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Colonization of *Serratia fonticola* on phylloplane of tomato and its impact on leaf cytoplasmic protein profile

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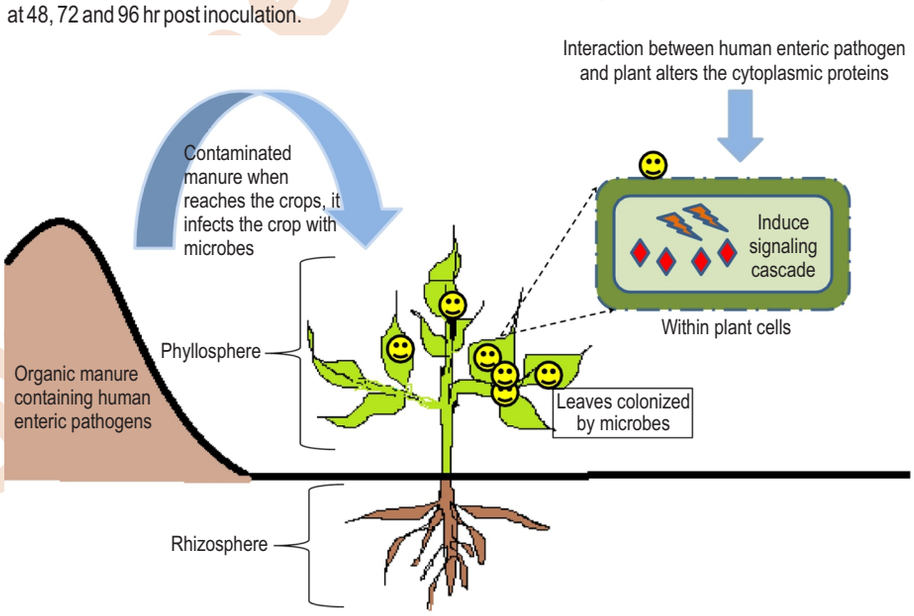
Abstract

Aim : The aim of the present study was to understand the impact of human enteric pathogen (*Serratia fonticola*) colonization on cytoplasmic protein profile of tomato leaves.

Methodology : Aseptically grown plants were inoculated with *S. fonticola* and sampled at different interval of time. Colonization pattern was studied by leaf impression technique. Changes in cytoplasmic protein profile were studied by protein-protein crosslinking method followed by separation through SDS PAGE.

Results : The colonization pattern study revealed that maximum colonies were found at 24 hr post inoculation which gradually decreased with time, showing the survival of the pathogen on phylloplane. Electrophoretic separation of cytoplasmic proteins showed crosslinking of some proteins in treated samples at 48, 72 and 96 hr post inoculation.



Interpretation : This study will help in understanding the molecular changes in cytoplasmic protein profile and survival of pathogen on phylloplane, thus limiting the growth of bacteria, *Serratia fonticola* on phyllosphere.

Key words: Cytoplasmic proteins, Human enteric pathogen, Phyllosphere, *Serratia fonticola*

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Introduction

Contamination of fresh farm produce with enteric pathogens is today a global concern. Numerous outbreaks associated with the consumption of contaminated lettuce, spinach, tomatoes, berries, and various types of sprouts have been reported in the United States, Canada, Asia and Europe (Hedberg *et al.,* 1999; Beuchat 2002; CDC 2006; Martínez-Vaz *et al.,* 2014). Pathogens beside colonizing human gut are also able to colonize plant parts. The pathogens for these outbreaks were reported to be *Salmonella enterica*, *E. coli* O157:H7, *Campylobacter, Yersinia* spp. etc. (Heaton and Jones, 2008; Brandl, 2006). Human diseases caused due to colonization of preharvest crops by *S. enterica* has been reported more in post-harvested crops. Bacterial species naturally colonizing plants tend to protect invading human bacterial species from desiccation which ensure their survival on leaf surface (Poza-Carrion *et al.,* 2013).

The main source of human enteric pathogens on fresh farm produce is due to irrigation with sewage treated water and application of animal dung as manures. In a recent study, the presence of *Klebsiella pneumoniae, Serratia fonticola, Enterobacter ludwigii, Stenotrophomonas maltophilia, Chryseobacterium jejuense* were reported to colonize fruits and leaves of *Solanum lycopersicum* and roots of *Raphanus sativus* (Gaur *et al.,* 2016). Interaction of human enteric pathogen (*K. pneumoniae*) with *P. syringae* pv. *tomato* has been reported to reduce the ability of colonization of *P. syringae* pv. *tomato* on plants (Gaur *et al.,* 2017). Information on the molecular interaction of these microbes with plant tissue is meagre; however, such studies are important for understanding the adaptation of these microbes to survive. Human enteric pathogens can survive on plant surface along with resident microbes and are able to establish cell to cell communication. This behavior amongst the bacteria is density dependent and named as "Quorum sensing" (Schuster *et al.,* 2013). Previous studies on colonization of *Salmonella enterica* and *E. coli* O157:H7 on tomatoes and lettuce have revealed that these pathogens induce expression of genes, important for biofilm production, modification of cell surface structures, virulence and for binding onto the leaf surfaces (Carey *et al.,* 2009; Martínez-Vaz *et al.,* 2014). These cellular structures are used by pathogens to colonize plant surface. Surface polysaccharides like colanic acid and LPS produced by *E. coli* O157:H7 differentially induce plant defense response, thus affecting the survival of human pathogen on plants, including leafy vegetables like *Arabidopsis thaliana* and romaine lettuce as a model plant (Jang and Matthews, 2018).

Serratia fonticola has been reported to colonize plant surfaces without causing any damage to plant (Vleesschauwer and Hofte, 2007). *Serratia fonticola,* isolated in 1979, is an opportunistic human pathogen reported to cause diarrhea in immuno-compromised patients, skin and soft tissue infections followed by trauma (Gavini *et al.,* 1979; Muller *et al.,* 1986; Aljorayid *et al.,* 2016). It is resistant to Penicillin-G, Cephalotoxin and Colistin class of antibiotics. It has been reported that the human enteric pathogens can survive in seeds for long time (Vander Linden *et al.,* 2013). Interaction of resident microbes of the plant with a human enteric pathogen is known but their mechanism of survival on the leaf is not much studied. Seeds, seedlings and subsequently fruits infected with pathogens can be a potent cause of enteric disorders. The molecular mechanism on survival of human enteric pathogen on tomato leaf has not been much studied. Therefore, the objective of the present study was to understand the colonization pattern of *Serratia fonticola* on phylloplane of tomato and its impact on the profile of cytoplasmic proteins.

Materials and Methods

Plant material: Seeds of *Solanum lycopersicum* (Tomato, Var. Pusa Ruby) were procured from National Seed Corporation, New Delhi, India. Seeds were surface sterilized with 0.1% sodium hypochlorite solution. Sterilized seeds were washed three times with sterilized distilled water to remove traces of hypochlorite. Seeds were sown in sterilized soilrite in plastic trays (35cmx25cmx6cm; LxWxH). Plants were grown at 25±1°C and 70% relative humidity with 12 hr (L/D) photoperiod in the culture room under aseptic conditions. Plants were watered daily with sterile distilled water and weekly with sterilized 100% Hoagland's solution.

Preparation of inoculum: Sterilized nutrient broth (50 ml) was inoculated with 1 ml of *Serratia fonticola* stock preserved in glycerol -20°C and incubated overnight at 37±1°C in an orbital shaker incubator. The inoculum was prepared from this overnight stock of *Serratia fonticola* by adjusting their concentration to 10^8 cells ml^{-1} (optical density of 0.1 at 600 nm) and was used to treat the plants.

Treatment of plants and sampling: Eight-week-old tomato plants were divided into 2 groups, 1 and 2. Each group had 3 replicates. Each replicates had 25 plants. Plants of group 1 (experimental) were inoculated with bacterial culture of *Serratia fonticola.* The plants of group 2 were sprayed with sterile distilled water and served as control. Plants were sprayed only once on day 1 (0 hr). The 3rd node leaf from each replicates of treated and control groups were sampled at 0 hr (day 1), 24 hr (day 2), 48 hr (day 3), 72 hr (day 4) and 96 hr (day 5) respectively.

Study of colonization pattern: Five leaf samples were collected from each sampling group. Nutrient agar media was prepared and sterilized. Media was poured in sterile petriplates as 20 ml portions. Leaf impression was taken by pressing the abaxial and adaxial surfaces of leaves separately in petriplates containing media with a glass spreader. This was to ensure there was a contact between the phylloplane and media. The plates were incubated at 37±1°C for overnight in a BOD incubator. The colonization pattern from each sample was studied by techniques described by Aneja (2003). The same method was repeated for all the samples.

***In-vivo* crosslinking of cytoplasmic proteins:** For *in-vivo* crosslinking, the control and *S. fonticola* inoculated leaves were

divided into two groups. One group from each was treated with formaldehyde (crosslinker) and the other was treated with distilled water (control). The samples were named as follows: (a) Control plant leaves (Non-crosslinked), (b) Control plant leaves (Crosslinked), (c) Treated plant leaves (Non-crosslinked) and (d) *S. fonticola* inoculated plant leaves (Crosslinked). One gram leaf sample for each sampling interval (0, 24, 48, 72, 96 hpi) were collected and washed twice with sterile distilled water. *In-vivo* crosslinking of cytoplasmic proteins was carried out following the method of Bhuvaneshwari *et al.* (2015).

The leaves were cut into small pieces, followed by the addition of 10 ml of 1X PBS and infiltrated for 10–20 min in a vacuum desiccator. Freshly prepared formaldehyde (18.5%) was added to bring the final concentration to 1 %. The leaf pieces were submerged followed by capping and poking with a needle and placed in vacuum desiccator for 10 min. After 10 min, the vacuum was released slowly. The submerged leaves appeared translucent. A 0.65 ml of 2 M glycine (final concentration 0.125 M) was added to reverse the process of cross-linking and kept in vacuum again for 5 min. On completion of time, the vacuum was released and air bubbles were removed. The infiltrated leaves were rinsed three times with ice-cold 1X PBS, dried and stored at -80°C for further use.

Extraction of cytoplasmic proteins: Cytoplasmic proteins from replicates of each sample (a-d) was extracted (Opentwetware). One gram of leaf sample was homogenized in cytoplasmic extraction buffer containing 10 mM Tris HCl buffer (pH 8.0), 0.4 M sucrose, 40 mM KCl, 2mM $MgCl_2$, 1 mM NaPPi, 1µg ml^{-1} Leupeptin, 1 mM $NaVO_4$, 1 mM NaF, 1 % (w/v) PVP, 5 mM 2-mercaptoethanol, 1 mM PMSF at 4°C, 10% glycerol and 10 mM DTT. PVP, PMSF and DTT were added just prior to use. The extract was centrifuged at 10,000×g for 20 min at 4°C. The pellet containing cellular debris was discarded and the supernatant was collected and centrifuged again at 100,000×g for 1hr at 4°C. The supernatant so obtained was concentrated by addition of acetone (4:6) and incubated at 4°C for overnight. The precipitate obtained was centrifuged at 15,000×g for 40 min at 4°C. The protein pellet thus obtained was dissolved in 10 mM Tris HCl buffer (pH 6.8). The protein concentration was quantified by Bradford's assay (Bradford, 1976) and isocratically separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue (CBB) staining (Laemmli, 1970).

Statistical analyses: Statistical test was carried out by GraphPad software. Significance was analyzed using t- test. Two tailed P values were calculated at 95% level of confidence. Mean value of each sampling interval was calculated followed by standard deviation (SD) and standard error of mean (SEM).

Results and Discussion

The colonization studies revealed that at 0 hr post inoculation (hpi), the leaf surface had a large number of bacterial colonies. Seventeen proteins were separated electrophoretically from 0 hr samples. The control and *S. fonticola* inoculated samples at 24 hpi, showed no changes in protein profile but the numbers of colonies in *S. fonticola* treated leaves were found to be maximum (91 CFU per cm^2 , $p \leq 0.0001$, Fig. 1a). After 48 hr, the colony count was 87.2 CFU per cm^2 ($p \leq 0.0001$) and a protein of ~11 kDa was prominently observed in the crosslinked sample (Fig. 1b; Fig. 2). The colonization on leaves was significantly higher near the midrib region, near margins and veins.

The number of CFU decreased significantly to 63 ($p \leq 0.0001$) and 46 CFU per cm^2 at 72 and 96 hr, respectively (Fig. 1b). The two tailed P value was 0.0066 and results were found to be statistically significant at 95% level of confidence (t = 6.7473 and df = 3). Table 1 shows the mean and SEM value of each sampling interval. The crosslinked samples had ~92 kDa protein. Proteins of ~48 and ~49 kDa possibly crosslinked at 96 hrs and appeared as a single protein of ~51 kDa. The colonization study suggests that there exists an interaction of human enteric

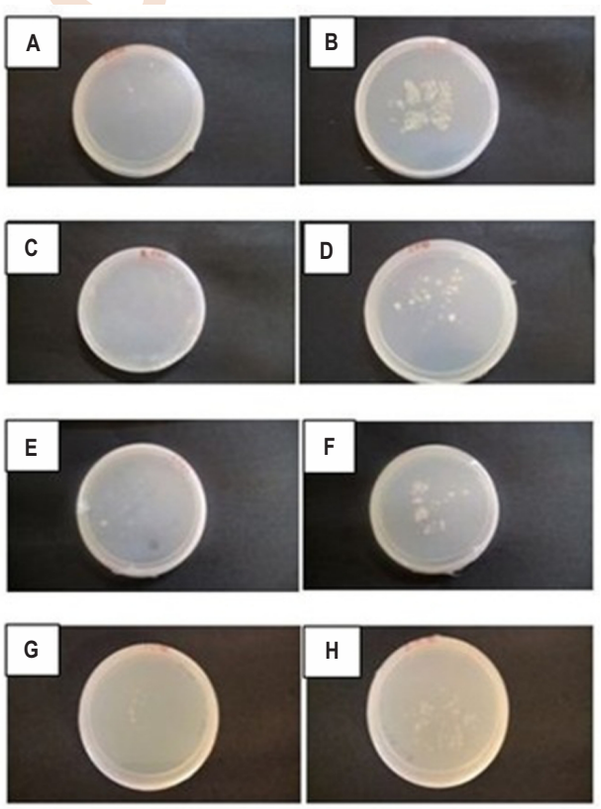

Fig. 1a: Colonization of human enteric pathogens on the phylloplane of leaves. A, B = Leaf Impression at 24 hr post inoculation (Control and *S. fonticola* inoculated); C, D= Leaf Impression at 48 hr post inoculation (Control and *S. fonticola* inoculated); E, F = Leaf Impression at 72 hr post inoculation (Control and *S. fonticola* inoculated) and G, H = Leaf Impression at 96 hr post inoculation (Control and *S. fonticola* inoculated).

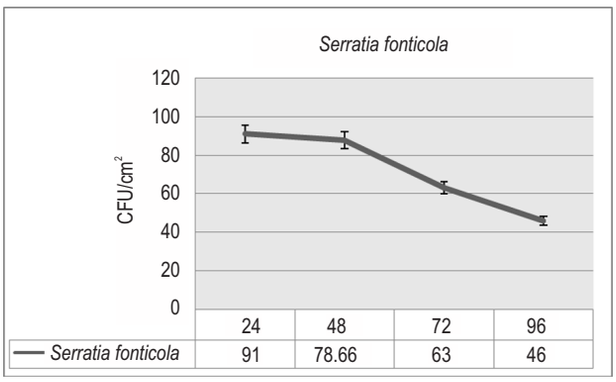

Fig. 1b: Colonization pattern of *Serratia fonticola* on tomato phylloplane. Vertical bars represent standard error.

pathogens with the leaf surface. HEP tends to make an entry and fit onto the surface, despite not being a resident of phylloplane of tomato. Tomato plants irrigated with contaminated water were found to be more infected with *Salmonella enterica* as compared to plants grown from seeds in pre-infested soil (Barak *et al.,* 2011).

Several other bacteria from the enterobacteriacae family have previously been isolated from fruits and vegetables (Sahilah *et al.,* 2010; Tunnug *et al.,* 2011; Whipps *et al.,* 2008). Beattie and Lindow (1999) demonstrated that there are different ways of colonization of bacteria onto the leaf surface. HEPs interact with the local environment and resident bacteria of phylloplane through signal molecules. Environmental factors favor attachment of HEPs on to the leaf surface (Lindow and Brandl, 2003). Human enteric pathogen like *Salmonella* sp. has ability to internalize within cell after attachment on the plant surface (Golberg *et al.,* 2011). In the studies reported so far, the cellular structures of bacteria like curli, fimbriae or flagella play a major role in colonization. This plant-microbe interaction facilitates the plants for acquiring nutrients, disease resistance and tolerating stresses (Jalil and Ansari, 2018). Metabolic activities in the plants have been reported to influence the capability of enteric bacteria to colonize the leafy greens (Klerks *et al.,* 2007; Quilliam *et al.,* 2012). The gene expression studies in *E. coli* and *Salmonella enterica* on lettuce and basil leaves showed the expression of genes to produce curli and flagella (Lim *et al.,* 2014).

In the present study, the colonization results suggested that maximum growth were observed at 24 hpi after which the population stabilized with time which is supported with the modification in cytoplasmic proteins profile. Certain proteins were found to be expressed at 48, 72 and 96 hpi which possibly arises due to *in vivo* crosslinking amongst proteins. At 48 hpi, a ~ 11 kDa (kilodalton) protein was observed which may be due to the crosslinking of ~ 11 and ~ 10 kDa proteins. In pathogen treated

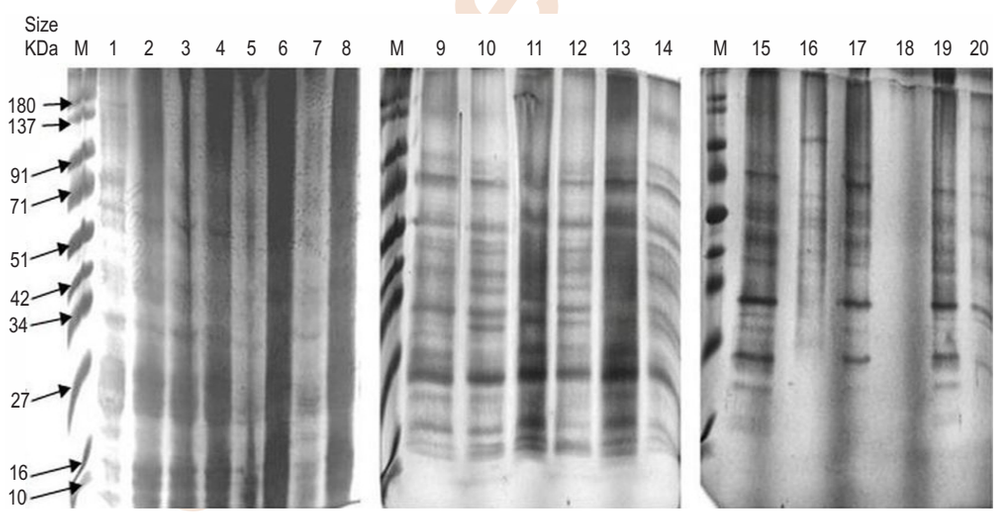

Fig. 2: Cytoplasmic protein profile of leaves treated with *Serratia fonticola* at different time intervals.

M=marker; lane 1= control leaves at 0 hpi crosslinked; lane 2= control leaves 0 hpi non-crosslinked; lane 3= leaves inoculated with *S. fonticola* at 0 hpi crosslinked; lane 4= leaves inoculated with *S. fonticola* at 0 hpi non-crosslinked; lane 5= control leaves at 24 hpi crosslinked; lane 6= control leaves 24 hpi non-crosslinked; lane 7= leaves inoculated with *S. fonticola* at 24 hpi crosslinked; lane 8= leaves inoculated with *S. fonticola* at 24 hpi non-crosslinked; lane 9= control leaves at 48 hpi crosslinked; lane 10= control leaves at 48 hpi non- crosslinked; lane 11= leaves inoculated with *S. fonticola* at 48 hpi crosslinked; lane 12= leaves inoculated with *S. fonticola* at 48 hpi non-crosslinked; lane 13= control leaves at 72 hpi crosslinked; lane 14= control leaves 72 hpi non-crosslinked; lane 15= leaves inoculated with *S. fonticola* at 72 hpi crosslinked; lane 16= leaves inoculated with *S. fonticola* at 72 hpi non-crosslinked; lane 17= control leaves at 96 hpi crosslinked; lane 18= control leaves 96 hpi non-crosslinked; lane 19= leaves inoculated with *S. fonticola* at 96 hpi crosslinked; lane 20= leaves inoculated with *S. fonticola* at 96 hpi non-crosslinked.

Table 1: Statistical parameters of *Serratia fonticola* count taken in replicates

Time interval	Replicate 1	Replicate 2	Replicate 3	Mean	SD	SEM
24 hr	91	93	89	91	2.00	1.15
48 hr	87	87.66	88.32	87.66	0.66	0.38
72 hr	62	65	62	63	1.73	1.00
96 hr	43	49	46	46	3.00	1.73

sample at 72 hrs interval, there was a significant change in crosslinked samples wherein a ~92 kDa protein was present. Proteins of ~48 and ~49 kDa possibly crosslinked at 96 hr and appeared as a single protein of ~51 kDa.

Proteomic analysis helps in the identification of differently expressed proteins in bacterial colonization (Knief *et al.,* 2011). The maximum growth in bacterial population was at 24 hpi with *S. fonticola* after which the internalization of bacteria or bacterial metabolites occurs. Erikson *et al.* (2012) reported that *E. coli* O157:H7 internalizes through spinach leaves. These metabolites are responsible for modulations in cytoplasmic proteins. The changes in protein profile was observed after 48 hrs and sustained till 96 hrs which supports the survival of HEPs on phylloplane. Alteration in the protein expression of control and pathogen treated leaf samples show that the enteric pathogen alters the physiology of plant, and are involved in foodborne outbreaks. In *S. fonticola* inoculated leaf samples, expression of different proteins were observed at different time intervals, which may be due to overexpression of certain specific gene.

The differential expressions of protein in control and *S. fonticola* inoculated samples demonstrate the effect of *S. fonticola* in inducing or suppressing specific genes. However, detailed proteomic analysis reveal the functional aspect of these proteins. These differential gene expressions are sometimes involved in biofilm modulations and are linked with the colonization potential of HEPs which has been reported to reduce the effectiveness of sanitation of fresh produce post-harvesting (Niemira *et al.,* 2010). Since *S. fonticola* does not cause disease in the plants, therefore the plant is able to defend itself by expressing some defense response enzymes (Melotto *et al.,* 2014). These changes help the HEPs to survive on the leaf surface and cope up with the nutrient scarcity and other harsh environmental conditions (Martinez *et al.,* 2014). The cytoplasmic protein expressions in crosslinked inoculated leaf samples suggest that possible internalization of *Serratia fonticola* modulates gene expression in plants.

This could be due to Pathogen Associated Molecular Patterns (PAMP) in the host plant (Zhang *et al.,* 2014). PAMPs are conserved molecules responsible for the survival of pathogens on the plant surface. So, the study of protein profile helps in understanding the plant–pathogen interactions from a systems perspective. The difference in protein profile supports the fact that HEP can invade plant cells and trigger signaling cascade of the host plant (Schikora *et al.,* 2008). Thus, the interaction of human enteric pathogens on leaf surface possibly follows internalization of pathogen and modulation of cytoplasmic proteins. Quenching of this phenomenon can possibly limit the contamination of fresh produce with human enteric pathogens, thereby limiting foodborne disorders. *Serratia fonticola,* a human enteric pathogen colonizes on the phylloplane of tomato. This colonization is, however, not latent. It is evident from colonization and protein profile that the pathogen effectively interacts with the phylloplane. *S. fonticola* effectively induces expression of specific proteins in the cytoplasm of inoculated leaves. The data supports the fact that survival of HEPs is related with the expression of proteins, thus limiting their expression may limit the population of HEPs.

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