



Review of Literature

2.0 REVIEW OF LITERATURE

Leaf spot caused by *Alternaria alternata* is a severe foliar infection of *Stevia rebaudiana*. Management of foliar diseases by means of fungicides was found to be effective in many *Stevia* growing areas. Extensive fungicide application incurs added costs to producers and repeatedly has selected for fungicide tolerant strains and reduces the biodiversity of soil microbes. Avenot *et al.* (2008) reported that *A. alternata* causing *Alternaria* late blight of Pistachios has developed resistance to pyraclostrobin, boscalid and pristine® fungicides in California.

Development of fungicide resistance by the pathogen have encouraged for the exploitation of non chemical means of disease management. Some plant growth promoting rhizobacteria (PGPR) have the capacity to improve the ability of plants to ward of pathogen infections. Use of biocontrol agents has been shown to be eco-friendly and effective against many plant pathogens. For instance, *Pseudomonas* spp. and *Bacillus* spp. are known to induce systemic resistance to a variety of phytopathogens.

2.1. *Stevia rebaudiana*

Stevia rebaudiana Bertoni is a natural sweet herb. It has been recognized world wide for its excellent sweetening property. *Stevia* opens up new avenues for crop diversification and a viable alternative to sugarcane. There is a potential to use *Stevia* (sugar leaf) in soft drinks,

confectionery, bakery, etc. instead of sweeteners with known adverse side effects in the long run. Stevia provides a whopping Rs.11.05 lakh returns in a cycle of three years (http://www.nabard.org/newsletter/the_sweet_secrets_of_stevia).

Stevia may be used in the form of dry leaves, dry leaves powder, tablets and also in crystal forms. It has zero calorie energy therefore, its products have medicinal properties and useful for the people suffering from diabetes, hypoglycemia, blood pressure, hypertension, etc. It may also be utilized for industrial and household usage, mainly in soft drink and beverage sector (Singh *et al.*, 2007).

2.2. *Alternaria alternata*

Alternaria alternata has been recorded as a saprophytic or a weak pathogen causing so called "indefinite or opportunistic disease" on a number of crops (Nishimura, 1980; Ibraheem *et al.*, 1987). In India, Maiti *et al.* (2007a) reported *A. alternata* causing leaf spot in *S. rebaudiana*.

Symptoms initially appeared as small circular spots, light brown in colour. Later, many became irregular and dark brown to grey, while others remained circular with concentric rings or zones. In severely infected leaves, several spots coalesced to form large necrotic areas. In older leaves concentric spots were more common at the tips. Leaf spots varied from 2-18 mm in diameter. Conidial dimensions varied from 10-40 × 6-12 µm, mild to dark brown or olive-brown in colour, short beaked, borne in long chains, oval and bean shaped with 3-5 transverse septa.

The other crops affected by *A. alternata* includes cotton (Bashan and Hernandez-Saavedra, 1992), rough lemon (Masunaka *et al.*, 2005), glory lily (Maiti *et al.*, 2007b), chickpea (Mandhare *et al.*, 2008), *Aloe barbadensis* (Kamalakannan *et al.*, 2008), *Withania somnifera* (Pati *et al.*, 2008) and Basil (Taba *et al.*, 2009).

2.3. Biological control of plant diseases

With growing public interest in reducing chemical pesticide residues in food and the environment, it has become a national research priority to develop new pest management technologies that reduce use of chemical pesticides. The discovery and development of microbial antagonists naturally present in agricultural ecosystems should provide environmentally compatible tools to employ in place of or in conjunction with traditional chemical methods for reducing plant diseases (Schisler *et al.*, 2009). There are strict regulations on chemical pesticide use and there is political pressure to remove the most hazardous chemicals from the market (Pal and Gardener, 2006).

Plant Growth Promoting Rhizobacteria (PGPR) are a group of free-living saprophytic bacterial microorganisms that live in the plant rhizosphere and aggressively colonises the root system. They are studied as plant growth promoters for increasing agricultural production and as biocontrol agents against plant diseases (Kloepper *et al.*, 1992; Chen *et al.*, 2000; Saravanakumar *et al.*, 2007b).

Microorganisms aimed at being used as biological control agents must survive and be able to antagonize co-occurring pathogens when applied

to crops or postharvest commodities. The phylloplane (leaf) constitutes an important terrestrial habitat populated by numerous microbial species that has been considered an extreme habitat for microbial colonists because of continuously fluctuating physical and chemical microenvironments. Native microorganisms from the phyllosphere are natural candidates to develop biocontrol strategies because they have evolved mechanisms such as efficient nutrient uptake in oligotrophic conditions, antimicrobial compound production, exopolymer synthesis and UV-shielding pigmentation to overcome these constraints and establish themselves under the harsh conditions present in this habitat (Fernando *et al.*, 2007b). *Bacillus* and *Pseudomonas* species are known for inducing resistance in plants against foliar pathogens (van Loon and Bakker, 2005).

2.3.1. *Pseudomonas fluorescens* as potential biocontrol agent

Many strains of *P. fluorescens* show potential for biological control of phytopathogens. These bacteria have become prominent models for rhizosphere ecological studies and analysis of bacterial secondary metabolism and in recent years knowledge on their plant beneficial traits has been considerably enhanced by widening the focus beyond the case of phytopathogen directed antagonism (Couillerot *et al.*, 2009). Certain rhizosphere fluorescent pseudomonads strains have received much attention as potential biocontrol agents given their ability to produce antibiotics, such as 2,4-diacetylphloroglucinol (DAPG), that confer a measure of plant protection (Siasou *et al.*, 2009).

Fluorescent pseudomonads have often been isolated from soils that were suppressive to ward a plant pathogen, meaning that the plant remains healthy even though the pathogen is present. Among other commercially available biocontrol organisms, strains of *P. fluorescens* are often used in agriculture to control soil borne plant pathogens in order to enhance productivity and plant health (Haas and Defago, 2005).

Soil borne fluorescent pseudomonads have received particular attention because of their catabolic versatility, excellent root colonising ability and their capacity to produce a wide range of enzymes and metabolites that favour the plant to withstand varied biotic and abiotic stress conditions (Radjacommare *et al.*, 2004a; Saravanakumar and Samiyappan, 2007).

2.3.2. *Bacillus subtilis* as potential biocontrol agent

Bacteria of the genus *Bacillus* considered very important in agricultural applications because of their ability to produce a variety of secondary metabolites. Enhancement of plant growth by *Bacillus* is well documented and several mechanisms have been suggested for the phytostimulatory activity of this group of plant growth promoting bacteria (PGPB). *Bacillus* spp. have several advantages over other PGPB in that they possess several growth promoting traits such as production of phytohormones, secretion of antibiotics, induction of systemic resistance and their use as biopesticides (Reva *et al.*, 2004; Swiecicka *et al.*, 2008).

Studies have reported that *Bacillus* species may effectively colonize the rhizosphere and control root diseases (Sari *et al.*, 2006, 2007). PGPR

based products mostly contain strains of *Bacillus* that may have direct agricultural application because of long term viability of these sporogenous bacteria. Therefore, *Bacillus* spp. has the potential to be applied as booster inoculants to increase the efficacy of plant growth promotion and elicitation of systemic disease protection in the field (Kloepper *et al.*, 2004).

Published results show that several specific strains of species *B. pasteurii*, *B. amyloliquifaciens*, *B. subtilis*, *B. cereus*, *B. pumilus*, *B. mycoides* and *B. sphaericus* elicit significant reduction in the incidence or severity of various diseases on a diversity of hosts (Choudhary *et al.*, 2007).

2.4. *In vitro* biocontrol analysis of *Alternaria alternata*

Spurr and Knudsen (1985) used bacteria from soil and other habitats for the control of *Alternaria* leaf spot of tobacco. Hebbar *et al.* (1991) found that the bacteria associated with leaves and roots inhibited *in vitro* growth of *A. helianthi*. The biological control agents such as *P. fluorescens* and *Trichoderma viride* significantly reduced the mycelial growth, spore germination, spore production and germ tube formation of *A. solani* and *A. alternata* (Veerasingam, 1997).

Sid *et al.* (2003) documented antagonistic activity of *B. subtilis* (HS93) against *A. alternata*. *Pseudomonas putida* IsoF colonizes tomato roots and produce *N*-acyl-L-homoserine lactone (AHL) in the rhizosphere. It increases systemic resistance of tomato plants against the fungal leaf pathogen, *A. alternata* (Schuhegger *et al.*, 2006). *P. fluorescens* was

found to be more efficient in inhibiting the colony growth of *A. alternata* to 64.9% (Indira *et al.*, 2006).

Hou *et al.* (2006) reported that *B. subtilis* strain LEV-006 was antagonistic to four major fungal pathogen of canola including *A. brassicae*. Out of 18 isolates of *Bacillus* and *Pseudomonas* tested against *A. triticina*, nine isolates showed inhibitory effects (Siddiqui, 2007).

2.5. Mode of action of biocontrol agents

Microbial inoculants can elicit physiological changes in plants that are mainly mediated by microbial production of secondary metabolites especially phytohormones. Bacteria can influence plant growth directly through the production of phytohormones and indirectly through the production of biocontrol agents against soil borne pathogens (Glick *et al.*, 2007). *Bacillus* and *Pseudomonas* are known to suppress diseases by inhibition of pathogens by competition of Fe (III), inhibition of pathogen by diffusible or volatile products, induction of resistance in plants and aggressive root colonization and stimulation of plant growth (Siddiqui, 2006).

2.5.1. Hydrogen cyanide (HCN) production

Cyanide is a secondary metabolite that is synthesized by some Gram-negative bacteria, such as *Chromobacterium violaceum*, *P. fluorescens* and *P. aeruginosa* (Knowles and Bunch, 1986). HCN does not take part in growth, energy storage, or primary metabolism but is

generally considered as a secondary metabolite that has an ecological role and confers a selective advantage to the producer strains (Vining, 1990).

Kavitha (2004) reported that among the different antagonistic bacteria tested for HCN production, the intensity of HCN production was strong in *P. chlororaphis* isolate PA23 which showed maximum inhibition against *P. aphanidermatum* causing rhizome rot of turmeric.

Sixteen isolates of phosphate solubilizing rhizobacteria (PSRB) isolated from different rhizospheric soil samples of tomato collected from tomato growing regions of Karnataka were negative for HCN production (Hariprasad and Niranjana, 2009). The production of HCN is one of the major factors in the control of *Rhizoctonia solani* by *P. fluorescens* CHAO (Jamali *et al.*, 2009).

2.5.2. Salicylic acid production

Salicylic acid is an important signalling molecule involved in both locally and systemically induced disease resistance responses. Certain PGPR strains are capable of producing salicylic acid and responsible for the induction of ISR in plants (Maurhofer *et al.*, 1994). Salicylic acid production has been observed in several bacterial strains and exogenously applied SA can induce resistance in plant species (Bakker *et al.*, 2003).

Foliar application of SA at the concentration of 1 mM significantly reduced the leaf blight disease in groundnut plants against *A. alternata* and increased the pod yield under glasshouse conditions (Chitra *et al.*, 2008). Exogenous applications of salicylic acid (400 μ M) induced

systemic acquired resistance against tomato stem canker caused by *A. alternata* (Esmailzadeh *et al.*, 2008).

2.5.3. Siderophore production

Free-living nitrogen-fixing bacteria in soils need to tightly regulate their uptake of metals in order to acquire essential metals (such as the nitrogenase metal cofactors Fe, Mo and V) while excluding toxic ones (such as W). They excrete compounds previously identified as siderophores that bind a variety of metals in addition to iron. At low concentrations, complexes of essential metals (Fe, Mo and V) with siderophores are taken up by the bacteria through specialized transport systems (Kraepiel *et al.*, 2009).

Competition for iron is also a possible mechanism to control the phytopathogens (Tokala *et al.*, 2002). Manwar *et al.* (2000) have reported that siderophore producing *P. aeruginosa* was capable of inhibiting the growth of *Aspergillus niger*, *A. flavus*, *F. oxysporum* and *A. alternata*. Studies in biological control have focused mainly on fluorescent pseudomonads, producing fluorescent pigments and siderophores like pyoverdine (Haas and Defago, 2005). Among the 41 isolates obtained from rhizosphere soil, the isolates R33 and R13 exhibited positivity towards siderophores and were identified as *B. subtilis* (Lee *et al.*, 2007). Idris *et al.* (2009) showed that five bacterial isolates, two from sorghum rhizosphere and three from the rhizosphere of grasses, were able to produce siderophores.

2.5.4. Indole acetic acid (IAA) production

Indole 3 acetic acid (IAA) is quantitatively the most abundant naturally occurring auxin produced by bacteria (Lambrecht *et al.*, 2000; Bloemberg and Lugtenberg, 2001). The proportion of IAA producing bacteria in the rhizosphere may be of relevance for plant growth and previous studies have shown that IAA producing bacteria can be stimulated in the rhizosphere (Weisskopf *et al.*, 2005).

IAA biosynthesis is also wide spread in plant associated bacilli and considered to be directly involved in plant growth promotion (Tsavkelova *et al.*, 2007; Idris *et al.*, 2007). Ali *et al.* (2009) reported that auxin production potential of plant associated *Bacillus* spp. can be effectively exploited to enhance the growth and yield of *Vigna radiata*. Rhizospheric isolates exhibit relatively more IAA synthesis than histoplane and phyllosphere isolates. Karnwal (2009) tested 30 isolates of fluorescent pseudomonads for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan at 50, 100, 200 and 500 µg/ml. Among which two strains, *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2, showed increased indole production with increase in tryptophan concentration.

2.6. Induced systemic resistance

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance wherein plant defenses are preconditioned by prior infection or treatment that results in resistance against subsequent challenge by a pathogen or parasite.

Selected strains of PGPR suppress diseases by antagonism between the bacteria and soil-borne pathogens as well as by inducing a systemic resistance in plant against both root and foliar pathogens. Rhizobacteria mediated ISR resembles that of pathogen induced SAR in that both types of induced resistance render uninfected plant parts more resistant towards a broad spectrum of plant pathogens (Choudhary *et al.*, 2007).

Induction of systemic resistance by antagonistic bacteria has earlier been reported by several workers (Kamalakaran *et al.*, 2003, Revathi, 2004; Abeysinghe, 2007; Bakker *et al.*, 2007). Result from Domenech *et al.* (2007) showed that PGPR isolated from the rhizosphere of *Nicotiana glauca* L., stimulates growth and induce systemic resistance against *Xanthomonas campestris* CECT 95 in *Arabidopsis thaliana*. Vleesschauwer *et al.* (2008) demonstrated the ability of *P. fluorescens* WCS374r to trigger ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*.

2.6.1. Mechanism of induced systemic resistance

The action of ISR is based on the defense mechanisms that are activated by inducing agents. It activates multiple potential defense mechanisms that include increased activity of chitinases, β -1,3-glucanases and peroxidases (Xue *et al.*, 1998; Dalisay and Kuc, 1995; Maurhofer *et al.*, 1994; Schneider and Ullrich, 1994) and wide spectrum of pathogens can be controlled by a single inducing agent (Hoffland *et al.*, 1996; Wei *et al.*, 1996; Dean and Kuc, 1985).

2.6.1.1. Phenylalanine ammonia lyase (PAL)

PAL is the first enzyme involved in the phenylpropanoid pathway which plays a significant role in regulating the accumulation of phenolics, phytoalexins and lignin, the three key factors responsible for disease resistance (Vidhyasekaran, 1988). Nakkeeran *et al.* (2006) reported that pretreatment of hot pepper seeds with either *B. subtilis* strain BSCBE4 or *P. chlororaphis* strain PA23 challenged with *P. aphanidermatum* induced earlier and higher accumulation of PAL compared to non-inoculated control. Activity of PAL in watermelon plants pretreated with biocontrol agents was induced upon challenge inoculation with *A. alternata* (Umamaheswari *et al.*, 2007). Renuka *et al.* (2008) observed increased activity of PAL in chrysanthemum plants against *Alternaria chlamydospora* pretreated with bacterial antagonist *P. fluorescens* Pfl.

2.6.1.2. Peroxidase (PO)

Peroxidase is a key enzyme in the biosynthesis of lignin and other oxidised phenols (Bruce and West, 1989). PO catalyzes the oxidation of hydroxy-cinnamyl alcohols into free radical intermediates, which subsequently are coupled into lignin polymers (Gross, 1980). Besides, PO itself was found to inhibit the spore germination and mycelial growth of certain fungi (Joseph *et al.*, 1998).

Kavitha *et al.* (2005) reported higher PO activity in chilli plants treated with *B. subtilis* (CBE4) and *P. chlororaphis* (BCA+) challenged with *P. aphanidermatum*. Increased activities of PO in *P. fluorescens* Pfl pretreated groundnut plants challenged with *S. rolfsii* was observed by

Karthikeyan *et al.* (2006a). The groundnut plants treated with biocontrol agent and challenge inoculated with *A. alternata* recorded significantly increased activity of PO isozyme (Chitra *et al.*, 2006). Anand *et al.* (2009b) recorded higher accumulation of PO in Pfl treated cucumber plants challenged with *Pseudoperonospora cubensis*.

2.6.1.3. Polyphenol oxidase (PPO)

PPO usually accumulates upon wounding in plants. Biochemical approaches to understand PPO function and regulation are difficult because the quinonoid reaction products of PPO covalently modify and cross-link the enzyme. The increased activation of PPO could be detected in the cucumber leaf in the vicinity of lesions caused by some foliar pathogens. Bacteria produced more toxic quinone along with those of inducibly synthesized polyphenol due to the oxidation process. Polyphenol oxidase and PO carry the oxidation of polyphenols. PO and PPO were reported to be capable of oxidizing phenols to quinones (Mansfield, 1983) and they were associated with disease resistance in plants.

The higher PO activity was noticed in peppermint plant treated with *P. fluorescens* isolate PFMMP challenged with *Rhizoctonia solani* (Kamalakkannan *et al.*, 2003). Increased activities of PPO were observed in Pfl pretreated ragi plants challenge inoculated with the *Pyricularia grisea* and remained at higher levels up to 9 days has been reported by Radjacommare *et al.* (2004b). The groundnut plants treated with biocontrol agent and challenge inoculated with *A. alternata* recorded

significantly increased activity of PPO isozyme (Chitra *et al.*, 2006). Renuka *et al.* (2008) observed increased activity of PPO in chrysanthemum plants against *Alternaria chlamydospora* pretreated with bacterial antagonist *P. fluorescens* Pfl. Increased activities of PPO in *P. fluorescens* pretreated chilli plants challenged with anthracnose and powdery mildew pathogens was observed by Anand *et al.* (2009a).

2.6.1.4. Total phenols

Plant phenolics are the well-known antifungal, antibacterial and antiviral compounds and they play an important role in determining resistance or susceptibility of a host to parasite infection. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi (Vidhyasekaran, 1988; Sequeira *et al.*, 1991; Bennett and Wallsgrove, 1994; Southernton and Deverall, 1990; Cahill and Mc Comb, 1992).

Girish and Umesha (2005) observed that the rate of reduction in the bacterial canker disease incidence was directly proportional to the amount of increased level of total phenol content. Karthikeyan *et al.* (2006b) found that soil application of biocontrol agents (*P. fluorescens*, *T. harzianum* and *T. viride*) in combination with chitin enhanced the phenolics concentration in palms that led to induced resistance to *Ganoderma lucidum*. Dutta *et al.* (2008) recorded increased level of phenol content in the leaves of pigeon pea against *Fusarium* pretreated with PGPR.

2.7. Bioformulation

The success of biocontrol eventually depends upon development of suitable formulations in which the antagonistic microorganisms can survive for extended periods of time. It should be borne in mind that most biological control agents are affected by prolonged storage compared to fungicides. Development of stable formulations of biocontrol agents is of great importance to developing countries where subsistence farming is prominent and where fungicide treatments are unaffordable. Seed treatment of tomato with formulations (talc-based powder, lignite-based powder, lignite/fly ash based powder, wettable powder, bentonite paste, polyethylene glycol (PEG) paste and a water-dispersible tablet) of a strain of *Bacillus subtilis* AUBS-1 resulted in effective control of damping-off caused by *P. aphanidermatum* and also enhanced plant biomass under glasshouse and field conditions (Jayaraj *et al.*, 2005).

A talc-based powder formulation developed by Vidhyasekaran and Muthamilan (1995) was effective for the control of rice blast (Vidhyasekaran *et al.*, 1997a), rice sheath blight (Vidhyasekaran and Muthamilan, 1999) and pigeon pea wilt (Vidhyasekaran *et al.*, 1997b).

Survival and multiplication of *P. fluorescens* strain Pf1 in the phyllosphere of groundnut after foliar application of the talc powder formulation may explain the effectiveness of the foliar sprays in the control of leaf spot and rust diseases (Meena *et al.*, 2002). Mathiyazhagan *et al.* (2004) reported that delivering of talc based formulation of *B. subtilis* (BSCBE4) through seedling dip and foliar application

effectively reduced stem blight disease incidence and increased the dry matter production under pot culture and field conditions.

Spray application of *P. chlororaphis* (PA-23), *Pseudomonas* sp (DF41) or *B. amyloliquefaciens* (BS6) helped to control stem rot of canola by reducing petal infestation of *Sclerotinia* through both direct antimicrobial action and/or induction of plant defense enzymes (Fernando *et al.*, 2007a). Lamba *et al.* (2008) observed that application of *T. harzianum* and *P. fluorescens* has led to an increase in the dry matter content, starch, total soluble sugar and reducing sugar contents in leaves of sunflower when done as seed treatment or coupled with spray.