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COLONIZATION ABILITY OF *ESCHERICHIA COLI* AND *LISTERIA MONOCYTOGENES* IN THE ENDOSPHERE OF SWEET PEPPER *(CAPSICUM ANNUUM* VAR. *GROSSUM)*

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Fruits and vegetables can be transmission vehicles of human opportunistic and obligate pathogenic bacteria, persisting in inner tissues for shorter or longer periods or colonizing the plants as facultative endophytes. In this study we investigated the ability of commensal *E. coli* and pathogenic *L. monocytogenes* strains to internalize sweet pepper seedlings via seed bacterization, as germinating seeds and roots are important infiltration sites for entry of enteric bacteria. By combining cultivation dependent and independent (PCR and FISH-CLSM) techniques we could not detect stably or transiently colonized inoculated bacteria in 6–7 weeks old pepper seedlings, suggesting that there is low risk associated with internalized enteric or human pathogenic bacteria via germinating seeds in sweet pepper.

Keywords: *Escherichia coli*, *Listeria monocytogenes*, sweet pepper, internalization

Currently, in the background of high and increasing incidence of illnesses associated with consumption of fresh fruits and vegetables, there were defined bacterial human pathogens (Teplitski et al., 2009; Mendes et al., 2013; Martínez-Vaz et al., 2014; Van Overbeek et al., 2014). It is now an evidence that fruits and vegetables can be the transmission vehicles of some human pathogenic bacteria living inside plants as facultative endophytes (Hallmann & Berg, 2006; Berger et al., 2010). Recently Van Overbeek and co-workers (2014) suggested to use the term "phytonosis" for the transmission of otherwise zoonotic human pathogenic bacteria via plants.

Foodborne pathogenic bacteria can internalize the plants either preharvest or postharvest through uninjured or damaged plant tissues (stomata, hydathodes, trichomes or leaf tip burn lesions, disrupted plant cuticle, crevices, respectively). Germinating seeds and roots are also considered as important infiltration sites; lateral root junctions are the "hot spots" for entry of enteric pathogens (Mendes et al., 2013). Fate of the internalized cells depends on the persistence and colonization within the plant tissues, what is highly influenced by the interaction with the native plant-associated microbiota. Tyler and Triplett (2008) hypothesized that plants may serve as alternative hosts for certain human pathogenic bacteria for surviving the unfavourable environmental conditions, and during animal ingestion they are released for re-colonizing the original host.

Enteric pathogens comprising different *Salmonella enterica* serovars and pathogenic *E. coli* strains were responsible for outbreaks associated with the consumption of fresh vegetables and fruit in most cases, but less frequently *Shigella*, *Campylobacter* spp., *Yersinia*

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enterocolitica, *Clostridium botulinum*, *Vibrio cholerae*, *L. monocytogenes*, and *Staphylococcus aureus* were also involved (Warriner, 2005; Beczner & Bata-Vidács, 2009; Berger et al., 2010; CDC, 2013a, 2013b).

Martínez-Vaz and co-workers (2014) referred to different pathogenic *E. coli* serotypes and *Salmonella enterica* serovars as identified causative agents of outbreaks, initiated by the consumption of leafy greens and sprouts between 2006 and 2012.

Enteric pathogens, especially those belonging to the family *Enterobacteriaceae*, get into contact with roots, stems, leaves, fruit, and seeds of cultivated plants from the environment via water, soil, raw or inadequately amended manure (Martínez-Vaz et al., 2014; Van Overbeek et al., 2014). Enteric bacteria, especially *Salmonella* and *E. coli* O157:H7 have enough survival capacity in soil to contaminate plants and adapt to the plant environment (Teplitski et al., 2009; Berger et al., 2010; Martínez-Vaz et al., 2014).

L. monocytogenes is an important Gram-positive foodborne pathogen, although *L. monocytogenes* infections are much more rarely associated with consumption of plant-derived foods than that of the dairy and meat products. *L. monocytogenes* is a highly tolerant and adaptive pathogenic bacterium (Brandl, 2006) being present in many environmental niches like soil, water, and plants (Vivant et al., 2013). Domesticated animals can carry the pathogen without any symptoms of infection and may contaminate milk and meat. Transmission of *L. monocytogenes* from farm animals to fruits and vegetables can take place via manure used for fertilization. Contaminated soil could be an effective mediator, as *L. monocytogenes* has a long survival time in soil at a wide range of temperatures (Jiang et al., 2004).

Difficulty in the identification of *L. monocytogenes* as causative agent of foodborne outbreaks could be attributed to the fact that a high ratio of listeriosis is sporadic, what could be ascribed to the long incubation period of the illness (Mililllo et al., 2008). A systematic analysis of listeriosis in the USA between 2000 and 2003 (Varma et al., 2007) supported the sporadic nature of listeriosis and revealed that *L. monocytogenes* infections were associated with the consumption of commercially produced melons and hummus in this period. Centers for Disease Control and Prevention (CDC (2016)) reported recently that frozen vegetables were responsible for an outbreak in the USA. Palmai and Buchanan (2002) demonstrated that *L. monocytogenes* was able to proliferate during germination of alfalfa sprouts and survived at a constant level at refrigeration temperature for seven days. Another food safety risk comes from the induction of a metabolically arrested, viable but non-culturable (VBNC) state of both *E. coli* and *L. monocytogenes* cells under stressful conditions (Dreux et al., 2007; Dinu & Bach, 2011).

In our studies we intended to check whether non-pathogenic (commensal) *E. coli* and foodborne human pathogenic *L. monocytogenes* strains are able to infiltrate and colonize sweet pepper via seed inoculation under similar conditions as the endophytic bacteria internalize host plants.

1. Materials and methods

1.1. Strains, culture conditions, and maintenance

E. coli ATCC 8739, *L. monocytogenes* CCM 4699, *L. innocua* 1010, and *L. grayi* CCM 4029 were used in the experiments, which were maintained in PGYS (peptone 5 g l^{-1}; glucose 1 g l^{-1}; yeast extract 2.5 g l^{-1}, NaCl 5 g l^{-1}) agar at 4 ºC.

1.2. Pepper seed bacterization, plant breeding, and sample processing

Seeds of Karpia cultivar of sweet pepper (*Capsicum annuum* L. var. *grossum*) were surface-disinfected (Belák et al., 2014) and inoculated with *E. coli* ATCC 8739 and *L. monocytogenes* CCM 4699 separately, as described in Niranjan Raj and co-workers (2003). Non-inoculated seeds served as sterile controls, while non-disinfected ones without bacterization were used as non-sterile controls. Seed germination was a slightly modified version of the paper towel method according to the International Seed Testing Association protocol (ISTA, 2016).

After seed germination, 9 seedlings with two cotyledons were selected from each of the bacterized and control samples, planted to rock wool cubes and cultivated in beakers under aseptic condition. Rock wool cubes were moisturized with 1.2 g l^{-1} Ferticare™ I fertilizer and seedlings were irrigated with 2.2 g l^{-1} of this solution. Phytolite Agro Grow-Bloom HPS 400 W lamp (PHT Trading SA, Ticino, Switzerland) supplied adequate light for indoor cultivation for 35–40 days.

1.3. Detection of the inoculated bacteria in the endosphere of pepper seedlings

After the cultivation process 9 seedlings (those from the bacterized and control samples) were divided into 3 equal groups and treated as follows.

1.3.1. Cultivation of bacteria from different organs of the seedlings. Small pieces of tissue samples were placed onto Chromocult® coliform (Merck KGaA, Darmstadt, Germany) and *Listeria* selective ALOA (Microgen Bioproducts Ltd., Camberley, UK) agar media, respectively, and incubated for 5 days at 30 °C.

1.3.2. Direct amplification of bacterial DNA by PCR. Plant tissue samples were homogenized in 1x PBS buffer (Daims et al., 2005) by IKA Ultra Turrax® T25 (IKA® – Werke GmbH & Co. KG, Staufen, Germany). DNA was isolated with the PowerSoil® Pro DNA Isolation Kit (MO BIO Laboratories, Qiagen, Carlsbad, USA). Specific PCR reactions were applied to detect strains used for seed bacterization (Table 1).

Table 1. PCR primer pairs used in this work: sequences, amplicon sizes (AS), and annealing temperatures (AT)

Target/specificity	Primer name	Primer sequence 5'→ 3'	AS bp	AT °C	References
16S rDNA/all bacteria	27f 1492r	AGAGTTTGATC**M**TGGCTCAG TACGG**Y**TACCTTGTTACGACTT	1467	58	Edwards et al., 1989; Weisburg et al., 1991
16S rDNA/all bacteria	1070f 1492r	TCAGCTCGTGT**Y**GTGA**R**A TACGG**Y**TACCTTGTTACGACTT	448 (863)	52	Shakya et al., 2013;
16S rDNA/ *Listeria*	27f Lis659R	AGAGTTTGATC**M**TGGCTCAG CACTCCAGTCTTCCAGTTTC	659	68	Lis659R: this work
16S rDNA/*E. coli* and *Shigella*	27f Ec/Sh473R	AGAGTTTGATC**M**TGGCTCAG AAAGGTATTAACTTTACTCCCT	466		Ec/Sh473R: this work

Nucleotides marked bold indicate degeneracy. M: A or C; R: A or G; Y: C or T

1.3.3. Detection of the colonized bacteria by FISH-CLSM. Plant organs were sliced into 10–15 mm long pieces and treated with a fixative solution (4% formaldehyde in 1x PBS) overnight at 4 °C. After fixation, samples were rinsed three times in 1x PBS and stored in a solution containing 1x PBS and 96% ethanol (1:1) at –20 °C. For specimen preparation, fixed

samples were embedded into Shandon™ Cryomatrix™ (Thermo Fisher Scientific Inc., Waltham, USA) solution at –23 °C for 20 min, sliced into 10–20 μm thick sections with Microm HM 505 NP Cryostat Microtome (MICROM International GmbH, Walldorf, Germany) and were placed onto microscopic slides. Citiflour AF1 (Citifluor Ltd., London, UK) was used as antifadent mounting agent. FISH analysis was performed as described by Daims and co-workers (2005). Image capturing was performed with Zeiss LSM 710 (Carl Zeiss AG, Oberkochen, Germany) confocal laser scanning microscope (CLSM).

1.4. PCR analysis

Oligonucleotide primers for PCR reactions were designed by aligning the 16S rDNA sequences of the targeted and the closest reference (mostly type) strains belonging to the same genus, using MEGA6 program (Tamura et al., 2013). Characteristics of the PCR primer pairs (melting temperature, GC content, and chance for secondary structure formation) were evaluated by OligoAnalyzer 3.1 IDT SciTool program (Integrated DNA Technologies Inc.). For evaluation of the primer specificity, NCBI Nucleotide BLAST tool (i) was used (Blast). PCR primers shown in Table 1 were applied for amplification of 16S rDNA as well as for detection of the seed-inoculated *E. coli* ATCC 8739 and *L. monocytogenes* CCM 4699 strains.

1.5. Designing and testing of FISH probes

New oligonucleotide probes based on the 16S rRNA gene sequences of the target strains were created by using the ARB© 6.0.2 program package (Ludwig et al., 2004). Specificity of FISH probes was tested by using the probeCheck web server (Loy et al., 2008), while thermodynamic properties of the probes (hybridization efficiency, change in Gibbs free energy, and the formamide dissociation profile) were evaluated by the mathFISH webtools (Mathfish).

E. coli/Shigella-specific Ec/Sh_453 probe described by Jansen and co-workers (2000) was adapted for labelling *E. coli* ATCC 8739 cells. Lis-637 probe for targeting *L. monocytogenes* CCM 4699 strain was selected for FISH analysis, which hybridizes to most *Listeria* species, except *L. grayi* (Schmid et al., 2003). However, because weak mismatching to some *Peanibacillus* and *Bacillus* strains was detected, competitive probe Lis-comp was also designed. Hybridization probes and conditions are shown in Table 2.

Table 2. FISH probes used in this work: sequences, labelling, and formamide (FA) concentrations

Specificity for bacteria	Probe names	Sequences 5' → 3'	5' end labelling	FA %	References
Most bacteria	EUB338	GCTGCCTCCCGTAGGAGT	6-FAM/Cy3	15-35	Amann et al., 1990
Planctomycetales	EUB338 II	GCAGCCACCCGTAGGTGT	6-FAM/Cy3	15-35	
Verrucomicrobiales	EUB338 III	GCTGCCACCCGTAGGTGT	6-FAM/Cy3	15-35	Daims et al., 1999
All bacteria	EUB mix	EUB338 : EUB338II : EUB338 III = 1:1:1	6-FAM/Cy3	15-35	Bathe & Hausner, 2006
Listeria spp., except *L. grayi*	Lis-637	CACTCCAGTCTTCCAGTTTCC	6-FAM	35	Schmid et al., 2003
Listeria competitor	Lis-comp	CACTCCAGTCTCCCAGTTTCC	no labelling	35	This work
E. coli/Shigella	Ec/Sh_453	GCAAAGGTATTAACTTTACTCCC	6-FAM	15	Jansen et al., 2000

Combinatorial labelling with the application of EUB mix and specific probes was used for simultaneous detection of the inoculated (target) and natural endophytic (non-target) bacteria (Table 3).

Table 3. General and specific probes used for combinatorial labelling

Sample name	General probes → labelling	Specific probe → labelling
L. monocytogenes – inoculated seedlings	EUB mix → Cy3	Lis-637 → 6-FAM Lis-comp
E. coli – inoculated seedlings	EUB mix → Cy3	Ec/Sh_453 → 6-FAM
Sterile and non-sterile control seedlings	EUB mix → Cy3	Lis-637 → 6-FAM Lis-comp
	EUB mix → Cy3	Ec/Sh_453 → 6-FAM

FISH test of bacterial pure cultures was implemented with suspensions of strains grown on PGYS agar for 24 hours. FISH-CLSM analysis was performed as described in section 1.3.

2. Results and discussion

2.1. Infiltration of pepper seeds by E. coli *and* L. monocytogenes

In order to analyse colonization ability of the commensal *E. coli* ATCC 8739 and the human pathogenic *L. monocytogenes* CCM 4699 strains, sweet pepper seeds were separately inoculated with these bacteria. Bacterized seeds germinated in good rate and seedlings had similar development to the control plants (grown from non-inoculated seeds) under aseptic conditions.

2.2. Detection of seed inoculated bacteria in pepper seedlings by cultivation

Persistence and colonization of seed-inoculated bacteria *E. coli* ATCC 8739 and *L. monocytogenes* CCM 4699 in sweet pepper were checked by incubating the plant organ samples (roots, stems, and leaves) on selective culture media.

Detection of any typical *E. coli* or *L. monocytogenes* colonies on coliform selective Chromocult® or Listeria selective ALOA agar media, respectively, gave negative results. This indicated that neither *E. coli* ATCC 8739 nor *L. monocytogenes* CCM 4699 were able to colonize plant tissues through seed bacterization.

2.3. Detection of seed inoculated bacteria in pepper seedlings by direct amplification from the extracted DNA

PCR amplification with species-specific primers was used in order to detect the presence of the inoculated bacterial strains in DNA extracted directly from the plant samples. For PCR detection of *E. coli* ATCC 8739 and *L. monocytogenes* CCM 4699 strains, 27f -Ec/Sh473R and 27f -Lis659R primer pairs were applied, respectively (Table 1). As shown in Figure 1, PCR detection of any *E. coli* and *L. monocytogenes* in DNA extracted from the inoculated

pepper plants failed, which confirmed the negative results of the cultivation-based detection on selective media.

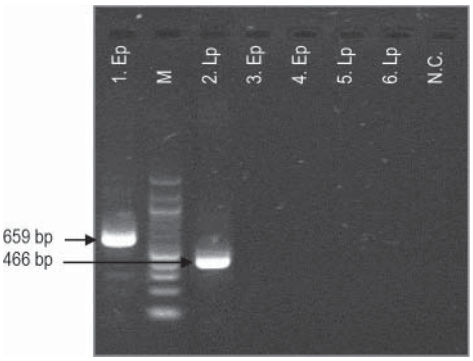

Fig. 1. PCR detection of *E. coli* ATCC 8739 and *L. monocytogenes* CCM 4699 in DNA extracted from pepper seedlings grown from inoculated seeds
1. Ep: PCR product of *E. coli* ATCC 8739; **2. Lp**: PCR product of *L. monocytogenes* CCM 4699; **3. Ep**: PCR detection of *E. coli* in root; **4. Ep**: PCR detection of *E. coli* in shoot; **5. Lp** PCR detection of *L. monocytogenes* in root; **6. Lp**: PCR detection of *L. monocytogenes* in shoot
Legend: **Ep**: 27f, Ec/Sh473R primers; **Lp**: 27f, Lis659R primers; **N.C.**: negative control. **M**: 100 bp DNA ladder (100–1517 bp, Catalog #: N3231S, New England Biolabs® Inc., USA)

2.4. Detection of seed inoculated bacteria in the pepper seedlings by FISH-CLSM technique

Visualization of seed-inoculated bacterial cells in the internal pepper tissues was performed by FISH-CLSM technique as described in Materials and methods. In FISH analysis general probes were used for detection of any kind of bacteria, while specific probes were applied in order to study the presence of bacteria used for seed bacterization (Table 3).

Hybridization efficiency of the *E. coli/Shigella*-specific Ec/Sh_453 probe (Table 2), as tested on the cells of *E. coli* ATCC 8739 under stringent condition, gave satisfactory result (Fig. 2), therefore it was used for targeting this strain in FISH-CLSM analysis.

Lis-637 probe also seemed to be suitable for detection of the inoculated *L. monocytogenes* strain by FISH-CLSM, as Lis-637 gave positive hybridization signal with cells of the tested species belonging to the *Listeria* genus, except *L. grayi*. In order to avoid false positive results as the consequence of potential mismatching of the probe, the competitive Lis-comp probe was used in combination with the Lis-637 probe (Table 2). In silico FISH testing of the combined Lis-comp and Lis-637 probes resulted in strong fluorescence of *L. monocytogenes* and *L. innocua* but not *L. grayi* cells (Fig. 2).

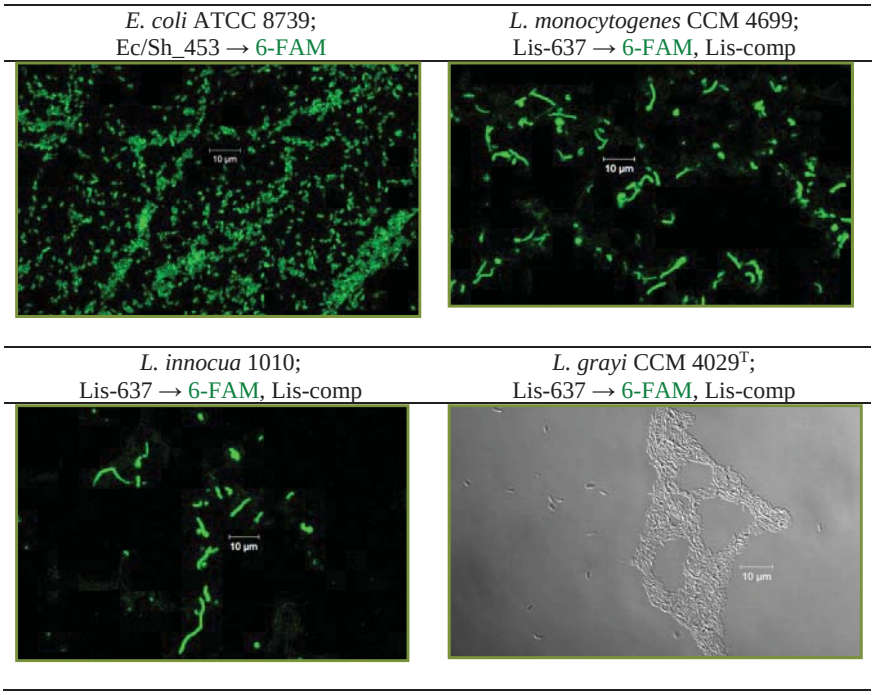

Fig. 2. Representative FISH-CLSM images of bacterial cells. Fluorescence of *E. coli* ATCC 8739 cells indicates hybridization with the Ec/Sh_453 probe, while fluorescence of *L. monocytogenes* CCM 4699 and *L. innocua* 1010 cells is the consequence of binding the Lis-637 probe. *L. grayi* CCM 4029 represents a negative control emitting no fluorescence. Probe legends: Probe name → labelling

Images of Fig. 3A illustrate that only a few red fluorescent cells appeared in plant sections (roots and shoots) inoculated with *E. coli* ATCC 8739. Red colour of the cells indicated hybridisation with the EUB mix probe alone, therefore these cells do not belong to *E. coli*, not even to the genera of *Escherichia* or *Shigella*. As shown in Fig. 3B, it was not possible to detect any bacterial cells in plant sections inoculated with *L. monocytogenes* CCM 4699. Seedlings grown up from non-inoculated, sterilized or non-sterilized (control) seeds were also analysed by hybridization with the combined EUB mix and specific probes. Results shown in Figures 3C and 3D indicate that no bacterial cells could be detected in most cases. There was only one root section of a pepper plant originated from sterilized seed (Fig. 3D), where a few cells emerged in the EUB mix emitting red colour. These cells probably belonged to the natural endophytic bacteriobiota and not to any of the bacterial strains used for seed inoculation.

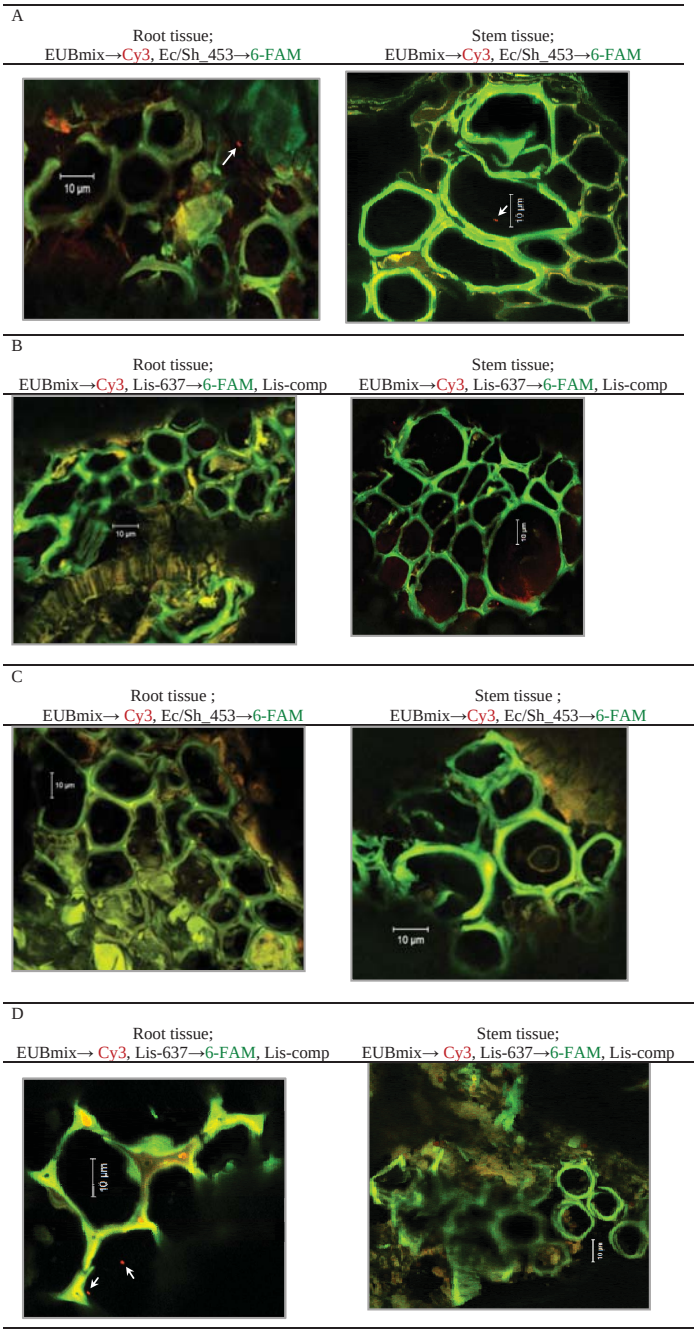

Fig. 3. Representative FISH-CLSM images of different tissue specimens prepared from inoculated and non-inoculated (control) pepper seedlings. A: surface sterilized seeds were inoculated with *E. coli* ATCC 8739; B: surface sterilized seeds were inoculated with *L. monocytogenes* CCM 4699; C: seeds were not sterilized and not inoculated; D: surface sterilized seeds were not inoculated. Fluorescent cells are indicated by arrows. Probe legends: Probe name → labelling

Absence of the inoculated bacterial cells in the endosphere could be explained by the incapability of the inoculated bacteria to infiltrate the seeds or migrate into the internal plant tissues. Even if internalization was successful, they were not able to survive plant cultivation period in an adequate amount to be detected by FISH-CLSM as it has a relatively high detection limit (approx. 10^3 cells ml^{-1} or $gram^{-1}$).

According to our results, the applied *L. monocytogenes* strain is unable to internalize sweet pepper seedlings. This is different from the results obtained by Milillo and co-workers (2008), who found that *L. monocytogenes* strains were able to attach to seeds and internalize intercellular spaces of the plant model *Arabidopsis thaliana*. In this case, although bacterization of the seeds considerably decreased seed germination frequency, *L. monocytogenes* was recovered from the leaves of plants originated from the inoculated seeds. Sweet pepper seems therefore more resistant to the infiltration by *L. monocytogenes* than *A. thaliana*. Jablasone and co-workers (2005) reported that both *L. monocytogenes* and *E. coli* O157:H7 were able to establish and persist in young (9 days old) seedlings of several vegetables (carrot, cress, lettuce, radish, spinach, and tomato) but were not recovered from tissues of mature plants. Our results confirm their conclusion, emphasising that the risk associated with internalized human pathogenic bacteria at harvesting time is low.

Pathogenic and non-pathogenic (K-12) *E. coli* strains are using the same strategies in the first steps of interaction with plants (attachment by curli fibres and flagellae to the surface, suppression of biofilm formation, and a downward shift in the energy metabolism), as it was demonstrated by Fink and co-workers (2012), who compared the whole genome transcriptions by microarray analysis. Hou and co-workers (2012) came to similar conclusions, when they analysed colonization and growth of *E. coli* K-12 in the lettuce rhizosphere, by combining transcriptional analysis, colonization assays, and CLSM. Genes coding for the curli and flagellum synthesis were crucial in the attachment, and genes involved in protein synthesis and stress responses (biofilm modulation) were up-regulated. Dong and co-workers (2003) found differences among *E. coli* O157:H7 and K-12 strains in colonization of the rhizosphere and endosphere of alfalfa seedlings depending on the inoculum levels. However, colonization of the endosphere by K-12 was weak, independently of the inoculum levels, indicating that genotypes of *E. coli* strains have an influence on the internalization. Our results suggest that even if the non-pathogenic (commensal) *E. coli* was able to attach to sweet pepper seeds, colonization in the 6–7 weeks old seedlings could not be demonstrated by cultivation dependent and molecular techniques.

3. Conclusions

Seed bacterization and combination of cultivation dependent and independent (PCR and FISH-CLSM) techniques provide efficient tools for studying the infiltration, persistence, and colonization of harmless *E. coli* and foodborne pathogenic *L. monocytogenes* in sweet pepper, especially because hydroponic cultivation is becoming a more and more popular agrotechnique for this freshly consumed vegetable's production. Sweet pepper seems to be at low risk in this respect, because no stable colonization of the inoculated strains in the inner tissues could be demonstrated, not even a transient persistence could be suspected by PCR amplification of DNA being present in dead or VBNC cells.

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