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Internalization of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in biologically damaged fresh produce

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Abstract

Consumption of fresh fruits and vegetables has steadily increased since they are considered as an important part of healthy diet. However, the consumption of fresh produces posses' greater risk of pathogenic infections because most fruits and vegetables host endophytic human pathogens and eaten raw. Most of the fresh outbreaks were related to *Escherichia coli* O157:H7 and *Salmonella typhimurium.* In present study, the effect of phytopathogen infection on internalization of enteric human pathogen was also evaluated. For this study, all the fresh produces were artificially challenged with predominantly existing plant pathogen *Xanthomonas campestris.* After disease induction the infected plants were surface inoculated and then the internalization capacity were monitored. The results indicated that *Xanthomonas campestris* infected plants bear significantly higher endophytic pathogenic population as compared to healthy plant (control). These results revealed that, phytopathogen promotes the internalization of bacterial human pathogens by providing favorable conditions with respect to nutrition, water availability and through wound produced by pathogens; human pathogen can get easily infiltered.

Key words : Fresh produce, Internalization, *Escherichia coli* O157:H7, *Salmonella typhimurium.*

INTRODUCTION

A continuous rise in number of outbreaks of food borne illnesses linked to fresh fruits and vegetables challenges the notion that enteric pathogens defined mostly by their ability to colonize the intestinal habitat. For number of these produce-related outbreaks, the origin of contamination has been traced back to the farms. The fresh produce can become contaminated with bacteria pathogens in the field through soil, feces or water used for irrigation, through application of manure, biosolids, pesticides and fertilizers and through insects and animals or during post harvest practices and by food handlers in food services establishments. Regardless the source of contamination in field, pathogen finds a way to survive and reproduce on the surface of salad ingredients and even worse, inside the plant tissue and become endophyte.

Environmental changes have facilitated microbial evolution, including that in human pathogens, to enable them adapt to the environmental stress and survive longer in phyllosphere, which will eventually increase the possibility of crop contamination and risk of food outbreaks. Phytopathogen infection, which occurs frequently in field cultivation, can affect the internalization of human pathogen in plants [1]. Thus, it was worthwhile to investigate the effect of phytopathogenically infected plant on internalization of human pathogens.

It has been frequently reported that, Infection with *Xanthomonas campestris* causes the most common disease in all tested salad ingredients all around the world. Hence, in present investigation *Xanthomonas campestris* was selected and used to induce plant infection in order to mimic the plant stress caused by phytopathogen infection during field growth and to explore whether it can affect *Escherichia coli* O157:H7 and *Salmonella typhimurium* internalization in different salad ingredients.

MATERIALS AND METHODS

Isolation and identification of bacterial plant pathogen:

The frequently encountered plant diseases in vegetables viz, spinach, tomato, cucumber, cabbage, radish, carrot, cauliflower and green onion were screened during cultivation period. The screened diseased plants were uprooted and the infected parts were separated and sealed into plastic bags and transported to Microbiology research laboratory, Washim. The diseased plant parts especially from the foliar region were cut with the help of sterile razor. The infected plant parts were surface sterilized by sequential immersion in 80% ethanol for 5 sec. and in 0.1% (w/v) mercuric chloride for 10 min [2,3]. The surface sterilized tissues were further peeled off and minced in sterile distilled water. A loopful of this suspension was then inoculated into nutrient broth and incubated at $37^0 \pm 5^0$C overnight for enrichment. The enriched cultures were then serially diluted and used for the isolation of pathogens on solid media. The serial dilutions were inoculated on *Xanthomonas* isolation media and plates were maintained at $37^0 \pm 5^0$C for 24 h. Further identification of the pathogens was done on the basis of colony, morphological and biochemical characteristics followed by comparing with available standard literature[4] adopting the method described previously. The isolated pure culture was maintained at 4^0C with periodic sub-culturing on specific media.

Collection of test pathogens:

The test pathogens viz., *Salmonella typhimurium* and *Escherichia coli* O157:H7 were collected from MTCC Chandigarh and Promochem, Banglore, and further used for present investigation.

Induction of plant infection and internalization studies in phytopathogen damaged fresh produce:

The pure culture of *Xanthomonas campestris* isolated from respective plants and having density was used for the induction of

plant infection adopting procedure suggested by [5]. The test plants viz., spinach, tomato, cucumber, cabbage, radish, carrot, cauliflower and green onion were spray inoculated with suspension of *Xanthomonas campestris* (10^9 CFU/mL) until runoff. The treatment was applied to 6-7 week (~45 days) old plants. At the same time, plants without *Xanthomonas campestris inoculation were maintained as control.* The misting was carried out with deionized water and it was discontinued once necrotic lesions were observed (~8 days after inoculation). After lesion formation, test and control vegetables were evenly swabbed using cotton applicator that was dipped in overnight *Escherichia coli* O157:H7 culture (10^9 CFU/mL). A new applicator was used for each plant and each treatment. Similarly *Salmonella typhimurium* was also surface inoculated on test and control plants. The treated plants were further sampled after 3, 4, 5 and 6 days of inoculation i.e. on 57, 58, 59 and 60 days after sowing. All the samples were collected aseptically in the sterile plastic bags and transported to microbiology Research Laboratory, Washim. Tissue homogenate of the surface sterilized samples were inoculated in sterile peptone broth and enriched overnight at $37^0 \pm 5^0$C.The enriched samples were further serially diluted and evaluated for presence of both test pathogens. The enumeration of test pathogens was carried using selective media, Sorbitol MacConkey agar and Bismuth Sulphite agar for *Escherichia coli* O157:H7 and *Salmonella typhimurium* respectively. The results of standard plate count were further transformed to $\log_{10}$ values. Whole experiment was performed in triplicate.

Statistical analysis: The observations were recorded in the form of $\log_{10}$ CFU/g or mL and further results were expressed as the mean ± SD. The data was further analyzed for the statistical significance using paired t test. The p-value <0.05 was considered to be statistically significant. Statistical analysis was carried out using SPSS software (version19.0).

RESULTS

In order to explore the effect phytopathogen on internalization *Escherichia coli*O157:H7 and *Salmonella typhimurium* in different salad ingredients under study, the screened diseased plants were collected from local farm and transported to laboratory. The isolation and identification of bacterial phytopathogen was carried out by adopting standard conventional methods. Based on the characteristics and available literature, the commonly encountered isolate was tentatively identified as *Xanthomonas campestris*.

The pure culture of *Xanthomonas campestris* isolated from different plants under study and having standardized inoculation density (10^9CFU/mL) was used for induction of plant infection in respective plants. Thereafter, the infected plants (lesion formation) were inoculated separately with test pathogens viz,. *Escherichia coli*O157:H7 and *Salmonella typhimurium*. All the plants were further analyzed for internalization of test pathogens. The results obtained for the internalization of *Escherichia coli*O157:H7 and *Salmonella typhimurium* are presented in figure (1,2)

From the results it was observed that, *Escherichia coli* O157:H7 population in *Xanthomonas campestris* infected spinach was log $_{10}$ 5.79, 4.67, 4.61 and 4.37 CFU/g on 57th, 58th, 59th and 60th days after sowing (DAS) respectively. Whereas, in control, the recorded population of *Escherichia coli* O157:H7 was 5.76, 4.32, 4.00 and 2.27 log $_{10}$ CFU/g respectively for the same sampling time. The *Salmonella typhimurium* recovered in test plant have population density 5.48, 4.96, 4.75 and 4.19 log $_{10}$ CFU/g while in T7 it was found to be 4.11, 4.00, 3.27 and 1.66 log $_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively. The results provide strong evidence that, *Xanthomonas campestris* infection promote internalization of both *Escherichia coli* O157:H7 and *Salmonella typhimurium* in spinach.

In case of tomato, *Escherichia coli* O157:H7 population recorded in phytopathogen damaged plant was 4.97, 3.94, 3.84 and 2.77 where as in control plants, the population was 3.27, 2.41, 2.20 and 1.95 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively. *Salmonella typhimurium* count recovered from *Xanthomonas campestris* infected plant on 57th, 58th , 59th and 60th DAS have population density, 5.53, 5.47, 4.90 and 4.81 $\log_{10}$ CFU/g respectively. At the same time, in control, it was 4.41 3.85,

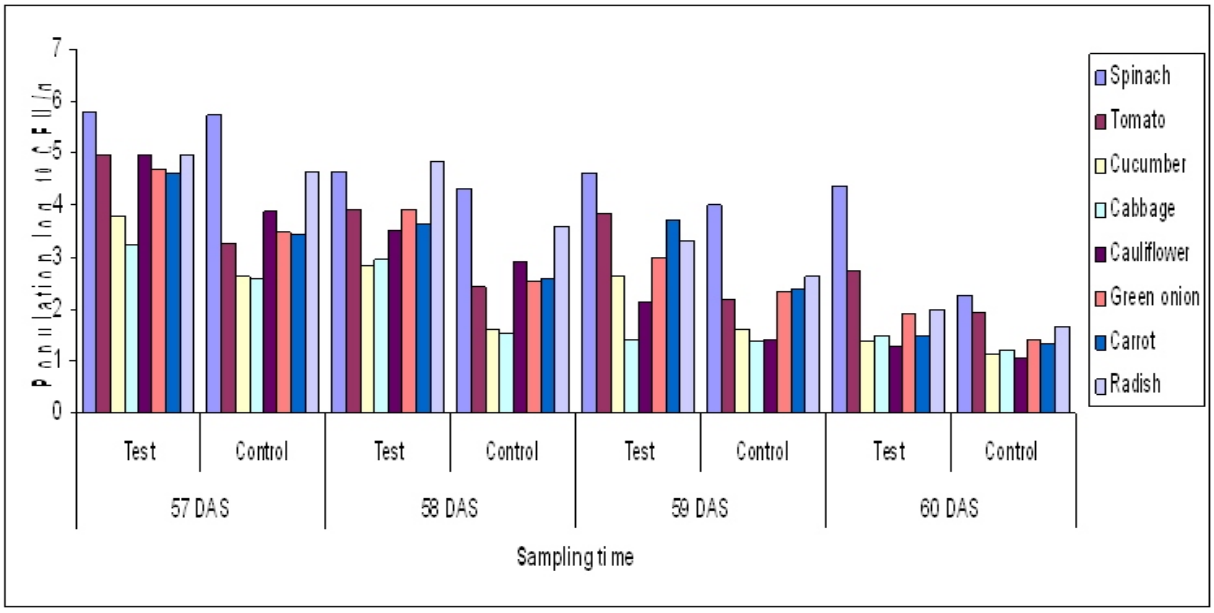

Figure 1: Studies on internalization of *Escherichia coli*O157:H7 population (Mean log 10 CFU/g) in *Xanthomonas campestris* infected fresh produce.

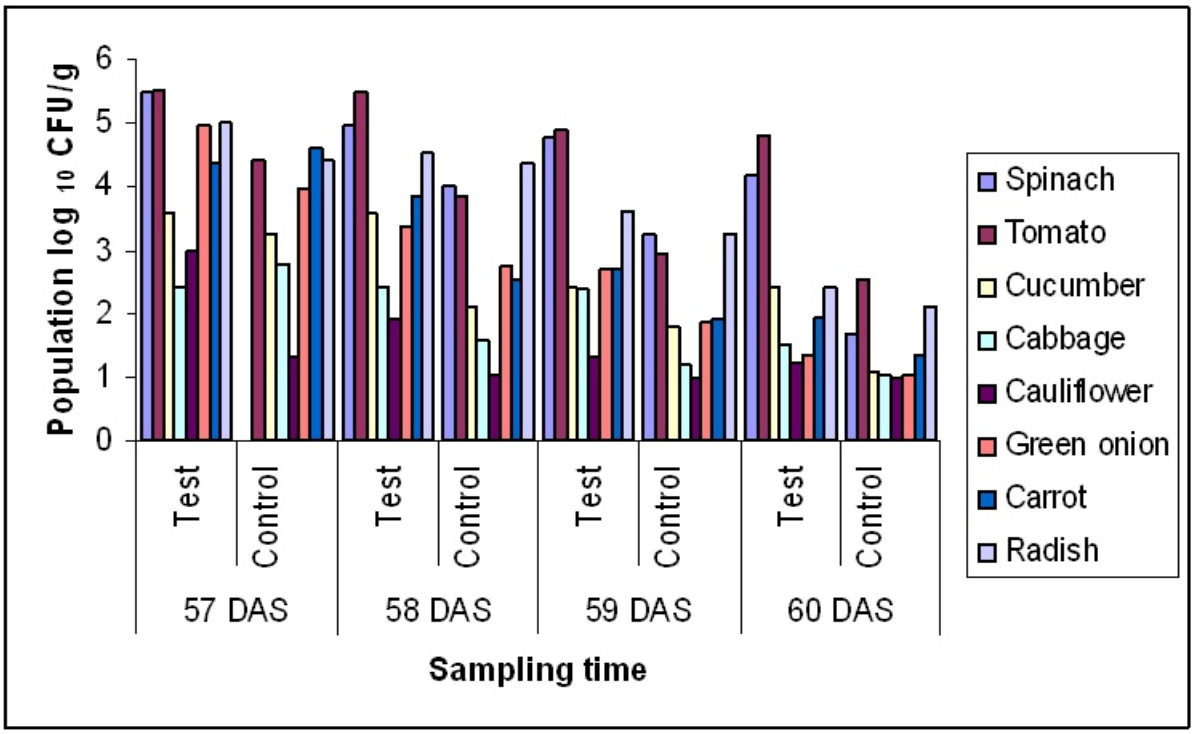

Figure 2: Studies on internalization of *Salmonella typhimurium* population (Mean log 10 CFU/g) in *Xanthomonas campestris* infected fresh produce.

Table 1: Paired t test for comparing the effect of phytopathogen *Xanthomonas campestris* infection on internalization of *Escherichia coli*O157:H7 in different fresh produce.

Treatment	Paired Differences					t	df	Sig. (1-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair 1 Test57 - Control57	.9296000	.5329955	.1884424	.4840046	1.3751954	4.933	7	0.000
Pair 2 Test58 - Control58	1.0977875	.4195948	.1483492	.7469975	1.4485775	7.400	7	0.000
Pair 3 Test59 - Control59	.8272250	.4908872	.1735548	.4168330	1.2376170	4.766	7	0.001
Pair 4 Test60 - Control60	.5932750	.6391173	.2259621	.0589596	1.1275904	2.626	7	0.017
Remark	Control <Test							

2.93, and 2.53 $\log_{10}$ CFU/g respectively.

In cucumber, *Escherichia coli* O157:H7 population recorded in test plant was 3.81, 2.85, 2.61 and 1.39 $\log_{10}$ CFU/g while in control it was 2.61, 1.60, 1.60 and 1.12 $\log_{10}$ CFU/g on 57th, 58th, 59th and 60th DAS respectively. In case of *Salmonella typhimurium* in test plant population density recorded were 3.57, 3.56, 2.42 and 2.41 $\log_{10}$ CFU/g and in control it was 3.27, 2.11, 1.77 and 1.07 on 57th, 58th, 59th and 60th DAS respectively. The results revealed that, in cucumber after *Xanthomonas campestris* infection *Escherichia coli*O157:H7 and *Salmonella typhimurium* population was more than the respective control.

In present study, the effect of *Xanthomonas campestris* infection on internalization of human pathogen in cabbage was also investigated. From the results it was observed that, *Escherichia coli*O157:H7 population detected in test plant was 3.23, 2.96, 1.43 and 1.50 $\log_{10}$ CFU/g while in control it was 2.60, 1.54, 1.39 and 1.20 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively. Similarly , the *Salmonella typhimurium* population density in test plant was recorded as 2.44, 2.43 2.40 and 1.50 $\log_{10}$ CFU/g whereas in control the population was recorded as 2.77, 1.60, 1.21 and 1.04 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively. The results revealed that high degree of internalization of *Escherichia coli*O157:H7 and *Salmonella typhimurium* in *Xanthomonas campestris* infected cabbage than the respective control in all the samples analyzed.

The internalization studies in cauliflower demonstrated that, the *Escherichia coli*O157:H7 population density encountered in teat crop was 4.99, 3.51, 2.14 and 1.29 $\log_{10}$ CFU/g while in T7 it

Table 2: Paired t test for comparing the effect of phytopathogen *Xanthomonas campestris* infection on internalization of *Salmonella typhimurium* in different fresh produce

Treatment		Paired Differences					t	df	Sig. (1-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Test57 - Control57	.6856250	.7362734	.2603120	.0700850	1.3011650	2.634	7	0.016
Pair 2	Test58 - Control58	1.0453750	.4515311	.1596404	.6678855	1.4228645	6.548	7	0.000
Pair 3	Test59 - Control59	.9494000	.5671665	.2005236	.4752370	1.4235630	4.735	7	0.001
Pair 4	Test60 - Control60	.9317375	.8290180	.2931021	.2386611	1.6248139	3.179	7	0.007
Remark		Control<Test							

was found to be 3.57, 2.93, 1.40 and 1.05 $\log_{10}$ CFU/g on 57th, 58th, 59th and 60th DAS respectively. Similar trend of internalization was also observed in case of *Salmonella typhimurium* internalization in cauliflower. *Salmonella typhimurium* population was recorded as 2.99, 1.89, 1.30 and 1.23 $\log_{10}$ CFU/g following test plant whereas in control it was 1.30, 1.04, 1.00 and 1.00 $\log_{10}$ CFU/g on 57th, 58th, 59th and 60th DAS respectively. The results showed that, more population density of both the test pathogens in *Xanthomonas campestris* infected plants as compared to respective control.

In case of Green onion, at 57th, 58th, 59th and 60th DAS the *Escherichia coli*O157:H7 population was found to be 4.70, 3.93, 2.98 and 1.89 $\log_{10}$ CFU/g respectively in test plant, whereas for the same sampling time; in control the population of *Escherichia coli*O157:H7 was found to be 3.50, 2.56, 2.36 and 1.41 $\log_{10}$ CFU/g. In green onion, in test plant the *Salmonella typhimurium* population was observed as 4.95, 3.39, 2.70 and 1.57 $\log_{10}$ CFU/g while in Control the count recorded was 3.98, 2.75, 1.85 and 1.03 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively. The results enlights the high risk of *Escherichia coli*O157:H7 and *Salmonella typhimurium* internalization in phytopathogenically damaged plant.

In carrot at 57th, 58th, 59th and 60th DAS after test plant the population of *Escherichia coli*O157:H7 was recorded as 4.60, 3.64, 3.71 and 1.51 $\log_{10}$ CFU/g and in control it was found to be 3.43, 2.60, 2.39 and 1.32 $\log_{10}$ CFU/g respectively. The *Salmonella typhimurium* internalization in carrot was also investigated. The results showed that in test plant the population was 4.36, 3.85, 2.71 and 1.93 $\log_{10}$ CFU/g it was recorded as 4.60, 2.54, 1.91 and 1.34 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively in control. Results showed that in the root vegetables like carrot phytopathogen damage enhances the possibility of internalization of human pathogens.

In case of radish, the *Escherichia coli* O157:H7 population following test plant was found to be 4.96, 4.85, 3.31 and 2.00 $\log_{10}$ CFU/ g on 57th, 58th , 59th and 60th DAS respectively. For the same sampling time, *Escherichia coli*O157:H7 population was recorded as 4.66, 3.61, 2.64 and 1.66 $\log_{10}$ CFU/ g respectively in control. Similarly, *Salmonella typhimurium* population in radish in test plant was recorded as 5.00, 4.54, 3.60 and 2.42 $\log_{10}$ CFU/ g while in control, it was 4.40, 4.38, 3.24 and 2.12 $\log_{10}$ CFU/g on 57th, 58th , 59th and 60th DAS respectively.

The data recorded during the internalization of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in test and control salad vegetables under study was processed for the statistical analysis and expressed as average mean ± SD. In *Xanthomonas campestris* infected fresh produce the average maximum (4.63±0.78) population of *Escherichia coli* O157:H7 was recorded at 57th DAS followed by (3.79±0.71) and (3.08±1.01) on 58th and 59th DAS respectively. The average minimum (2.09±1.03) *Escherichia coli* O157:H7 population was recorded at 60th DAS. In case of control, maximum population (3.70±1.03) was found at 57th DAS followed by (2.70±0.93) on 58th DAS and (2.25±0.85) on59th DAS. On 60th DAS, minimum population of *Escherichia coli*O157:H7 was recorded having population density (1.05 ± 0.43).

On the other hand, in *Xanthomonas campestris* infected fresh produce the average maximum (4.29±1.16) population of *Salmonella typhimurium* was recorded at 57th DAS followed by (3.83±1.21) and (3.10±1.23) on 58th and 59th DAS respectively. The average minimum (2.40±1.18) *Salmonella typhimurium* population was recorded at 60th DAS. In case of control, maximum population (3.60±1.12) was fond at 57th DAS followed by (2.78±1.20) on 58 th DAS and (2.15±0.89) on59th DAS. On 60th DAS, minimum population of *Salmonella typhimurium* was recorded having population density (1.47±0.58).

The data recorded during present investigation was further subjected to paired t test to compare the population status the endophytic test pathogen in *Xanthomonas campestris* infected plants with that of control. The results obtained for *Escherichia coli*O157:H7 are presented in Table (1). The results revealed that, population density of *Escherichia coli*O157:H7 in test plants was statistically superior over control for all sampling time viz., 57 58 59 and 60 DAS. Paired t test was also computed for *Salmonella typhimurium* and results obtained are presented in Table (2). The

results showed that, there was significant difference between *Salmonella typhimurium* population in test and control plants on 57, 58, 59 and 60 DAS. Test plants have superior *Salmonella typhimurium* population density over control.

DISCUSSION

The improved status of internalization of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in diseased spinach plant may be due to the internal tissue disintegration. The results are in accordance with [5], who demonstrated the proliferation and dissemination of *Escherichia coli* O157:H7 on biologically damaged lettuce plants. *Salmonella typhimurium* can also be internalized in the lettuce via leaf surface contamination. The present results revealed that, in test tomato plants the population of both the test pathogens was higher as compared to both the respective control among all the samples analyzed. Similar observations were also recorded previously [6].Moreover, authors also projected that, *Escherichia coli*O157:H7 count was greater in *Xanthomonas* infected plants than on *Pseudomonas* infected plant. This indicates that as compared to *Pseudomonas* infection, *Xanthomonas campestris* infected plants are more prone to human pathogen infection.

The results revealed that, in cucumber, cabbage, cauliflower and green onion after *Xanthomonas campestris* infection *Escherichia coli*O157:H7 and *Salmonella typhimurium* population was more than the respective control. Similar to the present investigation there are certain evidences that suggest damaged tissues are more likely to be affect by proliferation and internalization of *Escherichia coli*O157:H7 [7-9].

The data recorded revealed that, more internalization of human pathogens in carrot and radish occur in phytopathogenically damaged plants as compared to control.The results of the present investigation are in agreement with [10], who demonstrated the prevalence of *Salmonella* was significantly high in fresh fruits and vegetables. Similarly, higher incidences of *Salmonella poona* internalization was observed in cantaloupe when the fruits were co-inoculated with *Erwinia trachephila*, a common cucurbit wilt bacteria. [11]

The possible reason of increased internalization may be the predominant sugars leached from the surface of plants. These are normally glucose, sucrose, and fructose[12]. This might provide nourishment to the transient microorganisms. [13] found rhizosphere microorganisms are in the largest number near the root tips where the highest concentration of sucrose diffuses into soil. Plant also leach minerals, amino acids, sugar alcohols, pectic substances, proliferation hormones and vitamins [14].

Secondly, Plant pathogen caused decay to leaves, **which further contributed to** leaching and other micro environmental changes on plant surface. Injury to the leaves might increase the amount of leaching on leaf surfaces. These leachates can supply the carbon and nitrogen sources for microbial flora present on/in plant tissue. That would favor the survival and proliferation of human pathogens and this might promote the internalization of human pathogens in plant tissues.

Besides this, most of plant pathogen use enzymes to degrade cell wall components. The byproducts of catabolic reactions can create new sources of carbon for human pathogens or co-habitants. Cellulases include a broad range of enzymes that degrade cellulose completely to glucose. Pectolytic enzymes degrade pectic substances in the cell wall. Pectolytic activity is common in many plant pathogens. There are different pectic enzymes that act on the pectin molecules. Pectin is composed mostly of pollygalacturonic acids but also contains rhamnose, arabinose and galactose. There are also small amounts of other carbon-based molecules. Many of these compounds can be used by enteric bacteria as carbon and energy sources and makes the environment favorable and thus subsequent internalization might be possible.

The population size and distribution of phyllosphere microorganisms might be altered by amount of usable water present on plant surface [15]. Spatial experiments showed the dominance of epiphytic bacteria in the areas with wettability (or with more water activity). There is also evidence that high relative humidity increases bacterial communities on plant surfaces [16], so besides nutrients, water is also an important factor in bacterial proliferation.

It has been reported that some plant pathogens can release biosurfactants that changes leaf wettability in a way that makes it easier for epiphytes to use water. It has also been demonstrated that even after biosurfactants- producing microorganisms have become less dense on the leaf surface, the biosurfactants activity continues, thus water availability is still increased for remaining bacteria [15]. This is an important food safety issue because plants that have been previously diseased by pathogens capable of producing biosurfactants have increased leaf wettability. Thus this increased wettability will favors proliferation and internalization of enteric human pathogens. This might also be the reason for high level of internalization of *Escherichia coli*O157:H7 and *Salmonella typhimurium* were observed within the salad ingredients tested in present study.

Bacterial attachment to the plant surface was considered to be the preliminary step for bacterial internalization. Plant cuticle contains waxes that make it difficult for microorganisms to attach to plant tissue. For the bacterial attachment to the plant followed by internalization, the cuticle must be penetrated [17]. The plant pathogens might help for the destruction of this cuticle which may support to the bacterial internalization. This could be another reason for higher internalization in phytopathogen damaged plants.

Wounds produced by phytopathogen can also act as sites of co-infection with other microorganism that can alter the microenvironment. It is possible that *Escherichia coli* O157:H7 and *Salmonella typhimurium* adjusted better to the micro environment in the wounds allowing it to reproduce more rapidly and could easily be disseminated to the internal tissues and hence increases the chances of fresh produce related illnesses

CONCLUSION:

The results of the present investigation provide strong evidence that, phytopathogenically damaged plant support the growth and internalization of human pathogens. These results also revealed that, phytopathogen promotes the internalization of bacterial human pathogens by providing favorable conditions with respect to nutrition, water availability and through wound produced by pathogens; human pathogen can get easily infiltered.

The results of present study enlights the ability of human pathogens to get internalize in fresh produce. The existence of endophytic bacterial human pathogens in fresh produce compromises the efficacy of conventional washing and sanitizing treatments due to poor penetration of disinfectants. Hence, the fresh produce harboring pathogenic bacterial endophyte can act

as an occulant source of infection. To minimize the risk of fresh produce contamination in farm, the crop should be protected from any kind of biological injury.

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