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REGULAR ARTICLE

Bacterial niches inside seeds of *Cucumis melo* L.

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Abstract
Background and aims Seeds are inhabited by diverse bacterial and fungal taxa whose colonization patterns are little understood. We hypothesized, however, that specific niches within seeds host microbes.
Methods In this study, the putative presence of bacteria, inhabiting the seed endosphere of an angiosperm, the melon *Cucumis melo reticulatus* group cv. 'Dulce', was examined by scanning electron microscopy (SEM) and confocal laser-scanning microscopy coupled with double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH).
Results SEM images showed microbial-like structures in different tissues and FISH revealed endophytic bacteria colonizing the outer and inner seed parts, on perisperm/endosperm envelope, inside the cotyledons as parts of the embryo, and, to a lesser extent, inside embryonic hypocotyl-root axis tissues. *Alphaproteobacteria* were shown to inhabit the seed coat and the envelope surrounding the embryonic hypocotyl-root tissues, but could not be seen in the cotyledons, whereas *Betaproteobacteria* were only detected in the outer seed coat. Some *Gammaproteobacteria* were also seen in the outer seed coat, but were mainly visualized in the cotyledons with a few inside the seed's embryonic hypocotyl-root tissues, among other bacteria. *Firmicutes* were visualized inside the seed coat, but mostly inside the cotyledon tissues, on the perisperm/endosperm envelope and inside the embryonic hypocotyl-root axis tissues. Microscopy revealed *Actinobacteria* inside the inner and outer seed coat and inside the embryonic parts such as cotyledons, with a few inside the hypocotyl-root axis.
Conclusions This is the first demonstration of niches for the most active groups of bacteria inhabiting different seed tissues of an angiosperm.

Keywords Seed · Disseminating organ · Melon · Bacteria · Microhabitat

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Introduction

Plant bacterial microbiota are diverse, and are an intimate component of different plant parts and species. Different microbial taxa belonging to the plant microbiota can be found not only in the phytosphere but also as active inhabitants of the plant's endospheric compartments (Hardoim et al. 2015). This is especially true for the inner tissues of roots,

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stems and leaves (Turner et al. 2013; Compant et al. 2010; Berg et al. 2014). Studies describing the dynamics of such bacteria have revealed that most derive from the soil environment, including the rhizosphere and the spermosphere, but also from external stem environments known as the caulosphere or laimosphere (Compant et al. 2010). Recent data have shown, however, that plant parts such as fruits, seeds and caryopses also host specific bacteria (Compant et al. 2010; Truyens et al. 2014; Glassner et al. 2015). It is now known that these microbes derive from the anthosphere and carposphere as part of the phytospheric environment, or from the soil compartment, from which they migrate through the whole plant (Compant et al. 2011b). Microbes associated with reproductive or disseminating organs have not been studied in depth and there are gaps in our understanding of which plant niches they colonize and how they colonize them. This is especially true for disseminating organs such as seeds and caryopses, where endophytic bacteria have been found in organs such as the seeds of Norway spruce, tobacco, the caryopses of rice and maize (Cankar et al. 2005; Mastretta et al. 2009; Okunishi et al. 2005; Johnston-Monje and Raizada 2011), and inside the seeds of grape, Styrian oil pumpkin and papaya (Compant et al. 2011a, b; Fürnkranz et al. 2012; Krishnan et al. 2012). Most of these studies used various methods to determine localization patterns. However, direct microscopic analyses are still scarce. A more detailed knowledge is needed of the specific habitats colonized by endophytes, as well as their taxa.

Over the years, it has been postulated that the main niche for bacterial colonization is the outer part of the seeds; bacteria have rarely been localized to other seed tissues (Truyens et al. 2015). Some authors consider seeds to be a sterile environment due to the low number of bacteria isolated from them (Hallmann 2001). However, it has been shown that these organs can host microbes, and bacteria inhabiting seeds or other kinds of disseminating organs form an important group among the plant-associated bacteria (Truyens et al. 2015). They may play a role during their hosts' germination, seedling development and plant nutrition (Johnston-Monje and Raizada 2011; Truyens et al. 2015). Apart from bacteria present in the seed's surroundings, bacteria that are already present inside disseminating organs can be important for the evolution of seedling-associated microbiota (Johnston-Monje and Raizada 2011; Truyens et al. 2015).

We hypothesized that several tissues within the seed can host bacteria and that different taxa colonize different niches within seeds. To better understand endophytic colonization of seeds, we tracked microbial-like structures using scanning electron microscopy and analyzed the different taxa of bacteria living inside seeds of *Cucumis melo reticulatus* group cv. 'Dulce'. This cultivated melon variety was chosen because melon plants, like other members of the Cucurbitaceae, have the unique feature of fleshy fruits and a pericarp that surrounds and protects the seeds, which might influence the endophytic population. In a recent study, we identified the endophytes localized inside cucurbit fruits, and found *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria*. Culturable bacteria were further isolated and identified from fruit tissues of 'Dulce', and other cultivated and wild-field-grown Cucurbitaceae (Glassner et al. 2015). Substantial differences were observed between the wild and cultivated cucurbit taxa with regard to both the number of colonized fruits and the genera of endophytes. Here we performed a detailed investigation of their colonization niches in the different seed tissues using double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) coupled with confocal laser-scanning microscopy (CLSM). The resultant information is expected to help further characterize which tissues can be colonized by bacteria, particularly addressing active bacterial cells.

Materials and methods

Plant material and sampling procedure

The cultivated melon *Cucumis melo reticulatus* group cv. 'Dulce' was sampled in a commercial field, where fruits were grown from seeds originating from the Newe Ya'ar collection (Burger et al. 2006). Melons were seeded in April 2012 in an experimental plot at Newe Ya'ar Research Center, Israel (32°42′30.7″N 35°10′47.7″E). 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 as described by Glassner et al. (2015). Plants were fertilized with six units of NH_4NO_3 and H_3PO_4 per 0.1 ha of land, and fully developed fruits were harvested in mid-July. Between 10

and 20 samples were collected by harvesting the fruits upon ripening and kept at room temperature (20 °C) for no more than 5 days until processing. To avoid contamination of the samples by environmental bacteria, the melon fruits 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.

SEM, DOPE-FISH and CLSM of seeds' bacterial inhabitants

For microscopy analysis, seeds were harvested from fruits sliced under aseptic conditions in a laminar-flow hood. When melon seeds were surface-sterilized according to common procedure (i.e., as described in Glassner et al. 2015), no bacteria could be isolated (data not shown), probably due to the unique morphology of melon seeds, which contain a micropyle. Therefore, seeds surrounded by gelatinous tissue were collected, and cut into four small parts (each 0.5 cm long). Seeds were fixed overnight at 4 °C in a paraformaldehyde solution (4% *w/v* in PBS, pH 7.2) in Eppendorf tubes, then samples were rinsed thrice with PBS. Treatment with lysozyme solution (1 mg mL^{-1} in PBS) was applied for 10 min at 37 °C, samples were rinsed again and followed by an ethanol dehydration series (25, 50, 75 and 99.9%; 15 min each step). DOPE-FISH was performed with probes from Genecust (Luxembourg) labeled at both the 5′ and 3′ end positions according to Compant et al. (2013) and Stoecker et al. (2010). An EUBmix targeting all bacteria (with equivalent mixture of EUB338, EUB338II, EUB338III) coupled with the fluorochrome FLUOS (Amann et al. 1990; Daims et al. 1999), and a *Firmicutes* probe (LGC; Küsel et al. 1999), *Alpha-*, *Beta-* and *Gammaproteobacteria* probes (ALF1B, BET42a and GAM42a, respectively; Manz et al. 1992), and an *Actinobacteria* probe (HGC69a; Roller et al. 1994) coupled with Cy5 were used (Table 1). These bacterial taxa were specifically targeted because they have been shown to dominate melon fruit tissues (Glassner et al. 2015). In addition, a review of different plant-bacteria systems reported that *Proteobacteria*, *Actinobacteria*, and *Firmicutes* comprise about 90% of the total number of endophytic prokaryotic sequences (Hardoim et al. 2015). Moreover, most inhabitants of plant seeds belong to the *Proteobacteria* (*Gammaproteobacteria*), *Actinobacteria* and *Firmicutes* (Truyens et al. 2015). A NONEUB probe (Wallner et al. 1993) coupled with Cy5 or FLUOS was also used independently as a negative control (Table 1). 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 placed on slides in a 50-mL moist chamber (also housing a piece of tissue imbibed with 5 mL hybridization buffer). Post-hybridization was conducted at 48 °C for 30 min with a pre-warmed post-FISH solution containing 20 mM Tris–HCl pH 8.0, 0.01% 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 then observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 and HeNe(G)laser FV10-LAHEG230–2). X, Y, Z pictures were taken at 405, 488, 633 nm and with 10X, 20X or 40X objectives and then merged (RGB) using Image J software (Schneider et al. 2012). Z Project Stacks were then used to create the pictures (as described in Campisano et al. 2014). Pictures were cropped and due to the convolution process in the microscope, whole pictures were sharpened and the light/contrast balance improved to better observe the image details, as seen when samples are observed in the dark under the microscope (as described in Glassner et al. 2015). All experiments were repeated on 3–5 seeds from six plants and FISH was performed three independent times on different seed sections from at least three independent plants for each probe combination. Images presented in this publication are the average of colonization.

Some cut seeds were also observed with a Hitachi TM3030 tabletop ESEM (Metrohm Inula GmbH, Vienna) using 15 kV accelerating voltage in charge-up reduction mode. The samples were frozen with a cooling stage from Deben UK Ltd. (London) at −20 °C directly in the specimen chamber and pictures were acquired at different magnifications. Pictures were also cropped and due to the convolution process in the microscope, whole pictures were sharpened and the light/contrast balance improved to better observe the image details.

Results

Bacterial-like structures within seeds using SEM

Seed samples were examined using SEM (Fig. 1a) and microbial-like structures were detected in the outer seed

Table 1 Names, accession numbers and targets of probes used for DOPE-FISH/CSLM

Probe names	Accession numbers	Targets	References
EUB338	pB-00189	Most bacteria	Amann et al. 1990
EUB338II	pB-00160	*Planctomycetes*	Daims et al. 1999
EUB338III	pB-00161	*Verrumicrobia*	Daims et al. 1999
NONEUB	pB-00243	Control probe	Wallner et al. 1993
ALF1B	pB-00017	*Alphaproteobacteria* Some *Deltaproteobacteria* Some *Spirochaetes*	Manz et al. 1992
BET42a	pB-00034	*Betaproteobacteria*	Manz et al. 1992
GAM42a	pB-00174	*Gammaproteobacteria*	Manz et al. 1992
LGC	pB-01040	*Firmicutes*	Küsel et al. 1999
HGC69a	pB-00182	*Actinobacteria*	Roller et al. 1994

coat (Fig. 1b and c), inner seed coat (Fig. 1d) and within the nearby xylem element (Fig. 1d and e). Within the cotyledons, some zones were densely colonized by bacterial-like structures (Fig. 2a). Some of these structures were also detected at the perisperm/endosperm envelope layer (Fig. 2b), along with a few within the hypocotyl-root embryo, especially at the hypocotyl level (Fig. 2c) and the rest of the embryonic hypocotyl-root axis tissues (Fig. 2d).

Bacterial inhabitants of seeds

The hybridization of mixed EUB probes (EUBmix) targeting all bacteria to the seed parts further proved the presence of bacteria within the seeds and revealed their localization in the outer and inner surfaces of the seed coat as single cells or clusters (Fig. 3a). Some bacteria were also seen to colonize tissue near xylem vessels (Fig. 3a). In the embryonic cotyledon tissues, large

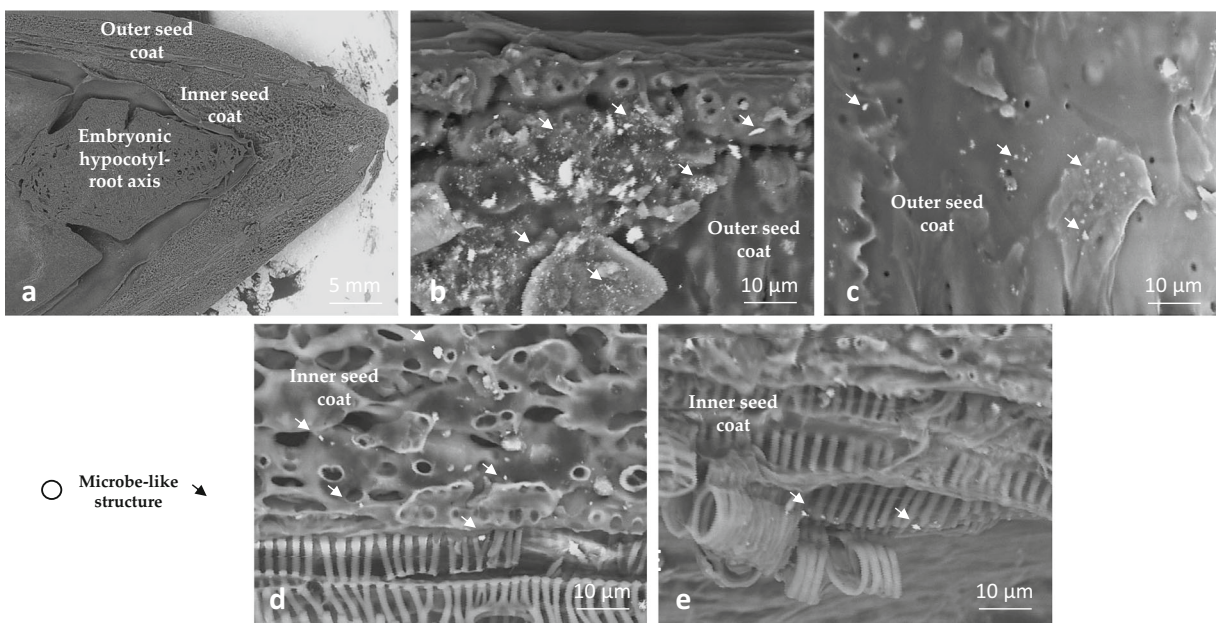

Fig. 1 SEM of seed coats of the cultivated melon *Cucumis melo reticulatus* group 'Dulce'. Within the seed sections (**a**) bacterial-like structures were detected inside the outer part of the seed coat (**b-c**), the inner seed coat (**d-e**), and nearby xylem vessels (**e**)

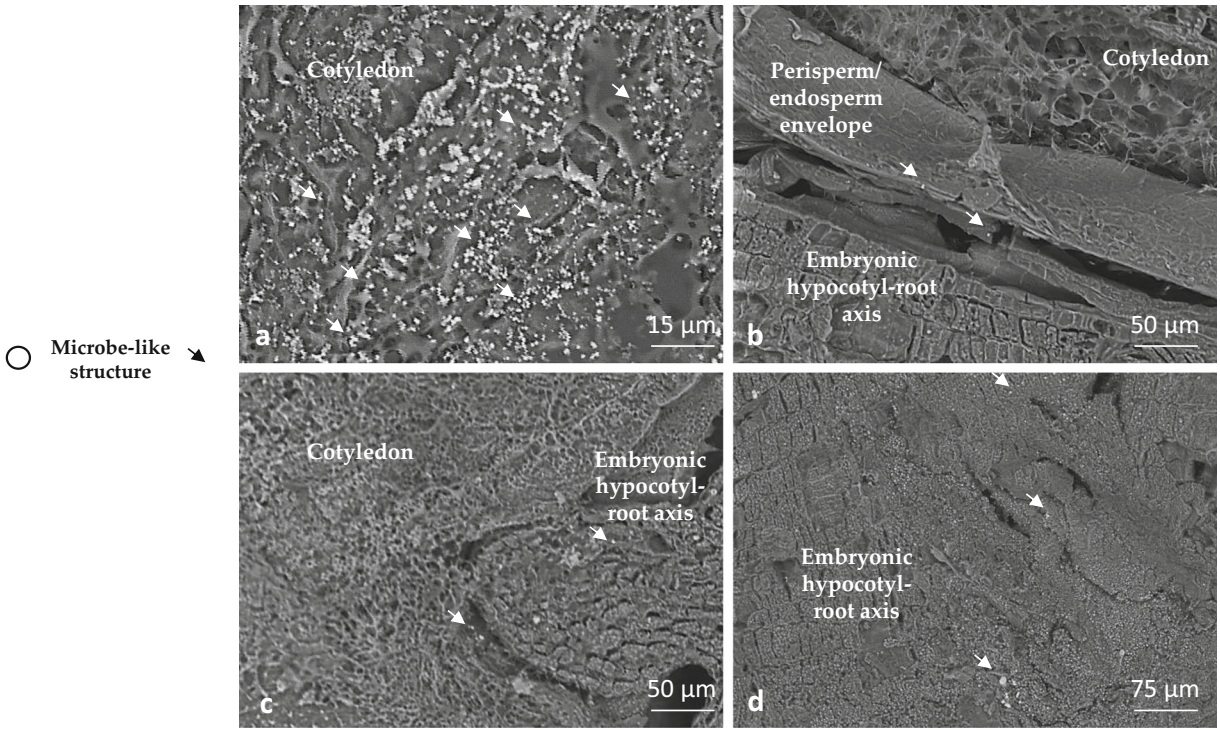

Fig. 2 SEM of cotyledons and other parts of the embryo of the cultivated melon *Cucumis melo reticulatus* group 'Dulce'. Bacterial-like structures were detected in the cotyledons (**a**), perisperm/endosperm envelope (**b**), and embryonic hypocotyl-root axis (**c-d**)

amounts of bacteria were visualized as single cells or also clusters of cells (Figs. 3b and c). Several bacteria were also detected near the embryonic hypocotyl-root axis tissues, especially at the border between these tissues

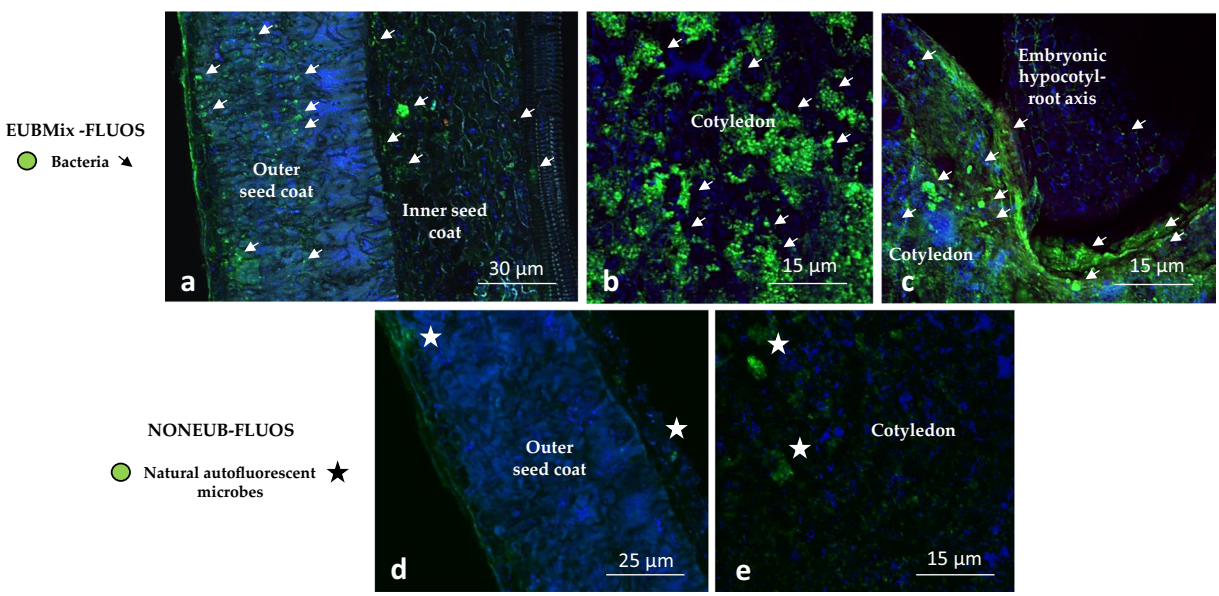

Fig. 3 CLSM with DOPE-FISH hybridization of seeds of the cultivated melon *Cucumis melo reticulatus* group 'Dulce' and use of EUBmix coupled with FLUOS fluorochrome**.** Bacteria were seen inside the outer and inner parts of the seed coat (**a**), cotyledons (**b**), embryonic hypocotyl-root axis and at the limit to the cotyledon tissues (**c**). Naturally autofluorescent microbes were slightly detected in tissues such as the outer seed coat (**d**) and the cotyledons (**e**) using NONEUB–FLUOS

and the cotyledon parts (Fig. 3c). Inside the embryonic hypocotyl–root axis tissues, bacteria were scarcely seen as single cells (Fig. 3c). The use of NONEUB probes on seed tissues confirmed that the EUBmix allows visualizing bacteria inside seed tissues, as only a few microbes were visualized with natural green fluorescence which might correspond to their natural autofluorescence; background fluorescence was also observed, but its intensity was lower than obtained with the EUBmix (Fig. 3d and f). This was also seen when no probe was used (data not shown), so care was taken to verify the presence of bacterial shapes prior to image acquisition. No red, but a few orange autofluorescing microbes were recorded in the seed coat (Fig. 3a and d).

Proteobacteria inside seeds

When the taxon-specific probe ALF1B–Cy5 was applied together with the EUBmix, *Alphaproteobacteria* could be seen inside the seed coat among other bacteria (Fig. 4a). They were especially noticeable in the outer seed coat (Fig. 4a), inside the cotyledons tissues (Fig. 4b) and at the level of the perisperm/endosperm envelope around the embryonic hypocotyl-root axis (Fig. 4c), where other types of microbes—including naturally blue–cyan-autofluorescing microbes—were observed together with reddish plant structures (Fig. 4c). No bacteria corresponding to *Alphaproteobacteria* were visualized inside the embryonic hypocotyl-root axis although a few other bacteria were visualized (Fig. 4c). Very few *Betaproteobacteria* were detected using the EUBmix probes with FLUOS and BET42a–Cy5; these were mainly in the outer part of the seed coat (Fig. 4d and e), whereas none were visualized inside the cotyledons or in the embryonic hypocotyl-root axis, although other bacteria were detected. *Gammaproteobacteria* were additionally detected inside the seeds using GAM42a–Cy5/EUBmix–FLUOS, but not generally inside the seed coat (Fig. 4f), although some samples showed a small amount of such microbes (data not shown). These bacteria were visualized mainly in the cotyledons together with others (Fig. 4g). Rarely, a few additional *Gammaproteobacteria* were observed inside the embryonic hypocotyl-root axis tissues (Fig. 4h), which were mainly colonized by other types of bacteria (Fig. 4h).

Firmicutes inside seeds

As for other taxa, *Firmicutes* were detected as seed inhabitants. They were visualized using the LGC probe and EUBmix in the outer and inner parts of the seed coat (Fig. 5a and b), the largest amount being in the outer part. Cotyledon tissues were colonized by *Firmicutes* (Fig. 5c). *Firmicutes* were also observed at the perisperm/endosperm layer interface (Fig. 5d) and few were seen inside the embryonic hypocotyl-root tissues among other bacteria, particularly intracellularly (Fig. 5e).

Actinobacteria inside seeds

The last group of bacteria studied was *Actinobacteria*. Using probe HGC69a together with EUBmix, they were detected inside the outer part of the seed coat (Fig. 6a) but also in the inner seed coat (Fig. 6b). Cotyledons that were strongly colonized by bacteria revealed *Actinobacteria* as well (Fig. 6c). *Actinobacteria* were not generally detected inside the embryonic hypocotyl-root axis tissues (Fig. 6d), but some samples showed a few of them.

Discussion

This study shows that bacteria inhabit the seed of the melon *C. melo reticulatus* group cv. 'Dulce', especially endophytic *Alpha-*, *Beta-* and *Gammaproteobacteria*, as well as *Firmicutes* and *Actinobacteria*, providing further support for our previous findings in fruits (Glassner et al. 2015). The presence of endophytic bacteria inside seeds has been recently demonstrated in plants such as grapevine (Compant et al. 2011a, b), tobacco (Mastretta et al. 2009), eucalyptus (Ferreira et al. 2008), rapeseed (Granér et al. 2003), coffee (Vega et al. 2005), ash (Donnarumma et al. 2010), soybean (Oehrle et al. 2000), sugarbeet (Dent et al. 2004), pumpkin (Fürnkranz et al. 2012), peanut (Sobolev et al. 2013), cauliflower (Pleban et al. 1995), wild mustard (Pleban et al. 1995), bean (Rosenblueth et al. 2010), tomato (Xu et al. 2014), strawberry (Kukkurainen et al. 2005), *Arabidopsis thaliana* (Truyens et al. 2013) and various grasses and weeds (Mundt and Hinkle 1976). Caryopses comprising seed tissues have also been reported to contain endophytic bacteria (e.g., in rice – Ebeltagy et al. 2000 and maize – Johnston-Monje and Raizada 2011). It should be noted that in addition to bacteria, seed microbial communities may contain fungal endophytes, whose presence in seeds and seed-borne transmission have been well documented in different plants (Hardoim et al. 2015), such as the fungus-grass symbioses (Clay and Schardl 2002; Schardl et al. 2004). It is reasonable to

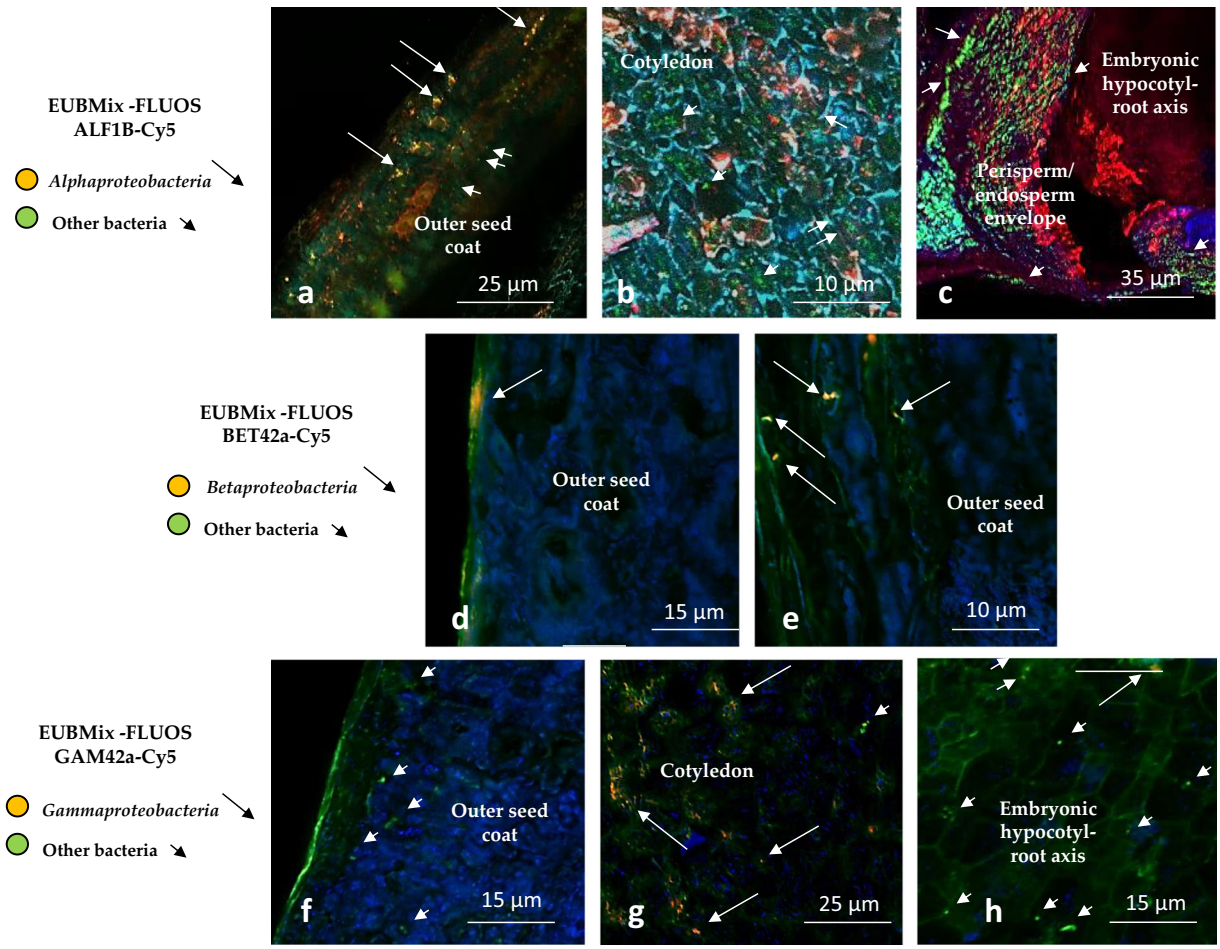

Fig. 4 CLSM with DOPE-FISH hybridization of seeds of the cultivated melon *Cucumis melo reticulatus* group 'Dulce' and use of specific probes for *Alpha-*, *Beta-*, and *Gammaproteobacteria*. *Alphaproteobacteria* were seen inside the seed coat (**a**), cotyledons (**b**) and perisperm/endosperm envelope surrounding embryonic tissues (**c**), while *Betaproteobacteria* were detected inside the seed coat (**d, e**) but not inside the cotyledon tissues or the rest of the seed embryo. In comparison, *Gammaproteobacteria* were not detected, for the most part, in the seed coat, but were detected inside the cotyledons and a few were found inside the embryonic hypocotyl-root axis with other bacteria (**f-h**)

assume that such fungi reside in melon seeds, but they were not addressed in the present study.

Bacteria were found in all seed tissues. Similar observations have been made in other plants, where microorganisms were reported to be localized inside the seed husk, coat, cortex, endosperm and embryonic cells (e.g. Cankar et al. 2005; Puente et al. 2009). However, those studies were performed mainly using culture-dependent methods. The SEM method used here allowed us to identify bacterial-like structures within all seed tissues and the applied FISH analysis indicated variations in the presence of endophytes in melon seeds among the different tissues, with most bacteria visualized near the seed coat, within the cotyledons as part of the embryo, and only a few localized inside the embryonic hypocotyl-root axis. Depending on the material, microscopy revealed several bacterial taxa in the perisperm/endosperm layer as well. This layer acts as a barrier to apoplectic permeability and radicle emergence and is mainly composed of endosperm and layers containing lipid and pectin/callose surrounding embryonic tissues (cotyledons and hypocotyl-root axis) (Salanenka et al. 2009). Bacteria may be attracted to compounds in these layers, and therefore colonize them. However, it was difficult to focus on this layer along the entire seed length due to the sectioning, which only revealed parts of it. The analysis, in parts such as the seed coat, cotyledons and embryonic hypocotyl-root axis, showed that

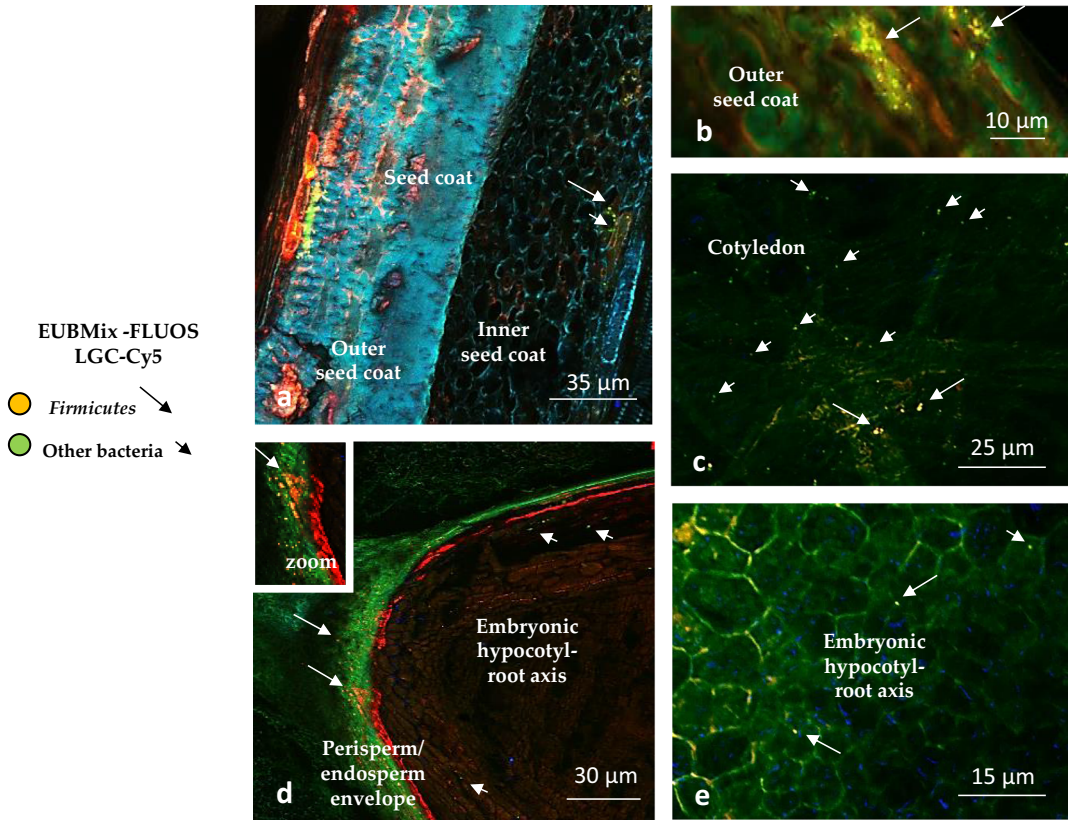

Fig. 5 CLSM with DOPE-FISH hybridization of seeds of the cultivated melon *Cucumis melo reticulatus* group 'Dulce' and use of a probe specific for *Firmicutes.* Members of this bacterial taxon were seen inside the seed coat (**a, b**), cotyledons (**c**), on the perisperm/endosperm envelope (**d**) and inside the embryonic hypocotyl-root axis (**e**)

each taxon exhibits a different distribution pattern in the various seed parts (Fig. 7); microscopic analyses suggested that the bacteria were alive and possibly active. In general, only bacteria with high ribosome content are detected by FISH. Furthermore spores or resting cells are not usually stained. In addition, some of the bacterial cells may not be sufficiently permeable to allow penetration of fluorescing oligonucleotide probes. Therefore, not all bacteria can be visualized by FISH. However, using the DOPE-FISH tool, the outer seed coat, and cotyledon seed parts were seen to be occupied by the five phyla examined using the specific probe sets, while the embryonic hypocotyl-root axis appeared to be poorer in both population size and diversity, with only three phyla observed (Fig. 7). The FISH analysis localized *Gammaproteobacteria* mainly in the cotyledons (although a few could be seen inside the embryonic hypocotyl-root tissues as well as in the outer seed coat). *Pseudomonas* and *Rahnella,* members of the same class, have been previously identified in the endosperm and embryonic tissues of Norway spruce seeds (Cankar et al. 2005), but further work is required to verify the genera of the melon seed inhabitants. *Firmicutes* were visualized inside the seed coat, embryonic first leaves as cotyledons and hypocotyl-root tissues. *Bacillus* species are important members of the *Firmicutes*. This genus is considered to be one of the most important endophytic colonizers (Jacobsen et al. 2004); some species have also been visualized along cell walls inside grapevine seeds (Compant et al. 2011a, b), and were the most abundant isolates from seeds of different cucurbit members (Khalaf and Raizada 2016).

Despite the growing evidence of vertical transmission of bacterial endophytes, the mechanisms of penetration and survival within the seed have been determined in only a handful of cases (Truyens et al. 2015). In their recent review, Barret et al. (2016) discussed the penetration and compartmentalization routes of various seed-associated plant pathogens. They concluded that when a pathogenic bacterium enters through the vascular system or floral

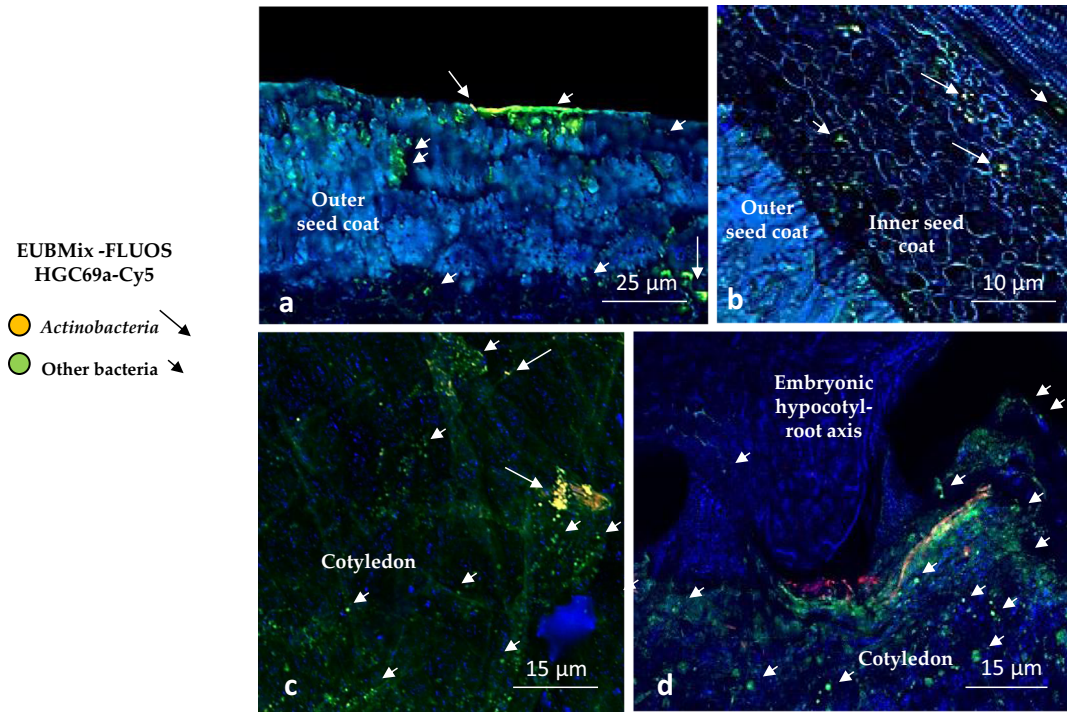

Fig. 6 CLSM with DOPE-FISH hybridization of seeds of the cultivated melon *Cucumis melo reticulatus* group 'Dulce' and use of probe specific for *Actinobacteria*. Members of this bacterial taxon were found inside the seed coat (**a, b**) and cotyledons (**c**), but generally not inside the embryonic hypocotyl-root axis, rather at the border of the cotyledons (**d**)

pathways, it colonizes all of the seed tissues, from the embryo to the seed coat (testa). In contrast, colonization of mature seeds through contact with microorganisms present on fruits or threshing residues is usually restricted to the seed coat. As this transmission pathway is more permissive than the internal or floral one, microbial assemblages associated with the seed surface are probably more diverse than the microbiota of the seed's internal tissues (Barret et al. 2016). Thus, the lack of *Betaproteobacteria* in the seeds observed by Glassner et al. (2015) may hint at colonization at a late developmental stage, whereas *Gammaproteobacteria* and *Firmicutes* localization may correspond with internal or floral colonization patterns. The muskmelon *C. melo L. reticulatus* group cv. 'Dulce', like other melon species, has a fleshy fruit and a pericarp that surrounds and protects the seeds (Burger et al. 2006). In *C. melo*, the presence of the taxa *Firmicutes* and *Proteobacteria* in the pericarp tissues (Glassner et al. 2015) and in the seeds taken directly from the fruit (this study) suggest that at least some of them were transmitted from the vegetative parts to the developing melon fruit and further on to the seed during maturation. Similar invasion routes of bacterial endophytes have been observed in cactus using culture-dependent methods (Puente et al. 2009). On the other hand, in muskmelon and several other cucurbit seeds, a thin envelope completely encloses the embryo with a covering that imposes a physical barrier (Yim and Bradford 1998; Ramakrishna and Amritphale 2005). The presence of such a seed covering may imply that the bacteria migrate in the earlier stages of seed maturation, or that endophytes are transferred to the seed parts directly through the gametes. Such specific localization patterns have been observed with *Acidovorax citrulli*, the causal agent of bacterial fruit blotch of cucurbits. Following pistil inoculation with *A. citrulli*, the bacterium was localized to the seed embryo, whereas pericarp inoculation resulted in bacterial localization under the seed coat but outside the perisperm-endosperm layer (Dutta et al. 2012).

If vertical transmission occurs through the seed itself, the specific bacterial localization observed throughout the different seed parts should be reflected in the amount and affiliation of the bacteria transmitted to the seedling. For example, *Betaproteobacteria* and *Actinobacteria*, which were detected mainly in the seed coat, might be found in the seedlings as they migrate to the seeds during fruit development, or they might have a role as seed colonizers, such as weakening the seed envelope in the process of

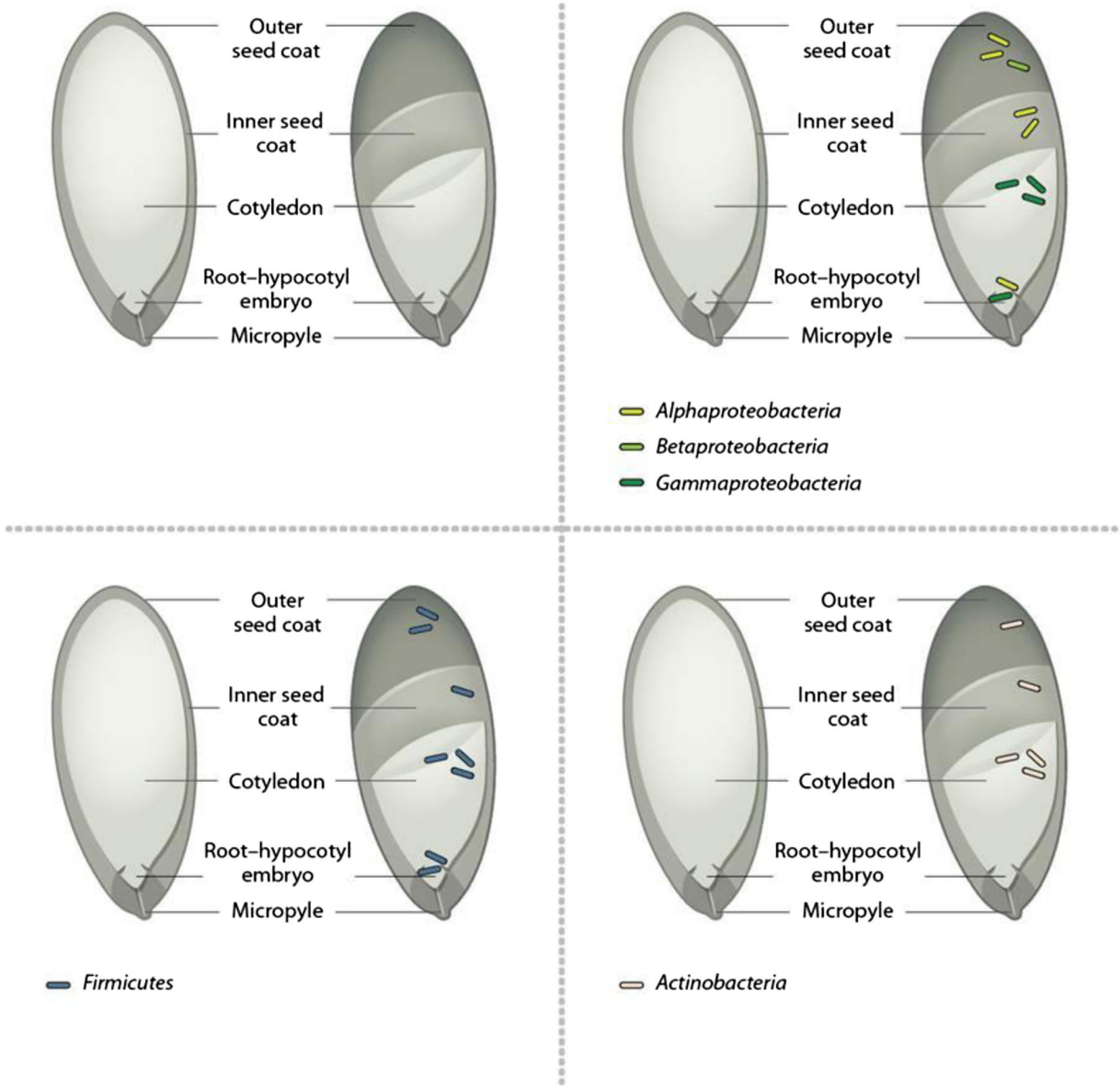

Fig. 7 Drawing summarizing the niches of endophytic bacteria inside seeds of cultivated melon *Cucumis melo reticulatus* group 'Dulce'

radical emergence. The seed is a junction between generations, and by being seed inhabitants, endophytes ensure their presence in new plants. Evidence from a seed microbiome revealed a core population that could be found in consecutive generations (Hardoim et al. 2012; Cope-Selby et al. 2016). In fungal endophytes, vertical transmission can be seen in grasses, such as for *Epichloë* and *Neotyphodium* (Clay and Schardl 2002; Schardl et al. 2004; Selosse and Schardl 2007). These fungi benefit their host by increasing their resistance to herbivores via the production of fungal alkaloids (Müller and Krauss 2005; Rudgers and Clay 2007), as well as enhancement of drought tolerance, plant vigor, and nutrient content (Malinowski et al. 2008; Kannadan and Rudgers 2008; Rudgers and Swafford 2009).

Endophyte transmission and presence in the seed coat might be affected by seed tissue structure. In contrast to disseminating plant organs such as grains of rice and maize, melon seeds are protected inside a fleshy fruit. It is only after fruit ripening that these seeds spread and are accessible to spermosphere bacteria. An example of such a transmission route was provided by

Ferreira et al. (2008), who inoculated a *gfp*-labeled *Pantoea* strain (*Gammaproteobacteria* member) onto the seeds of eucalyptus plants and found that the bacterium is carried into the embryo through breaks in the seed husk and continues on to colonize the seedlings.

It has been previously suggested that small amounts of bacteria with beneficial functions inside fruit and seeds, are sufficient for vertical transmission. Inoculation of alfalfa seeds with *gfp*-expressing *Salmonella* Stanley cells resulted in about 1000-fold increase in bacterial population during a 24 h germination period (Gandhi et al. 2001), suggesting that once established in the young plant, cell density increases. Other studies have reported a shift in the microbial community composition of emerging seeds and seedlings mostly due to an increase in the relative abundance of fast-growing bacterial and fungal taxa (Barret et al. 2015). However, it is reasonable to assume that the endophytes that are already in the seed can more easily establish themselves in the emerging seedling.

The intimate association between seed-colonizing endophytes and their plant host may result in the evolution of mutual adaptations. Although very few of such interactions have been studied, in *Miscanthus* for example, *Bacillus* species, which are a major component of many seed microbiota, have been found to form spores and other dense structures (Cope-Selby et al. 2016). This characteristic can be an important feature of seed colonizers that must endure a dormant period, as the spore protects them from long-term changes (Mano et al. 2006; Compant et al. 2011a), and provides a mechanism for survival and transmission (Cope-Selby et al. 2016). In addition, the ability to utilize starch may increase the survival of specific endophytic bacteria in seeds (Mano et al. 2006). Indeed, bacteria isolated from melon seeds could utilize starch in a plate assay (Yaron, unpublished data).

From the plant's perspective, seed-associated bacteria are of particular interest because their location suggests their importance for plant growth and health. For instance seed-associated endophytes have been found to promote growth and decrease stress damage in different plant species (Compant et al. 2005; Mastretta et al. 2009). Throughout the developmental stages, from pollination through maturation and finally ripening of the fruit, seed-endophytic communities are probably quite dynamic in terms of both identity and numbers. In this study, *C. melo* 'Dulce' seeds were sampled directly from the inside of sterilized mature fruits. The application of seed sterilization using a standard protocol can affect or destroy seed endophytes, resulting in an incomplete picture of the bacterial community (as shown for other plants, see Truyens et al. 2013). In addition, the elimination of seed-dwelling bacteria may have a deleterious effect on germination and further plant development (Holland and Polacco 1994). In our system, we also observed inhibition of seed germination following a standard seed-sterilization procedure (Glassner, unpublished data), suggesting that seed endophytes may be beneficial during germination and that this process decreases in the absence of bacteria. Similarly, Puente et al. (2009) demonstrated arrested seedling development in disinfected cactus seeds, and restoration of plant growth by inoculation with endophytes involved in rock weathering.

Culturable *Firmicutes*, *Alphaproteobacteria* and *Actinobacteria* were previously isolated from fruits and seeds of *C. melo*; several of those isolates, and in particular *Bacillus* species, exhibited antifungal and antibacterial activities against plant pathogens (Glassner et al. 2015), supporting the hypothesis that these microorganisms have an important function. It has been speculated that competitive colonization confines the later arriving bacteria to niches that are already occupied by taxa sharing similar metabolic requirement (Barret et al. 2016). In our system, it would be interesting to determine the influence of natural seed-associated endophytic communities on seed-colonizing pathogens such as *A. citrulli*.

Although it is presumed that some endophytes are especially well adapted to the particular environments in the plant reproductive and disseminating organs, very little is actually known about their origin, ecology or activity, or their interactions with the plant (Rosenblueth and Martínez-Romero 2006). Our current findings contribute to the growing body of evidence accumulated in recent years regarding the ecology and potential importance of seed microbiota.

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Compliance with ethical standards

Conflict of interest Stéphane Compant is Section Editor in Plant and Soil and Guest Editor of the special issue. This does not, however, interfere with the reviewing process.

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