



COMPARATIVE STUDY OF COLONIZATION OF DIFFERENT HUMAN ENTERIC PATHOGENS ON PHYLLOPLANE OF *SOLANUM LYCOPERSICUM*

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Abstract

Contamination of fresh farm produce with human enteric pathogen is a global concern. This leads to a number of gastrointestinal disorders worldwide. Various human enteric pathogens have been isolated from surface of spinach, lettuce, sprouts, tomato, radish, berries etc. These enteric pathogens are not endemic to plant surface but they survive on the phylloplane. In the present study, colonization of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* on tomato plants raised under aseptic conditions were studied. Colonization assay was studied by leaf impression method at time intervals of 24, 48, 72, 96 hours post inoculation. The study revealed that these microbes were able to establish themselves on phylloplane, and interact with the resident bacteria of the host. Number of colonies on the phylloplane increased significantly from 24 to 96 hours post inoculation. The study will help in understanding the growth thereby help in limiting the pathogen load on plant surface.

Key words: *Chryseobacterium jejuense*, Colonization, Human enteric pathogen, *Klebsiella pneumoniae*, Phylloplane, *Serratia fonticola*

Introduction

Gastrointestinal disorders due to consumption of human enteric pathogen contaminated fresh farm produce are on the rise. Human enteric pathogens contaminate crop plants through use of sewage treated water and animal dung as irrigation water and manure respectively. The major disease outbreak ever was reported in 1996 from Japan due to consumption of sprouts contaminated with *E. coli* strain O157: H7 (Brandl and Mandrell, 2002). Other disease outbreaks associated with consumption of enteric pathogen(s) infected Lettuce, Spinach, and different types of sprouts have been reported from United States, Canada, Asia, and Europe (Martínez-Vaz *et al.*, 2014). Most of these pathogens infect the human gut but are not common plant colonizers. They get associated with plants during cultivation and harvesting processes. The colonization of bacteria on the plant depends upon various factors which include nutrient availability, UV radiation, competition with other residents, toxic compounds released by the plant and other microorganisms, and desiccation (Beattie and Lindow, 1994; D. Aruscavage *et al.*, 2006). Earlier, colonization by *Klebsiella pneumoniae*, *Serratia fonticola*, *Enterobacter ludwigii*, *Stenotrophomonas maltophilia*, *Chryseobacterium jejuense* were reported on fruits and leaves of *Solanum lycopersicum* and roots of *Raphanus sativus* (Gaur *et*

al., 2016). These microbes multiply on the leaf surface, and form aggregates. Epiphytic fitness results from the aggregation of bacteria on the leaf surface (Lindow and Brandl, 2003). Studies based on colonization of *Salmonella enterica* and *E. coli* O157: H7 on surface Tomato and Lettuce have reported that these pathogens are responsible for biofilm production, modification of cell surface structures, virulence and for attachment of bacteria onto the leaf surfaces. (Carey *et al.*, 2009; Martínez-Vaz *et al.*, 2014).

Human pathogens used in this study are *Serratia fonticola*, *Klebsiella pneumoniae*, *Chryseobacterium jejuense*. *S. fonticola* is an opportunistic pathogen, which is known to cause diarrhea and soft tissue infections in immunocompromised individuals (Aljorayid *et al.*, 2016; Gavini *et al.*, 1979; Muller *et al.*, 1986). It is reported to be resistant against cephalotoxin and coistin class of antibiotics. *Klebsiella pneumoniae*, which is known to cause lung inflammation and sometimes, urinary tract and biliary infection (Caprioli *et al.*, 2005; Lopes *et al.*, 2005). Occurrence and survival of *Klebsiella pneumoniae* on the plant surfaces is of great concern. *Chryseobacterium jejuense* has been found adaptable on the phylloplane of tomato. Other members of this genus have been reported to cause diseases like nosocomial infections among neonates.

To reduce the load of human enteric pathogens on plant parts, it is important to understand what makes these microbes epiphytically fit. The study would be important to develop strategies for reducing biological load of those microbes by targeting their epiphytic colonization potential. The study is aimed to understand the comparative pattern of colonization of selected human enteric pathogens on phylloplane of tomato.

Materials and Methods

Plant Material

Seeds of *Solanum lycopersicum* (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 (35cmx25cmx6cm; LxWxH). Plants were grown at 25±1°C 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.

Preparation of Inoculum

Inoculum of *Serratia fonticola*, *Klebsiella pneumoniae*, *Chryseobacterium jejuense* were prepared from glycerol stocks maintained at -20°C. 50 mL of sterilized Nutrient broth was inoculated with 1 mL each of the selected pathogens and incubated overnight at 37°C on an orbital shaker incubator. The inoculum was prepared from this overnight stock of cultures by adjusting their concentration to 10⁸ cells/ml (optical density of 0.1 at 600nm). Combination of bacterial cultures was prepared by mixing respective cultures (1:1) and 0.1 OD maintained at 600 nm (10⁸cells/ml).

Treatment of Plants and Sampling

8 weeks old plants were divided into six groups. Each group had three replicates. Each replicate had 25 plants. The groups were treated as follows:

- Group 1- inoculated with *Serratia fonticola*
- Group 2- inoculated with *Klebsiella pneumoniae*
- Group 3- inoculated with *Chryseobacterium jejuense*
- Group 4 – inoculated with *Serratia fonticola* + *Klebsiella pneumoniae*
- Group 5- Inoculated with *Serratia fonticola* + *Chryseobacterium jejuense*
- Group 6- Inoculated with *Serratia fonticola* + *Klebsiella pneumoniae* + *Chryseobacterium jejuense*
- Group 7- sprayed with sterile distilled water (control).

The plants of each group were inoculated with the suspension of the respective bacteria using sterile

atomizer. The treatment was carried out under aseptic condition.

Study of Colonization Pattern

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 petridishes (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^o C for overnight in a BOD incubator. The process was repeated for each sample of each replicate for sampling intervals of each group. Colonies were counted and results expresses as CFU/cm² of leaf. Statistical test for significance was carried out by Graph Pad software.

Results and Discussion

The study revealed that colonization pattern was unique to each species and their combinations. 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. The 24 hours CFU count was 91(p ≤ 0.0001), which decreased to 46 CFU per cm² at 96 hours (p ≤ 0.0001). However in case of *Klebsiella pneumoniae* and *Chryseobacterium jejuense*, CFU count was observed to be gradually increasing. In *K. pneumoniae*, CFU count was 56 at 24 hpi (p ≤ 0.0001) and increased to 89 CFU at 96 hpi (p ≤ 0.0001). CFU count of *C. jejuense* was found to be 42 at 24 hpi (p ≤ 0.0001), and increased to 60 CFU at 96 hpi (p ≤ 0.0001). When these bacteria were inoculated in combination, similar pattern of colonization was observed. (Figure 1-3) Combination of *S. fonticola* and *C. jejuense* as well as combination of *S. fonticola*, *K. pneumoniae* and *C. jejuense* showed increasing pattern of growth (Figure 5-6). In both the cases, CFU was maximum at 96 hours post inoculation. However the combination of *S. fonticola* and *K. pneumoniae* had a different pattern of colonization. (Figure 4). CFU count first increased from 24 to 48 hour and then gradually decreased from 72 to 96 hours post inoculation. At 24 hpi, it was 50 (p ≤ 0.0001) which increased to 103 at 48 hpi (p ≤ 0.0001) and then decreased to 66 and 61 at 72 and 96 hpi respectively (p ≤ 0.0001). The number of colonies were significantly higher near the midrib region and of the leaves, near margins and veins. The colonization was found to be higher at abaxial surface as compared to adaxial surface of leaf.

Bacteria inoculated singly and in combination revealed varying results. On comparing the colonization pattern, the count of *Klebsiella pneumoniae* and *Chryseobacterium jejuense* were continuously increasing on the phylloplane, while colonies of *Serratia fonticola* gradually decreased and became static after 96 hrs. The combination of these pathogenic bacteria had an elevated CFU count from 24 to 96 hours post inoculation. Results suggest that bacteria are able to colonize and multiply on the phylloplane and therefore consumption such contaminated edible plant parts after several days of inoculation can lead to severe food-borne illnesses. Human enteric pathogens can persist in the plant environment and multiply to gain epiphytic fitness. Brandl and Mandrell (2002) have reported that *Salmonella enterica*, which is a natural colonizer of intestinal tracts of animals, can effectively colonize on the phyllosphere of Cilantro.

There are various factors which govern the attachment of enterobacters on the phylloplane, like UV radiations, plant secretions, nutrient availability, temperature fluctuations, humidity etc. (Beattie and Lindow, 1994; Suslow, 2002). Successful attachment of bacteria leads to their multiplication on the leaf surface resulting in to formation of aggregates. Microbial communities which are established on the phylloplane regulate their anabolic and catabolic processes, according to the conditions on the phylloplane. Since leaf surface is exposed to rapidly changing conditions of temperature, humidity and other factors, so it is considered as a harsh environment for survival of microbes. This is not because of extremities in the physical factors, but due to the dynamic fluctuations of these environmental conditions (Watanabe et al., 1999; Fonseca and Inácio, 2006). In addition to these factors, presence of other resident bacteria on the phylloplane also influences the colonization of HEPs on the phylloplane (Pollard et al., 2014; Poza et al., 2013). This is made possible by suppression of the Pathogen Associated Molecular Patterns (PAMP) by the human pathogen. It has been established that Human Enteric Pathogens moderate the local environment thereby making it suitable for itself to colonize (Potnis et al., 2014; Kwan et al., 2013). In the colonization study of *Salmonella enterica*, it has been reported that some genes are responsible for the attachment of bacteria on the surface (Saggers et al., 2008; Patel and Sharma, 2010). *S. enterica* mutant lacking bcsA gene, which is responsible for cellulose synthase, colonized to lower level than the wild type strains (Barak et al., 2007). A number of genes have been found to be regulated in the HEP colonization on leaves. Different bacterial pathogens have expression of different genes (Roy et al., 2013; Lim et al., 2014).

Significant increase in the bacterial CFU count is a matter of concern. The colonization of bacteria occurs in different patterns, but mostly they colonize near the tips and margins of the leaves. Lower surface of leaf has been found to colonize more number of bacteria than the upper surface. This is supported by the fact that presence of stomata, trichomes and difference in epidermal cells of lower and upper surface of the leaves make possible route of entry to these HEPs on the phylloplane (Bettie and Lindow, 1999). Since the HEPs are effectively colonizing on the leaf surface, consumption of these parts leads to serious health problems. Study of molecular mechanisms will help in understanding the adaptation on the phylloplane and fructoplane by these human pathogenic bacteria.

Conclusion

Human Enteric pathogens colonize on the phylloplane of tomato. It is evident from the colonization that the pathogen effectively establishes on the phylloplane. Pathogens like *Klebsiella pneumoniae*, *Chryseobacterium jejuense* and *Serratia fonticola*, which cause severe disorders adapt to their ecological and nutritional requirement on the phylloplane.

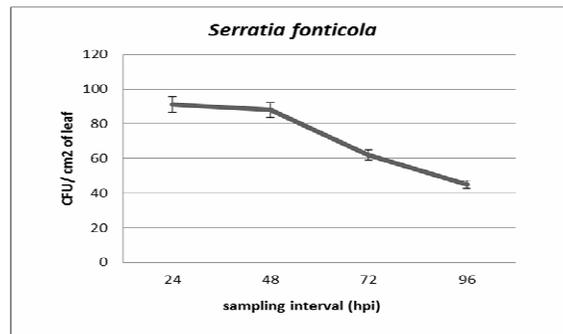


Fig. 1 : CFU count of *Serratia fonticola* from 24 to 96 hours post inoculation ($p \leq 0.0001$), Vertical bars represent standard error

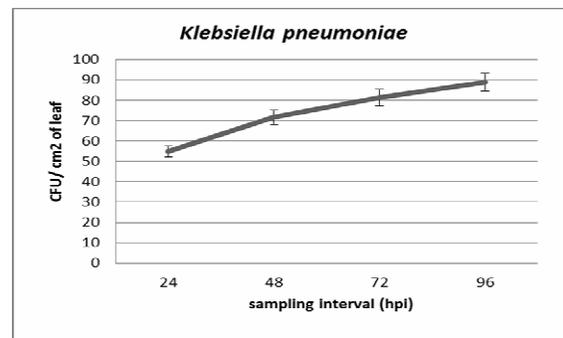


Fig. 2: CFU count of *Klebsiella pneumoniae* from 24 to 96 hours post inoculation ($p \leq 0.0001$), Vertical bars represent standard error

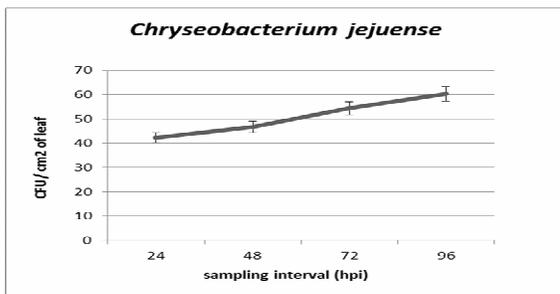


Fig. 3: CFU count of *Chryseobacterium jejuense* from 24 to 96 hours post inoculation ($p \leq 0.0001$), vertical bars represent standard error

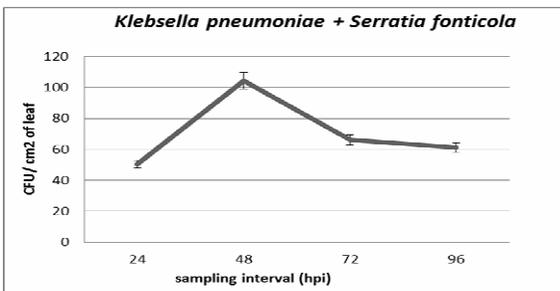


Fig. 4: CFU count of *Klebsiella pneumoniae* and *Serratia fonticola* from 24 to 96 hours post inoculation ($p \leq 0.0001$), vertical bars represent standard error

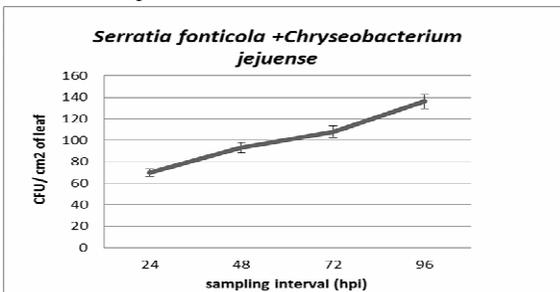


Fig. 5: CFU count of *Serratia fonticola* and *Chryseobacterium jejuense* from 24 to 96 hours post inoculation ($p \leq 0.0001$), vertical bars represent standard error

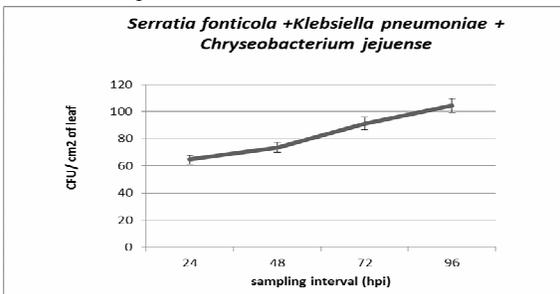


Fig. 6 : CFU count of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* from 24 to 96 hours post inoculation ($p \leq 0.0001$) Vertical bars represent standard error

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