Microbial Pathogenesis 115 (2018) 199–207

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Salmonella as an endophytic colonizer of plants - A risk for health safety vegetable production

Igor Kljujev[a,*], Vera Raicevic[a], Bojana Vujovic[b], Michael Rothballer[c], Michael Schmid[c]

[a] Faculty of Agriculture, University in Belgrade, Serbia
[b] Institute for the Development of Water Resources, Belgrade, Serbia
[c] Research Unit Microbe-Plant Interactions, Helmholtz Zentrum Munchen, Germany

ARTICLE INFO

Keywords:
Contamination vegetables
Salmonella spp.
Irrigation water
FISH
CLSM

ABSTRACT

Contamination of vegetables and fruits is the result of presence of human pathogen bacteria which can contaminate products in any part of production chain. There is an evidence of presence of: *Salmonella spp.* on the fresh vegetables and Salmonellosis is connected with tomato, sprouts, cantaloupe etc.

The goal of this research is transmission of pathogen bacteria from irrigation water to plants and studying/monitoring the ability of the *Salmonella spp.* to colonize the surface and interior (endophytic colonization) of root at different vegetable species.

Transmission of three *Salmonella spp.* strains from irrigation water to plants, as well as colonization of plants by these bacteria was investigated by using *Fluorescence In Situ Hybridization* (FISH) in combination with confocal laser scanning microscopy (CLSM).

All tested *Salmonella spp.* strains showed ability to more or less colonize the surface and interior niches of the root, stem and leaf of the investigated plant species. These bacteria also were found in plant cells cytoplasm, although the mechanism of their entrance has not been clarified yet.

1. Introduction

The consumption of vegetables is essential for healthy nutrition and it is an integral part of many diets which are recommended by various health organizations [61,65]. Since fresh produce is one of the major components, such as essential vitamins, minerals, and fiber, the consumption of fresh produce has increased worldwide in recent years [6,23].

The number of outbreaks associated with the consumption of contaminated fresh produce, especially those caused by Salmonella, has also increased. In EU countries excluding Spain, a total of 37 Salmonellosis outbreaks have been linked to the consumption of food of non-animal origin including fresh produce have been reported between 2007 and 2011 [21].

The vegetables could become contaminated [8] in contact with soil, manure, compost, irrigation water, contaminated water for washing and etc. Irrigation water contaminated with manure or animal waste is a common environmental source for the transmission of microorganisms into fresh produce [53].

The *Salmonella* is an important human and animal pathogen worldwide, mainly transmitted to humans through contaminated food [41]. Each year, nontyphoidal *Salmonella spp*. have been estimated to be responsible for 1.0 million [56] and 80.3 million human salmonellosis cases [48] in the United States and globally, respectively. The preharvest contamination vegetables by human pathogen is the most often cause of presence microbiologically unsafe products on the market [14,28,54,60]. There is evidence that outbreak of tomato-associated salmonellosis was caused by *Salmonella* Newport. This strain was isolated from water which was used for irrigation tomato plants [31]. Also, it was happened salmonellosis outbreak which was linked to consumption of alfalfa sprouts contaminated by *Salmonella* Newport in 2010. This *Salmonella* strain was isolated from seed which was considered as a source of outbreak. The similar outbreak was happened in 2010. with *Salmonella enterica* – contaminated alfalfa sprout, but the source of this *Salmonella* strain was run-off water (environmental sample) [16].

The *Salmonella* Saintpaul – outbreak, which was happened in more states, resulted in more than 1400 infected people in the USDA. This is an example how complicated it could be to detect source of contamination [15]. It was initially suspected that contaminated tomatoes are source of the outbreak, but investigation by CDC and FDA revealed that main source of Salmonella Saintpaul were jalapeno and serrano

* Corresponding author.
E-mail address: ikljujev@agrif.bg.ac.rs (I. Kljujev).

https://doi.org/10.1016/j.micpath.2017.12.020
Received 26 October 2017; Received in revised form 6 December 2017; Accepted 8 December 2017
Available online 14 December 2017
0882-4010/

peppers [44]. This failure resulted in huge economic loss to tomato growers. Also, some authors found out that about 86% of cases linked with eating raw jalapeno pepper and other similar food at Mexican restaurants [5].

The products might be contaminated during preharvest as well as postharvest process of production. Some authors examined routs of colonization tomato fruits in preharvest process by *S. enterica* [4,32,36]. Their point of view is that irrigation water could be a potential source of colonization fruit by pathogen bacteria. The entrance of *S. enterica* into the tomato plants through irrigation water is still inconsistent. There is evidence that *Salmonella enterica* serovar Newport could contaminate different tomato plant tissue via irrigation water [36]. The *Salmonella*-contaminated water was applied only in root zone and Salmonella was detected at tomato plants in different plant growth stages.

[32] showed that *Salmonella typhimurium* could colonize interior spaces of tomato plants via leaves and it can lead to high concentration of *Salmonella* in plant fruits but there are not any visible symptoms on the tomato plants.

[38] showed that using *S. typhimurium*-contaminated irrigation water can lead to contamination carrot and radish during the harvesting crops and *S. typhimurium* could survive 203 days in the soil after its due date. The *S. enterica* serovar *Thompson* can colonize whole leaves of coriander after 6 days of inoculation but the pathogen concentration on leaves largely depends on temperature and relative humidity [11] [51]. detected *S. enterica Typhimurium* in the leaves of rocket after 17 weeks of growing in the manure-contaminated soil. In the field growing conditions, *S. enterica* serovar *Typhimurium* can survive 63 days on lettuce and 231 days on parsley if the plants are irrigated with contaminated water and the similar results are obtained with carrot and radish [38] [39]. showed that *S. typhimurium* could entry in plant tissue. After surface sterilization plants, they used indirect method for quantification number of *S. typhimurium* inside plant tissue. The positive plant samples were appeared 9 days after inoculation plants, but number of *S. typhimurium* was not so high. The experiments of [3] showed that *S. enterica* strains easier adhere alfalfa sprouts than *E. coli* O157:H7. The tomato plants which were grown in hydroponic conditions could be colonized by *Salmonella spp.* if the water is contaminated with these bacteria. The one day after exposing tomato root to *Salmonella spp.*-contaminated water, the number of bacteria on hypocotyls, cotyledons and stem reaches 3,01 CFU/log10 - 3,40 CFU/log10 per gram [35].

Some experiments showed colonization plant damaged seed coat edge during the seed germination by *S. enterica*. The *S. enterica* is able to make huge microcolony (aggregations) on the alfalfa root hairs [17]. It is known that bacterial cells aggregation is associated with synthesis of cellulose fibrils [59]. The *S. enterica* also has ability to synthesize cellulose fibrils, but their function is not related with bacterial adherence or aggregation in the rhizosphere [66].

There is an experiment about localization human pathogen on the root of inoculated plants which are grown in the soil in lab conditions. During the colonization *Arabidopsis thaliana* root by *E. coli* Ol57:H7 and *S. enterica*, these bacteria were concentrated on the root top and in the places of branching lateral roots at an early stage of plant development. The *Arabidopsis thaliana* root was colonized by these bacteria uniformly at a later stage of plant development [18]. Same authors did seed inoculation by *S. enterica* mutants with reduced flagella number and observed much less colonization which means that bacterial motility has a significant role in plant colonization.

Confocal laser scanning micrographs showed ability of *S. enterica* serovar *Stanley* to enter inside undamaged alfalfa sprouts [25] [63]. detected endophytic colonization vascular system of bean sprouts by *S. enterica* after surface sterilization of plant samples and *in situ* staining plants. According to their opinion, bacteria enter in plant through the cracks in epidermis and place the growth of lateral roots. Some other researchers also showed interaction between alfalfa seedling and different *S. enterica* serotypes. The alfalfa seedlings were the most colonized by bacteria on the places the growth of lateral roots. Also, it was noticed differences in endophytic colonization at different *S. enterica* serotypes, which indicates a specificity of strain for adherence to plants [19].

[34,35] inoculated tomato plants at the stage before and after the start of flowering by *S. enterica*. They tested tomato fruit and out of 30 samples, 11 were positive for the presence of pathogen. The inner content of the tomato fruit in 6 out of 11 samples was contaminated by *S. enterica* which indicates the movement of the pathogen through the tomato plant. The same authors showed colonization tomato plants by *S. enterica* in hydroponic growing conditions. After 9 days of growing plants, the number of *S. enterica* was so high in leaves, stems, hypocotyls and cotyledons of seedlings. This indicates that *Salmonella sp*. could be transmitted into the plants if the plants are grown in contaminated environment.

The goal of this study is transmission of pathogen bacteria from irrigation water to plants and monitoring the ability of the *Salmonella spp.* to colonize the root surface and interior of different vegetable species.

2. Material and method

The ability of the surface and endophytic colonization of different plants by *Salmonella* strains was investigated in laboratory conditions with "monoxenic" model growing conditions. (one plant species inoculated by one bacterial strain).

2.1. Bacterial strains and plant species

The three strains of *Salmonella enterica* serotype *typhimurium* (LT2, S1 and ATCC14028) were used in this experiment. These strains are part of bacterial culture collection of Helmholtz Zentrum Munchen, German Research Center for Environmental Health (GmbH), Research Unit Microbe-Plant Interactions. The strain *Salmonella typhimurium* LT2 is commonly used in laboratory experiments and *S. typhimurium* S1 strain is isolated from organic waste of plants.

The experimental plants were: lettuce (*Lactuca sativa*) sort 2476; spinach (*Spinacia oleracea),* parsley (*Petroselinum crispum*); carrot (*Daucus carota* subsp. *sativus*); celery (*Apium graveolens*); tomato (*Lycopersicon esculentum*); sweet corn (*Zea mays* var. *saccharata*).

2.2. Inoculation experiments in an axenic model system

The *Salmonella* strains were cultivated on Xyline-Lysine-Desoxycholate Agar and SS Agar (Merck, Germany) and incubated on 37 °C overnight. After incubation, *Salmonella typhimurium* strains appeared as typical black colonies (Photo 1). The one single colony for each strain is transferred in separate falconen tube with 20 ml Luria Bertani Broth and incubated with shaking on 37 °C overnight. The tubes with incubated strains are centrifuged on 5000 rpm for 3 min, the supernatant is removed and added 20 ml PBS 1 × and each tube is vortexed. This step is repeated three times in total. The bacterial concentration (OD) is measured on spectrophotometer on 436 nm wavelength. The bacterial suspension is adjusted by PBS 1 × to reach ≈ 10^8 CFU/ml. The final concentrations for *S. typhimurium* LT2 and *S. typhimurium* S1 were ≈ 10^8 CFU/ml PBS (OD436 = 0.846/ml and OD436 = 0.846/ml) and for *S. typhimurium* ATCC14028 ≈ 10^7 CFU/ml PBS (OD436 = 0.710/ml).

It was used certified seeds (Germany) for this experiment. The surface sterilization of seeds was done according to the [12].

The sterile seeds (without washing) incubated in Petri-dishes with Nutrient Agar and LB Agar (Sigma-Aldrich) on 30 °C during the 24 - 48^h to start germination of seeds (appear cotyledons) and check its sterility before bacterial inoculation and planting (Photo 2).

The plants were grown in sterile quartz sand (particle size 1.0–2.5 mm) with Basal Salt Mixture (SIGMA). Basal Salt Mixture:

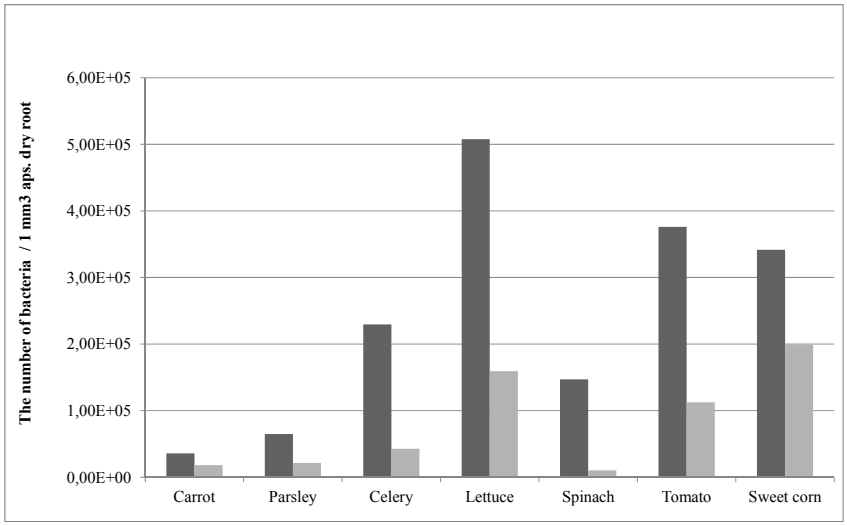


Fig. 1. The number of *Salmonella typhimurium* LT2 cells in the surface and interior layers of vegetable roots (■ root surface □ root interior).

4.3 g MS powder dissolved in 1000 ml H_2O_{dem} and autoclaved on 121 °C, 1.5 at, 20 min. The 200 g sterile quartz sand was placed in each sterile plastic dish with cap (SIGMA) and it is added 20 ml Basal Salt Mixture dilution. It was planted five same seedlings in one dish and after one day, the seedlings were inoculated by 1 ml of *Salmonella sp*. suspensions. The bacterial suspension was placed on the quartz sand surface close to seedlings. The control dishes with seedlings were inoculated by 1 ml sterile PBS dilution. The sweet corn seedlings were grown in large sterile glass tubes (3 cm width x 20 cm length) with sterile quartz sand (the tube was filled 7 cm in length) and 10 ml Basal Salt Mixture dilution was added. The one seedling was planted in each tube and inoculated by 1 ml bacterial suspension and tube is covered by sterile cap (Photo 3).

The inoculated plants were grown 21 days in absolute sterile conditions at room temperature and normal light mode.

After 21 days, the plants carefully removed from sand in sterile conditions without damaging the root system and root was cut. The attached quartz sand particles and weak attached bacterial cells were removed by washing root in sterile Phosphate-buffered saline dilution. The inoculated plant root samples were ready for Fluorescence *In Situ* Hybridization (FISH).

2.3. Fluorescence in SITU hybridization (FISH) and confocal laser scanning microscopy (CLSM)

The FISH was done for all plant root samples inoculated by *Salmonella sp.* strains (*Salmonella typhimurium* LT2; *Salmonella typhimurium* ATCC14028; *Salmonella typhimurium* S1).

The root samples were fixed in 4% (w/v) paraformaldehyde solution for 1.5^h at 4 °C [1]. The probe Salm-63-Cy3 used in this experiment and the strength of the deionized form amide used in the hybridization buffer and probe specificity is given in Table 1. The probe was synthesized and purchased from Thermo Electron, Division Interactiva (Ulm, Germany). Hybridization with fluorochrome-labelled oligonucleotide probe (Cy3) was performed according to the standard FISH protocol [2,49].

The confocal laser scanning microscopy LSM-510-META (Zeiss, Germany) was used for three-dimensional microscopic analyses of plant root system. The He–Ne laser provided excitation wavelength of 543 nm for Cy3 excitation with LP 560 long-pass filter. The Cy3 fluorescent dye is shown with its red fluorescence color. The Ar ion laser with 488 nm excitation wavelength and with BP 500–550 band-pass filter was used to show auto fluorescence and thus the structure of plant roots.

The number of *Salmonella sp*. which colonizes plant roots (surface and endophytic colonization) was determined by direct counting in 3D-micrographs by using Zeiss LSM Image Browser software. It was determined volume of plant roots and bacterial cells were counted on the

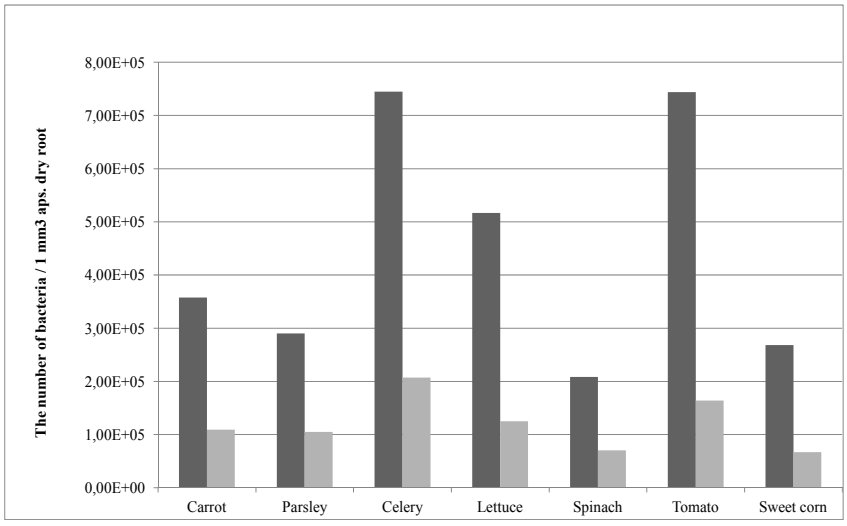


Fig. 2. The number of *Salmonella typhimurium* S1 cells in the surface and interior layers of vegetable roots (■ root surface □ root interior).

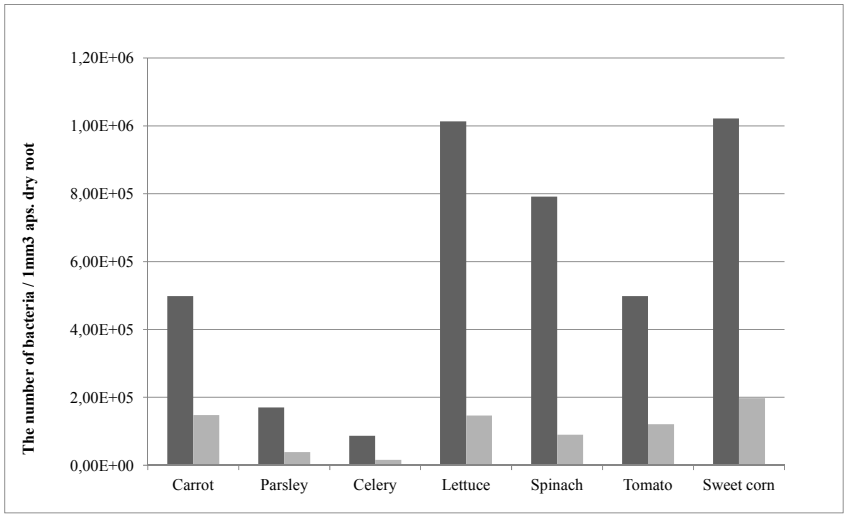


Fig. 3. The number of *Salmonella typhimurium* ATCC 14028 cells in the surface and interior layers of vegetable roots (■ root surface □ root interior).

surface and inside plant roots. It was taken root depth 0-5 μm as the surface area while the interior of the root was taken depth greater than 5 μm. The number of *Salmonella sp*. was calculated per unit volume and was shown as the number of cells/mm^3 absolutely dry plant root.

2.4. Statistical analyses

The statistical analyses were done by non-parametric Kruskal-Wallis Test. This test is alternative to the One-Way ANOVA test and it is able to assess if samples come from populations with the same median. The significance level α was 0.05 and the number of degrees of freedom was 2. This test is very useful when not all ANOVA assumptions are met. The reason for using Kruskal-Wallis Test is that our data (variables) were measured at the ordinal level and the assumption of normality was not met. In this test, it was used a null and the alternative hypothesis. The null hypothesis was that all measured samples have same medians. It means that there are not differences in colonization plant roots by different strains of *Salmonella*. The alternative hypothesis was that there are differences in colonization plant roots by different strains of *Salmonella*. It means that medians are unequal in all population.

3. Results

The detection of *Salmonella* cells was done using Zeiss LSM Image Browser software and all results are presented in Figs. 1–3. The obtained results showed ability of *Salmonella* strains for surface and endophytic colonization plant roots in the monoxenic model growing plants. Also, the micrographs of roots showed location of *Salmonella* cells on the surface and inside plant roots.

3.1. Inoculation experiments in the axenic system

The *Salmonella typhimurium* LT2 colonized root surface of lettuce in the highest degree (5.08×10^5 cells/mm^3 absolutely dry root). The high degree of surface root colonization was also detected at tomato and sweet corn roots (3.76×10^5 cells/mm^3 and 3.42×10^5 cells/mm^3 absolutely dry root). The roots of sweet corn (1.99×10^5 cells/mm^3), lettuce (1.59×10^5 cells/mm^3) and tomato (1.12×10^5 cells/mm^3 absolutely dry root) were the most endophiticly colonized by *S. typhimurium* LT2 (Fig. 1).

The surface and endophytic colonization plant roots by *Salmonella typhimurium* S1 was the largest at celery and tomato. The number of bacteria at celery root surface and interior was 7.45×10^5 cells/mm^3 and 2.07×10^5 cells/mm^3 absolutely dry root. Also, it was detected 7.44×10^5 cells/mm^3 (root surface) and 1.64×10^5 cells/mm^3 absolutely dry root (root interior) at tomato. The huge number of *S. typhimurium* S1 cells was detected on the lettuce root surface (5.17×10^5 cells/mm^3 absolutely dry root). The lowest level of colonization by this strain was noticed at spinach and sweet corn roots (Fig. 2).

The high level of colonization roots by *Salmonella typhimurium* ATCC 14028 was found out at leafy and fruitful vegetables but this strain colonized root-tuber plants less than others. Thus, the largest number of *S. typhimurium* ATCC 14028 was detected on the surface parts of lettuce and sweet corn roots (1.01×10^6 cells/mm^3 and 1.02×10^6 cells/mm^3 absolutely dry root). Also, these plants were endophytic colonized by this strain. In terms of root-tuber vegetables, the *S. typhimurium* ATCC 14028 colonized in the highest degree carrot root surface (4.98×10^5 cells/mm^3 absolutely dry root) and carrot root interior

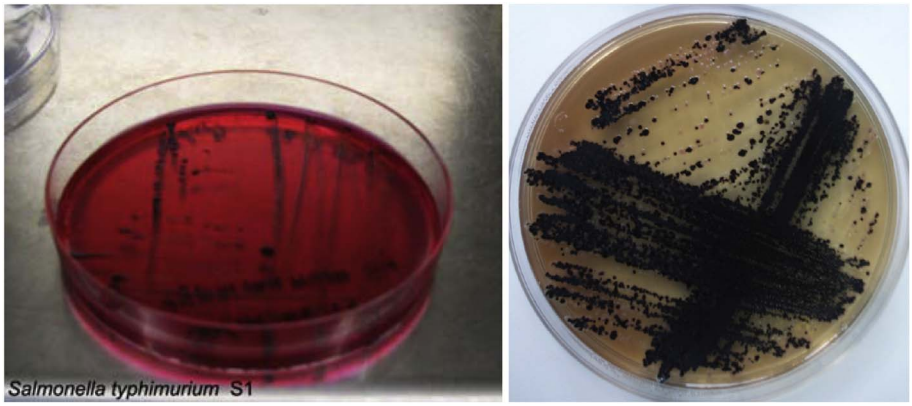


Photo 1. The characteristic black colonies of *Salmonella typhimurium* S1 strain on XLD Agar (left) and SS Agar (right) which was used for plant inoculation.

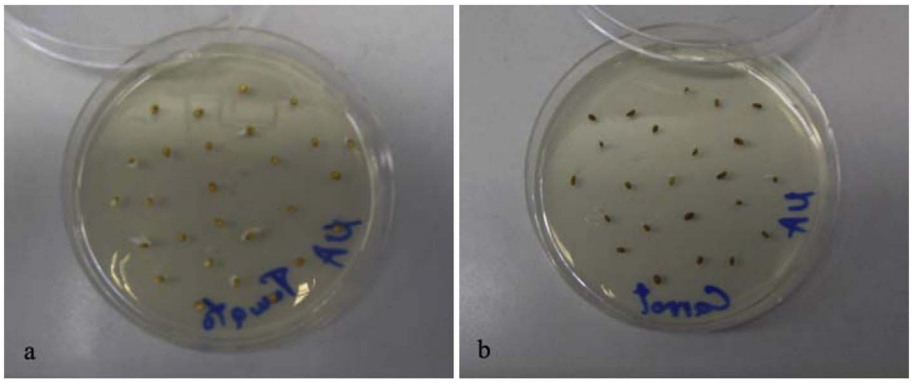


Photo 2. The seed germination in Petri-dishes with Nutrient Agar and checking of seed sterility (a – tomato seeds; b – carrot seeds).

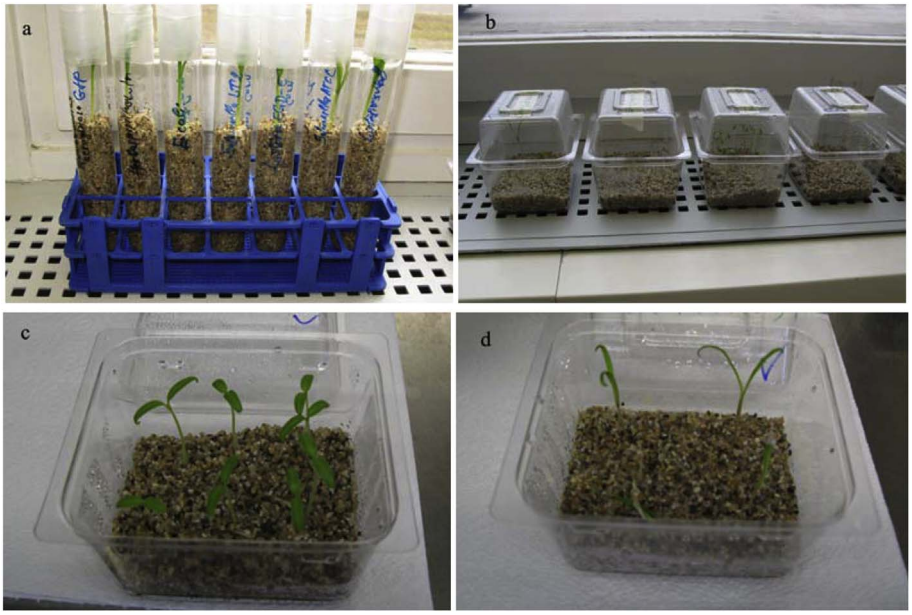


Photo 3. The plastic dishes and glass tubes with experimental plants inoculated by *Salmonella* strains (a – sweet corn in glass tubs; b – growing plants; c – tomato plants; d – spinach plants).

(1.48×10^5 cells/mm^3 absolutely dry root) (Fig. 3).

3.2. FISH/CLSM analysis of inoculated roots

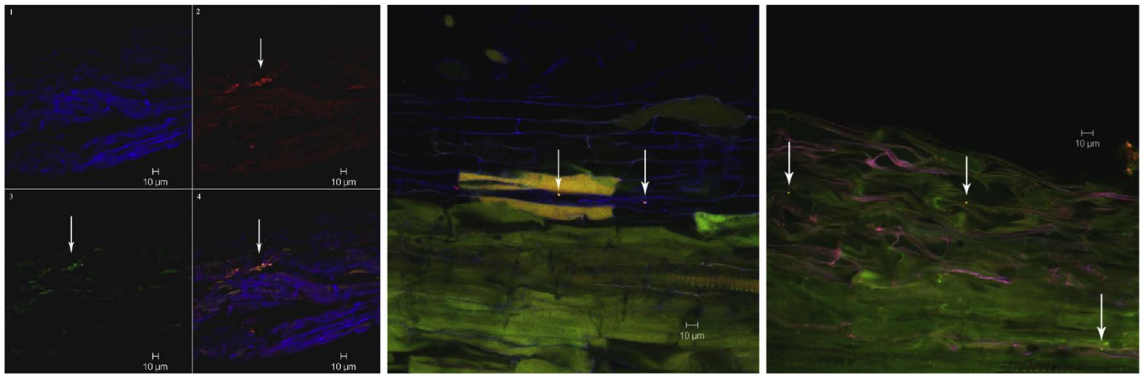
In the interior spaces of the lettuce root, the *Salmonella typhimurium* LT2 cells were seen as single or in the form of microcolonies. The bacterial cells were also located in intercellular spaces (niches) and they were found very close to vascular system of lettuce root (Micrograph 1). Also, it was detected that *Salmonella typhimurium* LT2 cells can reach very deep layers of interior at tomato and sweet corn roots. The bacterial cells were able to come close to root xylem (Micrograph 1). The *Salmonella typhimurium* LT2 cells could colonize root hairs and root surface in the very high degree (Fig. 1 and Micrograph 2).

The CLSM micrographs showed that *Salmonella typhimurium* S1 cells appeared in the form of microcolonies, aggregations in the intercellular niches at lettuce and parsley roots (Micrograph 3).

The *Salmonella typhimurium* ATCC 14028 cells formed aggregations on the sweet corn root hairs and appeared like biofilm. In the deeper layers of sweet corn root, bacterial cells were single and they were located in intracellular niches (Micrograph 4). It has been determined that bacterial cells formed microcolonies in the intercellular spaces that were located very close to xylem system of lettuce root. At the spinach root, *Salmonella* cells were predominantly attached to root hairs and they appeared in biofilm form (Micrograph 4). Regarding root-tuber plants, *Salmonella typhimurium* ATCC 14028 cells were dominantly located on the parsley root surface, but they were rarely found on root hairs. The bacterial cells also were found out in deep layers of carrot and celery root (intercellular niches) and they were located close to root xylem (Micrograph 5).

Table 1
The characteristics and specificity of oligonucleotide probes and the strength of the deionized formamide used in the hybridization buffer.

Bacteria	Probe	Specificity	Binding position		Sequence 50-30	% FA	Reference
			Target (rRNA)	*E. coli*			
S. typhimurium	Salm-63	*Salmonella sp. Plesiomonas shigelloides*	23 S	1742–1760	GCTGCCTCCCGTAGGAGT	35	W.Ludwig (not published)
All bacteria	EUB-338	*Bacteria*	16 S	338–355	GCTGCCTCCCGTAGGAGT	35	[1]



Micrograph 1. Colonization lettuce root (left), tomato root (middle), sweet corn root (right) by *S. typhimurium* LT2. The white arrows show bacterial microcolony (left picture) and single bacterial cells (middle and right picture) inside plant root. The bacterial cells are visible in yellow color.

3.3. Statistic test and decision about the null hypothesis

Based on experimental data, it was calculated the sum of ranks for each of the samples of surface colonization plant roots by *Salmonella* strains: $R_1 = 864$ (*Salmonella enterica* serotype *typhimurium* LT2); $R_2 = 924$ (*Salmonella enterica* serotype *typhimurium* S1); $R_3 = 1062$ (*Salmonella enterica* serotype *typhimurium* ATCC14028). The rejection region for this Chi-Square test was $R = \chi^2$: $\chi^2 > 5.991$ ($\alpha = 0.05$; df = 3–1 = 2). The result of Kruskal-Wallis Test was obtained according to formula:

$$H_s = 12 / N(N+1)(R_1{}^2/n_1 + R_2{}^2/n_2 + \cdots + R_k{}^2/n_k) - 3(N+1) = 12 / 75\ (75 + 1)\ (864^2/31 + 924^2/20 + 1062^2/24) - 3\ (75 + 1) = 11.501$$

It is observed that $\chi^2 = 11.501 > \chi^2_R = 5.991$, and it means that the null hypothesis is rejected. Also, the p-value is $p = .0032$, and since $p = .0032 < 0.05$, it could be concluded that the null hypothesis is rejected. There is statistical evidence about differences between investigated *Salmonella* strains in surface colonization plant roots at $\alpha = 0.05$ significance level.

The sum of ranks for samples of colonization interior spaces of plant roots by *Salmonella* strains are: $R_4 = 741$ (*Salmonella enterica* serotype *typhimurium* LT2); $R_5 = 859$ (*Salmonella enterica* serotype *typhimurium* S1); $R_6 = 956$ (*Salmonella enterica* serotype *typhimurium* ATCC14028). The rejection region for χ^2-Test was $R = \chi^2$: $\chi^2 > 5.991$ for $\alpha = 0.05$ and df = 3–1 = 2. The result of Kruskal-Wallis Test was:

$$H_i = 12 / N(N+1)(R_4{}^2/n_1 + R_5{}^2/n_2 + \cdots + R_k{}^2/n_k) - 3(N+1) = 12 / 71\ (71 + 1)\ (741^2/28 + 859^2/19 + 956^2/24) - 3\ (71 + 1) = 10.588$$

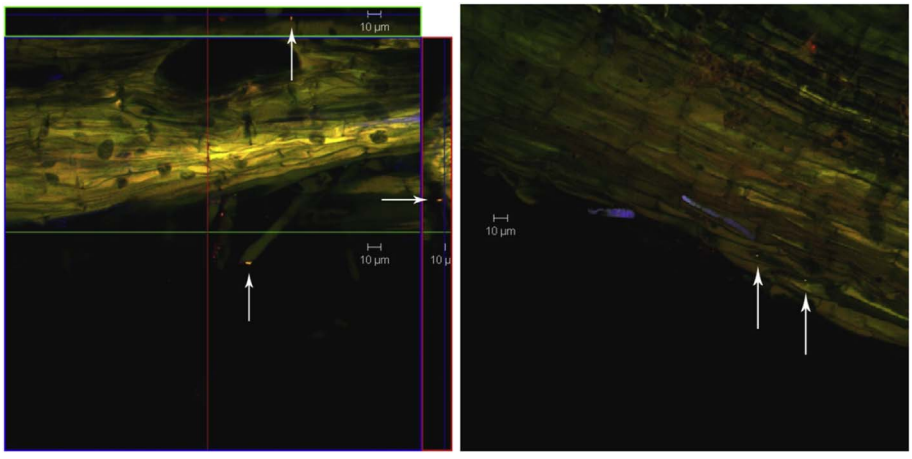
The Kruskal-Wallis Test showed that $\chi^2 = 10.588 > \chi^2_R = 5.991$, and the null hypothesis must be rejected. The p-value is $p = .005$, and since $p = .005 < 0.05$, it means that the null hypothesis is rejected. The statistical test showed significant differences between investigated *Salmonella* strains in interior colonization plant roots at $\alpha = 0.05$ significance level.

4. Discussion

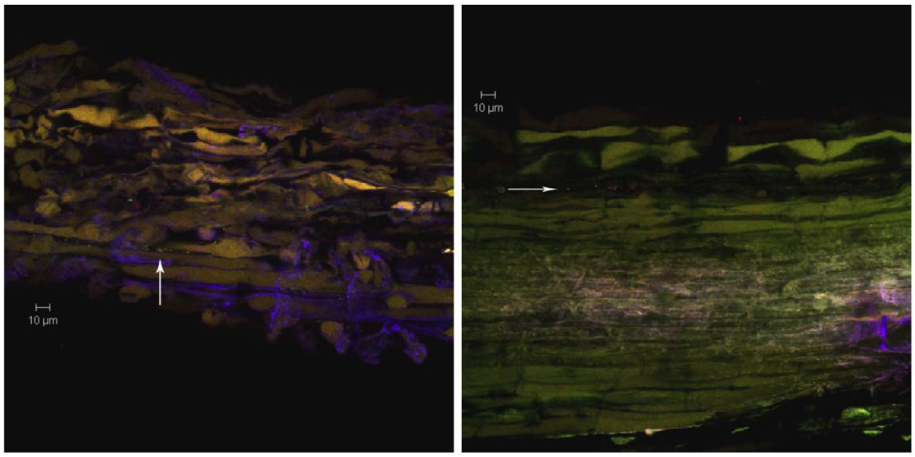
Our experiments showed that tested *Salmonella spp*. strains have different ability for plant colonization. Thus, ***Salmonella enterica* serotype *typhimurium* LT2** has the maximum capacity for surface and endophytic lettuce root colonization but the same strain has the weakest ability to colonize carrot root (Fig. 1). The obtained results showed that human pathogen, *Salmonela enterica* serovar *typhimurium* (*S. typhimurium*) could endophytic colonize different plant species very efficiently and also, these bacteria can actively enter into the root tissue and multiply in the intercellular niches of plant root.

There is evidence that *Salmonella* may exist epiphytically on the plants but it is also able to enter plant through different wounds, root, stomata, hydrathodes. The *Salmonella* could be found in fruits and seeds after contaminations plant flowers [33].). Also, trichomes, stomata, hydratodes allow presence of *Salmonella* into plant leaves [4,32,33].

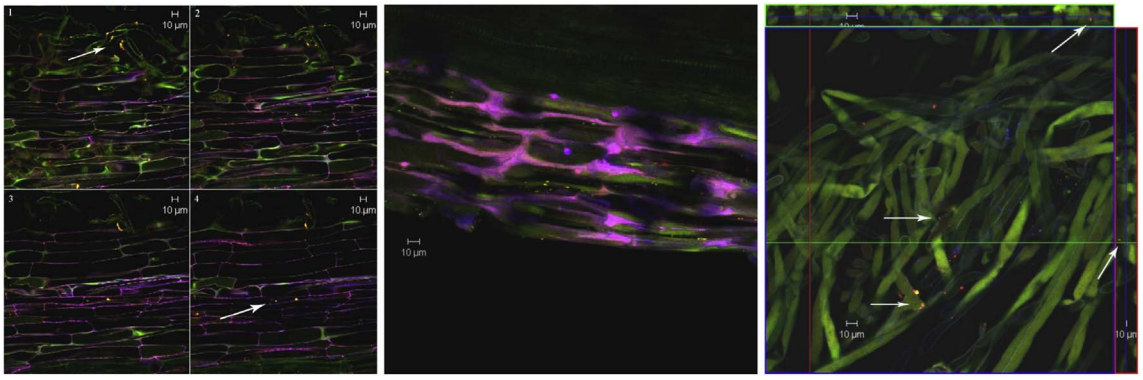
Schikora et al. (2008.) showed that *Salmonella typhimurium* reaches in interior of plant cells *Arabidopsis thaliana* and multiply in them. Although the infection of *Arabidopsis thaliana* activates plant immune response, which is similar as well as with other phitopathogen bacteria, it seems that *S. typhimurium* is able to overcome the host plant defense mechanisms and reproduces in plant. Our results are in accordance with literature and they unequivocally showed that *Salmonella typhimurium* could be typical plant endopathogen.



Micrograph 2. Colonization celery root (left) and carrot root (right) by *Salmonella typhimurium* LT2. The left picture is orthogonal view of celery root and white arrows show single bacterial cells which is attached to root hair. The attachment *Salmonella* cells to the surface of carrot root is visible on right picture. The *Salmonella* cells are visible in yellow color.



Micrograph 3. Colonization lettuce root (left) and parsley root by *S. typhimurium* S1. The white arrows show presence of *Salmonella* cells in intercellular spaces inside plant roots and bacteria cells are visible in yellow color.



Micrograph 4. Colonization sweet corn root (left), lettuce root (middle), spinach root (right) by *S. typhimurium* ATCC 14028. On the left picture, the *Salmonella* cells are clear visible as single inside sweet corn root and like microcolomies on the root hairs. On the middle picture, the bacterial cells are visible in deep layers of lettuce root very close to xylem. The right picture shows attachment *Salmonella* cells to spinach root hairs.

The native microbial population in the environment could have positive influence on ability of *Salmonella* to colonize plants. So, plant pathogens (viruses, fungi, and bacteria), wild and domestic animals could degrade plants and increase possibilities of colonization plants by *Salmonella* [27,40].).

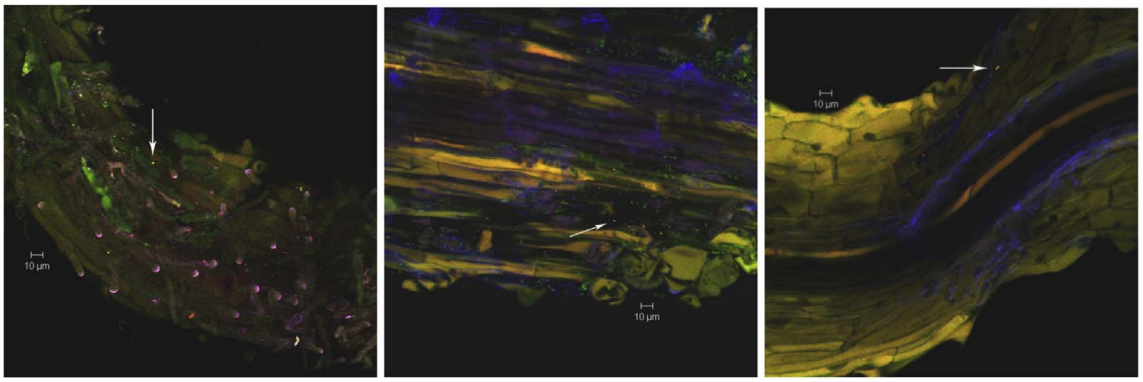
The *Salmonella* characteristics, like genetic features, presence of T3SS (type III secretion system), chemotaxis, motility, forming biofilm are very important in survival bacteria in the environment and colonization plants [58]. The experiment [58] showed that T3SS is essential for efficient plant colonization.

The plant root surface colonization by ***Salmonella enterica* serovar *typhimurium* S1** was the most prominent at tomato, lettuce and celery (more than 10^5 cells/mm^3 absolutely dry root) but this colonization was the smallest at spinach root. The root endophytic colonization by this strain was also the largest at tomato and celery and, it was detected more than 10^4 cells/mm^3 inside of spinach root (Fig. 2).

The results of other authors showed that *Salmonella* is able for colonization tomato plants through root system and leaves [32,36]. The entrance *Salmonella* cells through leaf surfaces (even they are modified with carborundum and surfactants) confirm that it is way for internal plant contamination [32].

In contrast, the experiment of [26] showed poorly colonization all tomato plant parts by *S. typhimurium* that suggest that contamination could be result of postharvest handling.

The literature data shows that *S. typhimurium* LT2 and DT104 strains are able to endophytic colonize seedlings and sprouts of barley which



Micrograph 5. Colonization parsley (left) carrot (middle), celery (right) by *S. typhimurium* ATCC 14028. The white arrows show surface attachment bacterial cells to parsley and celery root (left and right picture). On the middle picture, the *Salmonella* cells are visible deeply inside carrot root.

are grown in axenic model conditions. Using the FISH analysis, *Salomonella typhimurium* cells were detected inside plant tissue [46]. The investigations which were related to lettuce (*Lactuca sativa*) immune response to colonization by *Salmonella enterica* serovar Dublin showed that 43% surface sterilized and 93% none disinfected lettuce plants were colonized by this strain [43].

The ***Salmonella enterica serovar typhimurium ATCC14028*** has proven to be a very good in colonization root of investigated plant species. The highest number of bacterial cells was found out on the root surface at lettuce and sweet corn (10^6 cells/mm^3 absolutely dry root), but the large number of *Salmonella* cells was also detected at spinach, tomato and carrot root surface ($> 10^5$ cells/mm^3 absolutely dry root). The weakest root surface colonization was noticed at parsley and celery. It was detected $\approx 10^5$ *Salmonella* cells/mm^3 absolutely dry root, which colonized lettuce and sweet corn root endophyticaly. Also, inside the celery root, it was noted $\approx 10^4$ cells/mm^3 absolutely dry root (Fig. 3).

The Salmonella is able to form microcolonies near stomata and enter in mesophyll tissue of lettuce and arugula leaves. The lower degree of colonization was detected at red-lettuce and basil leaves but the marginal colonization was noticed at parsley and tomato leaves [29]. It is known that hydratodes and trichomes are very helpful in colonization leaves by *Salmonella* [4,32,33].

By studying colonization over time [43], found out that the highest number of bacteria was detected the twelfth day after beginning of inoculation. The same authors noticed the highest degree of bacterial colonization eighteenth day after inoculation at non disinfected plants.

Our research showed that *Salmonella typhimurium* was able to colonize all investigated plants (Figs. 1–3; Micrographs 1-5).

Also, our previous experiments with Gfp-transformed bacteria showed that if human pathogen bacteria get into the interior of plant leaf, they are able to multiply themselves and this is in accordance with literature [19,37]. Many studies showed that *Salmonella spp*. could be found in apoplast (intercellular spaces) and these bacteria are able to form biofilm in parsley root [47].

The human and animal infections by *S. typhimurium* depends on different factors and today it is known mechanism of penetration this bacteria in the epithelial cells of the mammalian host [24]. The viability of *Salmonella spp*. in the environment without host organism is not still completely known, but it is evident that these bacteria are very adaptable to low pH and high temperature [55]. According to [52]; *Salmonella spp*. is not able to survive in the soil more than 900 days after inoculation.

There is evidence that plants have response to colonization of human pathogen bacteria. It includes immune response, expression of pathogenesis related genes (PRG). The experiment with mutants in T3SS showed that wild-strain is able to exceed the plant immune response [58].

[64] showed that *Salmonella* attachment to plant surface is the most important step in colonization. There are some bacterial factors which are very helpful in this process such as lipopolysaccharides (LPS), fimbria and nonfimbrial adhesions, flagella.

Also, there is differences in attachment *Salmonella typhimurium* to plant leaves and it depends on plant growing stage, better attachment is at older than at youger leaves [45].

Some research shows that the contaminated soil, for growing crops which are eaten in raw, could be very serious cause of infections by pathogenic bacteria [10]. The *Salmonella spp*. is able to reach soil mainly through manure, waste water and other waste materials which have organic origin.

Our results show that plants, which were endophytic colonized by *Salmonela enterica* serovar *typhimurium*, could be good model for further research of colonization mechanisms and *Salmonella spp*. pathogenicity in plants.

Using Gfp-transformed bacteria in previous research as well as application FISH analysis, our research has proved surface and endophytic plant root colonization by *Salmonella spp*. The FISH method is used to detect nucleic acid sequences by means of a phylogenetic fluorescently labelled oligonucleotide probes that specifically hybridize to a complementary target sequence within the bacterial cell [9,22,50].

Some confocal micrographs (Micrograph 4) indicate the presence of bacterial cells in the interior of the plant root cells (cytoplasm) but the mechanism of penetration bacteria remains still unknown.

It is not much important surface and interior colonization leaves, but for consumers, it is important translocation bacteria through plant and reach the fruits. It was detected translocation bacteria in tomato plants [32]. These authors showed internalization bacteria in tomato fruits when the plant is systemically colonized.

It is known huge heterogeneity in colonization different plants by human pathogen. According to [30]; about 20% of plant population is colonization by bacteria, but this range could reach from 20% to 100% and it depends on plant species and bacterial strains.

[4] showed that when it is done inoculation one tomato cultivar with one *Salmonella* strain, the interaction between plant and bacteria could be different and it depends on plant species and bacterial strains.

Based on the experimental results [57], suggest that *Salmonella spp*. is able to inhabit the plant cells cytoplasm of *Arabidopsis thaliana* and using confocal microscopy, they noticed movement bacteria in the cytoplasm of plant cells. They found out *Salmonella* cells in the root hairs only 3 h after inoculation and 17 h after inoculation, the bacterial cells were noticed in rhizodermal plant cells. The caution in interpreting these results is necessary because it has still not clarified the exact mechanism of penetration human pathogen bacteria through the cell wall of plants.

5. Conclusion

All investigated *Salmonella* strains (*Salmonella typhimurium* LT2, *Salmonella typhimurium* ATCC14028, *Salmonella typhimurium* S1) showed ability in colonization vegetable plants. All strains were very good in surface and endophytic colonization plant roots. The *Salmonella* strains were frequently detected in intercellular spaces of roots all investigated plants. The strain *Salmonella typhimurium* ATCC 14028 was detected in the interior root cells at sweet corn but the mechanism of penetration bacterial cells through the plant cell wall is still unknown. The future research should be based on the mechanisms of plant response for bacterial colonization and mechanisms of bacterial entrance in plant cells.

Acknowledgment

This study was supported by the EU Commission (FP7-REGPOT-2012-2013-1 project AREA grant number 316004) and Serbian Ministry of Education, Science and Technological Development (project TR 31080).

I am very thankful to Prof. Anton Hartmann, Dr Michael Schmid, Angelo Weiβ and all people from Helmholtz Zentrum Munchen, Germany, AMP Research Unit Microbe-Plant Interactions who helped me to realize my research.

References

[1] R.I. Amann, L. Krumholz, D.A. Stahl, Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology, J. Bacteriol. 172 (1990) 762–770.

[2] R.I. Amann, B. Zarda, D.A. Stahl, K.H. Schleifer, Identification of individual prokaryotic cells by using enzymelabeled, rRNA-targeted oligonucleotide probes, Appl. Environ. Microbiol. 58 (1992) 3007–3011.

[3] D.J. Barak, L.C. Whitehead, A.O. Charkowski, Difference in attachment of Salmonella enterica serovars and escerichia coli O157:H7 to alfalfa sprouts, Appl. Environ. Microbiol. 69 (8) (2003) 4556–4560.

[4] J.D. Barak, L.C. Kramer, L.Y. Hao, Colonization of tomato plants by Salmonella enteric is cultivar dependent, and type 1 trichomes are pre-ferred colonization sites, Appl. Environ. Microbiol. 77 (2011) 498–504.

[5] B.C. Behravesh, R.K. Mody, J. Jungk, L. Gaul, J.T. Redd, S. Chen, et al., 2008 Outbreak of *Salmonella* Saintpaul infections associated with raw produce, N. Engl. J. Med. 364 (2011) 918–927.

[6] R. Betts, Microbial update: fruit and salad, Int. Food Hyg. 25 (2014) 9–12.

[8] L.R. Beuchat, J.H. Ryu, Produce handling and processing practices, Emerg. Infect. Dis. 3 (1997) 459–465.

[9] B. Blomme, A. Handler, Fluorescence in Situ Hybridization, (2009) Retrieved from Enviromental Microbiology http://filebox.vt.edu/users/chagedor/biol_4684/Methods/FISH.html.

[10] M.T. Brandl, Fitness of human enteric pathogens on plants and implications for food Safety, Annu. Rev. Phytopathol. 44 (2006) 367–392.

[11] M.T. Brandl, R.E. Mandrell, Fitness *of Salmonella enterica* serovar Thompson in the cilantro phyllosphere, Appl. Environ. Microbiol. 68 (2002) 3614–3621.

[12] B. Burun, E. Coban Poyrazoglu, Embryo culture in barley (*Hordeum vulgare* l.), Turk. J. Biol. 26 (2002) 175–180.

[14] CDC, Ongoing multistate outbreak of Escherichia coli serotype O157:H7 infections associated with consumption of fresh spinach – United States, September 2006, Morb Mortal Wkly Rep 55 (2006) 1045–1046.

[15] CDC, Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items, MMWR Morb Mortal Wkly Rep 57 (2008) 929–934.

[16] CDC, Multistate Outbreak of Human Salmonella I 4,[5],12:i:- Infections Linked to Alfalfa Sprouts (Final Update), (2011) URL http://www.cdc.gov/salmonella/i4512i-/index.html.

[17] A. Charkowski, O.J.D. Barak, C.Z. Sarreal, R.E. Mandrell, Differences in growth of *Salmonella enterica* and *Escherichia coli* Ol57:H7 on alfalfa sprouts, Appl. Environ. Microbiol. 68 (2002) 3114–3120.

[18] M.B. Cooley, W.G. Miller, R.E. Mandrell, Colonization of *Arabidopsis thaliana* with *Salmonella enterica* or enterohemorrhagic *Escherichia coli* Ol57, H7 and competition by Enterobacter asburiae. Appl. Environ. Microbiol. 69 (2003) 4915–4926.

[19] Y. Dong, A.L. Iniguez, B.M. Ahmer, E.W. Triplett, Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa and Medicago truncatula*, Appl. Environ. Microbiol. 69 (2003) 1783–1790.

[21] EFSA, Scientific opinion on the risk posed by pathogens in food of non-animal origin, ERA J. 11 (1) (2013) 3025.

[22] P. Eldor, Soil Microbiology, Ecology and Biochemistry, Academic Press is an imprint of Elsevier, Oxford, 2007.

[23] EU, Agricultural commodity markets—past developments: fruits and vegetables, (2007).

[24] P.I. Fields, R.V. Swanson, C.G. Haidaris, F. Heffron, Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent, Proc. Natl. Acad. Sci. U.S.A 83 (1986) 5189–5193, http://dx.doi.org/10.1073/pnas.83.14.5189.

[25] M. Gandhi, S. Golding, S. Yaron, K.R. Matthews, Use of green fluorescent protein expressing *Salmonella* Stanley to investigate survival, spatial location, and control on alfalfa sprouts, Food Prot. 64 (2001) 1891–1898.

[26] R. Garcia, J. Bælum, L. Fredslund, P. Santorum, C.S. Jacobsen, Influence of temperature and predation on survival of Salmonella enteric serovar Typhimurium and expression of invA in soil and manure-amended soil, Appl. Environ. Microbiol. 76 (2010) 5025–5031.

[27] C. Ge, C. Lee, E. Nangle, et al., Impact of phytopathogen infection and extreme weather stress on internalization of *Salmonella* Typhimurium in lettuce, Int J Food Microbiol 168–169 (2014) 24–31.

[28] I.A. Gillespie, Outbreak of Salmonella Newport infection associated with lettuce in the UK, Eurosurveillance Weekly 8, 2004. http://www.eurosurveillance.org/ew/2004/041007.asp, .

[29] D. Golberg, Y. Kroupitski, E. Belausov, et al., *Salmonella Typhimurium* internalization is variable in leafy vegetables and fresh herbs, Int J Food Microbiol 145 (2011) 250–257.

[30] E. Gorbatsevich, S. Sela Saldinger, R. Pinto, et al., Root internalization, transport and inplanta survival of *Salmonella enterica* serovar Newport in sweet basil, Environ Microbiol Rep 5 (2013) 151–159.

[31] S.K. Greene, E.R. Daly, E.A. Talbot, L.J. Demma, S. Holzbauer, N.J. Patel, et al., Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005, Epidemiol Infect 136 (2008) 157–165.

[32] G. Gu, J. Hu, J.M. Cevallos-Cevallos, S.M. Richardson, J.A. Bartz, A.H. van Bruggen, Internal colonization of Salmonella enteric serovar Typhimurium in tomato plants, PLoS One 6 (2011) e27340, , http://dx.doi.org/10.1371/journal.pone.0027340.

[33] G. Gu, J.M. Cevallos-Cevallos, A.H. van Bruggen, Ingress of *Salmonella enteric* Typhimurium into tomato leaves through hydathodes, PLos One 8 (2013) e53470.

[34] X.J. Guo, R.E. Chen, Brackett, L.R. Beuchat, Survival of salmonellae on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening, Appl. Environ. Microbiol. 67 (2001) 4760–4764.

[35] X. Guo, M.W. van Iersel, J. Chen, R.E. Brackett, L.R. Beuchat, Evidence of association of salmonellae with tomato plants grown hydroponically in inoculated nutrient solution, Appl. Environ. Microbiol. 68 (7) (2002) 3639–3643.

[36] L.D. Hintz, R.R. Boyer, M.A. Ponder, R.C. Williams, S.L. Rideout, Recovery of Salmonella enteric Newport introduced through irrigation water from tomato (Lycopersicum esculentum) fruit, roots, stems, and leaves, Hortscience 45 (2010) 675–678.

[37] L. Iniguez, Y. Dong, H. Carter, B. Ahmer, J. Stone, E. Triplett, Regulation of enteric endophytic bacterial colonization by plant defenses, Molecular Plant-Microbe Interactions 18 (2) (2005) 169–178 Pages.

[38] M. Islam, J. Morgan, M.P. Doyle, S.C. Phatak, P. Millner, X. Jiang, Fate of *Salmonella enterica* serovars Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water, Appl. Environ. Microbiol. 70 (2004) 2497–2502.

[39] J. Jablasone, K. Warriner, M. Griffiths, Interactions of Escherichia coli O157:H7, Salmonella Typhimurium and Listeria monocytogenes in plants cultivated in a gnotobiotic system, Int. J. Food Microbiol. 99 (2005) 7–18.

[40] C.S. Jacobsen, T.B. Bech, Soil survival of *Salmonella* and transfer to freshwater and fresh produce, Food Res. Int. 45 (2012) 557–566.

[41] M. Jay, M.J. Loessner, D.A. Golden, Foodborne Gastroenteritidis Caused by Salmonella and Shigella. Modern Food Microbiology, Springer Science, New York, 2005.

[43] M.M. Klerks, M. van Gent-Pelzer, E. Franz, C. Zijlstra, A.H.C. van Bruggen, Physiological and Molecular Response of Lactuca sativa to Colonization by Salmonella enterica Serovar Dublin, (2007) PhD.

[44] K.C. Klontz, J.C. Klontz, R.K. Mody, R.M. Hoekstra, Analysis of tomato and jalapeño and Serrano pepper imports into the United States from Mexico before and during a National Outbreak of *Salmonella* serotype Saintpaul infections in 2008, J. Food Protect. 73 (2010) 1967–1974.

[45] Y. Kroupitski, R. Pinto, E. Belausov, et al., Distribution of *Salmonella* Typhimurium in romaine lettuce leaves, Food Microbiol. 28 (2011) 990–997.

[46] S. Kutter, A. Hartmann, M. Schmid, Colonization of barley (*Hordeum vulgare*) with *Salmonella enterica* and *Listeria spp*, FEMS Microbiol. Ecol. 56 (2006) 262–271.

[47] A. Lapidot, U. Romling, S. Yaron, Biofilm formation and the survival of *Salmonella typhimurium* on parsley, Int. J. Food Microbiol. 109 (2006) 229–233.

[48] S.E. Majowicz, J. Musto, E. Scallan, F.J. Angulo, M. Kirk, S.J. O'Brien, T.F. Jones, A. Fazil, R.M. Hoekstra, The global burden of nontyphoidal Salmonella gastroenteritis, Studies international collaboration on enteric disease 'burden of illness', Clin. Infect. Dis. 50 (6) (2010) 882–889.

[49] W. Manz, R. Amann, W. Ludwig, M. Wagner, K.-H. Schleifer, Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions, Syst. Appl. Microbiol 15 (1992) 593–600.

[50] A. Moter, U. Gobel, Fluorescence in situ hibridization (FISH) for direct visualisation of microorganisms, J. Microbiol. Meth. (2000) 85–112.

[51] E.E. Natvig, S.C. Ingham, B.H. Ingham, L.R. Cooperband, T.R. Roper, *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure, Appl. Environ. Microbiol. 68 (2002) 2737–2744.

[52] F.A. Nicholson, S.J. Groves, B.J. Chambers, Pathogen survival during livestock manure storage and following land application, Bioresour. Technol. 96 (2005) 135–143.

[53] A.N. Olaimat, R.A. Holley, Factors influencing the microbial safety of fresh produce: a review, Food Microbiol. 32 (2012) 1–19.

[54] L. Pezzoli, R. Elson, C. Little, I. Fisher, H. Yip, T. Peters, M. Hampton, E. De Pinna, et al., International Outbreak of Salmonella Senftenberg in 2007. Eurosurveillance Weekly 12, (2007) (accessed on 15/June/07), http://www.eurosurveillance.org/ew/2007/070614.asp.

[55] J. Samelis, J.S. Ikeda, J.N. Sofos, Evaluation of the pH-dependent, stationary phase acid tolerance in *Listeria monocytogenes* and *Salmonella typhimurium* DT104 induced by culturing in media with 1% glucose: a comparative study with *Escherichia coli* O157: H7, J. Appl. Microbiol. 95 (2003) 563–575.

[56] E. Scallan, R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones, P.M. Griffin, Foodborne illness acquired in the United States—major pathogens, Emerg. Infect. Dis. 17 (1) (2011) 7–15.

[57] A. Schikora, A. Carreri, E. Charpentier, H. Hirt, The dark side of the salad: *Salmonella Typhimurium* overcomes the innate immune response of *Arabidopsis thaliana* and shows an endopathogenic lifestyle, PLos One 3 (2008) e2279.

[58] M. Schikora, B. Neupane, S. Madhogaria, et al., An image classification approach to analyze the suppression of plant immunity by the human pathogen Salmonella Typhimurium, BMC Bioinf. (2012) 13.

[59] G. Smit, J.W. Kijne, B.J. Lugtenberg, Involvement of both cellulose fibrils and a Ca2+-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips, J. Bacteriol. 169 (1987) 4294–4301.

[60] A. Soderstrom, A. Lindberg, Y. Andersson, EHEC O157 Outbreak in Sweden from Locally Produced Lettuce, August–september 2005. Eurosurveillance Weekly 10, E050922.1, (2005) http://www.eurosurveillance.org/ew/2005/050922.asp#1.

[61] United States Department of Agriculture, USDA and HHS Announce New Dietary Guidelines to Help Americans Make Healthier Food Choices and Confront Obesity Epidemic, USDA Office of Communications and HHS Press Office, Washington, DC, 2011.

[63] K. Warriner, S. Spahiolas, M. Dickinson, C. Wright, W.M. Waites, Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts, J. Appl. Microbiol. 95 (2003) 719–727.

[64] A. Wiedemann, I. Virlogeux-Payant, A.M. Chausse, et al., Interactions of *Salmonella* with animals and plants, Front. Microbiol. 5 (2014) 791.

[65] World Health Organization, Fruit and Vegetable Promotion, World Health Organization, Geneva, 2003.

[66] X. Zogaj, M. Nimtz, M. Rohde, W. Bokranz, U. Romling, The multicellular morphotypes of *Salmonella Typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix, Mol. Microbiol. 39 (2001) 1452–1463.