CHAPTER 5: DISCUSSION

The use of microbes for management of diseases in plants is gaining worldwide importance and acceptance. Previous studies have focussed upon physiological implications of phylloplane microbes but information is lacking on their inter-generic and interspecific interactions. The advent of human enteric pathogens and their nature of interactions with host plant and other phylloplane microbes have further complicated the phyllosphere biology. It is still an enigma as to how pathogens adapted to human gut can successfully colonise the leaf surfaces. Their interactions would be crucial in development of the phyllosphere microbiome and its subsequent impact on the food chain. In the present study an effort was made to understand such interactions of human enteric pathogens with other microbes and host plant, particularly the defence physiology of *S. lycopersicum.* The microbial colonisation pattern on the phylloplane and their correlation with trichomes was investigated, since trichomes are known to establish microniches for colonising microbes. The study is important as it helps to study the significance of trichomes in colonization of phylloplane by microbes and HEP, and their role in regulating the defence physiology of the host. The study demonstrated that phylloplane is a complex niche which provides adaptability to a diverse array of microbes which directly or indirectly influences host's defence physiology.

Leaf age is an important factor in the microbial colonization on phylloplane [De-Jager *et al* 2001], and the leaf surface topography along with the presence of trichomes and stomata help in the aggregation of epiphytes. In the present study it was demonstrated that leaf age was critical for colonization on both plant varieties. As the leaf ages, change in physiology leads to microbial colonization.

The knowledge on phylloplane colonization dynamics at different stages of leaf development is scanty [Saha *et al* 2013]. Study on the diversity of phylloplane microflora of tea cultivars in Assam revealed an existence of a comaparitively larger bacterial population than the fungal species [Tanti *et al* 2016]. Our study demonstrates 12 microfungi and 6 bacterial species and their possible adherence at trichome rich niches on the phylloplane. Earlier studies report of increase in phylloplane colonization by some fungal genera like *Cladosporium and Alternaria*, on old and senescent leaves [C. H. Dickinson 1983], while in our investigation both of these fungi were isolated from two to ten weeks old leaves and were not observed on older or senescent leaves. In our study,

population of certain microbes were abundant on mature leaves instead of young ones, for e.g. *Penicillium expansum, A. flavus, Serratia fonticola, Pseudomonas jessenii* which contradicts the observations made by Thompson *et al* [1993] that young leaves are known to harbor more bacterial species. The 9-11 weeks old plants were observed to be abundantly colonized. Some species (*Cladosporium cladosporioides, C. herbarum, Alternaria alternata, A. citrifolia, Klebsiella pneumoniae, P. koreensis, Sphingobacterium daejeonense)* appeared in large number on young leaves, their count reduced gradually with age and again increased towards the end of sampling period.

Carder [2010] demonstrated the variability of phylloplane bacterial microflora and its relationship with plant age. Throughout our study abaxial leaf surfaces was more susceptible to microbial colonization and supported more number of microbial populations as compared to the adaxial surface, whereas certain bacteria like *Pseudomonas jessenii* and *P. stutzeri* were found in higher number on the adaxial surface of ten and eleven weeks old plants which is in contrast with the findings of Korsten *et al* [1992] who suggested the adaxial surface of not being appropriate for microbial sustenance in his study on Avocado phylloplane. Yadav RKP *et al* [2011] demonstrated that mature leaves of *Arbutus unedo, Quercus coccifera, Pistacia lentiscus, Myrtus communis, Lavandula stoechas, Cistus incanus,* and *Calamintha nepeta* were highly populated compared to the younger ones. The abaxial and adaxial surfaces, however, demonstrated no difference in colonisation pattern. Our study revealed similar microbial colonisation pattern with leaf age, however, the adaxial surface was less colonised as compared to abaxial surface. The bacterial community composition on the phylloplane of *Populus deltoides* varied highly during the growing season; leaves sampled at weekly intervals were found to harbour significantly different communities [Redford and Fierer, 2009].

Bacterial and fungal colonies were found to be abundantly present on the midrib, veins of leaf, and near the tip as the leaves aged. The abaxial surface showed an increase in microbial colonization with leaf-age and a gradual decrease near senescence, although, *Sphingobacterium daejeonense, Pseudomonas koreensis, P.jessenii* and *P.stutzeri* deviated from this pattern. Similar findings suggested a decrease in susceptibility of groundnut to *Puccinia arachidis* with increasing plant age, probably due to the reduced ability of the host plant to recognize the pathogen [Savary, 1987]. Populations of the major microbial communities (pink and white yeasts, filamentous fungi and bacteria)

increased with time on the phylloplane of Spring wheat sampled in both glasshouse and field [Legard DE *et al*, 1994].

Bacterial and fungal colonization was more abundant on the local variety as compared to the F1 hybrid. There were specific microfungi and bacteria that repeatedly occurred at every stage of leaf expansion, for eg. *A.candidus, C.herbarum, P.expansum, K.pneumoniae, S.fonticola, S.daejeonense, P.koreensis, P.jessenii* and *P.stutzeri* which is in accordance with the findings of Last [1970]. Microbial colonies were found in abundance near the mid-vein as well as the periphery of the leaf blade whereas least number of colonies were isolated from leaf base. Because of the spatial diversity of nutrients and resources, phylloplane microflora apart from being affected by physical environment, is also influenced by the age of leaf, and is usually observed to positively correlate with it [De-Jager 2001]. The microbial communities we studied also exhibited this pattern, while some of them appeared in significantly large numbers on younger leaves and were not isolated from senescent ones. In contrast to our findings, previous reports describe of younger Chickpea leaves as more resistant to *Ascochyta rabiei* than the mature leaves [Dolar, 1997]. As the leaves matured, the microbial communities were reflected in high numbers around the veins, which gradually declined by senescence. This phenomenon possibly shows that microbial communities could show specificity towards the physiological age of leaf.

In addition to phytopathogens, plants could also be colonized by various human pathogens [Fletcher *et al* 2013]. *Serratia fonticola, Klebsiella pneumoneae,* both potent human pathogenic bacteria were isolated from the phylloplane of *Solanum lycopersicum.* The population densities of both microorganisms differed with leaf age where the former was not isolated from first three weeks of leaf development while latter was found to be ubiquitously present on different development stages although the distribution was found to be random. Populations of E. coli O157:H7 on Spinach leaves varied among different cultivars [Macarisin D *et al*, 2013].

The relative abundance of bacteria on Olive phylloplane was about 51% for *Pseudomonas syringae,* followed by *Xanthomonas campestris* (6.7%), *Erwinia herbicola* (6%), *Acetobacter aceti* (4.7%), *Gluconobacter oxydans* (4.3%), *Pseudomonas fluorescens* (3.9%), *Bacillus megaterium* (3.8%), *Leuconostoc mesenteroides* subsp.*dextranicum* (3.1%), *Lactobacillus plantarum* (2.8%), *Curtobacterium plantarum* (2.2%), *Micrococcus*

luteus (2.2%), *Arthrobacter globiformis* (1.4%), *Klebsiella planticola* (1.2%), *Streptococcus faecium* (1.2%), *Clavibacter* sp. (0.98%), *Micrococcus* sp. (0.82%), *Serratia marcescens* (0.81%), *Bacillus subtilis* (0.57%), *Cellulomonas flavigena* (0.4%), *Erwinia* sp. (0.37%), *Zymomonas mobilis* (0.3%), Bacillus sp. (0.29%), *Alcaligenes faecalis* (0.27%), *Erwinia carotovora* (0.08%), and *Pseudomonas aeruginosa* (0.04%) [Ercolani GL, 1991]. However, in our study 18-23% of *Aspergillus flavus* colonies were isolated from the mature leaves. The leaf bud of local variety had a 50% abundance of *Aspergillus candidus* and *Aspergillus flavus,* whereas it was 20-49% abundance on older leaves. 42% *C.herbarum* were isolated from the abaxial surface of 1 week old F1 variety leaves. *C.cladosporioides* and *C.herbarum* had relative abundance of 20% on 4 weeks old leaves of the local var. 12% *Fusarium oxysporum* and *Rhizoctonia solani* abundance was on 1 week old local variety leaves. 42.8 % *Penicillium expansum* colonies were isolated from 1 week old leaves, and then the fungus was found inhabiting the mature leaf surface till senescence. The relative abundance of human enteric pathogen *Klebsiella pneumoniae* was found to be 100% on the leaf bud and 20% on mature leaf surface of F1. *Sphingobacterium daejeonense* was isolated from abaxial surface of 3 weeks old leaves of F1 with a relative abundance of 46.1%, although it showed an upsurge on the adaxial surface of 10-13 week old leaves. 25 % relative abundance was observed on the leaf bud, and 9-21% abundance was recorded from older leaves before senescence.

Glandular and non-glandular trichomes were observed on the leaf surface of *Solanum lycopersicum* (cultivated/wild type) [Luckwill, 1943; Kang *et al*., 2010]. These include single and four-celled glandular head acting as a well-defined site for attachment and aggregation of microbial communities [Wilkens *et al* 1996; Anthony.L.Schilmiller *et al* 2010; Gabriela Lopez-Velasco 2011]. The chemical composition of glandular trichomes varies significantly within and between tomato species [Schilmiller *et al*., 2010; Besser *et al*., 2009]. In accordance to the previous reports, our findings confirmed the presence of both glandular and non-glandular trichomes on the leaf surface of *S. lycopersicum* from bud stage till senescence, and a positive correlation was observed between localization of trichomes and microbes on the phylloplane of *S. lycopersicum,* which may denote that trichomes possibly serve as specialized sites for microbial attachment. Majority of total trichomes was reported from abaxial leaf surface at all developmental stages.

Non-glandular trichomes were abundantly found on the mid-rib and veins, but glandular trichomes were observed in regions between the veins and were scarcely present on the mid-rib. The study confirms the findings of Ascensao L *et al* [1995] who suggested the presence of capitate and peltate trichomes on both abaxial and adaxial epidermis of *Leonotis leonurus*, predominately on the abaxial surface and particularly dense localization at the areas between veins, the non–glandular trichomes were however richly localised on the mid-rib and veins. Chmielewska and Chernetskyy [2005] confirmed that leaves of *K. beharensis*, *K. hildebrandtii*, *K. millotii*, *K. orgyalis*, *K. rhombopilosa*, and *K. tomentosa* are densely covered with non-glandular trichomes, whereas glandular trichomes were observed on both leaf surfaces of *K. manginii*, predominantly on abaxial side.

Results of the current study revealed a dense microbial localization at non-glandular and glandular trichomes rich sites. Phylloplane microbes along with human enteric pathogens *K.pneumoniae* and *S. fonticola* were isolated from midrib, veins and leaf periphery enriched with glandular and non- glandular trichomes. Yadav RKP *et al* [2004] earlier demonstrated that the hemicryptrophytes, *Melissa officinalis* and *Calamintha nepeta*, are also found to be covered with both glandular and non-glandular trichomes, sustaining high bacterial populations on their leaves. However, our study revealed 66% of isolated fungi (*Alternaria alternata, Fusarium oxysporum, Cladoporium cladosporioides, C. herbarum, Rhizoctonia solani*) were from trichome rich niches.

Foodborne pathogens (*Listeria monocytogenes* and *E. coli* O157:H7) harboured on the phylloplane of *Arabidopsis thaliana* and spinach, are also reported to be associated with trichomes [Milillo *et al* 2008; Mitra *et al* 2009]. Hooked and glandular trichomes are found to be associated with bacterial aggregates on leaf surface of beans [Monier and Lindow 2004]. The present study suggests that glandular trichomes were scarcely present on abaxial and adaxial epidermis at all leaf ages. Our observations thus contradicts earlier findings for *Lamiaceae* species that continuous initiation of new peltate glands results in a continuous increase throughout leaf expansion [Croteau *et al*., 1981; Maffei *et al*., 1986, 1989; Colson *et al*., 1993; Werker *et al*., 1993]. Gland formation stops with completion of leaf growth. In the present study, we also found that glandular trichome densities and regions of trichome formation are not uniformly distributed on the leaf surface, gland

initiation could be observed on 1st developmental stage and the highest number was reported on abaxial surface.

Crop plants acquire an enhanced defensive capacity that results in a faster and/or stronger defence reaction upon treatment with a resistance-inducing agent. On challenge with a microbe or its metabolites, plants can acquire resistance to a broad spectrum of pathogens and/or abiotic stresses [Balestra *et al* 2009]. The plant (*S. lycopersicum*)-pathogen (*Alternaria alternata, Fusarium oxysporum, Cladoporium cladosporioides, C. herbarum, Rhizoctonia solani, S. fonticola)* interactions were investigated in order to understand any possible role the phylloplane microflora could play in enhancing plant's defence physiology.

Bacterial alteration of the host plant environment has been associated with plant pathogenic bacteria [Lindow and Brandl, 2003]. Our study is an attempt to understand the effects of fungi, human pathogenic bacteria and non-pathogens on the defence physiology of a commercial crop, 'Tomato'. The defence related flavonoids are 'preformed' and 'induced', where preformed flavonoids are present in healthy plants, while the induced ones are a result of plant-pathogen interaction [Treutter D, 2006]. Our study demonstrated a distinct difference in the phenolic and flavonoid content of control, fungal and bacterial treated plants, where the inoculated leaves precisely contained elevated quantities of the metabolites. Previous reports indicated a direct association between the amount of simple and polyphenols, and microorganism invasion on plants [Tyagi VK, and Chauhan SK, 1982]. Similar studies inferred rise in total phenols and polyphenols of plants owing to infection by different bacterial species. *Pseudomonas koreensis, Sphingobacterium daejeonense,* and the human pathogens (*Serratia fonticola, P. jessenii, Klebsiella pneumonia*) have not been implicated in increase of plant's defensive metabolites.

Scagel CF, and Lee J, [2012] reported a differential alteration in the polyphenolic contents of Basil plants upon being inoculated with the arbuscular mycorrhizal fungus. In accordance with the findings, our study demonstrates significant ($P \leq 0.05$) changes in flavonoids and phenolic composition of *Solanum lycopersicum* leaves upon inoculation with *Aspergillus candidu, A.niger, A.flavus, Fusarium oxysporum, Rhizoctonia solani, Trichoderma viride, Alternaria alternata, A.citri, Cladosporium cladosporioides, C.herbarum, Curvularia lunata,* and *Penicillum expansum* isolated from the phylloplane.

Inoculation of a combined culture of *R.solani* and *C.herbarum* on the leaves increased the total phenolic composition to 5.996 mg/g as compared to the control after 48 hours. Similarly, highest amount of flavonoids was estimated from *Fusarium oxysporum* inoculated plants (4.287 mg/g) after 72 hours. Karthikeyan M *et al.,* [2006] have inferred the role of phylloplane microfungi *Trichoderma viride* at inducing higher levels of total phenolic compounds in the leaves of Rose to combat the black spot disease-causing microorganism *Diplocarpon rosae*. Present study confirms the finding as a four-fold increase in the total phenols of *Solanum lycopersicum* leaves was recorded upon being inoculated by *Trichoderma viride* after 48 hours. Similarly, synthesis of high amount of phenols by *Trichoderma viride*, compared to controls, suggests their role in inducing resistance against wilt and blight in legumes [Surekha CH *et al.,* 2014]. Wojakowska A *et al.,* [2013] studied changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (*Lupinus angustifolius*) plants to infections with the fungus *Colletotrichum lupine.*

The total phenolic profile of grape leaves increased after 168 hours of treatment due to the varietal impact of foliar and cluster powdery mildew infection caused by *Erysiphe necator* [Taware PB *et al.,* 2010]. However our study revealed maximum phenolic contents in the fungal-infected leaves after 72 hours of spraying. It clearly indicates that the phylloplane borne microfungal species used in this study are efficient in inducing a defence related response in plants than *E. necator*. Ruelas C *et al.,* [2005] reported a significant increase in the phenolic acids of Tomato fruit after being inoculated by *A. alternata.*

Similar studies inferred rise in total phenols and polyphenols of plants owing to infection by different bacterial species. Baker CJ *et al* [2014] demonstrated that the accumulation of apoplastic phenolics is stimulated *in planta* in response to bacterial inoculation. Jordan Vacheron [2013] reviewed several studies that have reported the metabolomic changes caused by PGPR inoculation, by analysing metabolite contents of root exudates, root tissues and shoots under normal or stressful conditions.

Some studies have shown that PGPR can elicit changes in the activities of root enzymes concerned with the synthesis of metabolites, especially flavonoids, leading to alterations in the pattern of root exudation [Lavania *et al*., 2006]. Priming of enhanced phytoalexin accumulation in grapevine leaves after root treatment with *Pseudomonas* sp. or their

corresponding extracts showed a pathogen-dependent activation of defence responses in grapevine plants [Verhagen *et al.,* 2010]. Results revealed significant ($P \leq 0.05$) augmentation in flavonoids and phenolic concentration in *S. lycopersicum* leaves upon inoculation with *K. pneumoniae, S. fonticola, P. koreensis,* and *S. daejeonense*. Foliar application of *Pseudomonas flourescens* strain Pf1 reduced the late leaf spot and rust disease of groundnut by increasing the Phenylalanine ammonia lyase activity and raising the total phenol content (Meena B *et al* 2000). Present study confirms elicitation of total phenolic concentration in *S. lycopersicum* leaves to 2.383 mg/g GAE as compared to control after 48 hours of inoculation with *Pseudomonas koreensis*. However, highest amount of flavonoids was estimated from *S.daejeonense* inoculated plants(4.187 mg/g) after 72 hours of inoculation. *Pseudomonas aeruginosa* N17.35 led to a decrease in the phenolic and flavonoid content of Blackberries by 28 and 33% respectively [Ramos-Solano B *et al* 2015]. In contrast, the phenolic and flavonoid content of leaves in our study was raised to 99% after inoculation with *Pseudomonas koreensis*. This indicated that *P. koreensis,* being non-pathogenic to plants, can positively prove to be an effective elicitor for plant's defensive strategies.

Previous studies have shown that increase in concentration of extracellular phenolics is triggered in plants in response to bacteria. Most of the plants have the ability to produce or accumulate structurally and functionally similar protective proteins under biotic/abiotic stress. Induction of peroxidases, polyphenol oxidases, catalases, lipoxygenases results in enhancing plant disease resistance. The present study emphasized on the role of the metabolites of phylloplane microflora in influencing the host defence physiology. Increased concentration of phenolic compounds in plants have been demonstrated to increase the disease resistance [Yao *et al.,* 1995; Geetha, *et al.,* 2005; Girish and Umesha, 2005; Ojha and Chatterjee, 2012]. Increase in phenol content after elicitor treatment may be due to increase of PAL activity, as PAL has been reported to be associated with the synthesis of phenolic compounds via phenylpropanoid pathway [Hahlbrock and Scheel, 1989; Guleria and Ashok Kumar, 2006; Raju *et al.,* 2008].

The rhizosphere has been extensively exploited with a range of consortia based microbial applications to elevate the defensive strategies of the plant [Solanki *et al.,* 2012; Ramos-Solano B *et al* 2015]. PGPR strains viz., *Achromobacter* sp. F2feb.44, *Streptomyces* sp. Zapt10 and *Bacillus licheniformis* either singly or in combinations were exploited to

induce systemic resistance in cucumber against the foliar disease of downy mildew caused by *Pseudoperenospora cubensis* and to enhance yield improvement [Sen K *et al*, 2014]. In contrast, our study demonstrated that the consortia of human enteric pathogens and phylloplane microflora significantly enhanced the plant's defence mechanism upon foliar application. Consortium of *T. viride, A. alternata, P. koreensis* and *S. fonticola* metabolites actively up regulated the activities of PAL, TAL, POX and PPO. This highlights the beneficial role these microbes play in facilitating plant's defence physiology when applied concomitantly.

The induction of PAL, PPO, POX and accumulation of phenolics was observed due to ISR in tomato plants (against early blight disease by *A. solani*) by biotic inducer [Latha *et al.*, 2009]. In banana, *P. fluorescens* induced accumulation of PR proteins (POX, PPO, PAL) in roots leading to reduced *Fusarium* wilt disease [Saravanan *et al.*, 2004]. The present study confirms previous findings of enhanced PAL, TAL, POX, and PPO activities upon inoculation with microbes. A significant rise in the activity of key defence enzymes was revealed in plants upon treatment with suspensions and metabolites of *T. viride, K. pneumonia, S. fonticola,* and *P. koreensis.* In *Arabidopsis*, priming for PAL expression could be mimicked by pretreatment with low doses of synthetic SAR inducer BTH, which caused rise in accumulation of PAL mRNA after infection with virulent *P.syringae* pv. *tomato* DC3000 [Conrath *et al.,* 2002]. Tomato treated with *Pseudomonas putida* strain BTP1 elicited priming of pathogen-dependent systemic resistance against *B. cinerea* [Adam *et al*., 2008]. Kosuge (1969) suggested rise in PPO activity due to infection by virus, bacteria, fungi, nematode or mechanical injury which possibly highlights that quinines formed by PPO act as toxic compound against the extracellular enzymes produced by the pathogens. Similarly, induction in POX activity is caused by the formation of barrier substances confined to the regions of pathogenic attack [Arun *et al.*, 2010]. In pearl millet, POX and PAL activities have been shown to be associated with reduction in the rate of pathogen multiplication and spread [Geetha *et al.,* 2005].

Tomato phylloplane exhibited superior defence during suppression of *R. solani* than the roots inoculated with *Bacillus* strains, leading to systemic rise in POX, PPO, PAL activity and total phenolic concentration [Solanki *et al.,* 2012]. In the present study *R. solani* metabolites affected the plant's defence physiology leading to a rise in PAL, TAL, POX,

and PPO activity. Madi and Katan [1998] found infiltration of *Penicillium janczewskii* and its filtrate into melon and cotton which induced enhanced peroxidase activity causing augmented protection against *R. solani* and elimination of the dampening-off symptoms. Similar observations were deduced in the present study when *Penicillium expansum* and its metabolites produced significant ($P \leq 0.05$) enhancement in POX activity and inhibited the foliar growth of *Pseudomonas stutzeri* and *Trichoderma viride* after 48 hours of treatment. Water extracts of dry mycelium of *Penicillium chrysogenum* (PEN) when applied to the roots of two *Gossypium hirsutum* cultivars (H552 and Vered) and two *G. barbadense* cultivars (PF15 and P906) increased POX activity and lignin deposition in hypocotyls at 16–48 and 24–48 hours after PEN treatment, respectively [Dong *et al.* 2003].

Gherbawy *et al*. [2012] demonstrated increase in POX activity in shoots of wheat infected with *Fusarium* spp. *Streptomyces setonii, Bacillus cereus* and *Serratia marcescens* efficiently reduced the *Fusarium* wilt symptoms on the tomato plant stems, which can be explained by the lower malondialdehyde concentration and the greater activities of peroxidases, polyphenoloxidases, glucanases, chitinases, phenylalanine ammonia-lyases and lipoxygenases, which are commonly involved in host resistance against fungal diseases [Ferraz *et al*, 2014]. In accordance with the observations, the present study also revealed the effectiveness of *F. oxysporum* and *Serratia fonticola* metabolites in effectively enhancing the PAL, TAL, POX, and PPO activity in treated leaves.

The proteins in the intercellular fluid play crucial functions in plant cell metabolism, responses to drought and salt stress and especially responses to pathogen stress [Zhang *et al*, 2009]. Alterations in the apoplastic proteome were studied during plant interactions with pathogenic bacteria (*Agrobacterium tumefaciens* and *Pseudomonas syringae*) and fungal pathogens, mainly *Verticillium longisporum* and*Magnaporthae oryzae* [Delaunois B *et al*, 2014]. In contrast to the previous findings, our study demonstrated upsurge in the intercellular fluid protein concentration in *S. lycopersicum* leaves upon interaction with a variety of fungi (*Rhizoctonia solani, Alternaria alternata, Alternaria citri, Penicillium expansum, Fusarium oxysporum, Cladosporium herbarum, C.cladosporioides, Curvularia lunata*, *Aspergillus flavus, A.candidus,* and *Trichoderma viride.*) and bacteria, including human enteric pathogen, *Klebsiella pneumonia*. A number

of new proteins like P14 have been observed in intercellular/apoplastic fluids of tomato leaves upon infection with *C. fulvum* [Wit *et al* 1986]. Shenton MR *et al* [2012] confirmed the presence of DUF26 domain-containing proteins as the early, pathogen stress-responsive proteins found in the intercellular fluid of rice leaves, induced by infection with *Magnaporthe oryzae.* However, in the current study intercellular proteins (ICP) concentration was significantly raised by 95% as compared to control upon treatment with *R. solani, A. alternata, A. citri, P. expansum, F. oxysporum, C. herbarum, C. cladosporioides, Curvularia lunata, Aspergillus flavus, A.candidus,* and *T. viride* metabolites. In addition to the fungal infections, apoplastic proteins were also estimated during rice-bacterial infections (*X. oryzae* interaction) led to the identification of 109 proteins of which only six were secreted from rice, highlighting higher abundance of bacterial-secreted proteins [Wang *et al*., 2013]. Similarly our investigations demonstrated *Pseudomonas koreensis, Pseudomonas stutzeri, Sphingobacterium daejeonense* and *Klebsiella pneumonia,* significantly ($P \leq 0.05$) augmented the concentration of intercellular fluid proteins in leaves either singly or in consortia. Combination of all the microbes with *Pseudomonas syringae,* also increased the total ICP concentration significantly (≤ 0.05).

It has been evident that among the substances present on the leaf surfaces are some anti-microbial agents. These may act directly to limit organisms on the leaves. However, it is possible that the concentration of such substances may be altered by microbial activity, either by direct breakdown or due to metabolites production that renders the toxic substances harmless [Kuc *et al*. 1956; Tokin, 1960]. The present study involving microbial interactions *in vivo* and *in vitro* confirmed previous observations. In accordance with the earlier studies as demonstrated by Whipps *et al*. [2008], the most prominently occurring fungal microflora belongs to the genera *Cladosporium, Alternaria, Penicillium, Aspergillus, Fusarium* as well as *Rhizoctonia.* Many of these fungi are liable to cause a plethora of diseases in plants and crops resulting in a large scale yield reduction [Estrella *et al*., 2007]. Use of synthetic fungicides often causes deleterious effects on plant's health and their long-term usage could also result in phytopathogens to become resistant **[**Živkovic *et al*., 2010]. In order to cope up with fungal pathogens, it is crucial to make use of biocontrol agents that may act against the pathogenic microorganisms without affecting plant health. Various studies have provided ample information about the use of bacteria as an effective biocontrol agent against different disease causing fungal

phytopathogens, and that does not lead to any hazardous implications on plant vigor [Thomashaw and Weller, 1996].

The purpose of studying microbial interactions was to evaluate inhibitory effects of isolated bacterial species against the cohabiting microfungi. The synthesis of antimicrobial compounds and competition for niches is important to pathogen establishment [Lindow & Brandl [2003] as one of the most important mechanisms for antagonistic effect amongst the microbial species on foliar surface. Previous studies report of *Bacillus* sp. as effective biocontrol agent against cucurbits powdery mildew, significantly inhibiting the growth of the fungus *Podosphaera fusca* colonising the watermelon phylloplane [Romero D *et al*, 2003]. In the current study, investigation of the antimicrobial activity of *P. koreensis, S.daejeonense, S. fonticola* and *K. pneumoniae* on foliar surface of *S. lycopersicum* was done. Significant ($P \leq 0.05$) inhibition of *C. cladosporioides, C. herbarum, T. viride*, and *F. oxysporum, C. lunata, P. jessenii, P. stuzeri*, and *A. alternata* by *P. koreensis* and human enteric pathogens was observed. Bacteria associated with the phylloplane, possessing antimicrobial properties have been of interest since long [Enya *et al*., 2006]. Our results highlighted significant ($P \leq 0.05$) antifungal activity by *P. koreensis* and *S. daejeonense*, against the phylloplane fungi.

Pseudomonas is a potent fungal antagonist against a wide array of fungi e.g. *P. aeruginosa* and its metabolites have been highly inhibitory to *A.flavus, A.niger,* and *R.solani* [Kishore *et al*., 2005]. Confirming the previous findings, our investigations indicated that *P.koreensis* and *S.daejeonense* are also significantly inhibitory against these fungi, however their metabolites did not produce any significant fungicidal activity. *P.koreensis* has been investigated as a bio-surfactant producer with phosphate solubilization and plant growth promoting activity and has been implicated in antimicrobial activities against *Phytophthora infestans* [Fernández *et al*., 2012; Naranasamy, 2013], but no antagonistic activity against any other fungi has been reported. *P.stutzeri* has significant antagonistic properties against variety of microfungi, e.g. *F.oxysporum, A.flavus, A.niger, R.solani in vitro* [Prasanna *et al*., 2014], whereas our study demonstrated negligent antifungal activity by it against *C. lunata, P. expansum* and *A. citri.* Culture filtrates of *P. stutzeri* negligibly inhibited the growth of *F. oxysoprum* however, it was inhibited by 90% when cultured juxtaposed with the bacterium in dual culture assay. It reduced the growth of *C. lunata* by 98% during *in vivo* interactions. In contrast to our investigation, Yadav *et al.* [2011] had demonstrated that metabolites of *T.*

viride and *A. flavus* were effective in inhibiting pathogenic effects of *Alternaria brassicae* in rabi crops.

The inhibitory effects of *K. pneumoneae* have not been investigated much against fungal microflora on plants. In our investigations, it limited the *in vitro* growth of *C.lunata, P.expansum, C.herbarum* and *C.cladosporioides* and its metabolites could reduce the total number of colonies of *C.lunata. Bacillus subtilis* obtained from the phylloplane of Brazilian yam produced significant antagonistic effects against *Curvularia eragrostidis,* acting as a biocontrol agent against yam leaf spot [Michereff SJ *et al*, 1994]. Our study also revealed the *in vitro* and foliar inhibition of *Curvularia lunata* by phyllobacteria and human enteric pathogens: *Klebsiella pneumoniae, Pseudomonas koreensis, Sphingobacterium daejeonense,* and *Serratia fonticola.*

Similarly, *Serratia plymuthica* isolates and its metabolites have been implicated in controlling fungal soil-borne and phyllopathogens. *S. plymuthica* strain IC1270 is an effective antagonist of *Penicillium expansum* (blue mould) on apple, has been used to control *Botrytis cinerea* and *Alternaria brassicicola* on Dutch white cabbage [De Vleesschauwer *et al* 2008]. However, in our study *Serratia fontiocola* and its metabolites showed negligent inhibition of the test fungi *in vitro,* though it significantly inhibited ($P \leq 0.01$) the growth of *P. jessenni, C. cladosporioides* and *C. herbarum in vivo.*

Alam *et al.* [2010] observed that increase in concentration of *Penicillium* spp EU0013 strain (isolated from tomato and cabbage roots) caused reduction in *Fusarium* wilt disease by pathogenic strains of *F. oxysporum*. In accordance with the finding, *P. expansum* completely inhibited the foliar growth of *T. viride* after 48 hours of concomitant application on *S. lycopersicum* leaves. As reported by Rajendiran *et al* [2010] *T. viride* could inhibit post-harvest pathogens of fruits and vegetables. Kuberan *et al.* [2012] also observed similar results while working with *T. viride* as biocontrol agent against *Glomerella cingulata* in tea. However, our study demonstrated complete inhibition of the fungus by *K. pneumoniae* upon inoculation on the leaves (*S. lycopersicum*). *A. niger, T. harzianum* and *Penicillium sublateritium* controlled leaf spot disease of *Spilanthes oleracea* caused by *Alternaria alternata* [Thakur and Harsh, 2014]. Our findings demonstrated that *A. flavus* and *P. koreensis* could significantly ($P \leq 0.01$) antagonise the growth of *A. alternata in vitro* and *in vivo*. However, some studies suggest that *Alternaria*

spp. were possibly found to be effective biocontrol agents against broad spectrum pathogens on a wide array of plants [Walker 1981; Fravel *et al* 1998].

The study confirms the microbial colonization patterns on phylloplane of field grown plants, tendency of young leaves to harbour high number of microbial colonies and the possible attachment of microbes to the trichomes present on leaf surface. It could also demonstrate the tendency of phylloplane microbes as consortia to produce antimicrobial activity, induce the accumulation of phenols and flavonoids, and to actively enhance the defence mechanism of the test plant by triggering increase in PAL, TAL, POX, and PPO activities. This is possibly the first report that suggests bio-elicitor role played by human pathogens (*K. pneumonia, S. fonticola, P. jessenii*), and the antimicrobial properties of *P. koreensis* and *S. daejeonense.* The antioxidative properties (if any) of these microbes could be explored further for enhancement of plant's defence mechanism.