

CHAPTER 1: INTRODUCTION

Tomato (*Solanum lycopersicum*) is a popular and widely grown vegetable ranking second in the world. It is also an important model system for research in plant science. The Tomato plant is prone to a large array of bacterial and fungal pathogens which tend to cause different fruit and foliar diseases, thereby causing major losses to the productivity of crops every year all over the world. Globally, tomato cultivation occupies maximum acreage among vegetable crops. The implications of pathogenic attack on the plant parts are usually high and lead to a major loss to the fruit and plant annually. Tomato is susceptible to *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium oxysporum*, along with various species of *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Phomopsis*, *Fusarium*, *Penicillium*. The surface of tomato leaf is characterised by the presence of trichomes whose role in phylloplane colonization is not well understood. As the leaf ages, they tend to vary in their physiology and consequently affect microbial colonization. Therefore it can well be considered that physiological age of the leaf could be crucial in regulating the phylloplane colonisation.

A number of microbes colonizing plant surface have been identified but no comprehensive information is available on inter-microbial interactions on the leaf surface. The population dynamics of phylloplane colonizers are expected to be impacted by these interactions. Inter-relationship between leaf age, human enteric pathogens and phylloplane colonising microbes are also not well understood. The inhibition of phytopathogens by phylloplane colonizers may help in controlling the occurrence of diseases on plants. Though human enteric pathogens colonise the phylloplane yet no information is available on antagonistic behavior of *K.pneumoniae*, *S.fonticola* *P. koreensis* and *S.daejonense*.. The present study is expected to fill in the lacunae in this information.

1.1. Phylloplane:

The term Phyllosphere was proposed by Last [1955] and Ruinen [1956] with regard to the leaf surface and its associated micro-environment. The surface of leaf acts as a suitable source of sustenance for a wide array of microorganisms [Hirano and Upper 2000]. It provides the epiphytes with an ample space to thrive and nutrients in the form of leaf exudates which contains sugars and amino acids as major sources of carbon and nitrogen

respectively needed for their growth and development [JP Blakeman 2006]. Various plants are habitat to fluctuating microbial colonists owing to different temperature conditions [Lindow and Brandl 2003]. Upper and lower leaf surfaces of different plants are known to be colonized by varying microbial communities [Yadav *et al* 2011].

1.2. Phylloplane microbes:

Microorganisms most abundantly isolated from the aerial plant parts belong to the leaf surface. The phylloplane harbours a significant variety of microflora termed as epiphytes surviving on the surface, whereas the microbes colonising the internal tissues are called endophytes. Every plant part hosts specific microbial communities. Majorly found microbes are bacteria along with fungi and yeast [Meyer and Leveau 2012, Prabakaran M. *et al* 2011]. They are usually sourced from air, soil, insects [S Raychaudhuri *et al* 2010], irrigation water, rain, and possess a particularity about the niches they survive on the plant's surface. Phylloplane microbes are specialized at surviving in inevitable conditions [Kishore *et al* 2005] like UV irradiation exposure, temperature variations, salt and water stress, polluted environment and lack of humidity. The solar radiations form a vital basis for selection and survival of phylloplane microbiota, selecting UV-resistant pigmented bacterial species [Stockwell *et al* 1999]. Phylloplane microbes are generally believed to alter the microenvironment of its host. The cuticle of leaves tends to wear off as they age. Changes in leaf surface wettability is one such physiochemical modification. *Pseudomonas fluorescens* has been observed to increase the wettability for their easy mobility and sustenance on phylloplane [Knoll and Schreiber 2000].

Microbes on the phylloplane are specialized at plant growth promotion by producing phytohormones [Vastakaite and Buzaitė 2011; Vorholt 2012; Limtong *et al* 2014], nitrogen and carbon di oxide fixation [Newell *et al* 1989; Favilli and Messini 1990; Freiberg 1998; Smith and Goodman 1999], cellulolytic activity [El-Said 2001], detoxification of pollutants, induction of systemic acquired resistance in plants and helping through the defence mechanisms of plants against phytopathogens [Harman *et al.*, 2004].

1.3. Leaf age and microbe aggregation:

Physiological age of leaf is a major factor affecting the development of epiphytes on phylloplane. Ageing of leaves is characterized by migration of microbes from leaves

leading to a reduction in the number of isolates. Certain microbial species tend to colonize the leaf surface more frequently as compared to the rest [Vorholt, Julia A. 2012]. Young and green leaves of plants support significantly less number of microbial flora as compared to old and mature ones [Hirano and Upper 2000, Saad A. Alamri 2008]. During senescence the frequency of epiphytic colonizers tends to reduce, high leaf wettability causes low amounts of nutrients leached on to the phylloplane which determines the survival of microbes [Knoll and Schreiber 2000]. In younger leaves the cuticle is firm while as the leaf ages, it tends to wither, eventually leading to a decrease in the establishment and sustenance of microbial communities on phylloplane [Lindow_and_Brandl 2003, Whipps *et al* 2008]. Changes in the nutrient availability with age and physiology of leaves often leads to variations in microbial colonization patterns and localization on the phylloplane because of growth and death of microbial species, and their migration from one niche to another [Peter Balint-Kurti *et al* 2009, Armando Cavalcante Franco Dias 2012]. Fungal endophytic infections on leaves of tropical forest trees is influenced by the leaf age [A. Elizabeth Arnold 2003]. Young lettuce leaves showed a rise in *Escherichia coli* O157:H7 colonization as compared to the mature ones [Brandl and Amundson 2008]. Various other researches have shown a lesser microbial colonization on the young leaves of lettuce, cabbage, sugar beet [Yadav, Karamanoli and Vokou 2011]. The availability of nutrients, sugars and other metabolites on leaves vary with the growth condition of plants and their age [Mercier and Lindow 2000]. Different temperature conditions alter the microbial colonization patterns on leaves due to the fluctuations in moisture levels and UV radiations causing variable nutrient availability [Natacha Bodenhausen *et al* 2013]. Moist and cool conditions led to the development of diverse bacterial colonies, where young leaves inhabited larger communities [Lindow and Brandl 2003]. All microbes are not able to multiply on the phylloplane. Sugars on leaves act as carbon sources making up for the nutrient need of microorganisms and get depleted as colonization occurs [Mercier and Lindow, 2000]

1.4. Trichomes:

Trichomes are the hairy structures found on the above-ground parts of plants. Usually observed on stem, flowers and leaves, they are formed from a single cell of the epidermis by a series of cell divisions [Kolb and Muller 2003]. Broadly categorized as glandular and non-glandular, trichomes have been observed in a variety of shapes and forms. They are widely studied for their peculiar features of imparting a line of defence for plants against

a variety of insects and pests [Kowalski *et al* 1992], for providing a characteristic aroma to plants [Anthony.L.Schillmiller *et al* 2010], and acting as a well-defined site for attachment and aggregation of microbial communities [Gabriela Lopez-Velasco 2011]. Non-glandular trichomes are considered useful for acting as a barrier for small insects [Wilfredo L. Gonzales 2008].

Glandular trichomes can be categorized into Capitulate and Peltate forms, where the former comprises of a one to two celled stalk and four celled glandular head, and the latter is formed of a short stalk and eight celled glandular head [Ascensao *et al* 1995]. They have been considered useful in terms of studying plant protection against aphids, insects, UV radiations and water loss. Production of phenols, peroxidases, polyphenol oxidases and various essential oils have been a crucial property of different types of trichomes [Kowalski *et al* 1992; Ascensao *et al* 1995]. Non- glandular and Glandular trichomes have been observed abundantly on different parts of Tomato plants. These include the ones with single cell and four-celled glandular head [Wilkens *et al* 1996; Anthony.L.Schillmiller *et al* 2010].

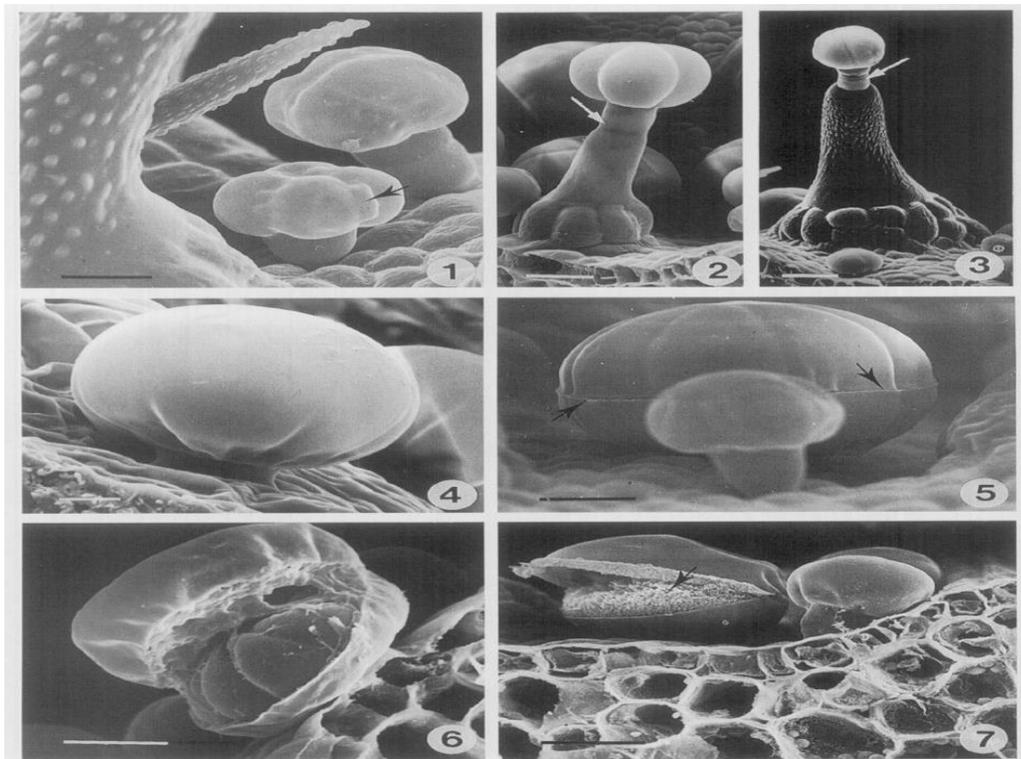
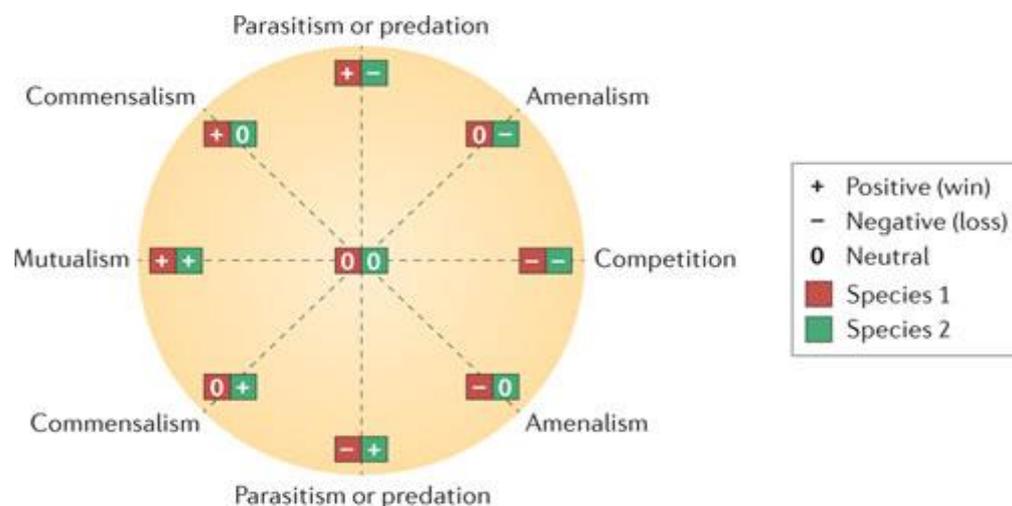


Fig.1.1. Glandular trichomes on the leaves of *Leonotis leonurus*. 1: trichome with four-celled glandular head, 2,3: Capitulate trichomes, 4-7:Peltate trichomes

[Source: Ascensao *et al* 1995]

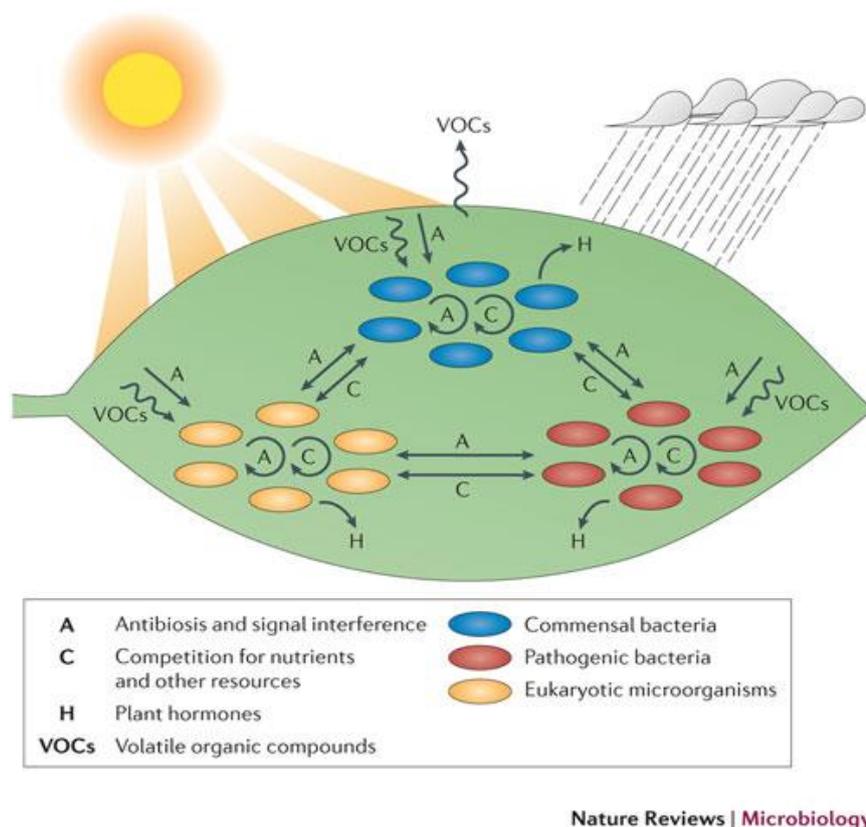
1.5. Microbe-microbe interactions:

Microorganisms found on the phylloplane are determined by genetic and environmental factors, depending on which they survive shaping complex communities, forming microbial ecosystems where they interact and influence each other's growth patterns [Gall LS 1970]. The epiphytic fitness of microorganisms defines the establishment of communication and interaction with one another in complex and specialized molecular and physiological mechanisms [Eddy J Smid and Christophe Lacroix 2013]. Microbial interactions are usually defined as symbiotic, intra cellular communications, or interactions upon direct surface contacts [Caroline Chagnot *et al* 2013]. Metabolic interactions help in the sustenance of microbial communities and biocontrol properties where secretion of toxins or metabolic compounds helps them in either suppressing or promoting each other's growth [M.J.Wolin *et al* 1997]. Inhibition of the epiphytic colonizers due to competition for survival amongst microorganisms is an outcome of various microbe-microbe interactions [Mercier and Lindow 2000]. Most of the volatile fungal metabolites secreted by various leaf-inhabiting microfungi are known for their mycostatic activity against the growth of several foliar pathogens [Upadhyay 1981]. Suppression of pathogenic growth on the phylloplane is achieved by interactions among epiphytes (saprophytic in nature) and pathogenic microflora, subsequently reducing foliar diseases [Blakeman and Fokkema 1982]. These interactions are necessary to understand their implications on disease management and crop productivity.



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Fig.1.2. Intermicrobial interactions on plants. [Source: Faust and Raes 2012]



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Fig.1.3. Interactions among Phyllosphere microflora. [Source: J.A.Vorholt 2012]

1.5.1. Antimicrobial activity of epiphytes:

Microorganisms often tend to restructure the colonization on plant's parts by antagonizing the growth of other microbial communities. These interactions are followed by the inhibition of plant pathogens by phylloplane colonizers which have been considered vital as they help in controlling diseases on plants [Patil and Kachapur 1998]. Various bacterial-bacterial, bacterial-fungal and fungal-fungal inhibitory interactions have been studied for crop protection and plant health [May *et al* 1997; Mohamed and Sater 2001]. Previous studies report bacterial species to either inhibit the growth of microfungi or promote the growth of others [Schrey *et al* 2012]. Kerr [1999] reported a number of bacterial genera like *Pseudomonas*, *Serratia*, *Klebsiella*, which have antifungal activities against a variety of fungi. *Pseudomonas* species are gram negative, aerobic, polar flagella bearing rods [Srivastava and Shalini 2008] and are widely accepted as potent fungal antagonists with substantial related studies undertaken in the area of rhizosphere [Alemu and Alemu 2013]. Earlier studies report of *Bacillus* strains isolated from phylloplane possessing antimicrobial activity and were capable of reducing foliar diseases

significantly [Vieira *et al* 2008]. *Pseudomonas* species are also known to inhibit the activity of phytopathogens like *Botrytis cinerea* and *Alternaria* species [Kong *et al* 1997; Swadling and Jeffries 2010]. The phylloplane bacterium *Ochrobactrum anthropi* BMO-111 was found significantly effective against blister blight disease of tea caused by *Exobasidium vexans* both *in vitro* and *in planta* [Sowndhararajan K *et al* 2013]. *Lactobacillus* sp. was also inhibitory to *Fusarium*, *Penicillium* and *Aspergillus* species [Magnusson *et al* 2003]. Antagonistic activity to *Drechslera dictyoides* (Drechsler) Shoemaker was shown by *Listeria denitrificans* (E2), *Pseudomonas fluorescens* (C37 and C92), and, *Xanthomonas campestris* (D119), isolated from the phylloplane of *Lolium perenne* (S24) [Brian Austin *et al* 1977]. *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum purpurascens*, *F. oxysporum* and *Myrothecium roridum* caused the inhibition of *C. gloeosporioides* whereas *Aspergillus terreus*, *C. roseo-griseum* and *P. oxalicum* antagonized the growth of *P. psidii* [R. R. Pandey *et al* 1993]. *Trichoderma harzianum* and *Trichoderma pseudokoningii* were found inhibiting the growth of *Colletotrichum destructivum* of cowpea [Akinbode and Ikotun 2011].

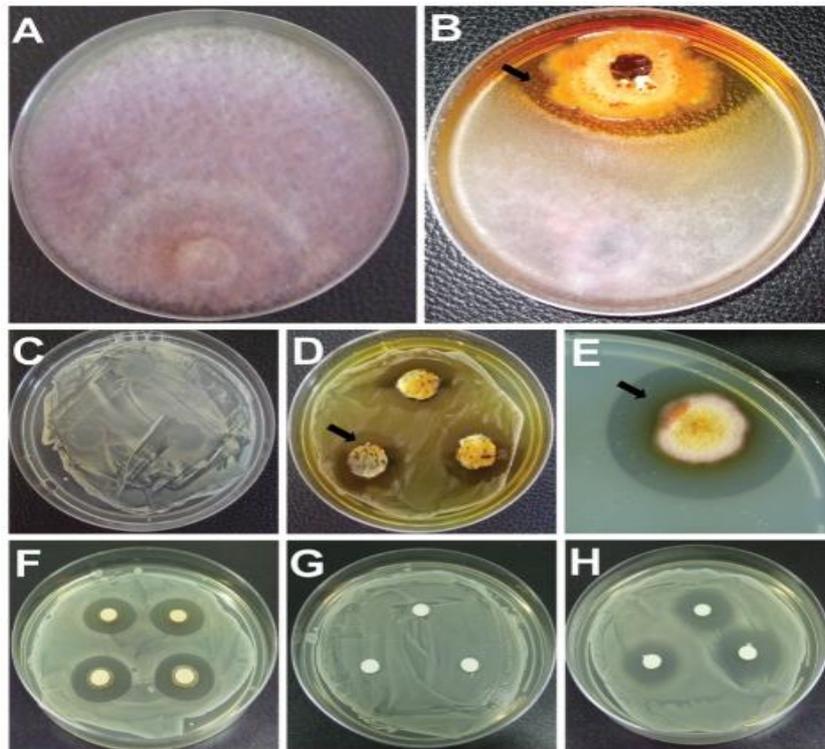


Fig.1.4. Antagonism of *E. nigrum* P16 against *F. verticillioides* (a–b). Inhibitory activity against *X. albilineans* (c–e). The *E. nigrum* P16 ethyl acetate extract also inhibited *X. albilineans* growth (f–h). [Source: Lima Favaro *et al* 2012]

In order to cope up with fungal pathogens, it is crucial to make use of biocontrol agents that may act against the pathogenic microorganisms, act as plant growth promoters and do not deteriorate plant health. Various studies have provided ample information about the use of bacteria as an effective bio-control agent against different disease causing fungal phytopathogens, and that does not lead to any hazardous implications on plant vigour [Thomashow and Weller, 1996].

1.5.2. Secondary metabolites of microbes:

Different bacterial-fungal interactions have been studied previously, and the biological mechanisms underlying this phenomena range from physical associations to molecular communications and antibiosis, in which bacteria-released deleterious compounds and exudates which may lead to the fungal growth inhibition [Warmink and Elsas 2009].

Microorganisms with antagonistic properties are often known to secrete a wide variety of metabolic products known to cause antibiosis. Bacterial microflora has long been recognized for the synthesis of certain volatile compounds like HCN, benzaldehyde, acetaldehyde [Laure Weisskopf 2013] and iron-binding compounds known as siderophores [Vandenbergh *et al* 1983], as well as lactic acid bacteria that produces cyclic dipeptides and phenyllactic acid which are implicated in imparting antimicrobial properties against different fungal microflora [Johan Schnürer and Jesper Magnusson 2005]. Anti-microbial peptides produced by microorganisms due to non-specific innate immunity response, play a key role in inhibiting the growth of pathogens [Cruz J *et al* 2014]. Various enzymes, bacteriocins and lipopeptides produced by the *Bacillus* strains possess tendency to antagonize the growth of different bacteria and fungi [Baruzzi *et al* 2011]. Chitin is the major component of fungal cell wall [Frändberg E. 1997]. Chitinases have potent antifungal activity. *Bacillus cereaus* exhibited the production of two antifungal chitinases against *Botrytis elliptica* causing leaf blight in Lily [C.J.Huaung *et al* 2004]. *Enterobacter agglomerans*, a chitinolytic microbe, antagonizes the growth of *Rhizoctonia solani* that causes leaf spot and root rot diseases in Tobacco plants [Chernin *et al* 1995; Gonzalez *et al* 2011]. It was also observed in previous studies that the bacterial-fungal contact inhibition also plays a vital role in limiting the microbial growth and may lead to physiological changes in the microbes, on the contrary, release of toxic compounds by them may also stimulate the diffusion of specific defensive compounds by

the microfungal species in order to protect themselves from the inhibitory action of the antifungals produced [Frey-Klett *et al* 2011].

1.6. Plant-microbe interactions:

The microbes settling on the plants benefit from the metabolites and nutrients leached from the surface, utilizing sugars like glucose, sucrose, fructose as carbon sources [Lindow and Brandl 2003]. The microbial colonization occurring on the host plants pertain to host-microbe interactions leading to pathogenesis, resistance to diseases and growth promotion that develop a cascade of secondary processes by the surface colonizers [Knoll and Schreiber 2000].

Changing the plant microhabitat is an outcome of the colonizers' interactions with the host [Lindow and Brandl 2003]. Frequent production of phytohormones by microbial flora has been abundantly investigated and only since few years the role of phylloplane associated microbes, has come into perspective, and their possible effects on the alteration of growth patterns of host plants [Vastakaite and Buzaitė 2011; Vorholt 2012; Limtong *et al* 2014]. Synthesis of hormones has been considered as a virulence factor, where the pathogens on plant surface may produce them at the site of infection or stress and quantities of phytohormones synthesized often play a key role in influencing the pathogenesis [Sheldrake 1973; Prasannath 2013]. During the course of infection a rise in the levels of IAA has been noticed [Fu and Wang 2011].

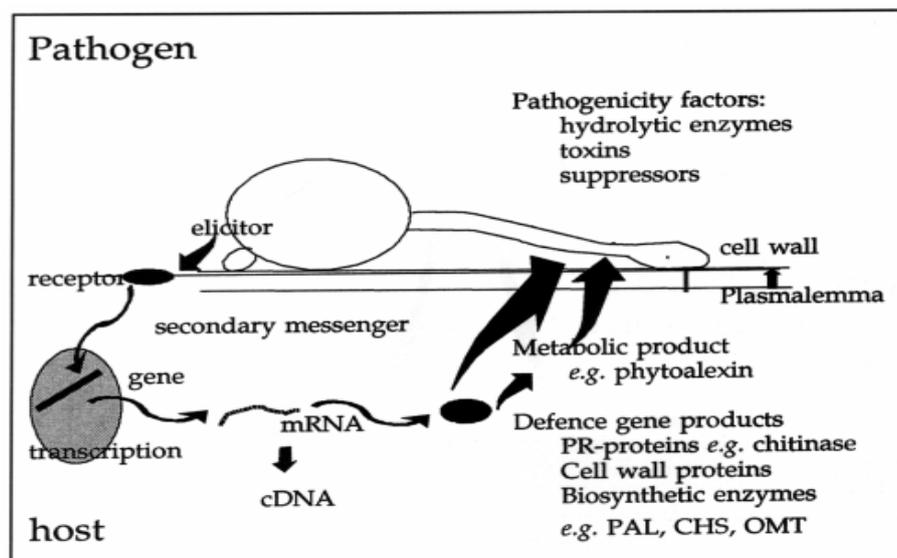


Fig.1.5 showing plant's response to a pathogen

[Source: Collinge DB *et al* 1993]

1.6.1. Phenols:

Phenolics that occur constitutively and function as preformed inhibitors are generally referred to as phytoanticipins, and those that are produced in response to infection by the pathogen are called phytoalexins and constitute an active defense response [Chérif *et al.*, 2007]. Owens [1953] suggested that the antimicrobial activity of phenolic compounds could be due to (a) binding of the quinone nucleus to –SH and NH₂ groups in the bacterial cell or (b) disturbance of electron transport system. Free phenols are likely to be much more toxic to the microorganism than the bound forms. Even if preformed antifungal phenolics are present in healthy plants at levels that are anticipated to be antimicrobial, their levels may increase further in response to challenge by pathogens. Infection in certain diseases is characterized by increased synthesis of certain precursors of phenolic compounds and oxidation products of phenolics, such as quinones, which exhibit more toxicity to microorganisms than their reduced forms [Gandia-Herrero *et al.*, 2005; Lattanzio *et al.*, 2006]. Studies have revealed of a positive correlation between the amount of phenolic content and degree of resistance to plant disease. Increase in phenolic synthesis in plants after attack by plant pathogens have been recorded in previous studies [Farkas, and Kiraly, 1962]. Phylloplane microfungi and bacterial species have not been largely reported for leading to increase in phenolic plant metabolites.

1.6.2. Flavonoids:

Flavonoids and their conjugates play an important role in plants [Gould, and Lister, 2006]. Besides imparting characteristic aroma to leaves and fruits, they are also known to be involved in the defensive strategies of plants, however, there is no detailed information available on the phylloplane microflora and human enteric pathogens on the leaf surface, in enhancing the flavonoid content of host/non-host. Among other functions, they are defence secondary metabolites synthesized as the result of fungal infection or stresses of other kinds [Bednarek *et al.*, 2003; Lozovaya *et al.*, 2004; Farag *et al.*, 2008, Schliemann *et al.*, 2008; Jasiński *et al.*, 2009]. Studies report of rise in total flavonoids upon being treated with microorganisms or their metabolites. Scagel CF, and Lee J, [2012] reported a differential alteration in the flavonoid contents of Basil plants upon being inoculated with the arbuscular mycorrhizal fungus.

1.6.3. Intercellular fluid proteins:

These are the proteins found in the apoplast which is the intercellular space that surrounds plant cells. This is a dynamic environment in which many metabolic and transport processes take place. Primary defence responses towards microbial pathogens also occur in the apoplast, namely the production of reactive oxygen species by extracellular peroxidases and oxidases and the strengthening of the cell wall through crosslinking and callose deposition. As a result of the above mentioned and other processes, a variety of proteins and amino acids are found in the leaf apoplastic fluid [O'Leary, B. M. *et al* 2014]. The intercellular fluid proteins quantities are known to fluctuate upon plant-pathogen interactions. A number of new proteins like P14 have been detected in intercellular or apoplastic fluids of tomato leaves infected with *C.fulvum* [De Wit *et al* 1986].

1.6.4. Phenylalanine ammonia lyase (PAL):

PAL is a crucial enzyme in the phenyl propanoid pathway. PAL catalyses the synthesis of phenols and Salicylic Acid involved in pathogen defence, stress response and secondary metabolism and is subject to post-translational phosphorylation [Nicholson and Hammerschmidt, 1992]. PAL activity in host tissues is induced following pathogen infection and by several biotic and abiotic elicitors [Ebel, 1986]. Concentration of PAL activity may differ widely according to the developmental stage of plant, genotype and environmental factors, and upon plant-microbe interactions [Dixon and Paiva, 1995]. PAL activity is often considered as indicators of resistance as it accumulates more rapidly and reaches higher levels within host plant during resistance responses (incompatible interactions) than in susceptible responses (compatible interactions) [Bhattacharyya and Ward, 1988].

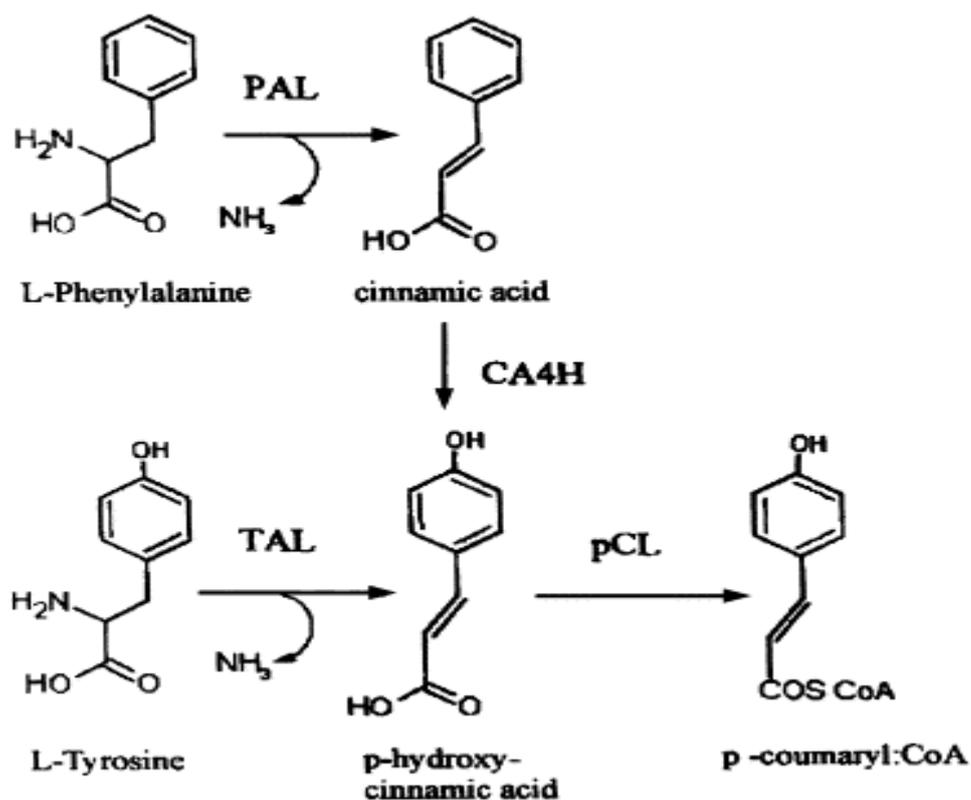


Fig.1.6. Crucial steps in the phenylpropanoid pathway in plants. CA4H: cinnamic acid 4-hydroxylase. [Source: Kyndt JA *et al* 2002]

1.6.5. Tyrosine ammonia lyase (TAL):

TAL is a member of the aromatic amino acid lyase family and a key enzyme of phenyl propanoid pathway. TAL is highly selective for L-tyrosine, and synthesizes 4-coumaric acid as a protein cofactor or antibiotic precursor in microorganisms [Watts K T *et al* 2006]. Previous studies report of changes in the TAL activity in plants due to abiotic/biotic stress, including pathogenic attack [Dixon and Paiva, 1995]. TAL (transcription activator-like) effectors constitute a novel class of DNA-binding proteins with predictable specificity. They are employed by Gram-negative plant-pathogenic bacteria of the genus *Xanthomonas* which translocate a variety of different effector proteins via a type III secretion system (T3SS) into plant cells where they serve as virulence determinants. Inside the plant cell, TALs localize to the nucleus, bind to target promoters, and induce expression of plant genes [Scholze and Boch, 2011]. The increase in TAL activity in compost treated tomato plants due to induced systemic resistance was found to be related to biosynthesis of lignin from tyrosine [Podile and Lami, 1998; Meena *et al.*, 1999; El-Zahaby, 2008; Fayzalla *et al.*, 2009; Abdel-Fattah and Al-Amri, 2012].

1.6.6. Peroxidases (POX):

POXs are haem-containing glycoproteins found in animal and plant tissues, and in microorganisms. The class III family of plant peroxidases (POX, EC 1.11.1.7) encoded by a large multigene family comprises of a number of peroxidase isoenzymes [Hiraga *et al.*, 2001].

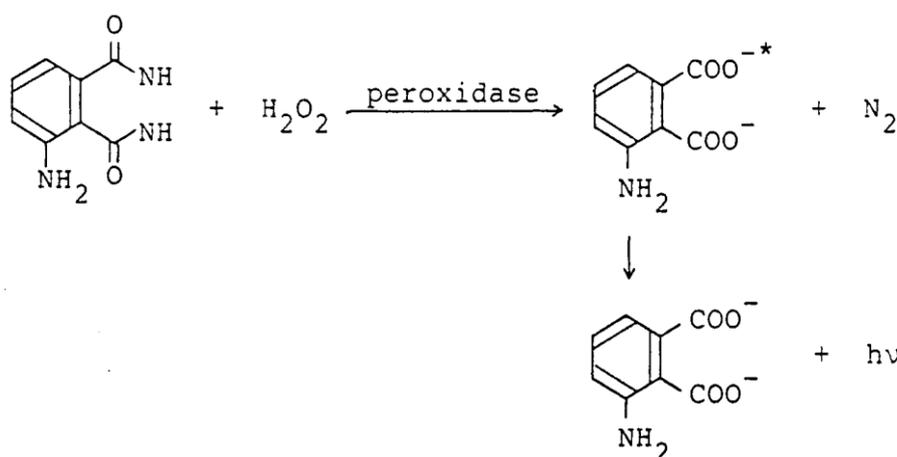


Fig.1.7. Conversion of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) to amino phthalic acid in the presence of peroxidase and hydrogen peroxide.

[Source: Akimoto K *et al.*, 1993]

Peroxidases are involved in a variety of plant physiological processes including cell wall modifications, developmental and defense mechanisms [Gaspar *et al.*, 1991]. Extracellularly secreted plant peroxidases are considered to catalyze the generation of reactive oxygen species (ROS) coupled to oxidation of plant hormone indole-3-acetic acid (IAA) and defence-related compounds salicylic acid (SA), aromatic monoamines (AMAs) and chitoooligosaccharides (COSs) [Kawanao T 2003]. The plant peroxidases (class III) are targeted to the outside of the plant cell or to the vacuole via the endoplasmic reticulum (ER) [Welinder *et al.*, 2002]. They can also generate active oxygen species as part of the oxidative burst during incompatible interactions [Gomez-Vasquez *et al.*, 2004]. They are believed to play a defensive role against the invading pathogen and is responsible for removal of toxic hydrogen peroxide from the host cells, thereby protecting the cells from damage. Rise in POX activity was observed in systemically infected leaves of three cucumber cultivars with the “W” strain of *Cucumber mosaic virus* [Wood and Barbara 1971]. The increase of POX activity due to AM fungi is involved in lignifications [Lagrimini *et al.*, 1987].

1.6.7. Polyphenol oxidases (PPO):

PPOs have been implicated in phenylpropanoid pathway [Kojima and Takeuchi, 1989] and has a defensive role since it is conspicuously present during wounding, pathogen infection or insect infestation, and due to the inducibility of PPO in response to various abiotic and biotic injuries or signalling molecules [Mayer and Harel, 1979; Constabel *et al.*, 1995; Thipyapong and Steffens, 1997]. They are ubiquitously present nuclear encoded copper-containing enzymes which catalyze the O₂-dependent oxidation of mono and *o*-diphenols to *o*-diquinones, highly reactive intermediates whose secondary reactions are responsible for the oxidative browning which follows plant senescence, and responses to pathogenic invasion [Thipyapong *et al.*, 2004].

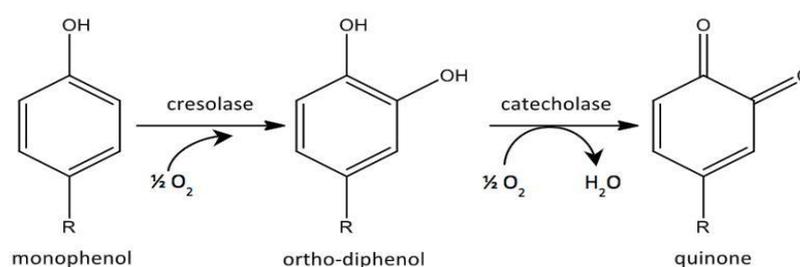


Fig.1.8. Activity of Polyphenol oxidase enzyme
[Source: Gertzen R and Escobar MA, 2014]

PPO activities increased in SA pre-treated onion plants inoculated with *Stemphylium vesicarium* [Abo-elyousr *et al.*, 2009]. Direct evidence about involvement of PPO in inhibiting pathogen ingress or growth has come from transgenic tomato plants with enhanced or suppressed PPO levels. When challenged by the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, PPO-overexpressing tomato plants showed reduced bacterial growth, whereas PPO suppressed lines had higher disease incidence [Constabel and Barbehenn, 2008]. The resistance induced by SA against *X.vesicatoria* in tomato led to increased PPO activities [Ibrahim, 2012].

1.7. Importance of work:

There are lacunae of information on the influence of age and leaf structure on microbe-microbe and plant-microbe interactions. The present study aims to provide an insight into the phylloplane colonization dynamics, and the inter-microbe and host-microbe interactions and their implication on host defence physiology.

The goals of this study are to understand the correlation between leaf age and trichome in phylloplane colonization leading to host defence.