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Biofilm Formation and Survival of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* on Tomato Phylloplane

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ABSTRACT

Various human enteric pathogens have been isolated from surface of spinach, lettuce, sprouts, tomato, radish, berries etc. These microbes are not endemic to plant surface but they adapt and survive by mechanism(s) which are still unknown. This study was aimed to understand the colonization pattern of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* by leaf impression method on tomato plants raised under aseptic conditions. The biofilm forming ability of these bacteria were also studied. The study revealed that the population of these enteric pathogens were significantly high (89 CFU/cm^2) on phylloplane of tomato after 96 hours of incubation. Each of these microbes had a distinct colonization pattern and could successfully form biofilm. The study throws light on the ability of human enteric pathogens to colonize phylloplane possibly aided by their biofilm forming capability on leaf surface. The study is significant since it shall enhance understanding of association of human enteric pathogens with plants to design strategies for their survival.

Keywords: Biofilm, *Chryseobacterium jejuense*, colonization, cross talk, human enteric pathogen, *Klebsiella pneumoniae*, *Serratia fonticola*

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INTRODUCTION

Increasing number of food borne gastrointestinal outbreaks is a global concern. Consumption of fresh produce as a part of organic and healthy diet has been reported to be associated with gastrointestinal disorders (Brandl & Mandrell, 2002; Martínez-Vaz et al., 2014). Important sources of human

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enteric pathogens are irrigation water and untreated organic manure in pre and post-harvest environment. *Escherchia coli* O157:H7 and *Salmonella enterica* have been reported to contaminate leafy greens (Bolton et al., 2011; Buchholz et al., 2012; Talley et al., 2009; Wachtel & Charkowski, 2002; Wasala et al., 2009). Other human pathogenic bacteria isolated from surface of tomato and radish include *Serratia fonticola*, *Klebsiella pneumoniae*, *Enterobacter ludwigi*, *Chryseobacterium jejuense* and *Stenotrophomonas maltophilia* (Gaur et al., 2016). A number of environmental factors affect the colonization and internalization of these bacteria on phylloplane. These factors include poor nutrient availability and aerobic conditions, exposure to ultra violet radiations. Further, presence of curli and fimbriae on bacteria facilitates their adhesion on the leaf surface (Brandl et al., 2005; Dinu & Bach, 2011; Erickson et al., 2010; Linden et al., 2013).

Bacteria which enter the plant system communicate and exhibit a density dependent behaviour. This environmental sensing system, which is used by bacteria to monitor their growth, is called quorum sensing (Fuqua et al., 1994). This system is established through small signaling molecules called autoinducers (Brelles-Mariño & Bedmar, 2001; Gowda et al., 2013; Holden et al., 2002). Gram negative bacteria secrete acylated homoserine lactone molecules (Waters & Bassler, 2005) and Gram positive bacteria secrete cyclic peptides as quorum sensing signals (Kleerebezem et al., 1997).

In the present study, bacteria isolated from phylloplane of tomato namely *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* have been chosen for study of colonization and biofilm forming capabilities. These bacteria have been reported to be potent human pathogens and cause severe diseases in immune-compromised individuals (Aljorayid et al., 2016; Caprioli et al., 2005; Gavini et al., 1979; Lopes et al., 2005). These bacteria have also been found to be resistant against broad range of antibiotics.

Although studies for Gram negative quorum sensing have been reported from plant associated bacteria, but the ability of human enteric pathogens to form biofilms on plant surface is not well elucidated. Tomato being the largest cultivated vegetable crop is widely consumed as fresh salad has been used in this study. The study is aimed to understand the biofilm forming capability of the selected human enteric pathogens which were found to be colonizing aerial parts of plants. Biofilms help microbes to adapt and survive on the substratum they colonize. The study of biofilm is important because effective formation of biofilm by a microbe ensures its survival under various conditions. In the present study, understanding the biofilm formation would help to unravel the intrinsic underlined aspects of its colonization on plant surface. The results would help us to device ways and means to disrupt the biofilm formation thereby making fresh farm produce safe for human consumption. The study would help to identify the capacity of these

microbes so that the colonization potential of these formidable human pathogens could be drastically reduced by disrupting their biofilm forming capacity or biofilm formation. This would enhance the safety of food products for human consumption. The procedure is unique as it provides a direct insight into the biofilm formation by human enteric pathogens on plants.

MATERIALS AND METHODS

Plant Material

Seeds of *Solanum lycoperscicum* (var. Pusa Ruby) were procured from National Seed Corporation, New Delhi, India. Seeds were surface sterilized using 0.1% Sodium hypochlorite solution followed by washing with sterilized distilled water to remove traces of hypochlorite. Seeds were sown in sterilized soilrite in plastic trays (35cm × 25 cm × 6cm; L × W × H). Plants were grown at 25±1ºC and 70% relative humidity with 12 hour (L/D) photoperiod under aseptic conditions. Plants were watered daily with sterile distilled water and weekly with sterilized 100% Hoagland's solution.

Bacterial Cultures

Inoculum of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* were prepared from glycerol stocks maintained at -20ºC. These bacteria were isolated from the fruits and leaves surface of field grown tomato, which used organic manure and underground water for irrigation. Fifty mL of sterilized Nutrient broth was inoculated with 1 mL each of the selected pathogens and incubated overnight at 37±1ºC on an orbital shaker incubator. The inocula of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* were prepared from this overnight stock of cultures by adjusting their concentration to 10^8cells/mL (optical density of 0.1 at 600nm), according to the method described by Cottenye (2010). Combination of bacterial cultures was prepared by mixing respective cultures (1:1) and 0.1 OD maintained at 600 nm (10^8 cells/mL).

Treatment and Sampling of Plants

Eight-weeks-old plants were chosen for the study. Plants were divided into seven groups. Experiment was conducted with three replicates. Each replicate had 25 plants:

Group 1- inoculated with *Serratia fonticola*

Group 2 - inoculated with *Klebsiella pneumoniae*

Group 3 - inoculated with *Chryseobacterium jejuense*

Group 4- inoculated with combination of *Serratia fonticola* and *Klebsiella pneumoniae*

Group 5 - inoculated with combination of *Serratia fonticola* and *Chryseobacterium jejuense*

Group 6 - inoculated with combination of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense*

Group 7 - sprayed with sterile distilled water (control).

The plants of each group were inoculated with 50 mL suspension of the bacteria using sterile atomizer. The treatment was carried out under aseptic condition.

Colonization Assay

The third node leaf from both control and treated plants were sampled at 0, 24, 48, 72 and 96 hours post inoculation (hpi) and placed in sterile polythene bags. Five leaf samples from each replicate were collected and colonization pattern was studied by leaf impression techniques as described by Aneja (2003). Nutrient agar media was prepared, sterilized and plated in petri dishes (9 cm diameter).

The adaxial and abaxial surfaces of the sampled leaves were pressed on surface of media. The leaves were subsequently removed. The plates were incubated at 37±1ºC for overnight in an orbital shaker incubator. The process was repeated for each sample of each replicate for sampling intervals of each group. Colonies were counted and results were expressed as CFU/ cm^2 of leaf. Statistical test for significance was carried out by GraphPad software. Number of CFU observed were analysed for statistical significance using t- test. Average of triplicates was calculated followed by standard error. The two tailed P values were calculated at 95% level of confidence.

Quantification of Biofilm Formation

Biofilm formation was determined and quantified by microtiter plate method (Boddey et al., 2006). Bacterial cultures grown overnight at 37±1°C with shaking were used for the estimation. 1 mL of nutrient broth media was added into the wells of 96-well plate and 10µL of cultures of *S. fonticola*, *K. pneumoniae* and *C. jejuense* respectively were added singly and in combination into the respective wells containing media. The plate was incubated at 37±1°C for 18 hours. After incubation, a fresh polystyrene plate was taken and 1 mL media was added. Ten (10)µL of respective bacteria from each well was transferred into the triplicate wells of the fresh plate containing media and incubated at 37°C for 24 hours without shaking. Wells containing only media served as control. After 24 hours the supernatant from the wells was carefully removed and 1% crystal violet was added to stain the wells and kept at room temperature for 30 minutes. Then stain was removed and wells were washed carefully twice with 175µL of deionized water followed by addition of 175µL dimethyl sulfoxide (DMSO) to solubilize the crystal violet. Then absorbance was recorded spectrophotometrically using ELISA reader at a wavelength of 570 nm. The assay was run separately for each strain in independent experiments.

RESULTS AND DISCUSSION

Colonization Assay

The study revealed that each bacterial species and their combinations had a unique colonization pattern on tomato leaves. No morphological or pathological changes were observed on the plants (Figure 1). The findings of the study strongly suggest that human enteric pathogens are able to

effectively colonize plant parts and thereby significantly increase the potential entry into humans through consumption of such contaminated fresh farm produce. The abaxial surface of leaf was more populated than adaxial surface. The colonies were concentrated around the midrib and veins of leaf (Figure 2). CFU count of *Serratia fonticola* was maximum at 24 hour post inoculation which gradually decreased with time and was found to be minimum at 96 hour post inoculation (Figure 3a). The CFU count at 24 hours was 91 CFU per cm^2

Figure 1. Plants inoculated with selected human pathogens. No pathological changes were observed on the plants

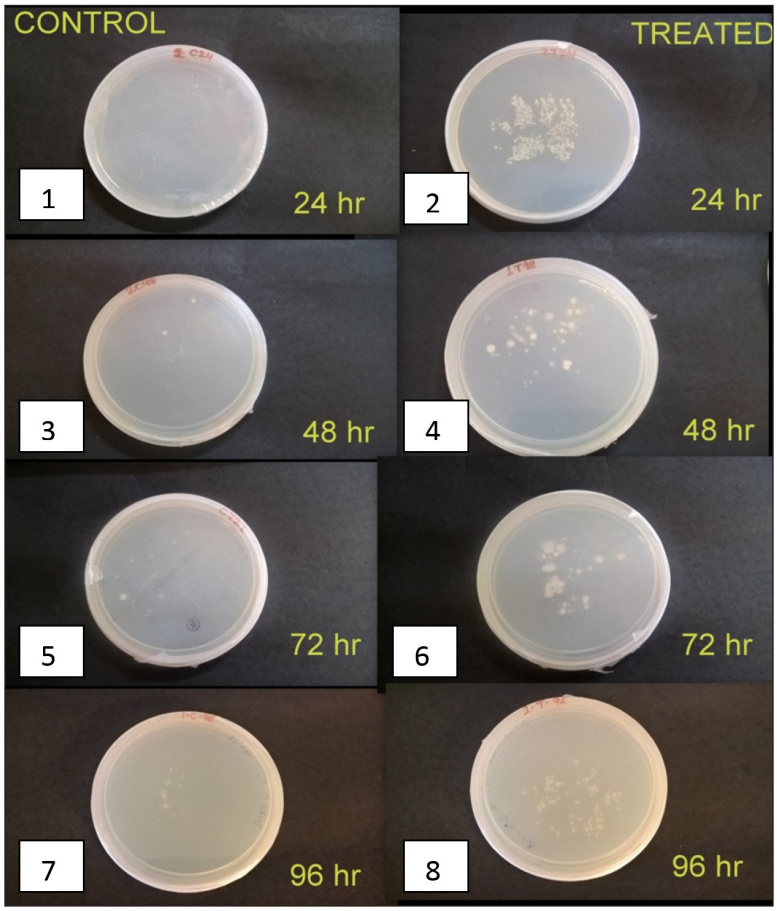


Figure 2. Bacterial colonization by leaf impression technique

($p \leq 0.0001$), which decreased significantly (46 CFU per cm^2) at 96 hours ($p \leq 0.0001$). However in *Klebsiella pneumonia*e and *Chryseobacterium jejuense*, CFU count gradually increased throughout the sampling period (Figures 3b and 3c). In *K. pneumoniae*, CFU count was 56 CFU per cm^2 at 24 hpi ($p \leq 0.0001$) and increased to 89 CFU per cm^2 at 96 hpi ($p \leq 0.0001$). For *C. jejuense*, it was found to be 42 CFU per cm^2 at 24 hpi ($p \leq 0.0001$) which increased to 60 CFU per cm^2 at 96 hpi ($p \leq 0.0001$). Inoculation of mixed bacterial colonies resulted in similar colonization pattern. Combination of *S. fonticola* and *C. jejuense* as well as that of *S. fonticola*, *K. pneumoniae* and *C. jejuense* showed similar pattern (Figures 3e and 3f). In both the combinations, maximum CFU was recorded after 96 hours post inoculation. However, the combination of *S. fonticola* and *K. pneumoniae* had a different pattern of colonization. (Figure 3d). CFU count initially increased during 24 to 48 hr sampling and then gradually decreased (72 to 96 hours post inoculation). The count was 50 CFU ($p \leq 0.0001$) and 103 CFU per cm^2 at 24 and 48 hpi respectively ($p \leq 0.0001$) and then decreased to 66 CFU and 61 CFU per cm^2 at 72 and 96 hpi ($p \leq 0.0001$) respectively. Bacterial inoculation either singly or in combination had varying results. More number of colonies were located on abaxial surface than on adaxial surface. Most of the colonies were around midrib and veins. It supports the fact that abaxial surface has more stomata which are the sites of leakage and accumulation of nutrients. Microbes generally tend to colonize where nutrients are available. Leben (1988) had also reported similar observations. Results suggest that bacteria are able to successfully colonize and significantly multiply on the phylloplane. It suggests that these human enteric pathogens have mechanisms to successfully adapt and colonize plant surfaces. Though these bacteria were isolated from the same niche, but all of them were found to have varying adaptation characteristics, when inoculated either single and in combination. The protocol followed for study is unique because it strictly helps in isolation and enumeration of human enteric pathogens from plant surface under aseptic conditions.

The findings are unique because the microbes are evolutionarily adapted to colonise human gut, but are able to successfully colonise, multiply and survive on phylloplane. The study highlights the possibility of HEPs entering human system through consumption of raw fruits and vegetables and thereby becoming major health hazard. *E. coli* O157:H7 has been reported on Alfalfa which persisted for long period (Cools et al., 2001; Gagliardi & Karns, 2002) but survival of *S. fonticola*, *K. pneumoniae* and *C. jejuense* on plant surfaces have not been studied much. The colonization of various enteric bacteria under field conditions is governed by numerous environmental factors like nutrient availability, fluctuation of temperature and humidity (Beattie & Lindow, 1994; Suslow, 2002). Short term changes in the weather conditions at the microscale level of leaves were earlier thought to be the reason for variation in population size of various bacteria on phylloplane.

Therefore modifications and optimum growth conditions on specific sites on leaves allow microbes to persist on plant for long time (Hirano & Upper, 2000). Similarly, in field grown parsley, the inoculum of *E. coli* and *S. enterica*, could colonize the plant surface for up to 177 and 231 days respectively (Islam et al., 2004). In lettuce

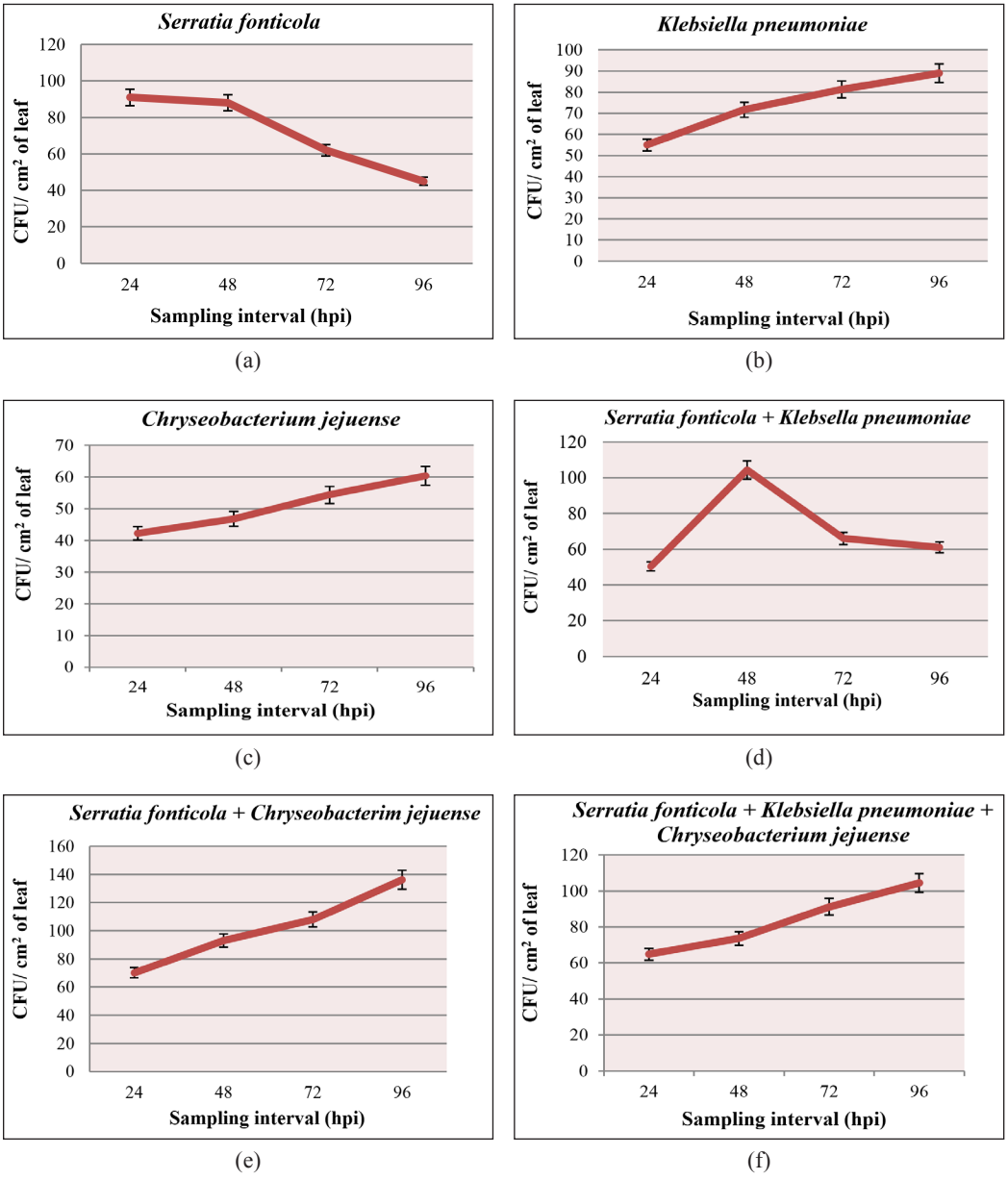


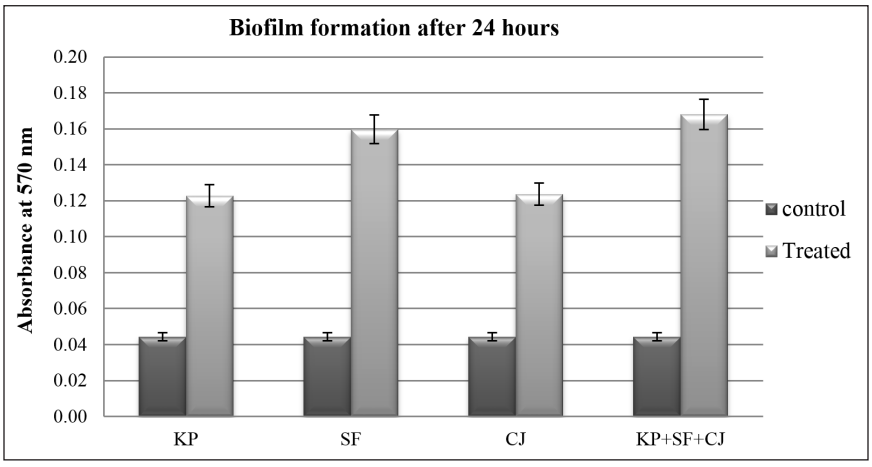
Figure 3. CFU count of (a) *Serratia fonticola*; (b) *Klebsiella pneumoniae;* (c) *Chryseobacterium jejuense;* (d) *Serratia fonticola* and *Klebsiella pneumoniae;* (e) *Serratia fonticola* and *Chryseobacterium jejuense; and* (f) *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense*
Vertical bars represent standard error

leaves the colonization and persistence of enteric pathogens was reported for upto 30 days (Solomon et al., 2003).

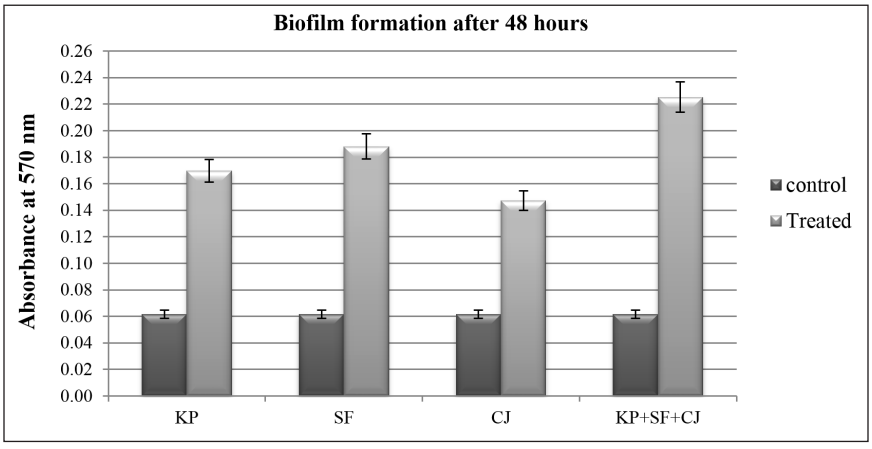
These human enteric pathogens modify the environment of the leaf surface, thereby making it suitable for their own colonization (Kwan et al., 2013; Potnis et al., 2015). The resident bacteria which are natural colonizers of phylloplane also influence the colonization of phylloplane by HEPs (Pollard et al., 2014; Poza-Carrion et al., 2013). Colonization of enteric pathogens is also regulated by expression of a number of genes. Barak et al. (2007) had reported that *Salmonella enterica* mutant which lacked bcsA gene, which was responsible for cellulose synthase production had a poor colonization than wild type strains. This is possibly because cellulose is one of the components for formation of biofilms. Any drop in biofilm formation would impact the colonization potential of the microbe. Lim et al. (2014) and Roy et al. (2013) had reported that differential gene expression was exhibited by different bacteria, in order to colonize plant surface. Hence in the present study, colonization by the pathogenic bacteria at certain specific sites is supported by the fact that these bacteria can modify the local environment so that they can persist and replicate on the phyllosphere. Gilbert et al. (2003) had proposed that microbial population of $<10^3$ on any edible plant surface were considered to be within the safe limits. According to this parameter, pathogens in present study colonize and persist on tomato phylloplane beyond the threshold concentration.

Quantification of Biofilm Formation

Colonization has been further studied by the ability of these human enteric pathogens to form biofilms. Bacteria which colonize on leaf surface, establish cell to cell communication through quorum sensing, which leads to aggressive growth of bacterial cells even after 96 hours post inoculation. Quantification of biofilm formation demonstrated that all the three bacterial species used in the present study could significantly form biofilm. Among the three bacterial isolates, *Serratia fonticola* had a significantly higher biofilm forming capacity as compared to *Klebsiella pneumoniae* and *Chryseobacterium jejuense*. The combination of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* exhibited highest biofilm formation capacity as compared to any individual species. Absorbance recorded for *S. fonticola* after 24 hours of incubation was 0.159 ($p \leq 0.0001$), while those for *K. pneumoniae* and *C. jejuense* was 0.122 ($p \leq 0.0001$) and 0.123 ($p \leq 0.0001$) respectively. However absorbance recorded for combination of three bacteria was 0.168 ($p \leq 0.0001$). Biofilm formation was found to be highest after 48 hours of incubation as compared to 24 hours (Figure 4a). Absorbance recorded for *S. fonticola* after 24 hours of incubation was 0.188 ($p \leq 0.0001$), while those for *K. pneumoniae* and *C. jejuense* was 0.169 ($p \leq 0.0001$) and 0.147 ($p \leq 0.0001$) respectively. After 48 hours, *Serratia fonticola* could significantly form biofilm as compared to the other two bacteria (Figure 4b). Absorbance recorded for combination of



(b)



(a)

Figure 4. Biofilm formation by bacterial isolates (a) after 24 hours; and (b) after 48 hours
Vertical bars represent standard error
Note:. KP = *Klebsiella pneumoniae*; SF = *Serratia fonticola*; CJ = *Chryseobacterium jejuense*; KP+SF+CJ = Combination of *Klebsiella pneumoniae*, *Serratia fonticola* and *Chryseobacterium jejuense*]

three bacteria was 0.225($p \leq 0.0001$), which was found to be highest among all. Results were found to be statistically significant.

Biofilms have been found to be associated with several molecules like acyl homoserine lactones**,** oligopeptides and amino acids (Loehfelm et al., 2007; McLean et al., 1997). Gram negative bacteria are able to produce acyl homoserine lactones which have been reported to control several genes for colonization (Costerton et al., 1999). Brandl and Mandrell (2002) had reported that bacteria formed biofilm or aggregates. Microbial biofilms can be formed on leaves roots, intercellular spaces of plant tissues. The microbes form biofilms to prevent themselves from desiccation, environmental stress, antimicrobial compounds etc. On the phyllosphere, biofilms impact the epiphytic fitness of the microbes (Costerton

et al., 1987; Morris et al., 1997; Zottola & Sasahara, 1994). It has been reported that biofilm help in their stability and persistence on phylloplane making them fit to survive. The studies reported so far, do not characterize the quorum sensing molecules produced by these human enteric pathogens on plant surface through the biofilm forming capacity exhibited by these human pathogenic bacteria is of a concern for the society as it possibly enhances their adaptability on plants and thereby enhance their survival in an otherwise harsh environment on leaf surface.

Niemira and Cooke (2010) reported that some human pathogenic bacteria were capable of modulating gene expression involved in biofilm formation. Certain biofilm forming components like curli and cellulose are involved in the quorum sensing. Bacteria produce signal molecules which diffuse from cells and get accumulated in the extracellular environment. Upon reaching a threshold concentration, these signals regulate the expression of certain genes for LuxR/LuxI, in turn regulating their transcription (Fuqua et al., 1996; Gray, 1997; Hardman et al., 1998).

In the present study, all three human pathogenic bacteria namely *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* have been found to form microbial biofilms. Identification of these molecules would help in limiting the bacterial colonization on phylloplane. Quorum sensing mechanisms of these bacteria are involved in antibiotic resistance against a broad spectrum of antibiotics and elimination of competitors from same niche (Brelles-Mariño & Bedmar, 2001). Pathogens like *Pseudomonas aeruginosa* have been studied for quorum sensing mechanism and virulence factors have been isolated and studied (Anzai et al., 2000).

The study of such factors in plant system would lead to limit the pathogen load on fresh produce. Developing quorum sensing inhibitors can prove to be a rational approach towards food safety.

CONCLUSION

The study revealed that human enteric pathogens namely, *Serratia fonticola*, *Klebsiella pneumoiae* and *Chryseobacterium jejuense* were able to form biofilm on the phylloplane of tomato. This possibly adapts them to survive and establish on the phylloplane and cause severe disorders.

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