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Niches and routes of transmission of *Xanthomonas citri* pv. *fuscans* to bean seeds

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Abstract

Aims Seeds are vectors of a diversified microbiota including plant pathogens. To better understand transmission of common bacterial blight (CBB) agents to bean seeds, we analyzed the role of non-pathogenic xanthomonads on seed transmission efficiency and investigated the location of *Xanthomonas citri* pv. *fuscans* (*Xcf*) into seeds and plantlets.

Methods Competition between CBB and NP strains was initially assessed in vitro and then extended *in planta* to monitor the impact of co-inoculation on *Xcf* seed transmission. Moreover, location of *Xcf* strains in seeds and seedlings was visualized using a combination of *gfp*-tagged strain and DOPE-FISH/CSLM.

Results Whereas CBB agent growth was inhibited in vitro by some seed-borne non-pathogenic xanthomonads strains, these strains did not transmit efficiently to seed through floral pathway and did not affect *Xcf* seed transmission. *Xcf* cells were observed entering seed through vascular elements and parenchyma of funiculus, but also micropyle and testa. *Xcf* cells were observed, moreover, among other bacteria on radicle surfaces, especially tip, in cotyledons, and plumules.

Conclusions CBB agents are more efficient than non-pathogenic xanthomonads in using the floral route to colonize seeds. CBB agents are located within different niches in the seed tissues up to the embryonic axis.

Abbreviations

Xcf	*Xanthomonas citri* pv. *fuscans*
GFP	Green fluorescent protein
NP	Non-pathogenic
CBB	Common bacterial blight
DOPE-FISH	Double labeling of oligonucleotide probes for fluorescence in situ hybridization
CSLM	Confocal laser-scanning microscopy

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Introduction

Seeds are remarkable organs that are not only involved in the transmission of the plant gene pool from one generation to another but also act as vectors of complex microbial assemblages. These microbial assemblages, or microbiota, contribute to seed preservation (Chee-Sanford et al. 2006), growth promotion (Cankar et al.

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2005; Mukhopadhyay et al. 1996; Truyens et al. 2015), but also to plant health (Gitaitis and Walcott 2007). Transmission to and by seed is, however, one of the most efficient means of dispersion for most plant-pathogenic bacteria (Baker and Smith 1966). These seed-borne bacterial plant pathogens are of particular concern due to very limited management strategies of bacterial diseases given the sparsity of chemical treatments available. Sanitary control of seeds remains the main method used to control seed-transmitted bacterial diseases, and includes seed testing and physical and/or biological treatments of infected seeds (Gitaitis and Walcott 2007). The efficiency of the seed testing methods relies in part in the efficiency of the detection method that is used that in turn depends on the bacterial location within seed tissues.

Bacterial location within seed tissues is linked to the transmission pathway used by bacteria to reach the seed. Three pathways of seed transmission have been reported: (i) internal transmission via the host xylem that usually results in the invasion of seed through the hilum, (ii) seed transmission via the pistil, where bacteria move from the stigma through the stylar tissues down to the ovule, and finally (iii) an external and/or sub-tegumental contamination of seed can occur as a consequence of contact of the seed with symptomatic fruit tissues or with threshing residues carrying large bacterial populations (Maude 1996). While the presence of bacteria within the embryo of seeds has been reported (Cankar et al. 2005; Glassner et al. 2017; Mitter et al. 2017; Mukhopadhyay et al. 1996), it has been postulated, however, that bacterial pathogens do not colonize the embryonic axis per se, being restricted to outer layers of embryo tissues (Maude 1996; Singh and Mathur 2004; Tancos et al. 2013). Bacterial plant pathogens are also frequently found in the endosperm, and inside and outside the testa (Dutta et al. 2012; Taylor et al. 1979; Zaumeyer 1932).

Bacterial location within seeds and mechanisms involved in seed transmission depends on the pathway of invasion. Although the relative importance of each pathway remains to be described for most pathosystems, some data have been obtained for specific phytopathogenic bacteria. For instance, internal transmission of *Xanthomonas citri* pv. *fuscans* (*Xcf*) to bean seeds is dependent of bacterial adhesins (FhaB and XadA) (Darsonval et al. 2009). In contrast, transmission of *Xcf* through the floral pathway does not required bacterial adhesins. Another example is the external invasion

of watermelon seeds by *Acidovorax citrulli* through the pericarp that results in colonization of the testa and the perisperm-endosperm layer, while ingress of *A. citrulli* via the pistil is associated with the surrounding cotyledons (Dutta et al. 2012; Dutta et al. 2014). Recently, investigation of endophyte niches inside seed tissues has been performed using double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) coupled with confocal laser-scanning microscopy (CSLM). Location of bacterial lineages in different tissues of the melon seed have been highlighted (Glassner et al. 2017). Most bacteria located in inner and outer seed coat and parts of the embryo such as cotyledons, and only a few inside the embryonic root-hypocotyl axis (Glassner et al. 2017).

It is widely accepted that plant-pathogenic bacteria co-exist in natural settings with other micro-organisms into aggregates or assemblages nowadays known as the microbiota (Barret et al. 2016). Interestingly, some plant pathogenic bacteria may co-exist with phylogenetically closely related non-pathogenic (NP) strains (i.e. strains that are unable to induce symptoms in their host of isolation following artificial inoculation and incubation under optimal conditions) in these assemblages. This co-existence has been reported for *Clavibacter* (Jacques et al. 2012) and *Xanthomonas* genera (Boureau et al. 2013; Essakhi et al. 2015; Grimault et al. 2014; Vauterin et al. 1996), highlighting the need for the development of specific detection methods.

Xanthomonads are plant-associated bacteria establishing neutral, commensal, or pathogenic relationships with plants. The common bacterial blight of beans (CBB) is a worldwide distributed disease (Vidaver 1993) caused by genetically diverse *Xanthomonas* spp.: *X. citri* pv. *fuscans* (previously *X. fuscans* subsp. *fuscans*), *X. citri* pv. *fuscans* NF2 (previously *X. axonopodis* pv. *phaseoli* NF2), *X. citri* pv. *fuscans* NF3 (previously *X. axonopodis* pv. *phaseoli* NF3), and *X. phaseoli* pv. *phaseoli* (previously *X. axonopodis* pv. *phaseoli* NF1) (Alavi et al. 2008; Constantin et al. 2016).

Seed contamination affects both bean production and the seed industry through direct and indirect costs. The bacterial pathogen colonizes both vascular tissues and parenchyma of its host and seed transmission occurs on plants and seeds that are symptomless. Co-existence of CBB agents with NP xanthomonads has been reported since decades (Goszczynska and Serfontein 1998), but very little is known on their respective niches in seeds.

In order to better understand transmission of common bacterial blight agents to bean seeds, we hypothesized that *Xcf* colonizes specific niches within bean and may interact with some other bacteria inside the seed tissues. We tested the role of NP strains in seed transmission of pathogenic strains, monitored transmission of GFP-tagged *Xcf* to seeds and seedlings of bean using CSLM and visualized *Xcf* in naturally contaminated seeds by DOPE-FISH/CSLM microscopy to have a global approach on colonization, ecology of *Xcf* within seed tissues, among other seed inhabitants.

Materials and methods

Bacterial strains, plasmids, and growth conditions The strains and plasmids used in this study are listed in Table S1. Xanthomonads strains were grown at 28 °C in 10% Tryptone-Soja Agar (1.7 g.L^{-1} tryptone, 0.3 g.L^{-1} soybean peptone, 0.25 g.L^{-1} glucose, 0.5 g.L^{-1} NaCl, 0.5 g.L^{-1} K2HPO4, and 15 g.L^{-1} agar) supplemented when appropriated with 50 mg.L^{-1} rifampin (Rif), 50 mg.L^{-1} kanamycin (Km), or 20 mg.L^{-1} gentamycin (Gm). *Escherichia coli* DH5α™ strain used for cloning purpose was grown at 37 °C on LB medium (10 g.L^{-1} peptone; 5 g.L^{-1} yeast extract; 15 g.L^{-1} agar). For quantification of bacterial population sizes in plant material, media were supplemented with 50 mg.L^{-1} cycloheximide and 10 mg.L^{-1} propiconazole to inhibit fungal growth. To prepare bacterial inocula, strains were grown for 48 h in 10% TSA. Then bacterial cells were scraped from plates and suspended in sterile distilled water. Suspensions were calibrated to 1×10^8 CFU ml^{-1} and adjusted to the desired final concentrations with sterile distilled water.

Competition assay Competitions between NP and phytopathogenic strains were monitored in microtiter plates (Microwell, Nunc, Denmark). Three wells per each strain or mixture, each containing 200 μl of 10%TSB (10% TSA without agar), were inoculated with dilutions of inocula to reach final concentrations at 1×10^7 CFU.ml^{-1} per strain and incubated at 28 °C for 3 days under agitation. Bacterial growth was recorded by dilution-plating on selective and non-selective 10%TSA to count colonies from pathogenic strains (Rif^R) and all colonies, respectively. Differences between these two counts provided counts for NP strains.

All experiments were performed in two independent biological replicates.

Tagging strains The GFP6 and mCherry sequences were PCR-amplified from pMDC43::*gfp6* and pRSET-B::*mCherry* with gfp6_SalI_F/ gfp6_EcoRI_R and mCherry_SalI_F/ mCherry_EcoRI_R primers (gfp6_SalI_F: GCGCGTCGACATGAGTAAAGGA; gfp6_EcoRI_R: GCGCATGAATTCGGCGCGCC TTTGTATAGTTCAT; mCherry_SalI_F: ATATATGT C G A C A T G G T G A G C A A G G G C G A G G A ; mCherry_EcoRI_R: GCGCGAATTCTACTTGTACA GCTCGTCCATGC) containing the *Sal*I and *Eco*RI restriction sites. PCR products were purified with Nucleospin Gel and PCR Clean up kit (Macherey nagel), digested with *Sal*I and *Eco*RI enzymes and introduced into the *Sal*I and *Eco*RI sites in the broad host ranged vectors pBBR1MCS-2 and pBBR1MCS-5 plasmids (Kovach et al. 1995), respectively using the pGEM®-T Easy vector system (Promega). The ligation products were electroporated in the *E. coli* DH5α™ strain (ThermoFischer Scientific) and inserts were sequenced by Genoscreen (Lille, France). Then pBBR1MCS-2::*gfp6* and pBBR1MCS-5::*mCherry* were extracted with the Nucleospin Plasmid kit (Macherey Nagel) and electroporated in *Xanthomonas* Rif^R strains. The stability of the constructions was assessed *in planta* by measuring population sizes of the various constructions 9 days after spray-inoculation of bacterial strains on bean leaves by dilution and plating on 10% TSA supplemented with the appropriate antibiotics.

Plant material and sampling procedure In planta experiments were performed with a variety of common bean (*Phaseolus vulgaris* L. cv. Flavert) susceptible to common bacterial blight using seed, trifoliate leaf, or flower bud inoculation.

Seed inoculation: seeds were disinfected 10 min in 1% chlorine and rinsed three times in sterile distilled water. For microbial analyses, seeds were inoculated as described in Darrasse et al. (2010). Briefly, seeds were soaked in 2 ml inoculum (1×10^5 CFU.mL^{-1}) per seed during 30 min and vacuum infiltrated for 3 min. Then, seeds were dried for 2 h in a sterile environment, at room temperature, and then directly used for microbial analysis or sowing in germination box or soil. For the analysis of seedling colonization, seeds were incubated in germination boxes up to four days as described in Barret

et al. (2016). Sets of five 3d–old seedlings per treatment from which cotyledons were removed were sampled for microbial analyses. Five pairs of cataphylls of 7 d-old plantlets per treatment were also sampled for microbial analyses. For microscopic observations, seeds were also inoculated by depositing 10 μL of 1×10^8 CFU.mL^{-1} inocula over the hilum area and observed after 2 h drying or up to four days after germination in germination boxes.

Leaf inoculation: seeds were sown in pots containing soil substrate (Tray substrat, NF U 44–551, Klasmann-Deilmann GmbL, Rippert France) and grown under controlled conditions with 16 h of light at 25 °C (28 °C for pathogenicity tests) and 8 h of darkness at 20 °C (22 °C for pathogenicity tests) as described in Darsonval et al. (2008). Beans at the first trifoliate leaf stage (V3, (Michael 1994); 10 d-old plants) were inoculated by dipping in bacterial suspensions at 1×10^7 CFU.ml^{-1} and with sterile distilled water as control. Inoculated plants were incubated under high (95%) relative humidity (RH) for two days post-inoculation, then RH was decreased to 60%. The first trifoliate leaf was collected 9 d post-inoculation. Three leaves were analyzed per treatment. The densities of the inoculated strains were determined on three leaflets per treatment by dilution and plating on selective media. The pathogenicity of the tagged strains was monitored on three leaflets per treatment by chlorophyll fluorescence imaging as described in Rousseau et al. (2015).

Flower bud inoculation: flower buds of bean plants (R5 stage, (Michael 1994)), grown as previously described, were inoculated with bacterial suspensions (2 drops of 10 μL each of a 1×10^6 cfu.mL^{-1} inoculum) of strains alone or in mixture (1:1). Seeds from inoculated flower buds (3 flower buds from 5 plants) were harvested 5 weeks after inoculation and analyzed following maceration and dilution-plating as described in Darsonval et al. (2008). For microscopic analyses of bacterial transmission to seeds, an inoculum at 1×10^6 CFU.mL^{-1} was spray-inoculated on bean plants at the flower-bud stage growing as described before. Immature seeds were collected from 3 d after inoculation up to maturity (40 d).

Microbial analysis of plant material To quantify seedling and leaf (cataphyll or trifoliate leaf) colonization, each leaf was weighed and ground individually (using Stomacher 80; Seward, London, United Kingdom) for 2 min at maximum power in 5 ml of distilled water.

Aliquots of 50 μl of every sample and appropriate dilutions were spotted on selective medium to enumerate the inoculated strain. To quantify seed contamination, seeds were weighed and soaked overnight at 4 °C in 2 ml of sterile distilled water per g of seeds (5.56 seeds.g^{-1}). Samples were then vigorously shaken. Aliquots of 50 μl of every sample and appropriate dilutions were spotted on selective medium to enumerate the inoculated strain.

Statistical analyses Statistical analyses were performed using Statbox Pro software (Grimmer Logiciels, Optima France). Log-transformed (population size) or arc sine-transformed (ratio of disease area) data were analyzed with Mann-Whitney tests.

CLSM of developing and mature seeds and of germinating seeds Microscopic observations were made on pods and seeds collected from 3 to 40 d after plant inoculation at the flower bud stage. This inoculation method was chosen in order to fit as closely as possible to natural transmission routes for seed contamination. Seeds (50 infiltrated, 50 spray-inoculated, and 15 healthy), and germinating seeds (40 contaminated by spray inoculation of mother plants, 45 inoculated after harvest, and 5 healthy) have been observed under CLSM. Seeds were cut in 120 μm-depth sections in sterile distilled water using a vibratome (Microm HM 650 V, Thermo Fisher Scientific, France). Slices were mounted on glass slides with deionized water and a coverslip. In order to confirm that tissue was contaminated before cutting and hence avoid false positive results due to bacterium displacement from a contaminated area to a healthy one by the razor blade or by water, observations were performed both at the surface and in the depth of the sample. For light-tight tissues, the observation of a co-existing disorder of tissue structure was used to confirm bacterial infection. Seeds were observed using a Confocal Scanning Laser Microscope (CSLM, Nikon A1, France). Seed cuts were observed under transmitted light to identify seed structures. Lasers at 405 and 488 nm were used to induce plant autofluorescence and excite the GFP, respectively. Images were generated by merging channels 488 nm and transmitted light to visualize GFP-tagged strain in seed tissues or using a hyperspectral detector mode (excitation at 405 and 488 nm and signal reception with all channels) to distinguish GFP-tagged cells from plant autofluorescence. NIS-element (Nikon, 4.20.00) was used as driver

for CLSM and imaging software for photographs and snapshots of 3D reconstructions from Z series of images (with one image per 0.75 μm).

DOPE-FISH and CLSM microscopy of Xanthomonas infected seeds For microscopy analysis of *Xanthomonas* and other seed inhabitants, seeds were harvested from bean plants spray-inoculated at the flower bud stage. Seeds were aseptically cut into two parts, and fixed overnight at 4 °C in a paraformaldehyde solution (4% *w/v* in PBS, pH 7.2) in Eppendorf tubes, before that the samples were rinsed three times with PBS. Treatment with lysozyme solution (1 mg.mL^{-1} in PBS) was applied for 10 min at 37 °C followed by an ethanol dehydration series (25, 50, 75 and 99.9%; 15 min each step). DOPE-FISH was performed with probes from Eurofins (Austria) labeled at both, 5′ and 3′ end, positions according to Glassner et al. (2015). An EUBmix targeting all bacteria (with equivalent mixture of EUB338, EUB338II, EUB338III) coupled with the fluorochrome ATTO488 (Amann et al. 1990; Daims et al. 1999) was used. A probe mix specific to *Xanthomonas* spp. 5′-CATTCAAT/CCGCGCGA AGCCCG-3′ was also created using Stellaris software, confirmed by NCBI blast, Probe check, RDP II gene, Oligo Calc, and Mathfish softwares. The probes, originally designed for *Xanthomonas campestris* but also targeting *Xanthomonas citri*, *X. fuscans*, *X. phaseoli* and several other *Xanthomonas* spp., were coupled with Cy5 at both 5′ and 3′ position, and further evaluated in vitro at different formamide concentration on pure culture a *Xanthomonas* isolate fixed with paraformaldehyde, treated with lysozyme, and dehydrated. A NONEUB probe (Wallner et al. 1993) coupled with Cy5or ATTO488 was also used independently as a negative control. 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 seed 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 HeNe(G)laser FV10-LAHEG230–2). X, Y, Z pictures were taken at 405, 488, 633 nm and with 10X, 20X or 40X objectives, analysed with Imaris software, 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 done on eight seeds and DOPE-FISH was performed three independent times on different seed sections for each probe combination. Images shown in this publication represent the average of colonization.

Results

Assessing the impact of non-pathogenic xanthomonads on transmission of common bacterial blight agents to seeds

To analyze the role of NP strains on seed transmission of CBB agents, we first tested the impact of nine NP strains on the growth of four pathogenic strains during competition assays performed in liquid culture (Table 1). The population sizes of pathogenic strains were significantly ($p < 0.05$) lowered when co-cultivated with four of the NP strains. These four strains (13,554, 13,555, 13,556, and 13,557) were further tested for their capacity to impact seed transmission of pathogenic strains through the floral pathway. This test was designed to be little invasive. For that, bacterial inocula were gently deposited in buds and great care was taken at harvest to avoid any seed contamination with pod tissue while collecting seeds. Efficient seed transmission of pathogenic strains was observed with this method with population density ranging from 6 to 8 Log10 CFU per g of seed (Fig. 1). In contrast, the population density of all NP strains tested was below the detection threshold (Fig. 1), therefore suggesting an absence of seed transmission. Moreover, none of the tested NP strains affected the seed transmission of pathogenic strains. Altogether, these results indicated that the floral pathway is efficient for seed transmission of CBB agents, but is not involved in transmission of closely related NP strains to bean seeds.

Table 1 Population sizes of pathogenic and non-pathogenic strains in competition assays. Values (Log10-transformed CFU.mL^{-1} and associated SEM) correspond to the pathogenic strain (a) and non-pathogenic strains (b) in the mix. Pathogenic and non-pathogenic strains were co-cultured in 10%TSB during 2 d at 28 °C under agitation

Non-pathogenic strain code	Pathogenic strains				
	7767-R	6546-R	6988-R	6996-R	none
	a Population sizes of pathogenic strains				
13,554	4.43 ± 0.10	6.40 ± 0.07	8.18 ± 0.07	8.10 ± 0.08	
13,555	6.22 ± 0.15	6.98 ± 0.05	8.59 ± 0.03	6.41 ± 0.35	
13,558	7.24 ± 0.04	8.10 ± 0.13	7.91 ± 0.48	7.53 ± 0.10	
13,560	8.08 ± 0.13	8.54 ± 0.17	8.55 ± 0.06	8.13 ± 0.24	
13,559	7.67 ± 0.11	8.21 ± 0.03	7.34 ± 0.01	8.00 ± 0.08	
13,561	5.86 ± 0.10	8.30 ± 0.13	8.31 ± 0.10	8.22 ± 0.10	
13,556	3.23 ± 0.23	8.03 ± 0.13	8.30 ± 0.07	3.76 ± 0.14	
13,557	6.33 ± 0.25	8.09 ± 0.14	6.85 ± 0.15	8.08 ± 0.12	
CFBP 7612	6.88 ± 0.11	8.35 ± 0.12	8.55 ± 0.25	7.75 ± 0.05	
none	7.98 ± 0.03	8.35 ± 0.04	8.04 ± 0.03	8.04 ± 0.43	
	b Population sizes of non-pathogenic strains				
13,554	8.55 ± 0.06	8.37 ± 0.04	7.76 ± 0.19	8.06 ± 0.13	9.20 ± 0.04
13,555	8.51 ± 0.06	8.42 ± 0.04	7.23 ± 0.23	8.24 ± 0.18	9.03 ± 0.06
13,558	8.70 ± 0.16	8.47 ± 0.26	8.38 ± 0.21	8.41 ± 0.25	8.79 ± 0.06
13,560	7.79 ± 0.40	7.23 ± 0.23	7.00 ± 0.00	8.16 ± 0.20	8.72 ± 0.24
13,559	8.26 ± 0.19	7.32 ± 0.32	6.27 ± 0.08	8.41 ± 0.06	8.90 ± 0.02
13,561	8.21 ± 0.07	7.63 ± 0.32	7.16 ± 0.16	8.07 ± 0.11	8.92 ± 0.05
13,556	8.17 ± 0.13	7.67 ± 0.33	7.60 ± 0.39	8.25 ± 0.12	9.01 ± 0.05
13,557	8.04 ± 0.31	7.63 ± 0.32	7.71 ± 0.26	7.35 ± 0.35	8.98 ± 0.33
CFBP 7612	8.11 ± 0.13	7.85 ± 0.19	7.91 ± 0.47	7.92 ± 0.23	9.30 ± 0.00

Non-pathogenic strains that impacted the population size of at least one pathogen are indicated in bold

Fig. 1 Population sizes of pathogenic and non-pathogenic bacteria in bean seeds. Bacteria were inoculated alone or in mixture (1:1) in flower buds. Population sizes were quantified in mature seeds (40 d-old seeds). Means and SEMs were calculated for seeds harvested from the 3 inoculated pods from 5 plants per treatment. Mean population densities followed by different letters are significantly ($P < 0.05$) different based on the Mann-Whitney test. The horizontal line represents the detection threshold for bacterial population sizes in seed samples
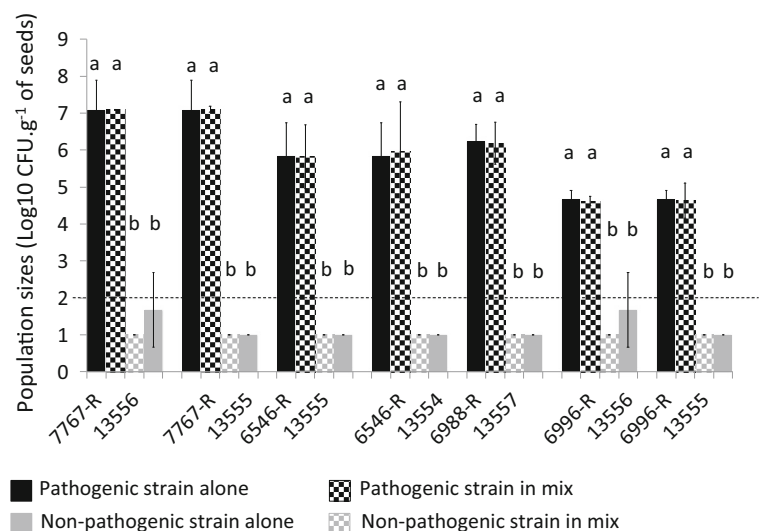
Since the NP strains employed in this study were isolated from bean seed samples, we tested whether these isolates could decrease the population size of CBB agents during emergence. When vacuum-infiltrated in seeds, both pathogenic and NP strains colonized efficiently seedlings and cataphylls (Fig. S1). However, while population sizes were highly homogeneous among strains on seeds, high discrepancies in population sizes were noticed among strains on seedlings and cataphylls with densities ranging from 4 to 7 Log10 CFU per g of tissue. Co-inoculations of pathogenic and NP strains in mixtures (1:1) resulted generally in lower population sizes for each strain in comparison to single inoculation. However, densities of pathogenic and NP strains in the mixtures were surprisingly quite similar, and no exclusion of any strain in the mixture was observed. This result illustrated the abilities of both pathogenic and NP strains to translocate efficiently from seeds to plantlets.

Tagging strains of common bacterial blight agents to assess niches in bean seeds

In order to assess the location of CBB agents within bean seeds, the four pathogenic strains employed in this work were electroporated with a self-replicating plasmid carrying gene encoding the GFP (pBBR1MCS-2::*gfp6*) or mCherry proteins (pBBR1MCS-5::*mCherry*). Pathogenicity and stability of the transformants were tested *in planta* following inoculation of the first trifoliate leaf of bean and incubation during nine days. Population sizes of the strains harboring the plasmids were most generally lower (up to ten times) than those of the wild-type strains indicating a fitness cost due to the plasmid itself and not to the tagging genes (Fig. S2). Plasmids were stable *in planta* as indicated by the absence of significant differences between population sizes quantified on selective vs. non-selective media. In agreement with a fitness cost of the construction, transformed and tagged strains were less aggressive than their relative wild-type (Fig. 2). Differences were lower for 7767-R pBBR1MCS-2::*gfp6* (*Xcf*::*gfp*) compared to its wild-type than for any other construction. Therefore, this strain was chosen then for the microscopic examination of bacterial transmission to seeds.

Location of *X. citri* pv. *fuscans* in bean seeds

Xcc::*gfp* cells were observed in intercellular spaces of testa parenchyma and in embryonic tissues of immature seeds. Of note, bean seed is non-endospermic and is composed of the testa and an embryo composed of the hypocotyl-root embryonic axis and the cotyledons. To locate niches of *Xcf*::*gfp* in bean seeds, the bacterium was spray-inoculated on bean plants at the blower bud stage and seeds were sampled in immature pods from 3 to 40 day-old. In immature pods (17 day-old), bacteria were observed on the surface of the seed coat and inside the seeds below the points of entry that are the micropyle, the hilum and the lens, and in the embryo (Fig. S3). Bacteria were located in the intercellular spaces of the parenchyma. Opacity of embryonic tissues prevented observing the sample in depth and thus did not allow to confirm that bacteria location was not a consequence of a displacement during sample preparation. In contrast, observation of bacteria among plant cells in disordered tissue confirmed that embryo was indeed infected (Fig. S3).

At 32 dpi, *Xcf*::*gfp* cells were observed in connections of vascular tissues from maternal tissues to seed. Connections of the vascular tissues of the maternal funiculus terminate in the chalazal region with the hilum, the scar of funiculus attachment. The central fissure in the hilum overlays the tracheid bar from the micropyle to the lens on the other side. The micropyle is an entrance pore for the pollen tube (Smykal et al. 2014). Fluorescent bacteria were observed in the vascular bundles entering the seed through the funiculus in the hilum groove of developing seeds (Fig. 3a). Bacterial cells were also visible in intercellular spaces of parenchyma cells of the funiculus and of the tracheid-like cells within the tracheid bar (Fig. 3b and c). The tracheid bar, found below the hilum, is an elongated cluster of tracheid-like cells surrounded by sheaths of elongate parenchyma. Bacterial cells were distributed in the width of the parenchyma surrounding tracheid bar and cotyledons on seed side (Fig. 3d). *Xcf*::*gfp* cells were abundant deeper in the seed in the parenchyma but remained outside the embryonic tissues that were rich in autofluorescent compounds (Fig. 3f and g).

Bacteria were also observed in symptomatic mature seed harvested from spray-inoculated plants (Fig. S4). Bacteria were detected on the surface of the testa and in necrotic areas that were associated with testa disruption.
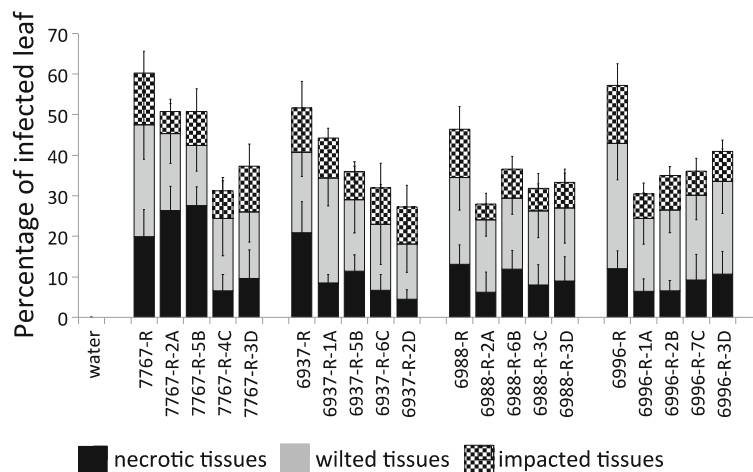
Fig. 2 Pathogenicity of selected GFP- and mCherry-tagged strains. Four strains representing the genetic diversity of common bacterial blight agents were tagged with GFP and mCherry. Pathogenicity of these strains was determined by chlorophyll fluorescence imaging. The percent of decayed leaf tissue (impacted, wilted and necrotic tissues as defined in Rousseau et al. 2015) was recorded for each strain after spray-inoculation of the strains on the bean phyllosphere and nine days incubation in growth chamber. *Xcf* strain 7767-R, *Xcf* NF2 strain 6988-R, *Xcf* NF3 strain 6996-R and *Xpp* strain 6937-R were transformed with pBBR1MCS-2::*gfp6* and pBBR1MCS-5::*mCherry*. Means and SEMs were calculated for 3 leaflets per treatment. No significant differences ($P < 0.05$) were observed in the percent of decayed leaf tissue between each strain and its transformants based on the Mann-Whitney test

Beneath this entry point, *Xcf::gfp* cells were observed in intercellular spaces of testa parenchyma, in cavity between testa and cotyledon, on the surface of and within the embryo.

Symptomatic seeds were further incubated in germinating box to allow water imbibition. Most seeds rotted and did not germinate. Among seeds that did not rot after 24 h-germination, *Xcf::gfp* cells were observed on the surface of the cotyledons, of the hypocotyl, of the plumules and in the folds of embryonic tissues, but not within embryonic tissues (Fig. S5). These locations of bacterial cells were also observed in seedlings issued from inoculated seeds. In this case, inoculum drops were gently deposited over the hilum area including the micropyle and the lens. *Xcf::gfp* cells were observed on the surface of the radicle, at the point of radicle emergence, and on the surface of plumules (Fig. S6).

Using DOPE-FISH and CSLM microscopy, *Xanthomonas* spp. fluoresced in yellow/orange (Fig. 4a) due to combination of general probes in green and specific in red, while other bacteria fluoresced in green (Fig. 4). *Xanthomonas* spp. were present among other bacteria on surface of the radicle (Fig. 4a-c and e), cotyledons (Fig. 4d and f-h), and in plumule (Fig. 4i-k) of bean seeds (Fig. 4a-k). Control seeds showed a few

Xanthomonas spp. because the probes were not specific at an infra-generic level, but concentration was lower than inside seeds from plant inoculated with *Xcf,* and concerned only few samples (Fig. 4l-o). Most bacteria

Fig. 3 CSLM images of *Xcf::gfp* in immature bean seeds (32 d-old). Fluorescent bacteria were distributed in the vascular bundles and in intercellular spaces of parenchyma cells of the funiculus (**a** - **b**). Transverse section of a seed at the funiculus level through tracheid bar. Fluorescent bacteria were located in intercellular spaces of parenchyma cells of the tracheid bar and in tracheid-like cells of the tracheid bar. Note fluorescent bacteria in intercellular spaces of the parenchyma (**c** - **d**). 3D–reconstructed image indicating the presence of the bacteria in the in the width of the tissues in funiculus and seed sides (**e**). Frontal section of a seed showing embryonic tissues embedded in parenchyma. Note the fluorescence of embryonic tissues. Fluorescent bacteria were restricted to the parenchyma and did not invade the embryo. (**f** and **g**). Images were generated by merging channels 488 nm and transmitted light (**a, c**, e and **f**) or under hyperspectral detector mode (excitation at 488 and 405 nm and signal reception with all channels) (**b, d** and **g**); plant compounds fluoresced in yellow allowing to confirm that the green objects are indeed GFP-tagged cells. White boxes correspond to a three times-magnification of the selected area; white arrows highlight GFP tagged-cells; tissues or structures are indicated by letters: chalaza (ch), funiculus (fu), testa parenchyma (tp), palisade of macroscereids (pm), tracheid bar (tb), hilar fissure (hf), cotyledon (co). Frontal section of the seed at the funiculus
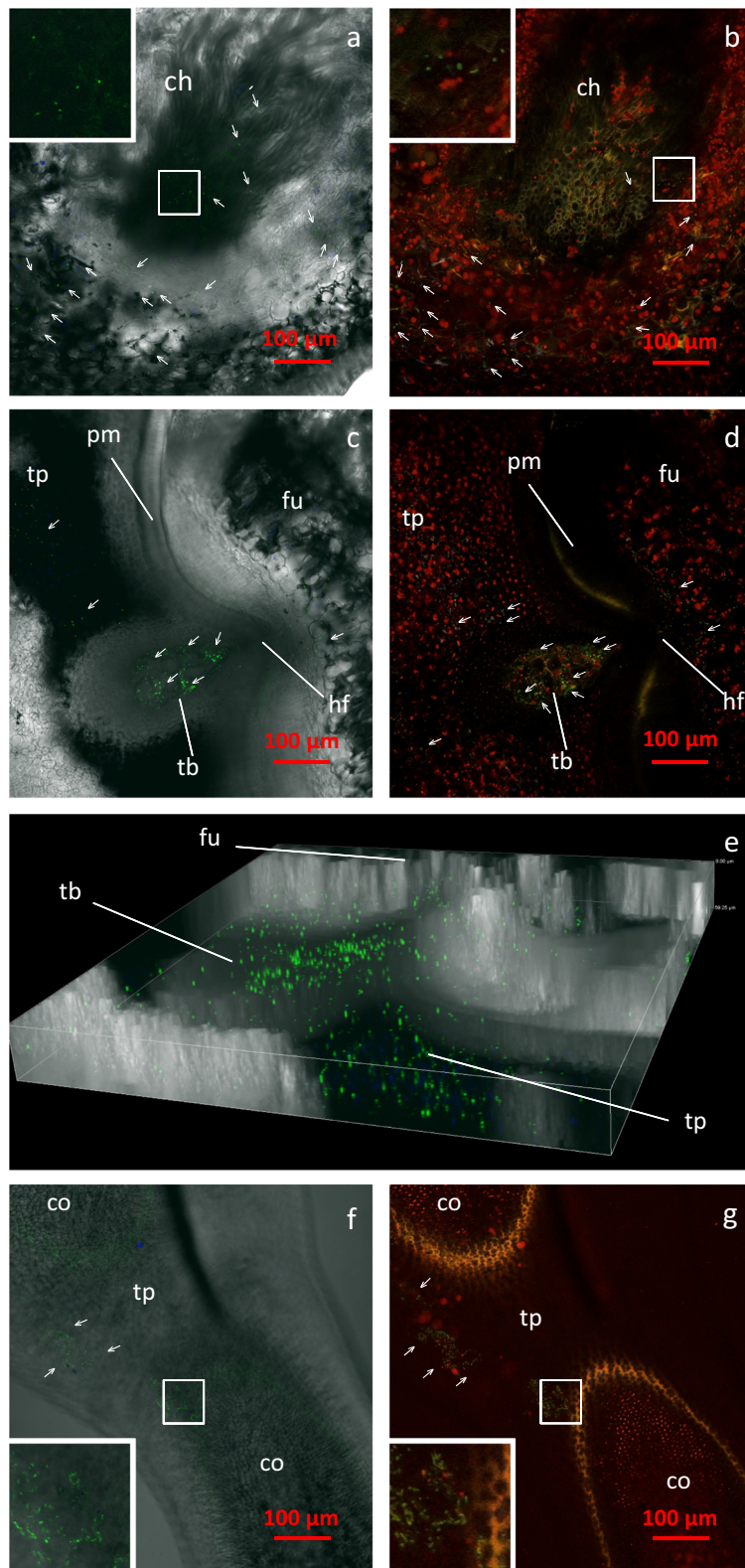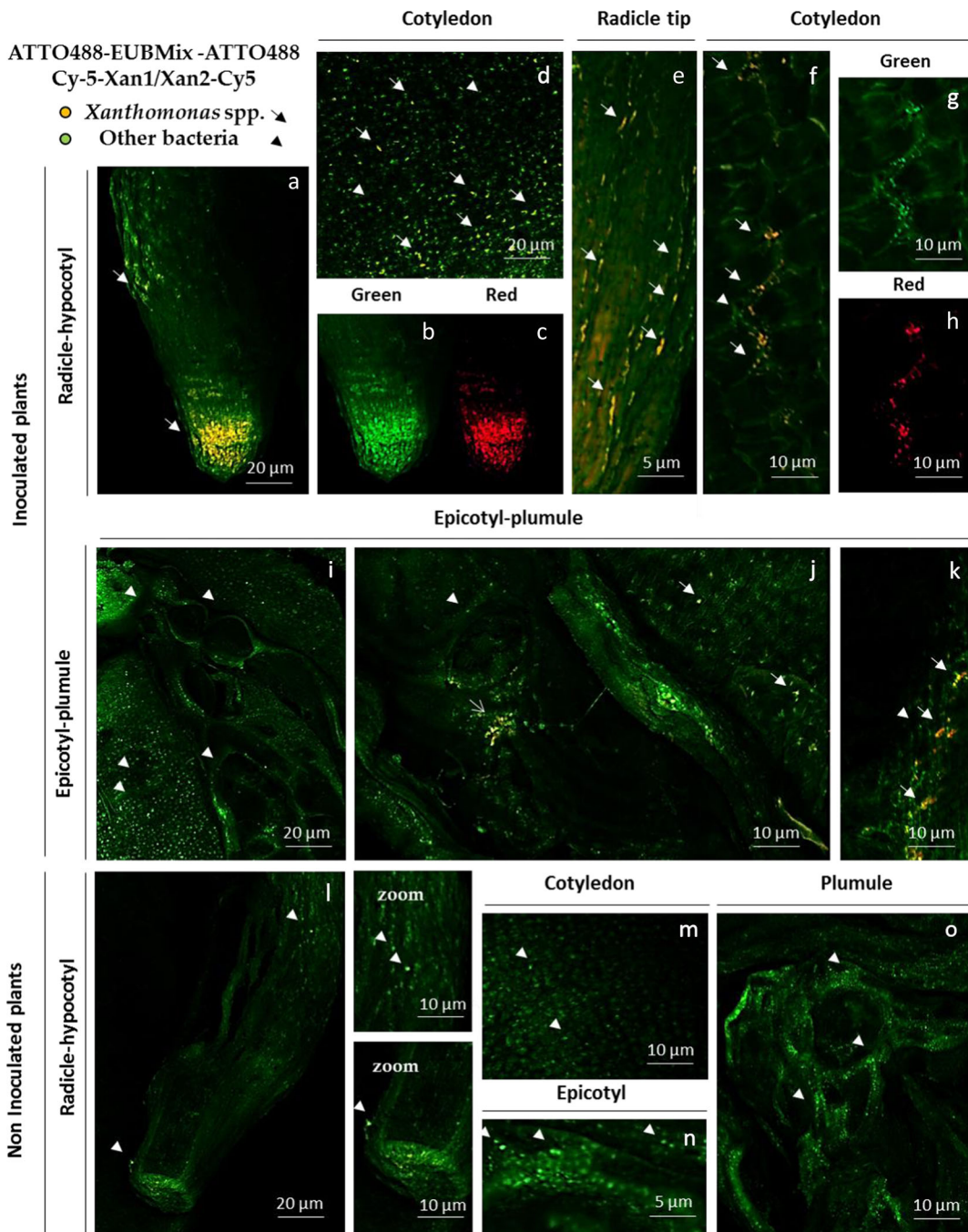
Fig. 4 CSLM microphotographs of seed tissues infested or not with Xcf 7767-R. DOPE-FISH was performed with generic bacterial probes (green, arrow heads) or specific *Xanthomonas* probes (red, arrows), which result in orange color when *Xanthomonas* is detected. *Xanthomonas* spp. were detected within seed tissues such as surfaces of radicle-hypocotyl (a-c; and e), cotyledon (d, and f-g), epicotyl-plumule (i-k) of inoculated plants. Non inoculated plants show presence of bacteria on radicle tip (l), cotyledon (m), epicotyl (n) and plumule (o)

were however green fluorescent and were detected at tip level of the radicle (Fig. 4l), cotyledon (Fig. 4m), and plumule (Fig. 4n-o). Use of negative NONEUB probe and no-fluorescent bacteria from seeds of inoculated or

non-inoculated plants (Fig. S7) further evidenced the presence of *Xcf* and endophytes of bean seeds revealed by DOPE-FISH/CSLM microscopy.

Discussion

Seed transmission is one of the most efficient ways to ensure dispersal and survival of plant pathogens. Therefore, decreasing the incidence of seed transmission is a key target for improving control methods. Given the paucity of chemical methods that are efficient and authorized against bacterial pathogens, control relies on physical methods such as thermotherapy, biological control, and seed health testing. Efficiency of these methods depends however on the location of bacteria in seeds and their ability to be efficiently disseminated by seeds. This work was developed to gain knowledge on effect of NP xanthomonads on transmission of CBB agents and location of these microorganisms within seeds.

CBB agents are genetically diverse and belongs to two separate species, *X. citri* and *X. phaseoli* (Alavi et al. 2008; Constantin et al. 2016). The first CBB causal agent was identified by Smith in 1897 (reported by Zaumeyer (1930)) and is the one known today as *X. phaseoli* pv. *phaseoli*. The biology and seed transmission of this bacterium were studied in details very early (Zaumeyer 1930). The fuscous CBB agent, which is known today as *X. citri* pv. *fuscans,* was first reported in 1930 from beans grown in Switzerland in 1924 (Burkholder 1930), and since this date, has been isolated from every bean production area worldwide. Albeit some papers reported vascular and floral routes of seed infection for this pathogen (Darsonval et al. 2009; Darsonval et al. 2008), no data has yet been available concerning its location within the seeds. Here, using an inoculation method allowing bacteria to reach seed through all pathways (floral, vascular and by contact), we show that *Xcf* 7767-R::*gfp6* cells enter the seed through vascular elements and parenchyma of the funiculus. In case of severe infection leading to water-soaked spots on pod, bacteria from the pod cavity enter the seed by degrading the testa. The bacteria were also seen in the micropyle and spreading in testa parenchyma, in tracheid bar, underneath lens and in micropyle. Contaminations were frequently limited to testa but, in some cases of heavily contaminated seeds, bacterial cells could be observed within damaged embryonic

tissues. This was also coherent with the germination failure of heavily contaminated seeds and with a location of the bacterial cells on the surface (and not within) of the organs emerging from a contaminated seed during imbibition. Zaumeyer (1930) reported that *Xpp* contaminates testa and embryo surfaces in bean seeds (Zaumeyer 1930). These locations were also demonstrated for another bean pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Zaumeyer 1932).

Using bioassays that were specifically developed to target either the floral or the vascular pathway (Darsonval et al. 2008), no convincing observations of bacterial locations within seeds could be obtained due to low bacterial densities within the seeds. When more concentrated inocula were used, organs aborted. In contrast, spray-inoculation of bacteria on plants at the flower bud stage allowed observations of high bacterial densities in and onto seeds. Using this inoculation method, bacteria appear reaching seeds using vascular, floral, and external pathways. It can be hypothesized that in that case seed infection resulted from the accumulation of bacteria from these different origins and that these accumulations succeeded to each other spatially and temporally. Indeed, bacterial location reflected vascular infection when bacteria were located in vascular bundles of the funiculus, floral route when bacteria were observed in the micropyle and external contact with bacterial masses in fruit when observed breaking the testa.

No transmission of NP xanthomonads was observed in bean seeds following flower bud inoculation. This was unexpected as floral transmission is thought to be less restrictive than vascular transmission to seed (Darsonval et al. 2008). This pathway is known to allow some transmission of pathogens in non-host situation and of NP strains, i.e. pathogenic strains depleted of their functional T3SS (Darsonval et al. 2008; Terrasson et al. 2015). This is also somehow in contradiction with the idea that vertical transmission (i.e. direct transfer from parent to progeny) should select against pathogenicity and favor mutualism (Rudgers et al. 2009). The NP xanthomonads we used are phylogenetically closely related to CBB agents and apart from being unable to secrete adequate effectors they share many determinants with their pathogenic relatives allowing partial recognition by the plant defense machinery either directly or indirectly following the use of plant components as substrate. Most bacterial plant pathogens secrete Type 3 effectors (T3Es) that are able to overcome plant defenses and this molecular dialogue

is engaged during seed infection (Terrasson et al. 2015). The two *X. axonopodis* NP strains 13,556 and 13,557 present genes encoding T3SS and T3Es, but their T3E repertoires are different from those of any CBB agent (data not shown) suggesting that these strains should not be able to efficiently overcome bean defenses. The two other NP xanthomonads (*X. arboricola* strains 13,554 and 13,555) are flagellated but depleted of any Type Three Secretion System and unable to secrete any T3E, as predicted by whole genome sequence analyses (data not shown), and as such should be recognized and controlled by the basal host defense pathway. Our results are in agreement with the optimal defense theory, which propose that under biotic threat, a plant will defend best those tissues or organs that contribute most to its fitness, including young and reproductive organs (Meldau et al. 2012; Neilson et al. 2013).

We focused our observations of GFP-tagged Xcf-contaminated seeds on immature seeds and germinating seeds, as these tissues have a high water content enabling bacterial metabolism and hence GFP-tagged cells monitoring. Indeed, in mature seeds bacteria are mostly metabolically inactive and hence very little GFP is regenerated and available for excitation and monitored in CSLM. DOPE-FISH/CSLM was the alternative method used to locate bacteria in seeds by targeting RNA within bacterial cells. This method allows to detect *Xanthomonas* spp. inside seeds on the radicle tip, hypocotyl, cotyledon, and plumules, among other bacteria, confirming some niches of xanthomonads within the seeds. Unlike a growing body of recently published studies (Compant et al. 2010); Rosenblueth and Martinez-Romero 2006, Mastretta et al. 2009; Hardoim et al. 2012; Cope-Selby et al. 2016), this study focuses on one particular component of seed-associated endophytes, the seed-borne plant pathogenic bacteria. This study describes the location of *Xcf*, a major bean pathogen, in bean seeds and indicates that these niches are highly similar to those already reported for the other bean blight pathogen, Xpp. Such internal locations as embryonic axis are mostly encountered in case of severe infection and symptomatic seeds that do not produce viable plantlets. Most often bacteria are observed from testa up to embryo surfaces. NP xanthomonads did not transmit from flower buds to seeds, while being initially isolated from seeds. Further experiments involving differential tagging and microscopic monitoring are needed to precise the route employed by NP xanthomonads to colonize seeds and give clues regarding the segregation of these organisms in seed niches. Our results are however coherent with the observation by DOPE-FISH/CSLM of a few xanthomonads within non-inoculated seeds, indicating that these xanthomonas should have reached seeds via the vasculature. This work contributes to refine the ecology of *Xcf* and of seedborne NP xanthomonads.

Compliance with ethical standards

Conflict of interest Matthieu Barret and Stéphane Compant are Guest Editors of the special issue. This does not, however, interfere with the reviewing process.

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