these results indicate that the plant geno- and phenotype effects are more pronounced than the growth conditions in the field. Cultivated plants have

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already been exposed to genetic processes aimed at improving fruits quality and sensitivity to different diseases. In addition, cucurbit fruits vary in shape, size, flesh texture, sugar content, pH and nutrient composition. Sugar content and acidity are examples of fruit habitat conditions that change during ripening and may have an influence on bacterial colonization and survival. Different fruits contain nutrients that may benefit selected microorganisms. For example, non-sweet cucurbit fruits such as cucumber and squash accumulate starch. In addition, all wild cultivars selected for this study contain only trace amounts of soluble sugars such as sucrose, glucose and fructose, whereas sweet fruits like melon and watermelon accumulate high levels of fructose, glucose, galactose, rhamnose and sucrose (Burger *et al.* 2006). 'Dulce' melons, for example, which have higher soluble sugar content and higher pH, harbored more bacteria than the wild *C. melo Momordica*, which has a low soluble sugar content and lower pH (Burger *et al.* 2009). Furthermore, the wild fruits of *E. elaterium, Ci. colocynthis* and *B. cretica* contain the plant substances cucurbitacins (Miro 1995). These are a group of bitter tasting, pharmacologically very active compounds that were shown to act as antifungal agents. The presence of these compounds combined with the low sugar content might explain the low number of bacteria associated with the fruits belonging to that bitter group.

The low density and diversity of bacteria in fruits may support the assumptions that only specialized endophytic strains are able to colonize and survive in plant disseminating organs, or that only specialized strains are able to move from the environment or other plant organs to the fruits (Compant *et al.* 2008, 2011; Compant, Clément and Sessitsch 2010). Macronutrients are probably not a limiting factor in the cucurbit fruits, because many cucurbit fruits accumulate carbohydrates, organic acids and other compounds needed to retain the osmotic pressure and flow of water during ripening. In melons, for example, amylose is degraded during ripening, and sucrose, raffinose and stachyose oligosaccharides are accumulated (Burger *et al.* 2009). As many microorganisms can use these carbohydrates as carbon and energy sources, there might be other factors apart of nutrition that control endophytes levels in the fruits. Since genes involved in the plant defense response and resistance undergo differential expression during development and ripening of watermelon fruits (Wechter *et al.* 2008), it is possible that the same mechanisms used by the plants to control pathogens are also used to restrict the establishment of non-pathogens, and thus endophytes need to have specific resistance properties and mechanisms to cope with the plant defense measures.

Bacillus species were dominant in most of the endophyte-containing fruits. Other bacteria, including *Pseudomonas*, *Brevibacterium*, *Alloactinosynnema*, *Burkholderia*, *Micrococcus*, *Massilia*, *Pantoea*, *Rhodobacter*, *Sphingomonas*, *Streptomyces*, *Tsukamurella*, *Vibrio* and *Xanthobacter* species, were also isolated from a few fruits belonging to different Cucurbitaceae taxa. *Bacillus* species have been described as common endophytes associated with different organs of various plants (Berg 2009). In many studies, *Bacillus* shows properties that benefit the host plant. Although we did not compare population diversity with that in other organs of the plants, it has been previously reported that *Bacillus* and *Staphylococcus* members identified as endophytes in berry fruits and seeds of grapevine were also found in roots or flowers, whereas others were unique to the fruits (Compant *et al.* 2011).

We investigated the antifungal and antibacterial activities of the isolated strains against common cucurbit pathogens. Most bacteria were able to inhibit the growth of at least one pathogen, but a few, mostly *Bacillus* isolates, showed a wide range of activities against all five tested pathogens. *Bacillus subtilis* relatives isolated from pumpkin fruits have also been shown to have antagonistic activity against the pumpkin pathogen *Didymella bryoniae* (Fürnkranz *et al.* 2012). The antagonistic activity of *B. subtilis* towards fungal and bacterial pathogens of cucurbits has been attributed to the production of antimicrobial lipopeptides such as bacillomycins, fengycins and surfactin. Bacillomycins and fengycins kill the pathogens' cells by damaging their membranes (Zeriouh *et al.* 2011), whereas surfactin probably elicits a plant defense response and induces biofilm formation by *Bacillus* (García-Gutiérrez *et al.* 2013; Zeriouh *et al.* 2013). Likewise, the antagonistic activity of *B. amyloliquefaciens* ssp. plantarum was attributed to the lipopeptides iturin, fengycin and surfactin, the polyketides difficidin, macrolactin and bacillaene and the dipeptide bacilysin (Compaoré *et al.* 2013). Starch, accumulated in non-sweet cucurbit fruits and fructose, glucose, galactose, rhamnose and sucrose accumulated in sweet fruits are digested by enzymes generally produced by *Bacillus* strains. At immature fruits stages, fructose and glucose are the predominant sugars in watermelon, and their levels decline as the fruits mature. In contrast, the content of sucrose, which is low in immature fruits, increases sharply in mature ones (Guo *et al.* 2011). Interestingly, high sucrose concentrations were reported to increase the growth rate of *Bacillus* (Peighamy-Ashnaei *et al.* 2007), which was by far the most abundant genus in the mature fruits, and to improve surfactin production by *Bacillus* (Abdel-Mawgoud, Aboulwafa and Hassouna 2008). A continuous study in the field is required to investigate whether the antimicrobial agents are synthesized in the fruits, and whether the observed antimicrobial activity can also be used to prevent disease and spoilage symptoms in the fruits before and after harvest, or to inhibit human pathogens such as *Salmonella*, which have been reported to be potentially associated with cucurbit fruits and may cause disease outbreaks (Mohle-Boetani *et al.* 1999; Yaron 2013).

In this study, we identified endophytes in cucurbit fruits with antimicrobial activity against major cucurbit pathogens. Even though bacterial numbers in the cucurbit fruits were relatively low, we demonstrated that colonization of fruits by potentially beneficial endophytes is a general phenomenon. Fruits are a yet unexplored resource of beneficial bacteria that can be implemented as biocontrol agents for melon growth. Further investigation of the identified bacteria, particularly *Bacillus* isolates but also others, with respect to their potential to improve growth and to protect plants not only in the field, but also post-harvest, is warranted. The latter might serve to increase the fruit shelf life by delaying softening and spoilage, or to ensure food safety.

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REFERENCES

Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NAH. Optimization of surfactin production by *Bacillus subtilis* isolate BS5. *Appl Biochem Biotech* 2008;**150**:305–25.

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Amann RI, Binder BJ, Olson RJ, *et al*. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 1990;**56**:1919–25.

Assmus B, Hutzler P, Kirchhof G, *et al*. In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Appl Environ Microbiol* 1995;**61**:1013–9.

Bent E, Chanway CP. The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Can J Microbiol* 1998;**44**: 980–8.

Berg G. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 2009;**84**:11–8.

Berg G, Krechel A, Ditz M, *et al*. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol Ecol* 2005;**51**:215–29.

Burger Y. Sucrose metabolism in melon fruits (*Cucumis melo* L.): the biosynthesis of sucrose from translocated stachyose and the role of the invertase enzyme in determining sucrose accumulation. *Ph.D Thesis*. Hebrew University, Jerusalem 2000.

Burger Y, Paris H, Cohen R, *et al*. Genetic diversity of *Cucumis melo*. *Hortic Rev* 2009;**36**:165–98.

Burger Y, Sa'ar U, Paris HS, *et al*. Genetic variability for valuable fruits quality traits in *Cucumis melo*. *Israel J Plant Sci* 2006;**54**:37–41.

Campisano A, Ometto L, Compant S, *et al*. Interkingdom transfer of the acne causing agent, *Propionibacterium acnes*, from human to grapevine. *Mol Biol Evol* 2014;**31**: 1059–65.

Cankar K, Kraigher H, Ravnikar M, *et al*. Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). *FEMS Microbiol Lett* 2005;**244**:341–5.

Cohen R, Horev C, Burger Y, *et al*. Horticultural and pathological aspects of *Fusarium* wilt management using grafted melons. *Plant Pathol* 2002;**37**:1069–73.

Compant S, Clément C, Sessitsch A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* 2010;**42**:669–78.

Compant S, Duffy B, Nowak J, *et al*. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* 2005;**71**:4951.

Compant S, Kaplan H, Sessitsch A, *et al*. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. *FEMS Microbiol Ecol* 2008;**63**:84–93.

Compant S, Mitter B, Colli-Mull JG, *et al*. Endophytes of grapevine flowers, berries and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb Ecol* 2011;**62**:188–97.

Compant S, Sessitsch A, Mathieu F. The 125th anniversary of the first postulation of the soil origin of endophytic bacteria—a tribute to MLV Galippe. *Plant Soil* 2012;**356**:299–301.

Compaoré CS, Nielsen DS, Sawadogo-Lingani H, *et al*. *Bacillus amyloliquefaciens* ssp. *plantarum* strains as potential protective starter cultures for the production of Bikalga, an alkaline fermented food. *J Appl Microbiol* 2013;**115**:133–46.

Daims H, Brühl A, Amann R, *et al*. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 1999;**22**:434–44.

Fürnkranz M, Lukesch B, Müller H, *et al*. Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. *Microb Ecol* 2012;**63**:418–28.

García-Gutiérrez L, Zeriouh H, Romero D, *et al*. The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. *Microb Biotechnol* 2013;**6**:264–74.

Gray E, Smith D. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol Biochem* 2005;**37**:395–412.

Guo S, Liu J, Zheng Y, *et al*. Characterization of transcriptome dynamics during watermelon fruits development: sequencing, assembly, annotation and gene expression profiles. *BMC Genomics* 2011;**12**:454.

Hallmann J. Plant interactions with endophytic bacteria. In: Jeger MJ, Spence NJ (eds). *Biotic Interactions in Plant–Pathogen Associations*. Wallingford: CABI, 2001, 87–119.

Hallmann J, Berg G. Spectrum and population dynamics of bacterial root endophytes. *Biol Res* 2006;**9**:15–31.

Hallmann J, Quadt-Hallmann A, Mahaffee WF, *et al*. Bacterial endophytes in agricultural crops. *Can J Microbiol* 1997;**43**: 895–914.

Hardoim PR, van Overbeek LS, van Elsas JD. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 2008;**16**:463–71.

Harel-Beja R, Tzuri G, Portnoy V, *et al*. A genetic map of melon highly enriched with fruits quality QTLs and EST markers, including sugar and carotenoid metabolism genes. *Theor Appl Genet* 2010;**121**:511–33.

James EK, Reis VM, Olivares FL, *et al*. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J Exp Bot* 1994;**45**:757–66.

Johnston-Monje D, Raizada MN. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. *PLoS One* 2011;**6**:e20396.

Krishnan P, Bhat R, Kush A, *et al*. Isolation and functional characterization of bacterial endophytes from *Carica papaya* fruits. *J Appl Microbiol* 2012;**113**:308–17.

Küsel K, Pinkart HC, Drake HL, *et al*. Acetogenic and sulfate-reducing bacteria inhabiting the rhizoplane and deep cortex cells of the sea grass *Halodule wrightii*. *Appl Environ Microbiol* 1999;**65**:5117–23.

Lodewyckx C, Vangronsveld J, Porteous F, *et al*. Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 2002;**21**:583–606.

Long HH, Schmidt DD, Baldwin IT. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One* 2008;**3**:e2702.

Loy A, Maixner F, Wagner M, *et al*. probeBase—an online resource for rRNA-targeted nucleotide probes: new features 2007. *Nucleic Acid Res* 2007;**35**:D800–4.

Manz W, Amann R, Ludwig W, *et al*. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* 1992;**15**:593–600.

Mastretta C, Taghavi S, van der Lelie D. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. *Int J Phytoremediat* 2009;**11**:37–41.

Miro M. Cucurbitacins and their pharmacological effects. *Phytother Res* 1995;**9**:159–68.

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Misaghi IJ, Donndelinger CR. Endophytic bacteria in symptom-free cotton plants. *Phytopathology* 1990;**80**:808–11.

Mitter B, Brader G, Afzal M, *et al.* Advances in elucidating beneficial interactions between plants, soil and bacteria. *Adv Agron* 2013;**121**:381–445.

Mohle-Boetani JC, Reporter R, Werner SB, *et al.* An outbreak of *Salmonella* serogroup Saphra due to cantaloupes from Mexico. *J Infect Dis* 1999;**180**:1361–4.

Morris CE, Glaux C, Latour X, *et al.* The relationship of host range, physiology, and genotype to virulence on Cantaloupe in *Pseudomonas syringae* from Cantaloupe blight epidemics in France. *Phytopathology* 2000;**90**:636–46.

Okunishi S, Sako K, Mano H, *et al.* Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*). *Microbes Environ* 2005;**20**:168–77.

Peighamy-Ashnaei S, Sharifi-Tehrani A, Ahmadzadeh M, *et al.* Effect of carbon and nitrogen sources on growth and biological efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Rhizoctonia solani*, the causal agent of bean damping-off. *Commun Agr Appl Biol Sci* 2007;**72**:951–6.

R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2009.

Roller C, Wagner M, Amann R, *et al.* *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiol* 1994;**140**:2849–58.

Rosenblueth M, Martínez-Romero E. Bacterial endophytes and their interactions with hosts. *Mol Plant-Microbe Interact* 2006;**19**:827–37.

Ryan RP, Germaine K, Franks A, *et al.* Bacterial endophytes: recent developments and applications. *FEMS Microbiol Lett* 2008;**278**:1–9.

Schaefer H, Renner SS. Phylogenetic relationships in the order Cucurbitales and a new classification of the gourd family (Cucurbitaceae). *Taxon* 2011;**60**:122–38.

Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;**9**:671–5.

Sessitsch A, Reiter B, Pfeifer U, *et al.* Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes. *FEMS Microbiol Ecol* 2002;**39**:23–32.

Shi J, Liu A, Li X, *et al.* Identification of endophytic bacterial strain MGP1 selected from papaya and its biocontrol effects on pathogens infecting harvested papaya fruits. *J Sci Food Agr* 2010;**90**:227–32.

Truyens S, Weyens N, Cuypers A, *et al.* Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environ Microbiol Rep* 2014;**7**:40–50.

Vakalounakis DJ. Root and stem rot of cucumber caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum* f.sp. nov. *Plant Dis* 1996;**80**:313–6.

Wallner G, Amann R, Beisker W. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* 1993;**14**:136–43.

Wechter WP, Levi A, Harris KR, *et al.* Gene expression in developing watermelon fruits. *BMC Genomics* 2008;**9**:275.

Weisburg WG, Barns SM, Pelletier DA, *et al.* 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173**:697–703.

Yaron S. Microbial attachment and persistence on plants. In: Matthews KR (ed). *The Produce Contamination Problem—Causes and Solutions*, 2nd ed. Amsterdam: Elsevier Press, 2013.

Zeriouh H, De Vicente A, Pérez-García A, *et al.* Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. *Environ Microbiol* 2013;**16**:2196–211

Zeriouh H, Romero D, Garcia-Gutierrez L, *et al.* The iturin-like lipopeptides are essential components in the biological control arsenal of *Bacillus subtilis* against bacterial diseases of cucurbits. *Mol Plant-Microbe Int* 2011;**24**:1540–52.

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