CHAPTER 2: REVIEW OF LITERATURE

2.1. Significance of epiphytes; tomato plants as host:

The surface colonisers of the phylloplane include bacteria, fungi, yeasts, algae etc. Bacteria are the dominant habitants. Distinct taxa of the microbial communities are found on the aerial parts of plants, where certain microorganisms are mostly localised on the Phyllosphere, for eg. pigmented bacteria [Lindow and Brandl 2003]. Competition arising among microbes for space and limited nutrition accounts for the microorganisms carrying capacity of host plants [Mericer and Lindow 2000].These microbes are specialized for possessing a broad range of adaptations that confer them the ability to survive in this unique milieu and also, provide the host with various beneficial properties i.e. nitrogen and carbon di oxide fixation secretion of plant growth promoters like IAA [[Lindow and Brandl 2003, Brandl *et al* 2001]. Beneficial microbes support plant growth and health, increase nutrient availability, induce plant defence mechanisms, produce antibiotics, outcompete pathogens, and provide growth-stimulating substances or enzymes of host plants [Compant *et al.,* 2005]. Though plant-microbe interactions have been extensively studied diversely, ranging from saprophytic, epiphytic commensals, mutualistic or pathogenic [Andrews and Harris, 2000], yet a lot of information is lacking about the role of epiphytic microbial associations in the life of the plant. An evident feature of phylloplane microbial population colonization is their variation in size on different leaves of the same plant species. The population sizes of phylloplane bacteria vary by approx. 10-fold from one leaf to another, even if leaves of same plant species are similar in appearance [Mercier and Lindow, 2000].

Different phytopathogenic and methylotrophic bacteria as well as yeasts and fungi growing on the phyllosphere can actively synthesize plant hormones. Methylotrophic bacteria are among the commonly found leaf epiphytes representing a stable and abundant phyllosphere microfloral community of a large number of crops and plants, which synthesize a variety of metabolites including phytohormones, which could be useful for promoting plant growth and yield [Kamlesh K.Meena *et al* 2012]. The biosynthesis of the plant growth regulator indole-3-acetic acid [IAA] is widespread among bacterial colonizers of the phyllosphere [Lindow and Brandl, 2003; Brandl *et al.,* 2001]. Frequently isolated from Phylloplane of many plants, they have also been observed to synthesize cytokinins like trans-zeatin [Robbin L. Koenig *et al* 2002]. The Methylobacterium spp.

isolated from sugarcane phylloplane was also found to produce high amounts of cytokinins as secondary metabolites in the culture filtrates and in a lot of cases they have been reported to help in the defence mechanism of plants against pathogens [Kamlesh K.Meena *et al* 2012]. *Erwinia herbicola*, is known to produce significant amounts of IAA and often leads to modulation in its habitats, and usually causes pear fruit russets [Susan S. Hirano and Christen D. Upper 2000]. Epiphytic bacteria able to produce IAA [Libbert *et al* 1966], and species such as *Bacillus megaterium* and *Pseudomonas aeruginosa* from the Phylloplane that may act as plant growth promoters [G.K.Kishore *et al* 2005]. Many nitrogen fixing strains such as *Azotobacter chrococcum* and *Azomonas macrocytogenes* obtained from the phyllosphere of the plants, were found to produce good amount of IAA *invitro* [Pati *et al* 1995].Other phyllosphere pathogens such as spp. belonging to the *Xanthomonas* genera [O.Pruvest *et al* 2002] were isolated that could produce significant amounts of IAA[Steven E.Lindow *et al* 1998]. Plants with phytopathogenic infections show traits like stem elongation, gall formation, features that are accompanied with enhanced IAA levels [Reeta Prusty Rao 2010]. Abscissic is also biosynthesized by phyllosphere microbes like *Botrytis* and *Cercospora* [B.Tudzynski 1997]. *Aspergillus niger* and *Cladosporium cladosporioides*, associated with the Qat phyllosphere were reported to synthesize ABA [Alhubaishi and Kadir 1971]. Phyllosphere diazotrophs, *Azotobacter chrococcum* and *Azomonas macrocytogenes* were shown to produce significant amounts of giberellins in their cultures [BR Pati *et al* 1995]. Growth promotion of groundnut plants as well as increase in the yield by phylloplane bacteria *Bacillus megaterium* GPS 55 and *Pseudomonas aeruginosa* GPS 21 was studied by Kishore *et al* [2005], which threw light on growth promotion in the rhizophere region by microbes inhabiting aerial plant parts. Improvement of soil structure, increase in the nutrient availability on plants, inducing plant defence mechanism, combating pathogens, producing antibiotics and acting as biocontrol agents, are some of the characteristics of epiphytic microflora [Compant *et al* 2005]. A Potential of reducing foliar disease of Citrus by Phylloplane *Bacillus*, *Pseudomonas*, *Aspergillus*, and *Trichoderma* spp. was discovered by Kalita *et al* [1996]. A number of epiphytic microorganisms are known for their hydrocarbon degrading properties. *Acinetobacter*, *Flavobacterium*, and *Micrococcus* are hydrocarbon degraders which are obtained in high percentages from the phylloplane of *Mangifera indica* and lowest from *Colocassia* sp. Such microbes can prove to be essential tools for bioaugmentation and treatment of polluted enviroments [LLori MO *et al* 2006].

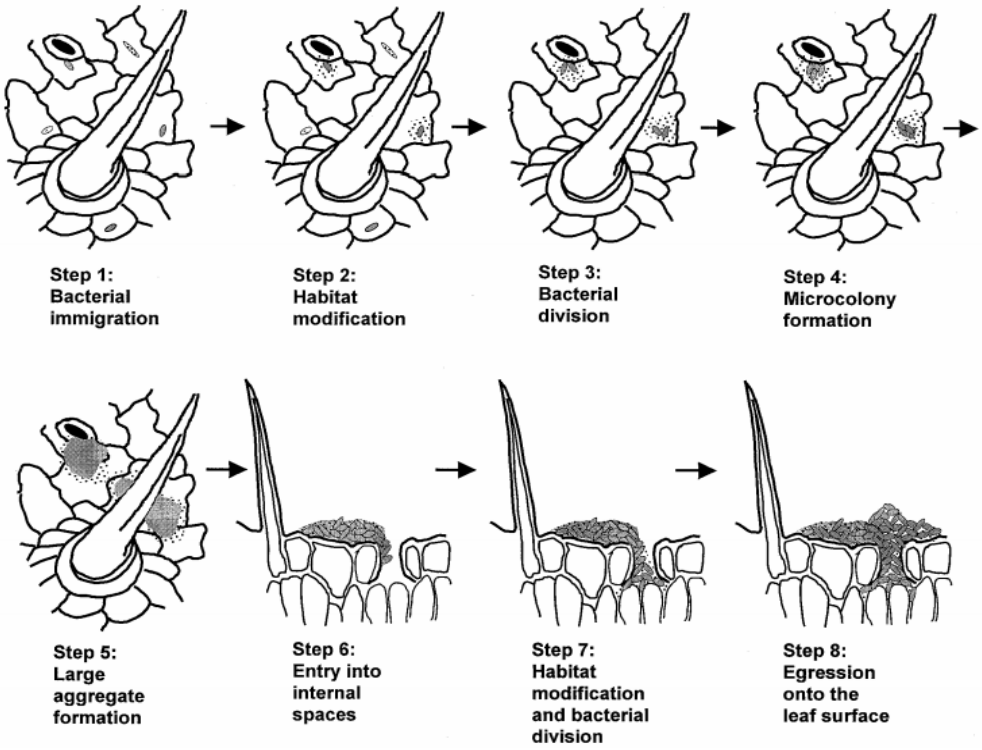

Fig.2.1 Pictorial depiction of microbial colonization strategies on the phylloplane. [Source: Beattie and Lindow 1999]

Tomato is one of the major grown crops around the world. The plants are hosts to large number of diverse microbial flora advantageous to the plants, and, to the pathogenic strains which can cause different diseases on fruits and the plant. Occurrence of diseases tends to cause loss of foliage, quality, and yield. Phylloplane bacteria with biocontrol activities can prove effective to overcome such agricultural losses. Two bacterial strains; *Novosphingobium capsulatum* and *Bacillus cereus* are reported to reduce late blight symptoms caused by *Phytophthora infestans* on leaves and fruits of Tomato [Halfeld-Vieira *et al* 2006]. *In vitro* and *in vivo* biocontrol activity of *Paenibacillus macerans* and *Bacillus pumilus* could effectively control *Xanthomonas vesicatoria* and *Alternaria solani* on Tomato plants [Filho *et al* 2010]. Bacterial speck of Tomato caused by *Pseudomonas syringae* pv. tomato, was suppressed by plant growth promoter bacteria *Azospirillum brasilense* by inducing reduction in the population sizes of the pathogen. Although, *A.brasilense* primarily belongs to the rhizosphere, yet some strains are also found on the phylloplane [Bashan Y and de-Bashan LE 2002]. *Bacillus cereus* isolated from healthy tomato phylloplane tends to cause ISR in tomato plants against *P. syringae* pv. *tomato* [Halfeld-Vieira *et al* 2006].

2.2. Leaf age and microbial colonization:

Microbial invasion of the phylloplane is often subjected to the rate of emigration, multiplication and the death of microorganisms [Kinkel LL 1997]. Owing to the exposure of aerial plant parts to environmental vagaries and other factors, diversity in the colonization patterns of microbes has been observed. The microbial colonization on plants varies from one to another, and often is subject to the physiology of different parts of the plants [Andrews and Harris 2000] and their distance from the soil. Phylloplane is a complex terrestrial milieu supporting the survival and growth of an array of microorganisms acting as pathogens, commensals or plant beneficiaries [Jackson *et al* 2013]. Some microbes migrate to the leaves but are not able to multiply for eg. casual fungi that land on the phylloplane owing to various migratory sources, but are unable to multiply, whereas the resident fungi are able to grow and multiply in same microenvironment [Prabakaran, M. *et al* 2011].The microorganisms adhere to the leaf surface and start spreading using different strategies. They settle and survive on the surfaces of leaves owing to the nutrient rich complexes [Lindow and Brandl 2003] usually localized at specific locations and niches on the phylloplane. The microbial colonization is thus said to be nutrient driven. The phylloplane ecology varies based on multiple factors including leaf-age [Ching-Hong Yang *et al* 2000]. Ageing of leaves may render plants with alternating microbial communities. Changes in the nutrient availability; depletion of nutrients with age and physiology of leaves often leads to alter the 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]. Microbiota of Olive leaves belonging to the same age at any time of the year was found to be similar, but, it differed significantly with the epiphytes obtained from leaves of the same age but at a different time and, with that on the leaves of different age at any particular time of the year [Ercolani GE 1991].

Colonization patterns of epiphytes is usually based on the shape and age of leaves and the spatial distribution of nutrients, because of which microorganisms are usually observed and isolated in a large number from and near the veins, stomata and trichomes [Lindow and Brandl 2003]. Different micro fungal species have been identified as prominent epiphytes of phylloplane, for example, *Cladosporium, Alternaria, Penicillium, Aspergillus, Fusarium* species [Whipps *et al* 2007] and the occurrence of filamentous form has been found to increase with leaf age [Sharma.P.D. 1984]. By the time leaves

reach senescence, the frequency of epiphytic colonizers tends to reduce, due to high leaf wettability that leads to low amounts of nutrients leached on to the phylloplane [Knoll and Schreiber 2000]. The loss and uptake of water and nutrients majorly decide the type and percentage of microfloral inhabitants of leaf surface as the increased amount of desiccation associated with senescing leaves may follow a less number of microbial communities [Fahmy & Ouf 1999]. The distribution of water and moisture along with the cuticular waxes on the surface get modified with leaf age, acting as factors determining microbial colonization [Lindow and Brandl 2003]. Furthermore, the microbial population sizes on phylloplane may get affected by the surface structure of leaves that includes ridges and troughs with the presence of trichomes, the base of which favors establishment of epiphytic aggregates. However, as the leaf topography changes due to ageing and the changes in microclimate there are significant alterations in the number of epiphytes [Gabriela Lopez-Velasco *et al* 2011].

Bacterial epiphytic densities on the leaves of *Apium nodiflorum*, *Nasturtium officinale*, and *Glyceria fluitans*, increased with age [Andrews and Hirano 1991]. Phylloplane microbial patterns on the leaves of cotton wood tree (*P.deltoides*) at different growth stages, varied considerably with age [Redford and Fierer 2009]. Age also plays an important role in colonization of leaves by fungal endophytes [Arnold and Herre 2003]. Jackson and Denney [2011] observed variations in the bacterial colony assembly patterns and structures in different seasons, where they found much distinct microbial flora from the phylloplane of magnolia [*Magnolia grandiflora*] tree in summers. In field grown Soya bean, the population of endophytic fungi decreased as plants aged whereas it increased in greenhouse grown plants [Pimentel IC *et al* 2006]. Population of epiphytic fungi and bacteria increased on the phylloplane of Dhak [*Butea monosperma* [Lamk.] Taub] with age [Chauhan D *et al* 2014]. Similar results were observed by Hurst JH and Pugh GHF [1983] from three sub-Arctic phanerogams where variations were observed in the quantitative and qualitative isolation of phylloplane microfungi. The young leaves were usually inhabited by sterile mycelia and the senescent leaves had primarily *Botrytis cinerea* colonies. Mature leaves of sugar beet (*Beta vulgaris*) harboured significantly high number of bacterial, yeast and, fungal colonies [Thompson *et al* 1992]. The filamentous fungi and yeast were maximum in summer [Thompson *et al* 1992]. Different irrigational practices are known to influence the growth of human pathogens like *E.coli* on lettuce plants. Rise in the growth patterns were observed with age increase [Williams TR *et al*

2014]. Exploring factors associated with the differential development of microbial communities on the phylloplane with age can be vital in effort for the management of crops and disease causing phytopathogens [Jacques MA 1996].

2.3. Relationship between leaf age and trichomes:

Leaf surface topography influences the spatial distribution of microbes and affects microbial diversity on the phylloplane. Epiphytic microorganisms are aggregated at various niches on the phylloplane, including stomatal openings and the base of trichomes [Lopez-Velasco *et al* 2011]. Trichomes are the epidermal structures out grown on various parts of many terrestrial plants, at times complex in structure, producing different shapes and fulfilling vital functions [Weryszko-Chmielewska *et al* 2005]. Associated with plant's defence mechanism, they are the synthesizers of alkaloids, terpenoids, flavonoids and defensive proteins, against a variety of insects [Kng JH *et al* 2010]. Karabourniotis *et al* [1995] established a relationship between trichome density and UV-B absorbing leaf phenolic constituents, which declined with an increase in the leaf age and trichome density, deducing a vital role played by leaf trichomes in protecting the underlying cells from UV-B damage. Glandular trichomes known to secrete various metabolic products, peltate trichomes release them after stimulation by external sources, and capitate release the metabolites naturally through micropores [Ascensao *et al* 1995].

Amme S *et al* [2005] observed short and tall glandular and tall non-glandular trichomes on the leaves of tobacco plants. Changes occurring in trichome development with the ageing phylloplane can be a vital path to understand their role in helping plants combat biotic and abiotic stress. Non-glandular trichomes found on the mature leaves of *Kalanchoë* sp. were dead epidermal structures, whereas trichomes with live protoplasts were found on the younger phylloplane. Their type and density did not change with age and secreted defence proteins which were found in abundance both from the young and old leaves. [Weryszko-Chmielewska and Chernetskyy 2005]. Kolb and Muller [2003] observed four different types of trichomes on the young leaves of Styrian pumpkin. The stipitate-capitate trichomes were half developed and full development was found on 4 cm long leaves. Large numbers of microbial communities were observed on live trichomes by Krimm U *et al* [2005]. Telfer A *et al* [1997] reported that the leaves of Arabidopsis thaliana rosette produced variations in the development of trichomes. Young leaves had no trichomes, later stages leaves had trichomes on the abaxial surface, but the leaves at

inflorescence had lesser or no trichomes present on the adaxial phylloplane. The distribution and densities of trichomes vary at different leaf ages. The GLABRA2 gene [GL2] is one of the genes known to play a key role in trichome development in Arabidopsis. Mutations occurring at this locus lead to the abnormal development of the trichomes [Rerie WG *et al* 1994]. Mutations in HDG2 gene alter mature trichome cell walls, and in *BLT* led to a loss of branching in trichomes. Mutations in *PEL3* resulted in the occasional tangling of developing trichomes [Marks MD *et al* 2009]. Studies done on the Birch leaves demonstrated change in trichome development with leaf age and varied biosynthesis of flavonoid-aglycones. Number of glandular type declined with leaf age and highest trichome density was observed during the younger stages of leaf development [Valkama E *et al* 2003].

Young leaves showed high growth rate of trichomes as compared to the older ones [Werker E *et al* 1993]. Different types of trichomes are found on the leaf surfaces of Tomato plants that act as the sites of microbial aggregation. Tomato plants inoculated with *S. enterica* were observed for the colonization patterns and *the aggregation levels on* tomato leaves were found to be cultivar dependent. Type 1 trichomes were colonized primarily. *S. enterica* levels on *Solanum pimpinellifolium* [West Virginia 700 [WVa700]] were lower as compared to *S. lycopersicum* cultivars [Barak JD *et al* 2011]. A rise in the density of trichomes on Tomato leaves was observed with an increase in the plant's age where 81-90 days old plants had the highest density and 66-70 days old plants bore the lowest densities of leaf trichomes [Oriani, Maria Auxiliadora de Godoy *et al* 2011].

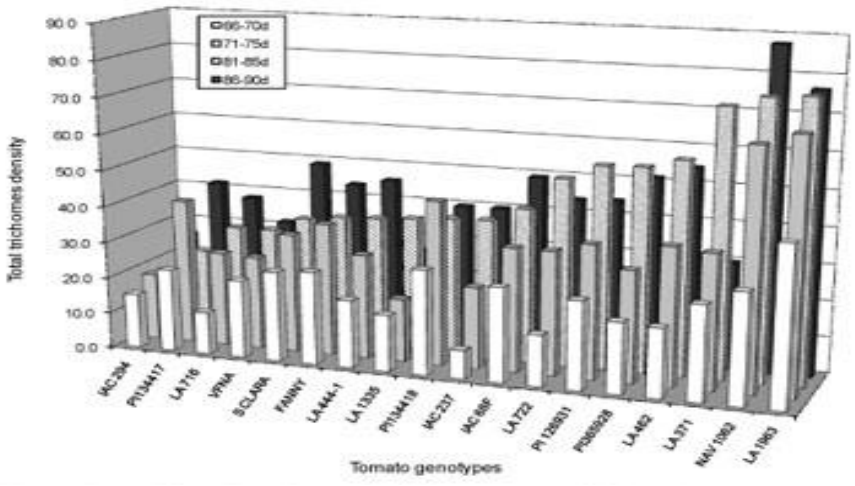

Fig.2.2. Dentisity of total trichomes on tomato

[Source: Oriani, Maria Auxiliadora de Godoy *et al* 2011

Type 6 glandular trichomes found on the *Lycopersicon sp.* and the concentration of insecticidal biochemical produced by them, showed variations with plant development and decreased quantitatively as the trichomes aged [Lin SYH *et al* 1987].

2.4. Inter-microbial interactions:

The growth of plants is largely affected by the resident fungal and bacterial microbiome. Microorganisms sustain in such habitats by allowing various interactions amongst them in order to compete for nutrients and space. An insight into the nature and effects of these microbial interactions is vital for enhancing the performance of these ecologies, which are essential, in such diverse processes as biological waste treatment procedures, water pollution reduction, and industrial fermentations, human or animal digestive processes and in soil [Gall L.S. 1970]. Microbial interactions can be parasitic, where one benefits at the cost of another, mutualistic, where both organisms are at advantage; or commensal, where one organism benefits at no cost or benefit to the other [Phelan V.V. *et al* 2012]. Nutrient and energy sharing is an integral part of inter-microbial interactions [Comolli LR 2014]. A wide array of bacterial and microfungal species possess properties, like secretion of metabolic compounds or toxins that either help them to interact with or suppress each other. The phylloplane microbial communities are known to sustain in extreme conditions and exhibit properties that may influence the colonization strategies of plant pathogens. Plants release nutrients in the form of exudates, that help the microbial colonists to aggregate and survive commensally or as saprophytes [Eberl *et al* 2007]. The heterogeneity of leaf surface colonizers is vastly considered as a factor dependent upon the mutual interactions amongst them [Lindow and Brandl 2003]. The microbial diversity on plant surfaces acts as a decisive factor for the plant's development and well-being. Various species of yeasts have been found to antagonize the activities of different bacteria [McCormack *et al* 1994]. Antagonistic properties of microbes against *B.*cinerea are largely focussed on competition and antibiosis, leaving aside mycoparasitisms. Adhesion of yeast and bacterial cells on *B.*cinerea inoculated on the excised stems of *Lycopersicon esculentum,* significantly degrades in the fungal hyphae. Such interactions lead to a direct effect rather than mediating through the phylloplane [Cook *et al* 1997]. These interactions occur via diverse set of channels through which genetic and molecular information is passed, thus, playing a vital part in microbial metabolic exchange and providing the basis for microbial survival. Although some of these interactions are dependent on cell-to-cell contact, many do not occur through physical contact. Contact-independent metabolic

exchange is advantageous because the signals are dispersed, enabling them to reach many neighbouring cells and communities as opposed to only one cell at a time. The dispersion of metabolic exchange factors allows them to serve as nutrients or cues to neighbouring microbes, thereby controlling the behaviour of the larger microbial community [Phelan V.V *et al* 2012]. However, these inter-microbial interactions are largely dependent upon the nature of the microenvironment in which these microbes sustain [Blakeman and Fokkema 1982]. A large number of studies have been undertaken in the area of rhizosphere regarding the investigation of antifungal qualities laden bacterial microflora [Pandey *et al* 1997]. Distinct patterns of production of secondary metabolites with antibiotic properties were associated with *Streptomyces*-microbe interactions causing inhibition of various mycorrhiza and phytopathogenic fungi [Schrey SD *et al* 2012].

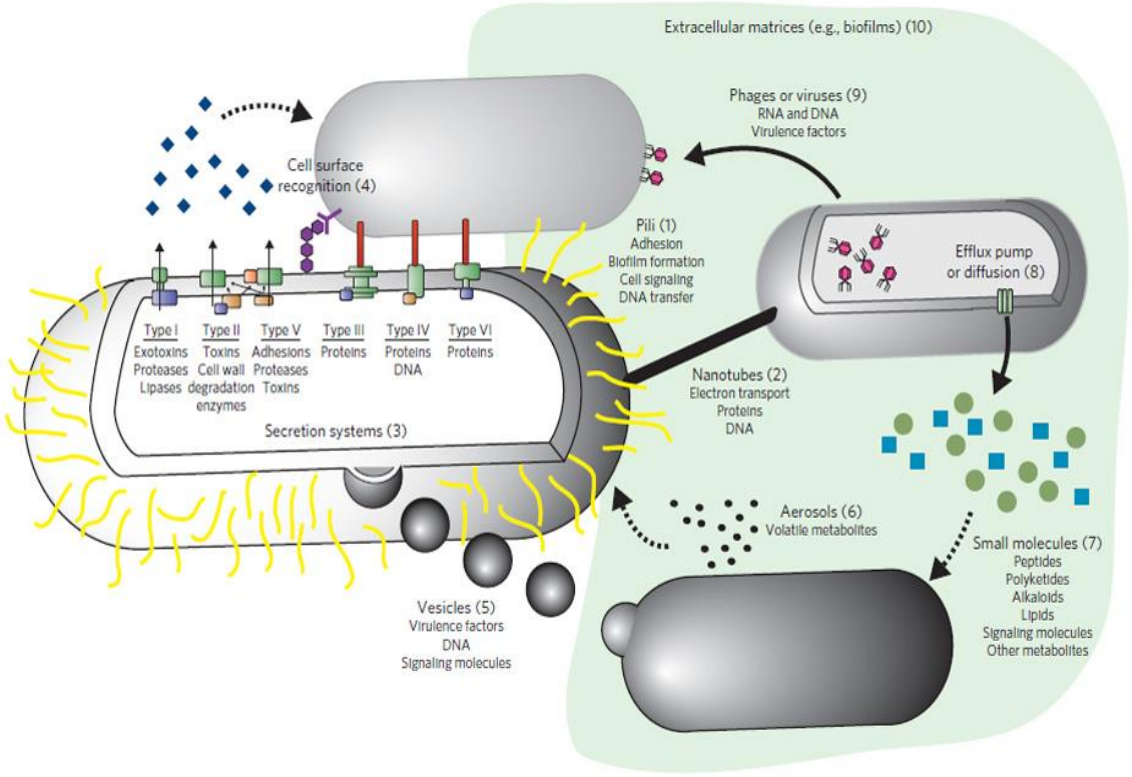

Fig. 2.3. Various interactive mechanisms amongst microbes.

[Source: Phelen V.V. *et al*, 2012]

2.4.1. Antimicrobial activity of Phylloplane bacteria:

A microbial ecosystem is composed of a fragile, yet balanced population of microbes each interacting with, and influencing the other members of the population in a number of ways. There are diverse, ubiquitous microbial interactions, such as commensalism, inhibition, food competition, predation, parasitism, and synergism, which either singly or in combination may confer a critical impact on the normal functioning of the microbial communities [Gall L.S. 1970]. Apart from interacting with the host, they also lead to inter-microbial interactions that could be a factor corresponding to the availability of nutrition or sites for colonization [Mandrell *et al* 2006]. The interactions between microbial populations are determined according to whether both populations or either of them benefit from the association, or one or both populations are negatively affected. The inhibition of phytopathogens by phylloplane residents has also gained importance and may help in affecting disease occurrence in plants [Patil and Kachapur 1998]. Various bacterial-bacterial [May *et al* 1997], bacterial-fungal and fungal-fungal antagonistic interactions have been studied for crop protection and plant health [Mohamed and Sater 2001]. In a number of studies, various bacterial species have been found to inhibit the growth of certain microfungal species and on the other hand, promoting the growth of others [Schrey *et al* 2012]. J.R.Kerr, [1999] reviewed the studies that have reported various bacterial genera like *Pseudomonas, Serratia, Klebsiella*, which were antifungal 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]. *Lactobacillus* sp. are also recounted to act against *Fusarium, Penicillium* and *Aspergillus* species [Jesper Magnusson *et al* 2003]. Chitinases are enzymes found in bacteria and other microorganisms, and is useful in the biodegradation of a polysaccharide ‘chitin’ abundantly found as the main component of the exoskeleton of yeasts and fungi [Hamid *et al* 2013].

Biological control of the phytopathogens is the suppression of microbial growth caused by the introduced microorganism via its establishment, stabilization, and antagonism. Fungal phytopathogens can be biologically controlled without interfering with the normal growth and development of plants [Viera *et al* 2008]. Microorganisms with antimicrobial properties could be considered as potent bio-control agents against a plethora of diseases caused by pathogens [Lim HS *et al* 1990]. Quality and quantity of available nutrients and

successful competition by antagonizing microbes, along with proper metabolic activity of the antagonists have been considered as an important factor governing inhibition [Thomashaw LS and Weller DM 1996]. Culturable bacteria associated with the phylloplane, possessing antimicrobial properties have been of interest since long [Enya *et al* 2006]. Various pathogens like *Rhizoctonia solani* and *Fusarium oxysporum* have also been found causing different diseases on commercially important crops like Tomato plants. Application of chemical fungicides for their treatment has been of least effectiveness [Rini and Sulochana 2007]. Bacterial species like *Enterobacter agglomerans* has been investigated to be active as a chitinolytic microorganism, antagonizing the growth of fungal phytopathogens like *Rhizoctonia solani* [Chernin *et al* 1995] that causes leaf spot and root rot diseases in Tobacco plants [Gonzalez *et al* 2011]. Sheath blight of Rice caused by *R.solani* could be reduced by application of *P. fluorescens, P. aeruginosa* and *P. asplenii* [Akter S *et al* 2014]. *Erwinia herbicola* and *Pseudomonas syringae* isolated from soybean leaves had antimicrobial activity against *Escherichia coli, Pseudomonas syringae* pv. glycinea, and *Geotrichum candidum* respectively, suppressing bacterial blight symptoms [Völksch B *et al* 1996]. 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]. Earlier studies report of many bacterial species like *Bacillus* strains isolated from phylloplane that are capable of reducing foliar diseases with their biocontrol activity to significant levels caused by other phytopathogens [Viera *et al* 2008]. *Bacillus* and *Pseudomonas* species are also known to inhibit the activity of phytopathogens like *Botrytis cinerea* [Swadling and Jeffries 2010] and *Alternaria* species [Kong *et al* 1997] that cause various plant diseases. Phylloplane bacterium *Ochrobactrum anthropi* BMO-111 has proved significantly effective against blister blight disease of tea, by ceasing the mycelial growth of *Exobasidium vexans* [Sowndhararajan K *et al* 2013].

2.5. Plant-microbe interactions:

Plant pathogens have evolved different strategies to infect their respective hosts, against which the hosts have evolved numerous defence strategies. The plant immune system recognizes conserved molecular patterns of the invading microbe, which initiate a set of

basal immune responses [Mishra *et al*, 2015]. The microbes- mediated defence strategies adopted by plants include activation of antioxidant status of the plant by reprogramming defence-related enzymes, modulation and activation of phenylpropanoid pathway leading to phenolics production [Mishra *et al*, 2015]. Induced plant defence enzymes/proteins, toxic compounds and antimicrobial proteins encounter a broad arsenal of pathogen-derived virulence factors [Doehlemann and Hemetsberger, 2013]. Plants often differ greatly in their resistance to microbial pathogens. These differences often lay in the speed and intensity of plants reactions [Shulaev *et al*., 1997; Ryals *et al*., 1996]. Resistant plants respond less to pathogens than susceptible individuals. Previous studies report on isolation of numerous plant resistance genes, recognised as R genes, functional in defence against fungi, bacteria and nematodes. Most of the R-genes are thought to encode receptors that recognise and bind specific molecules originating from pathogens and alert the plant to the pathogens presence. The specific pathogen molecules recognised, are referred to as elicitors which include protein, peptides, lipids etc. arising from the pathogen wall, the outer membrane or from a secretion process can induce phytoalexins production and activate other reactions leading to the production of defence enzymes [Ebel, 1986; Boller, 1989]. Plants accumulate a great diversity of natural products, many of which confer protection against phytopathogenic attack. The seeds treated with *Pseudomonas fluorescens* lead to accumulation of higher phenolic compounds and higher activities of POX, PPO and PAL which may play a role in defence of maize plants against *R. solani* f. sp. *sasakii* [Sivakumar and Sharma, 2003]. Accumulation of phenolics and lignin in high amounts, together with higher activities of major defense enzymes in response to the elicitors, may protect eggplants against *Ralstonia solanacearum* [Mandal, 2010]. Kagale *et al*. [2011] had reported that foliar application of the aqueous leaf extracts of *Zizyphus jujuba* and *Ipomoea carnea* followed by challenge inoculation with *R. solani* induces systemic resistance in rice, with significantly increased accumulation of POX, PAL and phenolic substances. The application of plant extracts can induce systemic resistance in many plants through accumulations of PR-proteins [Aboellil, 2007]. Protection of cucumber and tobacco plants against powdery mildew pathogens by leaf extracts of *Reynoutria sachalinensis* was accompanied by increased activities of POX, PPO, PAL [Herger and Klingauf, 1990].

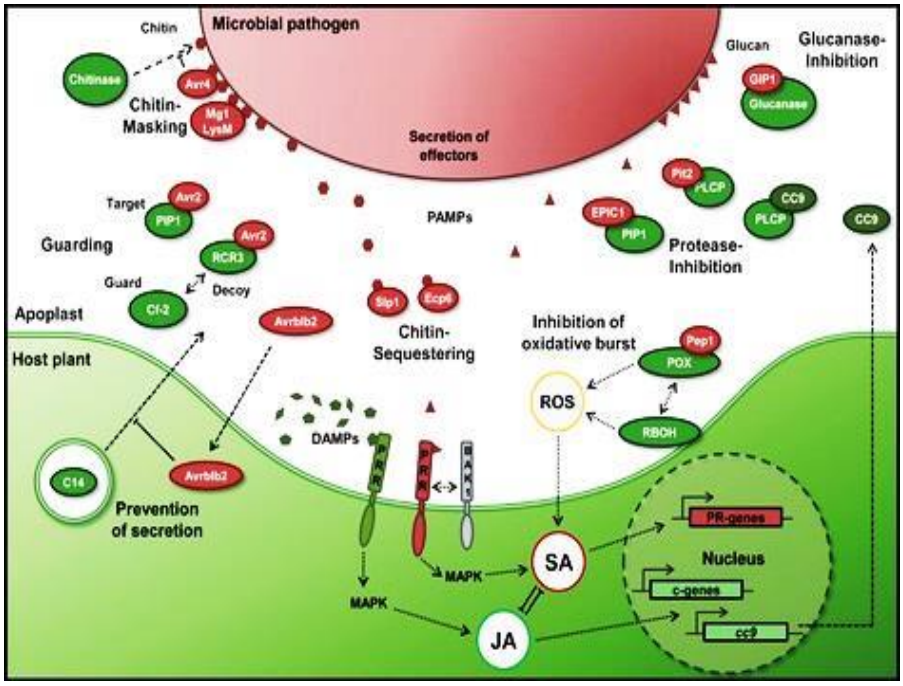

Fig.2.4. Schematic overview of a hypothetical plant infection by a filamentous microbial pathogen. Components of basic plant defense and interfering pathogen effectors. [Source; Doehlemann and Hemetsberger, 2013]

2.5.1. Changes induced in total phenols and flavonoids by microorganisms:

The metabolic activity of microorganisms present on the leaf surface modifies the nature and the quantity of biochemical substances on the phylloplane [Blakeman & Atkinson, 1981; Blakeman, 1985; Morris & Rouse, 1985]. Previous reports indicated a direct association between the amount of simple and polyphenols, and microorganism invasion on plants [Tyagi VK, and Chauhan SK, 1982]. However, not much data is available regarding their fluctuations in the foliage due to colonisation by phylloplane microbiota. Pradeep and Jambhale [2002] suggested that phenolic compounds and related oxidative enzymes are mostly considered as one of the important biochemical parameters for disease resistance to differentiate between resistant and susceptible genotypes. Though, the role of flavonoids in plant defence is not well studied, except in case of catechins and proanthocynadins [Freucht, and Truetter, 1999]. They have also been contributing to human health by being active principles of medicinal plants [Parr, and Bolwell, 2000; DeBruyene *et al.*, 1999; Kong *et al.*, 2003]. Plants possess high amounts of polyphenols and flavonoids and potent antioxidant activity leading to various defensive and disease fighting properties. 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]. Flavonoids play an important role in the protection of plants against plant feeding insects and herbivores. Their presence can alter the palatability of the plants and reduce their nutritive value, decrease digestibility or act as toxins [Harborne and Williams, 2000]. Flavonoids are very important in plant resistance against pathogenic bacteria and fungi. Antipathogenic properties of flavonoids can be non-specific and result, in part, from their antioxidative properties. They quench ROS, which are generated both by the pathogens and the plant as a result of the infection [Blount *et al* 1992; Dai *et al* 1996]. Flavonoid compounds are transported to the site of infection and induce hypersensitive reaction, and programmed cell death. They may also be directly involved in the inhibition of the pathogen's enzymes, especially those digesting the plant cell wall, by chelating the metals which are required for their activity [Treutter D, 2005]. The antifungal activity is often based on the inhibition of spore development and mycelium hyphae elongation [Blount *et al* 1992]. Studies on barley mutants showed that proanthocyanidins or even small amounts of dihydroquercetin are involved in the protection against *Fusarium* sp. [Skadhauge *et al* 1997].

Bhatia *et al*. [1972] concluded that the ability of tomato plants to resist infection caused by *A. solani* depends on the quantity of phenolics in the leaf, stem and roots of the plants which was found higher in the resistant variety than in the susceptible variety. Bhullar *et al*. [1972] observed an increase in phenolic content in the inoculated chilli plants of both the resistant and susceptible variety infected by early blight. Rice cultivar resistant to *Pyricularia oryzae* Cav. contain more of total phenols than the susceptible variety [Kotireddy, 1971]. Vidyasekaran [1974] reported that ragi resistant varieties contained more phenols than the susceptible varieties against *Helmenthosporium tetramera* Mc. Kinney. Similar observations were also made by many researchers in different plant tissues of resistant varieties than in those of the susceptible ones [Sathiyanathan, and Vidyasekaran, 1981; Anahosur *et al*., 1985; Reuveni and Cohen, 1978; Mandaviya, and Parameswaran, 1993; Kalappanavar, and Hirmath, 2000]. However, Venkateshvarulu and Sirohi [1976] found that brown rust infection increased the phenol content considerably in susceptible varieties under normal day conditions and decreased under long day conditions. Scagel and Lee, [2012] reported a differential alteration in the polyphenolic contents of Basil plants upon being inoculated with the arbuscular mycorrhizal fungus. Sharma *et al*. [1992] studied the quantity of phenolics in resistant and susceptible cultivars of maize with Turcicum leaf blight disease. The resistant cultivar showed an

increase in phenolics content indicating the post-inflection biosynthesis which may play an important role in resistance. 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*. The soil application of Salicylic acid and a biocontrol agent, *Trichoderma harzianum* induces resistance and higher phenolic accumulation in tomato plants infected with *F. oxysporum* f. sp. *lycopersici* [Ojha and Chatterjee, 2012].

2.5.2. Intercellular fluid proteins:

The apoplastic space represents an important contact area between host and pathogen. Crucial regulatory processes and protein–protein interactions take place in the apoplast/ intercellular spaces, and defined host–pathogen interfaces which are formed between the plant cytoplasm. The apoplastic plant immunity and its modulation by microbial pathogens has gained significance over the past years. Important components of the induced plant defence response against microbial pathogens are proteins that are produced on pathogen perception to restrict pathogen growth [Doehlemann and Hemetsberger, 2013]. Before entering a plant cell, pathogenic microbes need to pass the apoplast, where various defence compounds of the plant immune system form an efficient barrier. On pathogen recognition, attacked cell walls are remodelled and the apoplast is affected by means of antimicrobial low-molecular-weight compounds [Monaghan & Zipfel, 2012]. Parent and Asselin [1984] reported that in tobacco mosaic virus infected hypersensitive *Nicotiana* species, the pathogenesis-related [PR] or *b* proteins were found with other proteins in the intercellular fluid of leaf tissue. Ten proteins were detected in the intercellular fluid of tobacco mosaic virus infected *Chenopodium quinoa* Wild. These proteins were found in the inoculated tissue [Lauge *et al*, 1997]. PR-1, PR-2 and PR-5, were isolated from *Arabidopsis thaliana* apoplastic washing fluid after artificial induction of host defense by application of Salicylic acid analogue, 2,6-dichloroisonicotinic acid [INA] [Uknes *et al*., 1992]. The apoplastic PRs, which actually comprise all PR families except the ribonuclease-like PR-10, can be found in cell wall appositions in response to pathogen attack [van Loon *et al*., 2006]. De Wit and Spikman [1982] reported the existence of specific elicitors in the intercellular fluids obtained from compatible interactions of *C. fulvum* and tomato. The elicitors specifically induced chlorosis and necrosis in incompatible tissue. Accumulation of intercellular proteins and induction of leaf desiccation and abscission, occurred more quickly and severely in tomato after

inoculation with ECP1-, ECP2-, and ECP1/ECP2-deficient fungal strains than after inoculation with the wild-type strain indicating that the proteins play a role in virulence of *C. fulvum* on tomato and suggest that both are involved in suppression of host defence responses [Lauge *et al*, 1997]. The extracellular proteins ECP1 and ECP2 are abundantly secreted by the plant-pathogenic fungus *Cladosporium fulvum* during colonization of the intercellular space of tomato leaves [Lauge *et al*, 1997].

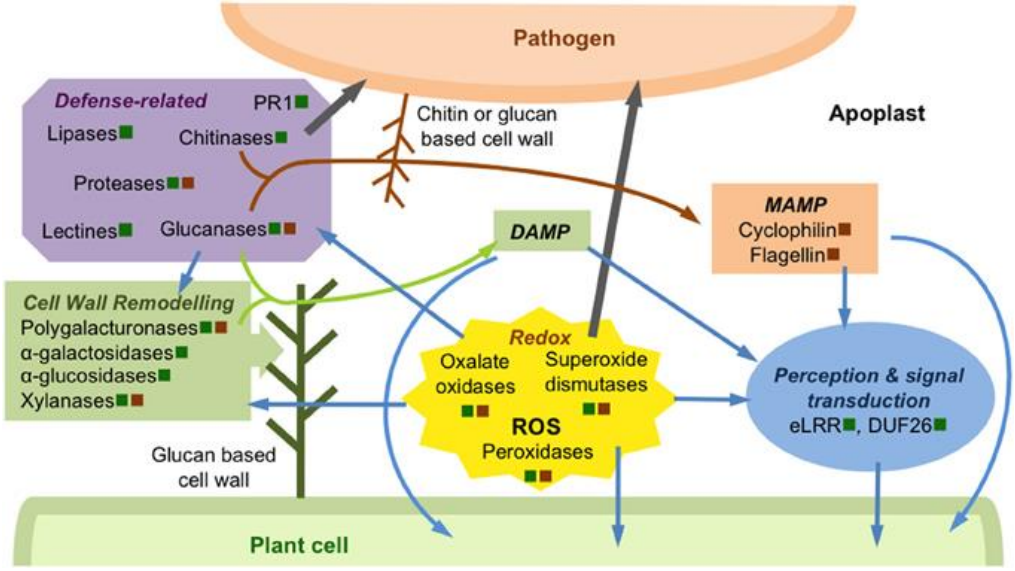

Fig.2.5. Schematic overview of some events occurring in apoplast during plant pathogen interactions. This illustration presents some examples of apoplastic proteins regulated during biotic stresses. These proteins are secreted by the plant cell (green square) and/or pathogen (brown square). [Source: Delaunois *et al*, 2014].

2.5.3. Induction of PAL and TAL activities:

It has been demonstrated in many plants species that both PAL and TAL activities reside on the same polypeptide [Rösler *et al*., 1997]. The products of PAL and TAL are modified, through phenylpropanoid metabolism, to precursors of secondary metabolites, including lignin, flavonoid pigments, and phytoalexins, all of which play key roles in a range of plant-pathogen interactions [Morrison and Buxton, 1993]. Hindumathy [2012] observed a sharp increase in TAL activity leading to induction of resistance in pearl millet seedlings post inoculation with *Sclerospora graminicola*. Banana plants challenged by treatments with *Pseudomonas flourescens* and *Fusarium oxysporum* f.sp. *cubense* showed increased enzymatic activities of PAL [Thangavelu *et al*, 2003]. Boonchitsirikul *et al*. [1998] recorded the time course changes of enzyme activities of PAL and TAL in young rice inoculated with the blast fungus *Pyricularia grisea* [Cooke] Sacc. for a five day

period after inoculation. Kale and Choudhary [2001] observed that in groundnut, resistant interaction was concurrent with early and rapid expression of PAL. Gupta and Kaushik [2002] reported enhanced specific activities of PAL and TAL in infected leaves of mustard as compared to healthy leaves. Fattah and Al-Amri [2012] confirmed that compost significantly increased the activity of TAL in tomato. Chakrabarty *et al*. [2002] concluded that induced rather than constitutive levels of PAL played crucial role in governing resistance in cotton genotypes. *B. subtilus* 174 showed great biocontrol potential and significant reduction in disease severity. Higher levels of PAL activities were observed upon inoculation of *B. subtilus* 174 in shoots of tomato plants as compared to *B. fortis* 162, thus showing its bio-control potential against *Fusarium* wilt [Akram and Anjum, 2011]. PAL activity increased rapidly after 2 h of inoculation with *Phytophthora megasperma* [Drechs.] f.sp. *glycinea* [Hildeb.] Kuan & Erwin race 1 in unwounded hypocotyls of soybean cv. Harosoy 63 [resistant] but did not change significantly in cv. Harosoy (susceptible) [Bhattacharya and Ward, 1986]. Kim and Hwang [2014] identified the pepper (*Capsicum annuum*) *PAL* (*CaPAL1*) gene, which was induced in pepper leaves by avirulent *Xanthomonas campestris* pv.*vesicatoria* (*Xcv*) infection. Das *et al*. [2003] studied the role of lignification and the enzymes involved in the biosynthesis of lignin i.e., PAL in the wheat spot blotch disease resistance using a resistant (Pusa-T 3336) and a susceptible genotype (Agralocal). Sharma [2003] reported high activity of PAL and TAL in resistant healthy (uninoculated) apple rootstock (MM115), but minimum activities were recorded from highly susceptible ones (MM103, MM104).

2.5.4. Elicitation of POX and PPO activities:

Reactive oxygen species (ROS) plays a major role in plant defence. POX and PPO have been reportedly involved in defence response of tomato [Fattah and Amri, 2012]. Generally, the POX and PPO enzymes have a defensive role against the invading pathogen. It is responsible for removal of toxic hydrogen peroxide from the host cells, thereby protecting the cells from damage. *Bacillus cereus* inoculated against *P. syringae* pv. *tomato* could significantly enhance activity of peroxidases in tomato plants [Halfeld-Vieira, 2006]. Harish *et al.* [2009] studied *Pseudomonas* and *Bacillus* isolates from the roots and corms of banana and tested their biocontrol efficiency against Banana bunchy top virus [BBTV]. *Bacillus cereus* strain BS 03 and *Pseudomonas aeruginosa* strain RRLJ 04 along with a rhizobial strain RH 2 could elicit SAR in pigeon pea against *Fusarium* wilt, both individually and in combination with increased activities of POX,

and PPO. Spraying of cacao plants with a heterogeneous chitosan suspension [MCp] from *Crinipellis perniciosa* mycelium showed a significant increase of oxidative POX and PPO activities [Cavalcanti *et al*., 2008]. The enhanced activitity of the POX may contribute to bioprotection of black gram plants against *B. tabaci* infestation [Taggar *et al*., 2012].

The induction of resistance in tomato plants against *Fusarium oxysporum* f. sp. *lycopersici* have been correlated to the increased activity of POX [Ojha and Chatterjee, 2012]. The activities of POX and PPO were positively correlated to the enhanced disease resistance against bacterial wilt in *Eucalyptus urophylla* [Longxian *et al*., 2004]. The increase of POX activity due to arbuscular mycorrhizal fungi is involved in lignifications [Lagrimini *et al*., 1987] and specific cell wall POX might be required to generate H_2O_2 [Van Huysatee, 1987]. Split root experiments confirmed that plants treated with combination of PGPR and *Rhizobium* can survive longer than individual treatments and control [Dutta *et al.*, 2008]. Wood and Barbara [1971] reported increased POX activity in systemically infected leaves of three cucumber cultivars with the "W" strain of *Cucumber mosaic virus*. *Trichoderma* produces a toxic compound with a direct antimicrobial activity against pathogens, and also secretes compounds that are able to stimulate the plant to produce its own defence metabolites [Nicot, 2011]. The expression of POX is induced to high levels in tissues responding to challenge by fungal pathogens, wounding, or exposure to either ABA or fungal elicitor preparations [Sherf *et al.*, 1993]. The induction of systemic resistance in tomato against *Phytophthora infestans* by pre-inoculation with tobacco necrosis virus was accompanied by an increase of POX activity in inoculated leaves as well as in upper non-inoculated leaf tissues [Anfoka and Buchenauer, 1997]. Resistant plants of tomato when inoculated with nematode *M. incognita* showed higher concentration of POX [Zacheo *et al.,* 1993]. The increase in POX activity was evident 22-24h after inoculation compared to control, followed by decline in POX activity in the later stages [Sarkar and Joshi, 1977]. Highly anionic POX of tomato are related to pathogenesis and imparts temporal and spatial effects on plants responding to pathogen attack, wounding, or exogenously applied abscisic acid or fungal elicitors [Sherf *et al.,* 1993]. Asha and Kannabiran [2001] observed increase in the activities of POX and PPO in chilli plants sprayed with 10% aqueous leaf extracts of *D. metel*. The seeds treated with *Pseudomonas fluorescens* lead to accumulation of higher phenolic compounds and higher activities of POX and PPO which may play a role in defence mechanism of maize plants against *R. solani* f. sp. *sasakii* [Sivakumar and Sharma, 2003].